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Cyclosporin A-Resistant Transactivation of the IL-2 Promoter Requires Activity of Okadaic Acid-Sensitive Serine/Threonine Phosphatases

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Expression of the IL-2 gene requires activation of T cells through stimulation of the TCR and costimulation through accessory receptors. We have found recently that okadaic acid-sensitive Ser/Thr phosphatases are involved in a cyclosporin A-insensitive pathway that selectively transmits costimulatory signals. In this study, we analyzed whether activities of these phosphatases are necessary for the expression of the IL-2 gene. In both activated peripheral blood T lymphocytes and activated tumorigenic T cell lines, IL-2 gene expression was blocked at the transcriptional level by okadaic acid. The transcription factors active at the IL-2 promoter were differentially influenced: upon down-modulation of okadaic acid-sensitive phosphatases, transactivation by octamer, NF-κB, and NF of activated T cells proteins was abrogated, while transactivation by AP-1 proteins was even enhanced. The Journal of Immunology, 1998, 161: 1803–1810.

Full activation of T lymphocytes requires two types of signals: those provided through the TCR/CD3 complex, and in addition, those induced by stimulation of coreceptors such as CD2, CD4, CD8, or CD28 (for review, see Ref. 1). T cell activation in the absence of costimulation leads to anergy or apoptosis (2–5). Signaling events induced by coreceptors overlap in part with the TCR signaling pathway. Recently, intracellular reactions clearly independent of the TCR have been identified (6, 7).

Expression of the IL-2 gene depends on an activation of both coreceptor- and TCR-triggered signal-transduction pathways (for review, see Ref. 8). These influence the activities of the transcription factors that mediate IL-2 transcription, namely Oct3, AP-1, NF-κB, and NF-AT proteins (for review, see Ref. 9). Protein kinase C is involved in the activation of the NF-κB transcription factor proteins, which requires their release from inactive cytoplasmic complexes (10–13). Members of the mitogen-activated protein kinase family regulate expression and activity of AP-1 proteins (14–16). The Ca/calmodulin-regulated Ser/Thr phosphatase 2B (PP2B)/calcineurin regulates the nuclear entry of the cytoplasmic components of the transcription factor NF-AT (17–19).

Additional phosphatases, namely okadaic acid-sensitive Ser/Thr phosphatases, were recently identified to be crucial for a cyclosporin A (CsA)-insensitive pathway, which is induced through the CD2 and CD28 coreceptors (6, 20, 21). Ser/Thr phosphatases are encoded by the gene families PPM and PPP. The PPP family comprises the subfamilies 1, 2A, and 5. The phosphatases of subfamilies 1, 2A, and 5 are sensitive to okadaic acid (22), while PP2B is inhibited by CsA.

During the CD2/CD28-induced signaling pathway, dephosphorylation of the essential phosphoprotein pp19/cofilin is catalyzed by okadaic acid-sensitive Ser/Thr phosphatases. Since dephosphorylation of pp19/cofilin correlates with IL-2 production, we addressed the question as to whether okadaic acid-sensitive Ser/Thr phosphatases influence the expression of the IL-2 gene. In this study, we show that indeed activities of okadaic acid-sensitive phosphatases are necessary to achieve transactivation of the IL-2 promoter by the NF-AT, NF-κB, and Oct transcription factor proteins.

Materials and Methods

Cell lines, tissue culture, and transfection procedure

Human primary T cells were prepared as described previously (21). EL4 cells and Jurkat cells were grown in RPMI supplemented with 5% (EL4) or 10% (Jurkat) FCS (Sigma, St. Louis, MO) and 1% penicillin/streptomycin (Life Technologies, Gaithersburg, MD). The Jurkat cell clone IL-2-luc stably transfected with the plasmid pIL-2luc (23) was a gift from B. Schraven Institute for Immunology, Heidelberg, Germany. EL4 cells were transfected, as described previously (24), using 1 μg of plasmid DNA per 2 × 10^6 cells. The constructs used were pnmoll2-2k-luc, pnmoll2-321-luc, and the control vector pcDNA3/luc; octp-luc, NFκB-luc, NFAT-luc, and the control vector pGL2 (25); 5× TRE-TATA CAT and the control vector TATA CAT (26); and 4× oct-luc and the control vector 4× oct mut-luc (27). Sixteen hours after transfection, cells were split in aliquots and treated with various stimulating agents. The concentrations used were 0.5 μM okadaic acid (Life Technologies); 0.1 μg/ml CsA (Sandoz, Basel, Switzerland); 10 ng/ml PMA (Sigma); 180 nM A23187 (Sigma); 10 μg/ml CD2 Abs M1, M2, and 3PT (28, 29); and 1 μg/ml CD28 Ab 9.3 (PharMingen, San Diego, CA).

Luciferase assay and CAT assay

Luciferase assays were performed with the Luciferase Assay System (Promega, Madison, WI). CAT assays were conducted using CAT ELISA (Boehringer Mannheim, Indianapolis, IN).

Isolation of RNA and Northern analysis

RNA was extracted from cells by a guanidinium isothiocyanate method. Before loading on a 1% agarose gel (1% in 1× MOPS; 1× MOPS equals 20 mM MOPS (3-N-morpholino)propanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA)), RNA samples were adjusted to 25% formamide, 1.1 M formaldehyde, 0.5× MOPS, 0.5% Ficoll, and 0.1% bromphenol blue.
After electrophoresis, gels were blotted onto Hybond N+ filters (Amer sham, Arlington Heights, IL). Prehybridization and hybridization were conducted at 65°C in solution HS (7% SDS, 1 mM EDTA, 0.5X phosphate buffer (1X phosphate buffer equals 2 M Na2HPO4;2H2O, 0.34% phosphoric acid)). For hybridization, cDNA probes (β-actin, human IL-2, murine IL-2 (30–32)) were labeled radioactively using a random hexanucleotide primer procedure (Stratagene, La Jolla, CA). Blot washes (at 65°C) were performed with 0.4X phosphate buffer, 1 mM EDTA, 5% SDS, and 0.04X phosphate buffer, 1 mM EDTA, 1% SDS. Finally, the blots were exposed to autoradiography.

Nuclear run-on analysis

Nuclear run-on analysis was performed, as described previously (33), using cDNA probes for the β-actin gene, the IL-2 gene, the c-jun gene, and the c-fos gene (30, 31, 34, 35) for the detection of the respective transcripts.

In vivo footprinting

In vivo footprinting was performed essentially as described (36). The oligonucleotide primers used to visualize the coding strand of the c-fos promoter were synthesized according to a previous report (37). Quantitation of band intensities was performed through scanning of the autoradiograms using a Saphir Ultra laser densitometer. From top to bottom of each lane of the autoradiogram, an intensity profile was produced using the Image Quant 3.0 software (Fuji). Subsequently, the intensity values of individual profile peaks were determined. Intensity values of affected bands were related to the intensity value of a nonaffected band in the same area of the gel to compensate for loading differences (relative intensity values). For graph representation, relative intensity values obtained from an in vitro methylated DNA band were set 1, and the relative intensity values of corresponding bands in the four in vivo DNA preparations were related to it. G 77 of the Oct site, G 84 of the OAP site, G 118 and G 120 of the NF-AT site, and G 158 and G 160 of the AP-1 site were analyzed as individual bands, while G 173 and G 174 of the ATGG site, G 206, and G 207 of the NF-kB site, and G 225 and G 227 of the TGGGC site were pooled with neighboring bands. At the ATGG, NF-kB, and TGGGC sites, in vivo methylated DNAs are hyperreactive when compared with in vitro methylated DNA (Fig. 7). This situation occurring at the coding strand of the IL-2 promoter has been described earlier. Its significance remains unknown (36).

Results

Okadaic acid prevents IL-2 expression in human peripheral blood T lymphocytes

Activation of human peripheral blood T lymphocytes (PBL-T) was performed using a combination of mitogenic Abs directed at the CD2 coreceptor or, alternatively, by phorbol ester and Ca ionophore (PMA + A23187). CD2 triggering provides an activation signal to T cells (28, 38). Phorbol ester induces the activation of protein kinase C, whereas Ca ionophores lead to an elevation of intracellular calcium. This treatment mimics intracellular signals induced by antigenic stimulation of T cells via TCR/CD3 and coreceptors (39).

As expected, Northern blot analysis showed that both stimulation protocols induced the accumulation of IL-2 mRNA (Fig. 1, lanes 3 and 5), which was prevented completely when okadaic acid was added 30 min before stimulation at concentrations known to inhibit the okadaic acid-sensitive Ser/Thr phosphatases of subfamilies I, 2A, and 5 (22, 27, 40, 41) (Fig. 1, lanes 4 and 6). This treatment did not generally affect the functional activity of the cells, since a nuclear run-on analysis (see below, Fig. 2) revealed that other transcriptional processes were ongoing throughout cell treatment.

These results show that inhibition of okadaic acid-sensitive phosphatases abrogates the expression of the IL-2 gene in activated PBL-T cells.

Okadaic acid blocks IL-2 expression at the transcriptional level

We then analyzed the mechanisms underlying the inhibition of IL-2 expression by okadaic acid. Specifically, we addressed the question as to whether okadaic acid treatment results in mRNA destabilization and/or reduced mRNA synthesis. To discriminate between these possibilities, the transcription rate of the IL-2 gene was determined in nuclear run-on experiments (Fig. 2).

To this end, PBL-T cells were activated through CD2 or by treatment with PMA + A23187, in the presence or absence of okadaic acid. Both CD2 triggering and PMA + A23187 treatment induced transcription of the IL-2 gene (Fig. 2, lanes 3 and 7, IL-2), which was inhibited by okadaic acid (Fig. 2, lanes 4 and 8, IL-2). This effect was not due to a general down-regulation of transcriptional processes by okadaic acid, since the β-actin gene was transcribed at similar levels in both groups of differently treated cells (Fig. 2, β-actin). Moreover, transcription of the AP-1 transcription factors c-Fos and c-Jun, detectable in resting and activated PBL-T cells (Fig. 2, lanes 5 to 8), was even strongly induced by okadaic acid (Fig. 2, lanes 6 and 8).

To further elucidate the mechanisms underlying okadaic acid-mediated inhibition of IL-2 transcription, we performed IL-2 promoter studies using transient transfections of reporter gene constructs or the in vivo footprinting technique. Since PBL-T cells could not be used due to their limited experimental manipulability, the tumorigenic murine T lymphoma line EL4 and the human T cell line Jurkat were employed.

As shown before in PBL-T cells, the high level of IL-2 mRNA expression detectable in PMA + A23187-activated cells (Fig. 3,
Okadaic acid inhibits IL-2 mRNA expression in the tumorigenic cell lines Jurkat and EL4. Northern blot analysis was performed with RNA isolated from differently treated Jurkat (lanes 1–4) or EL4 (lanes 5–8) cells. Cells were cultured in medium (lanes 1 and 5) or treated with okadaic acid (lanes 2 and 6). For stimulation, cells were incubated for 6 h with PMA/A23187 in the absence (lanes 3 and 7) or presence (lanes 4 and 8) of okadaic acid. Okadaic acid was added 30 min before stimulation. For detection of transcripts, cDNA probes of the human IL-2 gene (Jurkat) and the murine IL-2 gene (EL4 cells), and the β-actin gene were used.

Okadaic acid inhibits a CsA-insensitive pathway of IL-2 expression

Like okadaic acid, the PP2B/calcineurin inhibitor CsA prevents IL-2 expression via a transcriptional block (43). To exclude the possibility that the blocking activity of okadaic acid on IL-2 transcription was mediated via inhibition of PP2B/calcineurin, we analyzed whether the PP2B/calcineurin-independent pathway of IL-2 expression induced by a combination of PMA and the activating CD28 Ab 9.3 (44) could also be inhibited by okadaic acid.

To investigate this point, a Jurkat cell clone stably transfected with the IL-2 enhancer luciferase reporter construct pIL-2luc2kb (23) was stimulated by a combination of PMA plus CD28 Ab. This resulted in an induction of luciferase activity, while treatment with each agent alone led to no significant change in the basal level of luciferase activity (Fig. 5, compare lanes 1, 4, 5, and 6). Treatment with okadaic acid, but not with CsA, completely abrogated the PMA/anti-CD28-inducible promoter activity (Fig. 5, lanes 7 and 8). In contrast, luciferase activity, induced by a combination of phorbol ester and Ca ionophore (Fig. 5, lane 9), was prevented completely by both okadaic acid and CsA (Fig. 5, lanes 10 and 11).

These data show that okadaic acid inhibits a PP2B/calcineurin-independent pathway involved in IL-2 expression.

Oct-, NF-kB, NF-AT-, but not AP-1-mediated transcription depends on okadaic acid-sensitive Ser/Thr phosphatases

To determine whether okadaic acid differentially influences the transactivation by individual transcription factors regulating IL-2 promoter activity, luciferase reporter constructs driven by monomeric NF-AT, NF-κB-, or Oct-binding elements derived from the IL-2 promoter (25) or the respective control vector were transfected into EL4 cells. After transfection, cells were treated with okadaic acid (OA), PMA + A23187 (P/A), or okadaic acid and PMA + A23187 (P/A + OA), respectively. In the case of okadaic acid treatment of EL4 cells, predominantly the Oct reporter was stimulated, whereas the extent of NF-κB- and NF-AT-reporter induction was very low (Fig. 6A, OA). PMA + A23187 treatment activated the Oct-, NF-κB-, and NF-AT-reporter constructs (Fig. 6A, P/A), while application of okadaic acid inhibited the PMA + A23187-inducible promoter activities of all three constructs (Fig. 6A, P/A + OA).
and indicate SE of the mean. The data reflect results of four independent experiments. Error bars expressed as multiples of the value measured in untreated cells, which was the protein in each probe. The figure shows the normalized luciferase activities of different cell populations were measured and normalized to the amount of luciferase protein. The IL-2 promoter of cells treated with PMA and OA further enhanced the activity of the AP-1 reporter (Fig. 6A). Okadaic acid treatment of EL4 cells alone reproducibly induced a protection at G152 and G173 within the Oct site, at G84 indicative of protein binding to the AP-1 site of the IL-2 promoter (Fig. 7, lane 3). This observation correlated with the induction of low levels of IL-2 expression under these conditions (compare Fig. 3 and Ref. 42) as well as with the induction of AP-1 expression (compare Fig. 2) and activity (compare Fig. 6C) in okadaic acid-treated cells. Surprisingly, binding of Oct and AP-1 proteins seemed to be sufficient to induce a low level of IL-2 transcription.

The IL-2 promoter of cells treated with PMA and OA is sensitive to okadaic acid treatment. In the presence of okadaic acid showed no footprints at all (Fig. 7, lane 5). Thus, PMA and OA plus okadaic acid-treated EL4 cells also AP-1 proteins, although activated (compare Fig. 6C, P/A + OA), do not bind to the IL-2 promoter. The absence of footprints at the IL-2 promoter was gene specific since the cellular c-fos promoter was still occupied under these conditions. Thus, in vivo footprinting of the same DNA preparations, as used for the IL-2 promoter analysis, revealed the characteristic footprint pattern at the SRE binding region of the c-fos promoter (data not shown) (37).

These data demonstrate that okadaic acid completely prevents transcription factor binding to the IL-2 promoter in PMA + A23187-stimulated EL4 cells.

Discussion

IL-2 is produced by activated T cells. Its expression is regulated at the transcriptional level. In addition, posttranscriptional effects on IL-2 mRNA stability have been described (48). In this study, it is demonstrated for the first time that in activated T cells, the inhibitor of Ser/Thr phosphatases of the subfamilies 1, 2A, and 5, okadaic acid, prevents IL-2 expression through a transcriptional block. Okadaic acid treatment caused no general inhibition of transcription since the β-actin, c-fos, and c-jun genes (Fig. 2) and AP-1-dependent reporter genes (Fig. 6C) were still transcribed. Therefore, neither RNA polymerase II nor the basic transcriptional machinery is affected directly by inhibition of these Ser/Thr phosphatases.

Interestingly, okadaic acid treatment differentially affects the potencies of individual transcription factors regulating IL-2 transcription. Thus, while okadaic acid inhibits NF-κB, Oct-, and NF-AT-mediated transactivation processes during T cell activation, AP-1 activity is even enhanced.
Okadaic acid treatment enhances AP-1 activity through an induction of the expression of AP-1 proteins (Fig. 2; see also Ref. 49). Unexpectedly, this AP-1 expression was still detectable at late times after activation. In both activated and nonactivated PBL-T cells, okadaic acid treatment resulted in a high transcription rate of AP-1 genes 6 h after stimulation (see Fig. 2). Okadaic acid treatment rapidly induces expression of the AP-1 genes c-fos, c-jun, junB, and junD in EL4 cells. This expression was still detectable 8 h after stimulation (unpublished data). This kinetics of the AP-1 expression differs from that under other experimental conditions of T cell activation, in which AP-1 expression occurs as an early event followed by a rapid shut-off (see Ref. 9 for summary). At present, the regulation of this shut-off is not well understood. One explanation for the AP-1 expression pattern in T cells described in this study could be that okadaic acid-sensitive phosphatases are also involved in the down-regulation of AP-1 expression.

In nonactivated T cells, IL-2 transcription was inhibited completely by okadaic acid. AP-1 activity, which was enhanced under these conditions, was clearly not sufficient to overcome the inhibitory effect of okadaic acid on the IL-2 promoter. This could be based on the finding that IL-2 transcription seems to depend on the binding of a complete set of transcription factors to the promoter (36), most likely because the IL-2 promoter contains nonconsensus binding sites that favor cooperative transcription factor binding (24). Then, AP-1 proteins, although induced, could not transactivate the IL-2 promoter because their binding affinities toward the nonconsensus AP-1-binding elements would not be strong enough under conditions in which Oct, NF-κB, and NF-AT activities are missing. Indeed, in vivo footprinting analysis (Fig. 7) showed that the IL-2 promoter in okadaic acid-treated EL4 cells stimulated with PMA + A23187 is not occupied, indicating that transcription was blocked due to complete inhibition of activator binding. Moreover, no evidence for binding of repressor proteins to the IL-2 promoter was found.

In nonactivated EL4 cells, however, okadaic acid treatment led to low expression of IL-2. Note that this was the case only in EL4 cells, not in the other transformed and nontransformed T cells analyzed in this study. Okadaic acid-induced IL-2 expression in EL4 cells correlated with footprints only at the Oct site (G↓77), at the noncomposite AP-1 site at nucleotides G↓152 and G↓154, and at the OAP site (G↓84) that is bound by AP-1 proteins (46) (compare Fig. 7). Thus, in nonactivated EL4 cells, Oct and AP-1 proteins can induce transcription at the IL-2 promoter in the absence of other transcription factors.

One possibility to explain the opposite effects of okadaic acid on IL-2 transcription in activated and nonactivated EL4 cells could be that the accessibility of the IL-2 promoter is different. Since the binding of AP-1 proteins to the IL-2 promoter (Fig. 7) is distinct in activated and nonactivated EL4 cells while transactivating capacities of AP1 proteins are detectable in both states (Fig. 6C), perhaps this means that, comparable with the situation that is found at a growing number of cellular promoters (50, 51), the activity of...
the IL-2 promoter is regulated by its nucleosomal architecture as well. In this case, T cell activation would induce the establishment of an open form of IL-2 promoter packaging. The inhibition of okadaic acid-sensitive phosphatases during activation may either alter or disturb this process. At present, it is not clear whether, and if so how, okadaic acid treatment induces (in activated EL4 cells) or fixes (in other T cells) a promoter structure similar to the one in resting T cells that does not permit access of single transcription factors.

FIGURE 7. Determination of the in vivo occupancy of the IL-2 promoter in EL4 cells. In vivo methylated genomic DNA was isolated from EL4 cells cultured in medium (lane 2, black bars), treated with okadaic acid (lane 3, bars striped longitudinal), or stimulated with PMA/A23187 for 6 h in the absence (lane 4, bars striped diagonal) or presence (lane 5, cross-striped bars) of okadaic acid. Okadaic acid was added 30 min before stimulation. The coding strand of the IL-2 promoter was footprinted from in vivo methylated DNA preparations (lanes 2–5) and in vitro methylated DNA from untreated EL4 cells (lane 1, bars shaded) using the ligation-mediated PCR protocol. The experiment is representative of three repetitions. On the left, nucleotide positions relative to the transcriptional start point are shown. In the middle, hypersensitivities (open arrows) and protections (filled arrows) that have been reported to occur at the IL-2 promoter (36) as well as the corresponding binding transcription factors (NF-AT, TGGGC, NF-κB, ATGG, AP-1, NF-AT, OAP, Oct) are marked. Densitometric analysis of the autoradiogram is presented on the right-hand side. Relative intensity values of the bands were determined, as described in Materials and Methods. For graph representation, relative intensity values derived from in vivo methylated DNAs were normalized to that from in vitro methylated DNA, which was set 1. Densitometric analysis of the protected G\(^{285}\) and the hypersensitive G\(^{287}\) at the distal NF-AT site was impaired by the weak resolution on top of the gel.
factor activity, changes of the chromatin structure are probably involved in the regulation of IL-2 expression in T cells.

The signaling pathway investigated in this study does not involve PP2B/calcineurin. Thus, inhibition of okadaic acid-sensitive Ser/Thr phosphatases abrogated both the PP2B/calcineurin-dependent and independent pathways of IL-2 expression (Fig. 5). The similarities of the effects of okadaic acid and the PP2B/calcineurin inhibitor CsA are, however, remarkable: loss of activator binding to the IL-2 promoter was also observed when cells were stimulated in the presence of CsA (36). CsA is known to block NF-AT activation and, in addition, to exert inhibitory effects on Oct and NF-kB proteins (17, 46, 52). As shown in this study, okadaic acid also inhibits NF-AT, Oct, and NF-kB activities (Fig. 6A). One conclusion from these observations is to predict a convergence of the pathways depending on okadaic acid-sensitive Ser/Thr phosphatases and on PP2B/calcineurin. Perhaps this occurs downstream of PP2B/calcineurin, since there is evidence that PP2B/calcineurin may activate the type 1 Ser/Thr phosphatase PP1 by inactivation of the PP1 inhibitor 1 (53).

The dependency of IL-2 transcription on okadaic acid-sensitive phosphatases is conserved during tumorigenic transformation and evolution, since the inhibitory effect of okadaic acid was observed in nontransformed primary human T cells as well as in malignant T cell lines from mice and humans. This stresses the importance of these phosphatases for the regulation of IL-2 expression. Future work should focus on the identification of the individual phosphatases and proteins targeted by them. In this regard, the essential actin-binding protein pp19/cofilin has been identified as one of the substrates of okadaic acid-sensitive phosphatases. Dephosphorylation and nuclear translocation of pp19/cofilin as a consequence of costimulatory signals correlate with the induction of IL-2 production (20). Whether effects of cofilin on the nucleokleon are involved in changes of the chromatin structure influencing transcriptional activation remains to be determined.

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