NKRF phosphorylation regulates transcription elongation via its interactions with exoribonuclease (XRN2) and negative elongation factor (NELF)

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ABSTRACT

NF-κB repressing factor (NKRF) inhibits transcription elongation by binding to specific sequences in target promoters. Stimuli such as interleukin-1 (IL-1) have been shown to overcome this inhibitory action and enable the resumption of transcription elongation machinery by an unknown mechanism. Using mass-spectrometry and in vitro phosphorylation analyses, we demonstrate that NKRF is phosphorylated within three different domains in unstimulated HeLa cells. Phosphoamino acid mapping and mutation analysis of NKRF further suggest that only serine phosphorylation within amino acids (aa) 421-429 is regulated by IL-1 stimulation. In co-purification studies, aa 421-429 is required for interactions between NKRF, 5′-3′ exoribonuclease 2 (XRN2) and the negative elongation factor (NELF) complex in HeLa cells. ChIP experiments further show that IL-1 stimulation leads to decrease in NKRF aa 421-429 phosphorylation and dissociation of NELF-E and XRN2 by concomitant resumption of transcription elongation of a synthetic reporter or the endogenous NKRF target gene, Interleukin-8. Together, NKRF phosphorylation modulates promoter-proximal transcription elongation of NF-κB/NKRF regulated genes via direct interactions with elongation complex in response to specific stimuli.
INTRODUCTION

NF-κB repressing factor (NKRF, previously designated as NRF) is a constitutively expressed transcription factor that binds to a specific 11-base pair DNA sequence in several NF-κB-regulated cytokine genes and viral promoters (Fig 1) [1-4]. When binding adjacent to a target promoter, NKRF and NF-κB p65 were shown to interact through a minimal core sequence in NKRF (aa 204-308) and three unique protein motifs within the p65 Rel homology domain [5, 6]. NKRF knockdown experiments further confirmed a dual function for NKRF in transcription regulation. Specifically, NKRF repressed the basal transcription of its target genes in unstimulated cells; however, NKRF was also able to serve as a transcriptional co-activator after the specific stimulation of cells using IL-1 or different virus strains [1-5, 7-9]. The mechanistic role of NKRF has been best studied in the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) [1, 10-12]. We have previously shown that NKRF binding to HIV-1 LTR interrupts the elongation of proximally initiated transcripts in unstimulated cells [1, 5]. This mode of transcriptional regulation was generally known as promoter-proximal pausing; it occurs during RNA polymerase II (Pol II)-driven transcription of various genes [13]. The positive elongation factor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) sensitivity-inducing factor (DSIF) and the negative elongation factor (NELF) were previously identified as key regulators of promoter-proximal transcription pausing [14, 15]. At this stage, 5′→3′ exoribonuclease 2 (XRN2) has also been implicated in transcription elongation via the termination of initiated transcripts in many RNA Pol II dependent promoters [16]. Nevertheless, the majority of paused Pol II enzymes and initiated transcripts were found to remain in the gene bodies until a specific signal leads to the resumption of the transcription elongation complex, thereby increasing the elongation of initiated transcripts [13, 17].

In human T cells with integrated HIV-1 provirus, NKRF was shown to inhibit the recruitment of DSIF to the LTR by an unknown mechanism [1]. This effect was accompanied by a significant decrease in Pol II Ser 2 phosphorylation in the C-terminal domain (CTD), which is a hallmark of paused transcription elongation [18]. Following stimulation by the continuous binding of NKRF to the LTR, DSIF was shown to associate with the elongation complex and accelerate the elongation of initiated transcripts. However, NKRF by itself did not bind to DSIF or affect Pol II CTD Ser 2 phosphorylation in HIV-1 LTR [1]. Moreover, neither the affinity of NKRF for its target promoters nor its interaction with p65 was affected by different stimuli [1, 5, 19]. Thus, it was rather difficult to determine a direct role for NKRF in the termination and resumption of processive transcription elongation.

We hypothesized that a differential post-translational modification of NKRF regulates its interaction with other key elongation factors and results in disparate effects on the
transcription elongation complex upon stimulation. In the present study, we identify three different domains in NKRF that are phosphorylated; however, only one of these domains regulates the effect of NKRF on transcription events via protein-protein interactions with NELF-E and XRN2.

MATERIALS AND METHODS

Antibodies

The following antibodies were utilized in these experiments: p65 (F-6) (sc-8008, Santa Cruz Biotechnology, Dallas, Texas, U.S.A.); XRNR2 (ab172284, Abcam, Cambridge, United Kingdom); NELF-E (ab170104, Abcam, Cambridge, United Kingdom); DSIF SPT5 (H-300) (sc-28678, Santa Cruz Biotechnology, Dallas, Texas, U.S.A.); CBP (C-20) (sc-583, Santa Cruz Biotechnology, Dallas, Texas, U.S.A.); c-Fos (H-125) (sc-7202, Santa Cruz Biotechnology, Dallas, Texas, U.S.A.); c-Jun (H-79) (sc-1694, Santa Cruz Biotechnology, Dallas, Texas, U.S.A.); HDAC1 (H-11) (sc-8410, Santa Cruz Biotechnology, Dallas, Texas, U.S.A.); mSin3A (G-11) (sc-5299, Santa Cruz Biotechnology, Dallas, Texas, U.S.A.); Pol II (MMS-128P, Biolegend, San Diego, California, U.S.A.); phospho Ser2 Pol II (ab5095, Abcam, Cambridge, United Kingdom); goat anti-rabbit immunoglobulin/HRP (DakoCytomation, Fort Collins, Colorado, U.S.A.); LexA DNA binding region (ab14553, Abcam, Cambridge, United Kingdom); and GAL4 DNA binding domain Anti-GAL4 (ab135397, Abcam, Cambridge, United Kingdom).

Kinase inhibitors

The following kinase inhibitors were used in these experiments: SP 600125 (S5567, Sigma Aldrich, Hamburg, Germany); SB 203580 (S8307, Sigma Aldrich, Hamburg, Germany); and PD 98059 (P215 Sigma Aldrich, Hamburg, Germany).

Bacteria and cell lines

The bacterial strain XL1-Blue (DE3) (Stratagene, La Jolla, California, U.S.A.) was used during plasmid and PCR product cloning. Escherichia coli BL21 (New England Bio Labs, Ipswich, Massachusetts, U.S.A.) was used for the expression of GST fusion proteins. HeLa (CCL-2, LGC Promochem, Middlesex, United Kingdom). HEK-273-R1 (AMGEN, Thousand Oaks, California, U.S.A.) cells were maintained in DMEM with 5% fetal calf serum (FCS), transfected by calcium phosphate co-precipitation and stimulated with IL-1 or NDV as previously described [5].

DNA constructs
pL6G2 was constructed through pG and pTA-Luc recombination (Clontech, Mountain View, California, U.S.A.). p65LexA was constructed via pLexA-Rb-P [4] and p65 recombination as previously described [5]. pGALKRF was constructed via NRF1-690 and GAL4-VP16 recombination [4].

GST expression plasmids were cloned by direct PCR amplification of NKRF sequences from pNRF2722TAP [5]. Amplification products were directly cloned into the multi-cloning site of pGEX-4T-2 (Amersham Biosciences, Chalfont Buckinghamshire, United Kingdom).

**NKRF Mutations**

The following primer pairs were used to mutate NKRF protein sequences:

M1, 5´-AACACAGCCCACCTATCC ATCTGTCAAAAACCCAGAGGATCTGGAAAGAA-3´ and 5´-TTCTTT CCAGATCCTCTCG GGGTTTG ACAGATGGATAAGTGGGCTGTGTT-3´; M2, 5´-AAAGGTCAAAAAACGCCACCTC GCGGCATGCGATGGTCAAATAATCT-3´ and 5´-AGGATTTTGACCATCGATGCCCGAGGTCGTTTTTACCTTT-3´; M3, 5´-GAGACATCGCCCAAGATGCTACTCAAGA ACTCTTTGCTATACAAG-3´ and 5´-CTTGTATAGCGAAAGTCTTGGAGTACATTTGGCCATGCTC-3´; and M4SA, 5´-CCATCTGTC AAAGCTGCCACAATGCCATACAGGCGCTGCACCCC-3´ and 5´-GGGTGCAGCGCCTATGGCATTGTGCAGCTTTGACAGATGG-3´.

Inserted mutations were verified by enzymatic digestion and direct DNA sequencing.

**Protein purification**

Recombinant GST, TAP and STREP fusion proteins were expressed and purified as previously described [5, 20-22].

**Matrix-assisted laser desorption/ionization reflector time-of-flight (MALDI-ReTOF)**

The purified NKRF STREP fusion protein band was separated by SDS-PAGE. The excised protein band was sent to the PANTecs (Tuebingen, Germany) for further analysis. The protein band was reduced by dithiothreitol (DTT) and alkylated in iodoacetamide (Applichem, St. Louis, Missouri, U.S.A.) before trypsin (Promega, Madison, Wisconsin, U.S.A.) digestion. The digested sample was treated with trifluoroacetic acid (TFA) (Thermo Fisher-Scientific, Waltham, Massachusetts, U.S.A.) before mass spectrometry using Reflex IV MALDI-TOF (Bruker Daltonik GmbH). Data were analyzed using FlexAnalysis 2.4 (Bruker Daltonik GmbH, Bremen, Germany) and Mascot (www.matrixscience.com).

**In vitro phosphorylation assay**

HeLa or HEK293R1 cells were incubated in 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 0.3 mM Na₃VO₃, 20 mM β-glycerolphosphate, 400 nM okadaic acid, 10 μM E-64, 2.5 μg/ml
leupeptin, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM pepstatin, 5 mM DTT (pH 7.9) and 0.1% NP-40 for 10 minutes on ice and then centrifuged for 5 minutes at 10000×g. Next, the pellets were incubated in 20 mM HEPES, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% (v/v) glycerol, 5 mM DTT, 0.3 mM Na₃VO₃, 20 mM β-glycerolphosphate, 400 nM okadaic acid, 10 μM E-64, 2.5 μg/ml leupeptin, 0.3 mM PMSF and 1 μM pepstatin (pH 7.9) for 45 minutes on ice and then centrifuged for 5 minutes at 10000×g. The supernatants were maintained at -80°C for long-term storage. For the phosphorylation reactions, 5 µg cell extract, 2 µg GST fusion protein and 500 μM ATP/0.1 μCi/μl γ-32P ATP were incubated in 250 mM Tris (pH 7.4), 50 mM MgCl₂, 5 mM DTT, and 0.5% Triton X-100 for 30 minutes at 30°C. The phosphorylated fusion proteins were purified according to the manufacturer's protocol and analyzed by 10% SDS-PAGE. The total amount of protein was determined by Coomassie Brilliant Blue (CBB) staining, and the level of phosphorylated protein was determined by autoradiography. ImageJ 1.38x software was used to quantify the detected signals.

**Phosphoamino acid mapping**

For this experiment, 1 µg phosphorylated GST fusion protein was isolated from an SDS-PAGE gel and hydrolyzed by incubation in 100 μl 6 N HCl for 60 minutes. The samples were cooled to room temperature and evaporated together with 100 μl H₂O. The dried pellets were resuspended in electrophoresis buffer and subjected to phosphoamino acid mapping as previously described [23].

**Reporter assay**

Firefly and Renilla luciferase activities were determined using the Dual-Luciferase Reporter System (Promega, Madison, Wisconsin, U.S.A.) and normalized as previously described [21].

**RNA isolation and Quantitative real-time RT-PCR (qPCR)**

Total cellular mRNA was isolated as previously described [5]. First-strand cDNA was synthesized from total RNA obtained from HeLa cells using random primers and the SuperScript III Reverse Transcriptase kit (Life Technologies) according to the manufacturer’s protocol. For the quantification of specific RNAs, total RNA samples were reverse-transcribed using the Custom TaqMan® Small RNA kit (Life Technologies), as described in the manufacturer's instructions. Quantitative real time RT-PCR (qPCR) was performed using TaqMan Gene Expression Assays (Life Technologies) and ABI Prism 7900 HT Detector (Life Technologies). Data were normalized using both PPIA (cyclophilin A) and UBC (ubiquitin C). Results were analyzed with RQ Manager and DataAssist software (Life Technologies). Following primer sequences were used:
UBC (NM_021009.4; amplicon length 71 bp)

PPIA (NM_021130.3; amplicon length 98 bp)

Initiation (5´-GCUUGCAUCCGCUACUGUUGGUAAGCCACCAUG-3´; amplicon length 36 bp)

Elongation (5´-CUGGUUCCUGGAACAAUUGCUUUACAGAUGCACAUAU-3´; amplicon length 38 bp)

**Chromatin immunoprecipitation (ChIP)**

Cells were cross-linked in vivo with 1% formaldehyde for 10 min at 37°C. Cross-linking reactions were stopped by adding 125 mM glycine. Cells were washed in phosphate-buffered saline (PBS) and then incubated in radioimmunoprecipitation assay lysis buffer (40 mM Tris-HCl (pH 7.05), 120 mM NaPi, 200 mM NaCl, 1% Triton, 8 mM Na3VO3, 2 mM NaF, 80 mM β-glycerolphosphate, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 µM pepstatin) on ice for 10 min. After sonication and centrifugation, 5 µl of the soluble extract was analyzed as an input control. DNA fragments bound to various proteins were immunoprecipitated using 1 µg of the indicated antibodies. Specific reporter sequences were detected by PCR using the following primers: 5´-CGGACATTTCGAAGTACTCAG-3´ and 5´-AGCTCTTACGCGTGCTAGCTCG-3´. IL-8 promoter sequences were detected as described previously [5].

**RESULTS**

**NKRF is phosphorylated within three different domains**

Matrix-assisted laser desorption/ionization reflector time-of-flight (MALDI-ReTOF) mass spectrometry analysis (MS-analysis) of a trypsin-digested NKRF-STREP fusion protein from HeLa cells was initially performed to map possible phosphorylation sites within the NKRF protein. The resulting fragmentation range revealed that coverage was 34% (Supplementary Fig S1). A single peptide comprising amino acids (aa) 421 to 437 was identified with unphosphorylated (1475.68 m/z) and singly phosphorylated (1555.74 m/z) peptide peaks. Additionally, we performed a complete in vitro study using purified GST-NKRF fusion proteins from bacteria, cellular extracts from HeLa cells, and radiolabeled γ-32P ATP (Fig 2A). The GST477-690 protein contained a negligible radioactive phosphorylation signal, suggesting that NKRF aa 477-690 is not a major target of cellular kinases. In contrast, GST1-112, GST112-449 and GST1-389 were significantly phosphorylated following incubation with HeLa cell extract. The prominent phosphorylation signal of GST112-449 confirms the MS-analysis data. Strikingly, GST1-449 displayed the strongest phosphorylation signal compared to GST1-112, GST112-449 and GST1-389. Although in vitro studies cannot entirely reflect in vivo conditions, these results suggested that NKRF was simultaneously phosphorylated at multiple sites by constitutively active kinases in HeLa cells. To distinguish
between different phosphorylation sites, *in vitro*-phosphorylated GST1-112 and GST112-449 were next subjected to acid hydrolysis and phosphoamino acid mapping by two-dimensional thin-layer chromatography and ninhydrin staining (TLC) (Fig 2B). These experiments confirmed the presence of disparate phosphoamino acids within NKRF aa 1-449. GST1-112 was phosphorylated at Ser and Thr residues; GST112-449 was exclusively phosphorylated at Ser residues. Furthermore, no tyrosine phosphorylation was detected within the entire N-terminal domain of NKRF (aa 1-449).

**NKRF phosphorylation is impaired by IL-1 stimulation**

IL-1 was shown to affect NKRF transcription activity in HeLa cells [5]. Given that NKRF phosphorylation is necessary for its transcriptional activity, we examined whether IL-1 stimulation affects NKRF phosphorylation in HeLa cells [1, 5]. We prepared cellular extracts from HeLa cells before and after 15, 30 or 60 minutes of IL-1 stimulation. The phosphorylation of GST-Jun fusion proteins was monitored to assess the course of IL-1-induced signaling in HeLa cells. According to previous reports, the c-Jun protein was increasingly phosphorylated after 15 minutes of IL-1 stimulation [24, 25] (Fig 3A). In contrast, the level of GST1-449 phosphorylation decreased within 15 minutes of IL-1 stimulation and returned to the level observed in unstimulated cells after 60 minutes of stimulation (Fig 3A and B). This was a direct effect of IL-1 signaling, as we obtained identical data using cell extracts from HEK293R1 cells [26] expressing an exogenous IL-1 receptor (data not shown). Under identical conditions, GST1-112 phosphorylation was not significantly affected by IL-1 stimulation. We therefore assume that phosphorylation in this region plays a minor role in the regulation of NKRF function in IL-1 stimulated cells (Fig 3C). Analogous to IL-1 stimulation, HeLa cells stimulated with New Castle Disease virus (NDV) show a significant decrease in GST1-449 phosphorylation within 30 minutes (Fig 3D). This finding was not surprising; NDV stimulation of cells was shown to overcome the inhibitory action of NKRF, similar to IL-1 stimulation [3, 4]. The parallel effects of IL-1 and NDV suggest a mechanistic link between phosphorylation and transcriptional activity for NKRF. Interestingly, different mitogen-activated protein (MAP) kinases were shown to be transiently activated within 15 minutes of stimulation by IL-1 or NDV [27]. Therefore, we used specific MAP kinase inhibitors to further examine a possible involvement of the MAP kinase family in the regulation of GST1-449 phosphorylation. Cell extracts were incubated with different inhibitors before the kinase reaction was initiated. For a better comparison, the relative phosphorylation of GST1-449 without inhibitor was set to 1 (Fig 3E). Overall, marginal differences in GST1-449 phosphorylation due to the addition of inhibitors were observed (Fig 3E). Only JNK inhibitor (SP 600125) generated a minor decrease of 15% after 30 minutes of IL-1 stimulation. However, the same concentration of JNK inhibitor (SP 600125) led to 100% inhibition of c-Jun phosphorylation after 15 or 30 minutes of IL-1 stimulation (data not shown). This finding
suggests that MAP kinases are not directly involved in the observed IL-1-mediated decrease of NKRF phosphorylation in vitro.

Using the NetPhos 2.0 server, which predicts serine, threonine and tyrosine phosphorylation sites in eukaryotic proteins, we identified one potential threonine (t36) and 2 potential serine (s35 and s106) phosphosites within NKRF aa 1-112. In addition, 4 potential serine phosphosites (s421, s422, s428 and s429) were predicted within NKRF aa 112-449. Various mutations were introduced into GST1-449 (M1) and GST1-112 (M2 and M3). The M2 and M3 mutations decreased GST1-112 phosphorylation independently of IL-1 stimulation (Fig 3C, Supplementary Fig S1). These mutations also abolished phosphoserine and phosphothreonine signals in TLC experiments (data not shown). M1 mutation significantly interfered with the phosphorylation of GST1-449 in unstimulated HeLa cells. In contrast with M2 and M3 mutations, M1 mutation impaired the recurrent phosphorylation of GST1-449 after IL-1 stimulation (Fig 3A). Together, the results of the in vitro phosphorylation studies suggest a possible link between phosphorylation in the aa 421-429 region and the transcriptional activities of NKRF in unstimulated and IL-1 stimulated cells.

To specifically compare the transcriptional activities of NKRF with the NKRF M1 mutant, we utilized a synthetic two-hybrid reporter gene containing isolated binding sites for LexA and GAL4 fusion proteins (pL6G2). Additionally, the reporter contains a minimal reporter gene consisting of TATA-box and firefly luciferase coding sequences under the control of GAL4 and LexA binding sites. HeLa cells were stably transfected with the pL6G2 reporter plasmid; a single stable cell clone was used for further experiments. The pL6G2 reporter displayed the effects of additionally expressed NF-κB p65-LexA (p65Lex) and NKRF-GAL4 fusion proteins (GALNKRF and GALNKRFM1) exclusively. Endogenous p65 and NKRF proteins did not affect pL6G2 activity in unstimulated or IL-1 stimulated cells (control, Fig 4 A). GALNKRF alone had no effect on pL6G2 reporter activity (data not shown). Overexpression of p65Lex increased pL6G2 expression more than 13-fold in unstimulated cells and 32-fold in IL-1 stimulated cells. GALNKRF, but not GALNKRFM1, decreased expression of the relative reporter gene in unstimulated cells. This confirmed the significance of the aa 421-429 region in NKRF activity in unstimulated cells. In IL-1 stimulated cells, however, over-expression of GALNKRF and GALNKRFM1 had no inhibitory effect on p65 activity. GALNKRF shows a low but detectable co-activating effect in IL-1 stimulated cells, in agreement with previous observations [1-5].

Next, we performed a quantitative RNA analysis to determine the levels of initiated and elongated reporter transcripts in cells. As reported previously, p65 alone increased transcription initiation and the elongation of pL6G4 reporter transcripts (p65Lex, Fig 4B). IL-1 stimulation led to a 12-fold increase in transcription initiation and a 3-fold increase in
transcription elongation by p65 (p65Lex, **Fig 4B and C**). In agreement with previous reports, NKRF did not affect transcription initiation in unstimulated or IL-1 stimulated cells (**Fig 4B and C**). Most importantly, NKRF significantly decreased the level of p65Lex-derived transcription elongation in unstimulated cells (GALNKRF, **Fig 4B**). The M1 mutation compromised the inhibitory effect of GAL4NKRF, resulting in an increased number of elongated reporter transcripts in unstimulated cells (GALNKRF and GALNKRM1, **Fig 4B**). This emphasized the crucial role of phosphorylation in the aa 421-429 region of NKRF in transcription elongation in unstimulated cells. In IL-1 stimulated cells, GALNKRF expression had no significant effect on the number of elongated transcripts. This finding indicated that IL-1 stimulation abolished the inhibitory effect of NKRF on transcription elongation.

We utilized a previously described NKRF-TAP (tandem affinity purification) fusion system to determine a possible role for phosphorylation within aa 421-429 in the interactions between NKRF and major components of the transcription complex [5]. HeLa cells were transfected with equal amounts of NKRTAP or NKRFM1TAP plasmids and were either unstimulated or stimulated with IL-1. Equal amounts of whole cellular extracts and fusion proteins were incubated with TAP purification beads. The amounts of endogenous proteins were detected before (input) and following the second purification step (bound) (**Fig 5A**). As a control, equal levels of endogenous GAPDH and overexpressed TAP proteins were used in all experiments. We utilized a number of specific antibodies against endogenous transcription factors that have been previously associated with NKRF target promoters or general transcriptional elongation machinery [1, 5, 28]. Consistent with our earlier results, NKRF and NKRFM1 failed to bind to a number of transcription factors; these included RNA Pol II, p50, p52, c-rel, DSIF, CBP, c-Fos, c-Jun, HDAC1, 3Sin3A, DSIF and TFIID (data not shown). It was recently suggested that NKRF shares sequence homology with the PAXT-1 protein of *C. elegans*, which is known to interact with XRN2 [28]. We used a specific antibody against the human XRN2 protein and observed a strong interaction between NKRF-TAP and endogenous XRN2 in HeLa cells (**Fig 5A**). Considering the inhibitory effect of NKRF on transcription of the pL6G2 reporter, we further examined the interaction between NKRF and NELF-E. NELF-E is an essential component of the NELF complex. Similar to XRN2, we observed a strong interaction between NELF-E and NKRF (**Fig 5A**). NKRFM1 binding affinity to NELF-E and XRN2 was significantly reduced, demonstrating the importance of the phosphorylation site in the aa 421-429 region of NKRF in its interaction with the transcription elongation complex. The specificity of aa 421-429 phosphorylation in binding to endogenous NELF-E and XRN2 was confirmed by a comparative analysis of NKRFM2, NKRFM3 and combined substitutions of Ser residues 421, 422, 428 and 429 with alanine (NKRFM4SA, **Fig 5B**). Both single aa substitutions (NKRFM4SA) or the deletion of aa 421-429 (NKRFM1) significantly decreased XRN2 and NELF-E binding to NKRF (**Fig 5A and B**). Interestingly, IL-
1 stimulation revealed a similar effect; diminished interactions between NKRF and NELF-E and XRN2 (Fig 5A). We noticed a slight increase in XRN2 expression by IL-1 stimulation (Fig. 5, XRN2 input). When NELF-E and XRN2 signals were compared, IL-1 had a more drastic effect than NKRFM1. Neither NKRF mutations nor IL-stimulation had a detectable effect on the binding of endogenous p65 in these experiments indicating that NKRF phosphorylation was most likely not relevant for the interaction between NKRF and p65 NFκB. NKRF in IL-1 stimulated cells and NKRF containing mutations within aa 421-429 displayed decreased interaction capability. These findings, together with the decrease in aa 421-429 phosphorylation observed in response to IL-1, strongly suggest that IL-1 regulates interactions between NKRF and NELF and XRN2 through the restriction of specific aa 421-429 phosphorylation.

To determine the relevance of interactions between NKRF and elongation factors, we performed ChIP in HeLa cells stably transfected with the pL6G2 reporter. Equal amounts of cross-linked chromatin (input) were added to each PCR reaction (Fig 6A). Immunoprecipitation with rabbit immunoglobulin G served as a negative control; no detectable signal was observed. The data demonstrated that p65LexA, GAL4NKRF and GAL4NKRFM1 bound equally to the promoter in all experiments. NELF-E and XRN2 were associated with this complex through GAL4-NKRF, not GAL4-NKRFM1 (Fig 6A). IL-1 stimulation led to the gradual dissociation of NELF-E and XRN2 and an increased association with hyperphosphorylated RNA Pol II, which is involved in promoter-proximal transcription elongation. Consistent with TAP and reporter gene experiments, these data strongly suggested that decreased aa 421-429 phosphorylation by IL-1 stimulation selectively affects NKRF interactions with NELF-E and XRN2 in HeLa cells. We further examined the recruitment of NKRF, p65, NELF-E and XRN2 to the IL-8 promoter as an endogenous NKRF target promoter. We performed ChIP in non-transfected HeLa cells, as the transfection procedure itself can slightly enhance IL-8 gene transcription [5]. Similar to pL6G2 reporter, NKRF bound to IL-8 reporter continuously. IL-1 stimulation enhanced p65 binding to the promoter, whereas NELF-E binding was strongly decreased. These findings are in agreement with the decreased interactions observed between NELF-E and NKRF. Furthermore, XRN2 showed decreased but detectable binding to the IL-8 promoter following 60 minutes of IL-1 stimulation. Although this might be at least partially due to a small increase in XRN2 expression in IL-1 stimulated cells (Fig 5A), we assume that XRN2 is able to bind to the IL8 gene independent of its interaction with NKRF. Considering the complexity of endogenous promoters like IL-8, it is rather difficult to decipher the isolated effects of different participating transcription factors. Finally, this study reveals that NKRF, NELF-E and XRN2 pause transcriptional elongation at promoter-proximal regions via a unique mechanism.
DISCUSSION

The transition from transcription initiation into productive elongation is a critical limiting step for numerous human genes [13, 29, 30]. Pausing of transcription elongation appears to be a common control mechanism for genes that rely on constitutive restriction of their basal transcription activity, with the comparative advantage of increased inducibility and/or an immediate response to stimulation [15, 31, 32]. A number of studies uncovered that the transcription elongation process is a key regulatory stage in the NF-κB activation pathway in particular [31]. Our study provides several lines of evidence demonstrating that NKRF contributes to the regulation of NF-κB p65-derived transcription elongation under specific conditions. Moreover, phosphorylated NKRF can inhibit the elongation of initiated transcripts most likely via direct protein-protein interactions with two regulators of transcription elongation, NELF-E and the exoribonuclease XRN2.

Regulation of NKRF phosphorylation

This study identified three different phosphorylation domains within NKRF using different approaches. Two phosphorylation sites, Ser35/Thr36 and Ser106, were identified using in vitro experiments and single amino acid mutations. Further examinations, however, excluded the possible regulation of Ser35/Thr36 and Ser106 in response to IL-1 stimulation or their involvement in NKRF protein-protein interactions (Fig 3C). As in vitro studies cannot completely reflect cellular conditions, the significance of Ser35/Thr36 and Ser106 phosphorylation and their possible role in NKRF function remain to be determined.

The third phosphorylation domain, NKRF aa 421-429, was identified by direct MS-analysis of the NKRF protein under cellular conditions. Further in vitro phosphorylation and phosphoamino acid mapping experiments confirmed that serine phosphorylation occurs within aa 421-429, a region that contains 4 serine residues (Ser421, Ser422, Ser428 and Ser429). Complete deletion of this region (M1) or combined site directed mutations of all four serine residues (M4SA) abolished NKRF phosphorylation and its regulatory protein-protein interactions in cells (Fig 3B, 5A and B). Considering their adjacent positions, it is conceivable that all 4 serine residues can contribute equally to interactions with a cellular kinase and/or serve as alternative phosphorylation sites for cellular kinases. Therefore, further detailed studies will be required to ascribe NKRF interactions with NELF or XRN2 to a single serine phosphorylation site within aa 421-429.

Several observations in this study suggest that alterations in NKRF phosphorylation most likely belong to a general regulation mechanism of NKRF action. The basal level of NKRF phosphorylation in unstimulated cells was rapidly decreased in response to IL-1 stimulation and IL-1 receptor-mediated signaling pathways (Fig 3A and B). Moreover, the NRF phosphorylation level was equally decreased following NDV stimulation (Fig 3D). It is
important to note that IL-1 and NDV stimulation were previously shown to abolish the transcription silencing activity of NKRF without affecting the expression level of NKRF or its DNA-binding activity [3, 5, 19]. Thus, IL-1 and NDV exert indistinguishable effects on NKRF phosphorylation and its transcription activity in target promoters. Furthermore, the rapid decline in NKRF phosphorylation within 15 minutes of IL-1 or NDV stimulation suggested a direct involvement of induced intracellular signaling proteins. Previously, a family of IL-1-inducible dual specificity phosphatases (DSPs) was identified [33]. DSPs are localized to different subcellular compartments and selectively dephosphorylate critical phosphothreonine and phosphotyrosine residues within MAP kinases [33]. However, several observations cast doubt on the possible involvement of these cellular phosphatases in NKRF regulation. First, NKRF shares no sequence homology with MAP kinases. Second, NKRF phosphorylation was not affected by potent MAPK inhibitors (Fig 3E). Although participating cellular kinases or phosphatases remain to be identified, our study demonstrates that the regulation of NKRF phosphorylation can play a key role in the regulation of transcriptional events. Moreover, the identification of signaling proteins regulating NKRF phosphorylation will potentially describe a new regulatory link between NF-κB-directed transcription initiation and elongation.

**Impact of NKRF phosphorylation on promoter-proximal transcription elongation**

We have previously studied interactions between NKRF and several transcription factors, including members of the NF-κB factor family. In addition to specific protein-protein interactions with p65 NF-κB, NKRF exhibited no affinity for p50, p52, c-rel, DSIF, CBP, c-Fos, c-Jun, HDAC1, 3Sin3A and TFIID [5, 6]. This study confirmed that NKRF interacts with two additional regulatory transcription elongation factors, NELF-E and XRN2. Several observations suggested a high specificity for NKRF in forming complexes with NELF-E, XRN2 or p65 through distinct domains. First, deletion of aa 421-429 in the M1 mutation markedly interfered with NKRF binding to endogenous NELF-E and XRN2 proteins; however, interaction with endogenous p65 was not affected (Fig 5A). Second, ChIP experiments provided evidence for the simultaneous recruitment of NELF-E and XRN2, but not GALNKRFM1, to the GAL4 reporter via GALNKRF expression, confirming the crucial role of the aa 421-429 domain in the interaction between NKRF and NELF-E and XRN2 (Fig 6A). Third, substitutions of possibly phosphorylated serine residues with alanine revealed effects similar to the M1 mutation (Fig 5B). This finding indicates the importance of phosphorylation in the aa 421-429 region in interactions between NKRF, NELF-E and XRN2. Fourth, none of the introduced NKRF mutations in this study affected binding to the endogenous NF-κB p65 subunit in HeLa cells (Fig 5B). Mutations of all three NKRF phosphorylation sites are distant from the p65 binding domain, which was previously mapped to NKRF aa 204-308 [5, 6]. Thus, NKRF interacts through different domains with NELF-E and XRN2 and with p65.
simultaneously. These interaction boundaries may allow for an independently persisting complex of p65, NKRF, NELF-E and XRN2 in unstimulated cells, which needs to be explored experimentally. The ChIP and TAP interaction studies described here represent cellular conditions, given the use of whole cellular extracts. Therefore, these results do not exclude the participation of other cellular components that might contribute to the formation of such a regulatory complex.

In unstimulated HeLa cells, the simultaneous binding of p65, NKRF, NELF-E and XRN2 to the minimal pL6G2 promoter led to the promoter-proximal inhibition of transcription elongation (Fig 4B and C). This negative control was overruled by concomitant association with hyperphosphorylated RNA Pol II under different conditions. First, IL-1 stimulation increased the number of elongated transcripts (Fig 4C). Second, the M1 mutation increased transcription elongation at the reporter gene via the concomitant association with hyperphosphorylated RNA Pol II. Third, the absence of GAL4NKRF in unstimulated and stimulated cells resulted in significant increases of elongated transcripts (Fig 4B). Previously, a reduction in endogenous NKRF expression was shown to increase transcription elongation and association with hyperphosphorylated RNA Pol II at the IL-8 promoter and HIV-LTR in unstimulated cells [3, 5]. Thus, the role of NKRF and its interaction with NELF-E and XRN2 is the reduction in basal transcription elongation in unstimulated cells. The results obtained from NKRFRM1 and NKRFRM4A4 mutations further established the importance of the aa 421-429 domain and the phosphorylation of serine residues within it in the regulation of basal transcription.

IL-1 stimulation increased the level of transcriptional initiation and elongation at the pL6G2 reporter gene; this was accompanied by the dissociation of NELF-E and XRN2 and association with hyperphosphorylated RNA Pol II (Fig 6A). In the IL-8 promoter, IL-1 stimulation led the dissociation of NELF-E and XRN2 and association with hyperphosphorylated RNA Pol II (Fig 6B). This confirmed the regulation of NKRF, NELF-E and XRN2 interactions with pL6G2 and the endogenous IL-8 promoter in response to IL-1 stimulation. Notably, IL-8 and other endogenous NKRF target promoters contain a more complex 5’ regulatory region than the synthetic promoter of pL6G2. Thus, a number of additional transcription factors certainly contribute to fine tune transcription events at endogenous promoters. ChIP analysis of the IL-8 promoter confirmed that IL-1 stimulation resulted in the dissociation of NELF-E and XRN2 and increased transcription elongation by hyperphosphorylated RNA Pol II. Although these effects might partially be due to an IL-1-mediated decrease in NKRF phosphorylation, we cannot exclude possible additional effects of IL-1 on NELF-E or XRN2 proteins. Interestingly, IL-1 stimulation had a more significant effect on interactions with NELF-E and XRN2 than the M1 mutation (Fig 5A). Therefore, the ultimate structural role of aa 421-429 phosphorylation and its direct involvement in molecular
interactions between NKRF, NELF-E and XRN2 remains speculative. Further detailed mapping of the interaction domains is required. Recently, it was suggested that XRN2 and NKRF homologs from C. elegans to interact via a 92 aa DUF3469 homology domain within NKRF from Danio rerio [34]. However, the DUF3469 homology domain has not been precisely defined. Moreover, only the N-terminal 12 aa of the human NKRF protein sequence analyzed in the present study slightly matches the DUF3469 homology domain. Therefore, the precise boundaries of the XRN2 and NELF-E binding domains in NKRF remain undefined.

In summary, phosphorylated NKRF is able to recruit NELF-E and XRN2 to specific target promoters containing NKRF binding sequences in unstimulated cells. NKRF protein is ubiquitously expressed in all cultured mammalian cells examined thus far and silences the basal transcription of various pro-inflammatory cytokine genes [20, 21, 35]. This finding led to our initial assumption that NKRF down-regulation might be associated with pathologic conditions attributed to NKRF target genes. Two microRNAs, miR29 and miR301, have been recently shown to down-regulate NKRF gene expression in vivo; NF-kB driven gene expression is thereby increased under different pathological conditions, such as inflammation and cancer [7, 8, 12, 36-41]. Under these conditions, NKRF levels may be insufficient to mediate the termination of immature transcripts via XRN2. Indeed, XRN2 exoribonuclease was recently shown to target many endogenous pre-mRNAs and was associated with inflammation and cancer [42-44]. Intriguingly, the NKRF C-terminal domain shows significant homology to single- and double-stranded RNA binding proteins (Fig 1) and weak binding to IFN-γ mRNA [45]. Although NKRF was originally identified as a DNA binding protein, its C-terminal RNA binding domain might play a role in the recognition of gene-specific transcripts.

SUPPLEMENTARY DATA
Supplementary Data are available online.

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FIGURE LEGENDS

Figure 1. The human NKRF protein is designated by two different isoforms in the National Center for Biotechnology Information (NCBI) databank. Isoform 1 (NP_001166958.1) is based on hypothetical coding sequences in the genome via splicing of an upstream 12-kb intron. Isoform 2 (NP_001166959.1) lacks 15 additional N-terminal amino acid residues. In this study, we refer to the sequence of isoform 2, which was generated by directly cloning constitutively expressed cDNA sequences isolated from various human cell lines in our lab. The encoded protein sequence of NKRF isoform 2 is presented at the top, and the identified phosphorylation sites are underlined. The lower table summarizes the locations of the various functional domains within the NKRF protein sequence, including the nuclear localization signal, the p65 binding domain, and the DNA binding domain. The predicted double-strand RNA binding motif (Drm), G-patch (six highly conserved glycine residues) and R3H (single-stranded nucleic acid domain) homology regions are also presented.

Figure 2. NKRF is constitutively phosphorylated in HeLa cells. Purified GST-NKRF proteins were subjected to in vitro phosphorylation assays using HeLa cell extracts and analyzed by SDS-PAGE. (A) GST fusion protein levels were detected by CBB staining, and the phosphorylated protein levels were measured by direct autoradiography (upper panel). The detected CBB and radioactive signals were compared using densitometry analysis to estimate the relative phosphorylation factor for each GST fusion protein. The diagram summarizes the mean values ± the standard error of the mean (SEM) from three independent experiments. Statistical significance was calculated by t-tests, with p<0.05 considered significant. The relative phosphorylation level of GST1-449 was set to 1 in each independent set of experiments and was compared to the relative phosphorylation levels of the other GST fusion proteins. (B) NKRF aa 1-112 and aa 112-449 are differentially phosphorylated at Ser and Thr residues. Identical amounts of the indicated fusion proteins on the top were subjected to acid hydrolysis and phosphoamino acid mapping by two-dimensional thin-layer chromatography and ninhydrin staining (TLC). Arrows indicate the Ser, Thr and Tyr signals in a phosphoamino acid standard (left).

Figure 3. NKRF phosphorylation decreases upon IL-1 stimulation. (A) GST1-449 expression plasmids were modified as described in the Supplementary Data to create GST1-449M1. The GST-Jun expression plasmid was described earlier [3]. The purified GST fusion proteins were subjected to in vitro phosphorylation assays using extracts from unstimulated or IL-1-stimulated HeLa cells after 15, 30 and 60 minutes of stimulation. Then, the proteins were analyzed as described in the legend of Figure 2. Heavy frames indicate the amount of different GST fusion proteins that were detected simultaneously by CBB staining (upper panel) and by direct autoradiography (lower panel) in a single experiment. (B) The detected
CBB and radioactive signals of GST1-449 and GST1-449M1 were compared using densitometry analysis to assess the relative phosphorylation levels, as described in the legend of Figure 2. The diagram summarizes the mean values ± SEM from three independent experiments. Statistical significance was calculated by t-tests, with p<0.05 considered significant. The relative phosphorylation level of each GST fusion protein was compared with that of GST1-449 in a single experiment, which was set to 1. (C) NKRF1-112 phosphorylation is not affected by IL-1 stimulation. The GST1-112 expression plasmid was modified as described in Material and Methods to create GST1-112 M2 and GST1-112 M3. The purified GST fusion proteins were subjected to in vitro phosphorylation assays using extracts from unstimulated or IL-1-stimulated HeLa cells after 15, 30 and 60 minutes of stimulation. The detected CBB and radioactive signals (Supplementary Fig S2) were compared using densitometry analysis to measure the relative phosphorylation levels, as described in the legend of Figure 2. The diagram summarizes the mean values ±SEM from three independent experiments. Statistical significance was calculated by t-tests, with p<0.05 considered significant. The relative phosphorylation level of each GST fusion protein was compared with that of GST1-112 in a single experiment, which was set to a level of 1. (D) Purified GST1-449 fusion protein was subjected to in vitro phosphorylation assays using extracts from unstimulated or New Castle disease virus-stimulated HeLa cells after 15, 30 and 60 minutes of stimulation. Proteins were analyzed as described in the legend of Figure 2. The GST1-449 protein was detected by CBB staining and the amount of phosphorylated protein was simultaneously measured by direct autoradiography. The detected CBB and radioactive signals (Supplementary Fig S3) were compared by densitometry analysis to assess the relative phosphorylation level of each GST fusion protein. The diagram summarizes the mean values from three different experiments. The relative phosphorylation level of the GST1-449 fusion protein in stimulated cells was compared with that in unstimulated cells, which was set to a level of 1 in each experiment. (E) GST1-449 was phosphorylated using HeLa cell extracts after 15 or 30 minutes of IL-1 stimulation and supplemented with 1 µM JNK (SP 600125), 2 µM p38 MAP-Kinase (SB 203580), and 2 µM ERK-Kinase MEK (PD 98059) inhibitors. The resolving agent DMSO was added as a control for the inhibitors. The detected CBB and radioactive signals of GST1-449 were compared using densitometry analysis to assess the relative phosphorylation levels, as described above. The diagram summarizes the mean values ± SEM from three independent experiments. Statistical significance was calculated by t-tests, with p<0.05 considered significant. The relative phosphorylation level of each GST1-449 by presence of each inhibitor was compared with that of control experiments containing the resolving agent DMSO. The control reaction was set to 1 in each series of experiments.
Figure 4. NKRF phosphorylation at aa 421/429 regulates the transcriptional activity of NKRF in vivo. (A) HeLa cells were stably transfected with the pL6G2 reporter plasmid as described earlier [4]. After the expansion of a selected single clone, cells were transfected with the Renilla luciferase-expressing plasmid phRG-B (Promega) as the internal control, the p65-LexA expression plasmid (p65Lex), the GAL4-NKRF expression plasmid (GALNKRF) or the NKRFM1 expression plasmid (GALNKRFM1). At 48 hours after transfection, extracts of transfected cells were prepared and subjected to a dual-luciferase activity assay (Promega). The cells were stimulated with IL-1 16 hours before extract preparation (black bars) or were left unstimulated (white bars). Firefly luciferase activities were normalized to Renilla luciferase activities. The normalized firefly luciferase activity in cells transfected with the reporter plasmid alone (control) was set to 1 in each experiment. The mean activity ± SEM, which is indicated as the relative reporter activity on the Y-axis, is representative of five independent experiments. (B) Total mRNAs were isolated from cells at 48 hours after transfection of p65LexA and GAL4NKRF to analyze the initiated or the elongated reporter transcripts. Before RNA isolation, cells were left unstimulated or stimulated with IL-1 for 2 hours (C). Relative quantification of initiated and elongated transcripts was performed by TaqMan qPCR. The diagrams summarize the mean values ± SEM. Statistical significance was calculated by t-tests, with p<0.05 considered significant. The significance of most relevant experiments is indicated by **p<0.01.

Figure 5. NKRF phosphorylation at aa 421-429 regulates its direct interaction with endogenous XRN2 and NELF-E. (A) HeLa cells were transfected with equal amounts of expression plasmids encoding the TAP domain fused to NKRF and to NKRFM1. At 48 hours after transfection, the cells were stimulated with IL-1 for 15 minutes (+ IL-1) or were left untreated (-), and cellular extracts were immediately prepared. The amounts of XRN2 and NELF-E were detected by Western blot analysis using specific antibodies as input controls. Equal aliquots of cellular extracts were incubated with IgG-agarose. After several washes, 10% of the bound fraction of TAP fusion proteins was assayed by Western blotting using a rabbit peroxidase anti-peroxidase (PAP) soluble complex antibody. Following tobacco etch virus (TEV) protease digestion and a second purification step, the amounts of bound RNA Pol II, XRN2 and NELF-E were assessed by Western blotting using specific antibodies. (B) HeLa cells were transfected with equal amounts of expression plasmids encoding the TAP domain fused to NKRF, NKRFM2, NKRFM3 or NKRFM4SA. At 48 hours after transfection, cellular extracts were prepared, and the amounts of the indicated proteins were detected by Western blot analysis using specific antibodies as input controls. Equal aliquots of cellular extracts were incubated with IgG-agarose. After several washes, 10% of the bound fractions of the TAP fusion proteins was assayed by Western blotting using a rabbit PAP soluble...
complex antibody. Following TEV digestion and a second purification step, the amounts of bound NELF-E and XRN2 were monitored by Western blotting using specific antibodies.

**Figure 6.** NKRF phosphorylation at aa 421-429 regulates the recruitment of endogenous XRN2 and NELF-E to a target promoter. (A) HeLa cells were stably transfected with reporter plasmid (pL6G2) and equal amounts of expression plasmids encoding p65-LexA and GAL4-NKRF or GAL4-NKRFM1. ChIP assays were performed using soluble chromatin extract from cells before and after IL-1 stimulation for 15 or 30 minutes as indicated. The extracts were precipitated using the indicated antibodies or untreated as an internal quality control (input). The precipitated DNA was used for PCR with primers spanning the DNA sequence from -173 to +106 according to the transcription start site (+1). The results are representative of three to four independent ChIP experiments. (B) ChIP assays were performed using soluble chromatin extract from non-transfected HeLa cells before and after IL-1 stimulation for 15 or 30 minutes, as indicated. The extracts were precipitated using the indicated antibodies or untreated as an internal quality control (input). The precipitated DNA was used for PCR with primers spanning the IL-8 promoter region DNA sequence from -100 to +80 according to the transcription start site (+1). The results are representative of three independent ChIP experiments.