Expression and characterisation of biopharmaceuticals in heterologous expression systems

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Master of Science (Biotechnology)
Antje Pätz
aus Berlin

Referent: Prof. Rainer Fischer, Biologie VII, RWTH Aachen

Co-Referent: Prof. Klaus Ritter, Medizinsche Mikrobiologie, RWTH Aachen

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I.1 Human therapeutic proteins

One of the driving forces behind the biotech industry today is the development of new therapeutic drugs for the treatment of numerous life-threatening diseases. Many of these complex diseases, such as HIV, asthma and different kinds of cancer cannot be cured with conventional, simply structured molecules; here biopharmaceuticals offer the best promise for successful future treatment.

Biopharmaceuticals are essentially all pharmaceutical drugs, which are based on proteins (e.g. monoclonal antibodies, toxins, enzymes), recombinant DNA products, vaccine products, cultured cell and tissue/blood products (FROST&SULLIVAN(A) 2003). The trend for biopharmaceuticals is predicted to increase further as there are currently more than 350 products under evaluation in clinical trials (FROST&SULLIVAN(A) 2003). **M**onoclonal **a**nti**b**odies (**MAb**s) represent the fastest growing segment of the biopharmaceutical market. Currently there are 18 approved MAbs and eleven waiting to be approved within the next few years (GREENER 2005). MAb demand is calculated to rise up to 6000 kg/year by 2006. This indicates a trend towards specific antibodies produced in "high-titre" hosts to meet the annual product yield requirements at reasonable costs (COCO-MARTIN 2004).

I.1.1 Monoclonal antibodies for HIV-therapy

I.1.1.1 General features of viral replication and infection

Repeating outbreaks of viral infections worldwide such as **S**evere **A**cute **R**espiratory **S**yndrome (**SARS**), Ebola and Influenza have shown that infectious diseases present a continuing threat to the global health (HEYMANN 2005). Although modern advances, such as antibiotics and vaccines, have conquered some diseases new ones are constantly emerging.

A virus is defined as a noncellular genetic element containing either RNA or DNA, which replicates in cells but is characterised by having an extracellular state (BROCK *et al.* 1994). Viruses range in size from about 20 to 300 nm in diameter and generally consist of the viral genome and a surrounding protein coat (capsid). Some viruses possess an additional envelope around the protein coat resulting in a complete infection particle (virion; MODROW *et al.* 2003).

Immunity to viral infection is triggered by specific and nonspecific mechanisms. Early nonspecific responses (nonspecific inhibition by e.g. **n**atural **k**iller (**NK**) cell activity and interferon) limit virus multiplication during the acute phase of virus infection. Later specific

immune responses (humoral and cell-mediated) eliminate the virus and subsequently maintain specific resistance against reinfection.

Humoral immunity is induced by stimulated B-lymphocytes to produce antibodies. These antibodies can neutralise the virus by blocking viral-host-interactions or by recognising viral antigens on virus-infected cells. Antibody-coated viruses are bound by specific receptors on phagocytic cells leading to **A**ntibody-**D**ependent **C**ell-meditated **C**ytotoxicity (**ADCC**) or activation of complement-mediated lysis. Cell-mediated immunity involves cytotoxic T (T_c) lymphocytes, NK cells and macrophages, which recognise and kill virus-infected cells. Cytokines produced by monocytes, T helper (T_h) cells, and NK cells play important roles in regulating the antiviral immune functions. The target specific immune response starts after exposure and results in the production of antiviral antibody and cell-mediated immunity in 3 - 10 days.

During evolution many pathogens have evolved strategies to subvert the normal host defences. In these cases the virus is not cleared completely but remains in specific cells of the infected individual. Persistent infections may involve stages of both silent and productive infection without rapidly killing or producing excessive damage of the host cells. Adeno- and Herpes viruses for example reduce the number of **M**ajor **H**istocompatibility **C**omplex (**MHC**) class-I molecules on the surface of infected cells. This inefficient presentation of viral antigens complicates the recognition by T_c cells. **H**epatitis **B** **V**irus (**HBV**) produces large amounts of virus surface proteins (HbsAg) into the blood, which neutralise anti-HBV-antibodies. **H**uman **I**mmunodeficiency **V**irus (H**IV**) and Influenza virus constantly change sequence and structure of surface-exposed protein regions and therefore escape neutralising antibodies (MODROW *et al.* 2003).

I.1.1.2 The Human Immunodeficiency Virus (HIV)

Robert C. Gallo (National Institute of Health in Bethesda) and Luc Montagnier (Institute Pasteur in Paris) discovered HIV at the same time in 1981 as causative agent for the **A**cquired **I**mmune **D**eficiency **S**yndrome (**AIDS**). In 1983, the first HIV particle was isolated out of the lymphocytes of an AIDS-patient (BARRE-SINOUSSI *et al.* 1983). Subsequent research activity makes HIV one of the best-described viruses in respect to its molecular biology and pathogenesis (MODROW *et al.* 2003).

Recently, the findings of a 10-year long research study into the origin of HIV concluded that wild chimpanzees became infected simultaneously with two **S**imian **I**mmunodeficiency **V**iruses (**SIV**s) which formed a third virus capable of infecting humans and causing AIDS (BAILES *et al.* 2003). In January 2000, a computer model study suggested that the first case

of HIV-infection occurred around 1930 (with a 20 year error) in West Africa (KORBER *et al.* 2000). Since then HIV-infection spread worldwide and more than 20 million people died of AIDS. Estimated 38 millions currently live with HIV-infection and in 2003 alone, 3 million people became infected in sub-Saharian Africa (UNAIDS 2004).

The two types of HIV (HIV-1 and HIV-2) are transmitted by sexual contact, through blood and from mother to child and cause clinically indistinguishable symptoms. However, it seems that HIV-2 is less easily transmitted, and the period between initial infection and illness is longer (CLAVEL *et al.* 1986; NDOUR *et al.* 2000). Worldwide, the predominant virus is HIV-1, whereas the relatively uncommon HIV-2 type is concentrated in West Africa (NDOUR *et al.* 2000).

There are three branches in the phylogenetic tree of HIV-1 sequences, which comprise of the M (main), N (new or non-M and non-O) and the O (outlier) groups. Among these groups, the M viruses are the most widespread, being the variants of HIV-1 that are responsible for 99% of infections worldwide (MOORE *et al.* 2001). The M group is further divided into nine distinct genetic subtypes (A through K; MODROW *et al.* 2003).

HIV is a single-stranded (ss) RNA virus and belongs to the family of retroviruses, which depends on a reverse transcriptase to transcribe the viral RNA genome into DNA. The HIV genome contains about 9,500 nucleotides with nine genes that are flanked by long terminal repeats. As in all retroviruses the genome codes for the three major proteins gag (group associated antigen) for the capsid, pol for the enzymes involved in virus replication and integration and env for glycoproteins of the viral coat.

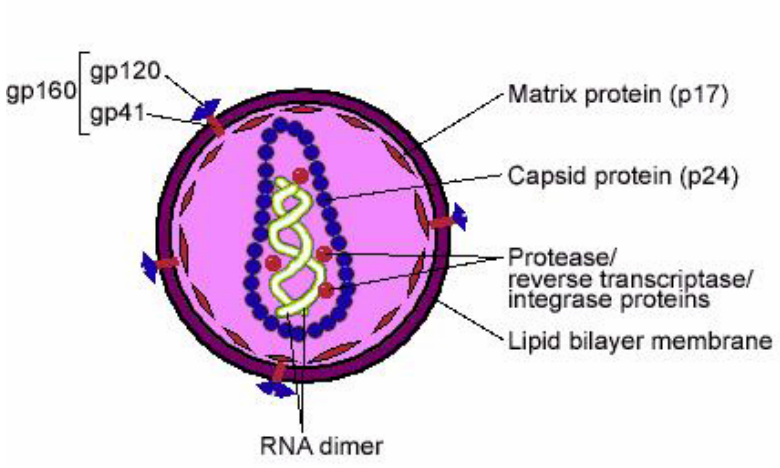


Fig.I-1: Schematic presentation of the HIV-particle. The virus particle is composed of a capsid (p24), which contains the RNA dimer and the viral proteins (protease, reverse transcriptase and integrase). The capsid is surrounded by a lipid bilayer membrane (the viral envelope) with the transmembrane glycoprotein gp160, a complex of gp120 and gp41. The inner side of the envelope is coated with matrix protein (p17); derived from www.mcld.co.uk/hiv/.

The virion (see Fig.I-1) contains two identical copies of the positive sense ss RNA, the viral enzymes (proteins integrase, protease and reverse transcriptase) inside of a conical capsid of capsid protein (p24). The virus envelope, a lipid bilayer membrane, derived from the host cell plasma membrane during virus budding, surrounds the capsid itself. The inner side of the envelope membrane is lined with matrix protein (p17). The envelope contains constituents of the host membrane and the viral transmembrane **g**lyco**p**roteins **gp**120 and gp41, which serve as the viral anti-receptor or attachment protein. These transmembrane proteins are named after their apparent molecular weight of 120 and 41 kDa. Gp41 traverses the envelope whereas gp120 is present on the outer surface and is non covalently attached to gp41. The precursor of gp120/41 (gp160) is synthesised in the endoplasmic reticulum and transported via Golgi bodies to the cell surface (MODROW *et al.* 2003).

By attachment of gp120 to **CD**4 (**c**luster of **d**ifferentiation, cell surface molecule) the virus infects exclusively the CD4-expressing T lymphocytes and macrophages (DALGLEISH *et al.* 1984). After fusion and penetration of HIV, the viral RNA is transcribed into double-stranded DNA by the reverse transcriptase. This proviral DNA travels to the nucleus of the cell, is integrated into the infected cell's chromosomal DNA by the integrase and may persist in a latent state for many years. Activation of the host cell due to unknown environmental influences results in the transcription of viral DNA back into RNA and translation into viral proteins. For this, HIV protease is required to process HIV proteins into their functional forms. Viral RNA and viral proteins are packaged and complete virions bud from the infected T cell (MODROW *et al.* 2003).

Three to four weeks following HIV-exposure, a phase of rapid viral replication starts, with high levels of virus in the plasma (viremia) and development of a "flue like" illness. Four to six weeks after exposure antibodies to HIV core (p24) and envelope (gp160, gp120, gp41) proteins appear. Six to eight weeks after exposure acute symptoms disappear and plasma viremia subsides (MODROW *et al.* 2003), while HIV-1 causes a chronic infection meanwhile with reservoirs of virus in T-cell-, macrophages and monocyte compartments. Additionally the virus diversifies during the infection, with repeated selection of mutants that escape both antibody and T-cell immune responses (McMICHAEL and HANKE 2003). Unless the HIV lifecycle is interrupted by treatment, the virus infection spreads throughout the body and results in the destruction of the body's immune system (BARON *et al.* 1994). Secondary (opportunistic) infections develop and as one characteristic for AIDS, these infections lead to the death of the individual in most cases. With current anti-viral medications, such as reverse transcriptase inhibitors (AZT, lamivudine, zidovudine, abacavir, 3TC, d4T, zalcitabine, ddC,

ddI, tenofovir, didanosine), protease inhibitors (amprenavir, nelfinavir, saquinavir, indinavir, ritonavir, lopinavir) and non-nucleoside reverse transcriptase inhibitors (delavirdinee, efavirenz and nevirapine) used in combination therapy, HIV-infection can be contained but resistance often occur (reviewed in MARCUS *et al.* 2002). Still no efficient vaccine is available on the market nor is anticipated for the near future.

I.1.1.3 Neutralising antibodies to HIV

Theoretically all pathogens (virus, bacteria, fungi, parasites) can be recognised by a B cell via a specific antibody. This requires a huge variety of different antibody specificities, which arises from recombinations of the variable, diversity, and joining genes (V, J and D), by junctional diversity and somatic mutations in affinity maturation of B cells (>10^8). Human **i**mmuno**g**lobulins (**Ig**) are composed of two identical **l**ight **c**hains (**LC**, 23kDa) with a length of around 220 amino acids and two identical **h**eavy **c**hains (**HC**, 50-70kDa) with around 440 amino acids. The light chain, belonging to the kappa or lambda class, consists of one **v**ariable and one **c**onstant region ($\mathbf{V}_L$, $\mathbf{C}_L$). The heavy chain, belonging to the alpha, gamma, mu, delta or epsilon class, contains one variable and three (or four) constant regions (V_H, C_H1-$C_H3/4$). This results in five different antibody classes (IgA, IgG, IgM, IgD and IgE) in combination with the L chains. The Ig classes are further divided into subclasses based on small differences in the amino acid sequences, e.g. human IgG1, 2, 3 and 4. The variable region of V_L and V_H consists of three hypervariable regions (**c**omplementarity **d**etermining **r**egions, **CDR**s) separated by four more conserved regions (frameworks; KABAT *et al.* 1990). CDRs of the heavy and light chain build the antigen binding site (paratope), which is stabilised by intramolecular disulfid bonds in the V and C region. These disulfide bonds lead to a three-dimensional folding into globular regions (domains). Igs and proteins with Ig-like domains [**T** **c**ell **r**eceptor (**TCR**), MHC, CD molecules and receptors] are forming the Ig-superfamily of related proteins all containing antiparallel β-sheet structure between invariant cystein residues. The domains are classified into variable (V) and constant (C)-like sets, with the C-set being divided into the C1-set (Ig-related molecules: Ig, TCR and MHC) and the C2-set (non-Ig related molecules; (SMITH and XUE 1997). Additional intermolecular disulfid bonds connect both heavy chains in the flexible region of the antibody (hinge) between C_H1 and C_H2 resulting in the typical Y-shape of an antibody molecule.

Enzymatic cleavage of antibodies by papain results in specific fragments, such as the **Fc** fragment (**f**ragment **c**rystallisable) consisting of the C-terminal halves of the two HCs linked to each other by the residual hinge region and the **Fab**-fragments (**f**ragment with **a**ntigen **b**inding) consisting of the LC and the N-terminal halve of the HC connected by an interchain

disulfide bond. A **F(ab)$_2$**-fragment, in which the two arms of the antibody molecule remain linked, results from pepsin cleavage.

Pathogens induce **anti**body **gen**eration (**antigen**) against an epitope, the local portion of an antigen recognised by an antibody. Usually viral capsid proteins and bacterial cell wall components have multiple epitopes. In relation to its strength to induce antibody production (immunogenicity), antibodies are produced to each epitope. The strength of the resulting interaction (affinity) increases with repeated exposure to the antigen. The dissociation equilibrium constant (K_D) describes the ratio between the rate constants for binding (on-rate) and dissociation (off-rate) of antibody and antigen. Typical affinities for IgG antibodies are 10^5-10^9 mol/L (JANEWAY *et al.* 2001).

At least three immunoglobulin classes have been demonstrated to exert antiviral activity: IgG, IgM, and IgA (JANEWAY *et al.* 2001). This antiviral activity caused by neutralisation of the virus can be achieved by different mode of actions. First, antibodies bind to the virus extracellular, either neutralising it immediately or blocking its interaction with host cells (JANEWAY *et al.* 2001). Aggregation of antibody-virus-complexes can also be formed, thus preventing the adsorption of virus to cells and decreasing the number of virions. Besides binding directly to the virus, antibodies may enhance phagocytosis due to three types of antibody interactions: direct binding of antibody to the surface of the phagocytic cells, uptake of antigen antibody complexes through Fc receptors, and uptake of antigen-antibody-complement complexes through the C3b receptor activating the complement system (BARON *et al.* 1994). It is suggested that the level of occupancy of binding sites by antibody molecules must exceed an antibody density threshold, after which the entire virion is neutralised (PARREN and BURTON 2001).

Neutralising antibodies are produced by the human body four to six weeks after infection. In general, these antibodies can clear virus infections in the following weeks. In case of HIV, most neutralising antibodies are effectless due to different reasons. Firstly, the main envelope glycoprotein complex gp160 has a trimeric structure composed of six individual subunits, three gp120s and three gp41s. This complex undergoes different conformational changes before the virus-cell membrane fusion occurs (KWONG *et al.* 2002). The CD4 binding site is devoid of asparagine-linked glycosylation acting as a decoy to stimulate largely irrelevant antibodies and additionally, the conserved regions are poorly accessible to antibodies (McMICHAEL and HANKE 2003). Secondly, the viral reverse transcriptase has no proofreading activity and resulting mutations change the HIV sequence and in consequence

the shape of proteins. As soon as the immune system produces neutralising antibodies, the target proteins have changed sufficiently to avoid antibody binding (MODROW *et al.* 2003). Nevertheless, studies of protecting vaccines against SIV indicate that antibody-mediated protection is possible (DESROSIERS *et al.* 1989). Sera from individuals infected with HIV have been analysed extensively for the presence of neutralising antibodies. Five human monoclonal antibodies **4E10** (STIEGLER *et al.* 2001), **1b12** (BURTON *et al.* 1994), **F105** (POSNER *et al.* 1991), **2G12** (TRKOLA *et al.* 1996) and **2F5** (PURTSCHER *et al.* 1994) have been isolated out of the serum of inapparent patients and were found to be capable of neutralising a broad range of primary B-clade HIV isolates, however high titre are required for this protection (MASCOLA *et al.* 1997; LI *et al.* 1998; PARREN *et al.* 1999). Their epitopes include regions on gp41 (2F5 and 4E10), the CD4-binding site of gp120 (1b12) and parts of the carbohydrate moiety of gp120 (2G12; reviewed in CALARESE *et al.* 2003).

Work in this thesis focused on the neutralising antibodies 2F5 and 2G12, which were generated and characterised by a team led by Hermann Katinger (TRKOLA *et al.* 1996). The neutralising antibody 2F5 of the isotype IgG3 was switched to an IgG1 by ligation of the 2F5 V_H to an IgG1 constant region, since this isotype is known for longer half-live in humans (KUNERT *et al.* 2000). Both antibodies are produced by recombinant expression in **C**hinese **H**amster **O**vary (**CHO**) cells as IgG1/κ (BUCHACHER *et al.* 1994; KUNERT *et al.* 2000). These antibodies target neutralising epitopes on the envelope of HIV-1, which are conserved among different subtypes (PURTSCHER *et al.* 1994; TRKOLA *et al.* 1996). Briefly, the 2F5 antibody recognises an apparently linear epitope (ELDKWA) near the C-terminal end of gp41 (MUSTER *et al.* 1993). 2G12 binds an unusually dense cluster of carbohydrate moieties designated as silent face. In general, oligosaccharides shield potential antigenic epitopes and oligosaccharides processed by the host are unlikely to be immunogenic. But in case of 2G12 first binding studies to recombinant gp120 without N-linked carbohydrates in the C2, C3, V4, and C4 regions of gp120 resulted in abolished binding (TRKOLA *et al.* 1996). Mutagenesis studies revealed the glycans at positions 295, 332 and 392 as the most critical for 2G12 binding (SCANLAN *et al.* 2002). Crystal structures of Fab 2G12 and its complexes with the di- resp. oligosaccharide (Manα1-2Man and $Man_9GlcNAc_2$) revealed that two Fabs assemble into an interlocked V_H domain-swapped dimer (CALARESE *et al.* 2003). Thus the 2G12 paratopes interact on gp120 with the N-glycans ($Man_9GlcNAc_2$) at the position 332 and 392. An additional interaction occurs between the unusual $V_H/V_{H'}$-paratope and one N-glycan at position 339. The 2G12 anti-HIV antibody is the only known antibody with a $V_H/V_{H'}$-interface leading to a third antigen-binding site (CALARESE *et al.* 2003).

Both antibodies, 2G12 and 2F5, potently neutralise the majority of HIV-1 primary isolates *in vitro* and protect macaques against intravenous (MASCOLA *et al.* 1999) and mucosal (BABA *et al.* 2000; MASCOLA *et al.* 2000) challenge with chimeric simian/human immunodeficiency virus. A first phase I open label study on seven patients has shown the safety, non-toxicity and non-immunogenicity of both antibodies with maximal plasma concentrations of 374 µg/ml [2F5] and 605 µg/ml [2G12] (ARMBRUSTER *et al.* 2002). The half-lives of 2F5 and 2G12 were 8 days and 16 days, respectively. Modest but significant short-term virologic and immunologic response was detected after MAb infusion of 2F5 and 2G12. Five months after the last antibody infusion, no antibodies were detectable in the circulation (STIEGLER *et al.* 2002). Conclusively, this first clinical trial of passive immunisation showed beneficial antiviral activity in humans chronically infected with HIV-1.

These findings suggest that neutralising antibodies should be further evaluated as an alternative therapeutic approach in HIV-1 mediated disease. However, enormous antibody concentrations will be needed in the coming years to potentially treat 38 millions of HIV-infected persons. Appropriate production and downstream processing platforms have to be developed and implemented for production of antibodies at clinical grade and considerable production costs.

I.1.2 Cell surface receptor as therapeutic target

I.1.2.1 FcγR receptors

Receptors that recognise the Fc portion of antibodies (FcRs) are found on most immune cells. FcRs were identified through the analysis of cytophilic antibodies and their mechanism of interaction with intact cells (BERKEN and BENACERRAF 1966). In immune regulation, FcRs link antibody-mediated immune responses with cellular effector functions. Specific FcRs exist for all classes of Igs, including IgA (FcαR), IgD (FcδR), IgE (FcεR), IgG (FcγR), IgM (FcµR).

The FcR for IgG (FcγR) comprises a multigene family (Fig.I-2), divided into three classes: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) based on differences in receptor structure, cell distribution and affinity for IgG (VAN DE WINKEL and CAPEL 1993; HULETT and HOGARTH 1994).

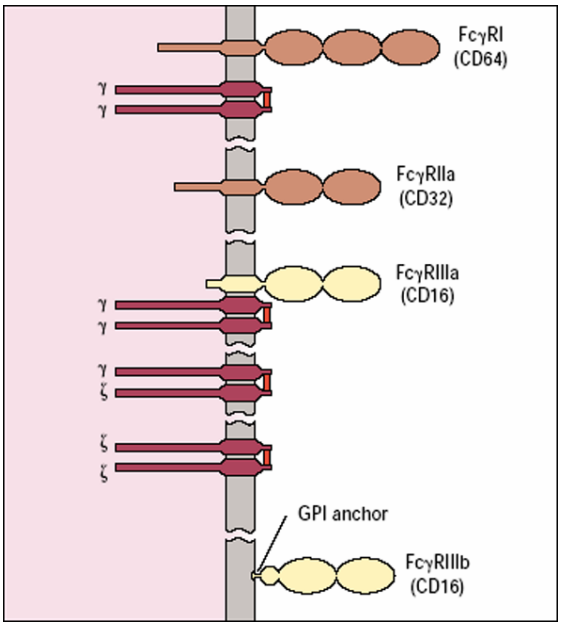


Fig.I-2: The human Fcγ receptor family. FcγRI, FcγRIIa, FcγRIIIa and FcγRIIIb with two or three extracellular immunoglobulin-like domains are presented with their corresponding signalling chains (γ, β, or ζ). (derived from ROITT *et al.* 2001)

The basic structure of FcγR consists of a ligand-binding α-chain with an extracellular part composed of two or three conserved Ig-like extracellular domains of the C2 class (SMITH and XUE 1997), a divergent transmembrane region and a cytoplasmic tail (QIU *et al.* 1990). FcγRI and -III require the association between the ligand-binding α-chain for signal transduction, and signalling γ, β, or ζ-chains (LANIER *et al.* 1989; RA *et al.* 1989; WIRTHMUELLER *et al.* 1992). These accessory chains contain signalling motifs in their cytoplasmic regions, named **I**mmunoreceptor **T**yrosine-based **A**ctivation **M**otifs (**ITAM**), which are involved in the recruitment and activation of specific tyrosine kinases upon receptor crosslinking (van den HERIK-OUDIJK *et al.* 1995). Members of the FcγRII class such as FcγRIIA and FcγRIIB are monomeric receptors containing either activatory (ITAM) or **i**nhibitory (**ITIM**) signalling motifs within their ligand-binding chains (DAERON 1995).

The structural heterogeneity of FcγR is reflected in a wide range of biological activities, including clearance of antigen/antibody immune complexes, regulation of antibody production, enhancement of antigen presentation, ADCC, phagocytosis, degranulation, and activation of inflammatory cells. Many of these responses are unique for selected cell types based on differences in tissue-specific expression of FcγRs (reviewed in RAVETCH and BOLLAND 2001;van der POL and van de WINKEL 1998). Additionally, diversity of individual FcγR receptor-mediated functions are related to genetically determined polymorphisms (DE HAAS *et al.* 1995).

FcγRs are expressed on monocytes, macrophages, neutrophils, basophils, eosinophils, platelets, B and T lymphocytes, NK cells, dendritic cells and Langerhans cells (van de WINKEL and CAPEL 1996).

The crystal structures of the extracellular domains for both FcγRII and FcγRIII have recently been solved, either as free molecule or in complex with IgG, contributing greatly to the understanding of these receptors' mode of action (MAXWELL *et al.* 1999; SONDERMANN *et al.* 1999; SONDERMANN *et al.* 1999; SONDERMANN *et al.* 2000; ZHANG *et al.* 2000). Considerable progress has been made since the high-affinity receptor for human IgG1 (FcγRI) was discovered, but detailed *in vitro* analysis failed so far due to lack of soluble FcγRI.

I.1.2.2 Fc gamma receptor I (FcγRI)

Human FcγRI (CD64) is a 72-kDa glycoprotein which binds with high affinity ($K_a = 10^8$–10^9 M^{-1}) to monomeric as well as aggregated IgG, with selectivity for human IgG1 and IgG3. The interaction with IgG4 and particularly with IgG2 is much weaker (van der POL and van de WINKEL 1998). FcγRI is a multi-subunit receptor consisting of the heavily glycosylated α-chain and two γ-chain subunits of the FcεRI (ALLEN and SEED 1989; KUSTER *et al.* 1990). The unique property of FcγRI is its high affinity for monomeric IgG, conferred by a third extracellular Ig domain not found in FcγRII or III (which only bind complexed IgGs; ALLEN and SEED 1989). Domain-swapping experiments involving FcγRI revealed a major site for IgG interactions in the second domain, whereas the third domain increases the binding specificity and affinity (van de WINKEL and CAPEL 1996). Mutagenesis studies on IgG molecules by Duncon *et al.* (1988) showed that Leu_{235} is the major determinant in receptor binding; additional interaction between the receptor and the region linking the CH2 domain to the hinge was only postulated. Later studies showed that the lower hinge region (residues 234–237) was one interaction site (LUND *et al.* 1991) but a second region contributing to receptor binding was identified in the hinge-proximal bend (Pro_{331}; CANFIELD and MORRISON 1991).

Three genes for human FcγRI have been identified (FcγRIa, FcγRIb and FcγR1c), all located on chromosome 1 (1p13 and 1q21; van de WINKEL and CAPEL 1993) encoding four transcripts: FcγRIa, FcγRIb1, FcγRIb2 and FcγRIc (van VUGT *et al.* 1999). The hFcγRIa transcript encodes the high-affinity IgG receptor. Transcripts FcγRIb1 and FcγRIc contain stop codons in the third extracellular region, and are predicted to generate secreted receptor forms (ERNST *et al.* 1992), although these have yet to be detected. The alternatively spliced hFcγRIb2 transcript encodes a product that is retained in the endoplasmic reticulum (van VUGT *et al.* 1999).

FcγRI is expressed constitutively on monocytic cells, macrophages and dendritic cells, whereas its expression can be induced by various elicitors on neutrophils, eosinophils, glomerular mesangial cells and mast cells (RAVETCH and KINET 1991; van de WINKEL and CAPEL 1993; HULETT and HOGARTH 1994; UCIECHOWSKI *et al.* 1998; OKAYAMA *et al.* 2000). Activation of FcγRI on macrophages induces the clearance of antigen/antibody complexes by endocytosis or phagocytosis, leading to enhanced antigen presentation on MHC I and II molecules (HEIJNEN *et al.* 1996; LIU *et al.* 1996; GUYRE *et al.* 2001). This triggers ADCC by T_c cells and superoxide generation, or the activation of T_h cells (van de WINKEL and CAPEL 1996; van VUGT *et al.* 1999; GUYRE *et al.* 2001). Inflammatory reactions can be induced by antibody binding to FcγRI on mast cells, leading to the secretion of amines, lipid mediator and cytokines (OKAYAMA *et al.* 2001; WOOLHISER *et al.* 2001). Surprisingly, despite the importance of FcγRI, one study identified four apparently healthy individuals within one family lacking phagocytic expression of FcγRI (van de WINKEL *et al.* 1995). Many other studies have linked variations in FcγRI activation to different disease patterns, e.g. overactivation of macrophages in acute and chronic inflammation (KIEKENS *et al.* 2000) or the inhibition of phagocytosis of **m**onocyte-**d**erived **m**acrophages (**MDM**s) in HIV-1 infection *in vitro* (KEDZIERSKA *et al.* 2002). The therapeutic value of FcγRI as a target in tumor therapy, allergic reactions and leukaemia has been extensively reviewed (CURNOW 1997; RAVETCH and BOLLAND 2001).

Since the characterization of the genes for human FcγRI in 1992 (ERNST *et al.* 1992), all attempts to produce a functional, soluble form of the receptor have so far failed (JEFFERIS and LUND 2002). Sondermann *et al.* (SONDERMANN and OOSTHUIZEN 2002), who crystallized soluble FcγRIIb and soluble FcγRIII in complexes with human IgG Fc, even suggested that the expression of soluble FcγRI is impossible. However, with optimised expression vector-host-systems new approaches might be developed to express the FcγRI receptor recombinantly. Expression of recombinant soluble FcγRI might allow in detail characterisation of the exact binding site for human IgG and potential modulation of this region to enhance IgG binding. Additionally, availability of recombinant soluble FcγRI will facilitate the investigation of FcγRI's therapeutic application.

I.2 Expression of human therapeutic proteins in heterologous expression systems

During the last years more and more biopharmaceuticals were approved by the regulatory US **F**ood and **D**rug **A**dministration (**FDA**) for clinical trials and commercial production resulting in a present market of about US$30 billion (COCO-MARTIN 2004). Estimated 4,400 biotech companies are globally involved, having brought more than 100 different biopharmaceutical products on the (U.S.) market (FROST&SULLIVAN(A) 2003). Actual lists are published by the Biotechnology Information Institute (http://www.bioinfo.com/).

Depending on their required characteristics, biopharmaceuticals are produced in different host systems. Although transgenic plants and animals are predicted to become commercially competitive for manufacturing therapeutic proteins in the future, every biopharmaceutical on the market today is produced in either a mammalian or microbial cell line (THIEL 2004). Currently the most important expression systems used in industry are the bacteria *E.coli*, yeast *Saccharomyces cerevisiae* and *Pichia pastoris* and mammalian CHO cells (DE NORONHA PISSARRA 2004). Production of proteins in plant systems will be an additional emerging expression system in the next years, but complete regulatory guidelines such as for the production in mammalian and bacterial cells, are not fully established (FISCHER *et al.* 2004; STOGER *et al.* in progress).

All expression systems offer advantages and disadvantages, thus the proper choice of a host organism for the production of a biopharmaceutical has to be made on a case-by-case basis and depends upon the protein under consideration.

I.2.1 Bacterial expression

Expression of recombinant proteins in *E.coli* has been investigated extensively over the last 20 years and is one of the most frequently used systems. Clear guidelines by the competent authorities (e.g. **E**uropean **A**gency for the **E**valuation of **M**edicinal Products (**EMEA**) and the FDA) for the production of recombinant proteins in *E.coli* have to be complied (DE NORONHA PISSARRA 2004). Some of today's blockbusters such as interferon (IFN-)alpha (Infergen by Intermune), recombinant insulin (Humalog by Lilly), recombinant human growth factor (Protropin by Genentech) and **g**ranulocyte **m**acrophage **c**olony-**s**timulating **f**actor (**GM-CSF**, Neupogen/Neulasta by Amgen) are expressed in *E.coli* (THIEL 2004).

E.coli grows on inexpensive carbon sources, offers rapid biomass accumulation and can be easily cultivated and scaled-up in high cell-density fermentations yielding protein

accumulation levels in the gram per litre scale (ARISTIDOU *et al.* 1999; BANEYX and MUJACIC 2004). An additional benefit is the well-characterised and established rapid cloning and expression procedure. Expression vectors containing a multiple cloning site downstream of an inducible promoter are developed to reach expression levels in excess of 50% of total cell protein (MIAO *et al.* 1995). Small polypeptide tags, including the hexa-histidine (his-tag), the FLAG or the anti-c-myc tag are added either N or C terminally to the protein for detection and purification (NOVAGEN 2002).

Expression of recombinant proteins can be performed in the cytoplasm or the periplasm of the bacterial cell (BANEYX 1999). The cytoplasm is known to yield much higher protein concentrations but it is not uncommon that overexpressed protein associates with each other and forms insoluble and inactive aggregates (inclusion bodies; SANCHEZ *et al.* 1999). Moreover, time and labour intensive refolding procedures are undertaken resulting in low amounts of functional protein.

Employing leader sequences (e.g. *pel*B or *omp*A leader), recombinant proteins are directed into the bacterial periplasm, where the oxidising milieu favours the formation of disulfide bonds by the disulfid-oxidoreductase (BRADWELL *et al.* 1991). Another advantage is the presence of chaperones leading to stabilisation of the recombinant protein (reviewed in WULFING and PLUCKTHUN 1994). Moreover, the periplasm contains less bacterial proteins than the cytoplasm and therefore less proteases, which facilitates the protein purification with less proteolytic degradation (TALMADGE and GILBERT 1982). The efficiency of periplasmic expression can be additionally enhanced by using compatible solutes and induction of protein expression through osmotic stress (BARTH *et al.* 2000).

Drawbacks and limitations for expression in *E.coli* include poor post-translational assembly (no glycosylation) and the high grade of inactivity of more complex proteins. Overexpressed proteins can also fail to fold correctly and undergo proteolytic degradation, and finally bacterial endotoxins can contaminate the purified preparations (PLUCKTHUN 1991). Consequently, testing of products derived from *E.coli* for endotoxin removal is mandatory.

The work described in this thesis focuses on the use of a pET-derived vector (Novagen) for expression in *E.coli* BL21(DE3)star cells. A *pel*B leader directs the expressed protein into the bacterial periplasm. An N-terminal penta-his tag facilitates the purification and detection of the expressed recombinant proteins.

The extracellular part of the human FcγRIIb was successfully expressed in *E.coli* cells (SONDERMANN and JACOB 1999). Activity of the unglycosylated form was proven by

binding of immobilized antibody as well as Fc fragment in solution. Thus, primary expression experiments of FcγRI would have to be performed in *E.coli*.

Generally, *E.coli* is the system of choice for the production of antibody fragments, such as scFvs (artificial construct comprising the V_L and V_H of an antibody linked by a stretch of peptides), Fabs or $F(ab)_2$ (reviewed in PLUCKTHUN *et al.* 1996). Only one publication described the successful expression of an aglycosylated full-size antibody (SIMMONS *et al.* 2002). As glycosylation contributes to the effector function of neutralising antibodies, the expression of the anti-HIV antibodies 2F5 and 2G12 in *E.coli* was not considered.

I.2.2 *Mammalian expression*

Since 1986, when Genentech announced the market approval of human tissue plasminogen activator (tPA), the first therapeutic protein from recombinant mammalian cells, this market has increased potentially to several hundred clinical candidates currently in company pipelines. Commercially manufactured products in CHO cells include Genentech's Herceptin (trastuzumab), Genzymes's Cerezyme (recombinant imiglucerase), Genentech's Avastin (bevacizumab) and Biogen Idec's Rituxan (rituximab; THIEL 2004). Sixty to seventy percent of all recombinant protein pharmaceuticals are produced in mammalian cells, as mammalian expression reveals the most authentic glycosylation leading to a high percentage of active complex proteins (WURM 2004). Most proteins are produced in CHO cells, but other cell lines were also approved by the FDA, like as mouse myeloma (NS0), **B**aby **H**amster **K**idney (**BHK**), **H**uman **E**mbryonic **K**idney (**HEK**-293) or human retinal cells (PER-C6; JONES *et al.* 2003). These cell lines can be or already are adapted to serum-free suspension media allowing large-scale production in bioreactor systems in well-defined media and at lower costs, since the expensive infrastructure as well as the low expression yields were one of the former drawbacks of mammalian expression. Additionally the risk of containing human pathogens can be reduced by using serum-free media. Extensive studies have yielded mammalian cell productivity in bioreactors to the gram per litre range (corresponding to a specific productivity of 20 - 90 pg/cell/day), which represents a more than 100-fold yield improvement in the last 25 years (WURM 2004). Employing leader sequences (e.g. Ig *kappa* leader), allows expression of the recombinant protein into the culture supernatant facilitating the purification procedure. Correct folding and functionality of the recombinantly produced proteins is achieved by chaperones and disulfidisomerases located in the ER (reviewed in FERRARI and SOLING 1999).

Drawbacks of mammalian expression include the long development time to reach stable expression clones. Initial cloning procedures are usually carried out in bacterial expression

vector, but the development of stably expressing eucaryotic clones is very time and labour intensive. Additionally, contamination with pathogens must be extensively removed in cleaning validation for clinical use of the recombinant proteins (MARTINEZ 2004), but this must be performed in bacterial expression as well.

For the work presented in this thesis the pSecTag-based system (Invitrogen) was used. An Ig-*kappa* leader directs the secretion of the recombinant proteins into the culture supernatant of transfected HEK 293T cells (STOCKER *et al.* 2003). The cell line HEK 293T expresses the large T-antigen of the SV40-virus enabling a high copy number of the transfected episomal plasmid in the transiently transfected cells. Different studies reported successful expression of different glycoproteins (MANNA *et al.* 2002; DU *et al.* 2003; NING *et al.* 2003; PATEL *et al.* 2004) as well as recombinant antibody fragments (GRUNBERG *et al.* 2003) in HEK 293 cells. For production of full-size antibodies in general hybridoma, NSO and CHO cells are used (BARNES *et al.* 2003) as in case of the human anti-HIV antibodies 2G12 and 2F5 (BUCHACHER *et al.* 1994; KUNERT *et al.* 2000).

Soluble human FcγRII was successfully produced in insect cells (SONDERMANN *et al.* 1999), whereas human FcγRIII is naturally expressed as soluble form in humans. Expression of soluble FcγRI will be tested in mammalian HEK 293T cells for productivity and functionality in comparison to the bacterial system.

I.2.3 Plant expression

Initially the idea to use plants and plant cells for the production of human proteins was "greeted with great scepticism" (SCHILLBERG *et al.* 2003). However, plant expression systems have the potential to provide virtually unlimited quantities of proteins for use as tools in human health care offering a simple and low-cost alternative to bacterial and mammalian systems (STOGER *et al.* 2002). The costs of producing recombinant proteins in plants might be 90-fold lower than the production in other systems (SCHILLBERG *et al.* 2003). Additionally, safe biopharmaceuticals can be produced in plants, since plants do not harvest human pathogens compared to mammalian cells (GIDDINGS *et al.* 2000). Commercial production of plant secondary products, such as codeine and morphine from *Papaver somniferum* and digoxin from *Digitalis sp.* has been used for centuries in human medicine (PAIVA 1999). Bioreactivity screens identified new compounds, especially anticancer compounds like as vincristine and vinblastine from *Catharantus roseus* and taxol from *Taxus sp*. Ginseng roots for example are used as whole whereas anthocyanins and flavours have to be extracted. Commercial production of shikonin is reported in the 750-L scale (CURTIN

1983), but plant cell suspension cells (TBY-2) were successfully grown up to 20,000-L cultures (NAGATA *et al.* 1992).

In the last 20 years, plants are gaining more and more acceptance as alternative production system for production of recombinant proteins in terms of cost, scalability and safety. Two of the world's most expensive drugs, glucocerebrosidase by CropTech Corp. (Blacksburg, USA) and GM-CSF by GeneMedix PLC (Newmarket, UK) have been produced in transgenic plants (GIDDINGS *et al.* 2000) and different companies will enter the market with their products in the coming years. Biolex (Pittsboro, USA) e.g. used *Lemna minor* (duckweed) as a system for the production of interferon-alpha currently in clinical phase I, Large Scale Biology Corp. (Vacaville, USA) completed clinical phase I studies with a Non-Hodgkin lymphoma vaccine produced in tobacco. Meristem Therapeutics (Clermont-Ferrand, France) used corn and tobacco for the expression of gastric lipase (clinical phase IIa), Plant Biotechnology (Hayward, USA) produced CaroRx against tooth decay (clinical phase II) and RhinoRx for common cold (preclinic) in tobacco, ProdiGene (College Station, USA) completed clinical phase I with an antibody for Traveler's diarrhea from corn and SemBioSys (Calgary, Canada) entered the preclinical phase with human insulin and apolipoprotein from transgenic oilseed (THIEL 2004).

A variety of different plants can be used as expression hosts for biopharmaceuticals. Hiatt *et al.* succeeded in 1989 with the first antibody and antibody fragment expression in transgenic tobacco plants (HIATT *et al.* 1989). Since then, many approaches have shown the expression of full-size antibodies (DURING *et al.* 1990; MA *et al.* 1994; VERCH *et al.* 1998), Fab-fragments (DE NEVE *et al.* 1993; DE WILDE *et al.* 1998), scFv-fragments (OWEN *et al.* 1992; FIEDLER and CONRAD 1995; FECKER *et al.* 1996), diabodies (VAQUERO *et al.* 2002) and bi-specific scFv fragments (FISCHER(C) *et al.* 1999) as well as complex proteins (CRAMER *et al.* 1996; TACKABERRY *et al.* 1999) in different cell compartiments of tobacco (SCHILLBERG *et al.* 1999), Arabidopsis (DE WILDE *et al.* 1998), rice, wheat (STOGER *et al.* 2000; STOGER *et al.* 2004) and alfalfa (KHOUDI *et al.* 1999).

The choice of the plant production system depends on factors such as intrinsic efficiency, suitability for scale-up, storage and downstream processing (STOGER *et al.* in progress). Tobacco for example is long established as a model system and offers advantages such as fast generation of large amounts of biomass, simple gene transfer and regenerations procedures as well as optimised vector systems. Large-scale purification of e.g. recombinant antibodies out of 200 kg batches of Tobacco leaves was already performed (VALDES *et al.* 2003).

As an alternative to whole plant expression, plant cells or organ cultures can be used. Compared to the production of recombinant proteins in transgenic plants, the generation and initial up-scaling of transgenic suspension cell lines is considerably faster and requires less resources (FISCHER(B) *et al.* 1999). Production of recombinant antibodies in plant cell suspensions allows sterile containment in compliance with GMP-conditions (FISCHER(B) *et al.* 1999; SHARP and DORAN 2001; SCHILLBERG *et al.* 2003; HELLWIG *et al.* 2004). Additionally, the cost of protein production in plant cell culture is still lower than that of mammalian cells (FISCHER(E) *et al.* 1999; STOGER *et al.* in progress). However, only two secondary metabolites, shikonin by Mitsui Petrochemical Company (Japan) and paclitaxel (Taxol) by Bristol-Myers Squibb (New York, USA) have been produced on a commercial scale in plant suspension cells (HELLWIG *et al.* 2004).

Different plant species have been used so far for the generation and propagation of cell-suspension cultures, such as *Arabidopsis thaliana* (DESIKAN *et al.* 1996) and *Cataranthus roseus* (CAREW and BAINBRIDGE 1976), but also important crop plants like rice (CHEN *et al.* 1994), soybean (UPMEIER *et al.* 1988), alfalfa (DANIELL and EDWARDS 1995), tomato (KWON *et al.* 2003) and tobacco (NAGATA *et al.* 1992). Over 20 different recombinant proteins have been produced in plant cell cultures, such as human lysozyme, anti-trypsin, human erythropoietin, bryodin 1, hGM-CSF, cytokines (IL-2, IL-4, IL-12) and antibodies/antibody fragments (HELLWIG *et al.* 2004). Until now only three reports have described the production of assembled full-size antibodies in plant suspension cells. An anti-TMV mouse IgG2b/κ was produced in suspension cells established from transgenic *Nicotiana tabacum cv.* Petit Havana SR-1 plants (FISCHER(B) *et al.* 1999) reaching an accumulation level of 15 mg/kg fresh weight. Guy's 13, an IgG1/κ recognising a 185 kDa cell-surface protein of *Streptococcus mutans*, was also produced with levels of 7.5 mg/L in a transgenic tobacco Nt-1 derived cell line (SHARP and DORAN 2001). The recently published work by Yano *et al.* (YANO *et al.* 2004) is the first report in which a human IgG1/κ against a HBV antigen was expressed in *Nicotiana tabacum* L. cv. **B**right **Y**ellow (**BY**-2) suspension cells and yields of 16.2 mg/L were achieved. In all studies the constitutive enhanced 35S promoter was used and the antibodies were targeted to the apoplast.

The tobacco suspension cell line BY-2 offers the advantage of direct transformation (co-cultivation with *Agrobacterium tumefaciens*) allowing detection of the foreign protein in transient expression after 2 – 3 days (SCHILLBERG *et al.* 2003). Recombinant proteins expressed in BY-2 cells can be secreted into the culture supernatant or retained within the cells. Chaperones responsible for correct folding and assembly are located in the ER of

plasma cells (MELNICK *et al.* 1994) and homologous proteins were also described for plants (FONTES *et al.* 1991; WALTHER-LARSEN *et al.* 1993). Since disulfide bridges are formed by the disulfidisomerases in the ER as well, plant produced recombinant protein should be targeted to the ER (DENECKE *et al.* 1992). Using a KDEL-retention signal can enhance the expression rate in the ER with a factor of 10 – 100 compared to secreted variants (WANDELT *et al.* 1992; SCHOUTEN *et al.* 1996; CONRAD and FIEDLER 1998).

Due to different glycan-modifying enzymes, plant produced proteins contain a different glycosylation pattern compared with mammalian cells, such as a high-mannose type consisting of N-Acetylglucosamine (GlcNAc) and mannose residues (CABANES-MACHETEAU *et al.* 1999). Carbohydrate moieties unique to plants may induce immune responses when regularly administered into humans. It is known for antibodies, that N-glycans at the Fc part of the IgG molecule contribute to effector functions (LUND *et al.* 1990; WRIGHT and MORRISON 1998; SHINKAWA *et al.* 2003). However, one plant produced anti-Rabies virus antibody with high-mannose type glycan exhibited the same neutralising activity compared with the mammalian derived antibody (KO *et al.* 2003).

For the work described in this thesis the pPAM-based system for expression in tobacco leaves (*Nicotiana tabacum* L. cv. Petit Havana) and plant suspension cells [*Nicotiana tabacum* L. cv. Bright Yellow (BY-2)] was used containing an IgG–*kappa* leader and a SEKDEL-tag for retention in the ER. Delivery of the vector-construct into the plant cells is mediated by *Agrobacteria tumefaciens*, which efficiently transfers DNA into the plant cell, where the gene of interest is transiently expressed (AN 1995; KAPILA *et al.* 1996).

The human anti-HIV antibodies should be expressed in BY-2 suspension cells to generate an attractive alternative system for the expression of these therapeutic antibodies to the CHO expression system at reasonable costs and yield. Human FcγRI should be expressed transiently in tobacco leaves to evaluate the potential of plant expression for functional production of this human molecule in comparison to the bacterial and mammalian expression systems.

I.3 Goal of this thesis

Two different biopharmaceuticals, one therapeutically relevant CD-antigen and one monoclonal antibody were selected for expression and functional characterisation in heterologous expression systems in this thesis.

The number of people infected by HIV is growing steadily and effective vaccines might not be available in the near future. With the current combination therapy using inhibitors of the viral reverse transcriptase and protease, the infection can be controlled but resistance develops very fast. Extensive studies resulted in the purification of five promising antibodies out of the sera of inapparent patients, which effectively neutralise a broad spectra of HIV primary isolates. Two of these antibodies, 2F5 and 2G12, produced in CHO cells (^{CHO}2F5/^{CHO}2G12) have been used successfully in clinical phase I studies. In order to satisfy the requirements of antibody in HIV therapy, alternative expression systems should be tested for their ability to produce clinical proteins in high amounts at reasonable costs. Plants represent an inexpensive, efficient and safe system for the production of therapeutic antibodies. Especially, plant suspension cells offer the advantages of bioreactor production in defined media under GMP compliance. Thus, protocols for the expression of anti-HIV antibodies expressed in plant suspension cells, more precisely in tobacco BY-2 suspension culture (BY-2; $^{BY\text{-}2}$2F5/$^{BY\text{-}2}$2G12) have to be developed both on a small-scale (1-L) and large-scale (100-L) feasibility. Available equipment for continuous downstream processing on industrial scale should be analysed and downstream processes to be developed to recover pure protein for biochemical and biophysical characterisation. Plant derived antibody preparations should be analysed concerning purity, integrity and stability of the anti-HIV antibody as well as antigen-binding property.

The second aim of this thesis was to investigate FcγRI, the high-affinity receptor for monomeric IgG, a potential therapeutic target in tumor therapy, allergic reactions and leukaemia. To achieve this the extracellular domain of the receptor headed to be amplified from peripheral blood, cloned and the most appropriate expression system needs to be determined. As a procaryotic expression system *E.coli* was chosen and for eucaryotic expression both HEK 293T cells and *N.tabacum* should be used. For the system yielding highest expression a protocol headed to be established for the expression and purification. Integrity and purity, as well as binding properties of the purified protein preparations will be tested.

A schematic overview of the structure of this thesis is presented in Fig.I-4.

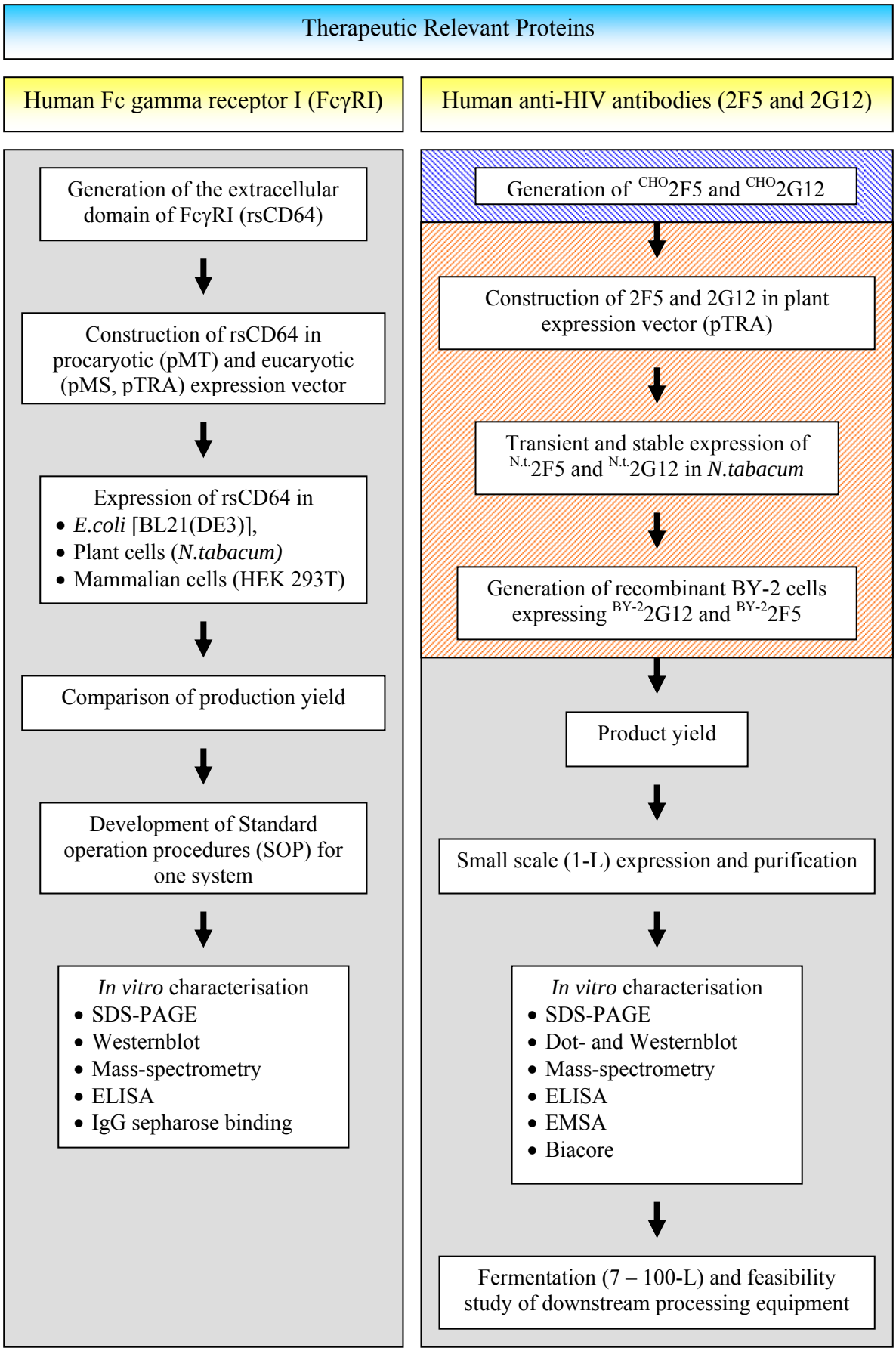


Fig.I-3: **Schematic outline of the subjects described in this thesis.** = CHO2F5 and CHO2G12 antibodies were provided by Polymun Scientific GmbH (Wien), = work performed by M. Bomble (BOMBLE 2004).

II. Material and methods

II.1 Material

II.1.1 Chemicals and consumables

Unless stated otherwise consumables and chemicals were purchased from Abcam (Cambridge, UK), Amersham Pharmacia (Freiburg), AP Biotech (Freiburg), BIACORE (Freiburg), Becton Dickinson Bioscience (Franklin Lakes, USA), Biochrom (Berlin), BioRad (München), Biozym (Oldendorf), CARR Separations Inc. (Franklin, USA), Ciphergen (Goettingen), Clontech (Heidelberg) Corning Inc. (Schiphol-Rijk, Niederlande), Cytogen (Obermörlen), Dynal (Hamburg), Eppendorf (Hamburg), ICN (Eschwege), Greiner (Solingen), Hewlett-Packard (München), Invitrogen (Eggenstein), Kodak (Stuttgart), KMF Laborchemie (St. Augustin), Millipore (Eschborn), MWG-Biotech (Ebersberg), NALGENE® (Neerijse, Belgium), News Brunswick Scientific (Nürtingen), New England Biolabs (NEB; Schwalbach), Nunc (Biebrich), Pall Filtron (Northborough, USA), Perkin-Elmer (Applied Biosystems, Foster City, USA), Pierce (Rockford), Promega (Mannheim), Qiagen (Hilden), Roche Molecular Biochemicals (Mannheim), Roth (Karlsruhe), Sarstedt (Nümbrecht), Schott-Glaswerke GmbH (Neufahrn/San Diego), Serva (Heidelberg), Sigma (Deisenhofen), Starlab (Ahrensburg), Whatman (Maidstone, England), VWR (Darmstadt), Werner GmbH (Leverkusen) and Zeiss (Oberkochen) were of the highest grade available. Culture media and additives were purchased from Duchefa (Haarlem, Niederlande), Invitrogen, Sigma and Roth.

II.1.2 Media, stock solutions and buffers

Standard media and stock solutions were prepared according to standard procedures (SAMBROOK and FRITSCH 1996; AUSUBEL and BRENT 1998; COLIGAN *et al.* 1998) using deionized water. Solutions were sterilised by autoclaving (25 min/ 121 °C/ 2 bar). Heat-sensitive components, such as antibiotics, were prepared as stock solutions, filter-sterilised (0.2 µm) and added to the medium/buffer after cooling to 50 °C.

II.1.2.1 General solutions

10x PBS (pH 7.4)

NaCl	1.37 M
KCl	27 mM
Na_2HPO_4 x $12H_2O$	81 mM
KH_2PO_4	15 mM

PBST

1x PBS (pH 7.4)	
Tween 20	0.05 % (w/v)

10x TBE Electrophoresis Buffer (pH 8.3)

Tris-base	900 mM
Boric acid	900 mM
EDTA	25 mM

50x TAE Electrophoresis Buffer (pH 7.5)

Tris-base	2 M
Glacial acetic acid	5.7 % (v/v)
EDTA	50 mM

10x DNA Loading Buffer (blue)

Bromphenolblue	0.1 % (w/v)
Xylencyanol	0.1 % (w/v)
Glycerol	50 % (v/v)
in TAE or TBE buffer	

5x OrangeG Loading Buffer

OrangeG	0.1 % (w/v)
Glycerol	30 % (v/v)
in TAE or TBE buffer	

5x SDS PAA running buffer

Tris-HCl (pH 8.3)	125 mM
Glycine	960 mM
SDS	0.5 % (w/v)

Blotting buffer

Tris-HCl (pH 8.3)	25 mM
Glycine	192 mM
Methanol	20 % (v/v)

5x Reducing Protein Loading Buffer

Tris-HCl (pH 6.8)	62.5 mM
Glycerol	30 % (v/v)
SDS	4 % (w/v)
Bromphenolblue	0.05 % (w/v)
ß-Mercaptoethanol	10 % (v/v)

5x Non-Reducing Protein Loading Buffer

Tris-HCl (pH 6.8)	62.5 mM
Glycerol	30 % (v/v)
SDS	4 % (w/v)
Bromphenolblue	0.05 % (w/v)

Coomassie Staining Solution

Coomassie bb. G-250	0.25 % (w/v)
Methanol	50 % (v/v)
Glacial acetic acid	9 % (v/v)

Coomassie Destaining Solution

Methanol	10 % (v/v)
Glacial acetic acid	10 % (v/v)

NuPAGE® SDS Running buffer

20x NuPAGE® MES	50 ml
dH_2O	950 ml
(200 ml + 500 µl Antioxidant)	

NuPAGE® Sample Preparation

Sample	x µl
NuPAGE LDS sample buffer	2.5 µl
NuPAGE reducing agent	1 µl
dH_2O	ad 10 µl

AP Buffer (pH 9.6)

Tris-HCl (pH 9.6)	100 mM
NaCl	100 mM
$MgCl_2$	5 mM

ELISA Coating Buffer (pH 9.6)

Na_2CO_3	15 mM
$NaHCO_3$	35 mM

II.1.2.2 Media for bacterial cultivation

LB (pH 7.0)		**2xTY (pH 7.4)**	
NaCl	1.0 % (w/v)	NaCl	0.5 % (w/v)
Peptone	1.0 % (w/v)	Peptone	1.6 % (w/v)
Yeast extract	0.5 % (w/v)	Yeast extract	1.0 % (w/v)
TB (pH 7.4)		**Media Supplements:**	
Peptone	1.3 % (w/v)	Ampicillin	100 µg/ml
Yeast extract	2.7 % (w/v)	Kanamycin	25 µg/ml
Glycerol	0.8 % (v/v)	Agar	1.6 % (w/v)
TfB-I (pH 5.8)		**TfB-II (pH 5.8)**	
Potassium Acetate	30 mM	MOPS	30 mM
$MnCl_2$	50 mM	$CaCl_2$	75 mM
$CaCL_2$	10 mM	RbCl	10 mM
Glycerol	15 % (v/v)	Glycerol	15 % (v/v)

II.1.2.3 Media for the cultivation of mammalian cells

Complex RPMI-Media [RPMI (C)]

RPMI-Media 1640 (Cytogen), serumfree media

Fetal Calf Serum (FCS)	10 % (v/v)		
Penicillin	50 µg/ml		
Streptomycin	50 µg/ml	**Media Supplements:**	
L-Glutamine	2 mM	Zeocin® (Invitrogen)	100 µg/ml

II.1.2.4 Media for the cultivation of *A.tumefaciens* and plant cells

MMA Buffer		**BY-2 Media (pH 5.8)**	
MS-salts	0.43 % (w/v)	MS-salts	0.43 % (w/v)
(Murashige & Skoog, basic salt mixture)		Myo-Inosite (SERVA)	0.1 % (w/v)
MES (pH 5.6)	10 mM	Sucrose	3 % (w/v)
Sucrose	2 % (w/v)	Thiamin-HCl	0.2 mg/l
(Acetosyringone	200 µM	2,4-dichloroph. acid	0.2 mg/l
was added directly before use)		KH_2PO_4 (pH 5.6)	0.2 % (w/v)

Media Supplements		**Extraction Buffer (pH 7.4)**	
Agar	0.8 % (w/v)	EDTA	5 mM
Kanamycin	100 mg/l	β-mercaptoethanol	5 mM
Claforan (cefatoxim)	200 mg/l		

YEB Media		**Induction Media**	
Nutrient Broth	0.5 % (w/v)	YEB media (pH 5.6)	
Yeast extract	0.1 % (w/v)	MES	10 mM
Peptone	0.5 % (w/v)	(Acetosyringone	20 µM
Sucrose	0.5 % (w/v)	added directly before use)	

Media Supplements		**Glycerol Stock Media (GSM)**	
$MgSO_4$	2 mM	Glycerol	50 % (v/v)
Rifampicin (Rif)	50 mg/l	$MgSO_4$	100 mM
Carbenicillin (Carb)	50 mg/l	Tris (pH 7.4)	25 mM
Kanamycin (Km)	25 mg/l		

II.1.3 Enzymes and reaction kits

Unless otherwise stated, enzymes were purchased from NEB, Roche Molecular Biochemicals, MWG-Biotech and Invitrogen. *Taq* DNA-polymerase from Roche was used for PCR amplification. The following kits were used:

QIAquick Gelextraction Kit	Qiagen
QiaExII Agarose Gel Extraction Kit	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
QIAquick „PCR-Purification“ Kit	Qiagen
Pre Analytix total-RNA Isolation from blood	Qiagen
One-Step-RT-PCR	Qiagen
PreAnalytiX Kit	Qiagen
PAXgene™ Blood RNA System	Becton Dickinson
BCA Protein Assay	Pierce
Amine Coupling Kit	Biacore
Sucrose, D-glucose, D-fructose Enzymekit	Roche

II.1.4 Antibodies and antigens

II.1.4.1 Antibodies

The following monoclonal primary and enzyme-labelled secondary antibodies were used:

Antibody	Epitope	Characteristics
mouse-anti-c-myc, [mAk9E10] (EVAN *et al.* 1985)	C-terminal c-myc-tag	monoclonal
mouse-anti-c-myc [α-cmyc^{HRPO}] (Abcam, England)	C-terminal c-myc-tag	monoclonal, conjugated to HRPO („horse radish peroxidase“)
mouse-anti-penta-his [α-pentaHis Ak] (Qiagen)	N- resp. C- terminal his-tag	monoclonal
mouse-anti-CD64 [M22] (Maine Biotechnology, USA)	human CD64	monoclonal
humanized-anti-CD64 [H22] (T.Thepen, BioVII, RWTH-Aachen)	human CD64	monoclonal
mouse-anti-FSH [scFv4813] (S.Hellwig, BioVII, RWTH-Aachen)	Follicle stimulating hormone (FSH)	monoclonal, single chain fragment (scFv)
chimeric mouse/human-anti-β-hCG [cPIPP] (M. Sack, BioVII, RWTH-Aachen)	β-hCG	monoclonal, chimeric mouse/human-IgG1
^{CHO}2F5IgG1-anti-HIV (Polymun Scientific GmbH, Wien)	gp41 of HIV-virus	monoclonal
^{CHO}2G12IgG1–anti-HIV (Polymun Scientific GmbH, Wien)	gp120 of HIV-virus	monoclonal
mouse-anti-CA19.9IgG1 (kindly provided by H.Abken, Klinik I für Innere Medizin, Cologne)	CA19.9	monoclonal
goat-anti-human [GαH^{AP} (α-IgGγ)] (Sigma)	γ-chain of human IgG	polyclonal, conjugated to alkaline phosphatase
goat-anti-human [GαH^{AP} (α-IgG *kappa*)] (Sigma)	κ-chain of human IgG	polyclonal, conjugated to alkaline phosphatase
goat-anti-mouse [GαM^{AP} (α-IgG Fc)] (Sigma)	Fc of mouse IgG	polyclonal, conjugated to alkaline phosphatase
goat-anti-mouse [GαM^{HRPO} (α-IgG Fc)] (Sigma)	Fc of mouse IgG	polyclonal, conjugated to HRPO („horse radish peroxidase“)

II.1.4.2 Antigens

Antigen hCG was kindly provided by M. Sack (BioVII, RWTH-Aachen). GαH-Fab serum was purchased from Sigma. Antigens for the antibody 2F5 and 2G12 were purchased from NIBSC (Centralised Facility for AIDS Reagents).

II.1.5 Synthetic oligonucleotides

Oligonucleotides used for DNA-sequencing or PCR were purchased from MWG Biotech:

Name	Sequence
pMS-5'	5'– AGC AGA GCT CTC TGG CTA ACT– 3'
pMS-3'	5'– CAT GCC CGC TTT TGA GAG GGA– 3'
pMT-5'	5'– GTC TGC TGC TCC TCG CTG– 3'
pMT-3'	5'– GGA TAT AGT TCC TCC TTT CAG– 3'
pS-5'	5'- GAC CCT TCC TCT ATA TAA GG-3'
pS-3'	5'- AAA GAG GTC TTA TTA CAC AC -3'
cd64-5'	5'- GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCA GTG ATC ACT TTG CAG CCT C-3'
cd64-cmyc-3'rc	5'-TCA GTC GCT CAG CCT ACA GAT CCT CTT CTG AGA TGA GTT TTT GTT CGA CAT GAA ACC AGA CAG GAG TTG-3'

II.1.6 Recipients of recombinant DNA

II.1.6.1 Bacterial strains

E.coli strain XL1-blue was used as a host cell for intermediate cloning constructs; BL21 (λDE3) was used for expression of recombinant proteins.

Strain	Genotyp
BL21 (λDE3)	F- ompT hsdS_R (r_B^- m_B^-) dcm^- gal lon^-(Novagen, Madison, USA)
XL1-blue	RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB lacIq ZΔM15 Tn10 (Tet^r)] (Stratagene, La Jolla (USA))
DH5α	*sup*E44 *lac*U169 (Φ80d*lacZ* ΔM15) *hsd*R17 *rec*A *end*A1 *gyr*A96 *thi*-1 *rel*A1 (HANAHAN 1983)

Table II-1 Name, supplier and genotypes of *Escherichia coli* strains used throughout the work**.**

II.1.6.2 Mammalian cells

HEK 293T (ATCC-Nr.:CRL-11268, SENA-ESTEVES *et al.* 1999), an adherent human, embryonic kidney (HEK) cell line, was used for expression of human rsCD64 (II.2.4). These cells were cultivated in complex RPMI-media (II.1.2.3) at 37 °C in a 5 % (v/v) CO_2 atmosphere.

II.1.6.3 Agrobacteria strain

Agrobacterium tumefaciens GV3101 [pMP90RK Gm^R Km^R Rif^R; KONCZ and SCHELL 1986] was used for *Agrobacterium*-mediated DNA delivery into the plant cell genome.

II.1.6.4 Plants and plant cells

Tobacco plants of the variety *Nicotiana tabacum* L. cv. Petit Havana resp. *Nicotiana tabacum* L. cv. Petit Havana SR1 were used for transient expression (I.2.3) and tobacco suspension cultures of the variety *Nicotiana tabacum* L. cv. Bright Yellow (BY-2; KATO *et al.* 1972) were used for expression of the human anti-HIV antibody 2F5 and 2G12 (II.2.5).

II.1.7 Vectors

II.1.7.1 pMT-M12

pMT-M12 (M. Tur, Fraunhofer IME, Germany) is a bacterial expression vector, derived from pBM1.1 (MATTHEY *et al.* 1999) containing an N-terminal his10-tag and a recognition site for an enterokinase to remove the his-tag was introduced.

For expression in *E.coli* (I.2.1) the cd64 gene (II.2.5) was cloned into this vector.

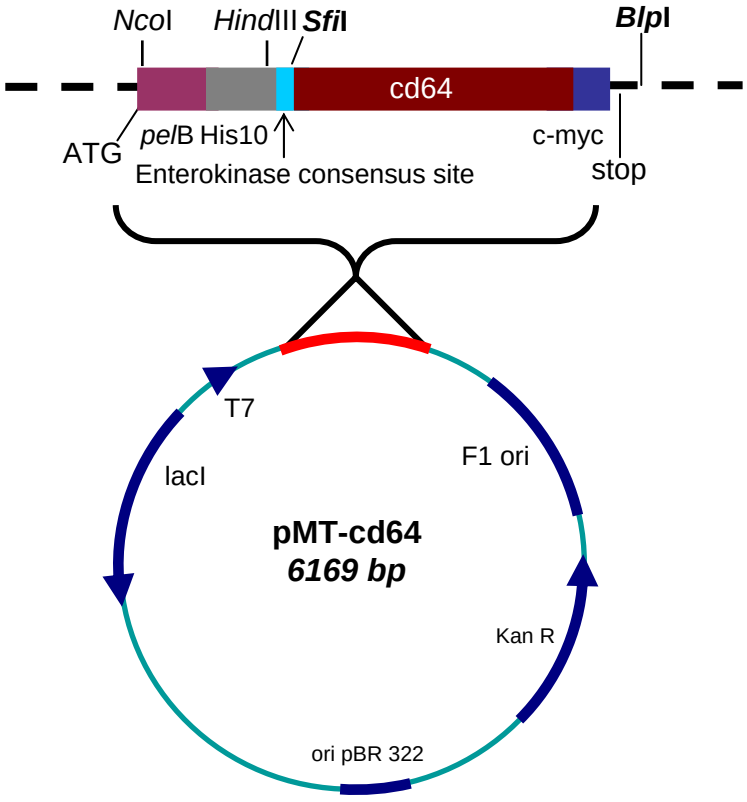


Fig.II-1: Vector map of pMT-cd64. *pel*B = signal peptide for transport into periplasma; his10/c-myc = sequence for detection and purification of the recombinant protein; cd64 = DNA sequence of extracellular domain of CD64; F1 ori = origin of replication for the production of single stranded DNA from M13 phage; Kan R = gene for kanamycin resistance; ori pBR 322 = origin of replication; lacI = Lac-repressor; T7-Promoter = IPTG inducable.

II.1.7.2 pMS-ILAng

pMS-ILAng (M. Stöcker, Fraunhofer IME, Germany) is a mammalian expression vector, derived from pSecTag2 (Invitrogen) that contains an **I**nternal **R**ibosomal **E**ntry **S**ite (**IRES**) in combination with a reporter gene for **e**nhanced **G**reen **F**luorescent **P**rotein (**eGFP;** STOCKER *et al.* 2003). The cytomegalovirus (CMV)-promoter enables constitutive expression of the recombinant protein which is secreted into the culture supernatant due to an Ig *kappa* leader sequence.

This vector was used for expression of rsCD64 (II.2.5) in HEK 293T cells (II.2.4).

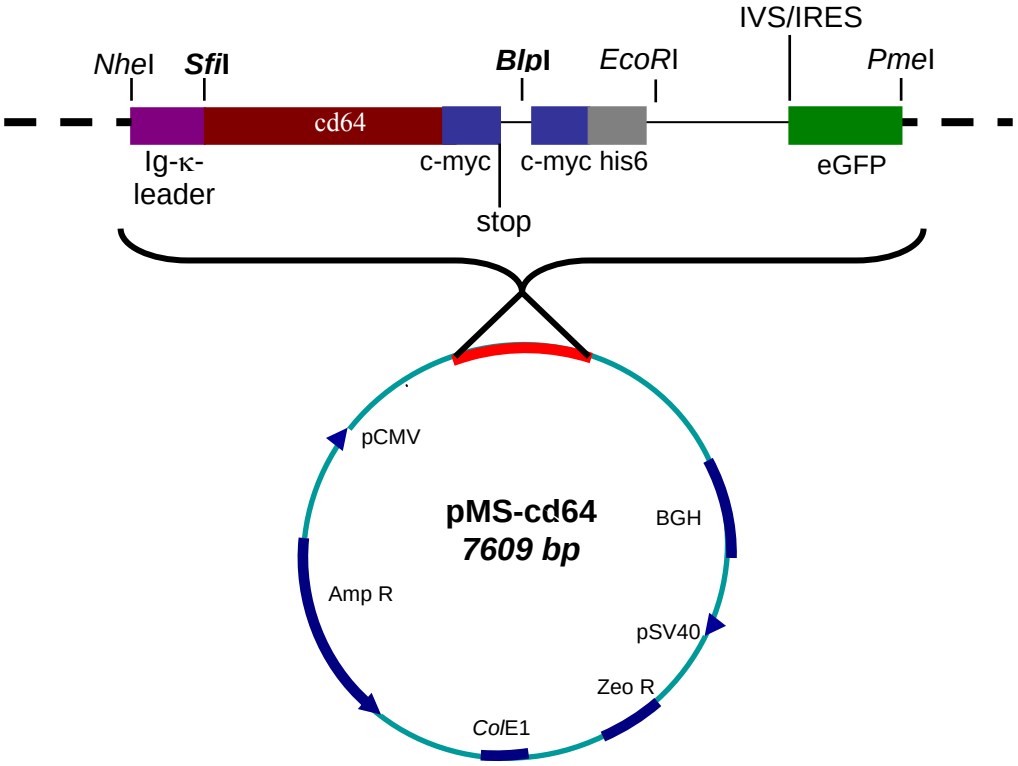


Fig.II-2: Vector map of pMS-cd64. Igκ-leader = murin signal leader sequence for secretion of protein into culture supernatant; cd64 = DNA sequence of extracellular domain of CD64; his6/c-myc = sequence for detection and purification of the recombinant protein; IVS = synthetic intron for stabilization of mRNA; IRES = internal ribosomal entry site; pCMV = cytomegalovirus (CMV)-promoter (constitutiv); eGFP = enhanced Green Fluorescent Protein; **BGH** = **B**ovine **g**rowth **h**ormone polyadenylation signal and transcriptional termination; pSV40 = SV40-Promoter, SV40-origin of replication; ZeoR = gene of Zeocin®–resistance for selection of transfected cells; ColE1 = ori of replication in prokaryotes; Amp R = gene of ampicillin resistance for selection in *E. coli*; pCMV = constitutive promoter of cytomegalovirus (CMV).

II.1.7.3 pTRAkc-AH

pTRAkc-AH (Dr. T. Rademacher, BioVII, RWTH-Aachen) is an optimised plant expression vector, derived from pPAM (genbank: AY027531) containing a his6-tag for purification and detection downstream of the **m**ultiple **c**loning **s**ite (**MCS**). This binary plasmid contains the *Col*E1- and the *RK2*-origin which enables replication in *E.coli* and in *A. tumefaciens*; the vir-region is separated from the T-DNA on the agrobacterium chromosome (HOEKEMA *et al.* 1984; HELLENS *et al.* 2000). This vector contains the **ca**uliflower **m**osaic **v**irus (**CaMV**)-

35S-promoter of the vector pRT101 (TOPFER *et al.* 1988) with a duplicated 35S-enhancer-region as well as the termination and polyadenylation sequence of CaMV. Two copies of **s**caffold **a**ttachment **r**egions (**SAR**) of the tobacco gene RB7 were introduced to improve transcription.

pTRA-ERH was used for transformation of *A.tumefaciens* (II.1.6.3) and translocation of the expression cassettes into the genome of *N.tabacum*. The vector was used for expression of rsCD64 (II.2.5) into the apoplast of tobacco leaves.

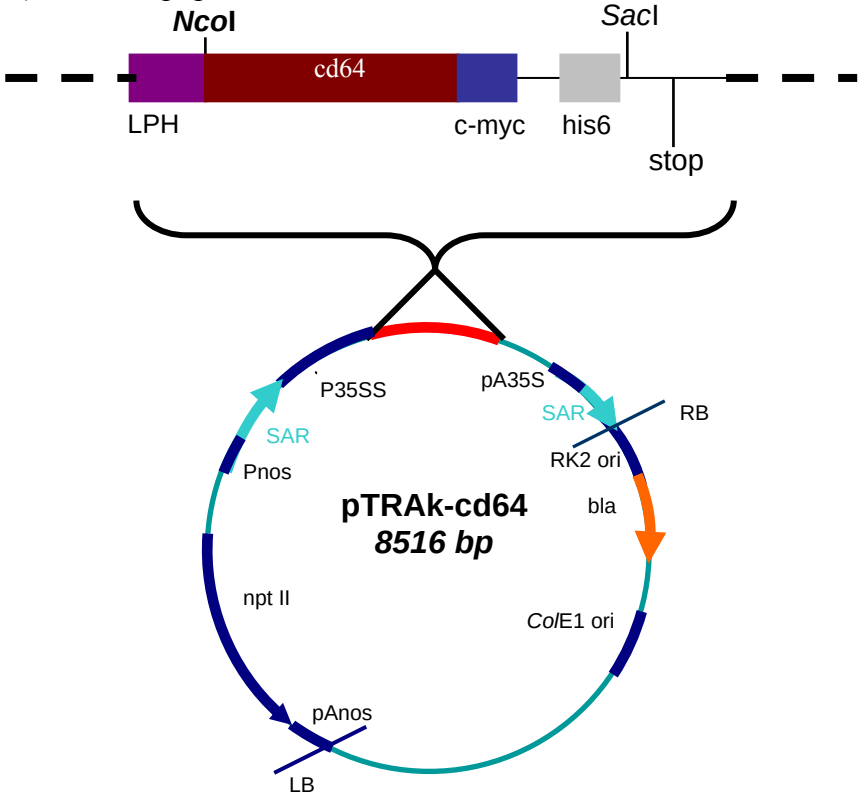


Fig.II-3: Vector map of pTRA-cd64. LPH = codon-optimised murine signal peptide of mAB24; cd64 = DNA sequence of extracellular domain of CD64; his6/c-myc = sequence for detection and purification of the recombinant protein; 35SS/pA35S = 35S-promoter with doubled enhancer and terminator of the cauliflower mosaic virus (CaMV) 35S gene; SAR = scaffold attachment region of *N. tabacum*; RK2 ori = origin of replication for *A. tumefaciens*; bla = ampicillin resistance *E.coli* / carbenicillin resistance *A.tumefaciens*; *Col*E1 ori = origin of replication for *E.coli*; LB und RB = right and left border of the nopalin-Ti-plasmid pTiT37; Pnos and pAnos = promoter and terminator of the nopaline synthase gene; nptII = coding sequences of neomycin phosphotransferase gene (kanamycin resistance).

II.1.7.4 pTRAkc-ERH

The pTRAkc-ERH is based on pTRAkc-AH containing an SEKDEL sequence for retention of the recombinant protein in the **e**ndoplasmatic **r**eticulum (**ER**). This vector was used for expression of rsCD64 (II.2.5) into the ER of tobacco leaves.

II.1.7.5 pTRAkt-rfp

The pTRA-rfp vector is based on the pTRAkc-ERH containing additionally an expression cassette for DsRed (JACH *et al.* 2001) fused to a transit peptide (Dr. T. Rademacher,

unpublished results) downstream the expression cassette of the recombinant proteins. This vector was used for the expression of the human anti-HIV antibodies 2F5 and 2G12 in BY-2 cells (I.2.3).

The cDNAs of the heavy and light-chains of the 2F5 and 2G12 antibody (BUCHACHER *et al.* 1994) were isolated each with their leader sequence out of the eucaryotic expression vectors p2F5IgG1and p2F5LC respectively (KUNERT *et al.* 2000). The sequence coding for a C-terminal ER retention signal (SEKDEL) was fused to the 3' end of both cDNAs by PCR. Each seqence was subcloned into an expression cassette consisting of the double enhanced CaMV-35S promoter, the TEV 5'-untranslated region and the CaMV-35S transcriptional terminator. For co-expression of the heavy and light-chain both cassettes were inserted in tandem (head-to-tail) into pTRAk. The expression units were separated by SARs of the tobacco RB7 gene (GenBank: U67919).

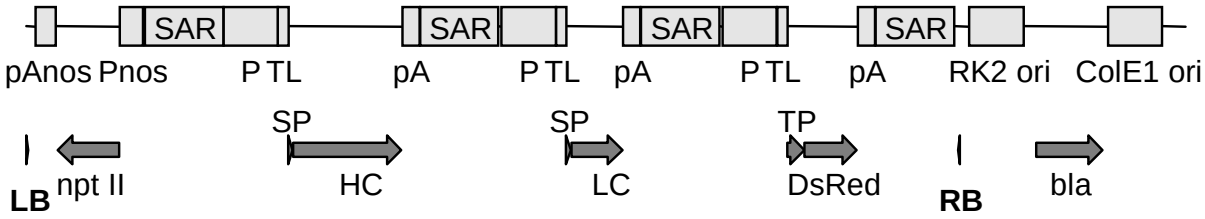


Fig.II-4 Schematic view of the T-DNA of the plant expression vector pTRAk-2F5ER-Ds. LB and RB = left and right border of the T-DNA; Pnos and pAnos = promoter and terminator of the nopaline synthase gene; npt II = coding sequence of the neomycin phosphotransferase gene; SAR = scaffold attachment region; P and pA = 35S-promoter with doubled enhancer and terminator of the cauliflower mosaic virus (CaMV) 35S gene; TL = 5'-UTR of the **t**obacco **e**tch **v**irus (**TEV**); **SP** = **s**ignal **p**eptide; HC and LC = coding sequence of heavy and light-chain of the 2F5/2G12 antibody with ER retention signal; **TP** = **t**ransit **p**eptide.

II.1.8 Equipment and applications

- Cameras: E.A.S.Y. 429K camera (Herolab).
- Centrifuges: Avanti^{TM} J-30, Allegra^{TM} 6KR and MicrofugeR (Beckman), Biofuge Pico (Heraeus Instruments), Eppendorf-centrifuge 5415D (Eppendorf), Picofuge (Stratagene), Rotilabo centrifuge with Butterfly-Rotor (Roth), centrifuge tubes (Beckman), CARR Powerfuge® P6 (CARR Separations Inc.).
- Chromatography equipment: Ni^{2+}-NTA Agarose (Qiagen), Protein-A Ceramic HYPERD F BioSepra (Ciphergen Biosystems), IgG-sepharose (Amersham Biosciences), XK26/20 column (Amersham Pharmacia), UFP-500-K-4X2TCA Hollow Fiber unit (500cut-off, 400cm^2, Amersham Pharmacia).
- DNA-sequencing machine: ABI Prism 3700 Capillary-Sequencer (Perkin-Elmer).
- Easypure UV/UF Watersystem (Werner GmbH).

- Electroblotting equipment: Mini Protean III gel chamber (BioRad), XCell II Blot Module (Invitrogen), Protein Gel-Apparatus and supplies.
- Electroporation apparatus: Gene Pulser, Pulse Controller Unit (BioRad) and 0.2 cm cuvettes (BioRad).
- FACSCalibur (Becton Dickinson Bioscience).
- Gel electrophoresis equipment: Mini PROTEAN II™, Gel Air Dryer (BioRad).
- Incubators: Innova™ 4340 incubator shaker (News Brunswick Scientific), 37°C Inkubator (Heraeus Instruments), Incubator (Heraeus Instruments).
- Laminar-Flow: Hera Safe HS12 (Kendro).
- Microscope: DM R Fluorescence Microscope (Leica), Leica KL 1500 LCD lamp with glass fiber optics (Leitz, Wetzlar) with green excitation (BP: 545/30) and color foil No 182 light red (colorfilter Lee).
- PCR-Thermocycler: Primus 96 Plus (MWG-Biotech), Programmable Thermal Controller PTC-200™ (MJ Research Inc, Watertown, USA).
- Photometers: Biophotometer (Eppendorf), Multichannel Photometer (SpektraMax 340, Molecular Devices).
- Probe Sonicator: UW2070 (Bandelin electronic, Berlin), Microtip Titan MS72, Microtip Titan MS73, Microfluidizer M110-L (Microfluidics Corporation, Newton, MA).
- Pumps: Vacuumpump Trivac S4A (Leybold-Heraeus, Osterode), peristaltic pump (MBR Bio Reactor AG, Member of the Sulzer Group).
- Software: Chromas 1.45 (C. McCarthy, Southport, Australia); Origin 5.0 (Microcal Software, Inc.); Clone Manager 5 (Sci-Ed Central), GCG (Wisconsin Package Tenetic Computer Group), clustalX 1.8 (NCBI), TreeView (Win32) 1.6.5 (Roderic D.M. Page, 2001), BioXpert 3.0 (Applikon).
- Surface plasmon resonance: BIACORE® 2000 (BIACORE®, Uppsala, Sweden) with software (BIAControl 1.3 and BIAEvaluation 3.0).
- Vacuum exsiccator (Glaswerk Wertheim).
- Vortex-Genie 2 (Scientific Industries Inc., Bohemia, USA).
- Warring Blender (VWR).
- 3- and 7-litre autoclavable glass bioreactor (BioBundle), 7-litre Steam In Place (SIP) stainless steel benchtop bioreactor (Typ BioBench7) and 140-litre bioreactor (Pilot System 140l), Bio Controller ADI 1030/1060, Bio Console 1035, Bio Bench ADI 1065, Control Console Interface 1040/21 (Applikon).

II.1.9 Approbation for the conducted work

The approbation for the conducted work at security level S1 was given by the "Landesumweltamt NRW", number of registration: 64-K-1.19/02.

II.2 Methods

II.2.1 Recombinant DNA technologies

General recombinant DNA techniques including DNA precipitation, restriction enzyme digest, DNA ligation and DNA agarose gel electrophoresis were performed according to the standard protocols described by Sambrook *et al.*, (SAMBROOK and FRITSCH 1996) or according to the manufacturer's protocol when using a kit.

II.2.1.1 Isolation of plasmid-DNA from *E.coli*

Plasmid DNA was purified using QIAprep® Plasmid Isolation Mini Kit (II.1.3) according to the manufacturers' manual. Quality and quantity of DNA was confirmed by spectrophotometric analysis (II.2.1.8) or analytical agarose gel electrophoresis (II.2.1.4). Isolated DNA samples were stored at –20 °C.

II.2.1.2 Polymerase-Chain-Reaction

Polymerase **c**hain **r**eaction (**PCR**), a procedure for rapid *in vitro* enzymatic amplification of a specific segment of DNA, was used for the amplification of genes of interest (SAIKI and GELFAND 1988) as well as for the insertion of restriction sites or short nucleotide sequences like purification-tags. The reactions were performed in 0.2 ml PCR tubes (Biozym), using a PCR thermocycler (MWG Biotech or MJ Research Inc.). PCR reactions were carried out in a total volume of 25 µl as described in the following table:

Components	Volume	Final concentration
Template DNA	0.5-1 µl	1-10 ng
10x PCR buffer	2.5 µl	1x
2.5 M Betain	2.5µl	250 mM
10 mM dNTP mixture	1 µl	0.4 mM each
100 pmol/µl forward primer	1 µl	10 pmol/µl
100 pmol/µl backward primer	1 µl	10 pmol/µl
Taq DNA Polymerase (5 U/µl)	0.25 µl	1.25 units
Destilled water	added to 25 µl	

Amplifications were carried out as follows:

	95 °C	5 min	initial denaturation
25x	95 °C	30 sec	denaturation
	T_P or gradient	30 sec	primer annealing
	72 °C	1 min/1 kb	elongation
	72 °C	7 min	final elongation

The optimal annealing temperature (T_P) of the primer was experimentally optimised (temperature gradient) or calculated by the empirical formula (WU and UGOZZOLI 1991)

$$T_P = \{22 + 1.046\ [2\bullet(G + C) + (A + T)]\}$$

PCR products were resolved on a 1-1.2 % (w/v) agarose gel (II.2.1.4) with appropriate DNA markers to confirm the successful amplification and integrity of the amplified product.

II.2.1.3 PCR based analysis of recombinant bacterial clones

Bacterial clones harbouring plasmid DNA containing the gene of interest were identified by PCR as described by Jesnowski *et al.* (1995). Single colonies were picked with sterile toothpicks and the bacteria loaden toothpicks were dipped into a PCR tube containing 10 µl sterile water. 15 µl of the PCR mix (II.2.1.2, 250 mM DMSO instead of Betain) were added to each 10 µl bacterial suspension giving a final volume of 25 µl. Specific primers annealing to the vector backbone were used for PCR reaction (II.1.5). Thermocycler conditions were used as described in II.2.1.2 but for effective lysis of the bacterial cells the time for initial denaturation was increased to 10 min for *E.coli*. For *Agrobacteria* 5 min were kept. Ten µl of the PCR product were analyzed on a 1.2 % (w/v) agarose gel (II.2.1.4).

II.2.1.4 Total RNA extraction from whole blood

For extraction of total RNA the PreAnalytiX Kit (Qiagen) was used according to the manufacturers' protocol after blood collection with the PAXgene™ Blood RNA System (Becton Dickinson). Briefly, 2x 2.5 ml blood were taken with the BD Safety-Lok™ Blood Collection Set. The tubes were gently inverted 10 times and stored at **r**oom **t**emperature (**RT**) **o**ver**n**ight (**o/n**).

In the first isolation step nucleic acids were pelleted (10 min/ 4000 g/ 4 °C). The pellet was washed with 360 µl RNase free water (BR1), resuspended and incubated in 300 µl optimised buffer (BR2) with 40 µl Protein-K for 10 min at 55 °C. In an additional centrifugation step (3 min/ 16,000 g/ RT) residual cell debris were removed and the supernatant was transferred to a fresh 1.5 ml microcentrifuge tube. Ethanol (350 µl) was added to adjust binding

conditions and 700 µl lysate was applied to a PAXgene RNA spin column. After a brief centrifugation (1 min/ 7500 g/ RT), RNA was selectively bound to the silica-gel-membrane while contaminants passed through. Remaining contaminants were removed in three washing steps (700 µl buffer BR3, 2x 500 µl BR4) and RNA was then eluted in 40 µl Buffer BR5. Concentration of RNA was determined photometrically (II.2.1.8).

II.2.1.5 Specific RT-PCR for cDNA synthesis

First-strand cDNA was synthesized using the One-Step RT-PCR Kit (Qiagen) and gene specific primers (cd64, II.1.5) according to the manufacturers' protocol. Qiagen OneStep RT-PCR enables efficient reverse transcription and specific amplification in one step. RT-PCR reactions were carried out in a total volume of 50 µl as described in the following table:

Components	Volume	Final concentration
Template DNA	x µl	<1 µg
5x Qiagen RT-PCR buffer	10 µl	1 x
10 mM dNTP mixture	2 µl	0.4 mM each
100 pmol/µl cd64-(L)-5'	3 µl	10 pmol/µl
100 pmol/µl cd64-(c-myc)-3'rc	3 µl	10 pmol/µl
Qiagen One Step Enzyme Mix	2 µl	
Rnase inhibitor	0.25 µl	5 U
Destilled water	added to 50 µl	

Amplification was carried out under the following conditions:

	50 °C	30 min	reverse transcription
	95 °C	15 min	inital PCR activation
25x	95 °C	60 sec	denaturation
	66 °C	60 sec	primer annealing
	72 °C	90 sec	elongation
	72 °C	10 min	final elongation

PCR products were resolved on a 1-1.2 % (w/v) agarose gel (II.2.1.4) with appropriate DNA markers to confirm the successful amplification and integrity of the amplified product.

II.2.1.6 DNA sequencing and sequencing analysis

DNA sequencing reactions were based on the Dideoxy-chain-termination-method depicted by Sanger *et al.* (1977). Fluorescent labelled dideoxynucleotides were used for sequencing of genes or DNA fragments of interest. Sequencing analysis was performed by using an "ABI

Prism 3700" Capillary-Sequencer (Applied Biosystems) and BigDye^{TM} cycle sequencing terminator chemistry. The extension products were detected by exciting the unique dyes attached to each dideoxynucleotide with a laser, followed by a measurement of fluorescent emission using a CCD camera.

pMT-5' and pMT-3' primers (II.1.5) were used for sequencing the cd64 gene cloned in the pMT vector. pMS-5' and pMS-3' primers (II.1.5) were used for sequencing of cd64 in the pMS vector.

Subsequently, the signals were interpreted by the Applied Biosystems Sequencing Analysis Program in order to determine the nucleotide sequence of the DNA template. Chromas software package (II.1.8) was used for displaying the chromatogram files. For evaluation of sequence data the sequences were exported to the GCG software packages.

II.2.1.7 Agarose gel electrophoresis

Undigested DNA (II.2.1.1), restriction enzyme digested DNA (II.2.1.9) and PCR fragments (II.2.1.2) were electrophoretically separated on 0.8-1.2 % (w/v) analytical agarose gels prepared in TBE or TAE buffer (II.1.2.1) containing 0.1 µg/ml ethidium bromide.

Known amount of DNA molecular marker such as *Pst*I-digested lambda DNA (MBI Fermentas) or 1 kb Marker (Invitrogen) were used for evaluation of sample size, integrity and determination of DNA concentration. The DNA was visualised on an UV transilluminator at 302 nm and documented by a black and white E.A.S.Y 429K camera (Herolab).

Preparative gels were used for isolation of PCR amplified DNA (II.2.1.2) or DNA fragments after restriction enzyme digestion (II.2.1.9) prior to cloning in the appropriate vectors. After electrophoresis the desired DNA fragments were excised from the gel and purified using the "QIAquick Gel Extraction Kit" (Qiagen; II.1.3) according to the manufacturers' protocol. The concentration of the recovered DNA was measured photometrically (II.2.1.8) or determined by analytical agarose gel electrophoresis.

II.2.1.8 Quantification of nucleic acids

The amount of RNA or DNA in a sample was estimated by measuring the OD_{260nm}. The OD_{260nm} of 1 corresponds to ~50 µg/ml of dsDNA or to ~40 µg/ml of ssDNA and RNA. Purity of the nucleic acid was ascertained by the OD_{260nm}/OD_{280nm} ratio of the measured optical density, which is 1.8 for pure DNA and 2.0 for pure RNA.

II.2.1.9 Restriction digest of DNA

Restriction endonucleases, appropriate buffers and BSA solution were obtained from NEB (Schwalbach). Restriction digest and double restriction digest of DNA were performed according to the manufacturers' protocol.

II.2.1.10 Dephosporylation

Dephosphorylation of restricted vector-DNA was done with **CIP** (**C**alf **I**ntestine **P**hosphatase, NEB) according to the manufacturers' protocol.

II.2.1.11 Klenow Fill-in

For the Fill-in of single-stranded DNA 5'-fragments, the Klenow Fragment of DNA-polymerase I of *E.coli* was used (JOYCE and GRINDLEY 1983). The Klenow-Fill-in reaction was carried out according to the manufacturers' protocol.

II.2.1.12 Ligation of DNA

Restriction enzyme digested DNA (II.2.1.9) was ligated using 80 U T4 DNA-Ligase or 1 µl Quick T4 DNA-Ligase (NEB) in buffer systems recommended in the manufacturers' protocol in a final volume of 20 µl. Sticky-end ligations were carried out at 22 °C for 30 min whereas blunt ligations were incubated at 4 °C (o/n). Ligation product was used for transformation of *E.coli* (I.2.1) or *A.tumefaciens* (II.2.3.2).

II.2.2 Bacterial expression system (*E.coli*)

II.2.2.1 Heat-shock competent *E.coli*

E.coli cells for $CaCl_2$-mediated heatshock transformation were prepared as described (HANAHAN 1983). Briefly, 5 ml of LB broth were inoculated with a single bacterial colony and cultured at 37 °C o/n. 500 µl of the o/n culture was transferred into 50 ml LB broth containing 20 mM $MgSO_4$ and 10 mM KCl. Cells were cultured at 37 °C for 3-4 h until the OD_{600nm} reached 0.5 and then transferred to an ice-cold tube. After cooling on ice for 10 min, the cells were recovered by centrifugation (10 min/ 2000 g/ 4 °C). The pellet was gently resuspended in 15 ml ice-cold TfB-I solution (II.1.2.2) and stored on ice for 10 min. The cells were sedimented down by centrifugation as described above and resuspended in 2 ml ice-cold TfB-II (II.1.2.2). Aliquots of 100 µl suspension were dispensed into prechilled 1.5 ml Eppendorf tubes, frozen immediately in liquid nitrogen and stored at –80 °C.

II.2.2.2 Heat-shock transformation of *E.coli*

The 'competent' cells (II.2.2.1) were thawed on ice and ice-cold plasmid DNA (~80 ng) or ligation product (II.2.1.10) were mixed gently with the competent cells and placed on ice for

30 min. A heatshock of 42 °C was given for 90 sec and the sample was cooled on ice for 10 min. Pre-heated (37 °C, 900 µl) 2xTB media (II.1.2.2) supplemented with 2 % (w/v) glucose were added to the sample and cells were incubated for regeneration at 37 °C for 30 min. Different samples (10 µl and 100 µl) were plated onto LB or 2xTY agar plates supplemented with appropriate antibiotics and incubated at 37 °C o/n.

II.2.2.3 Cultivation of *E.coli* and long-term storage

E. coli XL1-Blue bacterial cells (II.1.6.1) were grown at 37 °C o/n either in liquid LB medium in a shaker incubator or plated on solidified LB-agar (1.5 % (w/v) agar w/v) plates. *E. coli* BL21(DE3) were grown in 2xTY or 2xTB medium under the same conditions. The media was supplemented with appropriate antibiotics [25 µg/ml kanamycin (pMT) or 100 µg/ml ampicillin (pMS)] and 1 % (w/v) glucose for the growth of bacteria. The plates were stored at 4 °C no more than two weeks. For long-term storage of bacterial strains, glycerol was added to a final concentration of 15 % (v/v) to the liquid culture and cells were frozen at –80 °C.

II.2.2.4 Expression of rsCD64 in *E.coli*

rsCD64 (I.1.2.2) was expressed in *E.coli* and purified according to a modified protocol based on Bruell (BRUELL 2004).

Briefly, a freshly transformed single colony of *E. coli* strain BL21(λDE3; Novagen) was inoculated in 5 ml of LB medium supplemented with 1 % (w/v) glucose and 25 µg/ml kanamycin (pMT; LB-Km_{25}-$Glu_{1\%}$), and cells were cultivated o/n at 37 °C with vigorous shaking (225 rpm). The following day fresh LB-Km_{25}-$Glu_{1\%}$ media was inoculated 1:100 with o/n culture and grown (30 °C/ 225 rpm) to an OD_{600nm} of 0.6-0.8. After sedimentation of bacteria (15 min/ 5000 g/ 4 °C) the pellet was resuspended in the same volume of LB-Km_{25}-media and incubated (30 min/ 225 rpm/ 37 °C). Expression of recombinant proteins was then induced by addition of IPTG to a final concentration of 1 mM. Cells were cultured for 6 h at 28 °C. The cells were harvested by centrifugation (10 min/ 5000 g/ 4 °C) and the supernatant was discarded. The pellet was stored at -20 °C or was immediately resuspended in ice-cold PBS (II.1.2.1) supplemented with 1 M NaCl (extraction buffer) and sonicated on ice 3 times for 60 sec (50 %, power 9; Bandelin). Cell debris and insoluble components were removed by centrifugation (30 min/ 15000 g/ 4 °C). The supernatant (periplasmic fraction) and the filtered culture supernatant were subjected to further analysis.

Expression under osmotic stress and with compatible solutes was performed as described in Barth *et al.* (BARTH *et al.* 2000).

II.2.3 Plant expression system (*Nicotiana tabacum*)

To establish transgenic *N.tabacum* (II.1.6.4) plant cells *Agrobacterium*-mediated gene transfer was performed (II.1.6.3; KAPILA *et al.* 1996).

II.2.3.1 Competent *Agrobacterium* cells

A single colony of *Agrobacterium tumefaciens* strain GV3101 grown on YEB-agar plate (II.1.2.4) containing 100 µg/ml rifampicin (Rif) and 25 µg/ml kanamycin (Km; YEB-Rif_{100}-Km_{25}) was inoculated in 5 ml of YEB-Rif_{100}-Km_{25} medium (II.1.2.4) in a 100 ml Erlenmeyer flask and incubated at 28 °C for 48 h with shaking (250 rpm). 100 ml of YEB-Rif_{100}-Km_{25} medium were inoculated 1:100 and cultivated at 28 °C for 15-20 h with shaking (250 rpm) until the OD_{600nm} reached 1-1.5. The cells were chilled on ice for 15 min and spun down by centrifugation (5 min/ 4000 g/ 4 °C). The culture medium was decanted and the cells were washed three times with 10 ml of dH_2O by centrifugation and resuspended in 500 µl of sterile 10 % (v/v) glycerol. Aliquots of 45 µl suspension were dispensed into prechilled microcentrifugation tubes, frozen immediately in liquid nitrogen and stored at -80 °C.

II.2.3.2 Transformation of *Agrobacterium* cells

Plasmid DNA (0.2-1.0 µg; II.2.1.1) in sterile dH_2O was added to a thawed aliquot of electrocompetent *Agrobacterium* cells (II.2.3.1) and incubated on ice for 10 min. The cell/DNA mixture was transferred into a prechilled electroporation cuvette (0.2 cm) and assembled into a safety chamber. After application of the pulse (25 µF/ 2.5 kV/ 200 Ω), the cells were diluted in 1 ml of YEB medium and incubated (1 h/ 250 rpm/ 28 °C). Finally, 1-5 µl cells were plated on YEB-Rif_{25}-Km_{25}-$Carb_{50}$ agar (II.1.2.4) and incubated (48-72 h/ 28 °C).

II.2.3.3 Cultivation of *A.tumefaciens* and long-term storage

Single colonies of *A. tumefaciens* were examined for the presence of plasmids by control PCR (II.2.1.3). Positive colonies were inoculated in 10 ml of YEB-Rif_{25}-Km_{25}-$Carb_{50}$ medium and cultivated (48-72 h/ 250 rpm/ 28 °C). The culture was transferred to Falcon tubes and *Agrobacteria* cells were pelleted by centrifugation (10 min/ 4000 g/ 15 °C). The cells were resuspended in a 1:1 volume of YEB-Rif_{25}-Km_{25}-$Carb_{50}$ medium and glycerol stock media (II.1.2.4). The suspension was aliquoted (100 µl) and stored at –80 °C for further experiments.

II.2.3.4 Transient expression of rsCD64

Growth of recombinant *Agrobacteria* (II.2.3.3) and vacuum infiltration of tobacco leaves was performed as described by Kapila *et al.* (1996) and Vaquero *et al.* (1999). Expression levels of rsCD64 (I.1.2.2) were evaluated in transiently expressing *N.tabacum* leaves.

Briefly, 100 ml of YEB-Rif_{25}-Km_{25}-$Carb_{50}$ medium was inoculated with 100 µl of glycerol stock of the selected recombinant *Agrobacteria*. *Agrobacteria* culture for vacuum infiltration was grown at 28 °C o/n with shaking at 250 rpm after 24 h. The cells were pelleted by centrifugation (10 min/ 5000 g/ 15 °C), transferred into 250 ml of induction medium (II.1.2.4) and cultivated at 28 °C o/n with shaking at 250 rpm. *Agrobacteria* cells were centrifuged (15 min/ 4000 g/ 15-25 °C) and resuspended in 50 ml of MMA solution (II.1.2.4) and kept at RT for 2 h. The OD_{600nm} was measured after 1:10 dilution and the cell suspension was adjusted to an OD_{600nm} of 1. Diluted cell suspension (100 ml) was used for vacuum infiltration of plant leaves. Young *N.tabacum* cv. Petite Havana SR1 leaves (3 leaves for each construct) were placed in 100 ml of *Agrobacteria* suspension in a preserving glass and a continuous vacuum (60-80 mbar) was applied for 15-20 min. The applied vacuum was released rapidly, the leaves were briefly rinsed in tap water and kept on wet Whatman paper no.1 with adaxial side upwards. The plastic tray was sealed with saran wrap and placed at 22 °C with a 16 h photoperiod for 60 h. Leaves were weighed and stored at -80 °C till analysis.

For the extraction of transiently expressed proteins in vacuum infiltrated tobacco leaves, leaves were mixed with the extraction buffer (II.1.2.4) and homogenised using a motor driven stainless steel pistill or a Waring blender (VWR) for larger amounts of plant material. Total soluble protein was extracted using 2 volume of extraction buffer per gram leaf material. Cell debris were removed by two rounds of centrifugation (30 min/ 16000 g/ 4 °C) and the supernatant used for further analysis.

II.2.3.5 Expression of $^{BY\text{-}2}$2F5 and $^{BY\text{-}2}$2G12 in transgenic BY-2 cells

Transgenic BY-2 cells were used for expression of human full-size antibody $^{BY\text{-}2}$2F5 and $^{BY\text{-}2}$2G12 (I.1.1.3). Briefly, a 3-ml aliquot of 3 d-old *Nicotiana tabacum* L.cv. bright yellow 2 (BY-2) cells in BY-2 media with 200 µM acetosyringone (II.1.2.4) was inoculated with a recombinant *Agrobacteria* containing the antibody genes of interest to a final OD_{600} of 0.05 and incubated for 3 days at 26 °C (AN 1995). Selection of kanamycin-resistant transformants was performed on solid BY-2 agar medium supplemented with 100 mg/l kanamycin and 200 mg/l claforan (cefatoxim) at 26 °C for 4-6 weeks in the dark. Kanamycin-resistant calli appeared after 3-4 weeks of incubation and were screened for DsRed fluorescence (II.2.7.7) and transferred to plates with 0.8 % (w/v) Agar in BY-2 medium (II.1.2.4). After 3 weeks the expanded calli were screened for accumulation of recombinant protein. Expression levels of protein extracts were quantitified by SPR (II.2.7.6) and 4 lines were used to establish suspension cultures by subculturing the transformed callus in liquid BY-2 medium. The suspension cell lines were tested for accumulation of recombinant protein via Dotblot

(II.2.7.3) and the line with the highest antibody accumulation in suspension was used for scale up production. These genetically modified cells were cultivated in 500 ml Erlenmeyer flasks (working solution 300 ml/ 26 °C/ 180 rpm) and subcultured weekly with a 5 % (v/v) inoculum of a 7-day-old cell culture.

Calli were maintained on solid medium (plus 0.8 % (w/v) agar) in the dark at 26 °C and transferred as necessary. Long-term storage methods in liquid nitrogen are currently under investigation (K. Schmale, BioVII, RWTH-Aachen).

In order to achieve higher production volumes or product concentrations expressions on the 2-L, 5-L and 100-L bioreactor scale were carried out (II.2.6).

Extraction of soluble protein from BY-2 cells was performed essentially as described (FISCHER(A) *et al.* 1998). Briefly, cells were separated from the culture medium by paper/vacuum-filtration and stored at -20 °C. The frozen cells were thawed and resuspended in 2 volumes extraction buffer (II.1.2.4) and cells were disrupted in a Microfluidizer M110-L (Microfluidics Corporation). Insoluble material was removed by centrifugation (30 min/ 4000 g/ 4 °C) and the supernatant was adjusted to pH 8, stirred for 2 h at 4 °C and centrifuged (30 min/ 30000 g/ 4 °C). The clear supernatant was passed through a paper filter and applied to a Protein-A column (II.2.5.2).

II.2.4 Mammalian expression system (HEK 293T)

II.2.4.1 Transfection of HEK 293T cells

Transfected HEK 293T cells were used to evaluate expression levels of the human rsCD64 (I.1.2.2).

Transfection of eucaryotic cells was carried out using the synthetic kationic lipid reagent TransFast® (Promega) according to the manufacturers' protocol. The production cell line HEK 293T (II.1.6.2) contains the large-T-antigen of the SV40 virus which allows episomal replication of the introduced plasmids due to the early SV40-ori leading to high plasmid copy numbers over prolonged time periods.

Briefly, $1x10^5$ cells were seeded in 1 ml RPMI-(C)-media (II.1.2.3) into a 12-well plate and incubated o/n at 37 °C and 5 % (v/v) CO_2. The following day 1 µg plasmid DNA (II.1.7.2) was mixed with 300 µl RPMI-media and 3 µl TransFast® and incubated for 15 min at RT. The supernatant in the 12-well-plate was replaced with the 300 µl transfection mix and cells were incubated for 1 h at 37 °C and 5 % (v/v) CO_2, then 1 ml RPMI-(C)-media was added into each well.

Efficiency of transfections was controlled by eGFP fluorescence after 2-3 days and successfully transfected cells were transferred into cell culture flasks (75 cm^2) and cultivated under selection pressure (100 µg/ml Zeocin).

II.2.4.2 Cultivation and long-term storage of transfected HEK 293Tcells

Selection and proliferation of transfected cells was controlled every 2-3 days with the fluorescence microscope. Recombinant protein was constitutively secreted into the media. Accumulation levels were tested by Westernblot (II.2.7.2) and medium was changed every 2-3 days. Cells were subcultured as soon as a complete monolayer was formed in the cell culture flask. For Scale-up of production multi-bottom cell culture flasks were used (Labomedic) and cells were transferred to serum-free suspension media (CD 293, Invitrogen). Supernatant containing recombinant protein was collected, stored at 4 °C and used for further analysis by ELISA (II.2.7.4), Westernblot (II.2.7.2) and SPR (II.2.7.6).

For long-term storage $1x10^6$ cells were centrifuged and resuspended in RPMI supplemented with 20 % (v/v) FCS and 5 %(v/v) DMSO. Cells were frozen in a styrofoam box at -20 °C for 24 h and were then transferred to liquid nitrogen.

II.2.5 Purification of recombinant proteins

Recombinant proteins were expressed in *E.coli* (I.2.1), HEK 293T cells (II.2.4), *N.tabacum* leaves and BY-2 suspension cells, respectively (I.2.3). Expressions were scaled up using larger Erlenmeyer flasks (250 ml, 500 ml, 1-L) and multi-bottom cell culture flasks (175 cm^2) respectively on laboratory scale. In addition, feasibility studies were carried out in 5-L, 7-L and 100-L bioreactors (II.2.6).

Due to different leader peptides recombinant proteins can be directly secreted into the culture broth (*E.coli*; II.2.2, HEK 293T; II.1.7.2). Alternatively, protein is accumulated in specific cell compartments (periplasm for *E.coli* II.1.7.1, ER for BY-2 II.1.7.3). In the later case, cells have to be disrupted in order to extract all soluble protein (I.2.1, I.2.3, II.2.3.5). Recombinantly expressed rsCD64 containing a his-tag (II.1.7.1; II.1.7.2) was purified using the Ni^{2+}NTA matrix (II.2.5.1) which specifically binds to histidine.

Some proteins can be purified with specific agents binding to parts of the protein. Recombinantly expressed $^{BY\text{-}2}$2F5 antibody (I.1.1.3) was purified with Protein-A (II.2.5.2) which binds to the Fc-part of most antibodies.

II.2.5.1 Purification via Ni^{2+}NTA

Immobilized-**m**etal **a**ffinity **c**hromatography (**IMAC**) was first used to purify proteins (PORATH *et al.* 1975) using the chelating ligand **i**mino**d**iacetic **a**cid (**IDA**) in 1975. IDA is

charged with metal ions (Zn^{2+}, Cu^{2+}, Ni^{2+}), but has only 3 metal-chelating sites and therefore cannot tightly bind metal ions. In contrast, **n**itrilo**t**riacetic **a**cid (**NTA**) is a tetradentate chelating absorbent (JANKNECHT *et al.* 1991) and occupies four of the six ligand binding sites, leaving two sites free for interaction with the his-tag. Ni^{2+}NTA agarose has a binding capacity of approximately 5-10 mg of his-tagged protein per millilitre of resin.

Due to different amounts of contaminating proteins in the supernatant of sonicated *E.coli* (I.2.1) or homogenised *N.tabacum* leaves (I.2.3) compared to the supernatant of HEK 293T cells (II.2.4.2), different purification procedures were established.

His-tagged recombinant proteins from *E.coli* and *N.tabacum* leaves were purified using Ni^{2+}NTA affinity chromatography under denaturing conditions according to "The Expressionist 07/97" (Qiagen). The clarified supernatant (II.2.2.4) was desalted on PD10 columns (Amersham Pharmacia), loaded onto Ni^{2+}NTA columns (gravity-flow-type, BioRad) pre-equilibrated with extraction buffer (II.1.2.1), and passed through the column. After two washing steps with 2 x bed-volume of washing buffer, the bound protein was eluted with 1x bed-volume of elution buffer and protein concentration was determined by Bradford (II.2.7.1). The protein-containing fractions were pooled, imidazole was removed by passing the protein-sample through a PD10-column and purified samples were stored at 4 °C until further analysis.

Washing Buffer		**Elution Buffer**	
PBS (pH 7.4)		PBS (pH 7.4)	
NaCl	1 M	NaCl	1 M
Imidazole	10 mM – 40 mM	Imidazole	250 mM

His-tagged proteins in cell culture supernatant (HEK 293T cells) were purified according to a slightly different protocol. Cell culture supernatant was centrifuged (3 min/ 3000 g/ RT) to remove all cells and the clear supernatant was mixed with 3 volumes of 4x incubation buffer. The Ni^{2+}NTA column was pre-equilibrated with 1x incubation buffer and the supernatant was loaded onto the matrix with 1 ml/min with a peristaltic pump. After two washing steps with 2x column-volume of 1x incubation buffer, the bound protein was eluted with 1x column-volume of elution buffer and protein concentration was determined by Bradford (II.2.7.1). The protein-containing fractions were pooled, imidazole was removed by passing the sample through a PD10-column and the purified protein was stored at 4 °C until further analysis by SPR (II.2.7.6), Westernblot (II.2.7.2) and ELISA (II.2.7.4).

4x Incubation Buffer		**Elution Buffer (pH 8.0)**	
NaH_2PO_4	200 mM	NaH_2PO_4	50 mM
NaCl	1.2 M	NaCl	300 mM
Imidazole	40 mM	Imidazole	250 mM

II.2.5.2 Purification via Protein-A

Protein-A, a 43 kDa cell wall protein produced by the bacteria *Staphylococcus aureus* contains four binding sites for the Fc regions of IgG located in the interface between the C_H2 and C_H3 domains (WINES *et al.* 2000) and is commonly used for antibody purification. Protein-A was isolated and purified first by Sjoquist in 1972 (SJOQUIST *et al.* 1972).

For the purification of $^{BY\text{-}2}$2F5 antibody BioSEPRA Protein-A Ceramic HYPERD F (Ciphergen) with a binding capacity of 25 mg human IgG per millilitre Protein-A matrix was used (II.2.3.5).

The clear supernatant of homogenised BY-2 culture (II.2.3.5) was applied onto a Protein-A column (gravity-flow-type, BioRad) using a flowrate of 1 ml•min^{-1}•ml^{-1} (matrix). Non-specifically bound material was removed by extensive washing with PBS containing 5 mM EDTA and full-size antibody was eluted with 100 mM glycine (pH 2) containing 10 % (w/v) maltose. Elution fractions were immediately adjusted to pH 4.5 with 1 M unbuffered sodium acetate.

Several elutions were tested for reactivity of the binding the peptide ARP 7073 by SPR analysis (II.2.7.6). Fractions containing functional protein were pooled and dialysed extensively against degased 10 mM sodium acetate (pH 4.5) with 10 % (w/v) Maltose and 1 mM EDTA. The preparation was centrifuged at 30,000 g to remove residual insoluble material and used for further characterisation by ELISA (II.2.7.4), Westernblot (II.2.7.2) and EMSA (II.2.7.5).

II.2.6 Large-scale protein production in 7-L and 140-L bioreactors

To scale-up the expression of human anti-HIV antibody in BY-2 cells, feasibility studies were carried out in 7- and 140-litre stirred tank reactors with a height-to-tank diameter ratio of 2:1 (Applikon; II.1.8).

Set points and control circuits for the regulation of pH, temperature, amount of dissolved oxygen, foam and stirrer speed were adjusted by the Applikon ADI Bio Control Units (1030/1060; 1040/21). The control units are connected to a computer for data logging and supervisory control using the BioXpert software (II.1.8). Equipment required to operate the bioreactor system is situated in the ADI Bio Console 1035/ Bio Bench 1065. All bioreactors

are equipped with sensors for pH, dO_2 and temperature at the top or lower sidewalls. Data were collected and reported to the computer in specific time intervalls. Except for the 140-L Pilot System, for plant-cell-fermentation bioreactors were equipped with one three-bladed Marine impeller stirrer (Ø = 60 mm) and baffles were removed to minimize shear stress. The medium was aerated by membrane filtered air through a sinter disc at the bottom of the bioreactor. The agitation for the 140-L Pilot System was provided by three six-bladed Rushton impellers (Ø = 20 cm) and fitted with a sparger tube for aeration from the bottom.

pH electrodes were calibrated with pH 4.0 and pH 7.0 reference solutions and the working volume of BY-2 Media (II.1.2.4) was filled into the fermenter. This is typically 60 - 85 % of the fermenter total volume. The 7-L autoclavable glass reactor (Biobundle) was used with 5-L cell suspension media. The 7-L stainless steel bioreactor (Biobench 7) was filled with 4.5-L and the 140-L Pilot system with 110 L water to solve the solids of the BY-2 media components directly in the bioreactor. Glass bioreactors with media were autoclaved in the Varioklav (H+P Labortechnik AG), stainless steel reactors containing the media were sterilized by steam heated jacket. The antifoam Pluronic®L61 [0.01 % (v/v), BASF] was added separately into the bioreactor. pH was adjusted after autoclaving to pH 5.8, but not controlled during the fermentation. Temperature was maintained at 26 °C. The stirrer was operated at 100 rpm (50 rpm in the 140-L Pilot System). Aeration was constant at 0.1 vvm (0.2 vvm for 140-L Pilot System). Dissolved oxygen was controlled not to drop below 20 % (v/v) saturation using a DO control loop linked to aeration supply of max. 0.1 vvm. The bioreactors were inoculated with 5-10 % (v/v) of 5-7 d-old BY-2 cultures. The 140-L Pilot System was inoculated with the 11 d-old fermentation broth of a 7-L bioreactor. For transfer of the fermentation broth the 7-L bioreactor and the 140-L reactor were connected via a sterile silicon tube (ID 10 mm). Fermentation was performed for 10-17 days and samples were taken every 8-24 h.

II.2.6.1 Off-line data collection

Determination of fresh/dry weight and solid content were performed from 10 ml BY-2 culture in 14 ml polypropylen tubes (10 min/ 3000 g/ 4 °C). The PCV was read at the marking of the tube. 1 ml of supernatant was transferred to 1.5 ml Eppendorf tubes and stored at 4 °C until SDS-PAGE, Westernblot (II.2.7.2), analysis of glucose, fructose and sucrose (II.2.6.2) or determination of phosphate (II.2.6.3).

The remaining supernatant was resuspended with the cell pellet and vacuum filtrated through a 50 mm cellulose filter (Whatman, No 5). The sample was weighed immediately (fresh

weight) and after 24 h at 60 °C (dry weight). Samples of 0.5 g of the fresh weight was stored at -20 °C for analysis in SDS-PAGE or Westernblot (II.2.7.2).

II.2.6.2 Determination of substrate concentration

The substrate level is an important parameter to evaluate the growth behaviour of the cells and discover potential growth limitations.

Determination of glucose, fructose and sucrose in fermentation supernatants was performed with the "Sucrose, D-Glucose, D-Fructose" kit (II.1.3) according to the manufacturers' protocol.

II.2.6.3 Determination of Phosphate

Phosphate together with carbon and oxygen is an important nutrient for biological systems and determination of phosphate level in the supernatant of BY-2 cells reveals growth behaviour and growth limitations in fermentation processes.

The concentration of phosphate was determined by measurement of phosphor-molybdate. Hundred µl of supernatant (II.2.6.1) was mixed with 200 µl 10 % (w/v) ascorbic acid, 400 µl molybdate solution and filled to 10 ml with dH_2O. Reaction was stopped exactly 15 min after addition of the molybdate solution and absorbance was measured at A_{680nm} using the microplate reader Spectra Max 340 (Molecular Devices). For quantification samples were compared with a calibration curve of a dilution series (2.448 – 0.061 g/L) of phosphate. Calibration solution was prepared by solving 109.9 mg KH_2PO_4, dried at 75 °C o/n, in 500 ml of acidified dH_2O [0.4 % (v/v) of H_2SO_4 (40 %)].

Molybdate solution

$NH_4MO_7O_{24}\cdot 4\ H_2O$	2.20 mM
$K(SbO)C_4H_4O_6\bullet 0.5\ H_2O$	0.21 mM
Sulphuric acid [40 % (v/v)]	60 % (v/v)

II.2.6.4 Downstream processing of 100-L fermentation

For the purifcation of $^{BY\text{-}2}$2F5 antibody from 100-L fermentation broth, a process involving five **f**ull **t**ime **e**mployees (**FTE**) was implemented.

Purification of 100-L fermentation broth was carried out in two batches. Five-L samples were taken out of the fermenter and BY-2 cells were separated by vacuum filtration with one layer of Miracloth membrane 1R (Calbiochem-Novagen, Merck Biosciences). The BY-2 pellet was washed once with an equal volume of dH_2O and resuspended in 1 - 2 volumes of 200 mM borate buffer (pH 8.5) containing 5 mM EDTA and 5 mM β-Mercaptoethanol.

Cell disruption was performed in the Microfluidizer M110-L (Microfluidics Corporation) at lowest adjustable pressure (150 – 600 bar) and the homogenate was collected in 40-L NALGENE® closed dome tanks (Neerijse). Cells debris were removed by passing the homogenate through the CARR Powerfuge® P6 (CARR Separations Inc.) with 300 - 500 ml/min. The resulting supernatant was additionally clarified in a Hollow Fiber unit (UFP-500-K-4X2TCA, Amersham Pharmacia). The preparation was applied onto the 400 cm^2 Hollow Fiber column (500 kDa cut-off) with 30 - 35 ml/min o/n. The concentrated retentate was loaded onto a XK 26/20 column (Amersham Pharmacia) packed with 10 ml of Protein-A (BioSEPRA Protein-A Ceramic HYPERD F; Ciphergen) with 10-30 ml/min and eluted with pH-shift (II.2.5.2). Different elution fractions were tested for the presence of functional protein by SPR analysis (II.2.7.6).

II.2.7 Protein analysis

II.2.7.1 Quantification of proteins

The concentration of purified protein was determined by Bradford (BRADFORD 1976). Coomassie brilliant blue G-250, used in Bradford test, exists in three forms: cationic (470 nm red), neutral (650 nm green), and anionic (595 nm blue). The binding of the dye to protein causes a shift in the absorption maximum of the dye from 470 to 595 nm. The increase in absorption at 595 nm was monitored. Concentration measurements were done according to the manufacturers' instructions, using BSA as standard.

II.2.7.2 SDS-PAGE and Westernblot analysis

Discontinuous SDS-polyacrylamide gels (LAEMMLI 1970) were used for separation of protein samples.

Purified proteins (50 ng/ml – 1 µg/ml) were separated on 4-12 % (w/v) Bis-Tris NuPAGE™ gels (Invitrogen; II.1.2.1) under reducing conditions or on home-made 8 % (w/v) SDS-PAGE under non-reducing conditions (II.1.2.1). Proteins were visualised by staining with 0.25 % (w/v) Coomassie brilliant blue (II.1.2.1). For estimating molecular weights the SeeBlue Plus2 marker (Invitrogen) for reducing gels, the unstained standard MARK12™ (Invitrogen) and the "prestained protein marker broad range" (NEB) for non-reducing gels was used.

For Westernblot analysis proteins were electro-transferred to nitrocellulose membranes (Amersham Bioscienes). Membranes were blocked for 1 h at RT or at 4 °C o/n with 5 % (w/v) skimmed milk dissolved in PBS.

rsCD64 was detected with either anti-c-myc (1:5000), anti-his (1:5000), M22 (1:500) or H22 (1:2000) as primary antibody and corresponding anti-human or anti-mouse AP-coupled

secondary antibodies (αH^{AP}, αM^{AP}, II.1.4). Both, primary and secondary antibodies, were diluted in PBST (II.1.2.1) containing 0.5 % (w/v) skimmed milk. Bound full-size antibody 2F5 and 2G12 were detected by polyclonal $G\alpha H^{AP}$-antibodies (heavy and light-chain specific, 1 h, RT, 1:5000 diluted; Sigma).

Proteins were visualised by incubation with **N**itro **B**lue **T**etrazolium chloride/5-**b**romo 4-**c**hloro**i**ndol-3-yl **p**hosphate (**NBT-BCIP**, Pierce). Between the incubation steps membranes were washed three times with PBST.

II.2.7.3 Dot blot analysis

Twentyfive µl of supernatant containing soluble full-size antibody or 5 µl of homogenised BY-2 cells (II.2.3.5) were dotted onto Immobilon-P PVDF membrane using a self-made dot blot apparatus (Dr. T. Rademacher, BioVII, RWTH-Aachen). Immobilized antibody was detected as described (II.2.7.2).

II.2.7.4 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA (ENGVALL and PERLMAN 1971; CLARK and ADAMS 1977) analysis was used for quantification of purified rsCD64 and plant-expressed $^{BY\text{-}2}$2F5.

For quantifiaction or rsCD64 Microtitre plates (High-binding; Greiner Bio-One GmbH) were coated with approximately 1 µg/ml rsCD64 in 50 mmol/l bicarbonate buffer (pH 9.6) o/n at 4 °C. The plates were blocked with 5 % (w/v) skimmed milk dissolved in PBS for 1 h. Anti-HIV hIgG1/κ and mIgG1/κ anti-CA19.9 antibody as well as humanised anti-CD64 IgG1/κ (H22) were applied as two-fold serial dilutions starting with 1 µg/ml in PBST. Secondary antibodies [$G\alpha H^{AP}$(γ-chain), Sigma) / $G\alpha M^{AP}$(Fc), Sigma)] were diluted 1:5000 in PBST. Bound antibody was detected by incubation with nitrophenyl phosphate. Between incubation steps plates were washed once with PBST and two times with PBS. The OD_{405} was read using a microplate reader Spectra Max 340 (Molecular Devices) and data were evaluated with Microcal Origin 5.0.

For quantification of $^{BY\text{-}2}$2F5 plant-derived antibody Microtitre plates (High-binding; Greiner) were coated with 400 ng/ml ARP 7073 peptide (II.1.4.2) or GαH-Fab serum (Sigma) diluted 1:500 per well in coating buffer o/n at 4 °C. Two-fold serial dilutions of ^{CHO}2F5 (Polymun, Vienna) and $^{BY\text{-}2}$2F5 were applied to the coated and blocked wells, starting with 400 ng/ml of the ^{CHO}2F5. Bound antibodies were detected with polyclonal $G\alpha H^{AP}$-antibody (heavy-chain specific, 1:5000 diluted; Sigma). The ELISA was developed with 1 mg/ml *p*-nitrophenyl phosphate in AP buffer (II.2.7.2). Between incubation steps, plates were washed one time with PBS containing 0.05 % (v/v) Tween 20 and two times with PBS.

Resulting absorption was measured at A_{405nm} and evaluated using Microcal Origin 5.0. Data were displayed in graphs presenting the dilution/concentration on the x-axis and the absorption at 405 nm on the y-axis. The gradient of the linear range of each dilution series is proportional to the increase of the dilution of the known sample. Concentrations of assembled and functional antibody were calculated with the following formula:

$$C_{Sample} = \frac{m(S)}{m(C)} \cdot C_{Control} \cdot V$$

C_{Sample} = concentration of samples
m(S) = gradient of regression line of samples
m(C) = gradient of regression line of control antibody
$C_{Control}$ = concentration of control antibody
V = used volume

II.2.7.5 Electrophoretic mobility shift assay (EMSA)

The electrophoretic mobility shift assay (ANDREWS 1986) was performed according to Coligan *et al.* (1998).

The electrophoretic mobility of 1.6 µg of ARP 7073 peptide bound to 1 µg of purified antibody (at a ratio of 100:1) was compared with the electrophoretic mobility of purified antibody (^{CHO}2F5 and $^{BY\text{-}2}$2F5) and ARP 7073 peptide alone. For complex formation ARP 7073 peptide and antibody were incubated at 28 °C for 30 min.

The protein samples were diluted in 100 mM phosphate buffer (pH 6.5) containing 5 % (w/v) sucrose, loaded on native 10 % PAA gels, prepared with the same buffer and run at 100 V for 3 h at 4 °C. Proteins were visualised by Coomassie blue staining.

4x Phosphate Gel Buffer (pH 6.5)

$NaH_2PO_4xH_2O$	400 mM

Protein Loading Buffer

Sucrose	5 % (w/v)
In dH_2O	

PO_3 Nondenaturing PAA-Gel (10 %)

PAA	13.3 ml
4x phosphate gel buffer	10 ml
H_2O	16.48 ml
10 % (w/v) APS	0.2 ml
TEMED	0.02 ml

II.2.7.6 Surface plasmon resonance (SPR) analysis

Binding analysis of $^{BY\text{-}2}$2F5 and determination of kinetic constants of ^{293T}rsCD64 were calculated with SPR (MALMQVIST 1993) using BIACORE® 2000. This method allows real-time measurements of molecule interactions. The reaction partner were (covalently or none covalently) bound onto the chip-surface. Interaction leads to adsorption at the surface changing the fraction index which is displayed as **r**esonance **u**nits (**RU**). Data are presented in a sensogram placing the signal over time.

All injected samples were diluted in HBS buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005 % Surfactant P20) and subjected to centrifugation prior to injection (10 min/ 14000 g/ 4 °C) to remove insoluble components.

For antibody-capturing 100 µg/ml Protein-A was immobilized on a CM5-rg sensorchip following the standard EDC/NHS protocol (Biacore; (JOHNSSON *et al.* 1991)). After immobilisation the surface was conditioned with three 15 second pulses of 100 mM HCl. The fraction of active binding sites was determined by capturing about 1000 RU of ^{CHO}2F5 or $^{BY\text{-}2}$2F5 followed by injection of 50 µg/ml of peptide ARP-7073 using the kinetic mode. The assay was performed at 25 °C using a flowrate of 30 µl/min. The surface was regenerated by a 15 second injection of 30 mM HCl.

For detection of ^{293T}rsCD64 first hCG (II.1.4.2) was immobilised on the CM5-rg sensor chip. Secondly, cPIPP-IgG1 (kindly provided by M. Sack, BioVII, RWTH-Aachen) was bound to the immobilised hCG. In a third step, ^{293T}rsCD64 was injected to determine hte kinetic rate constants and active protein concentration.

The data were analysed using the BIAevaluation (3.0) software.

II.2.7.7 DsRed fluorescence

The fluorescence of the reporter gene DsRed (II.1.7.4) was controlled during incubation of transient infiltrated tobacco leaves (II.2.3.4) and in transgenic BY-2 (II.2.3.5) with an LCD lamp with glass fiber optic (Leika KL1500), an additional excitation filter for green excitation (BP545/30) and a colour foil (No. 182, light red). Strongest fluorescing cultures were used for scale-up expression and the accumulation of recombinant protein was determined by Dotblot (II.2.7.3).

II.2.7.8 Mass Spectrometry

SDS-PAGE of the target protein rsCD64 was performed (II.2.7.2). After Coomassie-staining (II.2.7.2), the protein band of interest was excised from the gel, the gel slice cut into small pieces and transferred to an Eppendorf tube. The in-gel reduction, alkylation and tryptic digestion was performed according to an in-gel-digestion protocol of EMBL Analytical Research Group (EMBL) and samples were analyzed by liquid chromatography electrospray ionization mass spectrometry.

III. Results

In this thesis two complex human proteins were expressed and characterised in heterologous expression hosts.

The first chapter of the result section focuses on human anti-HIV antibodies expressed in plant suspension cells. The broadly neutralising human anti-HIV antibodies 2F5 and 2G12 were already expressed in CHO cells and in clinical phase I, the safety, non-toxicity and non-immunogenicity of both antibodies was shown (ARMBRUSTER *et al.* 2002). Previously generated tobacco suspension cells BY-2 expressing the anti-HIV antibody $^{BY\text{-}2}$2F5 or $^{BY\text{-}2}$2G12 had to be evaluated in small-scale expression and purification. The integrity, stability and purity of the antibody preparation was tested and antigen binding activity was determined. Initial fermentations with one of the antibodies aimed in a feasibility study of a 100-L fermentation and establishment of a large-scale downstream processing protocol.

The second chapter focuses on the establishment of an expression and purification protocol for soluble expression of recombinant FcγRI (rsCD64). The therapeutic value of FcγRI as a target in tumor therapy, allergic reactions and leukaemia was extensively reviewed in the literature, and availability of a recombinant soluble form might allow in detail characterisation of the high-affinity receptor for monomeric IgG. As production of recombinant soluble FcγRI was postulated to be impossible (SONDERMANN and OOSTHUIZEN 2002), the expression of rsCD64 was optimised by investigating *E.coli*, mammalian and plant cells. The rsCD64 gene was amplified, cloned into three different expression vectors and expression levels were compared. Production protocols in the most suitable system were established and purification of functional protein will be performed to determine the stability as well as the IgG binding property.

III.1 Expression and characterisation of the $^{BY-2}$2F5 antibody

One of the human anti-HIV antibodies $^{BY-2}$2G12 and $^{BY-2}$2F5 ought to be expressed on a 100-L scale as a feasibility study. To initiate this, expression and purification protocols were established based on 1-L culture volumes for the $^{BY-2}$2F5. The cloning into the pTRA-rfp vector (II.1.7.4) and the generation of a 2F5 producing BY-2 suspension culture (II.2.3.5) was done by M. Bomble (BOMBLE 2004). Accumulation of ^{BY2}2F5 peaked at 30 % (v/v) PCV reaching 2.9 mg/kg fresh weight. No antibody was detectable in the culture supernatant, confirming the efficiency of SEKDEL mediated ER-retrieval (BOMBLE 2004).

III.1.1 Small scale (1.2-L) expression and purification

Small-scale expression was performed in 3x 400 ml culture contained in 1-L baffled shake flasks. Cells (340g) were harvested 8 days after "sub"-culturing and stored at –20°C. For purification purposes the cell pellet was thawn, homogenised in extraction buffer and cell debris were removed by centrifugation (II.2.3.5). SPR (II.2.7.6) was used to monitor the successive purification of $^{BY-2}$2F5 on-line during all steps on a Protein-A ligand (data not shown). The purification process was also followed by SDS-PAGE in a 10 % (w/v) gel (Fig.III-1).

No significant amounts of antibody were detected in the flow-through or the washing fractions. The affinity-purified antibody preparation displayed two distinct major bands of approximately 49 and 28 kDa corresponding to the $^{BY-2}$2F5 heavy and light-chain.

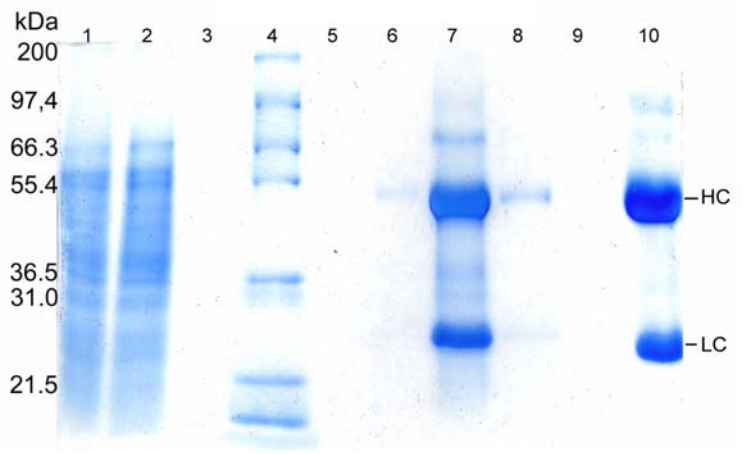


Fig.III-1: Monitoring of the purification process of $^{BY-2}$2F5 by SDS-PAGE. Transfected BY-2 cells were cultured and harvested (II.2.3.5). Cell pellet was thawn for purification, resuspended in extraction buffer (II.1.2.4) and homogenised (II.1.8)]. Cell debris were centrifuged (30 min / 4000 g / 4 °C) and clear supernatant was purified via Protein-A (II.2.5.2). Successive samples during purification were applied onto 10 % (w/v) SDS-PAGE and stained with Coomassie (II.2.7.2). Lanes: 1 = Protein-A load, clear supernatant of homogenised BY-2 cells; 2 = flow through of non-bound material through Protein-A column; 3 = washing fraction with PBS containing 5 mM EDTA; 4 = unstained protein standard marker (Mark12^{TM}, Invitrogen); 5-9 = subsequent elution fractions with 100 mM glycine and 10 % (w/v) maltose; 10 = 11 µg of ^{CHO}2F5; HC = heavy-chain of ^{CHO}2F5; LC = light-chain of ^{CHO}2F5.

^{BY2}2F5 was enriched more than 500-fold and 1.1 mg were retrieved from 340 g (wet-weight) of BY-2 cells giving a yield of 3.2 mg/kg or 0.92 mg/L suspension culture. The purified antibody was intact and contained only minor amounts of impurities. After extensive dialysis and subsequent centrifugation the degree of purity increased further probably due to precipitation of contaminants (Fig.III-2).

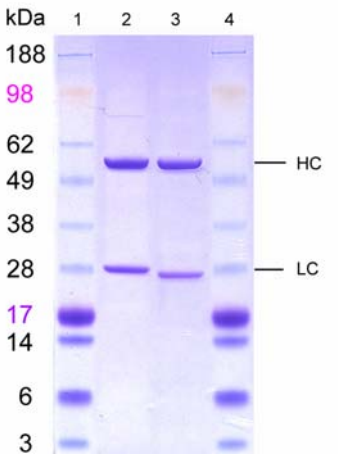


Fig.III-2: Reduced SDS-PAGE of dialysed $^{BY-2}$2F5 versus ^{CHO}2F5. Preparation of Protein-A purified $^{BY-2}$2F5 was subsequently dialysed and centrifuged (30 min / 30,000 g / 4 °C) to remove precipitates of contaminants. Integrity and purity of $^{BY-2}$2F5 was compared with the control antibody ^{CHO}2F5 on a 4-12 % (w/v) Bis-Tris NuPAGE^{TM} gel stained with Coomassie (II.2.7.2). Lanes: 1, 4 = prestained protein marker (SeeBlue Plus2, Invitrogen); 2 = 1 µg of Protein-A purified and dialysed $^{BY-2}$2F5; 3 = 1 µg of ^{CHO}2F5; HC = heavy-chain of 2F5; LC = light-chain of 2F5.

Degradation pattern can be revealed by specific detection of the $^{BY-2}$2F5 preparation in light-(κ)- and heavy-(γ)-chain Westernblots. Neither the γ-chain nor the κ -chain showed any protein degradations (Fig.III-3).

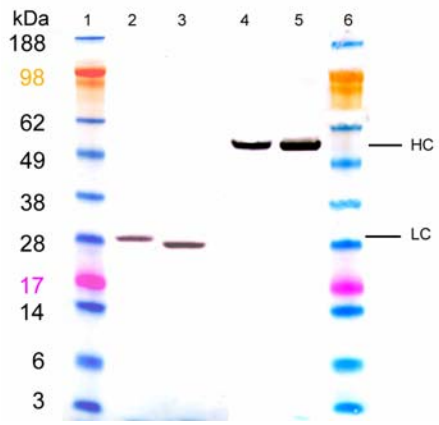


Fig.III-3: Reduced κ-chain and γ-chain Westernblot of $^{BY-2}$2F5 versus ^{CHO}2F5. $^{BY-2}$2F5 preparation purified with Protein-A and dialysis was checked for degradation in a reduced Westernblot analysis (II.2.7.2) versus the control antibody ^{CHO}2F5. 50 ng of each antibody were applied onto 4-12 % (w/v) Bis-Tris NuPAGE^{TM} gels and blotted onto nitrocellulose membrane (II.2.7.2). The Westernblots were detected with GαH^{AP} antibodies [1:5000; γ- and κ-chain specific; (II.1.4)]. Lanes: 1, 6 = prestained protein marker (SeeBlue Plus2, Invitrogen); 2 = 50 ng of $^{BY-2}$2F5 detected with GαH^{AP} (κ-chain specific); 3 = 50 ng of ^{CHO}2F5 detected with GαH^{AP} (κ-chain specific); 4 = 50 ng of $^{BY-2}$2F5 detected with GαH^{AP} (γ-chain specific); 5 = 50 ng of ^{CHO}2F5 detected with GαH^{AP} (γ-chain specific); HC = heavy-chain of 2F5; LC = light-chain of 2F5.

The electrophoretic mobility of the ^{BY2}2F5 LC is slightly lower than that of the ^{CHO}2F5 because of the C-terminal addition of the SEKDEL sequence. The electrophoretic mobilities of the HC do not show significant differences probably due to the smaller relative size difference or the different N-glycans. While the ER-retained ^{BY2}2F5 harbours high-mannose type N-glycans, the secreted ^{CHO}2F5 contains mammalian complex-type N-glycans. Non-reducing denaturing SDS-PAGE and Westernblot was performed to investigate the state of assembly of plant and mammalian produced 2F5 (Fig.III-4).

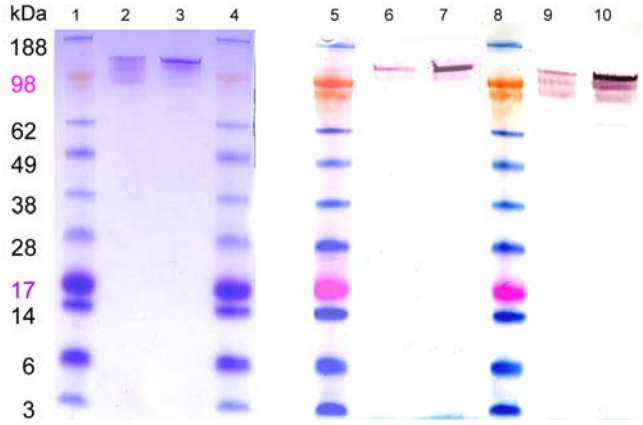


Fig.III-4: Non-reduced SDS-PAGE (A) and non-reduced heavy and light-chain Westernblot of $^{BY-2}$2F5 versus ^{CHO}2F5 (B). Protein-A purified $^{BY-2}$2F5 and control antibody ^{CHO}2F5 were checked for state of assembly in non-reduced SDS-PAGE and Westernblot analysis (II.2.7.2). For each antibody 1 µg was applied onto a 4-12 % (w/v) Bis-Tris NuPAGE^{TM} gels and stained with Coomassie. For each antibody 50 ng were applied onto 4-12 % (w/v) Bis-Tris NuPAGE^{TM} gels and blotted onto nitrocellulose membrane (II.2.7.2). Detection was carried out with GαH^{AP} antibodies [1:5000; γ- and κ-chain specific; (II.1.4)]. Lanes: 1, 4, 5, 8 = prestained protein marker (SeeBlue Plus2, Invitrogen); 2 = 1 µg of $^{BY-2}$2F5; 3 = 1 µg of ^{CHO}2F5; 6 = 50 ng of $^{BY-2}$2F5 detected with GαH^{AP} (κ-chain specific); 7 = 50 ng of ^{CHO}2F5 detected with GαH^{AP} (κ-chain specific); 9 = 50 ng of $^{BY-2}$2F5 detected with GαH^{AP} (γ-chain specific); 10 = 50 ng of ^{CHO}2F5 detected with GαH^{AP} (γ-chain specific).

No differences were found, showing that the antibody was efficiently assembled within the ER of BY2 cells. The GαH^{AP} (κ-chain specific) antibody detected the same three-band pattern (weakly visible in the Westernblot, Fig.III-4B).

III.1.2 Large Scale (7-L - 100-L) expression and purification

In order to express and purify one of the clinical relevant anti-HIV antibodies 2G12 or 2F5 in a 100-L feasibility study, protocols for expression and purification established in shake flasks cultures were first transferred to a 7-L fermentation.

III.1.2.1 7-L fermentation

One fermentation was carried out with BY-2 cells expressing $^{BY-2}$2G12 (II.1.2.4). Cloning of the 2G12 full-size antibody into the pTRAkc vector was done by M. Bomble (BOMBLE 2004). The final construct (pTRAkc-GD) contained the heavy- and light-chain of the human

antibody 2G12 and the DsRed gene and transient expression in *N.tabacum* yielded 77.8 mg/kg (BOMBLE 2004).

The $^{BY-2}$2G12 producing BY-2 suspension culture was developed by Dr. T. Rademacher (BioVII, RWTH-Aachen, II.2.3.5). Cultivation of BY-2 cells and supply of 500 ml 7-d-old BY-2 culture was performed by Dr. F. Schuster (BioVII, RWTH-Aachen) to inoculate the 7-L bioreactor.

The bioreactor was equipped as described (II.2.6) and data collection was started after inoculation with 5 % (v/v) BY-2 suspension culture. This initial fermentation was carried out without antifoam for maximal cell growth of BY-2 cells. Time flow of the fermentation included collected on-line and evaluated off-line data is presented in Fig.III-5.

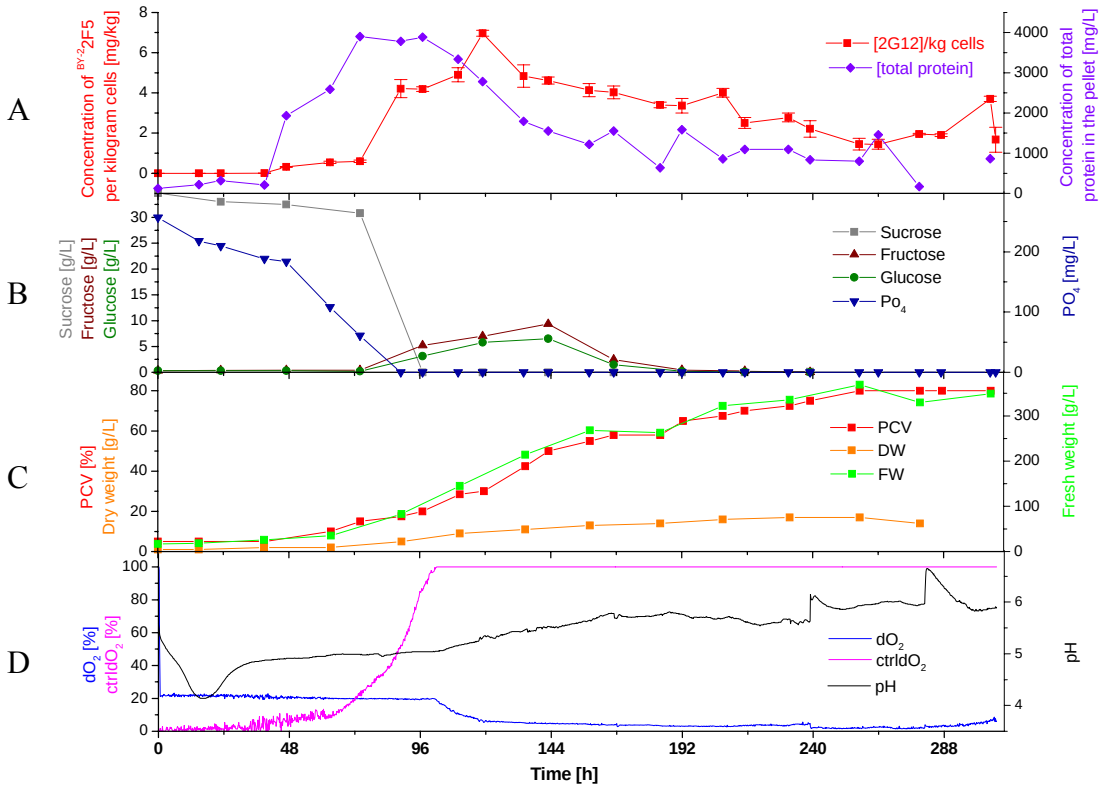
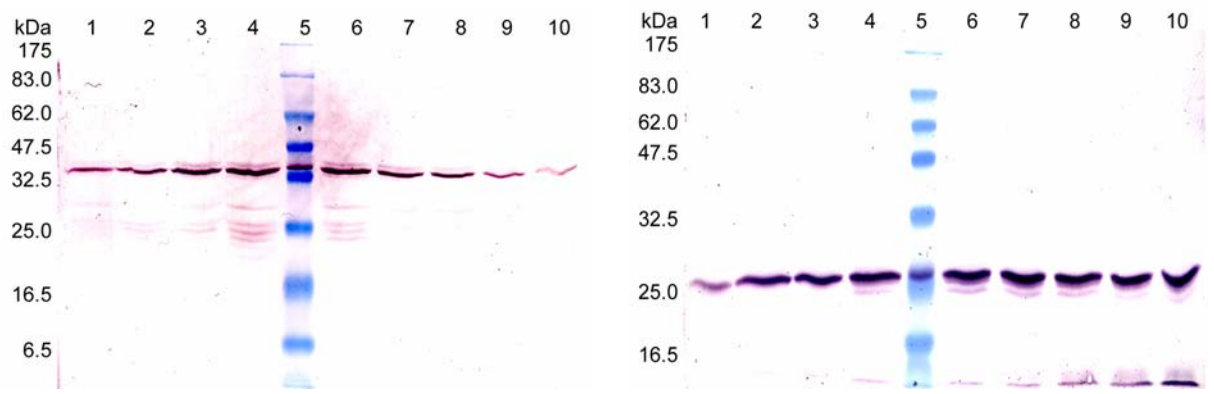


Fig.III-5: Batch fermentation of human anti-HIV antibody $^{BY-2}$2G12 in 7-L glass reactor. BY-2 cells expressing the anti-HIV antibody $^{BY-2}$2G12 were cultured in suspension in a 7-L glass reactor. Data for dissolved oxygen, control of oxygen and pH were collected on-line. Samples of supernatants and cell pellets were taken every 8-24 h (II.2.6.1) to determine off-line data such as packed cell volume, dry weight, fresh weight, substrate and phosphate concentration, expression yield of $^{BY-2}$2G12 and total protein concentration. On-line data: dO_2 = level of dissolved oxygen; $ctrldO_2$ = control of dO_2; pH = pH value in the media; Off-line data (II.2.6.1): PCV = packed cell volume; DW = dry weight; FW = fresh weight; Sucrose, Glucose, Fructose = sugar consumption in fermentation (II.2.6.2); PO_4 = Phosphate consumption (II.2.6.3); [2G12]/kg cells = concentration of human anti-HIV $^{BY-2}$2G12 antibody per kilogram cells; [total protein] = concentration of total protein in the BY-2 pellet determined by Bradford (II.2.7.1).

pH was not controlled during the fermentation and showed a typical time course (Fig III-19D, black line). During the first 24 h the pH dropped more than one unit from 5.8 to 4.5 and increased steadily to pH 6 over the next 150 h due to release of stored ions and uptake of nutrients such as ammonium (PAIVA 1999). With a maximal air input of 0.1 vvm of air (FigIII-19D, magenta line) the amount of dissolved oxygen was maintained at 20 % (v/v) for the lag- and the initial log-phase of the BY-2 culture but decreased to 1.5 % (v/v) during exponential growth of the cells (Fig-III-19D, blue line). The increase of biomass was illustrated by determining the packed cell volume (C, red line and squares), fresh cell weight (C, green line and squares) and dry cell weight (C, orange line and squares; II.2.6.1). After an initial lag-phase of approximately 24 h, constant cell growth was observed for 200 h. With a 5 % (v/v) inoculum, fermentation of 300 h resulted in a yield of 350 g fresh cell weight per litre reactor volume and an exceptional PCV of 80 % (v/v).

Phosphate- and sugar levels in the culture supernatant were determined to document the course of fermentation (II.2.6.2; II.2.6.3). Both nutrients were depleted after 90 h (FigIII-19B, blue down triangles; grey line and squares). The sucrose in the media was hydrolysed by BY-2 cells into the monosaccharides glucose and fructose (PAIVA 1999). Therefore levels of glucose (B, olive line and circles) and fructose (B, wine line and up triangle) increased in the early log-phase of the fermentation, whereas sucrose levels decreased.

Recombinant human $^{BY\text{-}2}$2G12 was constitutively expressed during the first 120 h of fermentation and detected during the whole fermentation process, as shown in Fig.III-5A (red line and squares) and in Westernblot analysis (Fig.III-6). Antibody expression level per kilogram cells was determined by ELISA (II.2.7.4; II.1.4). For Westernblot analysis and ELISA frozen pellet samples (II.2.6.1) were thawn and homogenised (II.2.3.5). Clarified supernatant was analysed first in Dotblots [(II.2.7.3); data not shown] to preselect samples containing protein for the Westernblot (II.1.4). $^{BY\text{-}2}$2G12 accumulated in the first 120 h of the fermentation to a maximum concentration of 7 mg/kg cells (FigIII-19A, red line and squares) and decreased slowly until the end of the fermentation to 2 mg/kg cells. For a maximum of antibody (7 mg/kg), the fermentation should have been terminated after 120 h. The Westernblot revealed a decrease in the amount of heavy-chain (Fig.III-6A), whereas the amount of the light-chain accumulated over the whole fermentation process (Fig.III-6B). The total protein concentration (Fig.III-5A, violet line and diamonds), determined by Bradford analysis (II.2.7.1) of the homogenised pellet samples (II.2.3.5) increased to 4 g/L in the first 70 h indicating the start of cell activity.



A B

Fig.III-6: Westernblot of $^{BY\text{-}2}$2G12 expression in a 7-L fermentation. Samples of BY-2 cells were taken every 8-24 h and stored at -20 °C (II.2.6.1). For analysis cell pellet was thawn, resuspended in extraction buffer (II.1.2.4) and sonicated (II.1.8). Cell debris were centrifuged (30 min / 4000 g / 4 °C) and clear supernatant was applied onto 12 % (w/v) reducing SDS-PAGE and blotted onto nitrocellulose membrane (II.2.7.2). A: Detection with γ-chain specific GαH^{AP} antibody (1:5000); B: Detection via κ-chain GαH^{AP} antibody (1:5000; II.2.6.2). Lanes: 1 = 38 **h**ours **p**ost **i**noculation (**hpi**); 2 = 63 hpi; 3 = 89 hpi; 4 = 110 hpi; 5 = prestained protein marker broad range (NEB) with 100 ng ^{CHO}2G12; 6 = 134 hpi; 7 = 158 hpi; 8 = 184 hpi; 9 = 207 hpi; 10 = 231 hpi.

Human full-size anti-HIV antibody $^{BY\text{-}2}$2G12 was expressed in a 7-L bioreactor. Expression level peaked at 7 mg/kg cells in the early growth phase. Light- and heavy-chain of the antibody showed minor degradations during the whole fermentation process.

Cells started to grow on nearly all surfaces in the bioreactor and cumulated on the media surface. The fermentation was repeated in the presence of 0.01% (v/v) Pluronic (data not shown) to achieve more homogeneous cell growth. Except for overall reduction of cell growth no differences were seen in comparison to the previous fermentation. Cells were more homogeneous building micro-aggregates of 2-4 cells even to a PCV of 80 % (v/v) with comparable antibody production rate (data not shown).

Proof of functional expression of $^{BY\text{-}2}$2G12 (binding to gp120) failed both in SPR and ELISA (data no shown). Thus a new cloning strategy was necessary to exchange an amino acid in the light chain of the 2G12 antibody sequence, which was different from the original sequence (BUCHACHER *et al.* 1994) and might have caused the non-functionality.

III.1.2.2 100-L fermentation

Results from the small-scale expression of $^{BY\text{-}2}$2F5 (3.2 mg/kg or 0.92 mg/L) and initial fermentations yielding in stable $^{BY\text{-}2}$2G12, prompted the feasibility study of a 100-L fermentation. For inoculation of the 140-L reactor the complete fermentation broth of a preceding fermentation in a 7-L reactor was used. This fermentation was carried out in a stainless-steel reactor to enable a transoculation into the 140-L reactor and based on the experience of the previous fermentations Pluronic®L61 anti-foam was added into the fermentation media. The time course of the 100-L fermentation including on-line and

evaluated off-line data is presented in Fig.III-7. The pH was adjusted to 5.8 before inoculation and the temperature was lowered to 28 °C after 3 days at 37 °C as sterile control. The bioreactor was equipped with three six-bladed Rushton impeller and stirrer was operated at 50 rpm to reduce shear forces. To ensure sufficient oxygen transfer the aeration was adjusted from 0.1 vvm after 48 h to 0.2 vvm (Fig.III-7D, blue line). The dO_2 was controlled to keep 20 % (v/v) with 0.2 vvm aeration.

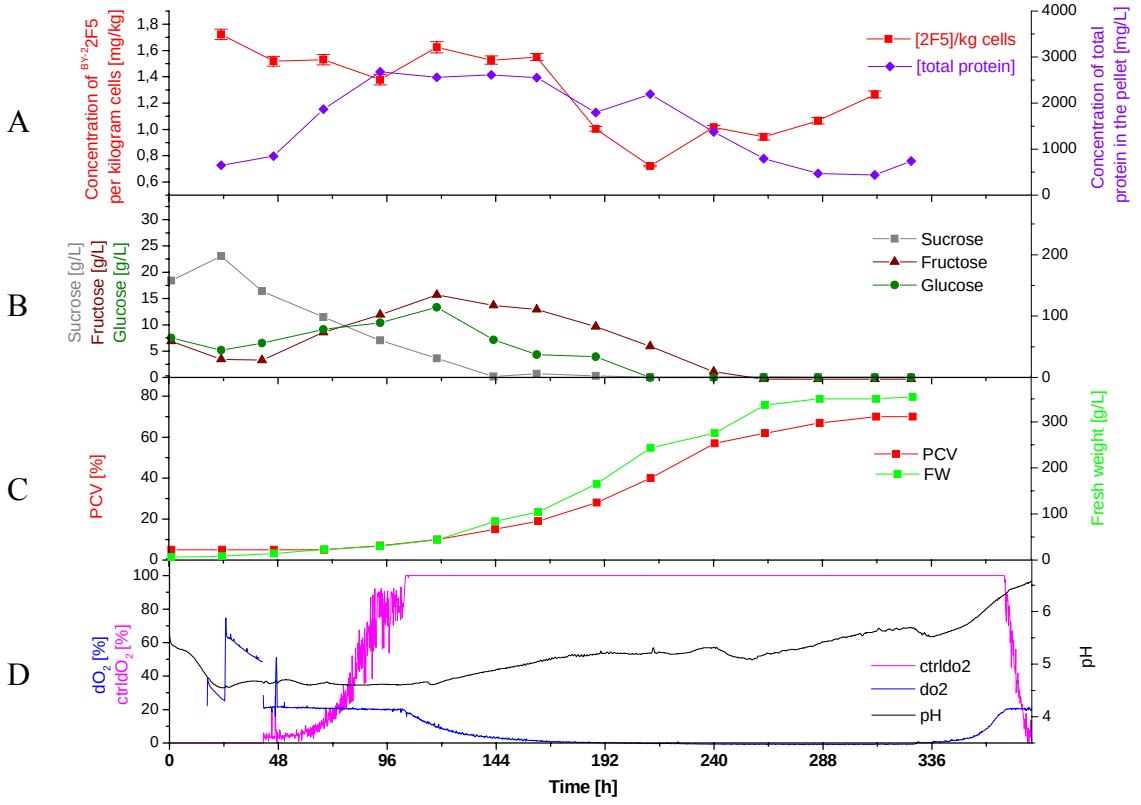
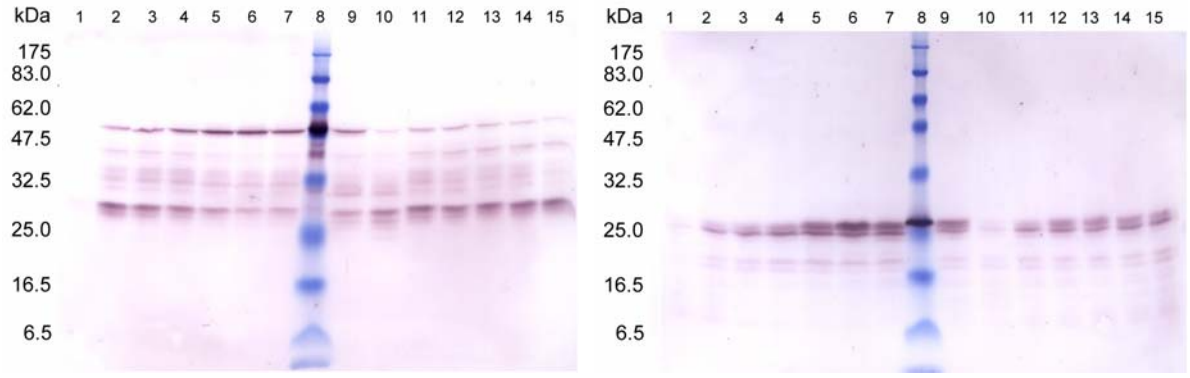


Fig.III-7: Batch fermentation of human anti-HIV antibody $^{BY\text{-}2}$2F5 in 140-L stainless steel reactor (Pilot System). BY-2 cells expressing the anti-HIV antibody $^{BY\text{-}2}$2F5 were cultured in suspension in a 140-L stainless steel reactor. Data for level of dissolved oxygen, control of oxygen and pH value were collected on-line. Samples of supernatant and cell pellet were taken every 8-24 h (II.2.6.1) to determine off-line data as PCV, DW, FW, substrate and concentration, expression yield of $^{BY\text{-}2}$2F5, and total protein concentration. On-line data: dO_2 = level of dissolved oxygen; $ctrldO_2$ = control of dO_2; pH = pH value in the media; Off-line data (II.2.6): PCV = packed cell volume; DW = dry weight; FW = fresh weight; Sucrose, Glucose, Fructose = sugar consumption in fermentation (II.2.6.2); [2F5]/kg cells = concentration of human anti-HIV $^{BY\text{-}2}$2F5 antibody per kilogram cells; [total protein] = concentration of total protein in the BY-2 pellet determined by Bradford (II.2.7.1).

Cell growth (Fig.III-7C) and demand of oxygen, measured in supply of oxygen (Fig.III-7D, magenta line), increased in the first 100 h and concentration of oxygen (Fig.III-7D, blue line) declined in early growth phase to 0 % (v/v) after 180 h. Cell growth was not affected by this oxygen limitation (Fig.III-7C). Sugar was consumed completely after 240h (Fig.III-7B).

Expression of $^{BY\text{-}2}$2F5 antibody in the cells decreased slowly over the fermentation process from 1.8 mg/kg to 1.3 mg/kg cells (Fig.III-7A, red line and squares).

The integrity and stability of the expressed $^{BY\text{-}2}$2F5 antibody in BY-2 cells during the fermentation process was checked in Westernblot analysis (Fig.III-8; III.1.2.1).



A B

Fig.III-8: Reduced Westernblot of $^{BY\text{-}2}$2F5 expression in a 100-L fermentation. Samples of BY-2 cells were taken every 8-24 h and stored at -20 °C (II.2.6.1). For analysis cell pellet was thawn, resuspended in extraction buffer (II.1.2.4) and sonicated (II.1.8). Cell debris were centrifuged (30 min / 4000 g / 4 °C) and clear supernatant was applied onto 12 % (w/v) SDS-PAGE and blotted onto nitrocellulose membrane (II.2.7.2). A: Detection with γ-chain specific GαH^{AP} antibody (1:5000); B: Detection via κ-chain GαH^{AP} antibody (1:5000; II.2.6.2). Lanes: 1 = 1 hpi; 2 = 23 hpi; 3 = 41 hpi; 4 = 68 hpi; 5 = 93 hpi; 6 = 118 hpi; 7 = 143 hpi; 8 = prestained protein marker broad range (NEB) containing 100 ng ^{CHO}2G12; 9 = 162 hpi; 10 = 188 hpi; 11 = 212 hpi; 12 = 240 hpi; 13 = 262 hpi; 14 = 286 hpi; 15 = 327 hpi.

Full-size heavy and light-chain were detected in the 100-L fermentation over the entire fermentation process (Fig.III-8). A double-band pattern was detected for the light chain until the end of the fermentation (Fig.III-8B). The amount of intact heavy-chain decreased over the fermentation process dramatically as cells grow under oxygen limitation and degradation accumulated (Fig.III-8A). At the time of harvest almost no intact heavy-chain was detected in the BY-2 cells. Nevertheless, a downstream processing was established.

III.1.2.3 Large-scale purification

The fermentation broth was processed stepwise in two batches starting after 340 h of fermentation. Successive purification of $^{BY\text{-}2}$2F5 was followed on-line during all steps by SPR (II.2.7.6) on a Protein-A surface and is illustrated in Fig.III-9.

The first 40-L batch for downstream purification was taken in 5-L samples out of the fermenter and cells were harvested by vacuum filtration. Resuspension of the BY-2 pellet was initially performed in one volume borate buffer and later in two volumes due to recurring plugging of the French Press. For the same reason the pressure in the French Press was adjusted from 150 bar to 600 bar. Thus, cells were disrupted into fines as observed in previous experiments (data not shown). Fibers and cell wall substances were solubilised and created a viscous homogenate.

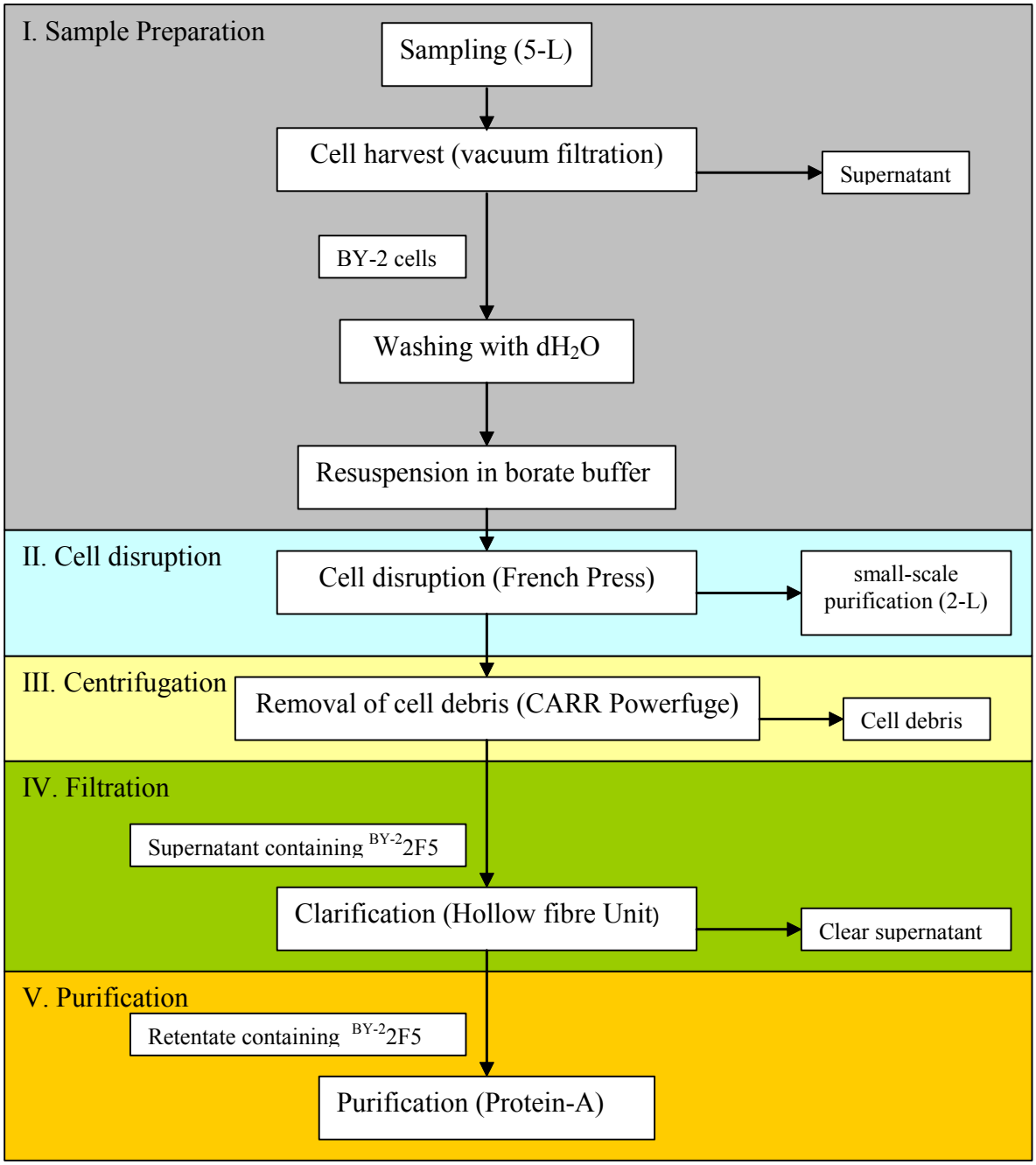


Fig.III-9: Flowchart for large-scale downstream processing. The flowchart illustrates the different steps in the downstream processing of a 40-L batch from the 100-L fermentation (III.1.2.2). 5-L samples were taken out of the fermenter (I) and cells were separated by vacuum filtration with Miracloth membrane (Merck Biosciences). The BY-2 pellet was washed with one volume of dH_2O and resuspended in 200 mM borate buffer. The suspension was disrupted in the Microfluidizer (II). Cell debris were separated in the CARR Powerfuge (III) and the supernatant (containing the antibody) was filtered using a Hollow Fiber Unit (IV). $^{BY\text{-}2}$2F5 antibody was purified on a Protein-A column using the retentate (V).

A 2-L sample was taken for small-scale purification following the established protocol (III.1.1) and the obtained data are summarised in Table.III-2 and Fig.III-10.

The large-scale batch was passed through the CARR Powerfuge and cloudy supernatant was subsequently clarified in a UFP-500-K-4X2TCA Hollow Fiber Unit. Despite the 500 kDa cut-off the antibody was detected in the retentate by SPR (II.2.7.6, data not shown). Hence, retentate was concentrated to 2-L total volume and centrifuged in two steps (1.

60 min/ 30,000 g/ 4 °C; 2. 30 min/ 100,000 g/ RT). Supernatant stored at 4 °C solidified after the first centrifugation. Thus, further purification steps including the ultracentrifugation were performed at RT. The clear supernatant was applied in 4 h (flow rate: 10 ml/min) onto a XK26/20 column packed with 10 ml Protein-A. Flow and elution fraction were tested via SPR (II.2.7.6 (see Table.III-2 and Fig.III-10).

Large-Scale

sample	volume [L]	mAB [mg]	mAB [mg/L]	yield [%]
FrenchPress Homogenate	25,000	38,250	1,53	100
CARR Supernatant	23,000	36,133	1,57	94
Hollow Fibre Filtrat	17,000	3,438	0,20	9
Hollow Fibre Retentat	2,750	32,184	11,70	84
Protein-A Feed	2,000	33,497	16,75	88
Protein-A Flow through	2,000	0,111	0,06	0,3
Eluate 3	0,005	3,350	670,00	9
Eluate 4	0,017	9,181	540,06	24
Eluate 5	0,023	4,268	185,57	11
Eluate 6	0,030	0,348	11,60	1
Eluate total	0,460	17,150	37,28	45

Small-Scale

sample	volume [L]	mAB [mg]	mAB [mg/L]	yield [%]
FrenchPress Homogenate	1,750	2,677	1,53	100
Supernatant after Centrifugation	1,750	2,320	1,33	87
Protein-A Feed	1,750	2,320	1,33	87
Protein-A Flow through	1,750	0,055	0,03	2
Protein-A Wash	0,300	0,005	0,02	0,2
Eluate 1	0,001	0,006	9,69	0,2
Eluate 2	0,001	2,239	1565,73	84
Eluate 3	0,001	0,008	14,21	0,3
Eluate 4	0,001	0,000	0,33	0,0
Eluate 5	0,001	0,000	0,02	0,0
Eluate total	0,002	2,252	1348,50	84

Table III-2: Comparison of antibody yield in small-scale and large-scale downstream processing. The table summarises the concentration of $^{BY\text{-}2}$2F5 antibody detected by SPR (II.2.7.6) after the different purification and concentration steps during purification and compared the yield of the 23-L large-scale purification with the 2-L small-scale processing. Downstream processing steps include French Press (Microfluidizer), CARR Powerfuge, Hollow Fiber Unit and Protein-A column for large-scale purification (II.2.6.4). Small-scale purification was separated after French Press and centrifugation and small-scale Protein-A purification was performed (III.1.1).

The antibody yield in the different purification and concentration steps is summarised in Table.III-2 and illustrated as yield in percent in Fig.III-10. The first antibody concentration measured after homogenisation in the French Press was taken as 100 %. During separation of cell debris in the CARR Powerfuge 6 % of antibody was lost. In the Hollow Fiber filtration step 9 % was detected in the filtrate and 84 % in the retentate. Thus, the retentate was purified further, the filtrate was discarded. Almost 50 % of antibody could not be rediscovered after the Protein-A purification step.

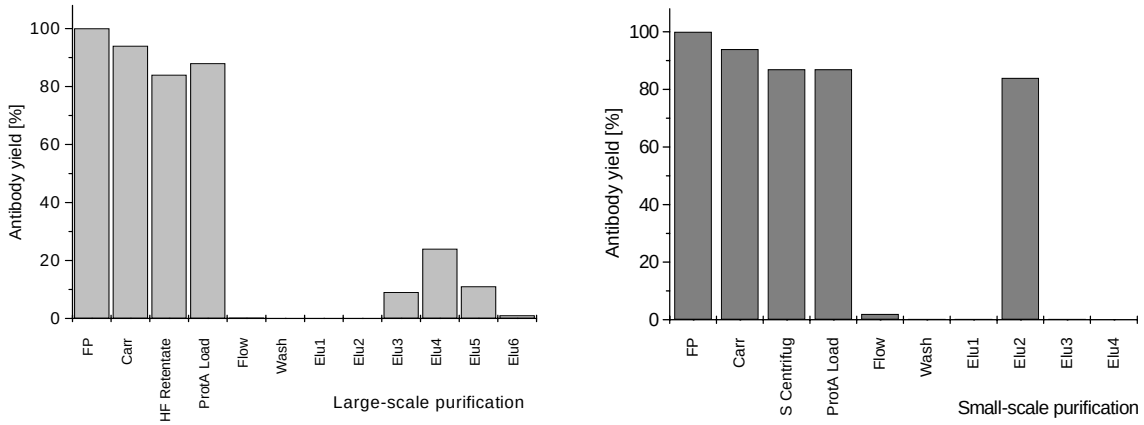


Fig.III-10: $^{BY-2}$2F5 yield in percent during large- und small scale purification. Antibody yield was determined by SPR (II.2.7.6, data not shown). For large-scale samples of the French Press (FP) homogenate, the CARR supernatant (CARR), the Hollow fiber retentate (HF retenate) and the Protein-A (Load, Flow and Elution fractions) are visualised (II.2.6.4). For the small-scale purification, samples of the French Press (FP), the CARR supernatant (CARR), the supernatant after centrifugation (S Centrifuge) and the Protein-A column (load, flow through, washing and elution fractions) are presented (III.1.1).

The small-scale purification was separately purified on a laboratory scale after removal of cell debris in the CARR Powerfuge. Centrifugation of the small-scale sample caused 7 % reduction of antibody yield. During Protein-A purification 84 % were recovered from the column.

Conclusively, an antibody yield of 45 % was achieved in the large-scale purification and 84 % in the small-scale sample. The critical step during large-scale downstream processing was the Protein-A purification as almost 50 % of the antibody disappeared. Only minor amounts of antibody were detected in the other washing fractions (not shown in Table.III-2). Thus, antibody was not completely eluted or denatured during the elution with pH 2.

The second batch was used to test some conditions and possible alternatives in purifying plant derived antibodies. Precipitation of fibers and plant polysaccharides with magnesium sulphate (200 mM $MgSO_4$) was successfully tested in small scale by M. Sack (BioVII, RWTH-Aachen). Magnesium sulphate was added to the fermentation broth at two different steps in the purification process. First BY-2 cells were resuspended with borate-buffer containing 200 mM $MgSO_4$ and homogenised in the Microfluidizer (II in Fig.III-9). Homogenate was centrifuged (30 min / 30000 g/ 4 °C) and stored at RT o/n. The clear supernatant after centrifugation changed colours to deep green but remained clear. BY-2 homogenate contained 0,954 mg $^{BY-2}$2F5/L without magnesium sulphate. After precipitation with magnesium sulphate 90 % corresponding to 0.86 mg/L were detected in the supernatant.

In the second approach plant fibers were precipitated after one CARR centrifugation step (III in Fig.III-9) by addition of 200 mM $MgSO_4$. The supernatant after a second CARR separation was less turbid but changed colour to dark green and precipitate became visible o/n. 91 % of

antibody were detected in the supernatant. Thus, $MgSO_4$ precipitation reveals a feasible method to specifically remove plant fibers without loosing antibody.

Additionally the capacity of the STREAMLINE™ (expanded bed chromatography by Amersham Pharmacia) was tested to directly purify recombinant antibodies out of crude extracts. The STREAMLINE™ column was loaded with 10-L of homogenised BY-2 suspension, but the expanded bed was slugged out at the end (data not shown).

III.1.3 Antigen binding activity of $^{BY\text{-}2}$2F5

Besides establishment of expression and purification protocols for the human anti-HIV antibody 2F5, the characterisation of the binding activity and comparison with CHO produced 2F5 was essential as well. Different types of experiments were performed to compare the antigen binding of $^{BY\text{-}2}$2F5 and ^{CHO}2F5 in detail. In theory all antibody binding sites in the preparation should be able to bind the antigen. Differences of functionality of $^{BY\text{-}2}$2F5 versus ^{CHO}2F5 indicates qualitative varieties in both preparations. For all these assays the peptide ARP7073 containing the epitope ELDKWAS recognised by the 2F5 antibody (II.1.4.2) was used.

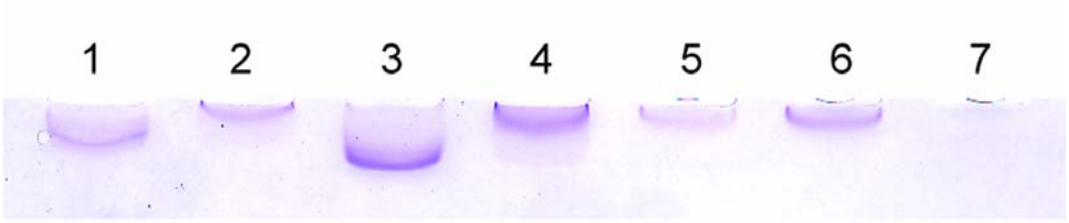


Fig.III-11: Electrophoretic mobility shift assay to determine antigen binding activity of $^{BY\text{-}2}$2F5 and ^{CHO}2F5. $^{BY\text{-}2}$2F5 antibody was purified as described (III.1.1). 1 µg of purified $^{BY\text{-}2}$2F5 antibody and ^{CHO}2F5 control antibody were applied as free antibody and complexed with 3.24 µg of ARP7073 peptide onto the native gels (II.2.7.5). 1 µg of ^{CHO}2G12 was used for negative control as free antibody and mixed with 3.24 µg ARP7073 peptide. Lanes: 1 = 1 µg ^{BY2}2F5; 2 = 1 µg ^{BY2}2F5 in complex with 3.24 µg ARP7073 peptide; 3 = 1 µg ^{CHO}2F5; 4 = 1 µg ^{CHO}2F5 in complex with 3.24 µg ARP7073 peptide; 5 = 1 µg ^{CHO}2G12; 6 = 1 µg ^{CHO}2G12 mixed with 3.24 µg ARP7073 peptide; 7 = 3.24 µg ARP7073 peptide.

An 100 M excess of the peptide ARP7073 was added to the antibody preparations in EMSA assays to reliably saturate all functional binding sites (Fig.III-11). The change in electrophoretic mobility is mainly due to a change of charge in the case of 2F5 (theoretical pI of 9.35) and ARP7073 (theoretical pI of 3,97). The difference in size of free (molecular weight without glycosylation: 149.85 kDa) and complexed (152,28 kDa or 154,71 kDa) antibody is neglectable. The EMSA was performed using PBS buffer at pH 6.5 in order to have near-physiological conditions. Under these conditions 2F5 migrated only a short distance into the gel. Nevertheless, small differences in the electrophoretic mobility of free ^{BY2}2F5 and ^{CHO}2F5 were observed (Fig.III-13, lane 1 and 3). The human anti-HIV antibody

^{CHO}2G12 recognising the antigen gp120 (II.1.4) but not the ARP7073 peptide in gp41 was used as negative control.

When excess of ARP7073 is present (Fig.III-11, lane 2 and 4) a clear shift was seen and no bands remained at the position of free antibody. Therefore both antibody preparations contained no detectable inactive molecules. No differences of free ^{CHO}2G12 and ^{CHO}2G12 mixed with the ARP7073 peptide were observed (Lane 5 and 6, Fig.III-11).

The sensitivity of the assay is however not good enough to reveal a small fraction of inactive or partially inactive molecules, i.e. antibodies with only one active binding site and therefore provides only qualitative or semi-quantitative information.

A quantitative comparison of the antigen binding activities of the plant and mammalian derived antibody preparations was done by ELISA. To achieve this, total and percentage of active antibody concentration was determined on the same plate. Total antibody concentration was determined by using GαH-Fab serum (II.1.4) as capture reagent (anti-Fab-ELISA) and antibody molecules with at least one active binding site were captured to coated ARP7073 (400 ng/ml, antigen-ELISA). This enables the use of GαH-IgG (Fc-specific) secondary antibody for both set-ups, minimizing possible error-sources and allowing the most direct comparison of the two antibody samples.

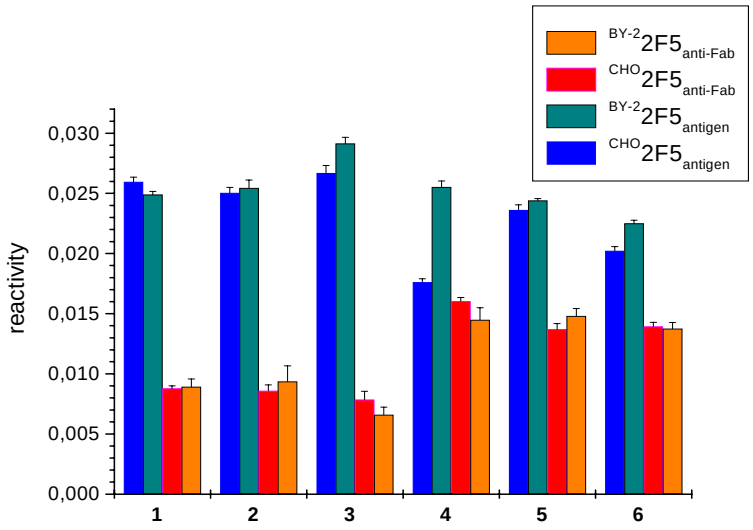


Fig.III-12: Reactivities of ^{CHO}2F5 and $^{BY-2}$2F5 determined in an anti-Fab- and antigen-ELISA. $^{BY-2}$2F5 was purified as described (III.1.1). GαH-Fab and ARP7073 antigen were used as capture reagent for ^{CHO}2F5 and $^{BY-2}$2F5 applied in two-fold serial dilutions (II.2.7.4). Detection was performed with GαH^{AP} (γ-chain specific) antibody (1:5000). The gradient of the linear range of every dilution series is displayed as reactivity of $^{BY-2}$2F5 in anti-Fab-ELISA (orange), ^{CHO}2F5 in anti-Fab-ELISA (red), $^{BY-2}$2F5 in antigen-ELISA (green) and ^{CHO}2F5 in antigen-ELISA (blue). Data column 1-6 represent different positions on the Microtitre plate.

ELISA reactivities obtained for ^{CHO}2F5 and $^{BY-2}$2F5 showed no significant differences while variation was observed between experiments (Fig.III-12). The relative antigen binding

activity of $^{BY-2}$2F5 can be derived by first dividing the peptide binding reactivity by the total antibody binding reactivity and then normalising it to the ratio obtained for ^{CHO}2F5. The mean relative activity of $^{BY-2}$2F5 was 108 % (+/- 20 % SD) of ^{CHO}2F5 and thus identical within experimental limits.

A Biosensor was developed by M. Sack (BioVII, RWTH-Aachen) to directly determine the percentage of active paratopes. The assay is based on first capturing 2F5 to a Protein-A surface followed by injection of the peptide ARP7073 using a peptide concentration high enough to achieve total saturation. The amount of antibody captured to the chip and the amount of peptide can be directly derived from the sensorgram. Moreover the maximal binding capacity is given by the molecular masses of the interacting molecules and the stoichiometry of the interaction. Finally, the sensitivity of the Biacore2000 instrument and the robustness of the assay is high enough to allow precise and accurate determination.

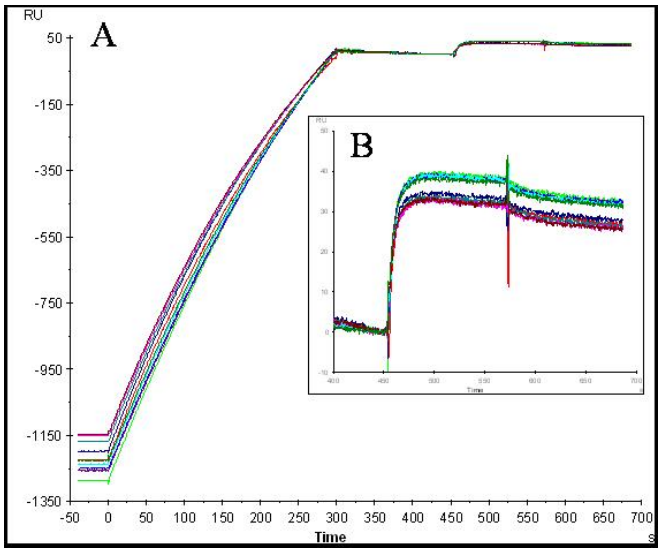


Fig.III-13: SPR assay of ^{CHO}2F5 and $^{BY-2}$2F5 for determination of active paratopes in the antibody preparations (II.2.7.6). $^{BY-2}$2F5 was purified as described before (III.1.1). In the first step 2F5 was bound to immobilised Protein-A. ARP7073 was subsequently injected for analysis. Five repetitive measurements were performed with ^{CHO}2F5 and $^{BY-2}$2F5 and displayed as overlayed sensorgrams over time. RU = resonance units, changes in fraction index due to binding of substances. A = overlay of 5 measurements of binding of ^{CHO}2F5 and $^{BY-2}$2F5; B = overlayed sensorgrams of 5 measurements of dissociation of ^{CHO}2F5 and $^{BY-2}$2F5 (Illustration and image editing provided by M. Sack, BioVII, RWTH-Aachen.).

For both antibody preparations 5 repetitive measurements were performed (Fig.III-13A), the amount of captured antibody and subsequently bound peptide were determined from the sensorgrams and the fraction of active binding sites was calculated (Fig.III-13B). The activity of 2F5 was 97 % (+/- 2 % SD) for the CHO and 88 % (+/- 5 % SD) for the BY2 derived product. Thus the activity of ^{BY2}2F5 was only slightly reduced.

III.2 Construction, expression and characterisation of rsCD64

Recent publications (SONDERMANN *et al.* 1999) describe the expression of the extracellular domain of FcγRII as soluble protein. Therefore, the published cd64 sequence (ALLEN and SEED 1988) was used as template to identify the extracellular domain of cd64 and synthetic oligonucleotides were designed to amplify this. To enable detection of the expressed protein, an anti-c-myc-tag sequence was added to the 3'-end by primer sequence (N- or C-terminal his-tag sequences are present in each of the expression vectors). Figure III-1 illustrates the origin of cd64 and subsequent cloning and expression into *E.coli*, HEK 293T cells and *N.tabacum* leaves.

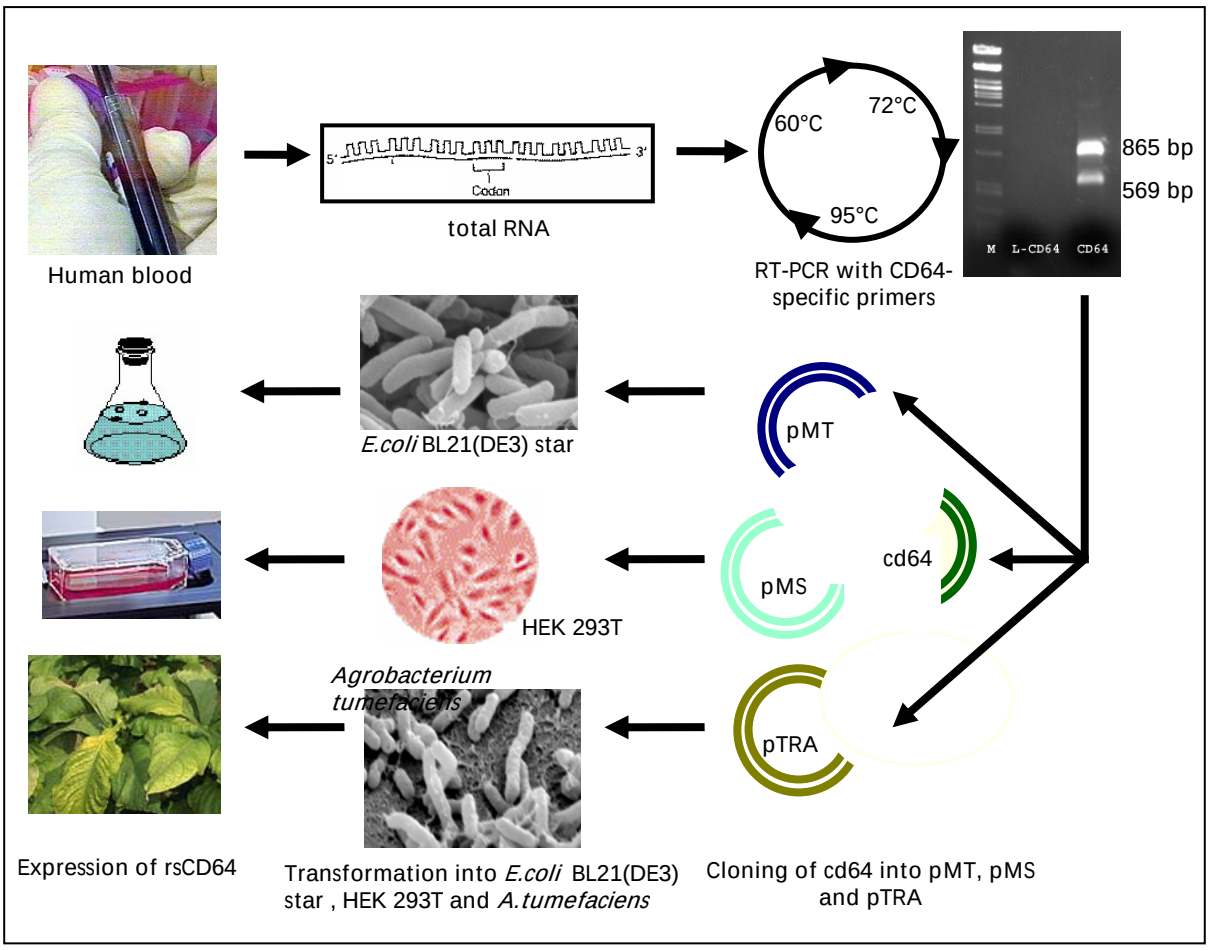


Fig.III-14: Schematic presentation of the cloning and expression of rsCD64 in different expression systems. Total RNA was isolated from human blood (II.2.1.4) and transcribed by RT-PCR (II.2.1.5) with cd64-specific primers (II.1.5) into cDNA. cDNA of cd64 was cloned into three different vectors enabling expression in *E.coli*, HEK 293T cells and *N.tabacum* (II.1.7). pMT = vector for bacterial expression in the bacterial periplasm, derived from pET27b (II.1.7.1); BL21(DE3) = *E.coli* strain for expression of rsCD64 (II.1.6.1); pMS = vector for mammalian expression into the culture supernatant, derived from pSecTag2 (II.1.7.2); HEK 293T = eucaryotic cell line for expression of rsCD64 (II.2.4); pTRA = plant expression vector derived from pBAM (II.1.7.3); *A.tumefaciens* = soil bacterium used for gene transfer into *N.tabacum* (I.2.3); cd64 = DNA sequence of extracellular domain of CD64; rsCD64 = protein of recombinant soluble CD64.

III.2.4 Total RNA extraction from whole blood and cd64-specific RT-PCR

Total RNA was isolated from 2 x 2.5 ml blood (II.2.1.4) and used for reverse transcription and amplification of cd64 (II.2.1.5). Primer pairs annealing at the 5' and 3' end of the extracellular domain [cd64-5'; cd64-cmyc-3', (II.1.5)] were designed and used.

Resulting PCR-products were electrophoretically separated on a 1.2 % (w/v) agarose gel shown in Fig.III-15. The PCR product of cd64 in lane 3 shows the expected size of 865 bp. The cd64 β-form was amplified as well, resulting in a PCR-product of 569 bp. Successive cloning was performed only with the FcγRI α-isoform (Lane 3, I.1.2.2).

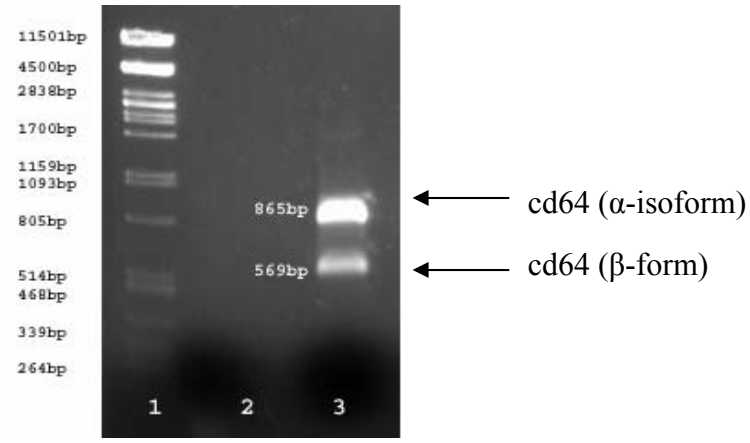


Fig.III-15: Agarose electrophoresis of RT-PCR product of amplified cd64. RT-PCR was performed with total RNA (II.2.1.5). 2 µl of the 50 µl PCR-reaction were applied onto a 1.2 % (w/v) TBE agarose gel (II.2.1.7). Lanes: 1 = λ-*Pst*I Marker (500 ng); 2 = negative control; 3 = PCR-product of cd64 (α- and β-form).

III.2.5 Bacterial expression system [BL21(DE3) *E.coli*]

First expression approaches were carried out in *E.coli* to prove whether the efficient and cost effective bacterial system is applicable for expression of the human rsCD64 protein.

III.2.5.1 Construction of pMT-cd64

The extracellular domain of the human receptor CD64 was cloned into the pMT-M12 vector for expression in *E.coli*. An overview of the stepwise construction of pMT-cd64 is presented in Fig.III-16.

Briefly, the cDNA fragment of 865 bp (III.2.4) was excised from the gel (Fig.III-15) and subcloned via *SfiI* and *BlpI* into pMT (II.1.7.1; Fig.III-16). The construct was transformed by heat-shock into *E.coli* XL1-Blue (II.1.6.1) and checked for presence of the insert in a restriction digest with *EcoR*I/*Blp*I. Subsequent sequencing analysis of three clones with the primer pMT-5'/pMT-3' (II.1.5) discovered one base pair replacement (T into G) at position 83 resulting in an aminoacid change from leucine to arginine. Until now no polymorphism with this exchange was described in the literature, therefore a transcription error of the polymerase in the PCR (II.2.1.2) was suspected (for final sequence see Appendix VII). *E.coli*

BL21(DE3) were transformed (II.1.6.1) with this pMT-cd64 construct for periplasmic bacterial expression (II.2.2).

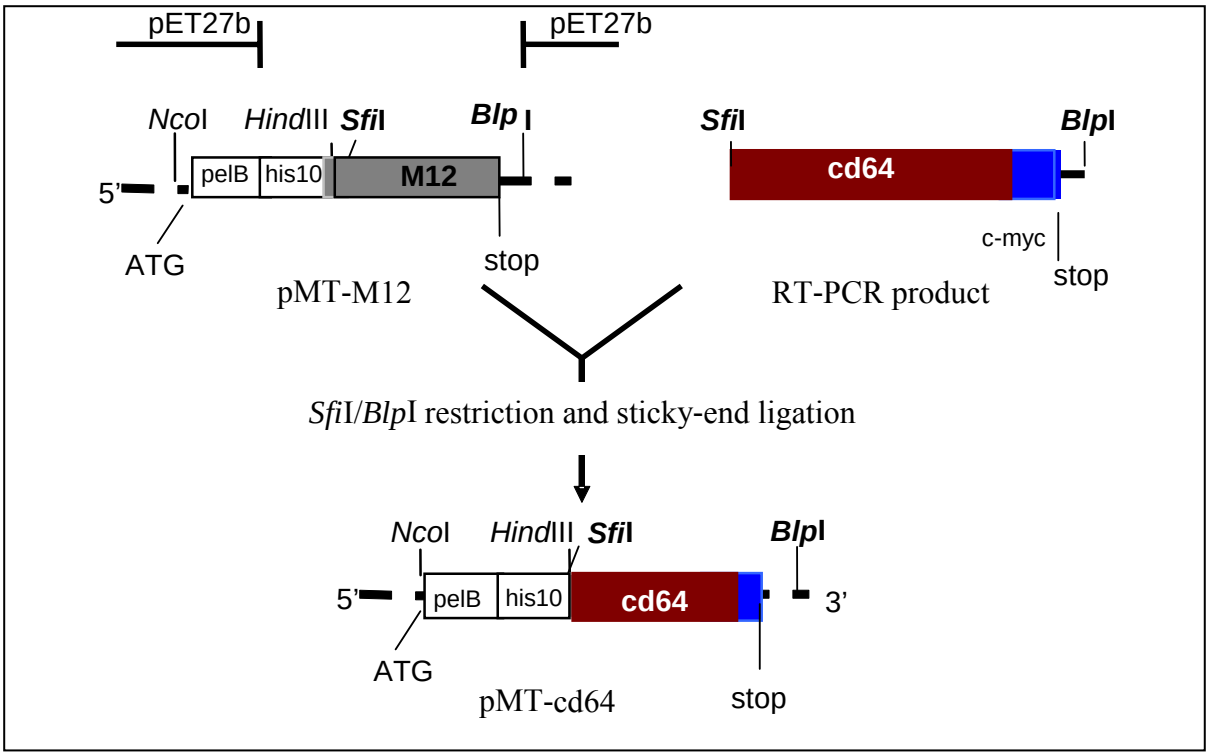


Fig. III-16: Schematic overview of cloning procedure of cd64 into pMT. pMT = bacterial expression vector derived from pET27b (II.1.7.1); his10 = coding sequence of polyhistidine-tag for detection and purification of the recombinant protein; *pel*B = signal peptide for transport into periplasm; c-myc = coding sequence of c-myc-tag for detection of recombinant protein; M12 = anti-MUC-1 scFv; cd64 = DNA sequence of CD64 extracellular domain.

III.2.5.2 Efficiency of rsCD64 expression in bacteria

Different IPTG concentrations (0.1 mM – 2 mM) and different induction time periods (6 h – 24 h) were tested, as well as expression with osmotic stress and compatible solutes. Soluble rCD64 could neither be detected in the supernatant of the culture nor in the disrupted *E.coli* cells. As control for IPTG induction scFv expression in BL21(DE3) *E.coli* was successfully performed in parallel (results not shown). Ni^{2+}NTA purification of the protein was carried out to concentrate the protein (II.2.5.1). Monitoring of the purification was performed in SDS-PAGE (results not shown), but a rsCD64 specific band could not be detected. Only corresponding Westernblots revealed a protein band at the expected size (Fig.III-17).

Two very faint bands of approximately 30 kDa and 35 kDa were visualised in the last washing and elution fraction. The theoretical molecular weight of bacterial expressed rsCD64 is 35.4 kDa, corresponding to the larger one of the detected bands. The other band probably represents a degradation fragment. Approximately 40 ng rsCD64 were purified per gram bacterial pellet (1.7 µg per litre bacterial culture respectively).

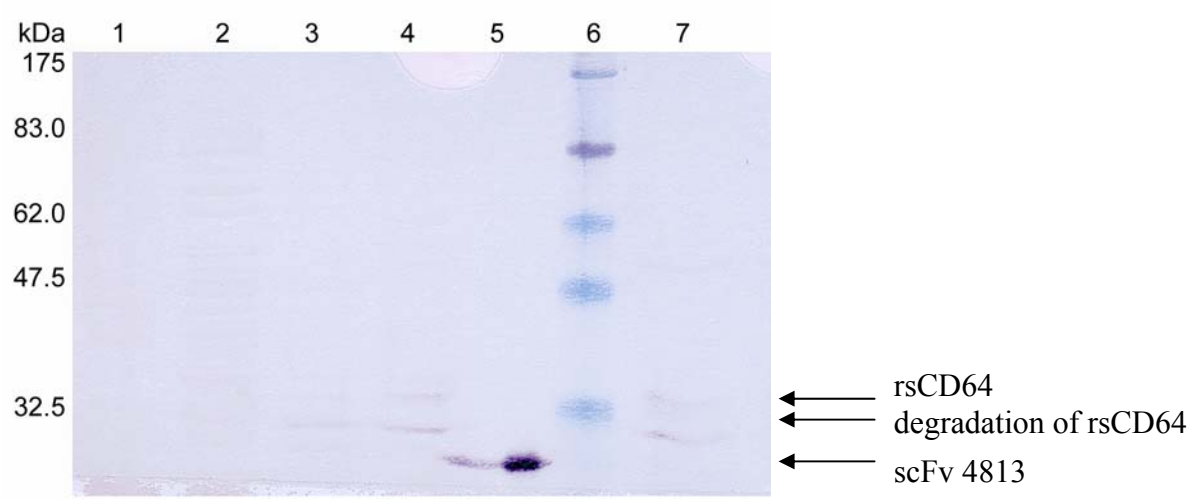


Fig.III-17: Westernblot illustrating the purification of rsCD64 via Ni^{2+}NTA out of *E.coli* BL21(DE3). *E.coli* cells were induced with 1 mM IPTG for 6 h to express rsCD64 in the periplasm. After expression cells were disrupted by sonication on ice and cell debris were removed by centrifugation (30 min / 15000 g/ 4 °C). Clear supernatant was desalted and purified via Ni^{2+}NTA column (II.2.5.1). Samples were applied onto 12 % (w/v) SDS-PAGE and blotted onto nitrocellulose membrane. Detection was carried out with α-his antibody (1:5000) followed by secondary $G\alpha M^{AP}$ antibody (1:5000; II.1.4). Lane: 1 = flow through of non-bound material through Ni^{2+}NTA column; 2 = washing fraction with PBS; 3 = washing fraction with PBS containing 10 mM imidazole; 4 = washing fraction with PBS containing 40 mM imidazole; 5 = 250 ng scFv4813 control for detection via anti-his and $G\alpha M^{AP}$ antibody; 6 = prestained protein marker broad range (NEB); 7 = elution fraction with PBS containing 500 mM imidazole.

III.2.6 Mammalian expression system (HEK 293T cells)

The feasibility of the HEK 293T mammalian expression system was evaluated for expression of human rsCD64.

III.2.6.1 Construction of pMS-cd64

The extracellular domain of the human receptor CD64 was cloned into the pMS vector for secretion into the culture supernatant of mammalian HEK 293T cells. The cloning strategy of pMS-cd64 is presented in Fig.III-18. Briefly, the cDNA fragment of 865 bp (III.2.4) was excised from the gel (Fig.III-15) and subcloned via *SfiI* and *BlpI* into the pMS vector (II.1.7.2; Fig.III-18). The construct was transformed by heat-shock into *E.coli* XL1-Blue (II.2.2.2).

The resulting construct pMS-cd64, was checked for presence of the insert in a restriction digest with *EcoR*I. Subsequent sequencing analysis of one clone with the primer pMS-5'/pMS-3' (II.1.5) revealed the same one base replacement (T into G) at position 83 as discovered in pMT-cd64 (III.2.5.1). Sequencing additionally revealed a loss of 60 bp at the C-terminal end of the pMS-cd64 construct. Thus one c-myc-sequence and the *Blp*I restriction site could not be detected. Restriction digest confirmed the loss of the *Blp*I restriction site (for final sequence see Appendix VII). HEK 293T cells were transfected with this pMS-cd64 construct for expression into the culture supernatant (II.2.4.2).

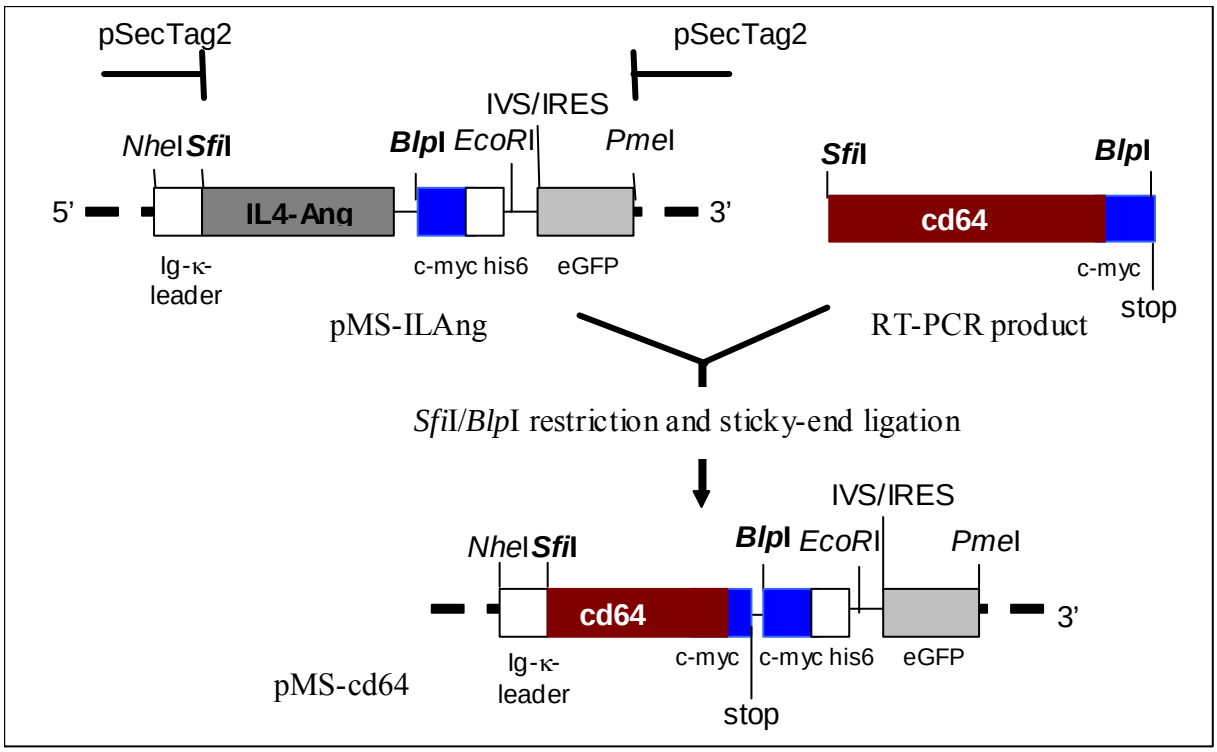


Fig.III-18: Schematic overview of cloning procedure of cd64 into pMS. pMS = mammalian expression vector derived from pSecTag2 (II.1.7.2); his6 = coding sequence of polyhistidine-tag for detection and purification of the recombinant protein; c-myc = coding sequence of c-myc-tag for detection of recombinant protein; eGFP = enhanced Green Fluorescent Protein; Igκ-leader = murine signal leader sequence for secretion of protein into culture supernatant; IRES = internal ribosomal entry site; IVS = synthetic intron for stabilization of mRNA; IL4-Ang = sequence of interleukine 4-Angeogenin; cd64 = DNA sequence of extracellular domain of CD64.

III.2.6.2 Efficiency of rsCD64 expression in mammalian cells

HEK 293T cells transfected with pMS-cd64 were cultured and production of recombinant protein was monitored by green fluorescence under the microscope. Culture supernatant was collected and 20 ml were purified via Ni^{2+}NTA (II.2.5.1). Efficiency of rsCD64 expression was analysed by Westernblot analysis (Fig.III-19).

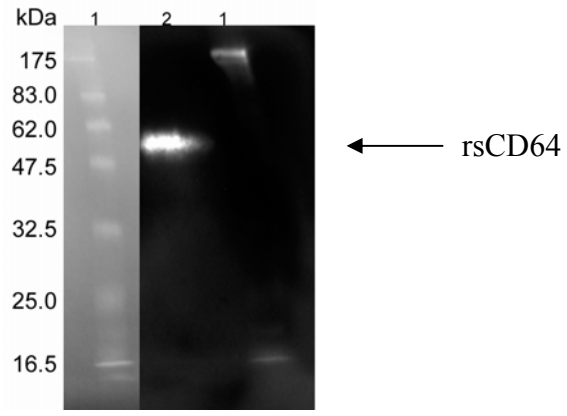


Fig.III-19: Reduced Westernblot of purified rsCD64 from cell culture supernatant of transfected HEK 293T. Supernatant (20 ml) of transfected HEK 293T cells was purified with Ni^{2+}NTA (II.2.5.1). Eluted rsCD64 was applied onto 12 % (w/v) SDS-PAGE and blotted onto nitrocellulose membrane. Detection was carried out with α-his antibody (1:5000) followed by secondary $G\alpha M^{AP}$ antibody (1:5000; II.1.4) and subsequent chemiluminescence reaction. Lanes: 1 = prestained protein marker broad range (NEB); 2 = elution fraction with 250 mM imidazole (II.2.5.1).

The Westernblot demonstrates clearly that rsCD64 can be expressed in mammalian cells. Approximately 20 µg rsCD64 were purified from 20 ml cell culture supernatant (Bradford result not shown), corresponding to ~1 mg/L. This translates in a productivity of 3 –15 pg/cell/day using a overall cell number of $5 \cdot 10^6$ - 10^7 cells per culture flask in 50 ml media.

III.2.7 Plant expression system (*Nicotiana tabacum*)

Transient expression in *N.tabacum* leaves was performed to evaluate whether the plant expression system is feasible for the expression of human rsCD64.

III.2.7.1 Construction of pTRA-cd64

Expression in *N.tabacum* plant leaves was performed by cloning the extracellular domain of human CD64 into the pTRAkc vector. A schematic overview of the cloning steps is presented in Fig.III-20.

Briefly, the vector pMS-cd64 was restricted with *Sau3A*I leading to 21 fragments of different sizes including one with the cd64 sequence (data not shown). The target fragment with the expected size of 835 bp was excised from the gel and purified (II.2.1.7). In parallel, the vector pTRA-ERH was digested with *Not*I and *BstE*II (II.2.1.9). For the fill-in of 5' overhangs, the Klenow fragment was used (II.2.1.11) resulting in blunt ends of the insert and the digested vector.

After dephosphorylation of the vector (II.2.1.10) and blunt-end ligation of vector and insert at the ratio of 1:8 (II.2.1.12), the ligation product was transformed into *E.coli* DH5α by heat-shock (II.2.2.2). The resulting construct, termed pTRA-cd64fi (for fill-in), was checked for correct insertion in a restriction digest with *Pst*I. The 5' end of the insert contained the amino acids MVTITL instead of the original MAVITL due to the fill-in reaction.

Additional subcloning was performed to obtain the 3' end of the cd64 sequence in frame with the his-tag. pTRA-cd64fi was digested with *Eag*I and *Asc*I, the vector pTRAkc-ERH and pTRAkc-AH with the enzymes *Not*I and *Asc*I. Restricted insert and vector DNA was purified (II.2.1.7), 5' overhangs were filled-in with the Klenow fragment (II.2.1.11) and handled as described above. This cloning procedure resulted in the 3' end EQKLISEEDRPAAHHHHH.

Positive clones were detected in a control restriction with *Asc*I and *Fse*I and three clones per construct were additionally checked by sequencing. Complete DNA- and aminoacid-sequences, respectively, are shown in the appendix (VII).

pTRA-cd64(AH) and pTRA-cd64(ERH) were transformed into *Agrobacteria* by electroporation (II.2.3.2). Three recombinant colonies were screened for the presence of the

cDNA insert by PCR (II.2.1.3). A recombinant *Agrobacteria* culture was prepared from a single colony (II.2.3.3) and used for transient expression of *N.tabacum* leaves (II.2.3.4).

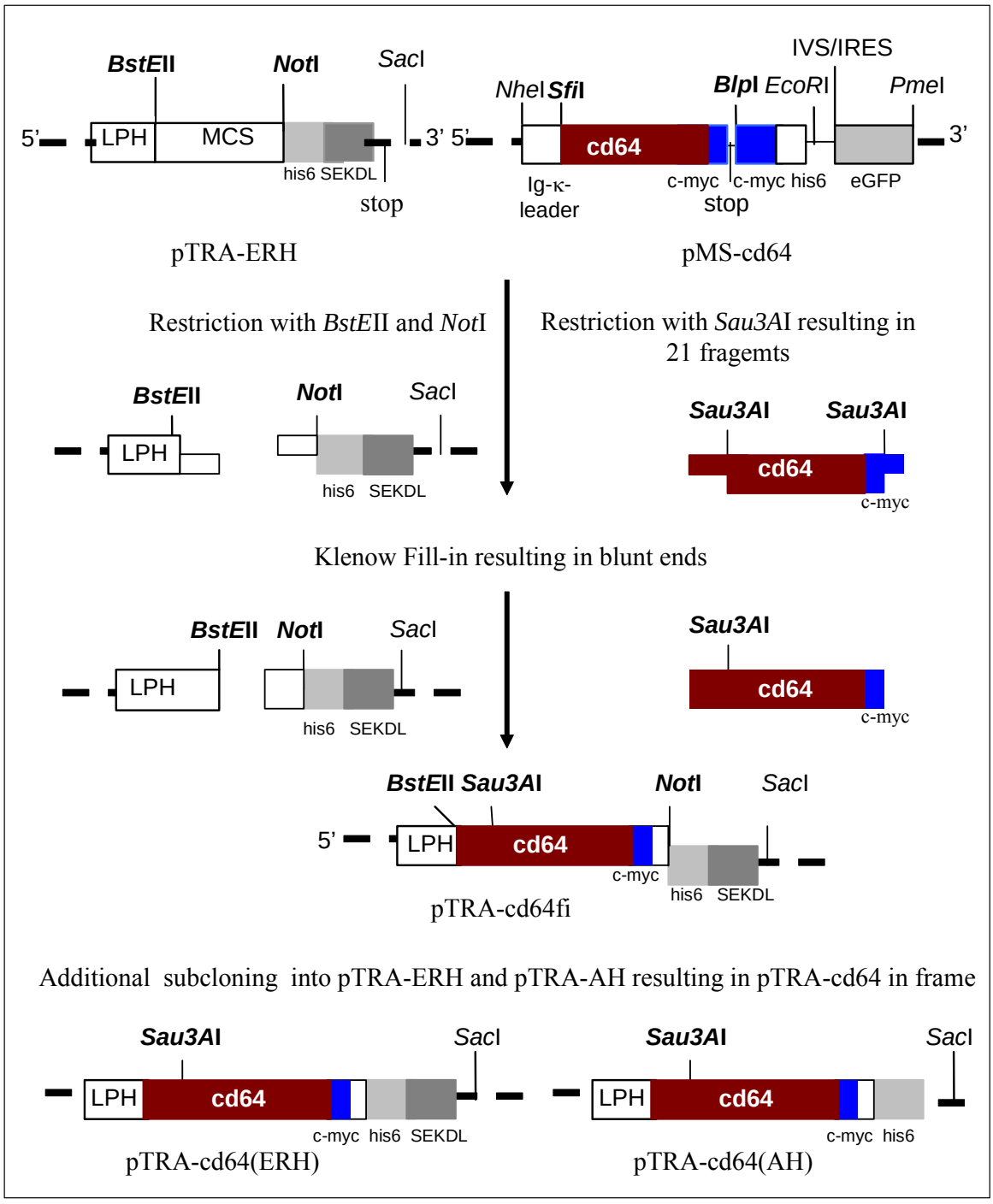


Fig.III-20: Schematic overview for cloning cd64 into pTRA-ERH and pTRA-AH. pMS = mammalian expression vector derived from pSecTag2 (II.1.7.2); Igκ-leader = murin signal leader sequence for secretion of protein into culture supernatant; IRES = internal ribosomal entry site; IVS = synthetic intron for stabilization of mRNA; his6/c-myc = sequence for detection and purification of the recombinant protein; eGFP = enhanced Green Fluorescent Protein; pTRAkc-ERH/AH = plant expression vector derived from pPAM (II.1.7.3); LPH = codon optimised murine signal peptide of mAB24; MCS = multiple cloning site; SEKDL = ER retention signal; cd64 = DNA sequence of extracellular domain of CD64.

III.2.7.2 Efficiency of transient rsCD64 expression in *N.tabacum*

Three *N.tabacum* leaves were transformed with pTRA-cd64(ERH) and pTRA-cd64(AH) by vacuum infiltration (II.2.3.4). Leave samples from each leave (Ø = 2 cm) were taken 3, 6 and 9 **d**ays **p**ost **i**nfiltration (**dpi**), homogenised (II.2.3.4) and analysed by SDS-PAGE und Westernblot (data not shown).

Highest rsCD64 expression was detected by Westernblot in 3 dpi leaves (data not shown), but the corresponding rsCD64 band was partially hidden by high amounts of ribulose-1,5-biphosphatcarboxylase/oxygenase. Thus purification via Ni^{2+}NTA was performed from 80g infiltrated leave material 3 dpi (II.2.5.1)] and purified rsCD64 was detected in Westernblots using anti-c-myc and anti-his (II.1.4) antibodies. Comparable expression level of rsCD64 were determined in the apoplast and the ER of *N.tabacum* leaves (results not shown), one Westernblot of purified rsCD64 out of the ER [pTRA-cd64(ERH)] is presented as example in Fig.III-21. Secondary GαM^{AP} antibody was used as a control (Fig.III-21, lane 2).

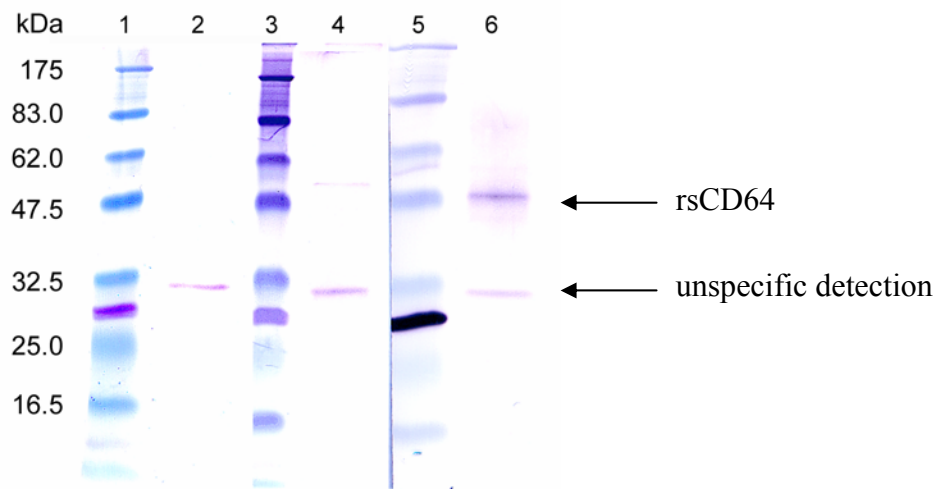


Fig.III-21: Westernblot of purified rsCD64 from 80 g homogenised *N.tabacum* leaves. Transformed *N.tabacum* leaves were homogenised 3 dpi (II.2.3.4) and cell debris were removed by centrifugation (30 min / 16000 g / 4 °C). Clear supernatant was purified via Ni^{2+}NTA (II.2.5.1) and rsCD64 preparations were applied onto an 12 % (w/v) SDS-PAGE and blotted onto nitrocellulose membrane (II.2.7.2). RsCD64 preparations were detected by α-his (1:5000) and α-c-myc (1:5000) antibody followed by secondary GαM^{AP} antibody (1:5000; II.1.4). Lane 1, 3, 5 = prestained protein marker broad range (NEB) containing 0.2 µg of scFv4813 (28 kDa); 2 = elution fraction with 250 mM imidazole detected by GαM^{AP} antibody; 4 = elution fraction with 250 mM imidazole detected by α-c-myc and GαM^{AP} antibody; 6 = elution fraction with 250 mM imidazole detected by α-his and GαM^{AP} antibody.

The Westernblot (Fig. III-21) revealed an unspecific 30 kDa band which appeared after incubation with the secondary GαM^{AP} antibody and a protein band of approximately 47 kDa that is specifically detected by anti-his and to a lesser degree with anti-c-myc antibody. 1 µg rsCD64 was recovered per gram leaf material from *N.tabacum*.

III.2.8 Summary of expression of rsCD64 in different expression systems

Three different expression systems (bacterial: *E.coli* and eucaryotic: HEK 293T mammalian cells and *N.tabacum* plant leaves) were tested for their feasibility to produce the extracellular domain of the human high-affinity receptor FcγRI. The expression yield in [mg/kg] cells/leave material and [mg/L] culture is summarized in table III-1.

construct	host cell	expression yield [mg/kg]	expression yield [mg/L]
pMT-cd64	*E.coli* BL21(DE3)	0.04	0.0017
pMS-cd64	HEK 293T	-	1
pTRA-cd64	*N.tabacum* leaves	1	-

Table.III-1: Summary of expression yield of different expression systems

Bacterial expression yielded lowest expression levels. Due to this, experiments of rsCD64 in *E.coli* were terminated.

Comparing plant and mammalian expression, 1-L cell culture supernatant is easier to produce and handle than 1 kg plant material. Thus, the mammalian expression was scaled-up in order to purify sufficient rsCD64 for proof of IgG binding property.

III.2.9 Initial characterisation of rsCD64 produced in HEK 293T cells

One litre cell culture supernatant of HEK 293T cells was collected and purified. Progress of the Ni^{2+}NTA purification of rsCD64 was monitored via SDS-PAGE (Fig.III-22A) and Westernblot (Fig.III-22B).

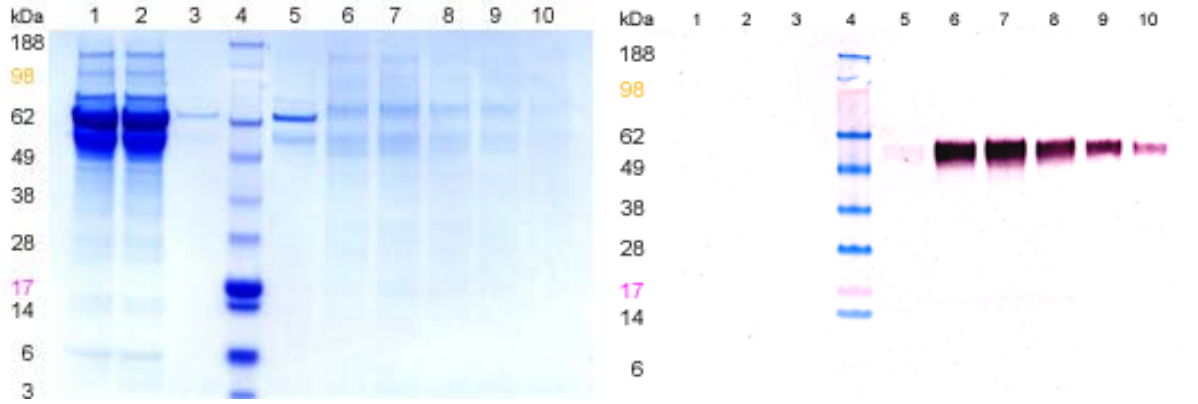


Fig.III-22: SDS-PAGE (A) and reduced Westernblot (B) monitoring the Ni^{2+}NTA purification of rsCD64 from 1-L culture supernatant of transfected HEK 293T (II.2.5.1). A = Coomassie stained NuPAGE™ 4-12% (w/v) Tris-Bis gel. B = Westernblot using α-his mAb/GαM^{AP} for detection. Lanes: 1 = cell culture supernatant, column load; 2 = flow through of non-bound material through Ni^{2+}NTA column; 3 = washing fraction with 1x incubation buffer (II.2.5.1); 4 = prestained protein marker (SeeBlue Plus2, Invitrogen); 5 – 10 = successive elution fraction with 250 mM imidazole (II.2.5.1).

One mg rsCD64 was purified from 1 litre cell culture supernatant. Additional purification was carried out with IgG sepharose. The high affinity IgG sepharose 6 (FF) from Amersham

Biosciences contains coupled human IgG in random orientation and functional rsCD64 can bind to free Fc-parts. Batch purification (Fig.III-23) showed binding of Ni^{2+}NTA eluates to the IgG-sepharose as the supernatant contained only marginal amounts of protein (Lane 3).

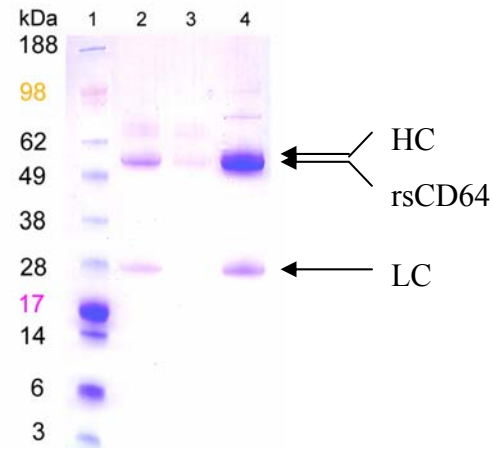


Fig.III-23: SDS-PAGE monitoring the purification and concentration of rsCD64 by IgG sepharose. rsCD64 preparation after Ni^{2+}NTA was subsequently purified via IgG sepharose (II.1.8) and concentrated in an ultrafiltration (10 kDa MWCO; PALL Filtron). Samples were applied on 4-12 % (w/v) Bis-Tris NuPAGE^{TM} gels and stained with Coomassie (II.2.7.2). Lanes: 1 = prestained protein marker (SeeBlue Plus2, Invitrogen); 2 = pooled and dialysed Ni^{2+}NTA eluates; 3 = flow through of non-bound material through IgG sepharose column; 4 = concentrated eluate from IgG sepharose.

SPR (II.2.7.6) analysis revealed that all IgG binding activity was removed from the supernatant (data not shown). Concentrated functional rsCD64 was eluted from the matrix (Lane 4). However, purity of the rsCD64 did not increase significantly as shown by the presence of the same contaminating band at around 30 kDa in the elution (Lane 2 and 4). It can be speculated that the preparation contained contaminating human IgG due to leaking from the IgG sepharose and the initial Ni^{2+}NTA elution may have contained bovine IgG from FCS, which was co-purified. Westernblots were performed to detect rsCD64 and to identify the contaminating proteins (Fig.III-24).

9E10 (Lane 2), M22 (Lane 4) and Penta-his (Lane 6) antibodies were used to specifically detect rsCD64. Identification of the contaminating proteins was performed by using $G\alpha H^{AP}$ specific for κ -chain and γ -chain in reducing (Lane 8 and 10) and non-reducing Westernblot (Lane 12 and 14).

The Westernblot analysis revealed that the affinity-purified rsCD64 is intact and stable. In addition the major contaminants are identified to be human IgG/κ from the IgG sepharose, which assembled to fully human antibody of approximately 150 kDa as detected in the non-reduced Westernblot.

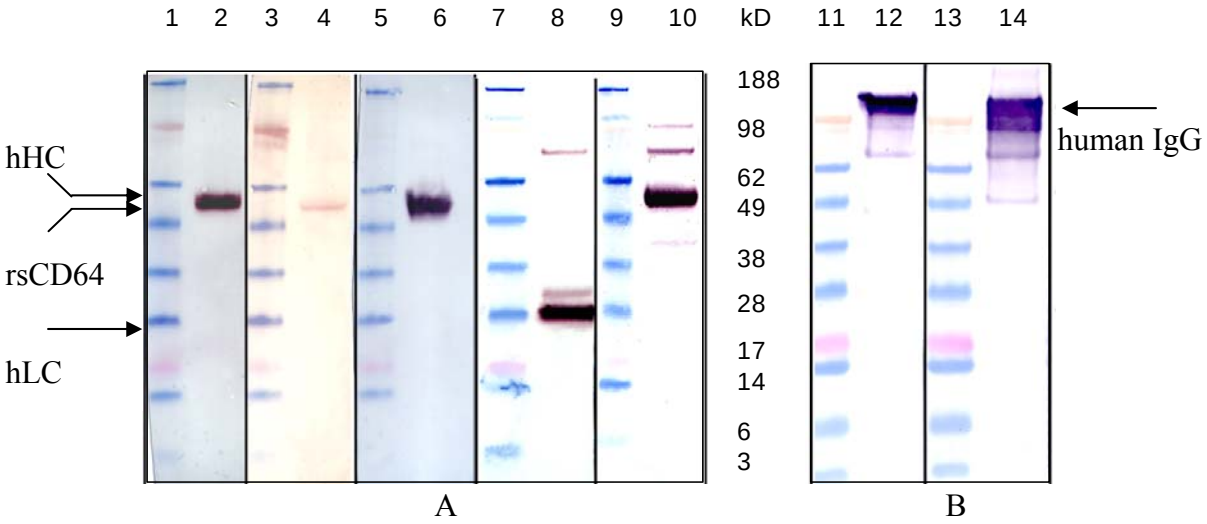


Fig.III-24: Reduced (A) and non-reduced (B) Westernblot analysis of purified rsCD64 secreted into the supernatant of HEK 293T cells. rsCD64 was purified via Ni^{2+}NTA, IgG-sepharose and subsequently concentrated by ultrafiltration with a 10kDa cut-off. Samples were applied onto an 4-12 % (w/v) Bis-Tris NuPAGE^{TM} gels and blotted onto nitrocellulose membrane (II.2.7.2). The rsCD64 was visualised by α-his (1:5000), α-c-myc (1:5000) or M22 (α-CD64; 1:500) primary antibodies and detected with GαM^{AP} secondary antibodies (1:5000). To identify contaminating proteins detection with GαH^{AP} secondary antibodies (γ- or κ-chain specific; 1:5000) alone under reducing (A) and non-reducing conditions (B) was performed (II.1.4). Lanes: 1, 3, 5, 7, 9, 11 and 13 = prestained protein marker (SeeBlue Plus2, Invitrogen); 2 = rsCD64 detected via α-c-myc-antibody and GαM^{AP}; 4 = rsCD64 detected via M22 (α-CD64) antibody and GαM^{AP}; 6 = rsCD64 detected via α-his antibody and GαM^{AP}; 8 = rsCD64 purification incubated with GaH^{AP} (κ-chain specific) antibody; 10 = rsCD64 purification incubated with GaH^{AP} (γ-chain specific) antibody; 12 = rsCD64 purification incubated with GaH^{AP} (κ-chain specific) antibody; 14 = rsCD64 purification incubated with GaH^{AP} (γ-chain specific) antibody.

III.2.9.1 Binding activity of rsCD64

Binding activity of purified rsCD64 to hIgG1/κ (^{CHO}2F5), mIgG1/κ (anti-CA19.9) and humanised anti-CD64 IgG1/κ (H22) was determined by a direct ELISA (see Fig.III-25; II.1.4.1).

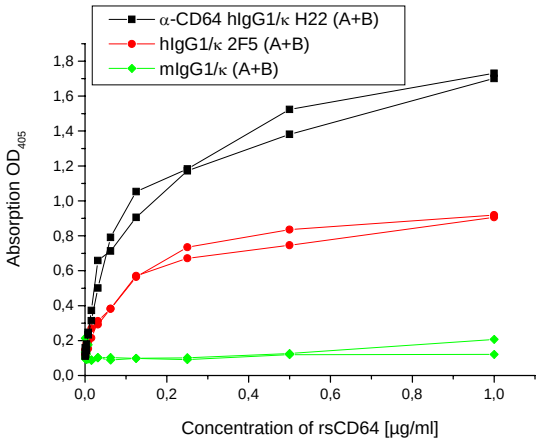
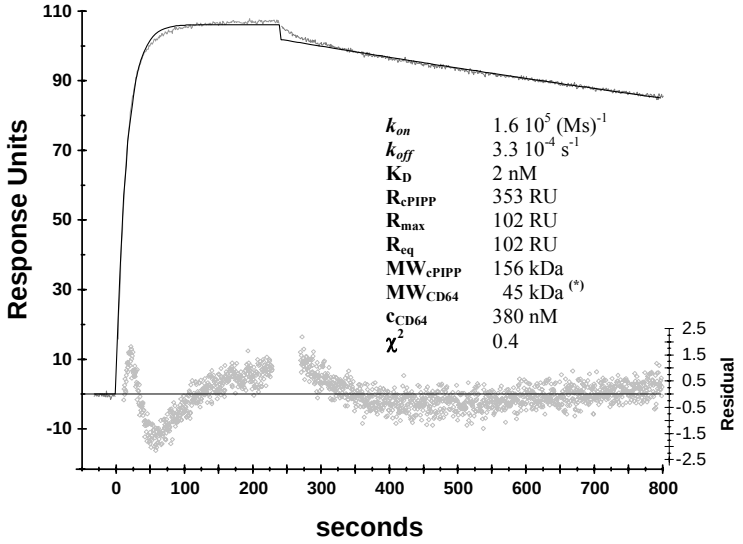


Fig.III-25: ELISA for determination of binding of different full-size antibodies to serially diluted coupled rsCD64. All samples were applied in duplicates (A+B). ■ = humanised anti-CD64 hIgG1/κ (H22); ● = human hIgG1/κ ($^{BY-2}$2F5), ◆ = murine IgG1 (anti-CA19.9).

HIgG1/κ was specifically bound by coated rsCD64 whereas mIgG1/κ was not bound at all. H22 showed highest binding activity offering two binding sites to the coated rsCD64: the

paratope binding and the human Fc part. Data of binding activity was confirmed by SPR (II.2.7.6) analysis. The SPR assay was designed to monitor the direct interaction of rsCD64 with human IgG1/κ cPIPP (Fig.III-26, done by M. Sack, BioVII, RWTH-Aachen).



(*) Derived from the sensorgram using the equation MWrsCD64 = MWcPIPP * Rmax / RcPIPP

Fig.III-26: Preliminary SPR (II.2.7.6) analysis of cPIPP-IgG1 and rsCD64 interaction. The anti-hCG mouse/human chimeric antibody cPIPP (IgG1/κ) was bound to immobilised hCG. Purified rsCD64 was injected for 4 minutes at a flow rate of 30 µl/min and dissociation was followed for 10 minutes. The binding curve was fitted using the Langmuir model for a simple monovalent interaction. k_{on} = binding constant of rsCD64 binding to cPIPP-IgG1; k_{off} = dissociation constant of releasing rsCD64 from cPIPP-IgG1; K_D = relative binding affinity, equilibrium dissociation constant between rsCD64 and cPIPP-IgG1; R_{cPIPP} = change of fraction index caused by binding of cPIPP-IgG1 onto previously coupled hCG onto the Biacore chip; R_{max} = maximal change of fraction index caused by binding of rsCD64; MW_{cPIPP} = Molecular weight of cPIPP-IgG1 antibody; MW_{CD64} = calculated molecular weight of rsCD64; c_{CD64} = estimated concentration of rsCD64 in the preparation; χ^2 = quality parameter (Measurement and calculations made by M. Sack, BioVII, RWTH-Aachen.).

First, anti-hCG cPIPP-IgG1 was bound to the immobilised hCG (II.1.4). RsCD64 was injected for analysis, i.e. to determine the active concentration and kinetic rate constants. Binding of rsCD64 to cPIPP was clearly demonstrated. Additionally affinity capture of cPIPP-IgG1 was stable and allows multiple analysis of rsCD64 binding, since rsCD64 was selectively eluted from the sensor surface without eluting cPIPP-IgG1.

The on- and off-rate (K_{off}/K_{on}) of the binding and dissociation of rsCD64 was determined and the K_D was calculated to be 2 nM. The RU-values of the IgG-cPIPP and rsCD64 and the known molecular weight of cPIPP were used to determine the molecular weight of glycosylated rsCD64 to be 45 kDa. Kinetic studies and the determination of parameters was performed by M. Sack (BioVII, RWTH-Aachen).

III.2.9.2 Initial MS analysis of trypsin digested rsCD64

MS analysis of the rsCD64 preparation was performed to get initial information about the glycosylation status. The rsCD64 preparation (III.2.6.2) was separated on an SDS-PAGE

(II.2.7.2) from visible contaminants (i.e. human κ-chain) and the protein band at approximately 50 kDa, containing rsCD64 and human heavy-chain, was digested with trypsin (II.2.7.8) and analysed in MS by M. Küpper (BioVII, RWTH Aachen; Fig: III-14).

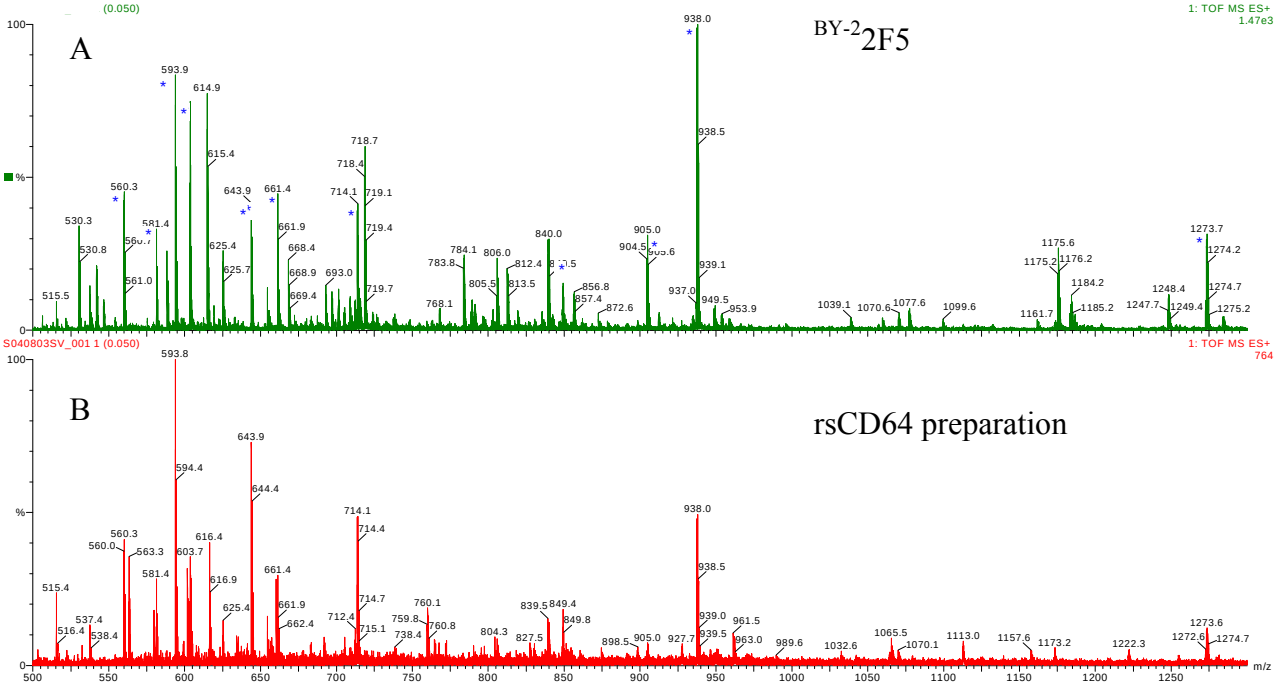


Fig.III-27: Initial MS analysis of the heavy-chain of human anti-HIV antibody $^{BY\text{-}2}$2F5 (A) and rsCD64 preparation (B). RsCD64 was purified via Ni^{2+}NTA, IgG-sepharose, concentrated by ultrafiltration with a 10 kDa cut-off and separated by SDS-PAGE (II.2.7.2). A 50 kDa protein band was excised and digested with trypsin prior to MS analysis (II.2.7.8). A = visualises the peak pattern of peptide fragments detected in the control samples containing human anti-HIV antibody $^{BY\text{-}2}$2F5; B = pattern of peptide fragments detected in the MS analysis of rsCD64 preparation.

In the upper MS graph (Fig.III-27A) the peak pattern of the heavy-chain of hIgG ($^{BY\text{-}2}$2F5) is shown. The lower MS graph (Fig.III-27B) illustrates the distribution of different peptide fragments in the preparation of rsCD64. Several prominent peaks (labeled with blue asterisk) corresponding to human IgG1 heavy-chain can be seen, showing that bleeding of IgG1 from the sepharose matrix is a serious problem during the purification procedure of rsCD64.

Additionally, MS/MS analysis was performed to verify the presence of rsCD64 in the preparation and the status of the N-glycosylation sites (data not shown). Five tryptic fragments of rsCD64 [T6 (GWLLLQVSSR), T7 (VFTEGEPLALR), T10 (LVYNVLYYR), T16 (YTSAGISVTVK) and T21 (NTSSEYQILTAR)] were unambiguously identified by MS/MS. One peptide containing an N-glycosylation motif (T21) was found without glycosylation. This demonstrates that this site is either not used at all or not always used for glycosylation. No other peptide containing an N-glycosylation motif has been found indicating that the other sites are utilised.

IV. Discussion

Two human biopharmaceuticals were expressed in heterologous host systems and characterised in this thesis.

Neutralising antibodies have shown therapeutic value in treatment of HIV infection (DESROSIERS *et al.* 1989). The human anti-HIV antibodies 2F5 and 2G12 produced in CHO cells were already tested in clinical phase I studies for mode of action (ARMBRUSTER *et al.* 2002). However, large quantities are needed and plant produced antibodies have already been proven to be useful for production at reasonable costs (FISCHER(B) *et al.* 1999; STOGER *et al.* 2002; SCHILLBERG *et al.* 2003). Plant suspension cells offer additional advantages since suspension cells can be contained under GMP compliance and might be approved by the FDA (HELLWIG *et al.* 2004). Therefore, the capacity of the tobacco suspension cell line BY-2 was evaluated for production of human anti-HIV antibodies 2F5 and 2G12 in high amounts. The $^{BY\text{-}2}$2F5 antibody was purified out of 340 g BY-2 cell material at a concentration of 3.2 mg/kg. Purity, integrity and assembly were proven and antigen binding property was comparable to the CHO produced control antibody. Established protocols from small-scale expression approaches were transferred to initial fermentation studies with the $^{BY\text{-}2}$2G12 antibody. During the fermentation process of 14 days intact and stable antibody was expressed. Subsequently a feasibility study with the $^{BY\text{-}2}$2F5 antibody in the 100-L scale was performed and available equipment was tested for application at industrial scale. Based on these results, a theoretical process was developed for the downstream processing of a 100-L fermentation broth of BY-2 derived anti-HIV antibody 2F5 in two days.

The high affinity receptor for monomeric IgG, FcγRI, was extensively investigated for its potential as therapeutic target in tumor therapy such as acute leukaemia and its role in allergic reactions such as rheumatoid arthritis (van de WINKEL and CAPEL 1996). The understanding of the interaction of IgG and FcγRI is crucial for the treatment of Fc-mediated diseases and this understanding will be enhanced with the availability of soluble FcγRI for biochemical studies. For detailed characterisation of the IgG binding and biochemical properties in future experiments, the receptor was expressed in recombinant, soluble form. The feasibility of three well established expression systems was evaluated for the expression of rsCD64. Bacterial expression yielded lowest expression (40 ng/kg), transient expression in *N.tabacum* leaves resulted in 1 mg/kg rsCD64 and mammalian expression was the system of choice with 1 mg/L of rsCD64 which was purified in one-step from the culture supernatant. Thus, mammalian expression was scaled-up to yield sufficient purified protein for

characterisation studies of the protein. Initial kinetic analysis determined the dissociation equilibrium constant of rsCD64 (2 * $10^9 M^{-1}$) to be in the same range as the K_D value described in the literature (van de WINKEL and CAPEL 1996). This clearly demonstrates the functionality and binding activity of rsCD64.

IV.1 Generation of the expression constructs

IV.1.1 Anti-HIV antibody 2F5

Cloning of the cDNAs of the heavy and light-chain genes in tandem into the binary vector pTRA for *Agrobacterium* mediated transformation was performed by M. Bomble (BOMBLE 2004).

IV.1.2 rsCD64

The easiest way to obtain the cDNA of a human protein, is the isolation of the corresponding mRNA out of cells expressing the protein such as body fluids. The mRNA of FcγRI was successfully isolated from blood cells (III.2.4). Primer were designed according to the published sequence (ALLEN and SEED 1988, II.1.5) and protein specific RT-PCR was performed resulting in cDNA of the extracellular domain of FcγRI (I.1.2.2; III.2.4). Cloning was performed into the bacterial (pMT) and mammalian expression (pMS) vector (III.2.5) and the cd64 gene was verified by sequencing (VII), resulting in two different discoveries. Firstly, sequencing revealed a one nucleotide mutation resulting in an aminoacid change from leucine into arginine at position 83 in all constructs. As no polymorphism is described in the literature, allelic variation can only be speculated. Otherwise this exchange might be explained by a PCR error introduced by the *Taq* DNA-polymerase (Roche), which has a mutation rate of $1.3 \bullet 10^{-5}$ (Roche, Technical information). Thus, subsequent PCR will be performed with a proof-reading polymerase to avoid this potential problem as it could lead to non-functional protein.

Secondly, in the pMS-construct 3' of the cd64 sequence 60 bp were missing (VII). This unexpected deletion of one c-myc-tag as well as the *Blp*I restriction site resulted in a pMS construct with c-myc **and** his-tag. This can only be explained as a result of homologous recombination by RecA (KO *et al.* 2002) in BL21(DE3) *E.coli*, the only *E.coli* strain containing the RecA protein. In spite of this deletion the sequence of cd64 with one c-myc-tag was still in frame downstream with the his-tag. Thus, this construct was used for further experiments.

For expression of rsCD64 in plant cells cd64 was subcloned into the plant expression vector (pTRA; III.2.6) by use of the restriction enzyme *Sau*3AI to avoid further PCR. Due to this restriction two amino acids at the 5' of the cd64-sequence were lost (III.2.7.2).

Expression of rsCD64 was tested in bacteria [BL21(DE3) *E.coli*], mammalian cells (HEK 293T cells) and *N.tabacum*. Evaluation of the expression yields in the different expression systems can only be an indication as the rsCD64 proteins are similiar but not identical. First the rsCD64 out of *E.coli* carries a N-terminal his10-tag and a C-terminal c-myc, whereas rsCD64 from mammalian cells and plants contain the c-myc- and his6-tag at the C-terminus. The different leader sequences (*pel*B for *E.coli* and IgG *kappa* for mammalian and plant expression) are cleaved at different recognition sites resulting in different aminoacid overhangs. The probability of cleavage sites can be predicted using SignalIP V2.0 available on the Internet (http://www.cbs.dtu.dk). The *pel*B leader is most probably cleaved two amino acids upstream of the histidine-tag resulting in a 32-aminoacid overhang at the N-terminus. The Ig *kappa* leader in the pMS construct is cleaved seven amino acids upstream of the protein start codon. Only the plant expression vector pTRA was constructed in such a way that cleavage of the Ig *kappa* leader results in expression of the protein alone.

Finally, at the C-terminal, the pMT-construct is terminated downstream the last amino acid of the myc-tag, the pMS-construct contains C-terminal five additional amino acids and the his6-tag. The pTRA-construct contains four additional amino acids, the his6-tag and the SEKDEL-tag. Therefore cd64 cDNA constructs yielding recombinant protein with different composition and molecular weights [$^{E.coli}$rsCD64 (942 bp = 35.6 kDa), ^{293T}rsCD64 (899 bp = 31.8 kDa) and ^{Nt}rsCD64 (894 bp = 33.8 kDa)] were expressed. As an alternative for procaryotic expression the pET-26b(+) vector can be used as it contains a C-terminal his6-tag. Moreover, only completely translated recombinant proteins will be purified by Ni^{2+}NTA. For mammalian expression, the expression cassette of the pTRA vector should be subcloned into the pMS plasmid, as all mammalian expression vectors commercially available and comparable with our system contain the same sequence downstream of the Ig *kappa* leader.

As the aim of this thesis was to compare functional expression of rsCD64 in different established expression systems, the available systems were used.

IV.2 Expression system E.coli

Expression of problematic proteins in the presence of compatible solutes under osmotic stress was successfully performed in our institute (BARTH *et al.* 2000; TUR *et al.* 2003).

IV.2.1 Anti-HIV antibody 2F5

Expression of aglycosylated full-size antibodies in *E.coli* was only described in one publication so far (SIMMONS *et al.* 2002). As the neutralising anti-HIV antibody 2F5 will be used in therapeutic approaches in the future and glycosylation contributes to the effector function of antibodies, expression in *E.coli* was not considered.

IV.2.2 rsCD64

Bacterial expression of rsCD64 was investigated under standard and osmotic stress conditions but both expression approaches resulted in minor amounts of recombinant protein. Approximately 40 ng rsCD64 were purified per gram bacterial pellet, corresponding to 1.7 µg per litre bacterial culture.

This low expression yield can be caused by problems in protein translation in *E.coli* as the sequence of the human proteins contain codons that are infrequently used by bacteria. High-level expression of a human gene causes depletion of the internal tRNA pool resulting in low or no expression. Certain codons for arginine, proline, isoleucine and leucine (SHARP and LI 1987) are known to be problematic. In case of rsCD64, 31 amino acids are coded by rare codons. Especially, at position 227-231 an accumulation of rare codons for leucine and arginine can be found in the cd64 sequence. It can be speculated that truncation in the protein translation occurred and resulted in an rsCD64^{-} with a size of approximately 28 kDa. Truncated proteins are purified as well by Ni^{2+}NTA purification using the N-terminal his-tag and this can explain the second band on the Westernblot (III.2.5.2) below 30 kDa.

To circumvent this, strains of *E.coli*, such as BL21-CodonPlus® (Stratagene), are available for AT- and GC-rich genomes which carry additional plasmids that encode and express these rare codons. Increase in production level of up to 100 times has been reported using these strains (KLEBER-JANKE and BECKER 2000).

Another possibility is a codon optimisation of the gene of interest. A complete compilation of the codon usage for different organisms can be found in the GenBank database at http://www.kazusa.or.jp/codon/, where e.g. the probability of specific codons used by *E.coli* and human is calculated. All rare codons will be exchanged by more frequent ones and oligo nucleotides covering the whole gene are joined to a new optimised gene in a number of **s**plice **o**verlap **e**xtension (**SOE**) PCRs. However, the time- and labour-consuming procedure does not guarantee a better expression yield, as experiments with a human scFv have shown (own data, not shown). On the other hand, in the literature some authors claim an increase of expression level up to 20 % (EJDEBACK *et al.* 1997) and 136-fold (TALARICO *et al.* 2001).

Even if rsCD64 is expressed, inclusion bodies can be formed or the protein itself is insoluble. In this case, the introduction of fusion tags can facilitate production of soluble proteins (NOVAGEN 2004) through the fusion partner who stabilises the protein and encourages the folding. Finally, expression can be enhanced by changing growth conditions, reduction of the temperature from 37 °C to 28 °C, feeding of complex media supplements to facilitate cell growth, and sufficient oxygen supply in order to avoid accumulation of stress signals to the cells.

Conclusively, many improvements and optimisation procedures can be employed to enhance the accumulation level of recombinant proteins in the bacterial periplasm. However, as other expression systems are also available, it was decided that labour and time consuming optimisation procedures should not be undertaken. Additionally, aglycosylated FcγRI expressed in bacteria may not lead to functional binding to IgG.

IV.3 Expression system N.tabacum

The successful expression of different complex proteins was published by Fischer *et al*. in the recent years (FISCHER(C) *et al.* 1999; FISCHER(F) *et al.* 1999; VAQUERO *et al.* 1999; STOGER *et al.* 2000; KATHURIA *et al.* 2002). The use of an SEKDEL sequence is known to increase accumulation levels of recombinant protein (WANDELT *et al.* 1992; SCHOUTEN *et al.* 1996; CONRAD and FIEDLER 1998), whereas it has been shown for secreted antibodies that stability can be dramatically reduced in the plant cell culture media (SHARP and DORAN 2001; TSOI and DORAN 2002).

IV.3.1 Anti-HIV antibody 2F5 in BY-2 cells

Generation and cultivation of BY-2 cells expressing the $^{BY\text{-}2}$2F5 was done by Dr. T. Rademacher. Previous experiments with different constructs of $^{BY\text{-}2}$2F5 showed that the SEKDEL tagged $^{BY\text{-}2}$2F5 accumulated approximately 10 times higher than the secreted $^{BY\text{-}2}$2F5 (3 – 4 mg/kg). This is consistent with publications describing 10 – 100 times enhancement of antibody accumulation (WANDELT *et al.* 1992; SCHOUTEN *et al.* 1996). Therefore experiments presented here were performed with the SEKDEL-construct and using Protein-A affinity chromatography 1.1 mg anti-HIV antibody $^{BY\text{-}2}$2F5 were purified from 340 g fresh weight grown in shake flasks (0.92 mg/L).

Only trace amounts of antibody were detected in the media supernatant (<1 %), which probably was released from damaged, or dead cells. Since the currently achieved accumulation levels for $^{BY\text{-}2}$2F5 are rather low and as all four chains of the assembled antibody exhibit a SEKDEL tag, it is not surprising that no secreted protein was detected.

Significantly higher accumulation has been obtained for the human mAb CL4 that is directed against HBV surface antigen (YANO *et al.* 2004). Although the antibody chains were not SEKDEL tagged, only between 36 – 63 % of the totally produced antibody was released into the culture medium. The maximal yields after Protein-A purification were 16.2 mg/L of suspension culture. Compared to the $^{BY\text{-}2}$2F5 antibody accumulation of approximately 1 mg/L, this represents a 16-fold increase. This may be due to the intrinsic properties of the variable domains of the 2F5 IgG1/κ antibody. Agrofiltration experiments of the $^{N.t.}$2F5 resulted in significantly lower accumulation of $^{N.t.}$2F5 (personal communication M. Sack) compared to other antibodies evaluated in our laboratory (up to 40 mg pure protein per kilo fresh leaf weight; KATHURIA *et al.* 2002).

Different strategies have been described to enhance antibody accumulation levels in plants. Addition of various agents to the culture medium, such as inorganic compounds, amino acids, **di**m**e**thyl**s**ulf**o**xide (**DMSO**), **p**oly**e**thylen **g**lycol (**PEG**), **p**oly**v**inyl**p**yrrolidone (**PVP**), gelatin, **b**ovine **s**erum **a**lbumin (**BSA**), gibberellic acid, haemin and Brefeldin A were tested (SHARP and DORAN 2001; TSOI and DORAN 2002). Providing amino acids and haemin can enhance productivity through faster protein synthesis. The other substances are used for stabilisation of the recombinant protein probably by protecting the protein from degradation and preventing precipitation or adsorption to available surfaces. Additionally, media composition and culture parameters, such as inoculum level, pH, carbon and nitrogen source, NH_4:NO_3-ratio, can be optimised (CHATTOPADHYAY *et al.* 2002; GORRET *et al.* 2004). Process engineering aspects such as batch, fed-batch, repeated batch, continuous and perfusion fermentation, can also enhance accumulation level (KIERAN *et al.* 1997; HELLWIG *et al.* 2004).

Even though accumulation of 2F5 in BY2 suspension cells was low, purification by single-step Protein-A chromatography was very efficient leading to intact and pure antibody. Purity of the antibody preparation was enhanced by extensive dialysis as shown by SDS-PAGE and Westernblot (III.1.1). More importantly antigen binding activity of ^{BY2}2F5 and ^{CHO}2F5 were indistinguishable by ELISA and EMSA (III.1.3). Only a high resolution single binding site SPR assay was able to reveal a lower activity of ^{BY2}2F5 of approximately 9 % compared to the ^{CHO}2F5. This is probably due to the higher purity of ^{CHO}2F5 since minor amounts of degradation products of ^{BY2}2F5 have been observed in over-developed Westernblots (data not shown).

IV.3.2 rsCD64 in *N.tabacum* leaves

RsCD64 was sucessfully expressed in tobacco leaves and could be unambiguously detected with anti-his and to less extent with anti-c-myc-antibody. 1µg rsCD64 could be purified per gram leave material for both constructs. Expression of rsCD64 was performed using pTRA plasmids with and without C-terminal SEKDEL tag but our initial results have not shown any differences in the expression level of rsCD64 with and without SEKDEL tag in *N.tabacum* leaves.

Experiments carried out in parallel in mammalian expression system lead to higher expression levels and since glycosylation pattern is closer to human, the validation of the mammalian expression was pursued.

IV.4 Expression system mammalian cells

Different complex proteins were successfully expressed into the culture supernatant of mammalian cells using the pMS-system (STOCKER *et al.* 2003; BRUELL 2004).

IV.4.1 Anti-HIV antibody 2F5

Highest specific productivity of CHO cells producing 2F5 was 22 µg/10^6cells/day (KUNERT *et al.* 2000) corresponding to 2.2 pg/cell/day. Based on own experiences this correlated to a production rate of approximately 1 mg/L (compared with results for rsCD64 III.2.6.2). Parallel expression with our established mammalian expression systems was not considered.

IV.4.2 rsCD64

Initial expression approaches of rsCD64 in HEK 293T cells succeeded with 1 mg/L of purified recombinant protein in a single-step purification out of 20 ml. Thus, the mammalian expression system was used to define a process for expression of rsCD64 in larger scale to yield sufficient protein (1 mg) for initial characterisation of the protein.

One interesting aspect concerning FcγRI was the investigation of the glycosylation status. The sequence of FcγRI contains 7 potential N-glycosylation sites (ALLEN and SEED 1988), but it is not known which sites are naturally glycosylated and how this contributes to the functional activity of FcγRI. One study showed that genetic variations between individuals in the extent of FcγRIII glycosylation led to differential ligand binding (reviewed in van der POL *et al.* 1998). The rsCD64 protein band on SDS-PAGE and Westernblot (III.2.6.2) was broad and diffuse probably probably reflecting the occupancy of different glycosylation sites. SPR (II.2.7.6) data revealed a molecular mass of 45 kDa (III.2.9.1). In general, glycosylated proteins are retarded in polyacrylamide gels, and our results showing a 55-kDa CD64-specific

band in the Westernblot confirm this. MS/MS analysis identified tryptic fragments of hFcγRI as well as of the human γ-chain (III.2.9.2). Mass spectrometry revealed on tryptic peptide containing a N-glycosylation site at position 219 in the third extracellular domain. It can be deduced that this site is not or not always used. As no other peptide was identified in the experiments, the other potential sites are glycosylated with high probability.

Initial purification using Ni^{2+}NTA matrix resulted in a co-purification of rsCD64 and a bovine antibody present in the fetal calf serum (III.2.6.2). There are no data addressing the binding of rsCD64 to bovine antibodies, but our results now suggest that this binding can occur. The use of IgG-Sepharose as a purification step failed due to the high leakage of bound human IgG from the matrix (III.2.9.1). The strong binding of monomeric antibodies during purification indicates a potential problem in recovering pure and active rsCD64, although the binding of rsCD64 to IgG-Sepharose did confirm the recombinant protein's IgG-binding activity. The use of serum-free medium could improve the efficiency of purification by avoiding immunoglobulin contamination early on, and facilitating a one-step purification using Ni^{2+}-NTA resin by decreasing the production cost without the expensive FCS.

Additional evidence for rsCD64 activity was provided by ELISA and SPR analysis (IV.4). Initial kinetic analysis determined the dissociation equilibrium constant of rsCD64 ($2 \cdot 10^9 M^{-1}$) to be in the same range as the K_D value described in the literature (van de WINKEL and CAPEL 1996). This clearly demonstrates the functionality and binding activity of rsCD64 despite of the aminoacid change in the sequence.

Data from the SPR (II.2.7.6) analysis revealed a simple stoichiometric interaction between rsCD64 and IgG. In theory, since IgG is a dimer, one IgG molecule can bind two Fc-receptor molecules, as was described for FcγRII (WOOF and BURTON 2004). In the case of FcγRIII, however, the binding of a second FcγRIII receptor molecule is prevented by steric hindrance (WOOF and BURTON 2004). This 1:1 stoichiometry was also predicted for FcγRI, since the IgG binding site in the hinge region is formed by both IgG heavy-chains (WOOF and BURTON 2004). From this work it was deduced that the purified rsCD64 preparation contained molecules that were either fully capable or fully incapable of binding to IgG, but none with intermediate binding activity.

Some aspects of the mammalian expression itself have to be optimised in the future. One drawback of the system is the random integration of the plasmid DNA into the cell genome. Transient expression for research purposes offers fast results as only few days lay between the DNA delivery and protein harvest. But protein production under GMP conditions requires reproducible and stable expression conditions and can only be achieved with site-specific

integration. Enzymes, like P1 Cre recombinase, lambda phage integrase and yeast Flp recombinase catalyse the exchange at high frequency if the donor and recipient DNAs are flanked by specific attachment regions (WURM 2004). The Flp-In™ System e.g. using the Flp recombinase offered by Invitrogen can be combined with HEK 293T cells and is currently under investigation in our laboratory. As soon as the system is adapted, stability of clonal cell lines will be proved over extended periods of time (several weeks or months).

Production of recombinant proteins in mammalian cell cultures is performed mainly in two formats today: cultures of adherent and suspension cultures. Cultivation of HEK 293T cells in CD 293 suspension media (Invitrogen) is currently under investigation. Preliminary bioreactor experiments with these cells did fail as cells did not grow as expected (personal communication D. Scheffler). Alternative procedures using Cytodex™ 1 microcarrier (Amersham Biosciences) based on a cross-linked dextran matrix to cultivate adherent HEK 293T in RPMI media are currently under investigation.

Like media, host cells can be genetically improved. Proto-oncogenes, cell cycle control genes (cyclins), growth factor genes (e.g. insulin-like growth factor) and antiapoptotic genes have been inserted into cell lines for the generation of superior production hosts (ARDEN *et al.* 2004). The implementation of strategies to control apoptosis and enhance culture productivities is also under investigation (ARDEN and BETENBAUGH 2004). Alternatively high-producer cell lines offer new perspectives, as the novel mammalian PER-C6 system, based on human retinal cells. For PER-C6 cells protein production of > 2 g/L in fed batch cultures was reported with corresponding viable cell densities of around 100 x 10^6 cells/ml (COCO-MARTIN 2004).

Microbial systems are surpassed by mammalian cells cultivated in bioreactors for the production of clinical relevant products in number and titre (WURM 2004). Future developments will improve the productivity by stable DNA delivery and site-specific integration, extended selection and cell line screening, host cell engineering and medium optimisation.

IV.5 Feasibility study: 100-L fermentation and downstream processing

IV.5.1 Large-scale expression in the 100-L bioreactor

Production of the BY-2 derived antibody 2F5 was scaled up from Erlenmeyer shake flasks to initial 7-L bioreactor approaches up to a feasibility study in a 100-L fermenter. The scale-up of a bioprocess itself is separated into three stages: (1) laboratory scale, where elementary

studies are carried out; (2) pilot scale, where the process optimisations are determined; and (3) plant scale of production, where the process is brought to economic results. In this thesis laboratory-scale data was determined and initial pilot-scale approaches were performed.

The following criteria must be considered during scale-up: mass transfer of oxygen, mechanical stress on cells, degree of homogeneity and circulation time in the bioreactor (STORHAS 2003). Predictable problems occur in larger reactor volumes, such as reduced mass transport, enlarged mixing times, less oxygen and heat transfer, higher power input and aeration rates. Less predictable are the performance of product quality, foaming and the by-product formation due to the altered conditions. It is impossible to keep all parameters constant during scale-up. The most frequently used criteria are constant specific power input (P/V), constant oxygen transfer (k_La), constant tip speed of the agitator and constant dissolved oxygen concentration (STORHAS 2003).

Constant specific power input cannot be realised in general as this would result in highly reduced stirrer speed. Constant stirrer speed cannot be realised, as this would require immense power input with corresponding high mechanical stress to the cells. Agitation must provide good mixing time to minimize the variation of dissolved oxygen, pH and nutrient concentration gradients.

For the scaling-up of an aerobic fermentation, the effect of gas liquid mass transport is the most significant factor (HENSIRISAK 1997). Therefore, scale-up in aerobic fermentation is often performed on the basis of keeping the value of k_La constant.

The oxygen transport coefficient (k_La), describes the oxygen transfer from the air bubble into the fermentation broth. This can be calculated by the **o**xygen **t**ransfer **r**ate (**OTR**):

$$OTR = (k_L \cdot a) \cdot (c_L^* - c_L)$$

OTR = oxygen transfer rate
k_L = oxygen transfer coefficient [$cm \cdot h^{-1}$]
a = gas – liquid specific area [$cm^2 \cdot cm^{-3}$]
c_L^* = oxygen concentration inside the air bubble [$mg \cdot L^{-1}$]
c_L = actual dissolved oxygen concentration in the broth [$mg \cdot L^{-1}$]

Fig.IV-1. Formula for determination of the oxygen transfer rate

The driving force for oxygen transfer is given by the concentration difference of actual dissolved oxygen in the broth and in the air bubble [$(c_L^* - c_L)$]. The area available for the transport [a] depends on the aeration rate and the size of air bubbles.

For a given volume of gas, more interfacial area [a] is provided if the gas is dispersed into many small bubbles rather than a few large ones. Since the efficiency of oxygen transport is approximately proportional to the ratio of the bubble surface area to the bubble volume, the

smaller size of micro bubbles increased oxygen transfer rate in the fermenter. In addition, smaller bubbles have a longer dwell time in the liquid because of their slower bubble-rise velocities allows more time for the oxygen to dissolve.

Oxygen demand of plant cells with 0.2 - 0.6 mmol•g^{-1}dw•h^{-1} (1 -3 mmol·L-1·h^{-1}; TATICEK *et al.* 1991) is relatively low compared with 5 – 90 mmol·L-1·h^{-1} in bacterial cultures (PAIVA 1999), thus fermentation with aeration below 0.3 vvm and slow stirrer speed is possible (HELLWIG 2000). However, with cell growth and increasing viscosity, oxygen transfer is becoming more and more inefficient, as a result of poor mixing and failure of air bubbles to disengage from the fermentation broth. Addition of antifoam can result in reduction of biomass attributed to a lower gas transfer rate, leading to a k_La value of up to 80 % lower with antifoam than without (TATICEK *et al.* 1991).

Shear stress must also be taken into account in fermentation of plant suspension cells. BY-2 suspension cells are more susceptible to shear stress than microbial cells because they are 10 - 100 times larger in size (20 – 40 µm in diameter and 100 – 200 µm in length), and especially older cells are almost completely filled with the vacuole. Additionally, plant cells contain a cellulose-based cell wall with high tensile strength (TATICEK *et al.* 1991).

As soon as elementary studies about the expression of the recombinant protein are finished the scale of expression can be enlarged. As the k_la value was not determined for the anti-HIV antibodies in shake flasks, the first fermentations were performed on the experience of K. Schmale (BioVII, RWTH-Aachen and SCHMALE 2002), keeping the concentration of dissolved oxygen at 20 % (v/v) by aeration of 0.1 vvm with air.

The first fermentation studies of an anti-HIV antibody were carried out with the $^{BY\text{-}2}$2G12 construct (III.1.2.1). BY-2 fermentation without antifoam resulted in a maximum concentration of 7 mg/kg cells in the first 120 h of fermentation. Intact antibody was detected over the whole fermentation process (III.1.2.1). During fermentation without antifoam, growth of BY-2 cells on all available surfaces was investigated, as observed before (FISCHER(B) *et al.* 1999; HELLWIG *et al.* 2004) as well as growth in cell-aggregates. As excessive foam formation can lead to blocked filter and probes and pressure builds up in the reactor, addition of 0.01 % (v/v) Pluronic®L61 into the sterilised media was performed for subsequent fermentations. BY-2 cells grew more homogeneous afterwards, as observed under the microscope (data not shown) resulting in the same concentration of intact antibody per gram cells and reactor volume. The 7-L bioreactor with 100 rpm and 0.01 vvm aeration supplied sufficient oxygen into the media, as levels of dissolved oxygen did not drop below

1.5 % (v/v) and cells were not affected by shearing (control of cells under the microscope, data not shown).

Due to these promising results, the $^{BY-2}$2F5 antibody was scaled-up to the 100-L scale. To reach sufficient number of cells for inoculation, one 7-L fermentation was carried out. However, this seed fermentation showed differences to the 7-L fermentation of the $^{BY-2}$2G12 before (data not shown). Cell growth stopped after 150 h as the carbon source was depleted (in difference to the $^{BY-2}$2G12 fermentation, where cells did grow for 300 h). Perhaps, continuous sampling of the fermenter did reduce the overall amount under a critical value. As soon as new media was added cell-growth started again and predicted PCV values of 50 % (v/v) were reached. The complete 7-L fermenter was used as inoculum for the 100-L bioreactor.

The 140-L pilot bioreactor could not be equally equipped as the 7-L fermenter, because marine impeller stirrer and a sinter disc did not exist for this reactor at this time point. Therefore, three six-bladed Rushton impeller and a sparger were adjusted into the bioreactor. To avoid as much shear stress as possible, baffles were removed and the stirrer was operated at 50 rpm. It was taken into account that reduced stirrer speed results in longer circulation times and less effective mass and heat transfer.

Stirrer speed of 50 rpm resulted in bubbles of up to 2 cm in diameter. If the impeller speed is to slow, the bubbles will not be broken down and will tend to rise directly to the surface due to their buoyancy. The fermentation broth before inoculation showed flooded impeller. Under these conditions, the bubbles accumulated and coalesced additionally under the impeller, leading to the formation of large bubbles and poor oxygen transfer rate. A similar phenomenon can be observed when the aeration rate is too high, but this can never be achieved in up scaling (STORHAS 1994).

The media density increased after inoculation, thus the stirrer speed resulted in better mixing of the solution, but a dispersion of the air bubbles could not be achieved. Aeration was set from 0.1 to 0.2 vvm, however the metabolic oxygen demand could not be satisfied for more than 150 h, when levels of dissolved oxygen dropped below 1 % (v/v). After 180 h of fermentation, oxygen limitation started with 0 % (v/v) dissolved oxygen measured in the media. Oxygen supply of 0.2 vvm air was maintained for the whole process, but the amount of oxygen supplied into the media was immediately used leading to areas in the bioreactor with anaerobic conditions.

In plants, a major route of ATP production under anaerobic conditions is ethanol fermentation (TADEGE *et al.* 1999). Lactic acid and ethanol fermentation was investigated in plant cell

fermentation under prolonged periods of anoxia (FARRES and KALLIO 2002). In a twelve-day fermentation of SR1-derived tobacco suspension culture expressing haemoglobin, ethanol level in the extracellular media reached up to 200 mg/L after 8 days, whereas the lactate production in the cell cytoplasm was around 1 µg/mg of dry weight. The concentrations of ethanol produced by plant cells under anoxia are insufficient to cause toxicity (reviewed in SOUSA and SODEK 2002). Lactate on the other hand is frequently associated with acidification of the cytosol and acidic pH is known to reduce antigen-binding activity (CASTLE *et al.* 2002) and to cause irreversible precipitation (JISKOOT *et al.* 1990). This can explain the high rate of degraded antibody in the 100-L fermentation.

If the oxygen level drops below the critical value of 1 – 2 % (v/v; SCHMALE 2002), oxygen consumption rate decreases with concomitant decrease in biochemical energy production, and as a result the cell growth rate also decreases (STORHAS 1994). For *E.coli* it is reported that limitation of oxygen supply reduced the cell viability since extra energy is required for plasmid synthesis and protein synthesis. On the other hand, at higher aeration rates, the recombinant strain showed highest growth rate, but low expression of recombinant protein (CHAVES *et al.* 1999). To our surprise, cell growth in the 100-L fermenter was not affected by this oxygen limitation.

IV.5.2 Downstream processing of 100-L fermentation broth

The downstream processing is the major time and volumetric bottleneck in the biopharmaceutical production process (FROST&SULLIVAN(A) 2004). The complete downstream processing of therapeutic proteins, including the downstream processing itself but also all necessary quality control checks to ensure product quality and prevent contamination, can last four to eight weeks (FROST&SULLIVAN(A) 2003). Thus, downstream processing is the highest cost in biopharmaceutical production (approximately 40 %; FROST&SULLIVAN(A) 2004). As the industry is heading to a situation of prolonged overcapacity in the manufacturing sector starting 2003, with the highest gap between supplied and needed capacity for 2005 and 2006 (FROST&SULLIVAN(B) 2004), fees for contract manufacturing are expected to fall at least 25 % over the next three years (FROST&SULLIVAN(A) 2003). Thus the production process must be highly standardized. To achieve greater volumetric throughput, increasing investment in automated systems must be undertaken which additionally reduces human resources, process development costs and increases the yield (FROST&SULLIVAN(A) 2004). The purification process of recombinant proteins depends on the protein itself and the source of the protein. Some aspects concerning purification of recombinant antibodies from plant-extracts were already investigated such as

the application of Protein-A, which can be used in a similar manner as in purification from ascites or hybridoma supernatants (FISCHER(D) *et al.* 1999). Based on this, a purification protocol for the human anti-HIV antibody $^{BY\text{-}2}$2F5 was established in small-scale from 1.2-L BY-2 cell suspension culture (III.1.1). Up scaling of the production volume to the fermenter scale is possible using special equipment for handling bulk quantities.

The aim of this downstream processing approach was to evaluate the existing equipment in respect to carrying out a process procedure of a 100-L fermentation in two days.

IV.5.2.1 Strategies to harvest cells from fermentation broth

Initial recovery of intracellular proteins can be done by separation of the biomass from the fermentation broth in centrifugation or filtration steps. Centrifugal separation is performed by two different types of centrifuges: disc-stack and tubular bowl centrifuges (FROST&SULLIVAN(B) 1999). As a result of different mechanism (rotating bowl versus rotating discs) the solids collected from the tubular centrifuge contain less water (LANGER 2004). Tubular centrifuges are available from Alfa Laval (AS16-VB) and CARR (Powerfuge P12). The CARR Powerfuge can be operated with 20,000 g with a maximum flow rate of 500 litres per hour and solids can be removed in an automated scraping cycle. Disc centrifuges can be purchased from Alfa Laval (BTP 205) and Westfalia (CSA 8), with maximum liquid capacities of 1,300 and 1,000 litres respectively (LANGER 2004).

For the filtration of fermentation broth, sufficient surface area must be provided. Thus filter membranes are packed in plate and frame stacks or Hollow Fibers. Manufacturers, such as Millipore, Pall, Amersham Biosciences and Sartorius offer a wide range of different membrane types (FROST&SULLIVAN(A) 1999). Hollow Fiber Systems are offered e.g. by Amersham Biosciences (GrandStand pilot) providing 30 litres per minute in cross-flow. A tangential flow filtration system e.g. from Millipore (ProFlux M 30 model) is supplied with Hollow fiber cartridges or flat membrane modules delivering up to 60 litre per minute. Plate and frame systems are provided by Pall (Centrasette 5 model) with 126 litre per hour and Sartorius (Sartoflow 20) with 150 litre per minute. Depth filters as single use disposables are offered e.g. by Sartorius (Sartoclear P) as capsules and modules in different sizes with different retention capacities (TARRACH 2004). The use of disposables has some advantages, such as reducing capital budget, less risk of cross-contamination and speeding of construction and production time (LANGER 2004). Flexibility is improved and process implementation times are shorter. Reduced labour (no cleaning/validation of cleaning and no chemicals for cleaning) and smaller space requirements must also be noted. On the other hand

time must be invested to test the biological and chemical compatibility of the disposables and the material costs per batch are rising.

IV.5.2.2 Initial cell harvest

The first step in the purification process is the separation of the biomass from its surrounding broth. Since the protein of interest is secreted into the culture supernatant, cell material can be discarded. However, more often the protein is expressed within the cell and must be released, as it was the case for the $^{BY-2}$2F5 construct.

As we decided to use the CARR Powerfuge for the separation of the cell debris after cell disruption (see Fig.III-9), this centrifuge could not be used for the cell harvesting due to time limitations. Initial time calculations for the 100-L purification with the available equipment, led to the decision to process the fermentation broth in two batches. It was decided to harvest the first batch discontinuously, taken 5-L samples from the 100-L fermenter and separate the cells from the fermentation broth by vacuum filtration. Resuspension into appropriate buffer was performed with 1 kg biomass samples.

The sampling in 5-L buckets, the vacuum filtration with Miracloth membrane and the homogenisation of the pellet in 5-L buckets was time- and man-power consuming but could be finished after almost three hours. For larger volumes, continuous harvesting and cell disruption should be the aim.

In conclusion: The CARR Powerfuge available at the institute can be used for initial harvest of BY-2 cells out of the fermentation broth. For taking continuous samples the fermenter must be connected to the Powerfuge.

IV.5.2.3 Strategies for cell disruption

Cell disruption methods can be divided into mechanical methods, such as sonication, French Press, Homogeniser and Ball mills and non-mechanical methods, like chelating or chaotropic agents, detergents, alkaline and enzymes (LANGER 2004). Wet (ball) milling is an efficient method, but heat is generated and especially in case of plant cells, wet milling can result in lysis of subcellular organelles, liberation of noxious chemicals (alkaloids, phenolics) and generation of fine cell debris, which are difficult to remove (SCHILLBERG *et al.* 2003). Sonication is the method of choice for small-scale purification, but wet milling and sonication are difficult to scale-up (HELLWIG *et al.* 2004). Ultrasonic industrial devices are offered by Hielscher (Teltow, Germany) with up to 16,000 Watts and a frequency of 20 kHz for industrial scale cell disruption. In a French Press cells can be opened efficiently, but the French Press (Microfluidizer M-110H) available at the institute can be operated for longer time periods only with diluted solutions thus enlarging the volume. The M-110H

Microfluidizer allows flow rates up to 450 ml/min with up to 1700 bar and contains a product heat exchanger, as well as automated **CIP** (**c**lean **i**n **p**lace) and **SIP** (**s**team **i**n **p**lace) equipment in compliance with GMP-regulations. Constant cell disruption by high pressure can be achieved additionally e.g. by the B&C Series of Constant Systems Ltd (Low March, UK). The GMP model is equipped with drain ports to allow CIP and SIP with flow rates up to 1.1 L/min at 1000 bar.

Partial digestion of plant cells was already performed with technical grade pectinase (FISCHER(F) *et al.* 1999). This method can be used for plant-expressed proteins, which are retained in the plant cell wall. For ER retarded protein, like the $^{BY\text{-}2}$2F5 construct, the cells must be disrupted completely. Therefore use of chelating/chaotropic agents and alkaline/detergents is not applicable.

IV.5.2.4 Cell disruption

For the disruption of BY-2 cells the French Press (Microfluidizer) was used. As investigated prior to the large-scale purification, cell disruption at low pressure (150 bar) was sufficient for complete release of the recombinant protein (evaluated in the SPR (II.2.7.6), data not shown). Higher pressures disrupted the cells into fine cell fragments enhancing the solubilisation of fibers and cell wall substances. This increased the viscosity of the homogenate and membranes were plugged more easily. Additionally, the solved fibers could not be removed with conventional centrifugation steps (data not shown).

In practise, cell disruption of 40-L in the French Press at low pressures did not work as expected since the pressure chamber plugged and had to be manually removed and reversal adjusted frequently. Thus the cell pellet was diluted in higher buffer volumes resulting in larger volume of homogenate containing more solved fibers than anticipated.

In conclusion: Different instruments must be tested for their feasibility using plant cells in small-scale experiments. Theoretically, the ultrasonic device by Hielscher, the M-110H Microfluidizer and the Constant Cell Disruption model by Constant Systems Ltd. seem to be applicable.

IV.5.2.5 Strategies for protein separation and purification

Separation and purification of the product can be achieved by filtration and chromatography. With appropriate membrane devices, the homogenate can be clarified in a tangential flow or dead-end filtration (DESAI 2000). For removal of cell debris different companies, like Millipore, Pall and Sartorius offer membranes with different pore sizes (FROST&SULLIVAN(A) 2004). Disposable single-use capsules by Sartorius can be

implemented e.g. for cross-flow filtration (MEYEROLTMANNS 2004), to reduce personal costs and enhance the reproducibility.

Adsorption of the protein to chromatography matrices is performed commonly with three types of functional compounds: charged, hydrophobic and affinity. Charged groups are divided into two groups: anionic and cationic. **A**nion **ex**change (**AEX**) chromatography can be used to capture DNA and impurities (DIERKS 2005). **C**ation **i**on **ex**change (**CIEX**) chromatography, e.g. at pH 5 captures 99 % of different antibodies to the column (DIERKS 2005). In **e**xpanded **b**ed **a**dsorption (**EBA**), the bed expands through the flow of the feed from below, increasing the interstitial spaces among the beads. Thus cruder samples can be applied. Genentech has developed an EBA approach, which can purify hybridoma supernatant in a single step (LANGER 2004). Resins with the dynamic capacity to allow protein adsorption at a high flow rate and short residence time are available from Amersham Pharmacia (STREAMLINE^{TM} media) and Ciphergen (Hyper Z) with different functional groups serving as cation/anion exchanger coupled with Protein-A (LANGER 2004). Clarification, concentration and initial purification of proteins from cell extracts or plant cell fermentation broth has been performed with the STREAMLINE^{TM} by Amersham Biosciences (BAI and GLATZ 2003). Initial trials of loading the BY-2 homogenate after the French Press directly to the STREAMLINE^{TM}-EBA column failed, as the expanded bed was slugged out after a while. However, binding of recombinant antibody could be determined by SPR (II.2.7.6, data not shown). It can be speculated that different cell disruption methods result in less viscous homogenates which can be applied onto the STREAMLINE^{TM}.

In **h**ydrophobic **i**nteraction **c**hromatography (**HIC**) hydrophobic compounds such as methyl, ethyl, isopropyl, n-butyl and phenyl are used for capturing of e.g. the hydrophobic Fc part of antibodies. This can be implemented as last purification step where the antibody is in the flow through but aggregates bind (DIERKS 2005).

Affinity chromatography uses ligands, which specifically bind the protein of interest. For purification of recombinant antibodies Protein-A matrices are applicable, e.g. Mab-Select by Amersham Pharmacia (DIERKS 2005), BioSEPRA Protein-A Ceramic HYPERD F by Ciphergen, Prosep-A Protein-A by Millipore and Styros-rA by Orachrom.Inc.. New emerging alternatives include membrane affinity chromatography by Sepracor Inc. (Trio Bioprocessing System), which build a Hollow fiber membrane (FROST&SULLIVAN(A) 2004). Membranes are thinner than chromatography column beds, thus the separation can be processed more rapidly. Since Protein-A resins are costly and require extensive cleaning and Protein-A cannot be used for purification of human IgG3, companies such as BioInvent International AB

(Lund, Sweden) and Medimmune (Gaithersburg, MD), have optimised the use of ion exchange and hydrophobic chromatography for protein-specific purification (LANGER 2004).

IV.5.2.6 Product separation and purification

The separation of cell debris from the initial 100-L fermentation was performed using the CARR Powerfuge in 2 h (with cleaning cycles). Higher rotation speed of the bowl may remove even smaller particles, since centrifugation with 10,000 g in the laboratory scale centrifuges resulted in a better separation and clearer supernatant (data not shown). For larger quantities, equipment for continuous separation can be connected.

For fine clarification of the supernatant, a Hollow Fiber Unit with a cut-off of 500kDa was used. Supernatant was applied with 30 -35 ml/min leading to over-night-recirculation of the preparation. The Hollow Fiber unit did no plug, but was unable to separate the remaining fibers and cloudy substances from the protein of interest [antibody was detected via SPR (II.2.7.6) in the retentate instead of the filtrate (data not shown)]. This can be explained by aggregation of antibody or adsorption to fiber particles, resulting in particles with a size larger than 500 kDa. Conclusively, increased volumes can be handled with larger Hollow Fiber Units but experiments with intact antibody preparations must be repeated before.

Affinity chromatography of plant extracts on a Protein-A medium was successfully applied for removal of contaminants and 100-fold concentration of the protein (FISCHER(F) *et al.* 1999). For selective capturing of the human anti-HIV antibody $^{BY\text{-}2}$2F5, BioSEPRA Protein-A Ceramic HYPERD F matrix was evaluated initially in small-scale experiments (III.1.1). Two-L clear supernatant were applied onto a XK 26/20 column packed with 10 ml Protein-A with 10 ml/min. For larger volumes the amount of Protein-A matrix can be increased, allowing higher flow rates.

Protein recovery was 50 % after Protein-A in contrast to the small-scale column, where 84 % were eluted from the matrix (III.1.2.3). Since all washing elution were tested (data not shown) and the flow-through did not include detectable amounts of antibody, the only explanation is, that the antibody still bound to the column or that elution with buffer at pH 2 denatured the antibody. The elution and subsequent pH shift to pH 4.5 with 1 M unbuffered sodium acetate was optimised in small-scale experiments (data not shown). But Westernblot analysis of the fermentation broth revealed only degraded antibody fragments. It can be speculated that these fragments were more susceptible to pH-shift and were denatured by elution with pH 2.

In conclusion: Loading the STREAMLINE^{TM} from Amersham Biosciences with crude extract or preclarified supernatant is the fastest way of purifying $^{BY\text{-}2}$2F5. Depending on the

isoelectric point of the protein of interest, cation or anion exchange matrices can be used. Alternatively, STREAMLINE™ adsorbent coated with Protein-A (STREAMLINE™ rProteinA) might be an alternative (THOMMES *et al.* 1996).

IV.5.2.7 Polishing step

Subsequent size exclusion chromatography (gel filtration) can be used as polishing step to remove antibody dimers and higher aggregates, as successfully performed for a full-size IgG by Fischer *et al.* [(F) 1999].

In general, viral contamination must be removed in additional steps by inactivation (heat, irradiation; DIERKS 2005) or ultrafiltration. One of the advantages in using plant cells is that plants do not harbour human pathogens (GIDDINGS *et al.* 2000). However, plant-derived biopharmaceuticals can contain biologically active plant metabolites or alkaloids (e.g. nicotine), plant macrometabolites (extraneous proteins, DNA, polysaccharides and lipids) and in case of whole plants additionally pesticides and herbicides (MIELE 1997).

Heat inactivation cannot be performed in most cases due to heat sensitivity of the protein of interest. UV-irradiation is a method commonly used in the food processing industry. Three different technologies can be used to irradiate food including gamma irradiation, electron beam (E-Beam) irradiation and X-ray irradiation. Companies such as Gray*Star, Inc., GUARDION and MDS Nordion offer irradiation equipment (FROST&SULLIVAN 2005).

Sartobind single-use modules (Sartorius; FISCHER-FRÜHHOLZ 2004) were developed for the removal of peptides, DNA, endotoxins, viruses and proteins. Sartorius also offers a concept for inactivation and removal of viruses and other contaminations by a combination of virus retentive membranes, viral inactivation by UV-irradiation and membrane adsorber (REIF 2004).

Final polishing steps will include thermal processing such as drying of the product with different dryer types (e.g. vacuum rotary dryer or fluidised bed drying; FROST&SULLIVAN(A) 2000) or protein crystallization (Bayer Technology Services; FROST&SULLIVAN(A) 2004) to improve stability and purity of the recombinant protein.

IV.6 Outlook $^{BY\text{-}2}$2F5 and large-scale fermentation/purification

Conclusively, BY-2 suspension cultures can be used to obtain intact and highly active human full-size antibody in a few months for detailed studies. The antibody yield in BY-2 cells is comparable to the published antibody levels in CHO cells. However, until now, there are no FDA-approved plant recombinant antibodies on the market and there might be considerable

consumer resistance. The safety of plant derived recombinant proteins, concerning the glycosylation pattern must be extensively proven before this antibody can enter clinical trials.

Initial fermentation of BY-2 cells expressing the $^{BY-2}$2F5 human anti-HIV antibody was performed in a 100-L scale. BY-2 cells were successfully grown in the 100-L fermenter but the heavy and light chain of the $^{BY-2}$2F5 antibody were degraded during the fermentation process. First demand for scale-up into the 100-L bioreactor is the appropriate equipment, as a marine impeller stirrer and a sinter disc. This will provide sufficient oxygen transfer at least at the fermentation start into the medium and reduces the shear stress onto the cells. Scale-up parameters can be calculated since bioreactor design is maintained.

However, the $^{BY-}$2F5 antibody yield must be increased dramatically to justify a scale-up into a 100-L bioreactor. Production of TMV-specific recombinant full-size antibody in plant cells yielded 15 mg/kg wet cell weight purified out of 1 kg BY-2 cells (FISCHER(F) *et al.* 1999). Highest yield of $^{BY-2}$2F5 antibody was 3.2 mg/kg in the small-scale purification (III.1.1). Additionally, the level of TMV-specific recombinant antibody was transiently increased threefold by supplementation of essential and non-essential amino acids (FISCHER(F) *et al.* 1999). To investigate the effects of oxygen limitation and amino acid supplementation onto BY-2 cells expressing the 2F5 antibody, three additional 5-L fermentations were carried out (data no shown). One fermenter was operated with unlimited oxygen and two with forced oxygen limitation after 120 h at a PCV of approximately 30 %(v/v). One of the oxygen limited fermentations was fed with 2.7 g/L casamino-acids after 120 h. Cell growth measured in PCV and FW was faster in the non-limited fermentation, reaching 60 % (v/v) PCV after 240 h with oxygen in contrast to 40 % (v/v) with limitation. Unlimited oxygen did not result in higher antibody yield and the degree of degradation was not tested. The amino acid feed seemed to have a stabilizing effect on the intracellular antibody, but these experiments have to be repeated.

Conclusively, the expression level of $^{BY-2}$2F5 antibody in BY-2 cells is not sufficient to repeat fermentation studies on a large scale. But the 100-L fermentation trial revealed important information to optimise the procedure of BY-2 cultivation in large-scale bioreactors.

With information from the large-scale purification employing the available equipment and taking data from the literature into considerations, suggestions can be made which equipment might be suitable to enable a 100-L downstream processing of BY-2 cells harbouring $^{BY-2}$2F5 antibody in two days. The following schematic overview shows a selection of basic units.

DSP step	Instrument	company	flow [ml/min]	duration [h]
Cell harvest	Powerfuge	Carr	300-500	3 ½ - 6 h
Cell disruption	Ultrasonication	Hielscher	?	?
	M-110H	Microfluidics	450	4 h
	Constant cell disruption	Constant Systems Ltd.	1100	1 ½ h
Protein Purification	Streamline 200	Amersham Biosciences	feed application	
			1050 - 2100	¾ - 1½ h
			elution sedimented bed	
			270 - 785	< 1 h

The eluted volume varies between 4.7 and 9.4 L. As prerequisite, cell disruption must be tested and optimised with different equipment to choose a useful device. Downstream processing of 100-L might be possible with this equipment in two days in compliance with GMP requests.

IV.7 Outlook rsCD64

Comparison of three different systems (*E.coli*, Mammalian HEK 293T cells and *N.tabacum* leaves) for the expression of functional rsCd64 revealed the mammalian expression system as the system of choice to produce functional protein at levels of 1 mg/L.

Successful expression of functional rsCD64 opens many perspectives for biochemical characterisation and analytical applications. Physiochemical analysis of the IgG-FcγRI interaction will be possible as well as the identification of the binding site of anti-CD64 antibodies outside the IgG binding site. The property of the unique high-affinity binding to monomeric IgG may be revealed and structure determination by crystallization of the soluble receptor will be possible.

A first step towards crystallization is the removal of glycosylation sites through the construction of deletion mutants. Expression levels of deletion constructs will provide more information about the need of all three domains for the high affinity binding. Rational protein design can guide in the construction of rsCD64 with irreducible size and unchanged or even enhanced binding affinity. It can be speculated that smaller constructs are expressed at higher levels. To further enhance the expression, hydrophobic amino acids will be removed at the C-terminus. The point mutation at position 83 (leucine to arginine) changes a hydrophobic amino acid into a basic one. It can be speculated, that this amino acid change contributes to the expression of rsCD64, since nobody has succeeded in the expression of soluble CD64 so far.

Analytical applications can be found primarily in fields where Protein-A/G is currently used.

Protein-A, G and FcγRI bind to the Fc part of human IgG. Protein-A is a 43kDa protein, isolated from *Staphylococcus aureaus* and Protein-G, a 35kDa protein is isolated from *Streptococcus sp.*. Both react with a large number of IgG isotypes. Protein-A binds human

IgG1, 2 and 4 with high affinity, whereas Protein-G strongly binds additionally to human IgG3. FcγRI binds to human IgG3, 1 and 4 with high affinity. Binding sites are different, thus FcγRI does not compete with Protein-A for binding to IgG1 (WINES *et al.* 2000).

Purification of recombinant antibodies is performed in general by Protein-A due to its high specificity and strong binding to the Fc part of immunoglobulins (DIERKS 2005). The equilibrium constant for Protein-G and Protein-A was determined to $1.13 \cdot 10^8$ M^{-1} and $2.9 \cdot 10^7$ M^{-1} respectively (SAHA *et al.* 2003). The kinetics of association and dissociation of human IgG1 to FcγRI on U937 cells was investigated with a ^{125}IgG1 monomer (SHOPES 1995). At 37 °C, the association constant was determined to $2.7 \cdot 10^5$ M^{-1} and the dissociation rate to $4.5 \cdot 10^{-4} s^{-1}$. The association and dissociation rate of rsCD64 to human IgG1 was determined in the SPR (II.2.7.6) analysis to comparable concentrations ($k_{on} = 1.5 \cdot 10^5$ M^{-1}, $k_{off} = 3 \cdot 10^{-4} s^{-1}$). The resulting equilibrium constant was calculated to be $2 \cdot 10^9$ M^{-1}. Thus rsCD64 shows comparable binding kinetics to human IgG1 as the membrane anchored receptor. Secondly, this binding affinity is 10 times stronger than binding to Protein-G and 100 times stronger than binding to Protein-A.

Bulk quantities of rsCD64 might replace Protein-A as the major substance for purification of recombinant antibodies. Stronger binding will lead to even more specific separation.

Other application may benefit from the oriented binding of full-size antibodies. This allows direct investigation of the functional activity of different full-size antibodies by binding of the target antigen, e.g. in SPR (II.2.7.6) analysis. Interaction properties with the target antigen could be investigated in detail. For *in-vitro* diagnostics, applications like the detection of low abundant antigens are imaginable. Interaction properties with the target antigen can be investigated in detail. This might also be interesting for *in-vivo* imaging by directed immobilization of disease-related full-length antibodies to rsCD64 conjugated to optically detectable labels.

IV.8 Outlook FcγRI and HIV

Cells of the monocyte/macrophage lineage exert crucial functions in innate immune response as well as in inducing and regulating cognate immunity against infectious agents. Macrophages can, however, be infected with any of several pathogens, including parasites, bacteria and viruses. In particular, macrophages are one major target of HIV-1. According to several studies, macrophages participate in initial transmission of HIV-1, in virus-spreading and cell-to-cell transmission within lymphoid tissues (PEREZ-BERCOFF *et al.* 2003). As HIV causes chronic infection of macrophages only with minimal cytopathology, these cells

can provide an important viral reservoir in HIV-infected persons, being even called the "Trojan horse" for the dissemination of virions (HOLL *et al.* 2004). Additionally, macrophages are an important reservoir for viral replication in late stages of AIDS, since HIV-1 infection of macrophages persists in the tissue for extended periods of time (months) with large numbers of virions contained within intracytoplasmic vacuoles (ROSENBERG and FAUCI 1991; CROWE 1995).

Infection of macrophages occurs via a combined role of CD4 and FcγR (JOUAULT *et al.* 1991). HIV complexed with human anti-HIV IgG can attach to FcγR of mononuclear phagocytes. Connor *et al.* postulated (1991) two hypotheses for FcγR acting in HIV-infection and identified the degree of antibody opsonisation as critical factor. Highly opsonised HIV will initiate high-affinity multi-valent interactions with FcγR and mediate endocytosis by FcγR and intracellular degradation of antibody-virus complex. Whereas few interactions on HIV lead to stabilisation of the virus at the cell-surface and **a**ntibody-**d**ependent **e**nhancement (**ADE**) of virus infection can occur subsequently (PORTERFIELD 1986). In particular, IgG bound to virus was reported to favour virus persistence when trapped by **f**ollicular **d**endritic **c**ells (**FDC**), a non-leukocyte cell population of unknown origin, mainly concentrated in follicles and specialised to capture antigen in form of immune complexes for long time periods (JANEWAY *et al.* 2001; SMITH-FRANKLIN *et al.* 2002).

Infected macrophages continuously release cytokines and chronically activate immune cells in the near environment. Dysregulation of the cytokine profile *in-vivo* and *in-vitro* was investigated as e.g. increased IFN-γ secretion and macrophage-colony-stimulating factor (M-CSF; reviewed in KEDZIERSKA and CROWE 2001). IFN-γ induces de novo expression of FcγRI on neutrophils and finally leads to chronically IFN-γ activated macrophages (CAPSONI *et al.* 1992). A significant increase in the expression of FcγRI on **a**lveolar **m**acrophages (**AM**) in the lung of HIV+ subjects in early infections was reported (GILBODY *et al.* 1997), as well as a continuous and significant increase on PMNs, which do not constitutively express FcγRI in late stages of HIV-infection (MOALLEM *et al.* 2000).

Despite of this, a number of monocyte/macrophage functions are impaired following HIV-1 infection *in vivo* and *in vitro*, including decreased accessory cell function (PETIT *et al.* 1988), chemotaxis (TAS *et al.* 1988; WAHL *et al.* 1989), phagocytosis (BIGGS *et al.* 1995; KEDZIERSKA *et al.* 2000; KEDZIERSKA *et al.* 2001), intracellular killing (BIGGS *et al.* 1995), and cytokine production (reviewed in KEDZIERSKA and CROWE 2001). These defects contribute to the pathogenesis of AIDS by allowing reactivation and development of opportunistic infections. Reduced phagocytic capacity for certain opportunistic pathogens,

including *Toxoplasma gondii* and *Candida albicans*, may be responsible for reactivation of these pathogens in persons with advanced HIV-infection (reviewed in CROWE 1995).

Bispecific antibodies targeting HIV-1 and FcγRI reduced the infectivity of HIV towards T cells (HOWELL *et al.* 1994) and mediated ADCC and virus neutralisation (MABONDZO *et al.* 1994). On the other hand, crosslinking of FcγRI and FcγRII activates HIV-1 gene expression in infected monocytes by inducing NF-*kappa* B, which binds to the LTR of HIV-1 (TSITSIKOV *et al.* 1995). Chronically infected macrophages have shown impaired phagocytosis (BIGGS *et al.* 1995) and the ability of HIV to persist in latently infected cells is the major barrier to eradication or cure of HIV. Thus, macrophages represent a therapeutic target which is, unlike to HIV, not highly mutable and directly targets the virus reservoir. One drug, WF10, is used as adjunct in **h**ighly **a**ctive **a**ntiretroviral **t**herapy (**HAART**). WF10 causes changes in macrophages function and activation of gene expression. It appears to down regulate inappropriated immunological activation (McGRATH *et al.* 2002) and increased the healthy activity of macrophages without increasing the amount of HIV in the blood.

FcγRI, the high-affinity receptor for monomeric IgG contributes highly to the degradation of pathogens by phagocytosis. In case of HIV-infection, high levels of IFN-γ and M-CSF in the sera of HIV-patients enhances the number of FcγRI on constitutive expressing cells further on and induces the expression on dendritic cells, neutrophils and mast cells. This leads to an overall increasing of FcγRI in immunological cells outnumbering FcγRII and FcγRIII. As FcγRI binds monomeric IgG with high-affinity, it can be postulated that HIV-virus opsonised with few antibodies is bound to macrophages predominantly by FcγRI. Possibly, FcγRI is the main contributor in the mechanism of ADE. Virus persistence by long-time binding of opsonised virus particles to FDCs maybe caused also by FcγRI. Perhaps, the interaction of FcγRI should be completely avoided in HIV-therapy but it can also be the major factor in virus neutralisation with anti-HIV antibodies.

Conclusively, it is not known whether FcγRI has a positive or negative effect in HIV pathogenesis. Binding of soluble FcγRI to human anti-HIV antibodies would specifically impair additional binding of the Fc part of these antibodies. How this contributes to virus neutralisation can be investigated in future experiments. Combination of rsCD64 and anti-HIV antibody 2F5 may reveal the underlying mechanism in neutralising action of anti-HIV antibodies.

V. Summary

The production of two complex human proteins, the therapeutically relevant anti-HIV full-size IgG1/κ antibody, $^{BY-2}$2F5, and a soluble form of the high-affinity receptor for human IgG, FcγRI (CD64), were successfully established and optimised for heterologous expression in plants, bacteria and mammalian.

Aim of this thesis was the establishment of a protocol for the expression and purification of the anti-HIV antibody, 2F5, in plant suspension cultures. Intact and pure antibody could be purified at levels of 1 mg/L per suspension culture out of 340 g BY-2 pellet. ELISA proved comparable antigen binding capacity of the $^{BY-2}$2F5 antibody, EMSA and SPR to the CHO derived control antibody. Neutralisation assays were performed in Vienna with this purification showing no influence of plant derived glycosylation.

Initial fermentation experiments have shown that BY-2 cells can be grown without limitations on 100-L scale, but conditions for stable antibody production and downstream processing have to be further investigated. For GMP implementation and expression in large-scale reactors under defined conditions protocols and procedures must be established first. Based on the experience in the 100-L downstream processing, feasible equipment was suggested for a purification procedure of 100-L BY-2 culture in approximately two days in compliance with GMP regulations.

The second aim of this thesis was to express FcγRI as soluble recombinant protein in an appropriate expression system. The extracellular domain of FcγRI was generated and cloned into vectors for the expression in *E.coli*, mammalian and plant cells. All three systems were investigated for their ability to produce functional rsCD64. Highest expression levels were reached in the mammalian expression system yielding 1 mg/L. The expression and purification protocol was optimised and purity as well as integrity of rsCD64 was confirmed. IgG binding activity was confirmed by ELISA and SPR studies.

Purified rsCD64 was functional as demonstrated by IgG binding, and the binding characteristic was comparable to that of the original membrane bound receptor. Functional, soluble protein is one of the prerequisites for intensive characterisation of the FcγRI. This characterisation, will lead to a better understanding of FcγRI functionality and development of new specific FcγRI targeted therapeutics or antibody affinity reagents.

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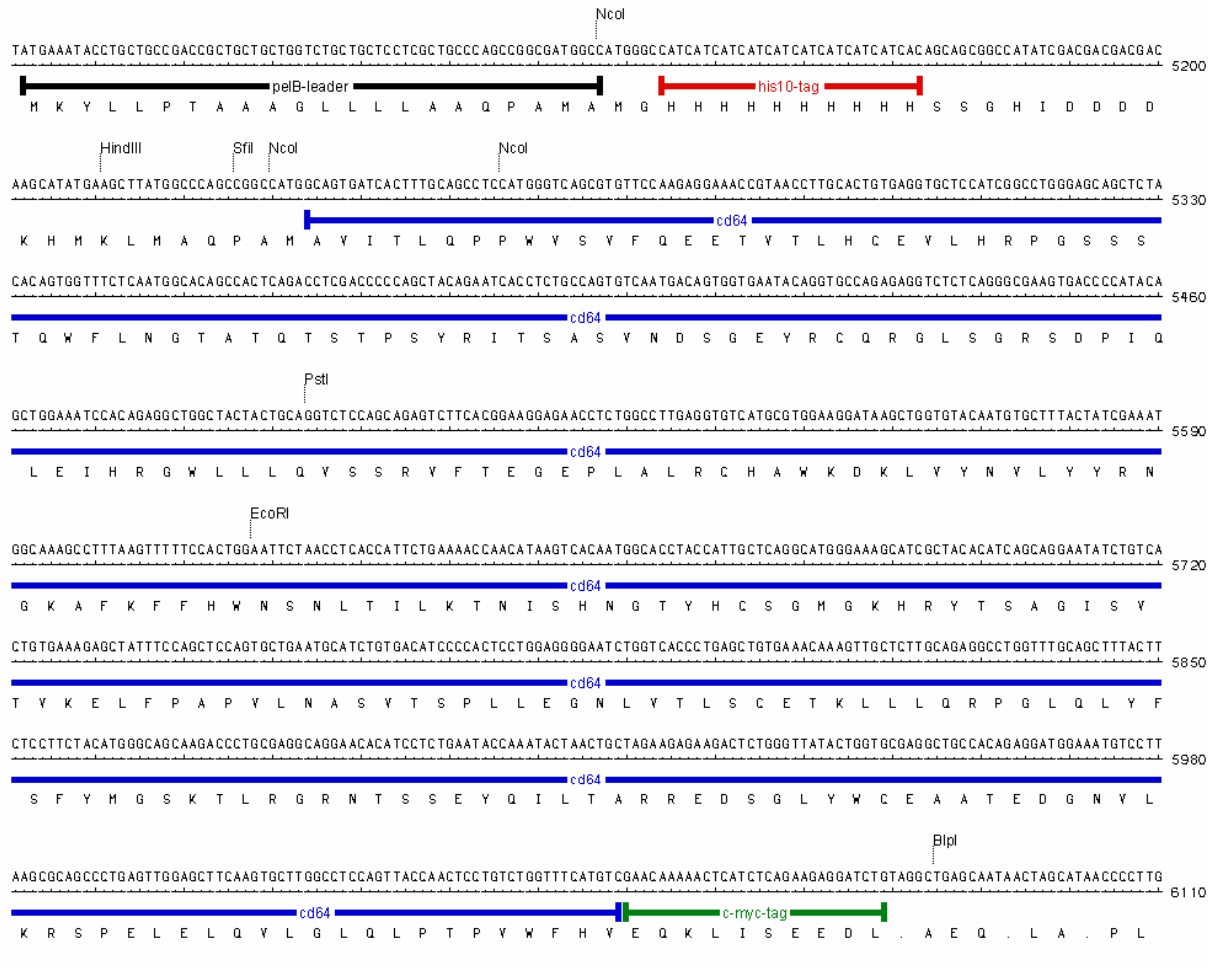
VII. Appendices

VII.1 Abbrevations

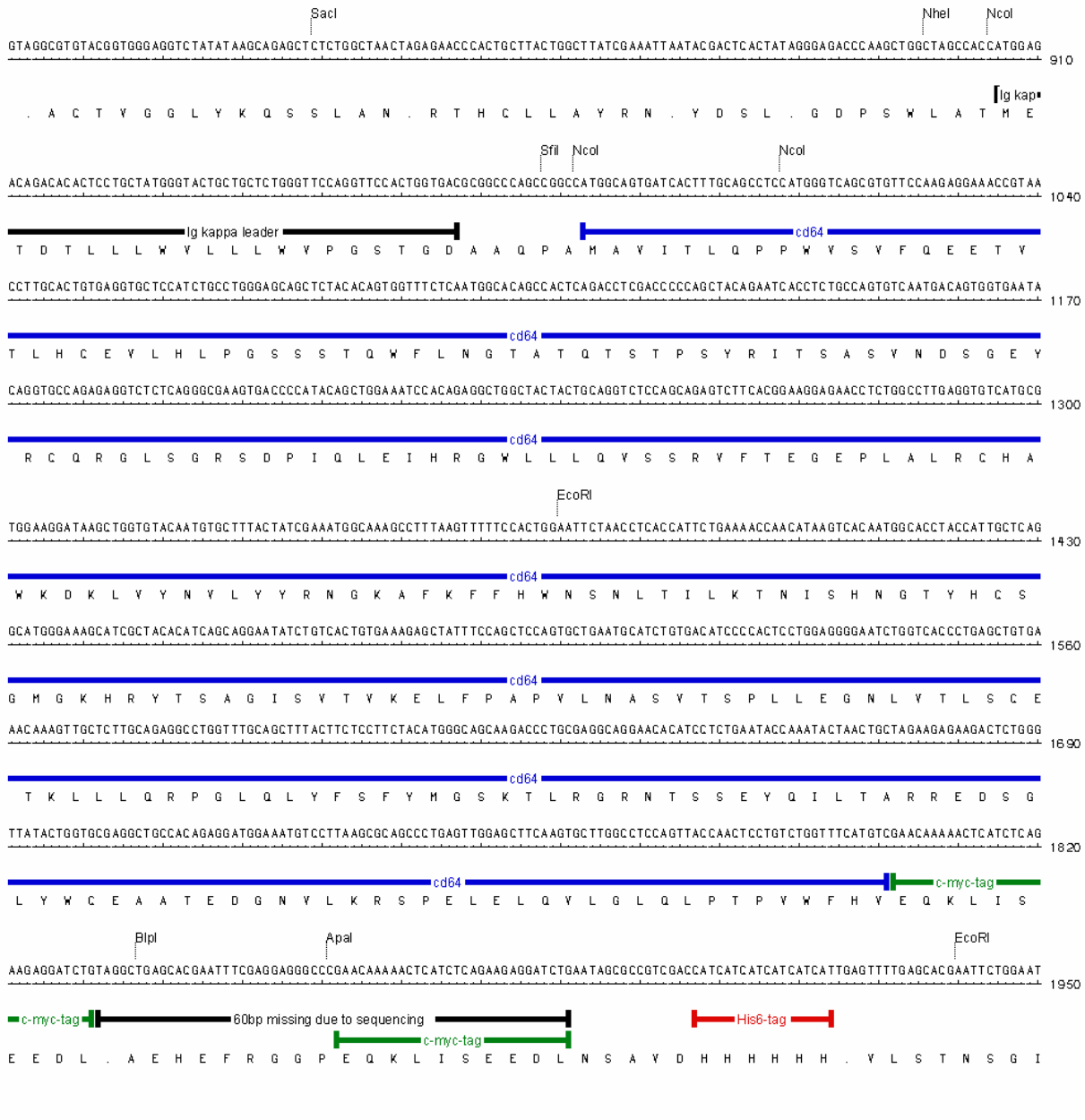
α	anti, used in antibody abbrevations
ADCC	antibody-dependent cell-meditated cytotoxicity
ADE	antibody-dependent enhancement
AEX	anion exchange chromatography
AIDS	acquired immune deficiency syndrome
AM	alveolar macrophages
BHK	baby hamster kidney, mammalian cell line
BGH	bovine growth hormone
BSA	bovine serum albumin
BY-2	bright yellow *N.tabacum* suspension cultures
C_L	constant region of light chain of an an antibody
C_H	constant region of heavy chain of an an antibody
CaMV	cauliflower mosaic virus
CDR	complementarity determining region
CHO	chinese hamster ovary, mammalian cell line
CIEX	cation ion exchange chromatography
CIP	clean in place
CIP	calf intestine phosphatase
DMSO	dimethylsulfoxide
dpi	days post infiltration
EBA	expanded bed adsorption
eGFP	enhanced green fluorescent protein
ELISA	enzyme linked immunosorbent assay
EMEA	European Agency for the evaluation of medicinal products
ER	endoplasmic reticulum
Fab-fragments	fragment with antigen binding (antibody fragment)
Fc fragment	fragment crystallisable (antibody fragment)
FDA	food and drug administration
FDC	follicular dendritic cells
FR	framework region
FTE	full time employees
GαH	goat-anti-human antibody
GαM	goat-anti-mouse antibody
gp	glycoproteins
GM-CSF	granulocyte macrophage colony-stimulating factor
HAART	highly active antiretroviral aherapy
HBV	hepatitis B virus
HC	antibody heavy chain
HEK-293	human embryonic kidney, mammalian cell line
HIC	hydrophobic interaction chromatography
HIV	human immunodeficiency virus
hpi	hours post inoculation
IDA	iminodiacetic acid

Ig	immunoglobulins
IMAC	immobilized-metal affinity chromatography
IRES	internal ribosomal entry site
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
kDa	kilo-Dalton
k_La	oxygen transfer coefficient
LC	antibody light chain
MAbs	monoclonal antibodies
MCS	multiple cloning site
MDM	monocyte-derived macrophage
MHC	major histocompatibility complex
NBT-BCIP	nitro blue tetrazolium chloride/5-bromo 4-chloroindol-3-yl phosphate
NK	natural killer
NTA	nitrilotriacetic acid
o/n	overnight
OTR	oxygen transfer rate
PEG	polyethylene glycol
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
rpm	rotation per minute
RT	room temperature
RU	resonance units
SAR	scaffold attachment region
SARS	severe acute respiratory syndrome
SIP	steam in place
SIV	simian immunodeficiency virus
SOE	splice overlap extension
SOP	standard operation procedure
SP	signal peptide
SPR	surface plasmon resonance
TCR	T cell receptor
TEV	tobacco etch virus
TP	transit peptide
w/v	weight per volume
V_L	variable region of light chain of an antibody
V_H	variable region of heavy chain of an antibody
v/v	volume per volume

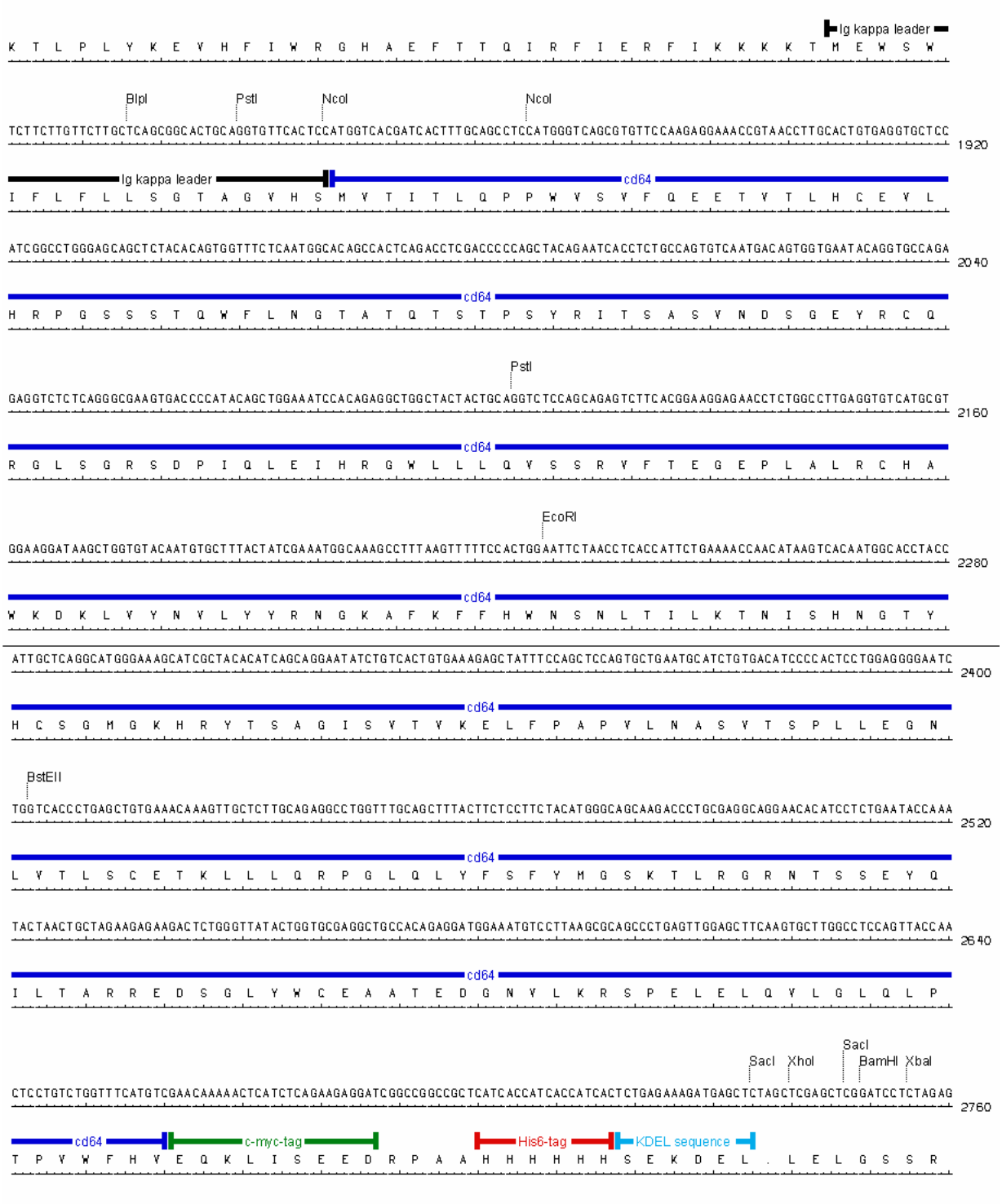
VII.2 Sequence of pMT-cd64



VII.3 Sequence of pMS-cd64



VII.4 Sequence of pTRA-cd64(ERH)



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Wege entstehen dadurch, daß man sie geht.
Franz Kafka

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Wer ein Problem definiert, hat es schon halb gelöst.
Julian Huxley

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unbekannt

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Lucius Annaeus Seneca

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Henry Louis Mencken

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Johann Wolfgang von Goethe

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Persische Weisheit

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Lebenslauf

PERSÖNLICHE DATEN

Name	Antje Pätz
Adresse	An der Junkersmühle 33-35 52064 Aachen
Tel.-Nr.	0241/9436595
Email	paetz@molbiotech.rwth-aachen.de

SCHULAUSBILDUNG

01/09/1982 – 30/06/1995	Gymnasium Blücherstrasse, Köln, Abschluss: Abitur (Note: 1,8)

HOCHSCHULSTUDIUM

01/10/1995 – 25/08/2000	Diplomstudium, Fachrichtung Bio- und Chemietechnik, Studiengang Biotechnologie, FH Mannheim, Hochschule für Technik und Gestaltung; Diplomarbeit: „Aufbau eines Fluoreszenz-Oberflächen-Assay für die Detektion von Interleukin-6 im Vergleich zu einem herkömmlichen ELISA“ bei der Firma MMI-Molecular Machines & Industries, Heidelberg (Note: 1,3); Abschluss: Dipl.-Ing. (FH) (Note: 1,9).
01/10/1998 – 27/09/2001	Study programme Master of Science in Biotechnology, Option Biomedical Sciences, Mannheim University of Applied Science; Masterarbeit: Investigation on effects of protein kinase B on ganglioside-triggered cell growth arrest and virus reactivation in EBV-genome positive B-lymphocytes, Institut für medizinische Mikrobiologie, Lehr- und Forschungsgebiet Virologie, Universitätsklinikum, RWTH Aachen (Note: 1,2); Degree: Master of Science (MSc) in Biotechnology (Grade: 1,4 [excellent]).
01/08/2001 – 30/08/2005	Promotion, Fraunhofer-Institut IME - Institut für Molekularbiologie und Angewandte Oekologie, Aachen; Doktorarbeit: Process optimization for the expression and purification of functional therapeutically relevant proteins in heterologous expression systems.

PRAKTIKA/AUSLANDPRAKTIKA

01/09/1996 – 31/01/1997	Labor für Qualitätskontrolle, DOM-Brauerei GmbH, Köln; Thema: Methoden zur Qualitätssicherung bei der Produktion von DOM-Kölsch.
03/03/1998 – 06/10/1998	Labor für Immunologie, Firma BIOSChile Ingenieria Genetica S.A., Santiago, Chile; Thema: Optimización de la produccion de una vacuna para el Síndrome Rickettsial de Salmonideros (SRS).
01/10/1998 – 30/11/1998	Studienarbeit, Firma LION Bioscience AG, Heidelberg; Thema: Feinkartierung eines 90kb-Fragmentes von *Arabidopsis thaliana* als Voraussetzung für eine gezielte DNA-Sequenzierung.

ZUSATZQUALIFIKATIONEN

01/12/1998 – 28/02/2000	Anstellung als studentische Hilfskraft, LION Bioscience AG, Heidelberg; Aufgabe: Computerunterstützte Aufnahme und Auswertung von Hybridisierungsdaten und computerunterstützte Erstellung und Auswertung physikalischer Karten genomischer DNA-Bibliotheken.
01/05/2002 – 30/04/2005	Anstellung als wissenschaftliche Hilfskraft, Fraunhofer-Institut IME, Aachen; Aufgabe: Organisation und Durchführung des Bestell-wesens für den allgemeinen Laborbedarf sowie Angebotsverhandlungen mit Außendienstmitarbeitern.

EDV-KENNTNISSE

Betriebssystem	Windows, Macintosh
Anwendungsprogramme	Windows Office, Origin, DNA-Star, Clone-Manager

FREMDSPRACHEN

Englisch	gute Kenntnisse in Wort und Schrift
Spanisch	Grundkenntnisse (schnell auffrischbar)
Französisch	Grundkenntnisse