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A common factor regulates both Th1- and Th2-specific cytokine gene expression

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Murine T helper cell clones are classified into two distinct subsets, T helper 1 (Th1) and T helper 2 (Th2), on the basis of cytokine secretion patterns. Th1 clones produce interleukin-2 (IL-2), tumor necrosis factor-β (TNF-β) and interferon-γ (IFN-γ), while Th2 clones produce IL-4, IL-5, IL-6 and IL-10. These subsets differentially promote delayed-type hypersensitivity or antibody responses, respectively. The nuclear factor NF-AT is induced in Th1 clones stimulated through the T cell receptor-CD3 complex, and is required for IL-2 gene induction. The NF-AT complex consists of two components: NF-ATp, which pre-exists in the cytosol and whose appearance in the nucleus is induced by an increase of intracellular calcium, and a nuclear AP-1 component whose induction is dependent upon activation of protein kinase C (PKC). Here we report that the induction of the Th2-specific IL-4 gene in an activated Th2 clone involves an NF-AT complex that consists only of NF-ATp, and not the AP-1 component. On the basis of binding experiments we show that this 'AP-1-less' NF-AT complex is specific for the IL-4 promoter and does not reflect the inability of activated Th2 cells to induce the AP-1 component. We propose that NF-ATp is a common regulatory factor for both Th1 and Th2 cytokine genes, and that the involvement of PKC-dependent factors, such as AP-1, may help determine Th1-/Th2-specific patterns of gene expression.

Key words: interleukin-2/interleukin-4/T cell activation/T helper 1 and T helper 2 cells/transcription

Introduction

The interaction of $CD4^+$, or helper T (Th), cells via the T cell receptor (TCR) complex with peptide-MHC complexes on antigen-presenting cells initiates a cascade of events culminating in an effective immune response. One consequence of this interaction is the synthesis and release of cytokines into the surrounding microenvironment.

Subsets of Th cells have been defined on the basis of their distinct cytokine secretion patterns and their concomitant immunomodulatory effects. Th1 cells produce the cytokines interleukin-2 (IL-2), tumor necrosis factor-β (TNF-β) and interferon-γ (IFN-γ) (Mossman and Coffman, 1989). These cytokines enhance antigen presentation, phagocytosis, Fc receptor expression, and nitric oxide and superoxide production (Murray, 1990), which result in an increased ability of macrophages to attack a wide variety of intracellular and extracellular pathogens (Murray *et al.*, 1985). Also, Th1, but not Th2, cells mediate delayed-type hypersensitivity (Cher and Mossmann, 1987), a complex inflammatory response. In contrast, Th2 cells produce the cytokines IL-4, IL-5, IL-6 and IL-10 (Mossman and Coffman, 1989). These cytokines stimulate the production of IgE by B cells (Coffman and Carty, 1986), and the development and degranulation of eosinophils and mast cells (Mossman and Coffman, 1989). These Th2-specific responses are associated with infections with metazoan parasites. Furthermore, evidence to date suggests that the cytokines produced by the Th cells themselves are important regulators of Th subset activation and differentiation (Mossman and Coffman, 1989).

The mechanisms responsible for the tissue-specific expression of Th1 or Th2 cytokine genes remain unknown. One explanation for Th1- and Th2-specific cytokine expression patterns may be differences involving the interactions of *trans*-acting factors with promoter/enhancer elements of cytokine genes. Exclusivity of expression of Th1 and Th2 cytokine genes might involve a Th1- or Th2-specific DNA binding factor(s), acting either positively or negatively on cytokine gene expression transcription. Alternatively (or additionally) tissue specificity could be due to the interaction of Th1- or Th2-specific co-activators with ubiquitous DNA binding proteins. Indeed, induction of the IL-2 gene in T lymphocytes has been shown to involve both ubiquitous and lymphocyte-specific factors (Serfling *et al.*, 1989). However, no factor conferring Th1-specificity on the IL-2 gene has been described.

Whereas many of the promoter/enhancer regulatory elements and *trans*-acting factors involved in the regulation of Th1-specific cytokine genes have been well defined (especially for the IL-2 gene), little is known of parallel mechanisms that drive transcription of Th2-specific cytokine genes. To elucidate the possible role of regulatory elements and *trans*-acting factors in determining Th2-specific cytokine gene expression, we chose to study the IL-4 gene using non-transformed murine Th clones. Here, we report that an element within the IL-4 promoter which is required for TCR-mediated inducible expression binds the lymphocyte-specific complex, nuclear factor of activated T cells (NF-AT) (Shaw *et al.*, 1988). Interestingly, however, the NF-AT complex we describe is different from that characterized to be required for the inducible expression of the IL-2 gene. Whereas the IL-2 NF-AT complex contains an AP-1 component thought to be required for IL-2 expression (Jain *et al.*, 1992a), the NF-AT complex required for inducible IL-4 expression lacks this AP-1 component. We conclude that NF-ATp serves as a common regulatory factor for both Th1- and Th2-specific

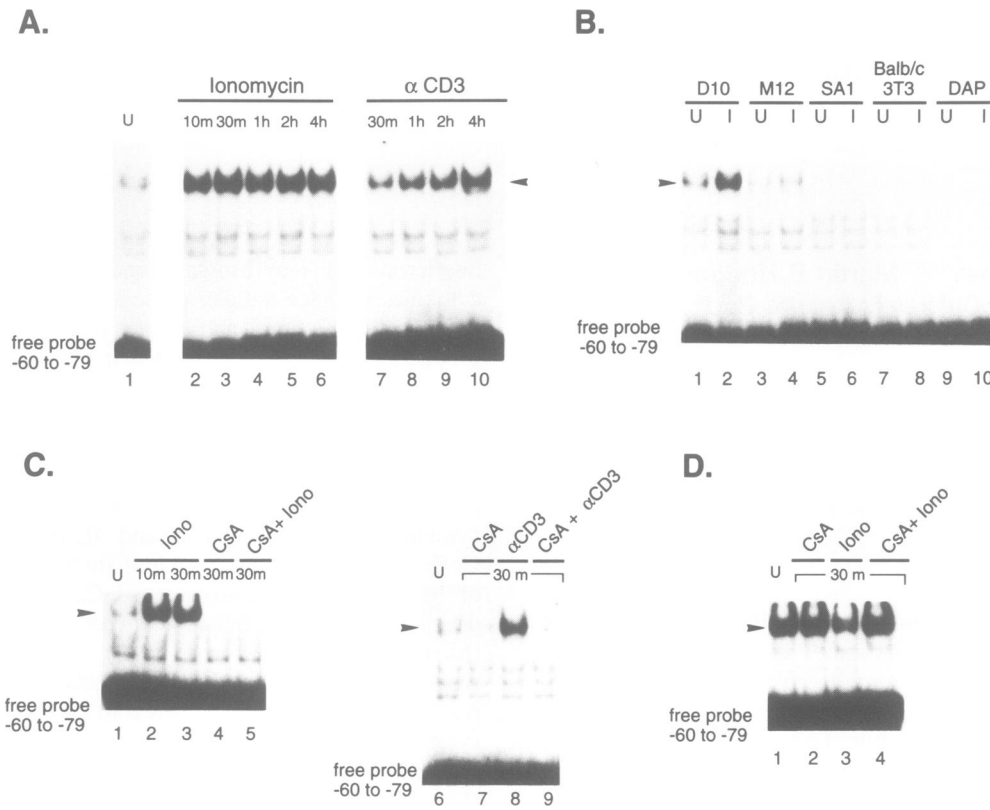

Fig. 1. Characterization of the IL-4 regulatory element binding complex. (**A**) Treatments with both ionomycin and anti-CD3 induce binding of a nuclear complex to an oligonucleotide encompassing an IL-4 regulatory element. Nuclear extracts from ionomycin- and anti-CD3-treated D10 cells were used in EMSAs with a radiolabelled oligonucleotide corresponding to −60 to −79 of the murine IL-4 promoter. Identical results were seen using the Th2 clone, CDC35. (**B**) The anti-CD3/ionomycin-inducible complex is present predominantly in T cells. The radiolabelled −60 to −79 IL-4 promoter oligonucleotide was used in gel shifts with nuclear extracts from the following murine cell lines treated with ionomycin for 30 min: the Th2 clone D10, the mature B cell lymphoma M12.4.1, the sarcoma cell line SA1, and the fibroblast cell lines BALB/c 3T3 and DAP. (**C**) Formation of the anti-CD3/ionomycin-inducible complex is inhibited by co-treatment with cyclosporin A. Nuclear extracts from D10 cells activated with ionomycin and anti-CD3 with and without cyclosporin A were used in gel shift assays using the radiolabelled −60 to −79 IL-4 promoter oligonucleotide. Identical results were seen using the Th2 clone, CDC35. (**D**) The anti-CD3/ionomycin-inducible complex is constitutively present in the cytosol of D10 cells. Cytosolic extracts isolated from untreated D10 cells or cells treated with ionomycin, or cyclosporin A, or both were used in gel shift assays using the radiolabelled −60 to −79 IL-4 promoter oligonucleotide. Identical results were seen using the Th2 clone, CDC35.

cytokine genes, and suggest that the role of AP-1 or other protein kinase C (PKC)-dependent factors may function alone, or as coactivators in conjunction with DNA binding factors such as NF-ATp, perhaps contributing to the Th1/Th2-specific expression of cytokine genes.

Results

A 20 bp region of the IL-4 promoter required for inducible promoter activity binds a Ca^{2+}-inducible, cyclosporin A-sensitive DNA binding factor

A previous study had identified a 20 bp region of the IL-4 promoter that was necessary and sufficient for inducible expression in the murine T cell clone, D10.G4.1 (D10) (Todd *et al.*, 1993). Alteration of the region from −60 to −79 by linker-scanning mutagenesis resulted in a >4-fold reduction of inducible promoter activity of an IL-4 promoter/CAT construct containing 157 bp of upstream promoter sequence in transfected D10 cells treated with antibodies to the TCR complex (anti-CD3) (Todd *et al.*, 1993). Additionally, when multimerized and placed upstream of a heterologous promoter/CAT construct, this region was sufficient to confer inducible expression in response to anti-CD3 and ionomycin (Todd *et al.*, 1993). To investigate the possibility that the linker-scanning mutagenesis had disrupted the ability of a DNA binding protein(s) to bind to a cognate *cis*-element(s) within this region, electrophoretic mobility shift assays (EMSAs) were done using nuclear extracts from the Th2 cell clone, D10 (Kaye *et al.*, 1983). Th cell clones offer the advantages of possessing true Th1 or Th2 cytokine secretion patterns and of being nontransformed, non-immortalized cells. Because several transformed cell lines have been reported to contain constitutively high levels of certain transcription factors, Th clones provide an opportunity of studying gene expression in the absence of tumorigenically altered levels of transcription factors associated with oncogenesis. Using an oligonucleotide corresponding to the region from −60 to −79 of the IL-4 promoter region as a probe, EMSA analyses identified an inducible DNA−protein complex in nuclear extracts prepared from stimulated (Figure 1A, lanes 2−10), but not unstimulated, D10 cells (lane 1). Treatments with either the calcium ionophore, ionomycin (lanes 2−6), or antibodies to CD3 (lanes 7−10) resulted in the rapid induction of this complex, suggesting that a rise in intracellular free calcium alone is sufficient for induction. Maximal levels of the inducible complex were seen within 10 min of treatment with ionomycin (lane 2), whereas treatment with anti-CD3

required up to 4 h until comparable levels were reached (lane 10). To determine the cell-type specificity of this complex, binding assays employing a variety of cell types were performed, some of which are shown in Figure 1B. The complex appeared to be lymphoid-specific and limited predominantly to T lymphocytes (lanes 1 and 2) with a low, constitutive level detected in a B lymphoma cell line (lanes 3 and 4). Inducibility of the complex was specific to treatments with ionomycin or anti-CD3, since treatments of T or B lymphocytes with other agents such as PMA, failed to induce the formation of the complex, nor did it augment the level of complex formation when used in conjunction with ionomycin (data not shown). Expression of the IL-4 gene in activated Th2 cells is inhibited by the immunosuppressive drug, cyclosporin A (Andersson *et al.*, 1992). Co-treatment of D10 cells with cyclosporin A and anti-CD3 (Figure 1C, lane 9) or cyclosporin A and ionomycin (lane 5) resulted in the complete inhibition of the inducible complex. To determine the subcellular localization of the inducible complex, cytosolic protein extracts were used in EMSA analyses (Figure 1D). The anti-CD3/ionomycin-inducible complex was present in the cytosol of both ionomycin-treated (lane 3) and anti-CD3-treated (data not shown) D10 cells, and was unaffected by co-treatment with cyclosporin A (lane 4). Slightly lower levels of the complex were seen in the cytosolic extracts of induced cells (lane 3), perhaps representing the translocation of the complex to the nucleus.

Inducibility of a CAT expression construct containing multiple copies of the 20 bp region parallels the inducibility of the Ca^{2+}-inducible, cyclosporin A-sensitive DNA binding factor

The *in vivo* significance of the characteristics of the Ca^{2+}-inducible, cyclosporin A-sensitive DNA binding factor was tested by transient transfection assays using a CAT expression construct containing three copies of the region of the IL-4 promoter spanning −60 to −79 (Figure 2). Addition of ionomycin or anti-CD3 to D10 cells transiently transfected by the multimer construct caused a >5-fold increase in CAT activity. Addition of PMA resulted in no increase of CAT activity over basal levels. PMA also failed to augment the CAT activity induced by ionomycin. Both ionomycin and anti-CD3 inductions could be completely reversed by adding cyclosporin A. Interestingly, co-transfection of an expression construct containing a constitutively active form of the Ca^{2+}- and calmodulin-dependent phosphatase, calcineurin, resulted in an 8-fold induction of the 20 bp multimer/CAT construct. Calcineurin has been shown to dephosphorylate NF-ATp *in vitro* (Jain *et al.*, 1993b; McCaffrey *et al.*, 1993a) and to be the target of cyclosporin A-mediated inhibition of cytokine gene expression (reviewed in Schreiber and Crabtree, 1992). These results suggest a strong correlation between binding of the Ca^{2+}-inducible, cyclosporin A-sensitive DNA binding factor, and the induction of promoter activity.

The anti-CD3/ionomycin-inducible complex binds to a site which bears a strong homology to the IL-2 NF-AT binding site

To delimit the region within the 20 bp promoter region that is necessary for binding of the anti-CD3/ionomycin complex, four oligonucleotides were designed containing serial 4 bp linker-scanning mutations and employed in binding and competition assays. The mut1 and mut4 oligonucleotides

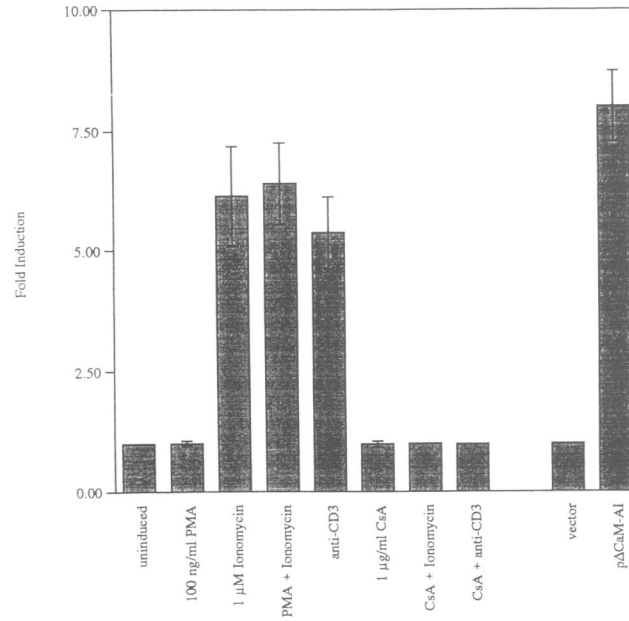

Fig. 2. The characteristics of inducibility of the IL-4 NF-AT multimer/CAT construct in transiently transfected D10 cells. The Th2 cell clone, D10, was transiently transfected with the IL-4 NF-AT multimer/CAT construct, p(IL-4 NF-AT)$_{3F}$-CAT, allowed to rest overnight, and then treated for 24 h with the indicated agents. Fold induction is normalized to the amount of CAT activity seen with cells transfected with pBL2CAT, the vector without the three IL-4 NF-AT sites. The last two bars represent a co-transfection experiment consisting of p(IL-4 NF-AT)$_{3F}$-CAT with either pSRα-296 (vector) or pSRα-ΔCaM AI. Inducibility of these co-transfections is normalized to the amount of CAT activity seen with cells transfected with p(IL-4 NF-AT)$_{3F}$-CAT plus pSRα-296 (vector). Three independent transfection experiments were performed.

bound (data not shown) and competed (Figure 3A, lanes 3−6 and 17−20) away the inducible complex, whereas the mut2 and mut3 oligonucleotides neither bound (data not shown) nor competed (lanes 7−10 and 13−16) for binding thus identifying an 8 bp region whose integrity is required for complex formation. That the inducible complex was indeed specific was shown by the ability of a 50-fold molar excess of unlabelled 'wild-type' −60 to −79 oligonucleotide to inhibit nearly completely the formation of a complex in EMSA analyses (lanes 2 and 12). In addition, methylation interference revealed that only the two guanine residues within the 8 bp element, and none of the neighbouring guanine residues outside of this element, were protected from cleavage by piperidine (summarized in Figure 3B).

To determine if the same region required for binding of the complex was also required for the inducibility of the IL-4 promoter *in vivo*, transient transfections were performed using IL-4 promoter/CAT expression constructs in which the same 4 bp linker-scanning mutations had been introduced into an IL-4 promoter fragment spanning −157 to +68 (Figure 4). [A previous IL-4 promoter dissection study had shown that inducibility by ionomycin and anti-CD3 is largely limited to the proximal IL-4 promoter region spanning this area (Todd *et al.*, 1993).] Whereas the inducibility of CAT activity by the IL-4 promoter/CAT constructs in transiently transfected D10 cells was relatively unaffected by the 4 bp mutations, mut1 and mut4, the introduction of the mut2 and mut3 mutations completely abolished the inducibility of CAT activity. These results indicate that, as with binding of the complex, inducibility of the IL-4 promoter is dependent on the 8 bp region spanning mut2 and mut3 (−74 to −67).

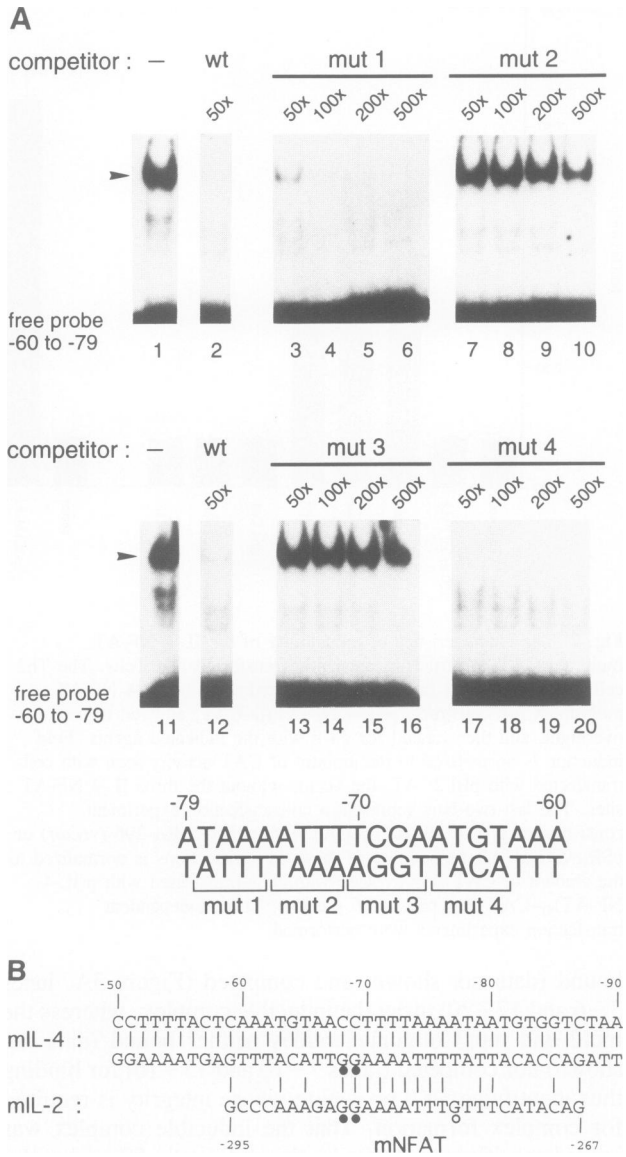

Fig. 3. Identification of the region involved in binding of the anti-CD3/ionomycin-inducible complex. (**A**) Competition using mutant oligonucleotides containing serial 4 bp linker-scanning mutations in gel shift assays identify an 8 bp region involved in binding of the anti-CD3/ionomycin-inducible complex. Nuclear extracts from activated D10 cells (2 h anti-CD3 treatment) were used in gel shift assays using a radiolabelled −79 to −60 'wild-type' oligonucleotide alone, or together with the indicated molar excesses of unlabelled mut1, mut2, mut3 or mut4 oligonucleotides (for sequences of oligonucleotides, see Materials and methods). Identical results were seen using the Th1 clone, D1.1, and the Th2 clone, CDC35. (**B**) Comparison of the IL-4 promoter element with the distal NF-AT element of the IL-2 promoter. Nucleotide sequences of the murine IL-4 promoter encompassing the anti-CD3/ionomycin-responsive element (in reverse orientation) and the distal NF-AT site of the murine IL-2 promoter (Bierer *et al.*, 1990). Filled and empty circles represent complete and partial protection, respectively, by methylation from cleavage by piperidine. Methylation interference of the IL-2 distal NF-AT site has been previously published (McCaffrey *et al.*, 1992).

The characteristics of the IL-4 anti-CD3/ionomycin-inducible complex are similar to those of the pre-existing component of NF-AT, NF-ATp (Flanagan *et al.*, 1991; McCaffrey *et al.*, 1993a), which has been shown to be involved in the transcriptional regulation of the IL-2 gene. Both complexes pre-exist in the cytosol, are inhibited from binding their cognate DNA elements by cyclosporin A, are limited predominantly to T cells (and found in low amounts in B cells) and are able to be induced by treatment with calcium-ionophore alone. Furthermore, closer inspection of the 8 bp inducible element in the IL-4 promoter identified by competition-EMSA and methylation interference revealed a 7 out of 8 bp homology with the distal NF-AT site of the murine IL-2 promoter (Figure 3B).

The anti-CD3/ionomycin-inducible complex which binds to the −67 to −74 region of the IL-4 promoter is NF-ATp

To determine the extent of similarity between the IL-4 anti-CD3/ionomycin-inducible complex and the IL-2 NF-ATp complex, 'cross-competition' EMSA analyses were performed using as both probe and competitor oligonucleotides containing the IL-4 anti-CD3/ionomycin-inducible element (herein designated as the IL-4 NF-AT site) and the IL-2 distal NF-AT site (Figure 5) in conjunction with nuclear extracts prepared from the Th1 clone, Ar-5 (Rao *et al.*, 1984), and the Th2 clone, D10. In EMSAs using nuclear extracts from ionomycin-treated D10 cells with an end-labelled oligonucleotide containing the IL-4 NF-AT site (Figure 5A), it can be seen that the IL-2 distal NF-AT site competes less efficiently than the IL-4 NF-AT site (compare in Figure 5A, lanes 3 and 8, and lanes 4 and 9), suggesting that the IL-4 NF-AT site has a higher affinity for the D10 (Th2) complex. Evidence for a differing affinity for the IL-4 NF-AT site and the IL-2 NF-AT site for the complex formed with nuclear extracts from ionomycin-treated Ar-5 cells was also found (compare in Figure 5B, lanes 3 and 8). Overall, the IL-4 NF-AT site had a higher affinity for the complex compared with the IL-2 NF-AT site when used as a labelled probe (Figure 5A) and as an unlabelled competitor (Figure 5A, lanes 6−10; Figure 5B, lanes 1−5). Thus, although the affinities for the NF-AT complex by the two NF-AT sites appear to differ, it is clear that both can function as efficient NF-AT binding sites.

In order to characterize more closely the Th2-inducible complex which binds to the IL-4 NF-AT site, and also to compare it with the Th1-inducible complex which binds to the IL-2 NF-AT site, proteolytic band-shift assays (PCBAs) (Schreiber *et al.*, 1988) were performed. Extracts prepared from anti-CD3-induced Ar-5 cells and anti-CD3-induced D10 cells were incubated with end-labelled oligonucleotides containing the IL-2 distal NF-AT site and the IL-4 NF-AT site, respectively, along with increasing amounts of four proteases with differing substrate specificities (Figure 6A). Although the binding affinities differ in the two reactions, it can be seen that the two DNA−protein complexes exhibit similar kinetics and products of limited proteolysis. It can also be seen that these two complexes comigrate and are therefore of similar molecular weights. The proteolytic patterns shown match those previously obtained using renatured NF-ATp purified from denaturing SDS−polyacrylamide gels (McCaffrey *et al.*, 1993).

To identify more stringently the presence of NF-ATp in the complex bound to the IL-4 NF-AT site, supershift-EMSA experiments were performed using an antiserum to a tryptic peptide of NF-ATp (McCaffrey *et al.*, 1993b) (Figure 6B). The antiserum to peptide NT72 of NF-ATp, when added to nuclear extract binding assays, was able to supershift both the complex which binds to the IL-4 NF-AT site using D10 nuclear extracts (Figure 6B, lane 2, upper filled arrow) and

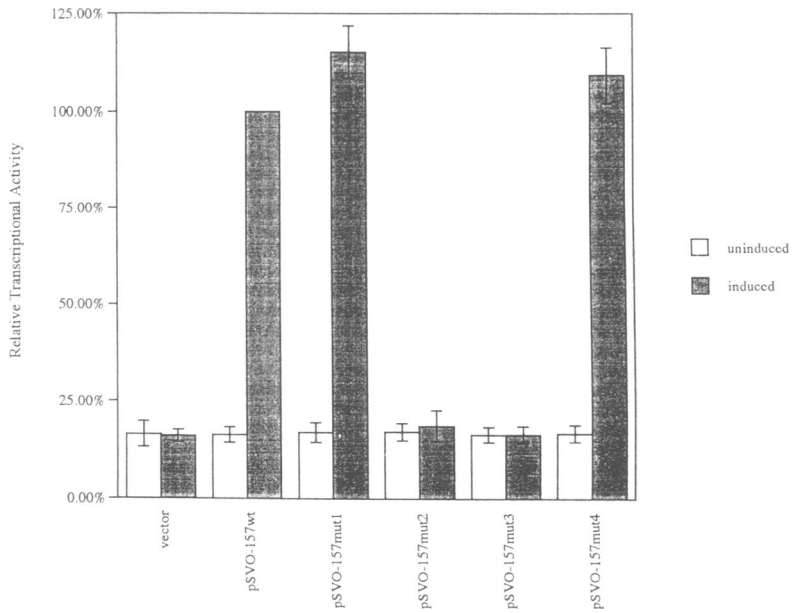

Fig. 4. Transient transfections using IL-4 promoter/CAT constructs containing the 4 bp linker-scanning mutations within the −79 to −60 region. Transfections were performed as in Figure 2 using the IL-4 promoter/CAT constructs containing from −157 to +68 of the IL-4 promoter in the tk promoter/CAT expression vector, pSVO-CATapoly (as described in Todd *et al.*, 1993). Linker-scanning mutations of 4 bp, identical to those shown in Figure 3A, were constructed and also used in transfections. D10 cells were transfected, allowed to rest overnight and either not induced or induced by plate-bound anti-CD3 for 24 h. Relative transcriptional activity is measured relative to the CAT activity from anti-CD3-treated D10 cells transfected with pSVO-157-CAT, which was set at 100%. Three independent transfection experiments were performed.

the complex which binds to the IL-2 NF-AT site using Ar-5 nuclear extracts (lane 8, upper filled arrow). That these supershifts are specific for the NT72 peptide of NF-ATp is shown by the ability of the cognate peptide, NT72 (lanes 3 and 9), but not an irrelevant peptide, NT48 (lanes 4 and 10), to reverse the supershift when added to the binding assays. These results show conclusively that the IL-4 anti-CD3/ionomycin-inducible complex, like the IL-2 NF-AT complex, contains NF-ATp.

The anti-CD3/ionomycin-inducible IL-4 NF-AT complex, unlike the IL-2 NF-AT complex, does not contain an AP-1 component

The NF-AT complex binding to the murine IL-2 distal NF-AT site in Th1 cells has been shown to contain, in addition to NF-ATp, an AP-1-like component (Jain *et al.*, 1992a). When specific EMSA binding conditions are used, the NF-ATp/AP-1 complex appears as an upper, more slowly migrating protein−DNA complex, as compared with the lower, faster migrating NF-ATp complex (Jain *et al.*, 1992a). The AP-1 component has been identified on the basis of the ability of antibodies against Fos and Jun, and oligonucleotides containing AP-1 sites, to compete for the formation of this slower, or upper complex (Jain *et al.*, 1992a). Using EMSA binding conditions which favour the appearance of this upper NF-ATp/AP-1 complex, the upper complex can be seen when extracts from anti-CD3-treated Ar-5 (Th1) cells are incubated with the IL-2 distal NF-AT site (Figure 7A, lane 2). That this upper complex contained an AP-1 component is shown by the ability of several AP-1 oligonucleotides (one of which, the human metallothionein AP-1 site, is shown in Figure 7A, lane 5) to compete for DNA binding of this complex, and also by the ability of an affinity-purified antibody against the Fos M peptide (data not shown) to 'supershift' this complex, as had been shown previously. However, when the identical experiment is carried out using extracts from anti-CD3 treated D10 (Th2) cells with the IL-4 NF-AT site, no upper complex is seen (Figure 7B, lane 7), and the lower complex is unaffected by either a 200-fold molar excess of unlabelled AP-1 oligonucleotides (Figure 7B, lane 10), or the aforementioned anti-Fos M antibody (data not shown). That the formation of the NF-ATp/AP-1 complex is dependent on the NF-AT binding site, and not the unavailability of AP-1 constituents in D10 nuclear extracts, is shown by the ability of an NF-ATp/AP-1 complex to form when D10 nuclear extracts are incubated with the IL-2 distal NF-AT site (Figure 7A, lanes 7 and 10), and by the inability of an NF-ATp/AP-1 complex to form when Ar-5 nuclear extracts are incubated with the IL-4 NF-AT site (Figure 7B, lanes 2 and 5) in EMSA analyses. In accordance with this is the finding that the formation of the upper NF-ATp/AP-1 complex with the IL-2 distal NF-AT site is dependent upon an adjacent 8 bp sequence bearing homology to a consensus AP-1 site (Jain *et al.*, 1993a). To test the possibility that a region required for binding of an upper AP-1/NF-AT complex could have been truncated in the −60 to −79 IL-4 NF-AT oligonucleotide, EMSA analyses were also performed using as probes longer oligonucleotides which extended 10 bp further upstream (−89 to −60), and 10 bp further downstream (−79 to −50) (data not shown). These oligonucleotides also failed to form upper AP-1/NF-AT complexes.

Discussion

In this study, we present evidence which suggests that a common factor, NF-ATp, is involved in the regulation of both Th1-specific (the IL-2 gene) and Th2-specific (the IL-4 gene) cytokine genes. Previous mutational analyses of the murine IL-4 promoter had implicated a 20 bp region from −60 to −79 as being functionally necessary and sufficient for inducibility by the TCR agonist, 2C11, an antibody

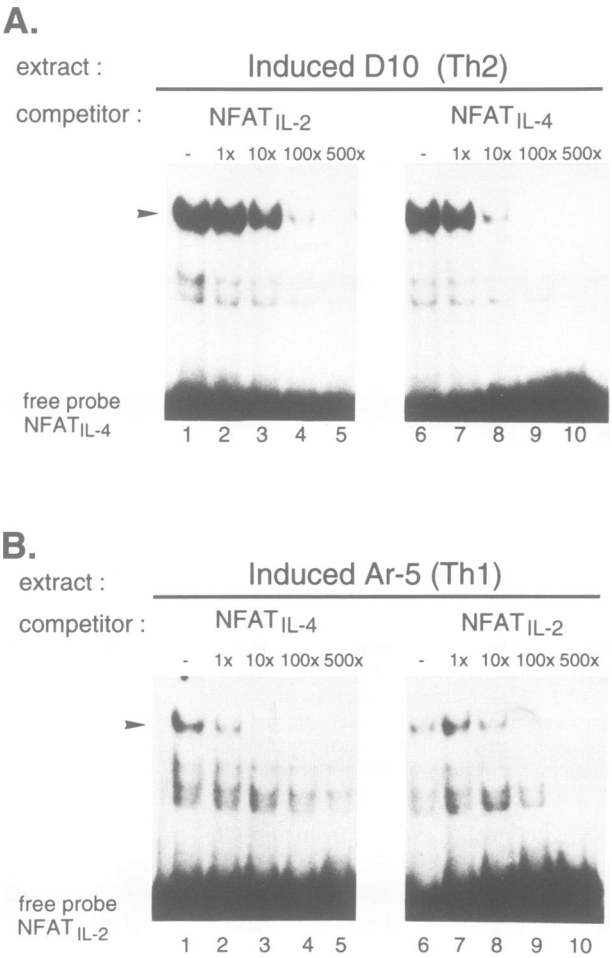

Fig. 5. Cross-competition of the Th2/IL-4 NF-AT and the Th1/IL-2 NF-AT complexes by excess unlabelled IL-2 and IL-4 NF-AT sites, respectively. (**A**) Competition of the Th2/IL-4 NF-AT complex by the IL-2 and IL-4 NF-AT sites. Binding assays were performed as previously described in Figure 1 using nuclear extracts from activated D10 cells (treated for 2 h with anti-CD3) and a radiolabelled IL-4 NF-AT oligonucleotide (−79 to −60, as in Figure 1) with the indicated molar excesses of unlabelled oligonucleotide containing the IL-2 NF-AT site (lanes 1−5) and the IL-4 distal NF-AT site (−295 to −267, as pictured in Figure 3B) (lanes 6−10). (**B**) Competition of the Th1/IL-2 NF-AT complex by the IL-4 and IL-2 NF-AT sites. Binding assays were performed as in Figure 1 using extracts from activated Ar-5 cells (treated with anti-CD3 for 2 h) and radiolabelled IL-2 NF-AT oligonucleotide with the indicated molar excesses of unlabelled oligonucleotides containing the IL-4 NF-AT site (lanes 1−5) or the IL-2 NF-AT site (lanes 6−10). Under these binding conditions, Ar-5 nuclear extracts produce only the lower complex containing NF-ATp, and no detectable upper complex containing AP-1 is detected using the radiolabelled IL-2 NF-AT site. Identical results were seen using the Th1 clone, D1.1, and the Th2 clone, CDC35.

against CD3 (Todd *et al.*, 1993). Linker-scanning mutagenesis of this region in the context of the proximal promoter region (−157 bp of upstream region) resulted in the 4-fold reduction of promoter activity (Todd *et al.*, 1993). Multiple copies of the same region were found to be sufficient to impart inducibility on a heterologous promoter/CAT construct (Todd *et al.*, 1993). It is clear, however, that other regions of the IL-4 promoter are required in order to confer maximal inducibility. Interestingly, in the human IL-4 promoter, linker-scanning mutagenesis and multimerization of a highly homologous region spanning −69 to −79, called the 'P sequence', yielded similar results in transient transfections using the human thymoma cell line, Jurkat (Abe *et al.*, 1992) but no statements were made regarding the significant homology between this region and the two NF-AT binding sites in the promoters of the human or murine IL-2 promoters. Using EMSA analyses employing an oligonucleotide spanning the region from −60 to −79, we identified an anti-CD3/ionomycin-inducible complex which shared many characteristics with the pre-existing component of the NF-AT complex, NF-ATp, which is involved in the inducible expression of the IL-2 promoter. The inducible factor was dependent solely on an increase in intracellular calcium for inducibility, for treatment with the calcium ionophore, ionomycin, was sufficient for complex formation, as has been shown for the binding of the pre-existing NF-ATp to the distal IL-2 NF-AT site. Treatment with PMA alone failed to induce complex formation, and the addition of PMA to ionomycin-treated cells resulted in no augmentation of levels of complex formed. The anti-CD3/ionomycin-inducible complex was constitutively present in the cytosol, and was present in the nucleus only after induction. Similar to its effects on NF-ATp, co-treatment of D10 cells with the immunosuppressive agent, cyclosporin A, prevented the appearance of the inducible complex into the nucleus. Finally, the inducible complex was restricted to T cells. Transient transfections using a heterologous thymidine kinase (tk) promoter/CAT construct driven by three copies of the −60 to −79 region of the murine IL-4 promoter showed the same characteristics of inducibility as the DNA binding complex. An increase in CAT activity was seen upon treatment of transfected cells with anti-CD3 or ionomycin, whereas no increase was seen when transfected cells were treated with PMA, and no augmentation of the ionomycin induction resulted from the addition of PMA. Treatment with cyclosporin completely abolished inductions of CAT activity by anti-CD3 and ionomycin, and the co-transfection of a constitutively active form of the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, alone was sufficient to result in maximal induction of CAT activity.

EMSA analyses using linker-scanning mutants of the −60 to −79 region of the IL-4 promoter as both probes and unlabelled competitors, and methylation interference analysis implicated an 8 bp region involved in binding of the inducible complex which bore a strong (7/8 bp) homology to the distal NF-AT site in the murine IL-2 promoter (Figure 3B). Transient transfections using IL-4 promoter/CAT expression constructs containing the same linker-scanning mutations confirmed that the same region necessary for complex formation was also necessary for the inducibility of the IL-4 promoter. Cross-competition EMSA analyses and PCBAs showed that the distal NF-AT site of the IL-2 promoter could both compete for, and function as a binding site for, the inducible complex from the Th2 clone, D10. Finally, an antiserum raised against a tryptic peptide of NF-ATp was able to supershift the inducible complex binding to the −60 to −79 region of the IL-4 promoter. Similar protein−DNA mobilities on EMSAs, similar PCBA profiles, the reactivity to an antiserum specific for NF-ATp, the sufficiency of ionomycin to induce complex formation and the sufficiency of ionomycin and co-transfection of calcineurin to induce promoter activity from the −60 to −79 multimer/CAT construct, and the inhibition of both complex formation and multimer/CAT expression by cyclosporin, strongly suggest that the inducible complex is the pre-existing component of NF-AT, NF-ATp.

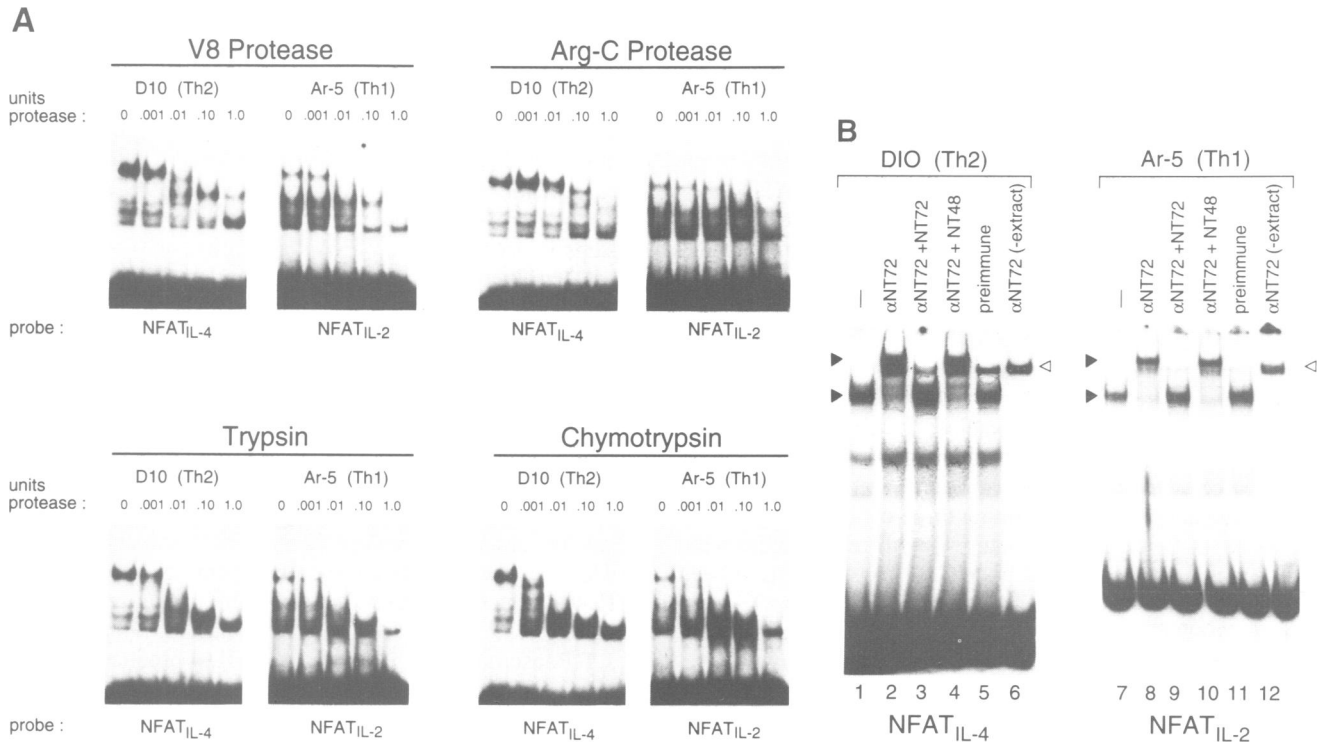

Fig. 6. The anti-CD3/ionomycin-inducible Th2/IL-4 NF-AT complex is NF-ATp. **(A)** PCBAs identify the Th2/IL-4 complex as the Th1/IL-2 NF-ATp complex. Binding assays were performed as in Figure 1 using nuclear extracts from activated D10 and Ar-5 cells (treated with anti-CD3 for 2 h) with radiolabelled IL-4 and IL-2 NF-AT oligonucleotides, respectively, with the indicated amounts, in units, of the proteases V8, Arg-C, trypsin and chymotrypsin. Identical results were seen using the Th1 clone, D1.1, and the Th2 clone, CDC35. **(B)** The Th2/IL-4 complex is supershifted by an antiserum against a tryptic peptide of NF-ATp. Binding assays were performed as in Figure 1 using nuclear extracts from activated D10 and Ar-5 cells (2 h anti-CD3-treated) with radiolabelled IL-4 and IL-2 NF-AT oligonucleotides, respectively, with the addition of antiserum raised against the NT72 peptide of NF-ATp alone (lanes 2 and 8), with 1 μg of its cognate peptide, NT72 (lanes 3 and 9), or with 1 μg of an irrelevant peptide, NT48 (lanes 4 and 10). An additional band indicated by an open arrow indicates a DNA binding activity present in the preimmune and immune antisera which is not present in the nuclear extracts.

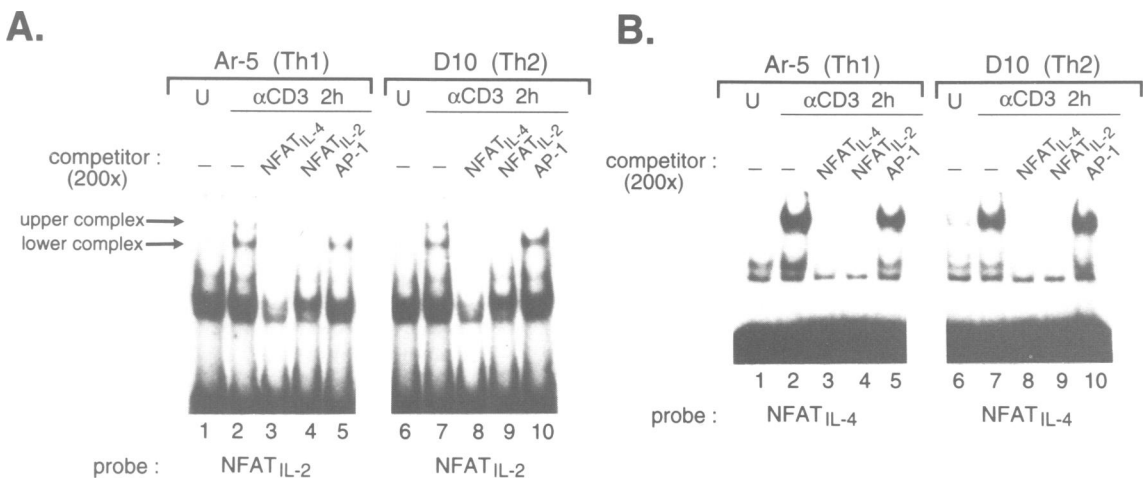

Fig. 7. The anti-CD3/ionomycin-inducible Th2/IL-4 NF-AT complex consists of the Th1/IL-2 NF-ATp complex but lacks AP-1. **(A)** Activated D10 cells contain an NF-AT complex containing AP-1 which binds to the IL-2 NF-AT site. Binding assays were performed using nuclear extracts from activated D10 and Ar-5 cells (treated with anti-CD3 for 2 h) with the radiolabelled IL-2 NF-AT oligonucleotide using binding conditions as in Figure 1 except that 200 ng of sheared poly(dI-dC) was used. Under these conditions, the upper NF-AT/AP-1 complex is obtained with both Ar-5 and D10 extracts (lanes 2 and 7). The addition of a 200-fold excess of an oligonucleotide containing the human metallothionein AP-1 site specifically competed for this upper complex (lanes 5 and 10). Identical results were seen using the Th1 clone, D1.1, and the Th2 clone, CDC35. **(B)** The Th2/IL-4 NF-AT complex lacks an AP-1 component. Binding assays were performed as in (A) except that an oligonucleotide containing the IL-4 NF-AT site was used. Nuclear extracts from both activated D10 and Ar-5 cells form a single complex which lacks an AP-1 component, as shown by the inability of the human metallothionein AP-1 oligonucleotide to compete (lanes 5 and 10).

Unlike the NF-AT complex which binds to the IL-2 promoter, however, the IL-4 NF-AT complex did not contain an AP-1 component as shown by the inability of AP-1 oligonucleotides to compete for DNA binding, and the inability of anti-Fos antibodies to supershift the complex. Nuclear extracts from anti-CD3-induced Th1 and Th2 cells

were competent for NF-ATp/AP-1 upper complex formation in EMSA analyses, indicating that the ability to form an AP-1-containing NF-AT complex is not Th1- or Th2-specific, but instead is dictated by the particular NF-AT binding site. This is in agreement with the dependency for formation of the AP-1-containing NF-AT complex on a sequence bearing homology to a consensus AP-1 site, TGTTTCA, located from −278 to −272, which overlaps the distal NF-AT site in the IL-2 promoter (J.Jain, personal communication), but is absent from the IL-4 NF-AT site (Figure 2B). Deletion of this AP-1 site from the IL-2 NF-AT site inhibits formation of the NF-ATp/AP-1 complex (J.Jain, personal communication).

Transient transfections of D10 cells using a CAT expression construct which contained three multimeric copies of the −60 to −79 region of the IL-4 promoter, when compared with similar experiments by other groups using an identical construct containing three copies of the murine IL-2 NF-AT site, revealed interesting differences. Both the IL-4 and IL-2 NF-AT multimer/CAT constructs have been shown to be inducible by treatment with anti-CD3 (Jain *et al.*, 1992a; Kang *et al.*, 1992; Woodrow *et al.*, 1993). However, different results are seen with treatments by phorbol esters. Whereas the IL-2 NF-AT multimer/CAT construct is strongly induced (to levels as high as with anti-CD3) with PMA (Woodrow *et al.*, 1993), no induction of the IL-4 NF-AT multimer/CAT construct by PMA is seen in our hands (Figure 2). Furthermore, treatment of cells transfected with the IL-2 NF-AT multimer/CAT construct with PMA and ionomycin results in a synergistic induction that is approximately twice that obtained with anti-CD3 (Woodrow *et al.*, 1993). Treatment with PMA and ionomycin resulted in no greater induction of the IL-4 NF-AT multimer/CAT construct than did treatment with ionomycin alone (Figure 2). Finally, when compared with the same results published by Woodrow *et al.*, the effect of ionomycin treatment on the induction of the IL-4 NF-AT multimer/CAT construct is considerably higher (~3-fold) than that obtained using the IL-2 NF-AT multimer/CAT construct (Figure 2). Similar differences are seen when one looks at the binding characteristics of NF-AT to the IL-2 and IL-4 NF-AT sites. The binding of NF-AT to the IL-2 NF-AT site is induced by PMA alone, and the binding induced by ionomycin is strongly augmented in the presence of PMA (Hivroz-Birgaud *et al.*, 1991). Using the IL-4 NF-AT site, however, no binding is seen upon treatment with PMA alone, and no augmentation of ionomycin-induced binding is seen by the addition of PMA. The differences of PMA-inducibility on NF-AT complex formation and NF-AT multimer/CAT construct expression would suggest that PKC activity may be involved in activation of the IL-2 NF-AT site but not the IL-4 NF-AT site, and are consistent with the ability of the IL-4 NF-AT complex to activate transcription from the IL-4 promoter in the absence of an AP-1 component.

The current model for NF-AT activation, based on the distal IL-2 NF-AT site, consists of two separate stimulation-dependent steps. One is cyclosporin-sensitive, and probably involves the modification of the pre-existing NF-ATp and/or its translocation to the nucleus (Flanagan *et al.*, 1991; McCaffrey *et al.*, 1993a). NF-ATp is a phosphoprotein, whose dephosphorylation and translocation to the nucleus are prevented by agents which inhibit the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, such as cyclosporin A and a specific peptide inhibitor (McCaffrey *et al.*, 1993a). The second step is insensitive to cyclosporin A and involves the PKC-dependent addition of a newly synthesized AP-1 component to the nuclear translocated NF-ATp in stimulated T cells (Flanagan *et al.*, 1991; Jain *et al.*, 1992a). In the case of the Th2-specific cytokine gene, IL-4, we propose a model in which only the first of the aforementioned two steps is necessary for NF-AT activation of IL-4 promoter activity. Increases in intracellular calcium alone, as a result of treatment with ionomycin, are sufficient for transcription mediated by the IL-4 NF-AT site. Similar to IL-2 NF-AT activation, the activation of transcription of the IL-2 gene involves a two-step mechanism requiring both calcium mobilization and PKC activation (Abbas *et al.*, 1991; Rao, 1991). In contrast, increasing evidence suggests that the transcriptional activation of the IL-4 gene, like activation by the IL-4 NF-AT site, may require only increases in calcium and may be independent of PKC activation. Several Th2 cell clones require only ionomycin, and not PKC, for IL-4 production (Gajewski *et al.*, 1990; Arai *et al.*, 1992; Muñoz *et al.*, 1990; M.Kubo, R.L.Kincaid, D.R.Webb and J.T.Ransome, in preparation). Also, IL-4 production by Th2 clones is 10-fold less sensitive to inhibition by cyclosporin A than is IL-2 production by Th1 clones (Gajewski *et al.*, 1990). Additionally, a recent study shows that the transient over-expression of a constitutively active, mutant form of calcineurin alone is sufficient to result in the large induction of IL-4 promoter activity (M.Kubo, R.L.Kincaid, D.R.Webb and J.T.Ransome, in preparation), whereas this was shown to be not sufficient for the activation of the IL-2 promoter, which required, in addition, activation of the PKC pathway by phorbol esters (O'Keefe *et al.*, 1992). We show here that this constitutively active form of calcineurin is also sufficient to activate an IL-4 NF-AT multimer/CAT construct in transiently transfected D10 cells (Figure 2). Both IL-2 promoter-driven and IL-2 NF-AT-driven transcription has been shown to be mediated by $p21^{ras}$, which in turn is dependent upon PKC activation (Downward *et al.*, 1990). Studies using constitutively active and inactive forms of $p21^{ras}$ have dramatically increased and decreased, respectively, IL-2 promoter (Rayter *et al.*, 1992) and IL-2 NF-AT (Woodrow *et al.*, 1993) activation. One might predict that the IL-4 promoter and IL-4 NF-AT site would, in contrast, be unaffected by these $p21^{ras}$ variants. Finally, whereas there exist several PKC-dependent AP-1 sites in the IL-2 promoter (Jain *et al.*, 1992b), a homology search of a region encompassing 800 bp upstream of the IL-4 transcription start site using the Transcription Factors Database of Regulatory Sites (Ghosh *et al.*, 1990) located only two potential AP-1 sites with homologies to known AP-1 sites of >60%: 5/8 (62%) at −760, and 7/10 (70%) at −711, regions whose importance for the inducibility of the IL-4 promoter has yet to be shown.

Taken together, these data suggest that PKC-dependent regulatory promoter elements, such as AP-1 sites, or the IL-2 NF-AT site, might play a role in Th1-specific, but not Th2-specific, cytokine gene expression.

Materials and methods

Mice

Female BALB/c (H-2^d) and AKR/J (H-2^k) mice were purchased from Taconic (Germantown, NY) and used between 6 weeks and 4 months of age.

Cell lines

The generation and maintenance of the cloned Th2 cell clone D10.G4.1 (D10) (American Type Culture Collection, Rockville, MD), specific for conalbumin in the context of I-A^k, and the Th1 cell clone Ar-5 [specific for *p*-azobenzenearsonate-ovalbumin (ARS-OVA) in the context of I-A^d] have been described (Kaye *et al.*, 1983; Rao *et al.*, 1984). Briefly, Th cell lines which had been allowed to rest and were not proliferating were stimulated every 2 weeks with antigen and irradiated syngeneic splenocytes as antigen-presenting cells. α-Methylmannoside-containing supernatant from rat spleen cells that had been stimulated with Con A for 48 h was added at 10% as a source of lymphokines, to complete medium consisting of RPMI 1640 medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol. M12.4.1 (murine mature B cell lymphoma), SA-1 (murine sarcoma), BALB/c 3T3 and DAP (murine fibroblasts) were cultured in the same complete medium except without rat Con A supernatant.

Stimulation of cell lines

Cell lines were treated in anti-CD3ε (2C11)-coated plates (coated at 1 μg/ml in 40 mM Tris−Cl, pH 9.5, overnight at 4°C, and then washed extensively with 1 × PBS), by addition of 1 μM ionomycin (Calbiochem Corp., La Jolla, CA), and/or 100 ng/ml PMA (Sigma) and/or 1 μg/ml CsA (gift from A.Abbas, Harvard Medical School). For kinetic experiments, T cells were placed in 2C11-coated flasks and were centrifuged on to the coated surface at 4°C to ensure synchronous activation. Cells centrifuged on to uncoated flasks were not stimulated (data not shown).

Transient transfections and CAT assays

Transient transfections by electroporation were performed as described previously (Todd *et al.*, 1993). CAT assays were performed using a modification of the method of Sleigh (1986) in which [^{3}H]acetyl CoA was used instead of [^{14}C]acetyl CoA. The plasmids pSRα-296 (Takebe *et al.*, 1988) and pSRα-ΔCaM-AI (O'Keefe *et al.*, 1992) were kindly provided by Randall Kincaid. The plasmids pSVO-157, pSVO-CATapoly and p(IL-4 NF-AT)$_{3F}$-CAT have been previously described (Todd *et al.*, 1993).

Electrophoretic mobility shift assays

Small-scale nuclear extracts were made from 2 × 10^7 unactivated or activated cells by a modification (Jamieson *et al.*, 1991) of the method by Dignam *et al.* (1983). Cytosolic extracts were made by extensive centrifugal dialysis against Solution D using Centricon-10 spin columns (Amicon Corp., Beverly, MA) at 4°C. Protein determinations were done by the method of Bradford (1976) using bovine serum albumin as a standard. Binding assays were performed at room temperature for 30 min using 2 μg (nuclear) or 5 μg (cytosolic) protein extracts and 0.25 ng radiolabelled oligonucleotides in a 10 μl volume containing 8 mM HEPES, pH 7.4, 0.3 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 0.8 mM EDTA, 0.5 mM DTT, 9% glycerol and 3 μg poly(dI-dC), of which 5 μl were loaded on a 0.25 × TBE/4% nondenaturing polyacrylamide gel. Formation of the upper NF-ATp/AP-1 complex was favoured by using 200 ng of sheared poly(dI-dC) per binding reaction. PCBAs were performed by the addition of varying concentrations of proteases to binding reactions which were carried out at 4°C.

Oligonucleotides used in EMSA analyses included from −79 to −60 of the murine IL-4 promoter, 5′-ATAAAATTTTCCAATGTAAA-3′, and the mutant variants mut1 (5′-AGCCCATTTTCCAATGTAAA-3′), mut2 (5′-ATAAACGGGTCCAATGTAAA-3′), mut3 (5′-ATAAAATTTGAACATGTAAA-3′) and mut4 (5′-ATAAAATTTTCCACGTGAAA-3′). The oligonucleotide containing the murine IL-2 distal NF-AT site encompassed the region from −295 to −267 (as shown in Figure 3B): 5′-GCCCAAAGAGGAAAATTTGTTTCATACAG-3′. The AP-1 site-containing oligonucleotides included the FS-TRE (5′-AAAAACATGACTCAGAGGAAAACATAC-3′), which corresponds to FSE2 sequences in the AP2 gene (Distel *et al.*, 1987), and the MT-TRE (5′-GAGCCGCAAGTGACTCAGCGCGGGGCG-3′), which corresponds to sequences in the human metallothionein IIA gene (Angel *et al.*, 1987).

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