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Nrf1 in a complex with fosB, *c*-jun, junD and ATF2 forms the AP1 component at the TNFα promoter in stimulated mast cells

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ABSTRACT

Cap 'n' collar-basic leucine zipper (CNC-bZIP) proteins are widely implicated in developmental processes throughout different species. Evidence is accumulating that some of them are also participating in induced gene expression in the adult. Here we show that the three CNC-bZIP members NF-E2, Nrf1 and Nrf2 are constitutively expressed in the murine mast cell line CPII and that they form transcription factor complexes with several AP1 binding proteins. Upon induction, complexes are observed at the 2 × NF-E2 consensus binding site and the extended κ3/AP1$^+$ site of the TNFα promoter. The interaction of Nrf1 with *c*-jun, junD, fosB and ATF2 in mast cells is in contrast to the recently reported binding of Nrf1 alone at the κ3/AP1$^-$ site in dendritic cells. We speculated that this may be the result of the expression of isoforms of Nrf1 in mast cells. Using a PCR cloning strategy, we have isolated six novel splice variants of this transcription factor. Some of them have deleted the translational stop codon, resulting in an Nrf1 protein with an altered leucine zipper region. Expression of this altered binding/interaction domain interferes with TNFα induction, indicating an interaction of this splice variant with the active AP1/NF-AT complex at this promoter.

INTRODUCTION

Cap 'n' collar-basic leucine zipper (CNC-bZIP) proteins are a subfamily of bZIP transcription factors characterized by an ~40 amino acid (aa) homology region that is immediately N-terminal to the basic leucine zipper region. In terms of evolution, proteins with this homology are widely implicated in developmental processes in *Caenorhabditis elegans* (Skn1; 1), *Drosophila melanogaster* (cap 'n' collar; 2), chicken (ECH; 3), mouse (Bach; 4) and man (5–7). The three currently known human members NF-E2, TCF11/LCR-F1/Nrf1 and Nrf2 (5–7), and their murine counterparts, were all isolated in the process of elucidating the mechanism leading to the lineage-specific expression of the β-globin gene locus (8). However, only NF-E2 is cell type-specific while Nrf1 and Nrf2 are ubiquitously expressed (4,9). Data on corresponding knockout mice (10,11), as well as the recent finding that binding sites for Nrf1 are important in the expression of the erythroid porphobilinogen deaminase gene, the heme-oxygenase 1 gene and the NAD(P)H:quinone oxidoreductase 1 gene, suggest a broader participation of these factors in mammalian gene regulation (12).

We have recently investigated promoter elements and transcription factors involved in TNFα induction in the murine mast cell line CPII [stimulation by IgE + antigen (Ag)] and the murine fetal dendritic cell line DC18 (stimulation by IgG + Ag; 13–15). In mast cells the IgE + Ag responsive element was mapped to a 28 bp oligonucleotide that interacted with the transcription factors AP1 and NF-AT (extended κ3/AP1$^+$ site A**TGAGCTCA**T**GGG-TTTCTCC**ACCACCAA; AP1 and NF-AT sites in bold; 14,16). The detailed composition of the AP1 component with respect to jun, fos, fra and ATF protein binding as well as the nature of the NF-AT family member at this probe have not been addressed so far in mast cells. A corresponding analysis in the murine dendritic cell line DC18 identified a very similar site (GAGCTCATGGGT-TTCTCCACCAA, extended κ3/AP1$^-$ site) which is 5′ 2 nt (AT) shorter and therefore has deleted parts of the AP1 binding site (15). Nrf1 binding to this probe was observed in the dendritic cell line, while in mast cells no interaction with any transcription factor was detected in a gel shift analysis (13,15). This difference is functionally supported by 5′ promoter deletions of reporter gene constructs which proved the requirement for a complete AP1 site for inducibility in mast cells but not in dendritic cells (13–15).

Here we show that in mast cells, Nrf1 or a closely related factor together with several of the classical AP1 transcription factors (*c*-jun, junD, fosB and ATF2) is found after stimulation by IgE + Ag in the AP1 complex at the TNFα promoter. Using a PCR detection/cloning strategy, we identified six novel splice variants of Nrf1 in this cell type. Several of them have deleted the currently assumed start and stop codon. Some splice variants, exemplified by clone D, contain an altered C-terminal domain with a change in the bZIP-region, as a result of the deletion of the stop codon. Expression of the altered leucine zipper region interferes with the transcriptional induction of TNFα, indicating that the new splice variant indeed is able to interact with the active AP1/NF-AT complex. These data suggest that distinct Nrf1 isoforms form different complexes with various cofactors and could explain the broad capability to heterodimerize with small Mafs, nuclear hormone receptors and classical AP1 proteins.

MATERIALS AND METHODS

Cell culture and stimulation of cells, electrophoretic mobility shift assays, gel shift probes and supershift assays, preparation of

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nuclear extracts and radiolabeled probes, transient transfections, protein purification and kinase reactions, FK506 treatment as well as western blot analyses were performed as recently described (14,15,17–20).

RT–PCR analyses

All primers were purchased from Gibco BRL-Life Technologies (Paisley, UK). The following oligonucleotides, specific for the classical murine Nrf1 sequence were used: 5′-GGTCCTTCAG-CAATGCTTTCTCTG-3′, 5′-CTCATGTCCATCATGGAAAT-GCAGG-3′, 5′-CCCAGGCTCACTTCCTCCGGTCC-3′ and 5′-CTGCCCTGCCCAGGGAGGAGG-3′. The oligonucleotides defining the junction between exon 3 and 6 (767 variant, position delineated from the human genomic structure) are 5′-CTTCCCTGCACAGGCTATGGAAG-3′ and 5′-CTTCCA-TAGCCTGTGCAGGGAAC-3′. The primers for the clone D splice variant and the clone 10 splice variant are 5′-CGATTGCT-TCGAGAAAAGGAAAATGAG-3′, 5′-CTCATTTTCCTTTTC-TCGAAGCAATCG-3′ and 5′-GGAGTTTGACTCTGAGC-ACAGAGC-3′, 5′-GCTCTGTGCTCAGAGTCAAACTCC-3′, respectively. The amplification of the fragments was carried out using the PCR advantage kit (Clontech, Palo Alto, CA) with the following parameters: 30 cycles at 94°C for 1 min, 65°C for 45 s, 68°C for 4 min with a final extension step for 7 min at 68°C. The amplification products were visualized by ethidium bromide staining after separation on a 1.2% agarose gel. Five µg total RNA of the murine mast cell line CPII were used as a source for the cDNA generated with the Superscript preamplification kit (Gibco). Reactions with murine β-actin primers (Clontech) served as a control for RNA integrity.

Quantitative RT–PCR analysis

Mimics were constructed using the PCR Mimic construction kit (Clontech). The composite primers for the generation of the mimics with a fragment of v-*erb*B and Nrf sequences specific for clone D (amplified fragment size 347 bp) are 5′-CGATTGCTTCGAGA-AAGGAAAATGAGCGCAAGTGAAATCTCCTCCG-3′ and 5′-CTGCCCTGCCCAGGGAGGAGGTTGAGTCCATGGGG-AGCTTT-3′, specific for clone 10 (amplified fragment size 497 bp) 5′-GGAGTTTGACTCTGAGCACAGAGCGCAGATGAGTA-TCTTGTCCC-3′, common for clone 10 and full length mNrf1 (clone G) 5′-CCCAGGCTCACTTCCTCCGGTCCTTGAGTCC-ATGGGGAGCTTT-3′, and specific for clone G (amplified fragment size 777 bp) 5′-CCTTCTCTGAGGAGGGTGCTGTT-GCAAGTTTCGTGAGCTGATTG-3′ (v-*erb*B sequences underlined). All primers were purchased from Gibco. Amplification, separation and visualization were done as described above. The cDNA used as an amplification source in the reactions is made from 5 µg total RNA from CPII mast cells or DC18 dendritic cells, respectively.

Cloning and sequencing

Gel-purified PCR fragments of the different splice variants were used for ligation into the pCR 3.1 vector (Invitrogen, Carlsbad, CA). The fragment from mNrf1 bp 1621 to 2270 (in the following termed Nrf1 DNA binding region) was cloned via *Bam*HI sites into the bacterial expression vector pQE-31 for protein expression (Qiagen, Hilden, Germany). The obtained clones were sequenced using the T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden) and additionally the deaza G/A sequencing mixes (Pharmacia Biotech).

Antibodies

All antibodies used for supershift assays and western blot analyses were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) except for *c*-jun, which was purchased from New England Biolabs (Beverly, MA).

In vitro transcription and translation

An aliquot of 1 µg of plasmid DNA was used either with the TNT T7 Quick kit (Promega, Madison, WI) or with the TNT T7 Coupled Wheat germ extract system (Promega) according to the manufacturer's protocol, using [^{35}S]methionine (Amersham, Little Chalfont, UK). The proteins were separated on SDS–PAGE 4–12 or 10–20% gradient gel (Novex, San Diego, CA), dried and subjected to autoradiography.

Reporter gene assay

The murine mast cell line CPII clone 12 represents a stable transfectant of the parental cell line with a TNFα promoter luciferase construct (21). Cells were preincubated with 0.1–100 µM 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, Calbiochem, San Diego, CA) for 1 h and stimulated for 3 h with 2 µg/ml anti-trinitrophenol/anti-dinitrophenol specific IgE (Pharmingen, San Diego, CA) plus 100 ng/ml Dinitrophenol-BSA (Calbiochem). After lysis with reporter lysis buffer (Promega) the luciferase values were determined.

Probes for electrophoretic mobility shift assays

The 2× NF-E2 consensus oligonucleotides [upper strand: 5′-CAAGCACAGCAATGCTGAGTCATGATGAGTCAT-3′ and lower strand: 5′-GCCTCAGCATGACTCATCATGACTCAG-CATTG-3′ (Gibco)] were annealed and filled in with Klenow polymerase and [α-^{32}P]dCTP (Amersham).

RESULTS

In mast cells, TNFα inducibility depends on an intact AP1 consensus binding site (extended κ3/AP1^{+} site), in contrast to dendritic cells, where Nrf1 binding to the extended κ3/AP1^{-} site alone is sufficient (15). The extended κ3/AP1^{+} site allows the formation of an AP1 complex which serves as a cofactor for NF-AT that is only detected and necessary at the TNFα promoter in mast cells but not in dendritic cells (14). We hypothesized that this AP1/NF-AT complex substitutes Nrf1 in the regulation of TNFα in this cell type which would be in line with recent findings that some CNC-bZIP family members are downregulated in expression upon full maturation in mast cells (22). However, western blot analysis showed that all three members of the CNC-bZIP family, NF-E2 (p45 and MafK p18) as well as Nrf1 and Nrf2 are strongly expressed at unchanged levels in non-induced and 2 h IgE + Ag induced CPII cells, which represent a mature effector mast cell line (23; Fig. 1A). This led us to conclude that a downregulation of CNC-bZIP proteins is not *per se* required for the final maturation to 'effector' mast cells. In gel shift analyses using the 2 × NF-E2 consensus binding site for this transcription factor family, a complex formation with nuclear extracts was detected which, in contrast to the western blots, is strongly

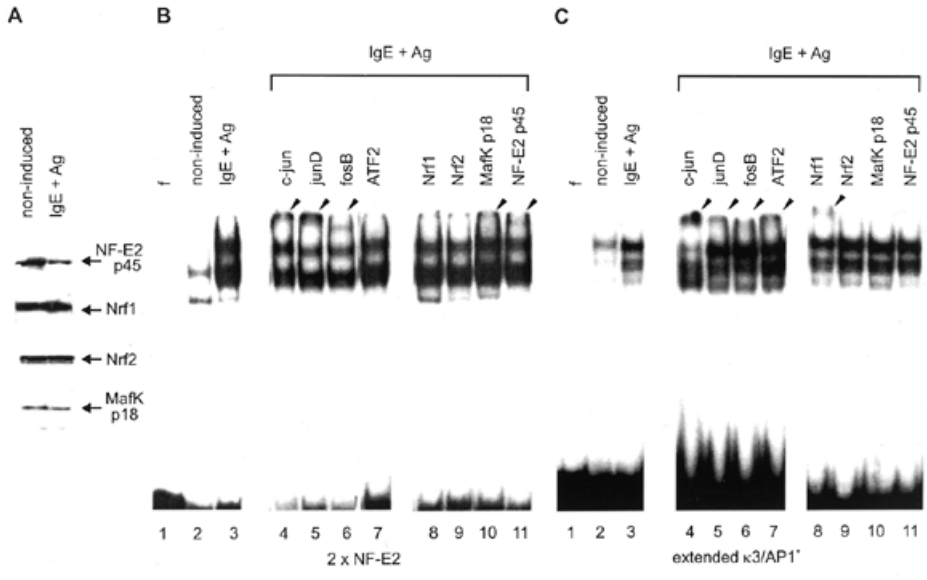

Figure 1. Expression of CNC-bZIP members in mouse mast cells and interaction with classical AP1 binding proteins. (**A**) Western blot analysis with extracts of non-induced and 2 h IgE + Ag induced CPII cells and antibodies corresponding to the CNC-bZIP family members. (**B**) Gelshift analysis with the 2 × NF-E2 site as a radiolabeled probe. Non-induced indicates nuclear extracts of non-stimulated CPII cells, induced extracts of 2 h IgE + Ag-stimulated cells. The antibodies used in the supershift analyses are indicated at the top. (**C**) Gelshift analysis with the extended κ3/AP1$^+$ site in an identical experimental setting as described in (B).

enhanced in stimulated mast cells (Fig. 1B). This is in agreement with the recent description that particularly NF-E2 binding activity significantly increases after activation of the ras/raf/MAP kinase pathway, a signaling cascade known to be initiated by crosslinking the FcεRI in mast cells (24). The composition of this complex in supershift analyses was determined as NF-E2 (p45 and MafK p18), together with *c*-jun, junD and fosB (Fig. 1B, lanes 4, 5, 6, 10 and 11). Nrf1 binding to this probe is not observed in such an experiment (Fig. 1B, lane 8). In gel shift/supershift analyses with the extended κ3/AP1$^+$ site of the TNFα promoter as a radiolabeled probe (28 bp oligonucleotide), again a complex of CNC-bZIP and classical AP1 proteins is detected, in addition to NF-AT (Fig. 1C). Here, *c*-jun, junD, fosB and furthermore ATF2 and Nrf1, but not Nrf2 and NF-E2 (p45 and MafK p18), build up the AP1 component (Fig. 1C, compare lanes 4–8 with lanes 9–11). This indicated that in induced CPII mast cells, dependent on the primary sequence of the probe, different CNC-bZIP proteins readily form dimers with classical AP1 factors. The interaction of Nrf1 with the extended κ3/AP1$^+$ site was further confirmed with a His-tag fusion protein containing the 216 AA CNC-bZIP region of this transcription factor (Fig. 2A). Binding of this protein fragment to the extended κ3/AP1$^+$ site but now also to the NF-E2 consensus site was seen, however, only after phosphorylation by CKII *in vitro* (15). The latter binding indicates that Nrf1 can, in principle, interact with this probe, but that in nuclear extracts its affinity is too low to successfully compete for such a binding with other classical and CNC-bZIP proteins. The requirement for CKII phosphorylation is further supported by applying DRB, a CKII inhibitor, which abolished the induction of TNFα in mast cells at the expected dose range (Fig. 2B; 15). In corresponding gel shift analyses, this treatment specifically altered the composition of the AP1 complex by preventing Nrf1 binding, without affecting other classical AP1 factors, such as junD, also detected at this probe (Fig. 2C, compare lanes 10 and 11 with lanes 12 and 13; lanes 1–4 show an SP1 gel shift for nuclear extract normalization). In addition, NF-AT binding, usually seen as the fastest migrating complex in such an analysis, was dramatically impaired by DRB, suggesting a potential link between Nrf1 and NF-AT (Fig. 2C, compare lanes 7 and 8; lane 9 represents a long exposure of lane 8). To further explore this fact, we analysed the composition of an AP1 complex at a canonical AP1 site (Fig. 3A). Similarly as at the NF-E2 consensus binding site, no Nrf1 interaction was detected at this probe in nuclear extracts [Fig. 3A, lane 14; only fosB (weakly), *c*-fos, junB (weakly), *c*-jun, junD and fra2 are seen; see lanes 6–10 and 12], as we speculated, most likely due to the absence of NF-AT. To prove this, we prevented the NF-AT activation by FK506 treatment, and readdressed the complex composition at the extended κ3/AP1$^+$ site (Fig. 3B). In contrast to all other investigated classical AP1 proteins, only Nrf1 (Fig. 3B, compare lanes 9 and 10 with lanes 11 and 12), besides NF-AT (Fig. 3B, compare lanes 7 and 8), which is severely reduced, disappeared after such a treatment from the complex at the extended κ3/AP1$^+$ site (Fig. 3B, lanes 1–4 show an SP1 gel shift for nuclear extract normalization). Taken together, these data imply that NF-AT and Nrf1 strongly depend on each other for stably interacting with the extended κ3/AP1$^+$ site, and therefore demonstrate a clear difference to the situation found in dendritic cells (15; Discussion).

One explanation for the binding of Nrf1 to the κ3/AP1$^-$ site alone in dendritic cells, and its role as a cofactor in binding to the κ3/AP1$^+$ site in mast cells, is the existence of different Nrf1 variants. This thinking is supported by the detection of multiple minor bands in a western blot analysis in mast cells with an Nrf1 specific antibody (V.Novotny, data not shown) and the description of variants of this protein in the human and the murine system (human: deletions of exon 1a, 1b, 3a, 4 and 6a; murine: deletion of the complete serine-rich region; 9,15). In an attempt to identify further isoforms in mouse, we used a PCR cloning strategy employing different primer pairs, including some covering the exon/intron boundaries delineated from the human genomic sequence (which is not described in mouse). A representative example of this type of analysis is shown in Figure 4A, where all the deduced variants found

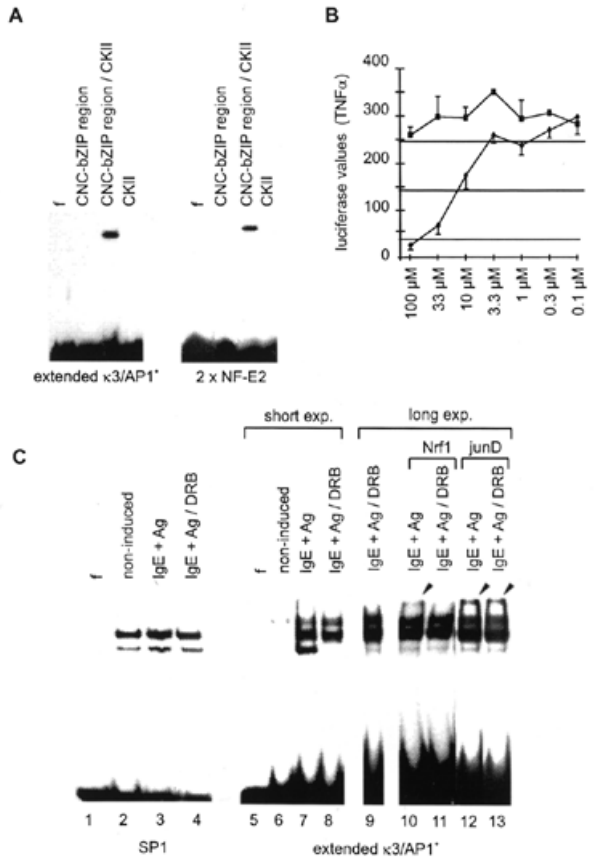

Figure 2. Interaction of the purified Nrf1 binding region with the 2 × NF-E2 consensus site and sequences of the TNFα promoter. (**A**) Gelshift analysis with the extended κ3/AP1$^+$ site of the TNFα promoter (left) and the 2 × NF-E2 consensus binding site (right) and a 216 aa purified His-tag Nrf1 binding region before or after phosphorylation with CKII. (**B**) Reporter gene assay of 3 h IgE + Ag-induced CPII clone 12 mast cells with and without DRB as an inhibitor. Concentrations of the CKII inhibitor DRB are given at the *x*-axis, luciferase values at the *y*-axis. Diamonds indicate values after drug treatment, squares indicate solvent controls. The IC_{50} is depicted by the dotted line. The experiment was done in quadruplicates, standard deviations are indicated. (**C**) Gel shift analysis/supershift analysis at the extended κ3/AP1$^+$ site (right panels) with extracts from DRB (30 µM) treated cells. SP1 gel shifts for normalization of the nuclear extracts are given to the left. Short and long term exposures are given, to result in the best resolution of the complexes and supershifts. Antibodies used in the supershift analysis are indicated at the top.

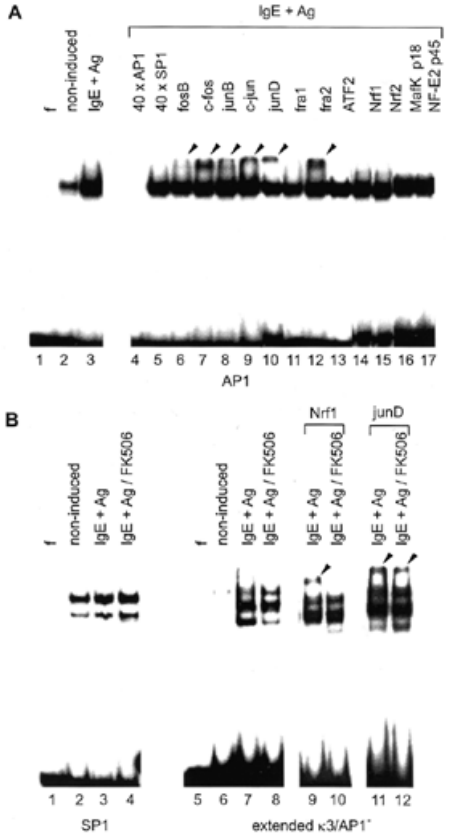

Figure 3. Nrf1 binding is dependent on NF-AT. (**A**) Gel shift analysis/supershift analysis using an AP1 consensus binding site and various classical and CNC-bZIP antibodies in a supershift analysis with nuclear extracts from 2 h IgE + Ag-induced mast cells. Oligonucleotides and antibodies used are indicated at the top. (**B**) Gel shift analysis/supershift analysis at the extended κ3/AP1$^+$ site (right panels) with extracts of FK506 (50 ng/ml) treated cells. SP1 gel shifts for normalization of the nuclear extracts are given to the left.

to be expressed in mast cells are given schematically in Figure 4B. While splice variant 10 represents the recently described isoform in murine dendritic cells (15), to our surprise there was the finding of two new splicing events, the 767 and D variant. These are of particular interest as they appear to have deleted the functional translational start (767) and the functional translational stop codon (D) of Nrf1 (in mouse it is assumed that the translational start codon in position 918–920 is used; 6,25). Interesting to us was splice variant D which not only has deleted the translational stop codon in position 2267–2270, but as a result of this, has a change in the second half of the leucine zipper motif. A complete variant D was cloned and sequenced (DDBJ/EMBL/GenBank accession no. AF071084). It contains an open reading frame with a coding potential for a protein with 458 aa in comparison to the 450 aa coding potential of full-length mNrf1 (clone G; start assumed at ATG position 918–920). The 72 C-terminal residues of clone G which are extremely positively charged (16 Arg and Lys versus 8 Asp and Glu) are exchanged for 80 aa, overall negatively charged in clone D (8 Asp and Glu versus 3 Arg and Lys). These 80 aa harbor a stretch of 42 aa with a clear homology to another bZIP protein, fra2, at the identical position in the leucine zipper (14/42 aa identical plus nine conservative exchanges).

Following our initial hypothesis that these Nrf1 variants are the basis for the observed differences at the TNFα promoter, we determined the expression levels of clone D (and related isoforms) and chose for comparison the complete Nrf1 sequence (clone G) as well as the recently described splice variant deleting the serine-rich region (clone 10). Corresponding mimics were constructed for all three isoforms (Fig. 5A) and quantitative RT–PCR analyses were performed (Fig. 5B, top panel). Clearly visible is that in mast cells, clone G is the most abundantly expressed variant, followed by clone 10 and the rarely expressed clone D. The relative expression ratios from this type of analysis can be calculated as 100:10:1 for clone G:clone 10:clone D. This ratio did not change if mast cells were stimulated by IgE + Ag for up to 4 h (time points tested 15, 30, 60, 120 and 240 min). To our surprise, these relative ratios of expression varied greatly between 6 cell lines (EL-4, Wehi, RBL1, DC18, L929 and 3T3) and three murine tissues (heart, testis and lung; V.Novotny, data not shown). Especially for dendritic cells (DC18) a pattern was established, resulting in a relative ratio (compared to mast cells)

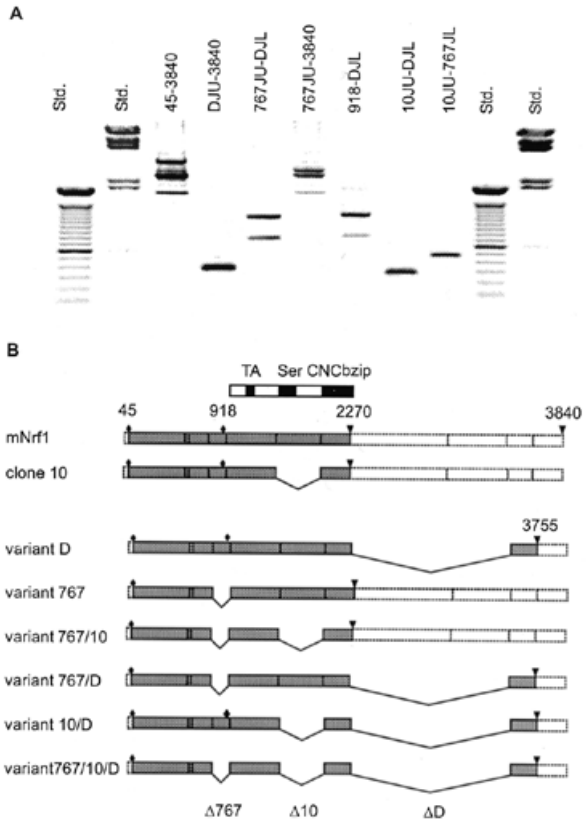

Figure 4. Different Nrf1 variants are expressed in CPII mast cells. (**A**) Agarose gel of RT–PCR analysis products with primers indicated (numbers give the position in the murine Nrf1 cDNA; U, upper strand; L, lower strand; J, junction oligonucleotide). Standards are 100 bp ladder and λ-*Hin*dIII fragments. (**B**) Scheme of the different possible Nrf1 variants found in CPII cells. Shaded boxes show the coding regions, start codons are indicated by diamonds, triangles indicate the stop codons; positions are given above. The transactivation domain (TA), the serine-rich region (Ser), the CNC-homology region (CNC) and the basic zipper region (bzip) are schematically indicated above.

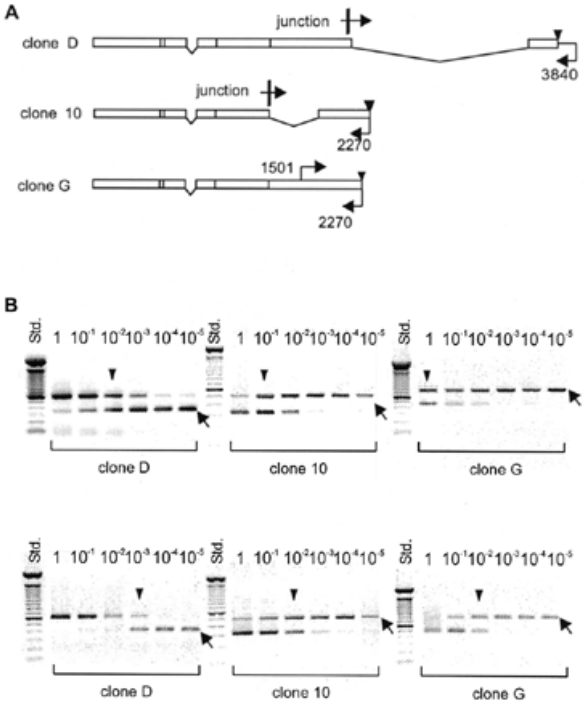

Figure 5. Different abundance of Nrf1 clone G (full-length mNrf1), clone 10, and clone D in CPII mast cells and DC18 dendritic cells. (**A**) Scheme of the mimic construction with the gene specific primers used for amplification in the quantitative RT–PCR. Stop codons are indicated by a triangle. (**B**) Top: agarose gels of the quantitative RT–PCR from mast cells, amount of mimic used (amol/μl) indicated at the top. Arrows show the specific Nrf1 products. The standard used is a 100 bp ladder. Bottom: same analysis as above for dendritic cells.

of 1:1:0.1 for clone G:clone 10:clone D (Fig. 5B, bottom panel; Discussion).

Due to the novelty of clone D, we used two strategies to investigate an involvement of this isoform in the TNFα regulation in mast cells. First, we applied a eukaryotic full-length expression clone (D) in transient transfections together with TNFα reporter gene constructs assuming that the levels of this transcription factor might be limiting and that overexpression would result in a further induction of TNFα. This strategy generally works for transcription factors which become upregulated in the process of cell activation. Second, we used a truncated version of clone D containing only the binding region and the leucine zipper domain in such transient transfections which should serve as a kind of transdominant negative mutation due to the lack of other (transactivation) domains. In this case, an abrogation of induction of TNFα should be observed, if clone D can interact with the AP1 complex (for constructs see Fig. 6A). Both constructs were checked in an *in vitro* transcription/translation system for their function, resulting in 110 and 25 kDa proteins, respectively (Fig. 6B). The truncated form uses for translational initiation presumably an internal AUG codon at position 1686–1688. As expected from the low but constant expression pattern, overexpression of a full-length clone D had no further stimulatory effect on TNFα induction. However, the 'transdominant negative' form, when overexpressed, abrogated TNFα induction, strongly indicating that it negatively interferes with the assembly and function of the active transcription factor complex at the TNFα promoter (Fig. 6C, Discussion).

DISCUSSION

Recently, two groups have generated Nrf1 knockout mice by targeted deletion of most of the coding region of Nrf1 or by disruption of the last exon alone (10,11). Surprisingly, there were fundamental phenotypic differences between the Nrf1$^{-/-}$ knockout mice generated via these two strategies. In the first case, development was arrested before day 7.5 in gastrulation and no mesoderm was formed with death *in utero* (11). In the second case, a much milder phenotypic alteration was observed with signs of anemia as a result of abnormal fetal liver erythropoiesis and animals dying at mid to late gestation (starting at day 12.5; 10). While this discrepancy is not solved, our isolation of multiple spliced isoforms of Nrf1 in the murine system suggests that a less dramatic gene disruption strategy, as taken by Chan and co-workers, might still result in the expression of certain isoforms in the knockout mice (10). This hypothesis is supported by the finding that the antibody they used to demonstrate the absence of Nrf1 expression in Nrf$^{-/-}$ mice was directed against a peptide deleted in the splice variants of clone 10, clone 767/10, clone 10/D and clone 767/10/D. In addition, as we have shown by *in vitro* transcription/translation and western blotting of clone D, a second commercially available Nrf1 specific antibody directed against a C-terminal peptide of Nrf1 does not recognize the protein of this variant and, as a logical consequence, also of clone

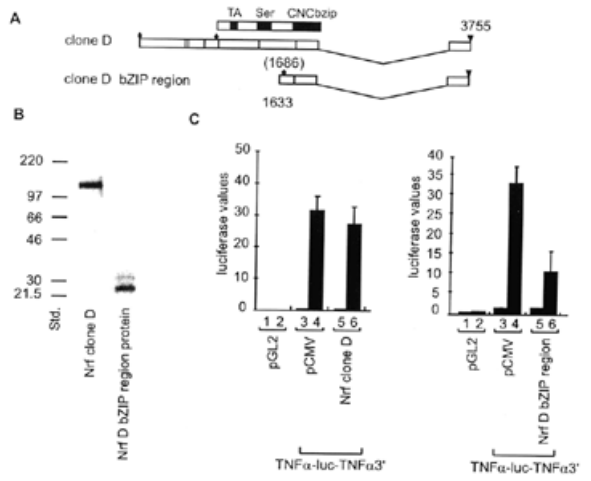

Figure 6. The clone D bZIP region interferes with the inducibility of the TNFα promoter in mast cells. (**A**) Scheme, showing the structure of the two eukaryotic expression constructs of clone D used. Position of the start codons indicated by diamonds, the number in brackets gives the position of the first aa of the assumed start codon. Triangles mark the position of the stop codons. The position of the transactivating domain (TA), the serine-rich region (Ser), the CNC domain (CNC) and the bZIP region within the proteins are indicated on top. (**B**) SDS–PAGE gel of an *in vitro* transcription and translation of the full-length Nrf clone D and the Nrf D bZIP region protein, using [^{35}S]methionine. (**C**) Transient transfections of the CPII mast cell line with the indicated plasmids and a TNFα reporter gene construct or pGL2 as the parental vector. Luciferase values are given to the left; uneven lane numbers indicate non-induced values, even lanes show luciferase values after 4 h stimulation with IgE + Ag. All experiments were done in triplicate, standard deviations are indicated.

767/D, clone 10/D and clone 767/10/D. This results in a situation where a number of Nrf1 isoforms, exemplified by our two variants 10/D and 767/10/D, have not and could not have been investigated at protein level so far, due to the lack of suitable reagents.

All CNC-bZIP proteins tested so far, including Nrf1, form homo- and heterodimers. Heterodimerization is described with nuclear hormone receptors (26) and preferentially with the family of small Maf proteins (MafK p18, MafF and MafG; 27). Our finding of complexes composed of CNC-bZIP proteins and classical AP1 factors involved together in the activation of a gene adds another level of complexity to the combinatorial possibilities of these transcription factors. The abrogation of TNFα induction, if only the leucine zipper region of the new variant is overexpressed, implies that this variant indeed does interact with the assembling of the activating AP1 complex. The concomitant loss of NF-AT, when Nrf1 is inhibited by DRB as well as the loss of Nrf1, when NF-AT is inhibited by FK506, however, suggests that Nrf1 is more dependent on NF-AT binding than on the other classical AP1 factors. This is supported by the absence of Nrf1 in the complexes generated at the consensus NF-E2 probe and the consensus AP1 probe.

Nrf1 (clone G) alone shows only a limited sequence specificity and upon heterodimerization a dramatic increase in DNA binding is observed (12). The finding of Nrf1 isoforms with an altered basic leucine zipper region suggests that the variations in the protein–protein interaction motifs of this factor might be used for heterodimerization with various partners. In addition, the different ratios of the Nrf1 splice variants in the various cell types would result in homo- and heterodimer formation with different participation of the various isoforms. The relatively low expression of clone G in dendritic cells, as estimated from the PCR data, thereby is the most striking difference between mast cells and dendritic cells. In this respect Nrf1 comprises a system that is reminiscent of NFκB p105, which is also differently spliced into p50 and p52 with various effects as homo- and heterodimers in the transcription machinery. This would suggest that the level of Nrf1 isoforms and the availability of adequate cofactors (in mast cells AP1 components and NF-AT) determine the site specificity and the potential to activate. This provides a basis to understand why Nrf1 can bind without a requirement for the full AP1 site in dendritic cells, while it shows such a restriction in mast cells.

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