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Nuclear Factor of Activated T Cells Is Associated with a Mast Cell Interleukin 4 Transcription Complex

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Interleukin 4 (IL-4), an immunoregulatory cytokine, is produced only by a subset of activated T cells and cells of the mast cell-basophil lineage. The production of IL-4 by mast cells likely represents a significant source of this protein in local immune-inflammatory responses in the skin, brain, gastrointestinal, and respiratory tracts, in which mast cells are prevalent. In the present study, the *cis*- and *trans*-acting elements that control inducible mast cell IL-4 gene transcription were examined and compared with those that function in T cells. We demonstrate that, as in T cells, sequences between bp −87 and −70 are critical for protein association and activation-dependent gene transcription and that this region (termed the activation-responsive element region) is the target of an inducible, cyclosporin A-sensitive, DNA-protein interaction. When assessed by electrophoretic mobility shift assays and UV cross-linking analyses, multiple proteins in both T- and mast cell nuclear extracts associate with the activation-responsive element in vitro, and some of these appear identical. However, distinct proteins are associated with each of the complexes as well. AP-1 family members are unique to the T-cell-stimulation-dependent complex, whereas mast cell complexes contain factors that are reactive with anti-nuclear factor of activated T cells p (NF-ATp) and anti-NF-ATc antibodies but have distinct molecular masses compared with those of T-cell-derived NF-AT. Furthermore, an anti-NF-ATp-reactive factor with a molecular mass of ~41 kDa is present in the nuclei of unstimulated cells and binds independently of cell activation, unlike the previously described NF-AT family members. These data support the idea that there are uniquely regulated, cell lineage-specific transcription factors related to T-cell-derived NF-AT that mediate inducible IL-4 transcription in mast cells. These differences likely reflect the distinct cell surface signalling requirements for IL-4 production in T and mast cells.

Interleukin 4 (IL-4) is a pleiotropic cytokine that exerts its diverse effects on a wide variety of target cells of hematopoietic and nonhematopoietic lineages (see references 3 and 29 for reviews). Originally defined as a B-cell growth and stimulatory factor, IL-4 is a critical immunoregulatory molecule. It influences the development and growth of distinct subsets of $CD4^+$ T helper cells that, in turn, determine the outcome of some infectious diseases (10, 16, 34, 35). It plays an important role in regulating the isotype of antibody produced by B cells and is essential for the production of immunoglobulin E (IgE). IL-4 also modulates the inflammatory response. This is due, in part, to its ability to affect adhesion molecule expression and cytokine production by endothelial cells and to influence the growth and/or activation states of neutrophils, mast cells, T cells, and eosinophils (3, 29).

The physiological production of IL-4 is tightly regulated; it has been detected only in a subset of activated T cells and cells of the mast cell-basophil lineage. The observation that IL-4 is produced by mast cells is particularly intriguing. Mast cells are best known for their role as the critical effector cells in IgE-dependent immediate-type hypersensitivity reactions (8, 11). In addition to mediators of allergic responses, it is well-established that activated mast cells are an important source of several other cytokines including IL-1, IL-3, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor alpha (5, 31, 41). They are widely distributed throughout the body and are located in direct proximity to many of the targets of cytokine action such as endothelial cells, bronchial smooth muscle cells, and connective tissue cells as well as recruited leukocytes in the skin, brain, and gastrointestinal and respiratory tracts. Mast cells remain fixed in tissues and can provide the high local concentration of cytokines that is critical in many immune responses. Thus, it has been proposed that mast cells, in part through their ability to produce cytokines such as IL-4, play a pivotal role in initiating and orchestrating both immediate-type hypersensitivity responses and late-phase inflammatory responses in these tissues (11). Mast cells are activated through cross-linkage of the high-affinity IgE receptor. On the basis of its ability to stimulate both IgE production and mast cell growth, IL-4 is likely a major regulator of mast cell function.

Many studies have focused on the signaling requirements that lead to expression of T-cell IL-4 (1, 2, 4, 7, 21, 23, 24, 26, 33, 36–38, 40). These investigations have provided information on some of the nuclear as well as early cytoplasmic changes that occur in response to cross-linkage of the T-cell antigen receptor and ultimately lead to an increase in IL-4 transcription. Several positive and negative *cis*-acting transcriptional regulatory elements in the 5′ region of the IL-4 gene have been defined. One sequence, between bp −88 and −60, regulates activation-dependent transcription of IL-4 in both murine and human T cells. It has been the termed the activation-respon-

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sive element (ARE), P, or the CS1 element by various groups (2, 4, 37). The ARE has significant sequence identity with the IL-2 nuclear factor of activated T cell (NF-AT) binding site that regulates the stimulation-dependent transcription of IL-2 (33–35). In fact, it is likely that the inducible ARE-protein interactions observed in activated T cells include some of the same components (or ones very similar to them) that mediate the IL-2 gene NF-AT transcriptional activity (33, 36, 38).

Recent evidence indicates that mast and T cells exhibit cell-specific differences in the regulatory elements that control IL-4 transcription. An enhancer in the second intron of the murine IL-4 gene functions only in IL-4-producing mast cell lines and is the target of transcription factors that are expressed in mast but not in T cells (13, 14). This difference may reflect the fact that mast cells are activated to produce cytokines through a different cell surface receptor than T cells and that cross-linkage of these two types of receptors generates distinct intracellular signals. A further comparison of the IL-4 transcriptional regulatory elements utilized by T and mast cells provides a model for examining cell-specific differences of a single gene in multiple cell types and may ultimately provide insight into more cell membrane-proximal signaling events in both cell types.

In this study, the contribution of the ARE to inducible mast cell IL-4 transcription was assessed. We demonstrate that as in T cells, the ARE mediates transcriptional activation in stimulated mast cells and is the target of an inducible DNA-protein interaction. Although several of the proteins associated with this element appear to be in common with those detected in T cells, there are also mast cell-specific factors. Some of these are related to the NF-AT family of transcription factors.

MATERIALS AND METHODS

Cells. CFTL 15 is a murine IL-3-dependent mast cell line derived from fetal liver and has been previously described (30). Low levels of IL-4 mRNA are expressed in unstimulated cells. Activation of these cells by cross-linkage of the high-affinity Fcε receptor (FcεRI) or treatment with the calcium ionophore ionomycin (1 μM) induces a significant increase in the amounts of IL-4 mRNA and protein (31). This increase is due in part to an increase in the level of transcriptional activity of the IL-4 gene (14). Cells were cultured in RPMI 1640 medium containing 10% bovine calf serum and penicillin-streptomycin and 25% WEHI-3 supernatant as a source of IL-3. Cyclosporin A (CSA) was used at a concentration of 200 ng/ml.

Transient transfections and CAT reporter gene assays. Most of the IL-4 chloramphenicol acetyltransferase (CAT) reporter gene constructs used in this study have been previously described (37). Constructs containing mutations within the ARE in the context of IL-4 5′ sequences were generated by in vitro mutagenesis by using either PCR-based methods or site-directed mutagenesis in M13 phage. Transfections and assays of CAT activity by a scintillation diffusion assay were performed as described elsewhere (37).

Nuclear extract preparation and EMSAs. Mast cell nuclear extracts in the presence of high concentrations of protease inhibitors were prepared by the protocol described by Dignam et al. (9). T-cell nuclear extracts were isolated by either the Dignam protocol or a modification of the procedure described by Fiering et al. as previously described (37). T-cell extracts prepared in either way gave identical results in electrophoretic gel mobility shift assays (EMSAs). Proteins were quantitated with the Bio-Rad protein assay kit (Bio-Rad, Richmond, Calif.).

EMSA binding reactions were performed in a total volume of 25 μl at room temperature for 45 to 60 min with 5 μg of nuclear protein and 0.1 ng of ^{32}P-labeled DNA (~20,000 to 40,000 cpm) in a buffer containing 10 mM Tris, KCl (10 mM with mast cell extracts; 50 mM with T cell extracts), 5 mM dithiothreitol, 1 mM EDTA, poly(dI-dC) (1.4 μg with mast cell extracts; 0.35 μg with T cell extracts), 0.1% Triton X-100, and 12.5% glycerol. Samples were electrophoresed on 5% polyacrylamide gels in a buffer consisting of 25 mM Tris, 190 mM glycine, and 1 mM EDTA. All binding reactions were performed under conditions of probe excess. To assess the association of NF-AT family members with the ARE in mast cells, EMSA binding reactions were performed in the presence of antibodies specific for human NF-ATc (28) and murine NF-ATp (27). The antibodies were added to the standard binding reaction mixture after 30 min of incubation. A monoclonal antibody that reacts with human brahma-related switch complex transcription factor, brg-1 (20), was used as a negative control in these experiments. These antibodies were the generous gift of G. Crabtree, Stanford University.

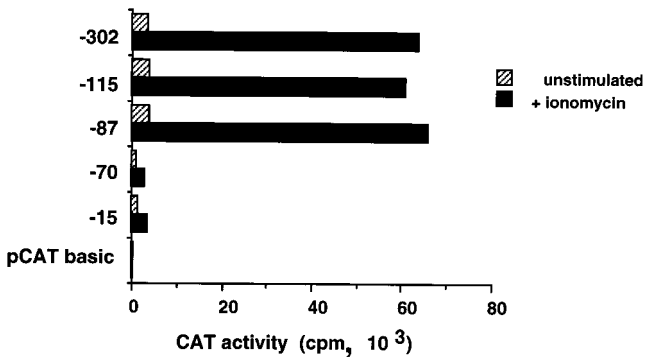

FIG. 1. Sequences between bp −87 and −70 relative to the transcription initiation site of the murine IL-4 gene mediate inducible CAT gene expression in stimulated mast cells. CFTL 15 mast cells were transfected with equimolar amounts of the indicated IL-4 CAT reporter gene constructs. After 18 h, half of the culture was stimulated with 1 μM ionomycin. CAT activity was measured on the following day. The results shown in this experiment are from at least five independent transfection experiments.

Oligonucleotides corresponding to the wild type and mutant forms of murine IL-4 ARE (−88 to −60) were synthesized on an Applied Bioscience Oligonucleotide synthesizer and are shown in Fig. 3B. The murine IL-2 upstream NF-AT sequence corresponds to sequences between −295 and −267 (32). The GATA binding site is derived from sequences within the second intron of the murine IL-4 gene that have previously been shown to have enhancer activity (13). Other oligonucleotides containing consensus AP-1 (5′CGCTTGATGAGTCAGCCG GAA) (22) and CTF (5′CCTTTGGCATGCTGCCAATATG) (6) binding sites were purchased from Promega, Inc. (Madison, Wis.).

UV cross-linking. An 80-μg amount of mast cell and T-cell nuclear extracts was incubated with 0.8 ng of a double-stranded bromodeoxyuridine-substituted ARE (one to five substitutions per strand) in a standard mobility shift assay mixture containing 11.2 μg (8.8 μg for T-cell reactions) of poly(dI-dC). The resulting DNA-protein complexes were resolved on a 5% native acrylamide gel as described above. The gel was then subjected to UV irradiation (250,000 μJ/cm^2) with a Stratalinker model 1800 apparatus (Stratagene, Inc.). After autoradiography, the DNA-protein complexes were isolated from the gel by electroelution, were concentrated, and were electrophoresed on a 5 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel.

Oligonucleotide affinity precipitation. Concatamers of the IL-4 5′ sequence corresponding to bp −88 to −60 (an average of 10-mers) were covalently attached to cyanogen bromide-activated Sepharose CL-4B. The DNA-Sepharose was equilibrated with EMSA binding buffer containing 5% glycerol. A 500-μg amount of crude nuclear extract [preincubated with 7 ng of poly(dI-dC) per μl] was incubated with 0.5 ml of the affinity matrix for 2 h at 4°C. The matrix was pelleted by centrifugation, and the unbound protein was collected in the supernatant. Bound protein was eluted by the addition of 1 M KCl (in binding buffer). NF-AT-related proteins in the unbound and bound fractions from both unstimulated and stimulated extracts were identified by Western blot (immunoblot) analysis.

Western blot analysis. Crude nuclear extracts (10 μg of T cells; 20 μg of mast cells) were electrophoresed on an SDS-8% PAGE gel and transferred to nitrocellulose by standard techniques. Murine anti-human NF-ATc ascites fluid (28) was added at a 1:2,500 dilution in 0.5% nonfat milk–0.5 M NaCl–0.1% Tween in phosphate-buffered saline. Rabbit antiserum reactive with murine NF-ATp (26) was added at a 1:1,000 dilution in 0.5% nonfat milk in TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.05% Tween 80). Appropriate secondary antibodies conjugated to alkaline phosphatase were used, and reactive proteins were visualized by treatment of the blot with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

IL-4 sequences between bp −87 and +5 are sufficient to promote inducible CAT transcription in stimulated mast cells. To examine the role of 5′ regulatory sequences in inducible mast cell IL-4 transcription, the activities of IL-4 CAT reporter gene constructs containing 5′ end deletions were assessed in transient transfection assays in CFTL 15 mast cells. As shown in Fig. 1, the constructs were inactive in unstimulated cells. Significant CAT activity was detected in stimulated cells trans-

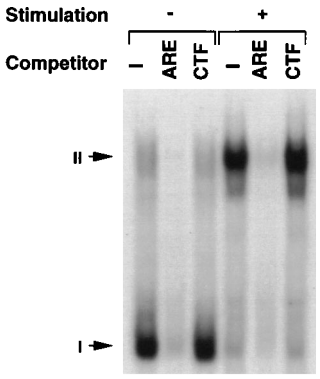

FIG. 2. The ARE is a specific target of both constitutive and inducible DNA binding proteins. EMSAs were performed with a ^{32}P-labeled probe corresponding to bp −88 to −60 of the murine IL-4 gene and nuclear extracts from unstimulated (−) and ionomycin stimulated (+) CFTL 15 mast cells. The specificities of the interactions were confirmed in unlabeled competition experiments in which a 100-fold molar excess of self (ARE) or unrelated (CTF) DNA was added to the binding reaction mixtures. Constitutive and inducible complexes are denoted by roman numerals. Free probe is not shown.

fected with constructs containing 302, 115, and 87 bp of upstream sequence. Deletion of sequences between bp −87 and −70 eliminated inducible CAT activity. These experiments demonstrate that, as in T cells, sequences between bp −87 and −70 are critical for activation-dependent gene transcription. The nucleotides spanning this region (bp −88 to −60) were designated ARE (37) and contain the previously defined P sequence (bp −79 to −69) (2, 36).

Mast cell nuclear proteins present in both unstimulated and stimulated cells specifically associate with the IL-4 ARE. To examine the nuclear proteins associated with this region of the IL-4 gene, an oligonucleotide probe (bp −88 to −60) spanning the critical region was used in EMSAs with nuclear extracts from unstimulated and stimulated mast cells (Fig. 2). Two complexes (complexes I and II) formed with the unstimulated extract. The amount of the lower-mobility complex (complex II) detected was dramatically increased upon stimulation. The specificities of these DNA-protein interactions were confirmed by the ability of a 100-fold molar excess of unlabeled ARE DNA, but not an unrelated oligonucleotide probe (CTF), to compete with the labeled probe for protein binding.

To define the nucleotides within the ARE that are critical for mast cell protein association, various base substitutions were introduced into the −88-to-−60 sequence (Fig. 3B). The abilities of these altered sequences to compete with the wild-type probe for binding of proteins in stimulated nuclear extracts were assessed in EMSAs. As shown in Fig. 3A, the addition of unlabeled oligonucleotides containing M1 and M6 had little effect on ARE complex formation. This result indicates that the nucleotides defined by these mutations are critical for protein association. In contrast, addition of M2, M3, M4, M5, and M7 to the binding reactions resulted in a significant reduction in signal, demonstrating an ability to compete with the wild-type probe. Titration experiments, in which increasing amounts of unlabeled mutant competitor (10- to 100-fold molar excess) were added to the binding reaction mixtures, revealed that some of these, notably M2 and M3, compete less well than the unsubstituted probe (not shown). Thus, the bases defined by these mutations are less important than M1 and M6 but do contribute to the in vitro association of mast cell proteins with the ARE. A similar pattern of competition was observed in experiments with unstimulated nuclear extracts, indicating that the constitutive binding activity requires the same ARE subsequence (not shown).

Functional analysis of the mutant IL-4 CAT constructs confirms the importance of nucleotides −76 to −68 in ARE-mediated CAT gene transcription. The effect of ARE mutations on the inducible CAT activity of −87-to-+5 IL-4 reporter gene constructs was assessed in transient transfection assays (Fig. 3C). With the exception of M5, a point mutation located at −78, all of the substitutions resulted in reduced CAT activity relative to that of the wild-type construct. The presence of M1 (with bp −76 to −71 altered) and M6 (with bp −70 and −69 altered) completely abolished inducible CAT activity, in keeping with their inability to bind protein. Constructs containing M2, M3, and M4 demonstrated diminished inducible CAT activity. Together, these data indicate that the nucleotides necessary for both protein association and transactivation function are located between −76 and −69. This is the precise region critical for inducible activity in T cells (7, 33, 36–38). Although the nucleotides defined by M7 did not have an effect on ARE

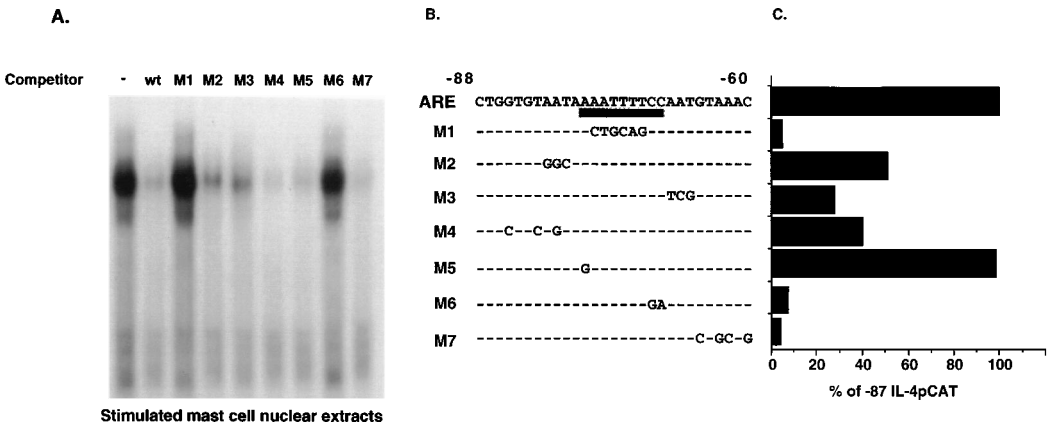

FIG. 3. Mutations within the −88-to-−60 sequence affect both ARE DNA-protein interactions and transactivation function. (A) Base substitutions within the ARE sequence affect the ability to compete with the wild-type probe for protein binding. A one-hundred-fold molar excess of the indicated oligonucleotides was added to standard binding reaction mixtures containing ^{32}P-labeled wild-type probe and nuclear extracts from stimulated CFTL 15 cells. (B) Specific mutations (M1 to M7) were generated within the context of the wild-type oligonucleotide from bp −88 to −60. (C) Equal amounts of IL-4 CAT (−87-to-+5) reporter gene plasmid containing the wild-type or mutated form of the ARE were transfected into CFTL 15 cells. Transient CAT production in stimulated cells was assayed as described in the legend to Fig. 1. CAT activities are expressed as percentages of that of the wild-type construct. These data from four independent transfection experiments.

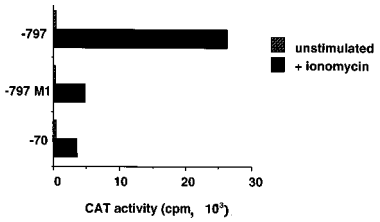

FIG. 4. ARE mutations within the context of bp −797 to +5 of the IL-4 5′ sequence abolish transient CAT activity in ionomycin-stimulated mast cells. Base substitutions defined by M1 (see Fig. 3B) were introduced into the −797-to-+5 IL-4 CAT construct (−797 M1). CAT activity from this mutated construct was assayed and compared with that of the wild-type construct (−797) in transiently transfected cells as described in the legend to Fig. 1.

complex formation in competition experiments (Fig. 3A), they profoundly decreased inducible CAT activity. This finding suggests that the nucleotides defined by this mutation contribute to inducible transcription, perhaps through involvement in other DNA-protein associations distinct from the ARE.

The M1 mutation was also introduced into an IL-4 CAT construct containing bp −797 to +5 of IL-4 sequence (−797 M1 IL-4 CAT) (Fig. 4). In transient transfection assays, this mutation reduced inducible CAT activity to the level observed in cells transfected with −70 IL-4 CAT. Although the −797-to-+5 sequence contains several other regions highly homologous to the ARE, termed P0, P2, P3 and P4 (36), these results

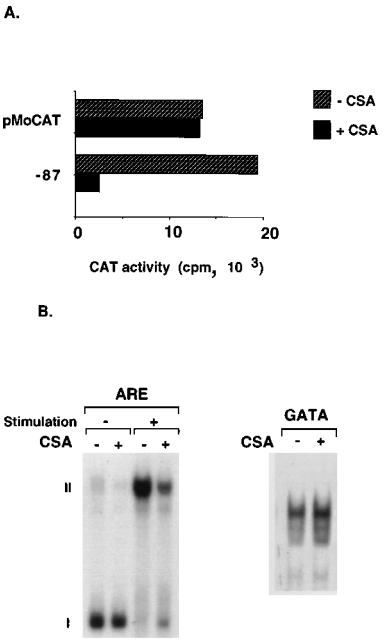

FIG. 5. The ARE is a specific target of CSA inhibition in mast cells. (A) CSA inhibits the activity of an IL-4 CAT construct containing the ARE. CFTL 15 cells were transfected with the indicated plasmids. After 18 h, cultures were divided in half and stimulated with either ionomycin (1 μM) or ionomycin plus CSA (100 μg/ml). CAT activity was measured 24 h poststimulation. These results are from four independent transfection experiments. A Moloney leukemia virus promoter-driven CAT vector was used as a positive control and is unaffected by CSA treatment. (B) CSA selectively inhibits the formation of IL-4 ARE-protein complexes. EMSAs were performed with an oligonucleotide corresponding to bp −88 to −60 of the murine IL-4 gene and nuclear extracts from unstimulated, ionomycin-treated, or ionomycin-CSA-treated cells. The stimulated extracts were used in EMSAs with a GATA transcription factor binding site probe. Specific IL-4 ARE complexes are denoted by arrows. The free probe is not shown.

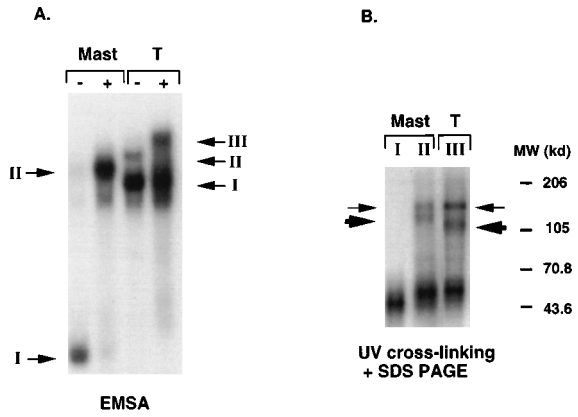

FIG. 6. Mast and T cells contain distinct ARE-binding proteins. (A) Mast cell and T-cell ARE-protein complexes have distinct mobilities. EMSAs were performed with unstimulated (−) and ionomycin-stimulated (+) CFTL 15 (mast) or PMA-stimulated EL-4 (T) cell nuclear extracts. Specific complexes are denoted by roman numerals. The free probe is not shown. (B) Some mast cell and T-cell proteins that directly associate with the ARE have distinct molecular masses. The mast cell complexes I and II and the T-cell activation-dependent complex III (see panel A) were subjected to UV cross-linking, excised from the EMSA gel, and electrophoresed on an SDS-PAGE gel. The migration of molecular mass standards is indicated on the right. The large arrows denote the species unique to each cell type.

indicate that the ARE is critical in the context of a larger segment of IL-4 sequence, further demonstrating the importance of the ARE in inducible CAT transcription in mast cells.

The ARE is a specific target of CSA inhibition of IL-4 gene transcription in mast cells. CSA inhibits the stimulation-dependent production of several mast cell cytokines including IL-4 (12, 19). In T cells, the ARE or P sequence is a specific target of CSA inhibition. CSA selectively represses both the inducible activity of ARE/CAT constructs and ARE-protein interactions (33, 37). Thus, we asked whether the ARE is a similar target of CSA inhibition in mast cells. As shown in Fig. 5A, CSA treatment of stimulated mast cells profoundly reduced inducible CAT activity in cells transfected with −87 IL-4 CAT. The activity of a murine leukemia virus CAT construct was unaffected by such treatment. This result indicates that the site of CSA inhibition in mast cells is also within 87 bp of the IL-4 transcription initiation site.

EMSAs were performed with nuclear extracts from CSA-treated mast cells to assess the effect of CSA treatment on ARE-protein interactions. As shown in Fig. 5B, CSA treatment of cells did not influence the formation of the constitutive ARE complex I. In contrast, the ability to form the inducible ARE complex II was dramatically reduced in extracts from stimulated, CSA-treated cells. The inhibition by CSA of ARE-protein interactions is selective; GATA binding activity in the same extracts was unaffected by CSA treatment. Together, these data demonstrate that the ARE and its associated proteins are a specific target of CSA inhibition in mast cells.

Cell-specific proteins associate with the ARE in mast and T cells. The observation that mast cells and T cells utilize the same IL-4 regulatory element for inducible IL-4 transcription suggests that the same or similar proteins mediate this response in both cell types. As a first step in comparing the nuclear factors associated with the ARE, EMSA were performed. As shown in Fig. 6A, the mobilities of both the constitutive and inducible mast cell complexes were distinct from those observed with T-cell nuclear extracts.

The components of these complexes were then analyzed by UV cross-linking and SDS-PAGE analysis, techniques that

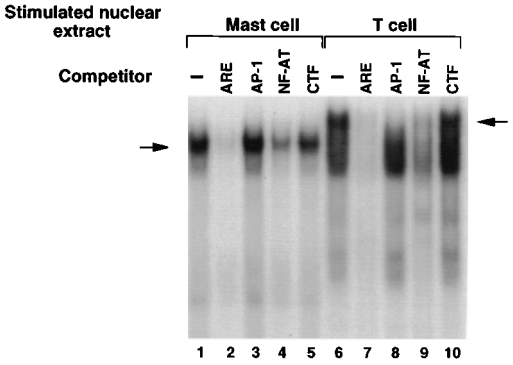

FIG. 7. AP-1 family members do not associate with the mast cell ARE DNA-protein complex. EMSAs were performed with stimulated nuclear extracts from mast and T cells and the ARE probe. Binding reaction mixtures contained no competitor (−) or a 100-fold molar excess of the indicated cold oligonucleotides. Specific complexes are indicated by the arrows.

evaluate proteins directly associated with the DNA. Our estimates of molecular mass by this technique included the contribution of the cross-linked 29-base oligonucleotide. Several proteins with apparent molecular masses of between 40 and 55 kDa appeared to be common to both the constitutive and inducible mast cell complexes (compare mast cell complexes I and II [Fig. 6B]). Complex II contains additional factors with molecular masses of ~135 and ~110 kDa indicating that these proteins form at least some of the inducible components of the mast cell ARE complex II.

A comparison of the inducible complexes from T and mast cells (complexes III versus II) also revealed similarities and differences. The ~135-kDa species and at least three proteins with molecular masses of between 40 and 55 kDa were observed in both the T-cell and mast cell complexes. However, unique cell-specific factors were also observed. A ~110-kDa protein is apparently mast cell-specific, and the ~105-kDa protein is T-cell specific.

AP-1 is not associated with the inducible mast cell ARE-protein complex. It was previously demonstrated that an oligonucleotide containing an AP-1 binding site competes with the ARE for the formation of the activation-dependent complex in T cells (7, 26, 38). In addition, jun and fos-specific antibodies react with proteins in this complex, indicating that AP-1 family members provide some of the T-cell ARE-inducible components (7, 38). The potential association of AP-1 with the mast cell-inducible complex (complex II) was examined in EMSAs with an AP-1 oligonucleotide as a cold competitor. As shown in Fig. 7, although addition of an AP-1 oligonucleotide eliminated the detection of the T-cell stimulation-dependent complex (lane 8), it had no effect on the inducible mast cell complex (lane 3). In some experiments, a very weak band with a lower mobility that was eliminated by the addition of an AP-1 oligonucleotide was observed. However, this was not a consistent result and was observed only if the concentration of nonspecific competitor DNA [poly(dI-dC)] in the binding reaction mixtures was dramatically lowered (>10-fold).

NF-AT is a component of the inducible mast cell ARE complex. The ARE shares 9 bp of sequence identity with the NF-AT binding site, a regulatory element that contributes to the activation-dependent transcription of IL-2 (33, 36, 38). This sequence identity is within the region critical for protein association and transactivation function of both elements (17, 33, 36, 38, 39). We and others have recently shown that the T-cell transcription factors associated with the ARE or P sequence are identical or highly related to those that make up the IL-2 NF-AT transcription complex (33, 36, 38). To examine the relationship of mast cell ARE binding factors to NF-AT, the ability of an NF-AT site oligonucleotide to compete with the ARE for binding of nuclear proteins was examined. As shown in Fig. 7 (lanes 2 and 4), addition of a 100-fold excess of unlabeled NF-AT or ARE significantly reduced detection of the complex.

Antibodies reactive with two defined NF-AT family members, NF-ATc and NF-ATp were used in EMSAs (Fig. 8). Addition of the NF-ATp antibody resulted in a clear supershift of complex I and the activation-dependent complex II. The NF-ATc antibody reacts only weakly with inducible complex II. These data indicate that factors reactive with both NF-ATp and NF-ATc antibodies are present in mast cells and associate with the IL-4 gene ARE in vitro.

Unique NF-AT-related proteins are present in mast cells. The reactivity of ARE complex II with both NF-ATc and NF-ATp antibodies suggested that mast cells express the same NF-AT transcription factors as T cells. Therefore, Western blot analyses were performed to compare these nuclear NF-AT-related proteins. In unstimulated mast cell extracts, a prominent ~56-kDa protein and trace amounts of at least two distinct proteins with molecular masses between 120 and 160 kDa reacted with anti-NF-ATc antibodies (Fig. 9, left panel). The same NF-ATc-related proteins are present in stimulated extracts; the amounts of the higher-molecular-mass species detected were dramatically increased, while the amount of the 56-kDa species remained constant. Surprisingly, these mast cell factors have molecular masses that are dramatically distinct from those present in stimulated T-cell extracts which migrate as three high-molecular-mass doublets.

This analysis was also carried out with anti-NF-ATp antibodies (Fig. 9, right panel). Proteins of ~145 and ~41 kDa are present in both unstimulated and stimulated mast cell extracts. Again, there is a dramatic increase in the amount of the higher-molecular-mass species observed in activated cell extracts, while the level of the 41-kDa factor remained unchanged. Although activated T cells express the higher-molecular-mass NF-ATp factor, they do not express the lower-mass, apparently

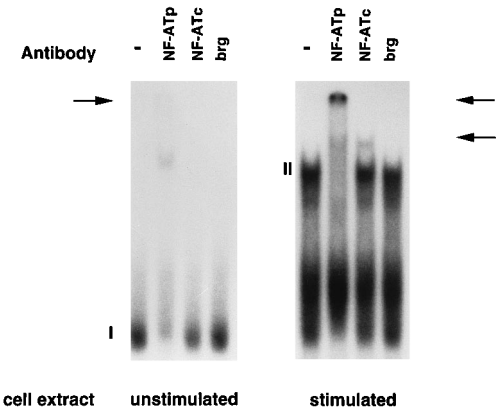

FIG. 8. Anti-NF-ATc and anti-NF-ATp antibodies react with components of the mast cell ARE-inducible complex. EMSAs were performed with unstimulated and ionomycin-stimulated mast cell nuclear extracts. Binding reaction mixtures contained no antibody (−) or 1 μl of the indicated NF-AT-specific antibodies. Anti-brg, a switch complex-specific antibody (20), was used as a negative control. The NF-AT antibodies did not form complexes when incubated with probe alone (not shown). Specific mast cell complexes are denoted by roman numerals. The NF-AT supershifted complexes are denoted by the arrows.

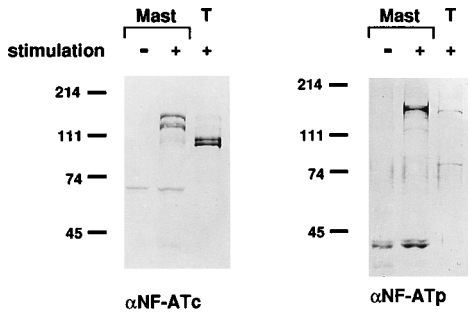

FIG. 9. NF-AT-related factors are present in both unstimulated and stimulated mast cell nuclear extracts. Western blot analysis was performed with crude nuclear extracts isolated from unstimulated (−) CFTL 15 mast cells (20 μg) and stimulated (+) CFTL mast cells (20 μg) and PMA-stimulated EL-4 T cells (10 μg). Proteins were separated on an SDS–10% polyacrylamide gel, transferred to nitrocellulose, and incubated with antibodies generated to either human T-cell NF-ATc or murine T-cell NF-ATp. The mobilities of the indicated molecular mass markers are shown on the left.

constitutive, 41-kDa species. A factor of ~74 kDa was also detected in EL-4 extracts in some experiments.

To determine which of the NF-AT-related proteins are directly associated with the ARE DNA, oligonucleotide affinity precipitation experiments were performed. Crude nuclear extracts from unstimulated and stimulated mast cells were incubated with Sepharose-linked concatamers of the −88-to-−60 sequence under standard binding reaction conditions. The affinity matrix was pelleted by centrifugation, and unbound proteins were collected in the supernatant. After several washes, the bound protein was eluted with high salt. The unbound and bound proteins were analyzed by Western blot assays. As shown in Fig. 10A, only a small amount of the higher-molecular-mass NF-ATc-related protein was detected in the eluted fraction of stimulated extracts, correlating with the weak supershift observed. Most of the NF-ATc antibody-reactive proteins in stimulated and unstimulated extracts, including the 56-kDa species, do not associate with the DNA in this assay. Similar amounts of the 41-kDa protein were observed in both fractions from the unstimulated extracts. In contrast, the bound fraction from stimulated extracts is highly enriched for the 41-kDa NF-ATp antibody-reactive protein. DNA affinity precipitation does not appear to enrich for the higher-molecular-mass species. These data indicate that the 41-kDa species is the major NF-AT-related protein associated with the DNA.

DISCUSSION

In most T cells and mast cells examined, high levels of IL-4 expression are detected only when the cells are activated, either through the T-cell antigen receptor or the high-affinity IgE receptor (FcεRI) on mast cells and basophils. Although the signals generated by the cross-linkage of both receptors lead to calcium mobilization, the protein kinase C (PKC) signal is not required for optimal mast cell cytokine production (31). In the present study, the 5′ elements that regulate inducible transcription in mast cells were examined to explore the possibility that the different cell surface signaling requirements leading to IL-4 production are reflected in the nuclear events that control inducible transcription in these two cell types. These data demonstrate that sequences between −87 and −70 (relative to the transcription initiation site) are critical for the activation-dependent transcription of IL-4 CAT reporter gene constructs. Mast cell nuclear proteins present in both unstimulated and stimulated cells specifically associate with an oligonucleotide spanning this region. Two DNA-protein complexes of distinct mobilities were observed in EMSAs with unstimulated nuclear extracts. Stimulation results in a dramatic increase in the amount of the lower-mobility complex (complex II) observed and a diminution of complex I. This suggests that some of the proteins associated with this important IL-4 regulatory region are preexisting and that cell stimulation results in enhanced binding to this region due either to modification of preexisting proteins and/or to synthesis of new proteins. Mutational analysis revealed that nucleotides between −77 and −69 are the most critical for protein association with the ARE and transactivation function. Some alterations outside this region also decrease ARE-mediated CAT transcription, suggesting an additional influence on transcription. Finally, it was demonstrated that the ARE is a specific target of CSA inhibition in mast cells; CSA treatment of stimulated cells resulted in a profound inhibition of the activities of both −87 IL-4 CAT reporter gene constructs and ARE-protein interactions.

The ARE sequence also contributes to the activation-dependent transcription of IL-4 in T cells (2, 4, 36–38). As the ARE in mast cells is the site of an inducible DNA-protein interaction, the same subsequences between bp −88 and −60 are critical for protein association and transactivation function and CSA inhibits ARE-protein association and function in T cells (37, 40). Thus, it is likely that some of the same proteins regulate ARE-mediated inducible IL-4 transcription in both cell lineages. The results of UV cross-linking experiments support this suggestion by demonstrating that some factors in both the inducible mast and T-cell complexes have the same molecular mass. Despite these similarities, there are striking differences in the mast cell- and T-cell-derived proteins that associate with this element. The complexes detected in EMSAs have completely unique mobilities. At least one nuclear protein with a distinct molecular mass, which was observed in UV cross-

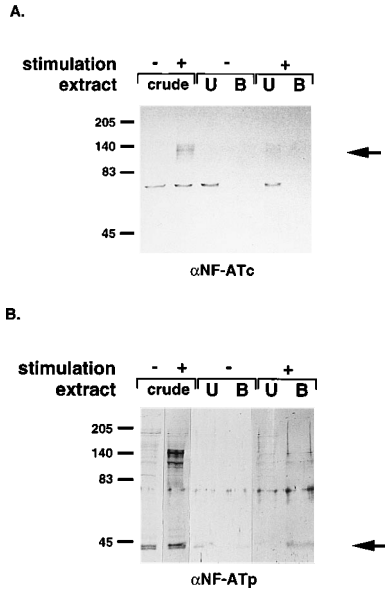

FIG. 10. NF-AT-related proteins that associate with the ARE are mast cell specific. DNA affinity precipitation was performed with ARE concatamers linked to Sepharose and nuclear extracts from unstimulated (−) and stimulated (+) CFTL 15 cells. Unbound (U) and bound (B) fractions were isolated and analyzed by Western blot analysis with antibodies reactive with T-cell-derived NF-ATc (A) and NF-ATp (B). The isolated proteins were compared with proteins present in crude nuclear extracts. Arrows denote the migration of the major bound protein species.

linking experiments, is unique to each cell type. In addition, although AP-1 family members are part of the T-cell stimulation-dependent ARE complex and form some of the inducible components of this complex, fos/jun family members do not readily associate with the inducible mast cell complex.

NF-AT is a complex of transcription factors that was first described on the basis of its ability to regulate expression of the IL-2 gene (17). This multiprotein complex consists of both preexisting (NF-AT) and stimulation-dependent factors belonging to the AP-1 family. According to a recent model, the assembly of an active IL-2 gene NF-AT transcription complex requires the calcium signal-mediated translocation of a preexisting cytoplasmic component to the nucleus. After translocation, NF-AT associates with newly synthesized proteins belonging to the AP-1 family of transcription factors. CSA, an immunosuppressive drug, inhibits IL-2 gene transcription by blocking NF-AT translocation. This model has been complicated by the recent discovery of at least four distinct NF-AT family members, NF-ATc, NF-ATp, NF-AT3, and NF-AT4. All proteins are expressed in T-lymphocyte lineage cells at various levels and are able to mediate transcriptional activation by the IL-2 promoter, although their relative contributions to IL-2 gene transcription in vivo have not been determined (15).

Several recent observations strongly implicate a component of NF-AT in ARE- or P-mediated IL-4 gene transcription in T cells. These include the presence of a common purine-rich sequence within the ARE and the NF-AT regulatory elements. The specific nucleotides critical for the function of both of these elements reside within this identity region (17, 38–40). CSA also inhibits ARE-mediated transactivation (33, 36, 38). Proteins with identical molecular masses are observed in both the ARE and NF-AT stimulation-dependent complexes in UV cross-linking experiments (33, 36, 38). Most compelling are the reactivities of NF-ATp- and NF-ATc-specific antibodies with components of the stimulated ARE or P complex (33, 38).

As the name implies, the expression of NF-AT was originally thought to be restricted to T cells. However, NF-AT proteins have recently been detected in several different cell types including B lymphocytes, monocytes, and NK cells, indicating a broader role for this factor in transcriptional regulation (18, 28, 32). Our findings clearly demonstrate that both NF-ATc- and NF-ATp antibody-reactive proteins are present in mast cell nuclear extracts. With the exception of a high-molecular-mass species that reacts with the NF-ATp antibody, these proteins have molecular masses distinct from those detected in T cells. DNA affinity precipitation experiments identified the 41-kDa NF-ATp-related species as the predominant ARE-associated protein. Because it is present in both unstimulated and stimulated extracts, this protein likely accounts for the reactivity of anti-NF-ATp antisera with both the constitutive and activation-dependent complexes in supershift experiments. Only small amounts of the higher-molecular-mass p- and c-related proteins were observed in the stimulated bound fraction, and this may reflect a lower affinity of these species for the ARE site. There were no 56-kDa αNF-ATc-reactive proteins in the bound fractions.

The 41-kDa proteins are present in unstimulated nuclear extracts in amounts equivalent to those detected in stimulated extracts. Thus, these proteins do not appear to be regulated by an activation-dependent translocation event. Furthermore, although association with the ARE appears to be activation independent, it is enhanced in stimulated extracts. It is possible that cell activation increases binding, either through a stimulation-dependent modification of the preexisting protein or through induction of another factor that facilitates the binding of these NF-AT-related factors.

The relationship of these NF-AT antibody-reactive proteins to those present in T cells is unclear. The 41-kDa factor was identified on the basis of its reactivity with an NF-ATp polyclonal rabbit serum. We have performed EMSA specificity controls with this serum, and although it does not react with T-cell NF-κB or AP-1 proteins (not shown), it has not been established that it reacts only with NF-ATp. Thus, this antiserum may have cross-reactivity due to similarities in a particular domain or epitope shared by NF-ATp and the 41-kDa protein. Nevertheless, these data, together with the recent identification of additional NF-AT family members (15), support the idea that the NF-AT family of transcription factors is much more extensive than originally described. There are clearly cell-type-specific differences in the particular family members expressed (15), and there may be differences in the way they are regulated as well. Alternatively, the species detected in mast cells are modified forms of the T-cell factors. These alternative forms may be the result of the mast cell-specific posttranslational modifications or alternative splicing which results in differential regulation. The question of whether these mast cell-specific NF-AT proteins contribute to the inducible ARE transcription remains. Efforts to isolate and characterize these apparently mast cell-specific factors, to determine their relationship to previously described T-cell-derived NF-AT family members, and to confirm their role in IL-4 transcription are currently under way.

The lack of an AP-1 component in the mast cell ARE DNA-protein complex likely reflects the fact that, unlike the requirement for IL-4 production in T cells, a calcium signal, but not activation of PKC, is sufficient for maximal induction of IL-4 (31). This observation also argues that the mast cell NF-AT factor(s) is distinct from the previously described NF-AT family members. All four previously described NF-AT proteins were found to readily associate with AP-1 in vitro (15). Our studies show that despite significant AP-1 binding activity in stimulated mast cell nuclear extracts (data not shown), jun/fos family members do not bind to the stimulation-dependent complex.

We have previously demonstrated that the regulation of IL-4 gene transcription in mast cells utilizes cell-specific regulatory elements (14). A sequence in the second intron of the murine IL-4 gene confers mast cell-specific enhancer activity. Two distinct elements within the intronic sequence are required for full enhancer activity. The molecular basis of this cell specificity is due to the differential expression in mast cells, but not T cells, of transcription factors belonging to the GATA and ets families that bind to these regions (13). The observations in the present study provide further evidence that there are distinct nuclear signalling molecules involved in T-cell and mast cell IL-4 transcription that reflect the different cell surface signalling requirements in these cells. Several models have been proposed to explain the regulation of a single gene in multiple cell types (25). Expression of a gene can be controlled by different *trans*-acting factors present in each cell. These factors could interact with separate enhancer elements, as is the case in the intronic enhancer. Distinct factors may also interact with the same element but may recognize different subsequences within that element. Such a mechanism appears to regulate the variable levels of activity of the simian virus 40 enhancer in different cell types (25). Alternatively, common *trans*-acting factors present in all cell types that express a particular gene may interact with the element in an identical manner. The finding that identical ARE subsequences are critical in both T cells and mast cells suggests that IL-4 gene regulation is governed at the ARE by both common and cell-specific factors that associate at the same site.

In view of the critical role that IL-4 plays in the regulation of immune-inflammatory responses and the potentially distinct contributions of mast cell- and T-cell-derived IL-4, it is of interest to understand the differences in the mechanisms that regulate the nuclear and more-cell-surface-proximal signalling events leading to IL-4 production. Such studies are essential for the development of strategies to selectively modulate the expression of IL-4 in T cells and mast cells for therapeutic applications.

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