Nuclear Factor κ B Activates Proenkephalin Transcription in T Lymphocytes

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Upon activation, T lymphocytes accumulate high levels of the neuropeptide enkephalin which correlate with high levels of proenkephalin mRNA in the cells. Here we investigated the transcriptional basis for these changes. The proenkephalin promoter contains a sequence GGGGACGTCCCC, named B2, which is similar to the κ B sequence GGGGACTTTCC, the binding site of the transcription factor nuclear factor (NF)- κ B. Activation of T lymphocytes induces an NF- κ B-like binding activity to the B2 site, concomitant with activation of the proenkephalin promoter. Mutations at the B2 site abolish this transcriptional activation. The purified homodimer (two p50s) of the DNA-binding subunit of NF- κ B binds the B2 site of proenkephalin relatively better than does the heterotetramer (two p65s plus two p50s) form of the factor. Thus, it appears that the T-cell-specific activation of the proenkephalin promoter is mediated by NF- κ B. However, as NF- κ B is ubiquitous and the transcriptional activation through the B2 site is T cell specific, yet another T-cell-specific factor which synergizes with NF- κ B should be considered.

Proenkephalin is a precursor for neuropeptides with a variety of functions in the neuroendocrine and nervous systems (1). Upon activation, T-helper lymphocytes were found to express high levels of proenkephalin mRNA and to secrete large amounts of the met-enkephalin neuropeptide, perhaps indicating an axis by which the immune and nervous systems interact (33).

Nuclear factor (NF)- κ B is a transcription factor originally found to be expressed in mature B lymphocytes, in which it enhances the expression of the immunoglobulin κ light-chain gene (28). Subsequently, NF- κ B was found to be present in an inactive form in many cell types in which, upon a posttranslational activation step mediated by protein kinase C (8, 19, 21, 22, 24, 29) or various other treatments (17, 23, 30), it becomes active. The biochemical basis for the activation of NF- κ B was shown to involve dissociation of an inhibitory subunit, termed I κ B, from the DNA-binding subunit(s) (3, 4). Mitogenic stimulation of T lymphocytes also converts inactive NF- κ B into its active form, which in turn induces the expression of κ B sequence-containing genes such as human immunodeficiency virus (21), interleukin-2 (IL-2) (14), and the α subunit of the IL-2 receptor (7).

The rat proenkephalin promoter region contains two sequences (B1 and B2, shown in Fig. 2a) that are similar to κ B, the binding site of NF- κ B. In this study, we show that NF- κ B can bind to the proenkephalin promoter (at the B2 site) and that this binding is the cause for the expression of proenkephalin in activated T lymphocytes.

MATERIALS AND METHODS

Protein extracts and EMSA. Whole-cell protein extracts were prepared as described previously (16). Briefly, 10-ml (2×10^6 to 4×10^6 cells per ml) cultures were washed once with phosphate-buffered saline and resuspended in 0.5 ml of 20 mM HEPES (N-2-hydroxypiperazine-N'-2-ethanesulfonic acid) (pH 7.9)-0.35 M NaCl-20% glycerol-1 mM

MgCl₂-1% Nonidet P-40-1 mM dithiothreitol-0.5 mM EDTA-0.1 mM EGTA containing the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 μ g of pepstatin A per ml, 10 μ g of leupeptin per ml, 0.1 mM *p*-aminobenzamidine, and 1 mg of aprotinin per ml. After 10 min of incubation on ice, the extract was centrifuged in an Eppendorf minicentrifuge for 15 min at 4°C and the supernatant was collected, frozen in liquid N₂, and kept at -130°C until thawed for the electrophoretic mobility shift assay (EMSA). For the EMSA, 6 μ l of protein extract (~20 μ g of protein) was incubated for 20 to 30 min at room temperature in 30 μ l of 10 mM Tris (pH 7.5)-20 mM KCl-1 mM EDTA-1 mM β -mercaptoethanol-4% glycerol-4 μ g of poly(dI-dC)-20,000 to 50,000 Cerenkov counts per minute (0.5 ng) of radioactively labeled DNA probe. An equal volume of loading buffer (16) was added to the binding reaction mixture before electrophoresis through a 4% native polyacrylamide gel (16).

Purification of NF- κ B and its assay by EMSA was as described previously (31, 32).

Plasmids and DNA fragments. The following DNA fragments were used: κ B, the *Xba*I-*Eco*RI fragment containing the κ B oligonucleotide subcloned into the *Bam*HI site of pUC18 (16); B1 and mB1, the *Xmn*I-*Ava*I fragments containing the relevant sequences of pEPCAT(wt) and pEPCAT (mB1B2), respectively (see Fig. 2a); B2 and mB2, the *Ava*I-*Pst*I fragments containing the relevant sequences of the same plasmids; κ -2, a fragment of the κ light-chain gene enhancer, which was prepared as described previously (28). In the experiments shown in Fig. 3, the B2 fragment was the oligonucleotide AGGGGACGTCCCCCTTAGCA cloned into the *Pst*I site of pGEM-2.

For construction of the proenkephalin promoter-*cat* gene reporter plasmid, the *Xba*I-*Sac*I fragment spanning nucleotides -501 to +51 relative to the rat proenkephalin gene cap site (26) was subcloned into pGEM-2. Mutations were introduced by the oligonucleotide-directed gap heteroduplex technique (20). The bacterial *cat* gene was subsequently inserted at the *Sac*I site downstream of the cap site of either

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FIG. 1. B2 site of the proenkephalin promoter is recognized by an NF- κ B-like protein. (a) Whole-cell extracts from ConA-PMA-activated (lanes 1, 3, 4, 6, 7, and 9) and nontreated control (lanes 2, 5, 8, and 10) Jurkat T cells were reacted with labeled DNA fragments containing the κ B sequence (lanes 1 and 2), the proenkephalin B2 site (lanes 3 to 5), the mutated B2 site (mB2) (lanes 6 to 8), and the κ -2 fragment (lanes 9 and 10), which contains the recognition site of the ubiquitous factor NF- μ E3 and was used to control for the amount of protein in the two types of extracts. Lanes 4 and 7 present reactions in which an excess (50 ng) of unlabeled κ B fragment (see below) was included in the reaction. Jurkat cells were treated for 24 h with PMA (100 nM, Sigma) and ConA (Sigma type IV, 50 μ g/ml), after which protein extracts were prepared from them as described in Materials and Methods. (b) EMSA with a whole-cell extract from S194 plasmacytoma cells. The extract was reacted with the κ B probe (lane 1), the B1 probe without (lane 2) or with (lane 3) an excess (50 ng) of unlabeled κ B fragment, and the B2 probe (lane 4) (see Materials and Methods). The lower basal levels of NF- κ B* (see text) in lane 2 (panel a) compared with those in lane 5 could be due to lower specific activity of the κ B probe in this experiment compared with that of the B2 probe. It also reflects the fact that the affinity of NF- κ B* for the B2 site is somewhat higher than that for the κ B site (see, for example, Fig. 3a).

the wild-type or the mutated proenkephalin promoter (see Fig. 2a).

Transfections. Jurkat cells were transiently transfected by the DEAE-dextran protocol (13). Twenty hours after transfection, each culture was split into two, and one aliquot was treated with concanavalin A (ConA) plus phorbol myristate acetate (PMA) (50 μ g/ml and 100 nM, respectively). Twenty hours later, the cells were harvested and assayed for chloramphenicol acetyltransferase activity (12). Incubation was for 4 h at 37°C followed by a 3- to 16-h exposure of the thin-layer chromatography plate to X-ray film.

RESULTS

NF- κ B-like protein binds B2 site of proenkephalin promoter. The T-cell line Jurkat was activated by a combination of PMA and the lectin ConA. Such treatments were reported (7, 14, 21) and were also shown here to activate NF- κ B DNA-binding activity as reflected by binding to the κ B sequence in the EMSA (9, 10) (Fig. 1a, compare lanes 1 and 2). The level of expression of a constitutive transcription factor, NF- μ E3, was not affected by the ConA plus PMA treatments (Fig. 1a, lanes 9 and 10).

The proenkephalin promoter contains two sites which are similar but not identical to the κ B sequence (16) (B1 and B2, Fig. 2a). We tested whether NF- κ B can recognize these sites of the proenkephalin promoter. An extract of the Jurkat T-cell line was reacted in an EMSA with the B1 or the B2 sequence of the rat proenkephalin promoter. A ConA-PMA-inducible NF- κ B-like activity was shown to bind specifically

to the B2 site (Fig. 1a, lanes 3 to 5) but not to the B1 site (data not shown). An extract of the plasmacytoma S194, which expresses high constitutive levels of NF- κ B (2), was also reacted with the B1 and B2 sequences and shown to bind only to the latter (Fig. 1b).

The B2 site is important for proenkephalin transcription in activated T cells. The observations that an NF- κ B-like activity recognizes the B2 site at the proenkephalin promoter (Fig. 1) and that similar activation treatments of helper T cells induce NF- κ B DNA-binding activity (Fig. 1a) and elevate the level of proenkephalin mRNA (33) raised the possibility that activation of NF- κ B is the cause for the expression of proenkephalin in activated T cells. To address this, we first constructed a plasmid containing the rat proenkephalin promoter in front of a reporter chloramphenicol acetyltransferase *cat* gene [see pEPCAT(wt) in Fig. 2a]. The plasmid was transfected into Jurkat cells, which were then treated with various concentrations of ConA plus PMA. Such treatments not only induced NF- κ B DNA-binding activity (Fig. 1a, lanes 1 to 5) but also produced significant transcriptional activations of the proenkephalin promoter (Fig. 2b).

Next, to determine whether the observed transcriptional activation is due to the action of NF- κ B, we mutated the B1 and B2 sites of the proenkephalin promoter. These mutations (marked mB1 and mB2 in Fig. 2a) abolished binding of the NF- κ B-like factor to the B2 site (Fig. 1a, compare lanes 3 to 5 with lanes 6 to 8). Because no NF- κ B-like binding was observed to the B1 site, mutating it did not affect the pattern of DNA binding (data not shown).

FIG. 2. Transcription activity of the proenkephalin promoter in Jurkat T cells. (a) Proenkephalin promoter-*cat* constructs used for transfections. The proenkephalin promoter (27) is attached to the *cat* gene through a *SacI* site [pEPCAT(wt)]. The B1 and B2 binding sites within the promoter are indicated. The mutations introduced into these sites are shown below the wild-type sequence (mB1 and mB2; × denotes an A base in the B2 site that was deleted during mutagenesis). Three combinations of the mutated sites were constructed: pEPCAT(mB1) with a mutated B1 (mB1) site and a wild-type B2 site, pEPCAT(mB2) containing a wild-type B1 site and a mutated B2 (mB2) site, and pEPCAT(mB1B2), in which both sites are mutated. (b) Proenkephalin promoter-mediated transcription in ConA-PMA-activated Jurkat T cells. Jurkat cells were transfected with pEPCAT(wt) and 24 h later treated for 20 h or not treated (lane 5) with PMA (100 nM) (lanes 1 to 3) and the indicated concentrations of ConA (micrograms per milliliter) (lanes 2 to 4). (c) Effects of mutating the B1 and B2 sites on proenkephalin promoter function in Jurkat T cells. Jurkat cells were transfected with the various proenkephalin promoter-*cat* gene constructs indicated in panel a: pEPCAT(wt) (lanes 1 and 2), pEPCAT(mB1B2) (lanes 3 and 4), pEPCAT(mB1) (lanes 5 and 6), and pEPCAT(mB2) (lanes 7 and 8). Even-numbered cultures were activated with ConA plus PMA. The basal activity of the (unstimulated) wild-type (wt) promoter appears lower in this experiment than in the one presented in panel b. This is due to a shorter exposure of the thin-layer chromatography plate to film in this experiment compared with the one in panel b.

The mutated B1 and B2 sequences were then introduced into the proenkephalin promoter-*cat* constructs (Fig. 2a) and transfected into Jurkat cells. The B1 mutation (mB1) had no significant effect on the ability of a ConA plus PMA treatment to activate the proenkephalin promoter in these T cells (Fig. 2c, compare lanes 1 and 2 with lanes 5 and 6). This result is consistent with the inability of the NF- κ B-like activity to bind to the B1 site (Fig. 1b). A construct containing the two mutated sites [pEPCAT(mB1B2)] and one which consists of a combination of the B2 mutant with wild-type B1 [pEPCAT(mB2)] exhibited reduced basal levels (data not shown) but most prominently an almost complete abolishment of the ConA-PMA-induced activity of the proenkephalin promoter (Fig. 2c, compare lanes 1 and 2 with lanes 3 and

4 and 7 and 8). It therefore appears that the native B2 site is essential for the inducibility of the proenkephalin promoter by the T-cell activators ConA and PMA.

Homodimeric form of NF- κ B is the major binder to the B2 site. The EMSA size of the NF- κ B-like complex which bound the B2 site (GGGGACGTCCCC) of the proenkephalin promoter appeared smaller than the complex formed with the canonical κ B sequence (GGGGACTTTC). This was true both for extracts from the ConA-PMA-induced Jurkat cells (Fig. 1a, compare lane 3 with lane 1) and for those prepared from the plasmacytoma S194, expressing constitutively active NF- κ B (Fig. 1b, lanes 1 and 4). To determine whether these size differences are significant, we first ran higher-resolution EMSA gels. Both in the Jurkat (data not

FIG. 3. Smaller form of NF- κ B (NF- κ B*) binds the proenkephalin B2 and IL-2 κ B-like sites. (a) Binding of S194 extract to the canonical κ B site and to the B2 site. The same extract was reacted in an EMSA with the κ B probe (lanes 1 to 3) or the B2 probe (lanes 4 to 6) in the presence of the indicated GTP concentrations. In this experiment, a 6% Tris-glycine gel was used. (b) Binding of S194 extract to the IL-2 κ B-like site. The extract was reacted in an EMSA with an end-labeled DNA fragment containing the IL-2 κ B-like site (sequence GGGATTTCACC; kindly provided by M. Karin). The binding was blocked either by the κ B sequence (20 ng; lane 2) or by the B2 sequence (20 ng of the *Ava*I-*Hind*III fragment from the plasmid described in Materials and Methods; lane 3). In this experiment, the native gel was 5% in Tris-borate buffer.

shown) and in the S194 (Fig. 3a) extracts, two DNA-protein complexes were observed to bind to the κ B sequence. Only the lower complex, marked NF- κ B*, showed strong binding to the proenkephalin B2 sequence (Fig. 3a, lanes 4 to 6). In agreement with the GTP-activatable nature of NF- κ B (4, 18), both NF- κ B and NF- κ B* were activated by GTP treatment, although NF- κ B* appeared to require higher GTP concentrations (Fig. 3a).

The DNA-binding activity of NF- κ B appears in two forms (4). The major and larger one is a heterotetramer consisting of two p65 and two p50 subunits (4); this form is sensitive to the action of the inhibitory subunit I κ B (4, 31). The second form appears to be a dissociation product of the larger complex and is a smaller homodimeric form consisting of only two DNA-binding subunits (two p50 subunits). NF- κ B*, which preferentially binds the B2 site, appeared to migrate in the EMSA to the same position as does the homodimeric form of NF- κ B. We therefore tested whether NF- κ B* is actually the homodimer of the p50 subunit of NF- κ B. Purified preparations of NF- κ B in either the heterotetrameric or the homodimeric form were reacted in the EMSA with the B2 sequence of the proenkephalin promoter. Both forms of NF- κ B recognized the B2 sequence (Fig. 4). However, when normalized to the levels of binding obtained for each form with the canonical κ B sequence (Fig. 4, lanes 1 and 5), it appeared that the homodimer binds the B2 site at least five times better than does the heterotetramer (Fig. 4,

compare the signal in lanes 1 to 3 with that in lanes 5 to 7). This result, obtained with purified proteins, correlates with the observation that in crude extracts (Fig. 3a) only NF- κ B* exhibits high levels of binding to the B2 site.

DISCUSSION

This study shows that the expression of proenkephalin in activated T cells (26, 33) is, to a large extent, the result of transcriptional activation caused by interaction of the transcription factor NF- κ B with the B2 site of the proenkephalin promoter. The induction of NF- κ B DNA-binding activity by ConA plus PMA paralleled the activation of the proenkephalin promoter (Fig. 1a, lanes 1 to 5, and Fig. 2b). Moreover, point mutations (Fig. 2a) that abolished the ability of NF- κ B to recognize the B2 site (Fig. 1a, lanes 6 to 8) rendered the proenkephalin promoter unresponsive to the T-cell activators ConA and PMA (Fig. 2c). The B2 site of the proenkephalin promoter can therefore be considered an additional, hitherto unrecognized (5, 6), regulatory element of this promoter. Its present characterization resolves the transcriptional basis for the enigmatic appearance of high levels of enkephalin in activated T lymphocytes (26, 33).

The NF- κ B-like activity (marked also NF- κ B*) that binds the B2 sequence appeared to migrate differently in the EMSA than did the complex that binds the canonical κ B sequence (Fig. 1 and 3a). The size difference of the com-

FIG. 4. Binding of purified NF- κ B to the proenkephalin B2 site. NF- κ B preparations which were purified as described previously (31, 32) were reacted in EMSA with either the κ B (lanes 1, 2, 5, and 6) or the B2 (lanes 3, 4, 7, and 8)-containing DNA fragment in the presence of unlabeled κ B competitor (lanes 2 and 6) or B2 competitor (lanes 4 and 8). Competitors were as described in the legend to Fig. 3b. In lanes 1 to 4, the larger heterotetramer (two p50 subunits plus two p65 subunits) of NF- κ B (arrowhead) was used (the lower band is probably a proteolytic product of the factor [4]). In lanes 5 to 8, the smaller homodimeric (two p50 subunits) form of the factor was used.

plexes can be attributed to the fact that the major activity binding to the proenkephalin B2 site is the faster-migrating homodimeric form of NF- κ B, whereas the κ B sequence is bound better by the slower-migrating heterotetrameric form of NF- κ B (Fig. 4). That indeed NF- κ B* is the homodimer of the p50 subunit of NF- κ B is supported by the following: (i) its coinduction with NF- κ B (Fig. 1a, lanes 1 to 5); (ii) the GTP-activatable nature of its DNA binding (Fig. 3a), characteristic of both forms of NF- κ B (4, 17); and (iii) most importantly, a comparison of its properties with those of the purified p50 homodimer (Fig. 4)—both proteins had similar electrophoretic mobilities in the EMSA, i.e., slightly faster than the heterotetrameric form of the factor (Fig. 3a and 4), and both NF- κ B* and the p50 homodimer had the same DNA-binding preferences. The two proteins bound the B2 site of proenkephalin as well as, if not better than, the canonical κ B sequence (Fig. 3a and 4). This is in sharp contrast to the heterotetrameric form of NF- κ B, which had at least fivefold higher affinity for the κ B site (Fig. 3a and 4). All cells that express significant levels of NF- κ B also express the p50 homodimer of the factor (4, 31). It is likely therefore that NF- κ B*, the major NF- κ B-like DNA-binding activity to the B2 site of the proenkephalin promoter, is the p50

homodimer of NF- κ B. Notably, recent cDNA cloning experiments have shown the p50 homodimer to probably be identical to the transcription factor KBF1 (11, 15). Thus, NF- κ B*, KBF1, and the p50 homodimer of NF- κ B appear to be the same entity, but whether additional (mainly posttranslational) modifications differentiate among these three proteins remains to be seen.

The B2 site, through which NF- κ B activates proenkephalin transcription in T lymphocytes, as well as the B1 site, which is not recognized by NF- κ B, are also binding sites for BETA, a brain-specific transcription activator expressed only in brain cells (16). But, curiously, in primary cultures of cerebellar neurons that express BETA, the mutations at the B1 and B2 sites did not have a marked effect on proenkephalin promoter function (data not shown). This suggests that although NF- κ B and BETA can both bind to the B2 site, they differ in their transcriptional activation capacity with regard to the proenkephalin promoter.

Furthermore, NF- κ B in both its forms is present in many cell types other than T cells, for example, the plasmacytoma S194 (Fig. 3). However, transfection of the proenkephalin promoter-*cat* construct into S194 cells that were either treated or not with ConA plus PMA did not result in transcriptional activation (data not shown). In addition, the mutation at the B2 site did not have any effect on the activity of the promoter in cells other than T lymphocytes. It thus appears that the action of NF- κ B in the context of the proenkephalin promoter is specific for activated T cells. A similar situation exists for the promoter of the lymphokine IL-2, which is activated through a κ B-like regulatory sequence only in activated T cells (25).

Notably, both in the proenkephalin (this study) and in the IL-2 promoter (14a), the major DNA-binding activity to the κ B-like sites was the homodimeric form of NF- κ B (Fig. 3a and 4). Consistent with this was a competition experiment showing that the IL-2 κ B-like sequence and the B2 site of proenkephalin are bound by the same protein (Fig. 3b). However, as both sequences were also recognized, albeit with lower affinity (Fig. 4), by the heterotetrameric form of NF- κ B, it is difficult to determine which of the two forms of the factor is responsible for the T-cell-specific activation of these two promoters. But the ubiquity of NF- κ B, as opposed to the T-cell specificity of the κ B-like site-mediated proenkephalin and IL-2 promoter function, argues that there might be yet another T-cell-specific factor which participates with (or modulates) NF- κ B to activate these promoters. One possibility is that the "naked" homodimeric form of NF- κ B that binds tighter to the B2 site of the proenkephalin promoter is the target for such a T-cell-specific interaction.

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