The mechanism of action of the immunosuppressive drug cyclosporin A (CsA) is the inactivation of the Ca\(^2\+)/calmodulin-dependent serine-threonine phosphatase calcineurin by the drug-immunophilin complex. Inactive calcineurin is unable to activate the nuclear factor of activated T cells (NFAT), a transcription factor required for expression of the interleukin 2 (IL-2) gene. IL-2 production by CsA-treated cells is therefore dramatically reduced. We demonstrate here, however, that NFAT can be activated, and significant levels of IL-2 can be produced by the CsA-resistant CD28-signaling pathway. In transient transfection assays, both multicopy NFAT- and IL-2 promoter-β-galactosidase reporter gene constructs could be activated by phorbol 12-myristate 13-acetate (PMA)/αCD28 stimulation, and this activation was resistant to CsA. Electrophoretic mobility shift assay showed the induction of a CsA-resistant NFAT complex in the nuclear extracts of peripheral blood T cells stimulated with PMA plus αCD28. Peripheral blood T cells stimulated with PMA/αCD28 produced IL-2 in the presence of CsA. Collectively, these data suggest that NFAT can be activated and IL-2 can be produced in a calcineurin independent manner.

Nuclear factor of activated T cells (NFAT)\(^1\) is required for IL-2 production after antigenic stimulation of T cells (1, 2). Upon stimulation of T cells, an NFAT-containing protein complex appears in the nucleus and recognizes two or more sites in the IL-2 promoter (2, 3). The complex is composed of a pre-existing cytoplasmic component, which translocates to the nucleus, and an inducible nuclear component composed of members of the AP1 family (4–6). Translocation of the cytoplasmic component involves Ca\(^2\+)—induced activation of the calcium/caldmodulin-dependent serine-threonine phosphatase calcineurin (5, 7). Inactivation of calcineurin by the immunosuppressive drugs CsA or FK506 results in the blockage of translocation and inhibition of IL-2 production (7, 8).

CD28 is a T cell surface molecule that plays a major role as a co-receptor in the optimal activation of T cells (9). One of the major effects of CD28 stimulation is increased production of cytokines, such as IL-2, interferon γ, granulocyte macrophage colony stimulating factor, and others, through transcriptional and post-transcriptional mechanisms (10, 11). One of the hallmarks of the CD28 costimulation is its insensitivity to the immunosuppressive drugs CsA and FK506 (10, 12, 13). We reasoned that if CD28 costimulation results in IL-2 production, and if NFAT is required for the expression of IL-2, there must be a CsA-resistant pathway for activation of NFAT. In this report, we demonstrate that NFAT can be activated in a CsA-resistant manner.

MATERIALS AND METHODS

Cells and Tissue Culture—Freshly purified human peripheral blood T cells were greater than 95% CD\(^3\) cells. PMA was used at 10 ng/ml, ionomycin at 1 μM, and αCD28 monoclonal antibody (kindly provided by Dr. Carl H. June) at 100 ng/ml. Cyclosporin A (500 ng/ml) was added 30 min before addition of the other stimuli. Plasmids—The thymidine kinase-β-galactosidase (TK-βgal) reporter plasmid was constructed by subcloning a HindIII/Xho fragment containing the TK promoter from the pBlCAT2 vector (14) into the pEgQ3 LacZ plasmid (15). The NFAT-TK-βgal plasmid was constructed by synthesizing three copies of the IL-2 distal NFAT site (AAAGAG-GAGAAAAACGTTTCATACG), containing HindIII (at the 5’ end) and BamHI (at the 3’ end) restriction sites, and subcloning into the TK-βgal plasmid. The NFATβ-TK-βgal reporter plasmid was constructed as above, using the Igs B sequence (ACAAAGGGACTTTCCGCT). The expression vectors for NFAT, and for dominant negative NFAT (16) were generous gifts from Dr. Gerald R. Crabtree. The pL2-568 (15) construct (kindly provided by Dr. Christopher B. Wilson) was generated by subcloning the HindIII fragment (~568 to ~500) of the IL-2 promoter into the pEgQ3 LacZ plasmid. Transfection and β-Galactosidase Assay—Cells were transfected by a modified DEAE-dextran method (17). In brief, Jurkat cells were washed once with RPMI containing 50 μl/ml (pH 7.4). Cells were resuspended in the same medium at a density of 8 × 10\(^6\)/ml. An aliquot of 0.5 ml cell suspension containing appropriate plasmids was added to 0.5 ml of Tris-RPMI containing 500 μM DEAE-dextran. The aliquot was incubated for 1 h with occasional stirring, then centrifuged, washed once with Tris-RPMI, and resuspended in 5 ml of RPMI (10% fetal calf serum) and plated in a six-well plate. The cells were stimulated after 24 h and harvested for β-galactosidase assay 18 h after stimulation. When multiple stimulations for a particular plasmid(s) were used, transfection was done in a single tube. After transfection, cells were divided into groups depending on the various stimulations. β-Galactosidase was assayed as reported (18). The results were expressed as activity, calculated as follows: (A\(_{420}\)–A\(_{405}\))/g of protein) × 10\(^6\).

Electrophoretic Mobility Shift Assay (EMSA)—The preparation of nuclear extracts and EMSA were performed as described previously (19). The digonucleotides used were: IL-2 distal NFAT site (5’-AAAAGAGAGAAAAACGTTTCATACG-3’), AP1 site from human collagenase gene (5’-agatgagctgacacccctgctttctggag-3’), SP1 site from HIV-1 long terminal repeat (5’-gatcggagagcggcgagggaga-3’).

Antisera—Antisemur αNFAT was raised against a peptide that is
highly conserved in all NFAT proteins described to date. The peptide is found in the Rel homology domain, and its sequence is NH2-SDIEL-RKGETDIGRKNTR. Antiserum aNFATp was raised against a peptide specific to NFATp. Its sequence is VPAIKTEPSDEYEPSLI (sequence in murine NFATp, but the antiserum also recognizes the human protein).

Cytokine Testing—Human IL-2 was measured by ELISA (enzyme-linked immunosorbent assay) test kits from Genzyme Corp. (Cambridge, MA). The assays were performed by the Clinical Immunology Services Program, SAIC, NCI-FCRDC, Frederick, MD.

RESULTS

Induction of NFAT Activity in a CsA-resistant Pathway—To investigate whether NFAT can be activated by CD28 costimulation, three copies of the distal NFAT site from the IL-2 promoter were subcloned into a TK-βgal reporter gene construct, designated as NFAT-TK-βgal. This construct was transiently transfected into Jurkat T cells, and NFAT activity was assayed after various treatments of the cells. Since we were looking for a CsA-resistant pathway, we could not use αCD3 as a stimulus, because one of the pathways it activates is Ca2+-dependent. Therefore, we chose to mimic the Ca2+-independent aspect of αCD3 signaling by PMA, as described previously (20). PMA alone had little effect on reporter activity (Fig. 1A) and αCD28 alone had no effect (data not shown), but the combination of PMA and αCD28 resulted in significant activity (about 4-fold induction relative to the vector alone). Strikingly, nearly all of this (PMA + αCD28)-induced activity was resistant to CsA. In contrast, NFAT activity induced by PMA + ionomycin was essentially completely CsA-sensitive (Fig. 1B). These results suggest that, in addition to the well-known Ca2+-dependent pathway, there is a second Ca2+-independent way to activate NFAT.

In the experiment described above, it was possible that the assay measured some other unknown factor able to bind to the NFAT sites in the reporter plasmid rather than bona fide NFAT activity. To test this, the assays were repeated in the presence of cotransfected NFATc. Consistent with the results of others (16, 21), NFATc had little effect on the multicopy NFAT reporter in untreated cells (Fig. 1C), but had a marked effect in cells stimulated with PMA and αCD28 (15-fold induction of NFAT activity relative to the vector alone). As before, this induction was insensitive to CsA. In the same assay, promoter activity induced by PMA + ionomycin was abolished in the presence of CsA (data not shown). These results indicate that overexpressed NFATc protein can be activated through a CsA-resistant pathway.

In order to determine whether the IL-2 promoter can also be activated by a CsA-resistant pathway, the 568-base pair IL-2 promoter-β-galactosidase reporter construct (designated as pL2-568) was tested in the transient transfection assay (Fig. 2). Like the multicopy NFAT-TK-βgal construct, pL2-568 showed much higher promoter activity after PMA/αCD28 stimulation compared to either the unstimulated control or to PMA alone (panel A). This activity was further increased in the presence of overexpressed NFATc protein (panel B). The vector alone did not have any activity (data not shown). The inducible promoter activity was completely resistant to CsA (panels A and B), whereas CsA suppressed PMA/ionomycin-inducible promoter activity (panel C). To test whether the promoter activity induced by PMA + αCD28 was mediated by NFAT, a dominant-negative NFATc protein (16) was tested in transient transfection assay. As shown in Fig. 2, A and B, overexpression
of the dominant negative resulted in 70–80% inhibition of PMA/CD28-inducible promoter activity, but, as expected, the dominant negative NFATc protein had very little effect on an PMA/CD28-inducible promoter activity, but, as expected, the dominant negative resulted in 70–80% inhibition of mediated by NFAT.

other antiserum raised against an NFATp-specific epitope proteins was able to block the inducible complex (serum raised against a peptide that is common to all NFAT we performed supershift analysis. As shown in Fig. 3A, an antiserum raised against a peptide that is common to all NFAT showed a partial supershift (lane 4), whereas an irrelevant antiserum failed to react with the complex (lane 3). As shown in Fig. 3C, a PMA/αCD28-inducible complex was also present in the nuclear extracts of peripheral blood T cells (compare lane 2 with 1). As in Jurkat cells, this inducible complex was insensitive to CsA, whereas the PMA/ιCD28-inducible complex was sensitive to CsA (compare lanes 10 and 11). Competition assays and supershift analysis revealed that the PMA/ιCD28 complex contained NFAT protein as well as the members of the AP1 family. These data show that in Jurkat cells and in peripheral blood T cells the NFAT DNA-binding complex can be induced in a CsA-resistant manner by PMA/ιCD28 stimulation.

Production of IL-2 by Peripheral Blood T Cells after Stimulation with PMA and αCD28 in the Presence of CsA—It has been shown previously that treatment of peripheral blood T cells with PMA + αCD28 leads to IL-2 production, and that this induction is largely resistant to CsA (10, 12, 13). We expected this to be true under our conditions as well, since reporter assays showed activation of the IL-2 promoter and EMSA showed induction of the NFAT complex. To test for IL-2 production, peripheral blood T cells were stimulated with PMA and αCD28 for 18 h with or without CsA. As shown in Fig. 4, stimulation in the absence of CsA resulted in the production of over 200 units/ml of IL-2; in the presence of CsA, IL-2 was produced at 70–85% of this level (the slight drop may be due to toxic effects from the high concentration of drug used (500 ng/ml)). In contrast, CsA drastically reduced IL-2 production (to 7% of the level produced without CsA) in cells stimulated with PMA + ionomycin. In absolute terms as well, CsA-resistant IL-2 levels are substantially higher in PMA-CD28-stimulated cells (140 units/ml) than in PMA/ionomycin-treated cells (24 units/ml). These data demonstrate the physiological significance of CsA-resistant NFAT activation.
DISCUSSION

Previous studies have shown that the immunosuppressive drug CsA drastically inhibits, but does not completely block, IL-2 production following stimulation by αCD3 and αCD28 (10). The drug-resistant activity can most likely be traced to the CD28 signaling pathway, since stimulation of cells with PMA (to mimic the Ca2+—independent aspects of αCD3 signaling) and αCD28 is entirely resistant to CsA (10). In this case, IL-2 is produced at significant levels in the continued presence of CsA or FK506. Since the transcription factor NFAT is required for expression of the IL-2 gene, these results imply that, in addition to the well-known Ca2+—dependent pathway, NFAT can be activated by a CsA-resistant pathway. Data presented here show that this is the case.

The involvement of NFAT in the activation of reporter gene constructs (both NFAT-TK-βgal and pIL2–568) after PMA/αCD28 treatment in the presence of CsA was demonstrated by the facts that (i) overexpressed wild type NFAT augmented reporter activity only after PMA/αCD28 stimulation, and (ii) the inducible reporter activity was essentially eliminated in the presence of a dominant negative mutant of NFAT. The presence of NFAT in the PMA/αCD28-inducible DNA-protein complexes was also demonstrated by the supershift assays. In agreement with the transient transfection assays, the peripheral blood T cells were also shown to produce IL-2 after PMA/αCD28 treatment, and this production was resistant to CsA. The established pathway for NFAT activation involves Ca2+, dependent activation of the serine-threonine phosphatase calcineurin. Subsequent dephosphorylation of NFAT leads to its nuclear translocation and, in conjunction with AP-1, DNA binding. The data presented here suggest an alternate pathway for NFAT activation that is calcineurin independent. Since dephosphorylation of NFAT appears to be necessary for its activity, a different phosphatase must be involved in this pathway. A candidate second messenger (instead of Ca2+) is ceramide, which has been reported to be involved in CD28 signaling (22, 23). Interestingly, a serine/threonine phosphatase has been reported recently that is involved in the sphingomyelin

FIG. 3. EMSA demonstrating the presence of inducible NFAT complex. A, EMSA with 32P-labeled IL-2 distal NFAT site using nuclear extracts of Jurkat cells treated with medium (lane 1), PMA/CD28 (lane 2), PMA/CD28/CsA (lane 3), PMA/ionomycin (PMA/Iono.; lane 4), and PMA/ionomycin/CsA (lane 5). Lanes 6, 7, and 8 represent competition analysis of PMA/CD28-treated nuclear extracts using unlabeled specific competitor (S.C.), AP1 oligonucleotide, and SP1 oligonucleotide as a nonspecific competitor (N.C.). B, supershift analysis of PMA/CD28-inducible complex (lane 1) using αNFAT (lane 2), αNFATp (lane 4), and an irrelevant antiserum (IR, lane 3). C, EMSA with labeled IL-2 NFAT site using nuclear extracts of peripheral blood T cells treated with medium alone (lane 1), PMA/CD28 (lane 2), PMA/CD28/CsA (lane 3), PMA/ionomycin (PMA/Iono.; lane 4), and PMA/ionomycin/CsA (lane 5). Lanes 6, 7, and 8 represent competition analysis of PMA/CD28-treated nuclear extracts using unlabeled specific competitor (S.C.), AP1 site, and SP1 as a nonspecific competitor (N.C.), respectively. Lanes 7, 8, and 9 represent supershift analysis of PMA/CD28-inducible complex using αNFAT, αNFATp, and an irrelevant antiserum (IR), respectively.

FIG. 4. Production of IL-2 by peripheral blood T cells. Peripheral blood T cells isolated from two different donors were stimulated with medium alone, PMA/CD28, PMA/CD28/CsA, PMA/ionomycin, and PMA/ionomycin/CsA for 24 h. IL-2 secreted into the medium was measured by enzyme-linked immunosorbent assay. CsA was added to the cells 30 min prior to stimulation.
pathway (24). It is an attractive hypothesis that this or another ceramide-induced phosphatase can lead to NFAT activation in a CsA-resistant manner. This idea is currently being tested.

The present finding may explain the ineffectiveness of CsA in the treatment of graft versus host disease following allogenic bone marrow transplantation (25, 26). The cytotoxic T cells thought to be responsible for graft versus host disease (27) express CD28 on their surface and are capable of producing cytokines responsible for immune reaction in the presence of CsA.

Acknowledgments—We thank Dr. Scott Durum for his critical comments, William Bere for technical assistance, Susan Charbonneau and Joyce Vincent for their secretarial and editorial assistance, and the Cytokine Testing Laboratory for their help.

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J. Biol. Chem. 1996, 271:7700-7704.
doi: 10.1074/jbc.271.13.7700

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