Identification and Characterization of a Critical CP2-binding Element in the Human Interleukin-4 Promoter*

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Expression of cytokine genes in T cells is thought to result from a complex network of antigen- and mitogen-activated transcriptional regulators. CP2, a factor homologous to Drosophila Elf-1 and previously found to be a critical regulator of several viral and cellular genes in response to developmental signals, is rapidly activated in T helper (Th) cells in response to mitogenic stimulation. Here we show that overexpression of CP2 enhances interleukin (IL)-4 promoter-driven chloramphenicol acetyltransferase expression, while repressing IL-2 promoter activity, in transiently transfected Jurkat cells. A CP2-protected element, partially overlapping the nucleotide factor of activated T cell-binding P2 sequence, was required for IL-4 promoter activation in CP2-overexpressing Jurkat cells. This CP2-response element is the site of a cooperative interaction between CP2 and an inducible heteromeric co-factor(s). Mutation of conserved nucleotide contacts within the CP2-response element prevented CP2 binding and significantly reduced constitutive and induced IL-4 promoter activity. Expression of a CP2 mutant lacking the Elf-1-homology region of the DNA-binding domain inhibited IL-4 promoter activity in a dominant negative fashion in transiently transfected Jurkat cells. Moreover, overexpressed CP2 markedly enhanced, while its dominant negative mutant consistently suppressed, expression of the endogenous IL-4 gene in the murine Th2 cell line D10. Taken together, these findings point to CP2 as a critical IL-4 transactivator in Th cells.

Interleukin (IL)-4 is a pleiotropic cytokine that modulates the differentiation and the biologic activities of virtually all cells of hematopoietic origin (1). IL-4 is typically expressed in and, at the same time, promotes the differentiation of the T helper 2 (Th2) functional subset of CD4+ T cells, thereby playing a pivotal role in the regulation of humoral and allergic responses. Conversely, Th1 cell-associated cytokines, such as IL-2 and interferon-γ, are central to the development of cell-mediated, delayed type, and autoimmune responses (2). The biochemical and molecular mechanisms accounting for polarized expression of cytokine genes in T cells have been the focus of a number of studies over the past few years (3). Calcineurin-mediated activation of members of the nuclear factor of activated T cell (NFAT) family of transcription factors has been associated with antigen-dependent cytokine gene expression in both Th1 and Th2 cells (4). However, NFAT-directed IL-4 transcription is preferentially induced in Th2 cells, presumably due to the involvement of Th2-restricted co-factors (5, 6).

It is likely that the commitment toward an IL-4-producing, Th2 phenotype is the outcome of a complex, dynamic interaction of multiple transcriptional regulators rather than the effect of a single protein. Irrespective of the cytokine milieu leading to preferential Th1 or Th2 differentiation (7), priming of T cells by antigen or mitogens is a requirement for IL-4 but not IL-2 production (7). In fact, the IL-4 gene is only transcribed in actively dividing T cells, while IL-2 expression occurs independently of the cell cycle (8). T cell priming affects the expression and/or activation of a number of factors presumably involved in the regulation of cytokine genes. For example, primed T cells express higher levels of NFAT-1 and support greater NFAT-directed transcription than naive T cells (9). An earlier study also showed rapid and marked up-regulation of the DNA binding activity of the transcription factor CP2 following mitogenic stimulation of peripheral blood naive T cells, suggesting the involvement of this protein in the activation of immediate and/or early genes in these cells (10).

CP2, also known as leader-binding protein (LBP)-1c (11) or late simian virus 40 factor (12), is the prototypical member of a novel family of mammalian proteins sharing a high degree of similarity to Elf-1, a Drosophila melanogaster tissue-specific factor encoded at the embryonic lethal locus Grainyhead (11, 13). By interacting with a hyphenated sequence composed of two directly repeated 4-base pair (bp) motifs separated by a 6-bp linker (CNRG-Nc-CNR(G/C)) (11, 14), CP2 has been reported to regulate transcription of a number of viral and cellular genes in response to developmental signals, including those encoding for rat γ-fibrinogen (15), mouse α-globin (16), simian virus 40 (12), human immunodeficiency virus-1 (11), herpes simplex virus-1 (17), γ- and ε-globin, and the human β-like globin gene cluster (18), major histocompatibility complex class
II Ea (19), and human c-fos and mouse thymidylate synthase (20). However, its contribution to cytokine gene expression in T cells has not been explored to date. In this study, we investigated the contribution of CP2 to the mechanisms regulating expression of the two pivotal T cell-restricted cytokines IL-2 and IL-4.

EXPERIMENTAL PROCEDURES

Cells—A line of Jurkat T cells, which constitutionally expresses IL-4 and produces IL-2 and interferon-γ following activation, was a gift by Dr. Jack L. Strominger (Harvard University). Cells were cultured in RPMI (Mediatech, Herndon, VA), containing 2 mM l-glutamine, 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, Calabasas, CA), and 50 μg/ml gentamicin (complete medium). Aliquots of cells frozen at early passages were recovered from liquid nitrogen and used for experiments between 1 and 6 weeks after thawing. The murine conalbumin-specific Th2 clone D10.G4.1 (D10; American Type Culture Collection, Manassas, VA) was maintained in culture by biweekly stimulation with 10 μg/ml concanavalin A in the presence of 2 mM l-glutamine, 10% heat-inactivated fetal bovine serum, 2 mM -glutamine, 100 units/ml penicillin, 0.1% streptomycin, and 50 μg/ml 2-mercaptoethanol. IL-4 expression in these cultures was verified using a sensitive commercial enzyme-linked immunosorbent assay (Cytoscreen-US®; BIOSOURCE International, Camarillo, CA).

Plasmids—The IL-4 promoter fragments used in this study were generated by polymerase chain reaction using human genomic DNA as a template and cloned into the HindIII and XhoI sites of a Bluescript vector (Stratagene Cloning Systems, La Jolla, CA). An XhoI-tailed oligonucleotide, corresponding to bp +36 to +55 of the human IL-4 gene, and one of eight HindIII-tailed oligonucleotides, corresponding to bp −741 to −722, −311 to −292, −265 to −246, −225 to −206, −175 to −156, −145 to −126, −95 to −76, or −65 to −46, were used to introduce the appropriate restriction sites at the invariant 5′- and at the 5′-ends, respectively. Synthetic oligonucleotides were cloned into the HindIII and XhoI sites of the chloramphenicol acetyltransferase (CAT)-expressing reporter vector, pCAT-Basic (Promega, Madison, WI) to construct the corresponding reporter plasmids IL-4:471, IL-4:311, IL-4:265, IL-4:225, IL-4:175, IL-4:145, IL-4:95, and IL-4:65 (21). An IL-4:225 plasmid carrying two point mutations within the CP2-response element (CPRE) was generated by site-directed mutagenesis (QuikChange®; Stratagene). The following mutagenic primer (mutations are underlined) and its complement were synthesized (Genosys Biotechnologies, The Woodlands, TX): 5′-CATTTTCTATTGTATTATTATTATTCCATTTTGG-3′ (225–176), 5′-AGTTGCTTTACATTGTTGTGCTGTTCTTACCAGAACATTTTACCTTTGTTT-3′ (195–146), 5′-TCGTATTTTGATGGCCAGAAATTTTGCTTTGTTT-3′ (156–105), 5′-TGCATTTGCAGCAGTATTGTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTAT
and of a Maxam-Gilbert G + A ladder of the same probe (27) were resolved by electrophoresis on an 8% acrylamide, 7 M urea gel.

RESULTS

Differential Effect of CP2 Overexpression on the IL-4 and IL-2 Promoters—Several potential CP2 elements are located within the proximal 300 bp of the human IL-4 promoter. We analyzed the effect of transient CP2 overexpression on CAT gene expression driven by an IL-4 promoter region included between bp −311 and +55 in the human T cell line Jurkat. In agreement with previous studies (24, 28, 29), we found that these cells are in a preactivated state with respect to IL-4 expression, characterized by constitutively elevated IL-4 mRNA accumulation (data not shown) and promoter activity (Fig. 1). Constitutive IL-4 promoter activity was enhanced up to 5-fold in Jurkat cells transiently transfected with a CP2 expression plasmid (Fig. 1). CP2 overexpression resulted in less noticeable increase of IL-4 promoter activity in cells stimulated with PMA (10 ng/ml) and the Ca²⁺ ionophore A23187 (1 μM), presumably due to the contribution of endogenous CP2 to IL-4 activation in these cells (see below). In striking contrast, PMA- and A23187-induced IL-2 promoter activity was reduced by up to 80% in CP2-overexpressing cells (Fig. 1).

Identification of a CRE in the IL-4 Promoter—To map the IL-4 promoter element(s) necessary for transcription by CP2, we generated a panel of deletional mutants for use in reporter studies (Fig. 2A) (21). Transiently overexpressed CP2 was more effective on promoter constructs truncated at bp −265 or −225 than on IL-4-311 or IL-4-741 (Fig. 2B). However, removal of the region spanning bp −225 to −176 decreased transcriptional activation in CP2-overexpressing cells by almost 4-fold, while constructs truncated at bp −145 through −65 were unresponsive to the factor (Fig. 2B). This indicated that a CRE is located between bp −175 and −146, although additional nucleotide contacts upstream of bp −175 might be required for full promoter inducibility by CP2.

The IL-4 promoter region included between bp −225 and −146, shown in Fig. 2C, harbors a number of elements contributing to a varying degree to IL-4 transcriptional activation (21, 30, 31). To verify whether CP2 can directly bind to elements located within this IL-4 promoter region, we generated three oligonucleotides spanning partially overlapping sequences for use in EMSA (Fig. 2C). rCP2, used at a concentration (100 ng/15 μl) sufficient for saturation of an α-globin consensus oligonucleotide (Fig. 2D, lane 1), did not bind to oligonucleotides 175–146 (lane 2) and 225–176 (lane 4). Since nucleotide contacts in both subregions might be required for CP2 binding, we also used as a probe an oligonucleotide (195–146) including the proximal 20 bp of the 225–176 segment in addition to 175–146 (Fig. 2C). This resulted in consistent formation of a CP2 complex of apparently lower affinity than that formed on the α-globin probe (Fig. 2D, lane 3). A complex of faster mobility also formed on the 195–146 oligonucleotide, which was accounted for by binding of monomeric CP2, as assessed by co-incubation with CP2-specific antibodies (data not shown).

To define the nucleotide contacts necessary for CP2 binding to this region of the IL-4 promoter, we analyzed the pattern of protection from chemical cleavage of a 195–146 oligonucleotide following EMSA with rCP2. In experiments using copper-phe- nanthroline as the cleaving agent (27), rCP2 (100 ng) protected a sequence extending from bp −177 to −158 (Fig. 2E). A CRE (−172CTGATTTCACAGG−162) is recognizable within this sequence. Its homology to CP2 elements identified within other cellular and viral promoters is shown in Fig. 2F.

Binding of Endogenous CP2 to the IL-4 CRE—A constitutive CP2-immunoreactive complex was formed in EMSA using a consensus α-globin CP2 oligonucleotide and nuclear extracts from Jurkat cells (Fig. 3, A and B). CP2 nuclear expression and/or DNA binding activity in these cells was not affected by stimulation with PMA and A23187 (Fig. 3A, lanes 2 and 3). Four complexes were detectable in EMSA with Jurkat nuclear extracts and a similar length oligonucleotide probe centered on the IL-4 promoter CRE (lanes 4–6). Differently from the α-globin complex, Ca²⁺-mediated stimulation did affect formation of the slower mobility complexes I and II, with consistently increased formation of complex II and diminished complex I formation (lane 5). Both complexes appeared to contain immunoreactive CP2, as indicated by the neutralizing effect of rabbit anti-CP2 antibodies (Fig. 3B, lane 6), while neither complex reacted to CBF-A-, NFAT-1-, or STAT6-specific antibodies (lanes 7 and 8 and data not shown). Complexes III and IV apparently consisted of unrelated constitutive nuclear protein(s) of unclear sequence specificity.

To elucidate the contribution of endogenously expressed CP2 to constitutive and inducible activity of the IL-4 promoter, we analyzed CAT expression in Jurkat cells transiently transfected with plasmids carrying point mutations within the IL-4 CRE that would selectively affect the formation of the CP2-binding domain (13) and referred to as elf-1 (−172ATATTTCACAGG−162) was sufficient to markedly decrease CP2 binding in EMSA using Jurkat nuclear extracts (Fig. 3C) or rCP2 (not shown). This was accompanied by an almost 50% decrease in constitutive and induced CAT expression in Jurkat cells transiently transfected with an IL-4-225 plasmid carrying the same mutation within the CRE (Fig. 3D).

IL-4 Transcriptional Repression in T Cells Expressing a CP2 Dominant Negative—To further assess the contribution of endogenous CP2 to IL-4 promoter activity, we generated, for use in transient transfection experiments, a plasmid encoding a CP2 polypeptide lacking the Elf-1 homology region of the DNA-binding domain (13) and referred to as Elf-1 mutant. In a previous study, this mutant did not bind to DNA and specifically inhibited binding of full-length CP2 in a dominant negative fashion (22). Its overexpression inhibited by >75% constitutive IL-4 promoter activity in Jurkat cells (Fig. 4A). A similar
FIG. 2. Identification of a CPRE in the human IL-4 promoter. A, schematic representation of the human IL-4 promoter and of the polymerase chain reaction-generated fragments bearing 5' deletions to the indicated bp upstream of the IL-4 gene transcription initiation site. Insertion into pCAT-Basic generated the reporter constructs IL-4.741 (not shown), IL-4.311, IL-4.265, IL-4.225, IL-4.175, IL-4.145, IL-4.95, and IL-4.65. Open and closed rectangles indicate the relative positions of positive and negative regulatory elements, respectively, identified to date in the IL-4 promoter. P0–P4, the five NFAT-binding P sequences (30); CCAAT, binding elements for CBF (31). Octamer-associated protein (OAP) and c-Maf response element (MARE) bind activation protein-1 family members (i.e., JunB) and the related proto-oncprotein c-Maf, respectively (5, 6). The negative regulatory elements (NRE-I and -II) bind as yet unidentified transcriptional repressor(s) (29). Interferon stimulation-response element (ISRE) is a negative regulatory interferon-responsive factor-2-binding element (31). Box II, A/T-rich region contributing to repression by high mobility group protein I(Y) (36). B, Jurkat cells were transiently transfected with 1 μg each of the indicated human IL-4 promoter constructs and 2 μg of a pRc/CMV-CP2 expression vector or its corresponding noncoding control (dashed line). Shown is mean ± S.E. fold induction of control CAT expression in three independent experiments. C, sequence of the region required for IL-4 promoter activation by overexpressed CP2. The relevant elements and their cognate transcription factors are indicated. Shown is, among other elements also indicated in α, the recently identified P2 negative regulatory element (NRE), binding the putative transcriptional repressor Rep-1 (21). Also shown is a schematic representation of three oligonucleotides (225–176, 195–146, and 175–146), spanning promoter subregions included between the indicated bp, that were used as probes in EMSA using rCP2. HMG I(Y), high mobility group protein I(Y); IRF-2, interferon-responsive factor-2; ISRE, interferon stimulation-response element. D, shown for comparison (lane 1) is the binding of rCP2 (100 ng) to an oligonucleotide probe including the proximal α-globin CP2 consensus site. The formation of a complex with CP2 homodimers is indicated. E, copper-phenanthroline footprinting of a 195–146 oligonucleotide,
degree of IL-4 promoter inhibition was seen in cells stimulated with A23187, while IL-2 promoter activity was fundamentally unaffected (data not shown).

Up-regulation of IL-4 promoter activity in CP2-overexpressing Jurkat cells was often paralleled by markedly increased IL-4 secretion, suggesting direct activation of the endogenous IL-4 gene (data not shown). However, due to low level expression of IL-4 and the poor transfection efficiency in these cells, the effect of overexpressed ΔElf-1 CP2 was far less apparent (not shown). To elucidate the role of CP2 in transcriptional activation of the endogenous IL-4 gene, we therefore assessed the effect of overexpressed CP2 polypeptides on IL-4 transcript accumulation in the murine Th2 cell line D10. As shown in Fig. 4, B and C, D10 cells transfected to higher than 50% efficiency with a full-length CP2-encoding plasmid expressed, upon CD3 ligation, markedly higher levels of IL-4 mRNA than cells transfected with the corresponding noncoding plasmid. In contrast, and in clear agreement with our findings shown in Fig. 4A, overexpression of the dominant negative ΔElf-1 mutant consistently interfered with CD3-dependent activation of the IL-4 gene in D10 cells.

DISCUSSION

In this study, we provide the first evidence of the involvement of CP2, the prototypical member of a novel family of transcription factors related to the Drosophila developmental protein Elf-1, in the regulation of cytokine genes expressed in T cells. CP2 has been involved in gene regulation in a variety of cell lineages, where it can act as both a transcriptional activator and a repressor (14, 22, 32, 33). We confirm here the dual nature of this factor, as we found that CP2 can repress IL-2 promoter activity, while its expression and function are required for IL-4 promoter-driven transcription in the human T cell line Jurkat (Figs. 1 and 4A) and for activation of the

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5'-end-labeled on the coding strand. Following EMSA with rCP2, free (F) and bound DNA (CP2) were cleaved within the gel matrix and then eluted and electrophoresed on an 8% sequencing gel. The alignment with a Maxam-Gilbert ladder (G+A) of the same probe is shown. Positions relative to the IL-4 transcription initiation site are indicated to the left. A hypersensitive site at bp −151 and a footprint extending from bp −177 to −158 are indicated to the right as an asterisk and a dashed line, respectively. F, the CP2-protected sequence is aligned with a series of CPREs identified in several cellular and viral promoters. The CNRG repeats (see “Discussion”) are indicated in boldface letters. Shown are the distal and proximal CPRE from the mouse α-globin promoter, the high affinity sites from the rat γ-fibrinogen promoter and the human immunodeficiency virus-1 long-terminal repeat, and a low affinity site from the major histocompatibility complex class II Ea proximal promoter. Also shown is an element within the mouse steroid 16α-hydroxylase gene (Cyp 2d-9) that interacts with the CP2-related protein LBP-1a (41).
endogenous IL-4 gene in the established murine Th2 line D10 (Fig. 4, B and C). These findings point to CP2 as a specific activator of the IL-4 gene in T cells.

We show that a CPRE is located between bp -178 and -158 of the human IL-4 promoter, a region previously shown to be required for maximal promoter activation in Th cells (30, 34). The IL-4 CPRE (−174CGATTTGCAGG−162) only diverges by 1 bp from the reported CP2 consensus (Fig. 2F), which was defined by assessing the effect of in-frame clustered mutations within high affinity CP2 sites, such as the proximal α-globin site or the γ-fibrinogen site, on rCP2 binding in EMSA (14). While the nucleotide composition of the linker sequence was found to only marginally affect CP2 binding, spacing of the two CNRG motifs was critical for the stability of the CP2-DNA complex (14). Thus, CNRG repeats separated by 5 bp, such as the α-globin distal CPRE (Fig. 2F), can bind CP2 with about 4-fold lower affinity than elements containing a linker of 6 bp (14). Consistent with this view, the IL-4 CPRE, featuring a 5-bp linker, bound rCP2 with apparently lower affinity than the proximal α-globin site (Fig. 2D).

CP2 represents a novel family of homo-oligomeric transcription factors binding direct DNA repeats (13, 22). However, heteromeric interaction of CP2 with Elf-1-related and nonrelated factors, depending on the promoter context, has also been described (13, 35). In a previous study, two classes of CPRE have been defined (35). Type I CPRE, including the high affinity α-globin and γ-fibrinogen sites, preferentially bind CP2 as a homodimer or homotetramer (20), while type II CPRE, including sites within the γ- and ε-globin promoters, bind CP2 as an obligate heterodimer (35). Our findings show that the IL-4 CPRE behaves as a type II site. In fact, while rCP2 homodimers bind to this site with relatively low affinity, native CP2 accounts for the formation of two complexes having noticeably slower mobility than the complex formed on an α-globin type I site (Fig. 3, A and B) or in EMSA using rCP2 (Fig. 2D). This suggests that additional factor(s) might contribute to CP2 interaction with an otherwise low affinity site. Consistent with this view, Ca2+-mediated stimulation of Jurkat cells, while not affecting CP2 binding to the α-globin site, led to increased formation of a major IL-4 complex, with decreased formation of an additional, slower mobility complex (Fig. 3A). This suggests the involvement of a Ca2+-regulated, as yet unknown heteromeric co-factor(s) in CP2 interaction with the IL-4 promoter.

The IL-4 CPRE is surrounded by binding elements for the factors NFAT, activation protein-1, CBF, interferon response factor-2, and high mobility group protein I(Y) (30, 31, 36) (Fig. 2C). A CCAAT box, binding the constitutive factor CBF, lies immediately upstream of its 5’-end (31). Mutation of a 5-bp cluster spanning bp −178 to −174, thereby disrupting this CCAAT box and the distal CNRG motif of the CPRE, resulted in 40% reduction of overall IL-4 promoter activity in Jurkat cells (31). We show (Fig. 3D) that the isolated disruption of the IL-4 CPRE is sufficient to produce a similar or greater decrease of promoter activity (−50%) in the same cellular host. Although CP2 and CBF, also known as CP1 or nuclear factor-Y, also bind to adjacent elements in the α-globin promoter (37), the two factors form mutually exclusive complexes on oligonucleotides including these elements (22). The 3’-half of the CPRE partially overlaps the NFAT-binding P2 sequence and a consensus STAT6 element (38). However, CP2 does not apparently interact with NFAT or STAT6 in EMSA using oligonucleotides including both the CPRE and the P2 sequence (data not shown).

Our finding of reduced transcriptional activity of constructs carrying a mutated CPRE demonstrated the critical involvement of endogenous CP2 in IL-4 promoter activity in Jurkat cells. This observation was confirmed using cells transiently expressing a CP2 mutant having disrupted DNA binding but intact dimerization domains. The DNA-binding domain of CP2 includes a region, featuring the highest Elf-1 homology, that has been reported to be essential for interaction of both CP2 and Elf-1 with their cognate sites (13, 14). This region, encoded by exon 6 of the CP2 gene and spanning amino acids 189–239, is missing in several non-DNA-binding CP2 variants, such as
LBP-1d, generated in several cell lines by alternative splicing of CP2 transcripts (11–13). Some studies have documented the ability of these polypeptides to function as CP2 antagonists, specifically inhibiting CP2-DNA interactions in vitro (13, 22). However, the expression in vivo of sufficiently high levels of these mutants to act as dominant negatives has not been demonstrated. Here we show that overexpression of the ΔElf1 mutant markedly represses IL-4 promoter activity in Jurkat cells as well as IL-4 gene expression in activated D10 cells (Fig. 4), stressing the role of CP2 in the activation of the IL-4 gene in T cells. Use of this dominant negative in vivo should provide a valuable tool to understand the role of CP2 in differential cytokine gene expression in Th cell clones.

CP2 is constitutively expressed in the nuclei of Jurkat (Fig. 3A) and D10 cells (not shown), and stimulation did not affect its expression and/or binding to an α-globin element in EMSA. Our data suggest that inducible co-factor(s) stabilize CP2 binding to the IL-4 CRE and contribute to IL-4 activation in stimulated cells. Jurkat cells are in a preactivated state with respect to IL-4 transcription and the nuclear expression of NFAT or other IL-4 promoter-binding factors (29, 39). Interestingly, both IL-4 expression and CP2 binding activity are induced by mitogenic stimulation of circulating naive Th cells in a cell cycle-dependent fashion (8, 10). Consistent with these findings, histone deacetylase inhibitors, such as butyrate, can suppress the cell cycle requirements for both IL-4 transcription and CP2 activation (8, 40). In conclusion, our findings support the hypothesis that CP2 expression and DNA-binding activity in T cells are correlated with IL-4 gene expression and point to CP2 as an important participant in the mechanisms regulating Th cell differentiation in the periphery.

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