A novel splice variant of the transcription factor Nrf1 interacts with the TNFα promoter and stimulates transcription

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ABSTRACT

Common signaling chains of various receptor families, despite some similarities, are able to provoke quite different cellular responses. This suggests that they are linked to different cascades and transcription factors, dependent on the context of the ligand binding moiety and the cell type. The ITAM (immunoreceptor tyrosine-based activation motif) containing γ chain of the FcεRI, FcγRI, FcγRIII and the T-cell receptor is one of these shared signaling molecules. Here, we show that in the context of the FcγRIII, the γ chain activates the transcription factor Nrf1 or a closely related protein that specifically interacts with the extended κ3 site in the TNFα promoter. A novel splice variant of Nrf1 with a 411 bp deletion of the serine-rich region, resulting in an overall structure reminiscent of the BTB and CNC homology (Bach) proteins, was isolated from the corresponding DC18 cells. In a gel shift analysis, this bacterially expressed splice variant binds to the TNFα promoter site after in vitro phosphorylation by casein kinase II (CKII). In addition, cotransfection studies demonstrate that this splice variant mediates induced transcription at the TNFα promoter after stimulation/ activation in a heterologous system.

INTRODUCTION

Immune complexes mediate the facilitated uptake of complexed antigen, thereby linking the innate and cognate immune system. In addition, they represent potent activators of a variety of cells that contribute to an inflammatory response, such as mast cells, monocytes, and macrophages, by triggering and initiating a signaling cascade via certain FcRs (1,2). Until recently, only FcεRI engagement and triggering was linked directly to a role *in vivo*, while immunoglobulin G (IgG)-triggered inflammation was mostly attributed to effects of the complement system. Targeted disruption of components of the complement system (3,4), the α subunit of the FcεRI (5), the FcγRII (6) and the FcγRIII (7), as well as similar disruption of the common γ chain (8), now indicate a dominant role for the FcγRIII in the anaphylactic mortality (type I reaction) and the cutanous Arthus reaction (type III reaction). This receptor, in contrast with the FcγRI, is only triggered by IgG complexes, and results in cell activation upon aggregation. The FcγRII, a second receptor responsive to IgG complexes in all three known isoforms in the murine system can only convey a negative (inhibitory) signal to cells (9,10).

We have recently elucidated signaling events and responsible transcription factors after triggering the receptor for IgE plus antigen (Ag), the FcεRI, in murine mast cells. In this cell type, a variety of lymphokines and chemokines were shown to be under the control of a member of the transcription factor family of nuclear factors of activated T-cells (NF-AT) and different cofactors after such a stimulation (11–13). At the TNFα promoter in particular, an interaction of NF-AT and AP1 at the κ3 site was demonstrated (13,14). This picture is reminiscent of T-cells where NF-ATp and ATF-2/jun, as the AP1 component, were identified (15). In other cell types, such as induced monocytes, NFκB without further cofactors was shown to bind to this promoter element and to control the induction of TNFα (13,15–21). These findings suggest that the κ3 site and sequences surrounding it constitute a versatile promoter element responding to different extracellular stimuli and signaling cascades by interacting with a variety of transcription factors. Similar investigations of the transcription factor(s) mediating TNFα induction after FcγRIII triggering of cells specialized in antigen presentation are still missing, despite its newly recognized pathological function and the fact that this receptor shares the signal transmitting γ chain with the FcεRI and the T-cell receptor. To address this issue, we used the murine DC18 cell line (22) as a model system to investigate the transcription factors linked to the FcγRIII, which is the only stimulating Fc receptor expressed on these cells.

MATERIALS AND METHODS

Cell culture, transient transfections, firefly- and renilla-luciferase readouts, electrophoretic mobility shift assays, supershift assays,

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preparation of nuclear extracts and radiolabeled probes, and western blot analyses were performed as described recently (11,12,14,23).

TNFα ELISA

A total of 1×10^5 cells in 200 μl medium per well were stimulated as outlined. TNFα concentrations in the supernatant were determined using the Genzyme mouse TNFα DuoSet System (Genzyme, Cambridge, MA) according to the manufacturer's instructions.

Stimulation and inhibitors

Cells were stimulated with 2 μg/ml anti-trinitrophenol/anti-dinitrophenol specific IgG2a (Pharmingen, San Diego, CA) plus 10 μg/ml dinitrophenol–BSA (Calbiochem, La Jolla, CA). Anti-FcγRII/III (2.4G2, anti CD16/32) monoclonal antibody, as well as the isotype control rat IgG2b kappa, were obtained from Pharmingen and used at 20 μg/ml. Murine IgG2a and goat anti mouse IgG F(ab′)$_2$ fragments were obtained from Sigma (Sigma Chemical Company, St Louis, MO) and used at 1 and 20 μg/ml respectively. Phorbol-myristate-acetate (PMA, Sigma) was used at a concentration of 20 ng/ml. 5-6-Dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB; Calbiochem), Apigenin (Calbiochem) and SB203580 (Calbiochem) were applied 1 h prior to stimulation in the concentrations indicated.

RT–PCR analyses

As primers specific for the FcγRs, the following oligonucleotides (Genset, Paris, France) were used. FcγRI, 5′-GGCTTCTAACA-ACTCTGCTAC-3′ and 5′-GGGACCAAGCACTTGGAGC-3′; FcγRIIb1, 5′-CCCATGGAGAGCAACTGGAC-3′ and 5′-GGAG-GATTGTATGGGCTGCTG-3′; FcγRIIb2, 5′-CCCATGGAGAG-CAACTGGAC-3′ and 5′-GGAGGATTGTCTGGAACCTGC-3′; FcγRIII, 5′-CCAGATGTTTCAGAATGCACAC-3′ and 5′-GG-GTGTCACTTGTCTTGAGG-3′ (24–27). As primers specific for nuclear factor erythroid-related factor 1 (Nrf1) clone 10S, the following oligonucleotides were used 5′-GGAGTTTGACTCT-GAGCACAGAGC-3′ and 5′-CCCAGGCTCACTTCCTCCG-GTCC-3′. For the FcγRs, the amplification was done in 35 cycles, with an annealing step at 55°C for 1 min, an elongation step at 72°C for 1 min and a denaturation step at 94°C for 1 min, using the PCR Supermix (Gibco-BRL, Gaithersburg, MD). For Nrf1 clone 10S, amplification parameters were as follows: 30 cycles at 94°C for 1 min, 65°C for 45 s and 68°C for 4 min, using the PCR advantage kit (Clontech, Palo Alto, CA). Amplification products were analyzed on a 1.8% agarose gel and visualized by ethidium bromide staining. As a source for amplification, cDNA generated from 5 μg total RNA (Superscript pre-amplification system, Gibco) of DC18 cells was used. Reactions with primers specific for murine β-actin (Clontech) served as a control for RNA/cDNA integrity.

Size separation of nuclear extracts

Nuclear extracts (~500 μg protein) were fractionated on a TosoHaas TSKgel SW Guardcolumn (7.5 mm ID × 75 mm) and a TosoHaas TSKgel G3000SW column (7.5 mm ID × 600 mm) with a flow rate of 0.5 ml/min on a Beckmann-System GOLD HPLC system. As a running buffer, 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl$_2$ and 0.2 mM EDTA, prefiltered on a 0.22 μm membrane (Nalge Company, Rochester, NY), was used. Fractions of 250 μl were collected and 10 μl of each fraction was tested in a gel shift analysis. The column was calibrated using M$_r$ standards (Bio-Rad, Hercules, CA) of 670, 158, 44, 17 and 1.35 kDa.

Antisera for supershift analyses

Antisera to nuclear factor erythroid 2 (NF-E2) p45 and p18, as well as to Nrf1 and Nrf2, were purchased from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Protein purification of Nrf1 10S

Nrf1 10S was cloned into the pQE 31 vector (Qiagen, Hilden, Germany). For protein purification, SG13009 (pREP4) bacteria were transformed and induced with 1mM IPTG for 4 h. Bacteria were lysed with 6 M guanidine hydrochloride, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris–HCl, pH 8. Purification was done according to the manufacturer's instructions. For renaturation, dialysis against 20 mM HEPES–KOH, pH 8, 5% glycerol, 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 0.005% (v/v) Tween in a stepwise gradient (8, 6, 4, 2 and 0 M urea) for 30 min each step, was performed.

Kinase reaction

An aliquot of 200 ng Nrf1 10S was incubated with 200 μM ATP and 1000 U CKII (New England Biolabs, Beverly, MA) in 20 mM Tris–HCl, pH 7.5, 50 mM KCl, 10 mM MgCl$_2$ at 30°C for 30 min. Ten microliters of the reaction were subsequently used in an electrophoretic mobility shift assay. For the erk2 (New England Biolabs) *in vitro* kinase assay, 100 ng Nrf1 10S were incubated with 25 μCi ATP (Amersham), 100 μM ATP and 2.5 U erk2 in 50 mM Tris–HCl, pH 7.5, 10 mM MgCl$_2$, 1mM DTT and 1 mM EGTA for 30 min at 30°C. The reaction was separated on a 4–20% gradient polyacrylamide gel (Novex), fixed in 40% methanol and 10% acidic acid for 20 min, dried and exposed to X-ray film (Kodak).

RESULTS

Investigations into the molecular mechanism of TNFα induction after FcγRIII triggering by IgG plus Ag complexes have not been performed so far. Several lines of evidence suggest that DC18 cells comprise a suitable model system for such an investigation (12). First, a RT–PCR analysis demonstrates that they are devoid of other positively signaling IgG-binding structures such as the high affinity receptor for monomeric IgG (FcγRI), which complicates the analysis in other systems (Fig. 1A). Secondly, stimulation of these cells via IgG plus Ag (IgG complexes) leads to a rapidly induced secretion of TNFα into the supernatant (Fig. 1B, left panel). Triggering with IgG complexes composed of IgG plus goat anti mouse IgG F(ab′)$_2$ fragments or directly with a mAb against CD16/32 (2.4G2) shows an identical result (Fig. 1B, middle panels). These stimulation conditions result reproducibly in a much stronger induction of TNFα release in those cells than the previously exploited PMA stimulation (Fig. 1B, compare the right panel with the left panel at the 4 h time point, and the middle panels; 13). Thirdly, the non-stimulating FcγRIIb2 (negatively regulating in the murine system), which is also expressed on DC18 cells (Fig. 1A), does not abrogate an activation via the FcγRIII, therefore allowing the investigation of positive signaling.
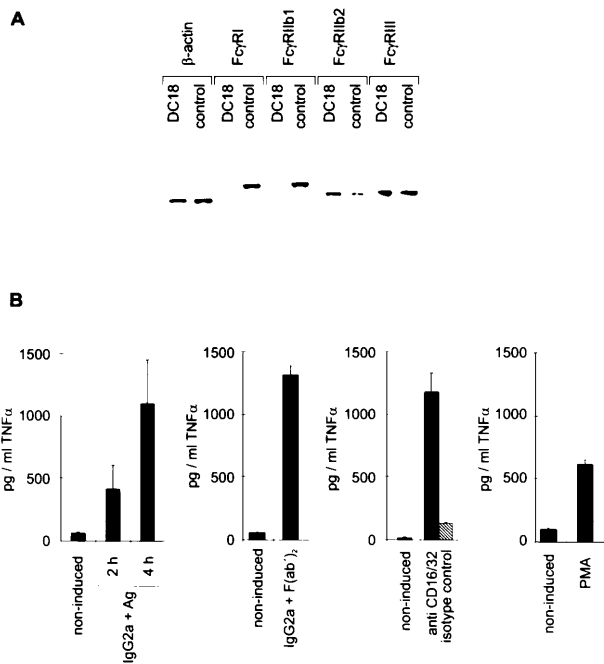
Figure 1. FcγRIII stimulation leads to TNFα production in DC18 cells. (**A**) RT–PCR analysis with total RNA of DC18 cells or control cells (CPII mouse mast cells for the FcγRI, FcγRIIb1, FcγRIII, FcγRIIb2) as indicated at the top. Primers specific for the different FcγRs and the β-actin control are given above. (**B**) TNFα ELISA after various stimulations with IgG plus Ag (time kinetic), with IgG plus (Fab')₂ (non-induced and 4 h induced), with anti CD16/32 (mAb 2.4G2, non-induced and 4 h induced) and with PMA (non-induced and 4 h induced) in black bars. Isotype antibody control for the mAb 2.4G2 is shown shaded. Stimuli and timepoints are indicated on the *x*-axis, pg/ml TNFα on the *y*-axis. All experiments were done in triplicate. SD is indicated.

Figure 2. A transcription factor binding to the extended κ3 site of the TNFα promoter mediates inducibility after IgG plus Ag stimulation. (**A**) Mapping of the TNFα promoter in DC18 cells. Top, a map of the TNFα promoter is given with putative transcription factor binding sites and their relative distance to the TATA box. Three different 5′ deletion constructs used in the analysis are shown below with the sequence defined by Del 2 and Del 3 given expanded. Below, transient transfection using three 5′ successive deletions of the TNFα promoter cloned upstream of luciferase as a reporter. DC18 cells were either left non-stimulated (odd lane numbers), or activated with IgG plus Ag (even lane numbers). Input DNA and stimuli are indicated on the *x*-axis, luciferase values on the *y*-axis. pGL2 represents the parental vector. All experiments were done in triplicate, SD is indicated. (**B**) Electrophoretic mobility shift assay with the extended κ3 site as a radiolabeled probe with nuclear extracts from either non-induced or 2 h induced DC18 cells. f indicates free probe.

The κ3 site interacts with an inducible factor after FcγRIII stimulation

For these reasons, we began to map the corresponding promoter elements of the TNFα promoter in transient transfections in DC18 cells after FcγRIII triggering using 5′ deleted promoter fragments linked to luciferase as a readout system. This analysis identified a 20 bp region spanning the κ3 site and extending to a few 5′ bases (extended κ3 site in the following) as partly responsible for the observed induction (Fig. 2A). At the 5′ end, this probe is identical to the κ3s probe used by Tsai *et al.* in the T-cell system, while at the 3′ end it is one nucleotide shorter (15). At the κ3s probe, the binding of two proteins was recently described in T-cells that were identified as NF-ATp or closely related factors (19). In DC18 cells, a similar picture is observed with two complexes detected to interact with the extended κ3 site in nuclear extracts of IgG complex-induced, but not in non-induced, cells in a gel shift analysis (Fig. 2B; specificity and competitions also discussed later).

Nrf1 or an antigenically related factor interacts with the κ3 site in DC18 cells

NF-AT (see above), in conjunction with AP1, and NFκB were already shown to interact with probes spanning the κ3 site (13,15–21). In addition, in DC18 cells treated with the artificial stimulus PMA, binding of NFκB p50 and p65 to the extended κ3
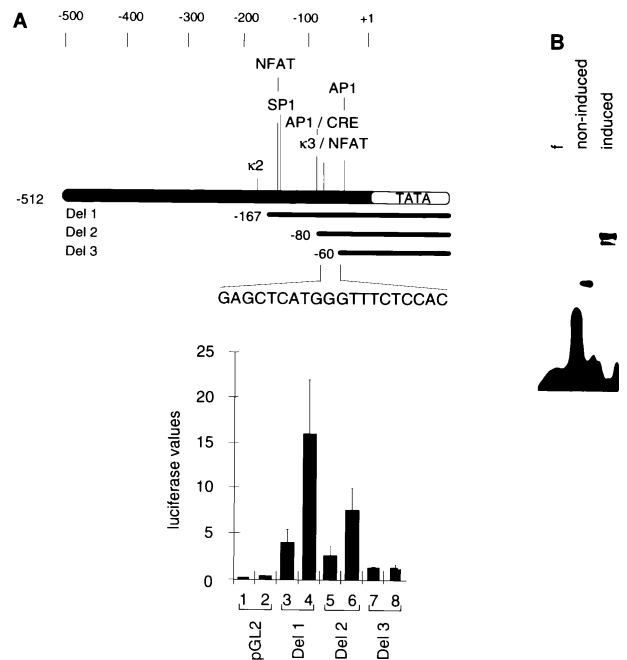
site was demonstrated recently (13). Therefore, we investigated these factors first. An NFκB consensus binding site not only failed to compete successfully for the complex formation in a 40-fold excess, but also the addition of the antisera to NFκB family members, used previously, showed no supershift (see below; 13). Similarly, an NF-AT consensus binding site failed to abolish complex formation. This finding is in agreement with the resistance of DC18 cells to FK506 treatment after IgG complex stimulation (data not shown). These results suggest that an as yet uncharacterized transcription factor is interacting with this promoter region. To elucidate the nature of this transcription factor(s) that interact(s) with the extended κ3 site in the IgG-complex-induced DC18 cells, we determined the molecular weight by size fractionating nuclear extracts prior to a gel shift analysis. Binding activity (one complex; see Discussion) was recovered in two fractions, leading to an estimated size of the factor of 36–45 kDa (Fig. 3A). The finding that DC18 cells express the transcription factor Nrf1 (28), and the similarity between the extended κ3 site and the binding sites of AP1 factors (i.e. fos, jun, fra), CRE binding proteins and Nrf1 [and other cap'n collar (CNC) family members like NF-E2], prompted us to further explore this factor. In contrast with an NFκB consensus site, an Nrf1/NF-E2 binding site strongly competed for the complex formation. In addition, a clear supershift was seen with an antiserum specific for this protein but not with antisera for the other
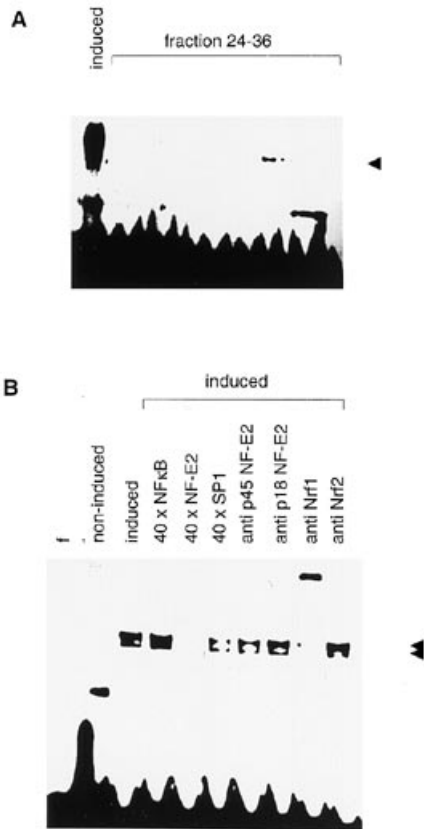
Figure 3. A transcription factor related to Nrf1 binds to the extended κ3 site. (**A**) Electrophoretic mobility shift assay with the extended κ3 site as radiolabeled probe using either nuclear extracts of 2 h induced DC18 cells (induced) or size-fractionated extracts of different fractions. The complex formation in the two fractions is highlighted by an arrowhead to the right. (**B**) Electrophoretic mobility shift assay with either non-induced or 2 h induced DC18 nuclear extracts. Induction, competitor oligonucleotides in 40-fold excess, as well as polyclonal sera against CNC family members and NF-E2 p18 (MafK), are indicated above the lanes. f indicates free probe. The double complex in nuclear extracts of induced cells is marked with two arrowheads to the right.

members of this family or its interaction partner (p18; Fig. 3B). These data suggested that Nrf1 or an antigenically closely related factor is interacting with the TNFα promoter. Therefore, we cloned and expressed the murine Nrf1 protein for further structural and functional analyses.

A novel splice variant of Nrf1 is expressed in DC18 cells

PCR amplification from position 918–2270 of the published murine sequence of Nrf1 that corresponds to the reported, expressed portion of human Nrf1, resulted in two different fragments (29). Cloning and sequencing revealed two types of mRNAs in DC18 cells (Fig. 4A). While clone 10L is identical to the published sequence except for a few nucleotide exchanges most likely due to mouse strain variations, clone 10S is a splice variant with a deletion of the complete serine-rich region plus additional COOH-terminal sequences. To exclude the possibility of a PCR artefact prior to cloning, the existence of this splice variant *in vivo* was verified by a PCR analysis using an oligonucleotide covering the 5′ splice site (Fig. 4B). Due to the novelty of this splice form, which is reminiscent of a similar splice variant described
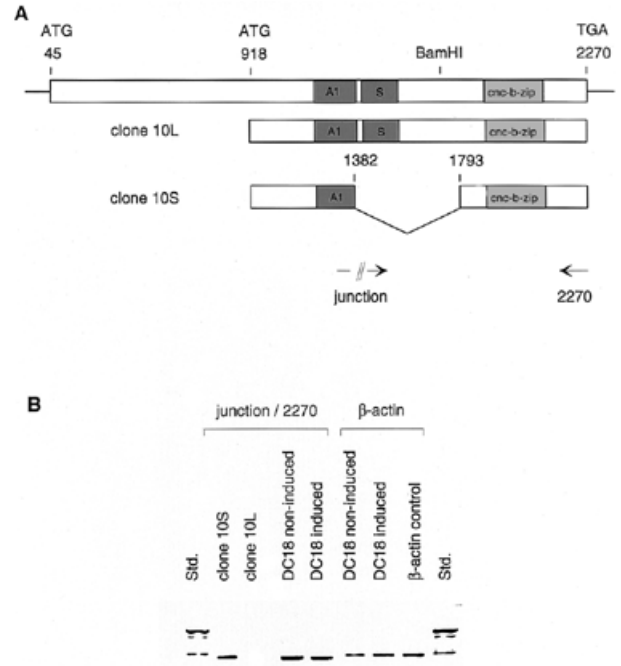

Figure 4. DC18 cells express a novel isoform of Nrf1. (**A**) Scheme showing the two isoforms of Nrf1 cloned. Start and stop codons are indicated at the top. The acidic domain (A1), the serine-rich region (S) and the CNC-basic leucine zipper region (cnc-b-zip) are shaded. Oligonucleotides used in a PCR analysis are indicated at the bottom. (**B**) PCR analysis using either 10 ng plasmid DNA of clones 10S and 10L, or total RNA of non-induced and induced DC18 cells with primers specific for clone 10S or β-actin as a control. Input DNA and primers are given at the top.

recently for Bach proteins (30) and the good correlation of the predicted molecular weight of the deduced protein with our size fractionation (~40 kDa), we concentrated on this cDNA.

Nrf1 is dependent on CKII phosphorylation for binding

Bacterial expression and purification of Nrf1 10S resulted in a 42 kDa N-terminal His tag fusion protein that was equally well recognized by an anti-His tag monoclonal antibody (data not shown) or by the Nrf1 antiserum (Fig. 5A). To our surprise, the bacterially expressed material did not bind to the extended κ3 site probe in a gel shift analysis, despite several different refolding procedures. While this still could be due to incorrect folding of the protein, another possibility was the requirement of a post-translational modification (i.e. phosphorylation) taking place *in vivo* either constitutively or in the process of cell activation after triggering the FcγRIII. A computer analysis for putative phosphorylation sites showed that Nrf1 10S contains six CKII, three MAP kinase and one abl phosphorylation sites. CKII is reported to modify the binding capacity of b-zip proteins such as jun or other transcription factors like the serum response factor (31), which prompted us to investigate this possibility. Phosphorylation of purified Nrf1 10S *in vitro* by CKII leads to a detectable complex formation in a gel shift analysis which is neither seen with the purified kinase nor the purified Nrf1 10S alone (Fig. 5B). Identical results were obtained with a 216 AA His tag-fragment of clone Nrf1 10L (from *Bam*HI to the end, data not shown). The specificity of this process was further underlined by the finding that several other kinases such as the MAP kinases erk2 and p38 as well as abl did not result in
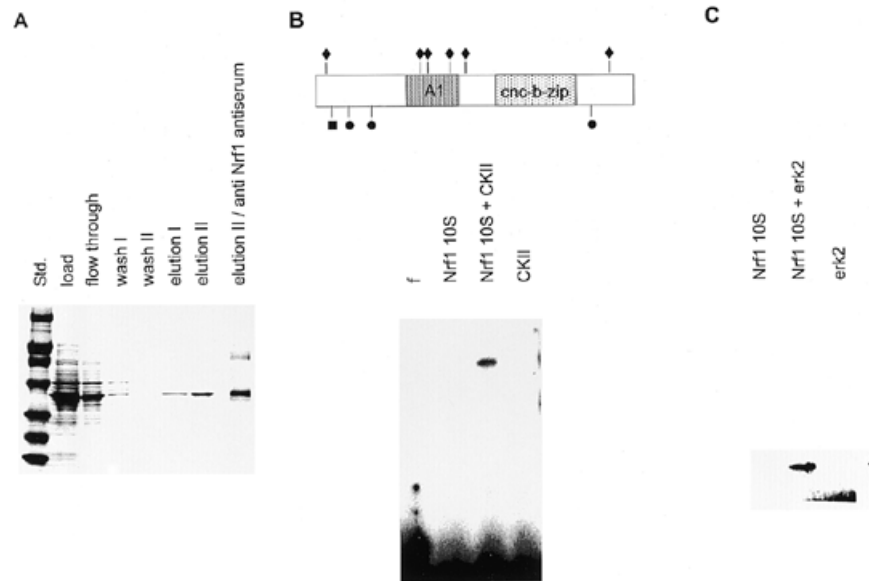
Figure 5. Binding and phosphorylation of *in vitro* purified Nrf1 10S. (**A**) Purification of recombinant Nrf1 10S by Ni–agarose chromatography. Standard (Std.) and fractions are indicated at the top. Right-most lane, western blot analysis of the recombinant purified Nrf1 10S with a polyclonal serum against Nrf1. (**B**) Binding of bacterially expressed Nrf1 10S protein after *in vitro* phosphorylation. The protein structure of Nrf1 10S is shown at the top with potential CKII (◆), MAPK (●) and abl (■) phosphorylation sites indicated. A1 corresponds to the acidic region, CNC-b-zip to the cap'n collar basic leucine zipper region. Electrophoretic mobility shift assay with purified Nrf1 10S protein either non-phosphorylated or phosphorylated with CKII *in vitro* or a CKII control reaction as indicated at the top using the extended κ3 site as radiolabeled probe. f corresponds to free probe. (**C**) Phosphorylation of Nrf1 10S by erk2. SDS–protein gel electrophoresis with purified Nrf1 10S protein, purified erk2 protein and purified Nrf1 10S and erk2 protein after a 30 min *in vitro* kinase reaction with radiolabeled [γ^{32}]ATP. The gel was dried and exposed to an X-ray film. The phosphorylated Nrf1 10S protein is highlighted by an arrowhead to the right.

a similar effect on Nrf1 10S binding. Erk2, however, was shown to phosphorylate Nrf1 in an *in vitro* kinase reaction with purified components (Fig. 5C), suggesting an involvement of this molecule in the signaling process.

To further prove an involvement of CKII and erk2 in the signaling cascades initiated at the FcγRIII, inhibitors specific for these kinases were applied prior to the stimulation of DC18 cells. DRB, a specific inhibitor of CKII, abolished TNFα induction in a dose-dependent manner in the expected dose range ($IC_{50} = 6$ μM for CKII, Fig. 6A). It strongly suggests that CKII is involved in the signaling process in these cells, most probably via facilitating Nrf1 binding to the promoter site, as demonstrated by our biochemcal data (see Discussion). An inhibitor of the MAP kinases, Apigenin ($IC_{50} = 25$μM*)*, also resulted in an abrogation of TNFα induction, suggesting that the observed phosphorylation of Nrf1 by erk2 plays a role *in vivo* (14; Fig. 6B, top panel). In contrast, a specific inhibitor of the MAP kinase p38 (SB 203580) had no effect (Fig. 6B, bottom panel; see Discussion). We next looked at the expression and activation of MAP kinases after FcγRIII triggering as the biochemical basis for the use of Apigenin as an inhibitor. We were unable to detect jnk 1,2 or jun expression in those cells in western blot analyses (data not shown). In contrast, both erk 1,2 and p38 are constitutively seen and become readily phosphorylated/activated after stimulation (Fig. 6C). These findings altogether strongly point towards the involvement of erk 1,2 activation in the overall induction process, thereby providing a link to recent findings in murine macrophages where the FcγRIII was shown to activate p42 MAPK (erk2) 5–10 min after triggering (32).

Nrf1 induces TNFα transcription

To prove functionally that Nrf1 10S confers transcriptional activation to the TNFα promoter, we used CPII mouse mast cells and HeLa cells as heterologous systems. Nrf1 10S and 10L were cloned in a eukaryotic expression vector under the control of the cytomegalovirus promoter and used in cotransfections in both cell types, together with the TNFα promoter reporter gene construct (13). While Nrf1 10S cotransfection has a marginal effect on the TNFα promoter activity in non-induced cells, it leads to an ~10- to 15-fold enhancement in IgE plus Ag stimulated mast cells and a 3-fold general enhancement in HeLa cells independent of the stimulation (Fig. 7A and B). This implies that upon activation of cells, Nrf1 10S can function as a transcriptional activator of this cytokine in the appropriate cell type. Clone Nrf1 10L, in contrast, has no influence on the transcriptional activation in both systems in an identical experimental setting but instead inhibits reproducibly the induction in mast cells at ~50% (Fig. 7A and B; see Discussion).

DISCUSSION

Several novel transcription factors with a specificity for AP1/AP1-like binding sites, and probably also for CRE binding sites, were recently described. These include the members of the CNC family of transcription factors, such as NF-E2, Nrf1, Nrf2, the erythroid cell-derived protein with CNC homology (ECH) and the more distantly related Bach proteins (28,30,33–35). In contrast with their mostly ubiquitous expression, only a few genes such as the
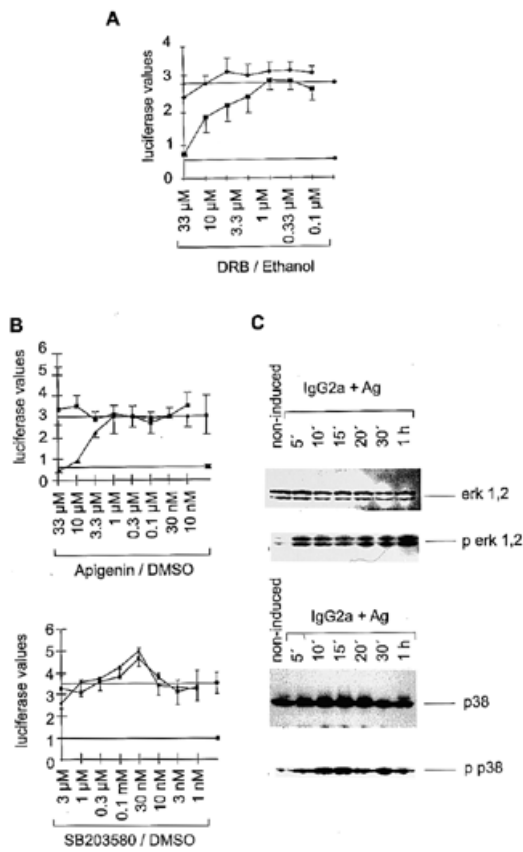
Figure 6. CKII and MAPK are involved in the signaling cascade initiated at the FcγRIII in DC18 cells. (**A**) DRB inhibits TNFα transcription in a dose-dependent manner after triggering by IgG plus Ag. DRB was tested on inhibition of TNFα reporter gene induction (luciferase values on the *y*-axis) at the concentrations indicated at the bottom (*x*-axis) after stimulation by IgG + Ag. (◆) Drug application, (■) solvent control application. All experiments were done in quadruplicate, SD is indicated. Solid lines are values of non-induced and induced cells. (**B**) Apigenin, but not SB 203580, inhibits the induction of TNFα by the FcγRIII. Drugs were tested on inhibition of TNFα reporter gene induction (luciferase values on the *y*-axis) at the indicated concentrations (*x*-axis) after stimulation by IgG + Ag. (◆) Drug application, (■) solvent control application. All experiments were done in quadruplicate, SD is indicated. Solid lines are values of non-induced and induced cells. (**C**) Western blot analyses for constitutive and activated (phosphorylated) erk 1,2 and p38. Time kinetic over the first hour of stimulation by the FcγRIII using whole cell extracts.

Figure 7. Expression of Nrf1 10S, but not Nrf1 10L, activates the TNFα promoter in heterologous systems. Transient transfection using a TNFα reporter gene construct and either Nrf1 10S or Nrf1 10L expression plasmids in a cotransfection experiment. CPII mouse mast cells (**A**) and HeLa cells (**B**) were either left unstimulated (odd lane numbers) or were activated with 2 μg/ml IgE plus 100 ng/ml Ag or 20 ng/ml PMA plus 100 ng/ml ionomycin (even lane numbers), respectively. Input DNA and stimuli are indicated on the *x*-axis, luciferase activity on the *y*-axis. pCMV resembles the parental expression vector control. Each experiment was done in triplicate, SD are indicated.

β globin gene and the NAD(P)H–quinone oxidoreductase1 gene are known to be controlled either directly or indirectly by these factors. Xenobiotics and antioxidants activate the NAD(P)H–quinone oxidoreductase1 gene from a basal level of expression through Nrf1 and Nrf2, indicating that these transcription factors are involved in induced gene expression (36). This confirms our finding that Nrf1 interacts with the extended κ3 site of the TNFα promoter after FcγRIII stimulation and participates in the induction of this cytokine. This interaction as demonstrated by the cotransfection of Nrf1 10S expression plasmids in the heterologous systems of activated HeLa and CPII mouse mast cells leads to a 3-fold enhancement of overall transcriptional activity and >10-fold enhancement of induced transcriptional activity of the TNFα promoter activity, respectively. The values are in agreement with the stimulation seen in transient cotransfections of Nrf1-expressing plasmi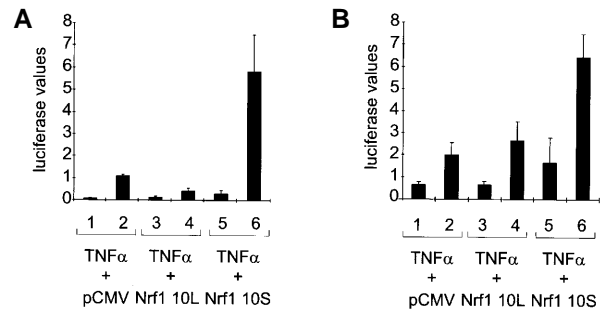ds with hARE element driven reporter gene constructs (4.8-fold in Hep-G2 and Cos cells; 36) and of Bach 1 expressing plasmids with NF-E2 consensus site driven reporter gene plasmids (3-fold in HD3 chicken erythroid cells; 30). It is noteworthy that only clone 10S has this potential in our hands, while clone 10L is either inactive or represses the transcriptional activation. This mimics the situation seen with the Bach proteins, where also the splice variant without the serine-rich region confers activation in transient transfections in HD3 chicken erythroid cells while the full length clone shows repression in such an experiment (30). This characterizes the extended κ3 site of the TNFα promoter as being the same as the previously described interaction sites for this factor family. In contrast with classic AP1 components such as jun, Nrf1 is obviously slightly different in its requirements for DNA contact points. While we and others never found classic AP1 components binding to the extended κ3 site (due to the missing T 5′), Nrf1 interaction at this probe is possible.

The isolation of Nrf1 10S with a 411 bp deletion covering the serine-rich region provides an interesting analogy to the Bach family of proteins. For both Nrf1 10S and the Bach family of protiens, isoforms with and without the serine-rich region (369 bp splice variant of Bach 2) that is implicated in protein–protein interaction and as a target of phosphorylation, have now been described. These serine-region-lacking variants, despite this lack, are able to confer transcriptional activation in transient cotransfections (30). The overall transcriptional induction upon stimulation, seen in mast cells but not in HeLa cells, suggests that this transcription factor has to fit into a cell-type-specific activation cascade. This fit can either mean certain activating molecules, like kinases, or transcriptional cofactors. The first possibility is underlined by the fact that the PKC activation via PMA in HeLa cells is a different signal to the erk 1,2 activation after IgG-complex stimulation in DC18 cells, and FcεRI triggering in mast cells. The second point is suggested by the fact that in nuclear extracts of stimulated DC18 cells, a double complex formation at the extended κ3 site probe is observed, indicating that homo- or heterodimerization has to take place at this promoter site. Homodimers of Nrf1 10S are unlikely because only one partial AP1 consensus binding site is present in the extended κ3 site, indicating an additional uncharacterized protein. This protein might be expressed in mast

cells but not in HeLa cells. Cloning and bacterial expression of mafK, a described cofactor for Nrf1 (29), however, did not result in binding to the κ3 site of this protein alone or in combination with Nrf1, while it clearly bound to an Nrf1/NF-E2 consensus binding site. In addition, in cotransfections no further enhancement of the induction at the TNFα promoter was observed, suggesting a less important role for mafK as a cofactor at the TNFα promoter. Third, our deletion studies indicate that an additional factor, binding in the region between Del 1 and Del 2, is required for full activation of the TNFα promoter (see drop of activity between Del 1 and Del 2). This factor was recently charactzerized as SP1 (13). Therefore, as in other systems (i.e. mast cells), the induced transcription is probably mediated by a complex of several transcription factors, including Nrf1, which becomes assembled around the κ3 site. In contrast, sequences further upstream contribute to the overall induction in DC18 cells, but not in mast cells (see drop between Del 1 and Del 2; 13,14).

The *in vitro* phosphorylation of Nrf1 10S by erk2 does not contribute to the binding of this transcription factor to the extended κ3 site, suggesting that Nrf1, as jun, is probably phosphorylated by this kinase in its potential transactivation domain. In contrast, *in vitro* phosphorylation of bacterially expressed Nrf1 by CKII strongly facilitates the interaction with the TNFα promoter site as well as with a consensus Nrf1/NF-E2 binding site (29). The specificity of these reactions is demonstrated by the fact that two additional kinases, abl and p38, do not phosphorylate and stimulate the binding capacity of this protein, even though putative phosphorylation sites are present in Nrf1, although autophosphorylation of both enzymes is observed. The post-translational modification of Nrf1 10S (and also Nrf1 10L) by CKII might also explain some recent difficulties with bacterially expressed Nrf1 protein in gel shift analysis (29). Our finding that an inhibition of CKII by application of DRB abrogates TNFα transcription in IgG plus Ag-activated DC18 cells, strongly indicates that Nrf1 phosphorylation by CKII is a step also required *in vivo* for the activation of this factor. Therefore, Nrf1 10S would be regulated in analogy with the serum response factor whose binding activity is also increased by phosphorylation at four serine residues by this kinase. In contrast, CKII phosphorylation of the b-zip protein, jun, results in the loss of its binding activity (31). This might provide an interesting link among the three 'AP1 binding proteins', fos, jun and Nrf1. While the binding capacities of Nrf1 and jun are regulated in an opposite way to fos by this kinase (31), the serum response factor, which activates fos transcription, is identically regulated. Fos, however, was recently reported to negatively regulate NAD(P)H–quinone oxidoreductase, while Nrf1 was positively activating. CKII might therefore comprise an essential component to balance different AP1/AP1-like binding proteins in a cell.

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