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Inhibition of Cyclic AMP Response Element-Binding Protein/Cyclic AMP Response Element-Mediated Transcription by the Immunosuppressive Drugs Cyclosporin A and FK506 Depends on the Promoter Context

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ABSTRACT

The immunosuppressants cyclosporin A and FK506 (tacrolimus) can block the phosphatase calcineurin, thereby inhibiting gene transcription directed by the cyclic AMP (cAMP)- and calcium-responsive transcription factor, cAMP response element (CRE)-binding protein, and its binding site, CRE, in various cell lines. This action is a novel molecular mechanism of cyclosporin A and FK506 action. Because inhibition of CREB/CRE-directed transcription by cyclosporin A and FK506 has previously been observed by using synthetic minienhancers, reporter fusion genes were constructed to examine the effect of cyclosporin A and FK506 on the transcriptional activity of CRE-containing natural promoters. In transient transfection experiments, cyclosporin A and FK506 inhibited the transcriptional activation by cAMP and the membrane depolarization of three CRE-containing promoters. However, cyclosporin A and FK506 failed to inhibit the activation by cAMP of another promoter, the rat insulin I gene promoter. The lack of cyclosporin A/FK506 sensitivity is not intrinsic to the insulin CRE because cyclosporin A and FK506 inhibited the activation by cAMP of the insulin CRE when isolated and used as a synthetic minienhancer. Rather, cyclosporin A/FK506 resistance may be conferred by specific promoter interactions because a mutational analysis of the insulin promoter revealed that inside this promoter, CRE activity depends on an adjacent control element. These data show that cyclosporin A and FK506 can inhibit CRE activity when the CRE resides in its natural promoter. However, the cyclosporin A/FK506 sensitivity depends on the specific promoter context. The results suggest that cyclosporin A and FK506 may alter target tissue function through the regulation of a subset of CRE-containing genes.

Cyclosporin A and FK506 (tacrolimus) are clinically important immunosuppressive drugs that are widely used to prevent graft rejection after organ transplantation. The introduction of cyclosporin A in the field of organ transplantation in the early 1980s resulted in extraordinary improvements of graft survival, and cyclosporin A has become a first-choice drug for patients with allograft organs. In addition, cyclosporin A is used in the therapy of an increasing number of autoimmune diseases. However, the therapeutic application of cyclosporin A and FK506 is limited by untoward effects that are shared by both drugs, including nephrotoxicity, hypertension, neurotoxicity, and impaired glucose tolerance (European FK506 Multicenter Liver Study Group, 1994; U.S. Multicenter FK506 Liver Study Group, 1994). The structurally unrelated drugs bind to their respective intracellular receptors, the immunophilins. These drug/immunophilin complexes directly target the calcium/calmodulin-dependent phosphatase calcineurin, thereby blocking its activity (Ho et al., 1996). To date, all of the therapeutic effects, as well as the toxic effects, of these drugs have been shown to be due to inhibition of calcineurin. Inhibition of calcineurin blocks the translocation of the cytosolic component of the nuclear factor of activated T cells (NFAT) into the nucleus, resulting in a failure to activate the genes regulated by the NFAT transcription factor, including those necessary for T cell proliferation, such as interleukin 2 (Ho et al., 1996; Rao et al., 1997; Rühlmann and Nordheim, 1997). Inhibition of NFAT-directed transcription may be important for the repression of early steps in T cell activation and, thus, immunosuppression induced by cyclosporin A and FK506 (Ho et al., 1996; Rao et al., 1997; Rühlmann and Nordheim, 1997). However, other targets of calcineurin are likely to play a role in this process.

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ABBREVIATIONS: CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; CREB, CRE-binding protein; NFAT, nuclear factor of activated T cells; bp, base pair.

Cyclic AMP response element-binding protein (CREB) is an ubiquitously expressed transcription factor that is activated by phosphorylation at Ser119 (in CREB-327) in response to elevated levels of cyclic AMP (cAMP), an increase in the intracellular calcium concentration and growth factors (Meyer and Habener, 1993; Xing et al., 1996; Montminy, 1997). CREB thereby confers cAMP, calcium, and growth factor responsiveness to genes that carry a CREB binding site, the cAMP response element (CRE), with the consensus octamer sequence TGACGTCA (Meyer and Habener, 1993; Xing et al., 1996; Montminy, 1997). It has been suggested that calcineurin functions as a negative regulator of CREB-directed transcription at subthreshold electrical stimulation of cultured hippocampal neurons (Bito et al., 1996). In contrast, cyclosporin A and FK506 can inhibit the activation of CRE-directed transcription in a great variety of, but not in all, cell types (Schwaninger et al., 1993a,b, 1995; Krüger et al., 1997). The effective concentrations are consistent with the reported affinities of both drugs to their distinct immunophilin receptors and are similar to those concentrations that inhibit calcineurin phosphatase activity and that are effective in T cell repression (Schwaninger et al., 1993a,b, 1995; Krüger et al., 1997). When inhibition of calcineurin by FK506 or cyclosporin A was reversed by rapamycin or by overexpression of calcineurin, CRE-dependent transcription was disinhibited (Schwaninger et al., 1993a,b, 1995). By using a GAL4-CREB fusion protein and phosphoCREB-specific immunoblotting, it was shown that cyclosporin A and FK506 inhibit the activation of CREB without blocking its phosphorylation at Ser119 (Schwaninger et al., 1993b, 1995; Krüger et al., 1997). Thus, through inhibition of calcineurin, cyclosporin A and FK506 can block the activation of CREB/CRE-directed transcription. Inhibition of CREB/CRE-directed transcription has been observed in cell lines that are derived from tissues in which shared adverse effects of the immunosuppressants develop (Schwaninger et al., 1993a,b, 1995; Krüger et al., 1997). It also has been observed in Jurkat T cells (Krüger et al., 1997), a cell line that faithfully mimics the early stages of T cell activation and that has been used to demonstrate the effect of cyclosporin A and FK506 on NFAT-directed transcription (Ho et al., 1996; Rao et al., 1997). Recent studies have shown that CREB seems to play a critical role in antigenic stimulation of T cell activation. In transgenic mice that express a dominant-negative form of CREB under the control of the T cell-specific cluster of differentiation 2 promoter/enhancer, T cells displayed a profound proliferative defect characterized by markedly decreased interleukin-2 production, G_1 cell cycle arrest, and subsequent apoptotic death in response to a number of different activation signals (Barton et al., 1996). Furthermore, CREB-null mice have an impaired fetal T cell development of the $\alpha\beta$ lineage (Rudolph et al., 1998). These findings indicate that CREB becomes phosphorylated and activated during T cell stimulation and that it is required for normal cytokine production and T cell proliferation. When the results showing inhibition of CREB/CRE-directed transcription by cyclosporin A and FK506 in Jurkat T cells are taken together with the evidence for an essential role of CREB in T cell activation and proliferation, they strongly suggest that inhibition of CREB/CRE-directed transcription is a molecular mechanism through which cyclosporin A and FK506 exert the immunosuppressive effect.

Thus, inhibition of CREB/CRE-dependent transcription represents a novel molecular mechanism of cyclosporin A and FK506 action that could underlie their pharmacological effects, both desired and undesired. However, it has remained unclear whether cyclosporin A and FK506 can inhibit the CREB/CRE-mediated activation of natural promoters. The inhibition of CREB/CRE-directed transcription by cyclosporin A and FK506 has been shown with synthetic minienhancers consisting of oligomerized CREs or oligomerized binding sites of the GAL4-CREB fusion protein (Schwaninger et al., 1993a,b, 1995; Krüger et al., 1997). Cyclosporin A and FK506 also inhibited the depolarization-induced activation of the glucagon gene promoter (Schwaninger et al., 1993b), providing one example of cyclosporin A and FK506 blocking CRE-mediated transcription when the CRE is integrated into a natural promoter. Extending this observation, the present study examined the effect of cyclosporin A and FK506 on the activation by cAMP and/or membrane depolarization of four CRE-containing promoters. It was found that cyclosporin A and FK506 failed to block the CRE-mediated activation of one of these promoters, the rat insulin I gene promoter. These data indicate that cyclosporin A/FK506 sensitivity of CREB/CRE-mediated transcription depends on the specific promoter context and suggest that only a subset of CRE-containing genes may be regulated by cyclosporin A and FK506 in their target tissues.

Experimental Procedures

Plasmid Construction. The plasmids −350GluLuc, −900SomCAT, −711c-fosLuc, −410InsCAT, −410InsLuc, −85InsLuc, and (4×InsCRE)−85InsLuc have been described before (Schwaninger et al., 1993c; Oetjen et al., 1994; Eckert et al., 1996; Eggers et al., 1998). The plasmids −167InsLuc and −193InsLuc were generated by polymerase chain reaction with the 5′-primers 5′-TAAGCCTCGAGTTCTGCAGACTTAGC-3′ and 5′-TAAGCCTCGAGAGTTGTTGACGTC-3′, respectively, and the 3′-primer 5′- TAAGCAGATCTACATACCTGCTTGCT-3′. The resulting amplificates were digested with *Bg1*II and *Xho*I and subcloned into the *Xho*I/*Bg1*II site of pXP2 (Schwaninger et al., 1993c). The plasmid −167InsLuc was used to construct −193(i−168/−167)InsLuc: an oligonucleotide containing the sequence of the rat insulin I gene CRE ranging from −193 to −168 with 5′-GATC overhangs (Oetjen et al., 1994) was cloned in the forward orientation into the *Bam*HI site of −167InsLuc. For −193(Δ−164/−161)InsLuc, the *Xho*I/*Bg1*II fragment of −193InsLuc was subcloned into the *Xho*I/*Bam*HI site of Bluescript (Stratagene Inc., La Jolla, CA), digested by *Pst*I, blunt-ended by T4 polymerase, and religated. After amplification by the polymerase chain reaction with the primers described above, the amplificate was digested with *Bg1*II and *Xho*I and subcloned into the *Xho*I/*Bg1*II site of pXP2. The plasmid −193(*m*−172/−165)InsLuc was constructed by subcloning the *Xho*I/*Bg1*II fragment of −193InsLuc into the *Xho*I/*Bam*HI site of Bluescript, digesting it with *Aat*II and *Pst*I, and ligating it with a double-stranded oligonucleotide with 5′-*Aat*II and 3′-*Pst*I ends, the sense strand of which reads as follows: 5′-CCAATGATACAGCGATGCA-3′ (the mutated base pairs are underlined). After amplification by polymerase chain reaction with the primers described above, the amplificate was digested with *Bg1*II and *Xho*I and subcloned into the *Xho*I/*Bg1*II site of pXP2. For the construction of the CRE+E3 construct, the following oligonucleotide was used: 5′-GATCCAGAGTTGTTGACGTCCAATGAGCGCTTTCTGCAGACTTAGCACTAGA-3′. All constructs were confirmed by sequencing.

Cell Culture and Transfection of DNA. HIT-T15 cells (Schwaninger et al., 1993c) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 5% horse serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. Cells were transfected

with 2 μg of indicator plasmid per 6-cm dish. RSV-CAT or RSV-Luc (0.4 μg/6-cm dish), respectively, was added as a second reporter to check for transfection efficiency. Cells were trypsinized and transfected in suspension by the diethylaminoethyl-dextran method as described previously (Schwaninger et al., 1993c). Cell extracts (Schwaninger et al., 1993c) were prepared 48 h after transfection. Cells were stimulated with forskolin (10 μM) 6 h before harvest. Cyclosporin A (5 μM) or FK506 (at concentrations as indicated) was added 7 h before harvest. A chromatographic CAT assay was performed as described (Schwaninger et al., 1993c). Thin-layer chromatography plates were scanned by a Fuji PhosphorImager (Fujisawa GmbH., Munich, Germany). The luciferase assay was performed as described previously (Schwaninger et al., 1993c).

Materials. Luciferin, Tween 80, and forskolin were purchased from Sigma Chemical Co. (St. Louis, MO). FK506 was a gift from Fujisawa. Cyclosporin A was a friendly gift from Sandoz (Basel, Switzerland). Forskolin was dissolved in dimethyl sulfoxide, and FK506 was dissolved in ethanol. A stock solution of cyclosporin A (10 mg/ml) was prepared in ethanol with 20% Tween 80 and further diluted in RPMI 1640. Controls received the solvent only.

Results

Cyclosporin A and FK506 Inhibit the Activation of Three CRE-Containing Promoters. To study the effect of cyclosporin A and FK506 on the activation of CRE-containing promoters, reporter-fusion genes containing the 5′-flanking regions of the rat glucagon, rat somatostatin, and human *c-fos* genes were used (Fig. 1A). These promoters contain CREs at different distances upstream of the start site of transcription (Fig. 1A). The glucagon and somatostatin gene CREs contain the consensus CRE octamer sequence TGACGTCA, whereas the CRE of the *c-fos* gene contains an imperfect CRE octamer, TGACGTTT. It previously has been shown that these promoters are activated by cAMP and membrane depolarization-induced calcium influx (Montminy et al., 1986; Knepel et al., 1990; Sheng et al., 1990; Schwaninger et al., 1993c; Oetjen et al., 1994). By studies of nuclear protein binding, by mutational analyses, and by overexpression of a dominant-negative CREB mutant, it has been demonstrated that the activation of these promoters depends wholly or largely on their CREs binding the transcription factor CREB (Montminy et al., 1986; Knepel et al., 1990; Sheng et al., 1990; Schwaninger et al., 1993c; Oetjen et al., 1994; B. Eckert and W. Knepel, unpublished observation). The reporter fusion genes were transfected into HIT cells. In this cell line, the stimulation of GAL4-CREB- and synthetic CRE minienhancer-directed transcription has been shown to be inhibited by cyclosporin A and FK506, with IC_{50} values of about 30 nM and 1 nM, respectively (Schwaninger et al., 1993b, 1995). As shown in Fig. 1B, cyclosporin A (5 μM) did not change basal activity but inhibited the stimulation of these three promoters by cAMP and membrane depolarization. Membrane depolarization resulted in a 2.7-fold increase in the transcriptional activity of the glucagon promoter, which was almost completely abolished by cyclosporin A. Forskolin stimulated transcription of the glucagon gene promoter by 9-fold; the addition of cyclosporin A caused a rate of inhibition of 32%. The synergistic stimulus of membrane depolarization plus forskolin led to a 22.5-fold rise of the transcriptional activity, which was diminished by 84% by cyclosporin A. Membrane depolarization led to a 2.6-fold increase in transcriptional activity of the somatostatin promoter. Cyclosporin A inhibited membrane depolarization-induced transcription completely and diminished the forskolin-stimulated (34-fold) and the calcium plus forskolin-enhanced (60-fold) transcriptional activity of the rat somatostatin gene by 72% and 85%, respectively. The transcriptional activity of the human *c-fos* promoter was enhanced by membrane depolarization, by forskolin, and by both stimuli together 2.7-, 5-, and 7.5-fold, respectively. The stimulated transcriptional activity of −711c-fosLuc was decreased by cyclosporin A by about 60%, 55%, and 50%, respectively. Thus, the CRE-mediated activation of these three promoters was inhibited by cyclosporin A (Fig. 1B). Similar results were obtained with FK506 (not shown). Extending the previously observed inhibition of depolarization-induced activation of the glucagon promoter (Schwaninger et al., 1993b), these results demonstrate that cyclosporin A and FK506 inhibit not only the activation of synthetic CRE minienhancers but also the transcriptional activation of CREs within natural promoters.

Cyclosporin A and FK506 Inhibit the CRE-Mediated Activation of Some, But Not All, Promoters. It is well

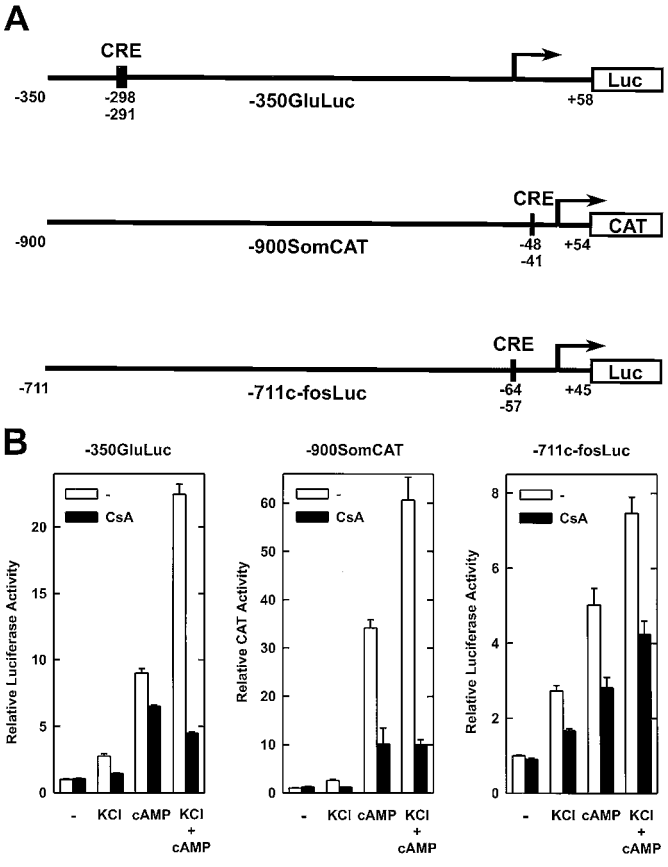

Fig. 1. Inhibition by cyclosporin A of the transcriptional activation by cAMP and membrane depolarization of three CRE-containing promoters. A, reporter-fusion genes containing the 5′-flanking regions of the rat glucagon, rat somatostatin, and human *c-fos* genes. The relative positions of the CRE octamers are indicated. Arrows indicate the start site of transcription (+1). Luc, luciferase; CAT, chloramphenicol acetyltransferase. B, Reporter-fusion genes were transfected into HIT cells and the cells were stimulated by high potassium-induced membrane depolarization and calcium influx (KCl, 45 mM), by the adenylate cyclase activator forskolin (10 μM), by cAMP, or by a combination of both (KCl + cAMP). CsA, cyclosporin A (5 μM). Reporter enzyme activity is expressed relative to the mean value in each experiment of the activity measured in the respective controls (no treatment). Values are means ± S. E. of three independent experiments, each done in duplicate.

known that the rat insulin I gene promoter is stimulated by cAMP (German and Wang, 1994; Oetjen et al., 1994). It has been shown recently that this stimulation is conferred by CREB binding to the rat insulin I gene CRE (Eggers et al., 1998). The CRE octamer is positioned from −178 to −185 within the insulin promoter (Fig. 2). HIT cells were transfected with the reporter gene construct −410InsCAT and incubated with either increasing concentrations of FK506 alone or with 10 μM forskolin plus increasing concentrations of the immunosuppressant. As shown in Fig. 2, the basal activity of the insulin promoter was not affected by FK506. Forskolin enhanced transcriptional activity about 2-fold. Increasing concentrations of the immunosuppressant had no effect on forskolin-induced transcription of the rat insulin I gene (Fig. 2). The same results were obtained by treating the cells with cyclosporin A (data not shown). Thus, cyclosporin A and FK506 failed to inhibit CREB/CRE-mediated activation of the rat insulin I gene promoter, indicating that the immunosuppressants inhibit the stimulation of some, but not all, CRE-containing promoters.

Cyclosporin A Inhibits Forskolin-Stimulated Transcription Directed by the Isolated CRE of the Rat Insulin I Gene. Because cyclosporin A and FK506 did not inhibit the activity of the CRE inside the rat insulin I gene promoter, it was investigated whether cyclosporin A inhibits transcription directed by the isolated insulin CRE. For this purpose, a luciferase reporter plasmid containing four copies of the CRE (from −193 to −168; Eggers et al., 1998) of the rat insulin I gene in front of the homologous minimal promoter was used (Fig. 3). It has been shown before that the rat insulin I gene CRE binds CREB and confers basal activity and cAMP responsiveness (Oetjen et al., 1994; Eggers et al., 1998). As shown in Fig. 3, the minimal homologous promoter (−85InsLuc) showed low basal activity and was not stimulated by the addition of 10 μM forskolin. Four copies of the CRE conferred basal activity to the promoter, which was further enhanced 2.4-fold by stimulation with forskolin (Fig. 3). Cyclosporin A had no effect on the basal transcriptional activity of the CRE (Fig. 3). However, forskolin-induced transcription was completely abolished by cyclosporin A (Fig. 3). Thus, in contrast to the rat insulin I gene, the isolated CRE is regulated by cyclosporin A. These results suggest that interactions of the CRE with other elements within the promoter render the rat insulin I gene CRE insensitive to cyclosporin A treatment.

cAMP Responsiveness of the Insulin Promoter Depends on an Interaction between the CRE and the E3 Element. The CRE of the rat insulin I gene confers cAMP responsiveness to the promoter (German and Wang, 1994; Oetjen et al., 1994; Eggers et al., 1998) and is sufficient to confer cAMP responsiveness to the minimal promoter (Oetjen et al., 1994; Eggers et al., 1998). However, at its native position within its promoter, the function of the CRE may depend on an interaction with other promoter elements (Nitsch et al., 1993; Roesler et al., 1995; Blackwood and Kadonaga, 1998). To identify such a promoter element, mutant insulin promoter constructs were prepared by 5′-deletion, internal deletions, insertions, and mutations. A 5′-deletion construct with all bases upstream of the CRE removed (construct −193InsLuc) was stimulated by cAMP in a cyclo-

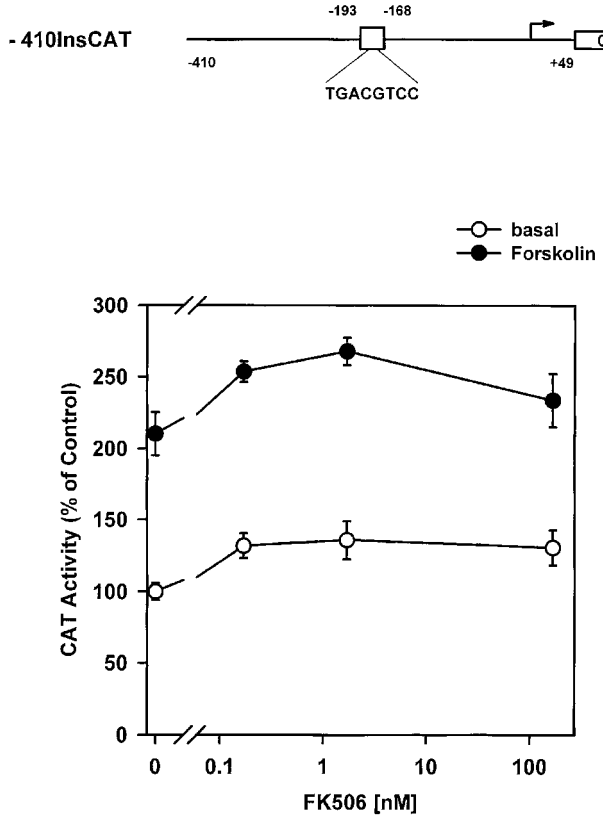

Fig. 2. Lack of inhibition by FK506 of the cAMP-induced activation of the CRE-containing rat insulin I gene promoter. The plasmid −410InsCAT was transfected into HIT cells and the cells were stimulated by forskolin (10 μM). Reporter enzyme activity is expressed as a percentage of the mean value in each experiment of the activity measured in the untreated controls. Values are means ± S. E. of three independent experiments, each done in duplicate.

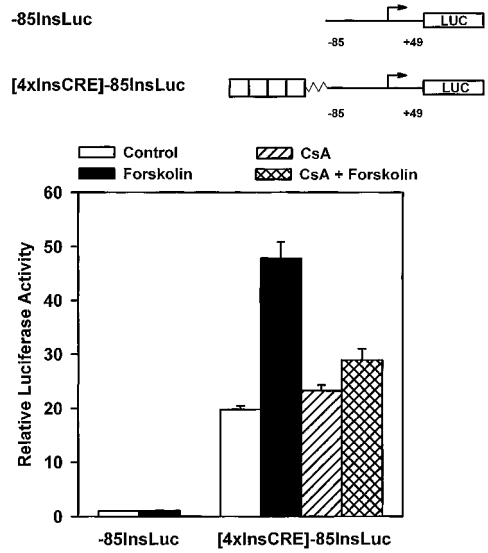

Fig. 3. Inhibition by cyclosporin A of the cAMP-induced activation of the isolated CRE of the rat insulin I gene. A synthetic minienhancer consisting of four copies of an oligonucleotide containing the CRE of the rat insulin I gene (from −193 to −168) was placed in front of the homologous core promoter. Constructs containing the core promoter (−85InsLuc) or the isolated CRE in front of the core promoter ([4×InsCRE]−85InsLuc) were transfected into HIT cells and the cells were stimulated by forskolin (10 μM). CsA, cyclosporin A (5 μM); LUC, luciferase. Luciferase activity is expressed relative to the mean value in each experiment of the activity measured in the −85InsLuc control. Values are means ± S. E. of three independent experiments, each done in duplicate.

sporin A-insensitive manner like the wild-type construct (Fig. 4), indicating that the promoter region upstream of the CRE is not essential for cAMP responsiveness and cyclosporin A resistance. In contrast, the insertion of 34 base pairs (bp), the deletion of 4 bp, and the mutation of 8 bp in a region just downstream of the CRE abolished cAMP responsiveness (Fig. 4). Basal activity of these constructs was 26 ± 4%, 80 ± 11%, and 35 ± 11%, respectively, of that of the wild-type construct. These alterations by insertion, deletion, and mutation fall into a previously defined control element, called E3 (Ohlsson and Edlund, 1986; Karlsson et al., 1987; Moss et al., 1988; Fig. 4). Therefore, the present results suggest that the CRE of the rat insulin I gene interacts with the adjacent E3 element to confer cAMP responsiveness to the promoter. This interaction may then be responsible for the lack of cyclosporin A/FK506 sensitivity of cAMP-induced activation. Cyclosporin A and FK506 treatment did not change nuclear protein binding to these elements as revealed by the electrophoretic mobility shift assay (not shown).

To further explore the role of the E3 site, oligonucleotide cassette constructs were prepared. As shown in Fig. 5, inclusion of the E3 element in a single CRE site construct enhanced cAMP inducibility of the CRE. cAMP responsiveness was conferred also by oligomerization of the CRE (4×CRE; Fig. 5). However, this latter stimulation was inhibited by cyclosporin A, whereas that conferred by the CRE plus E3 was not (Fig. 5). These data support the conclusion that the E3 element, in conjunction with the CRE, confers cAMP responsiveness in a cyclosporin A-insensitive manner.

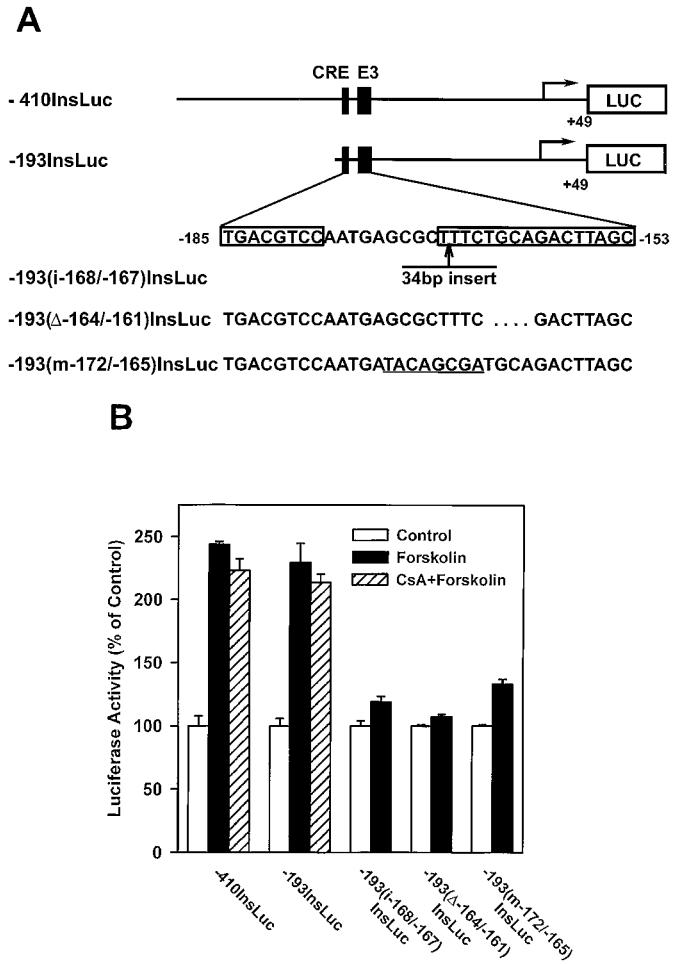

Fig. 4. CRE activity of the rat insulin I gene promoter depends on an interaction with an adjacent promoter element as revealed by mutational analysis. A, Wild-type and mutant reporter-fusion genes. The construct −193(i−168/−167)InsLuc contains a 34-bp insertion between −168 and −167; the construct −193(Δ−164/−161)InsLuc contains an internal 4-bp deletion from −164 to −161; the construct −193(*m*−172/−165)InsLuc contains an 8-bp mutation from −172 to −165 (the mutant bases are underlined); the internal insertion, deletion, and mutation fall into a previously described control element called E3. The sequences of the CRE octamer and E3 element are boxed. LUC, luciferase. B, Reporter-fusion genes were transfected into HIT cells and the cells were stimulated by forskolin (10 μM). CsA, cyclosporin A (5 μM). Luciferase activity is expressed as a percentage of the mean value in each experiment of the activity measured in the respective controls. Values are means ± S. E. of three independent experiments, each done in duplicate.

Discussion

The transcriptional activity of a gene depends on the synergistic interaction between multiple promoter and enhancer control elements dictating chromatin remodeling and the assembly of coactivators and the general transcription machinery (Blackwood and Kadonaga, 1998). In genes carrying a CREB binding site, such interactions also include the CRE, as exemplified by the tyrosine aminotransferase and the phosphoenolpyruvate carboxykinase genes, the cAMP responsiveness of which has been shown to require the interaction between the CRE and liver-specific control elements (Nitsch et al., 1993; Roesler et al., 1995). The present study shows that cyclosporin A and FK506 inhibit the CREB-dependent activation by cAMP and membrane depolarization of promoters from three different genes. Consistent with and extending a previous observation (Schwaninger et al., 1993b), these data indicate that cyclosporin A and FK506 can inhibit CREB/CRE-mediated transcription, not only when conferred by synthetic oligomerized CRE minienhancers, but also when a CRE is integrated in its natural promoter. In

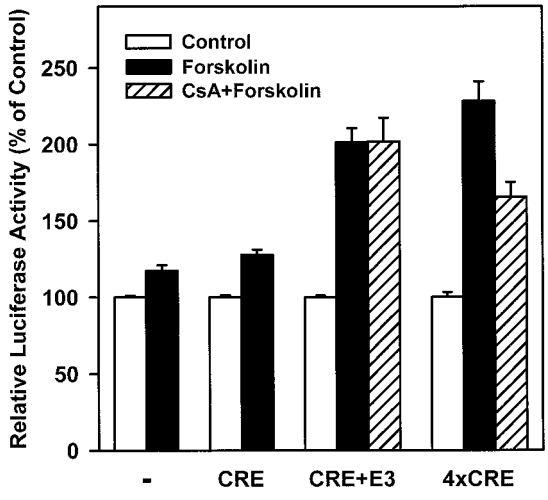

Fig. 5. The E3 element in conjunction with the CRE of the rat insulin I gene confers cAMP responsiveness in a cyclosporin A-insensitive manner. An oligonucleotide containing the CRE (from −193 to −168) or the CRE plus E3 (from −193 to −148), as well as four copies of the CRE (4×CRE), were placed in the forward orientation in front of the truncated promoter fused to the luciferase reporter gene. The constructs were transfected into HIT cells and the cells were stimulated by forskolin (10 μM). CsA, cyclosporin A (5 μM). Dash, promoter alone. Luciferase activity is expressed as a percentage of the mean value in each experiment of the activity measured in the respective controls. Values are means ± S. E. of four independent experiments, each done in duplicate.

contrast, cyclosporin A and FK506 failed to block the activation by cAMP of another promoter, the rat insulin I gene promoter. A mutational analysis (German and Wang, 1994; Eggers et al., 1998) and the overexpression of a dominant-negative CREB mutant (Eggers et al., 1998) have shown that the activation by cAMP of this promoter is mediated by CREB binding to the CRE. The lack of cyclosporin A/FK506 sensitivity is not intrinsic to this CRE because the activation by cAMP was inhibited by cyclosporin A and FK506 when the rat insulin I gene CRE was used in multiple copies as a synthetic minienhancer in front of the homologous core promoter. Therefore, the data suggest that the lack of cyclosporin A and FK506 sensitivity is secondary to specific interactions between this CRE and other promoter elements. Consistent with a facilitated tracking model for enhancer function, according to which an enhancer-bound complex containing DNA-binding factors and coactivators "tracks" via small steps along the chromatin (Blackwood and Kadonaga, 1998), the activation of the CRE of the rat insulin I gene was found to depend on an immediately adjacent control element. This element, E3, has been defined before through specific nuclear protein binding (Ohlsson and Edlund, 1986; Moss et al., 1988) and constitutive transcriptional activity (Karlsson et al., 1987), although the binding proteins have not yet been molecularly cloned. When taken together, our data indicate that cyclosporin A and FK506 inhibit the CREB-dependent activation of some, but not all, CRE-containing promoters, depending on the specific promoter context.

The molecular mechanisms through which interactions between the CRE and other promoter elements render the CREB-mediated transcriptional activation insensitive to cyclosporin A and FK506 remain unclear. Cyclosporin A and FK506 have been shown to inhibit stimulus-induced, but not basal, CRE activity without preventing the phosphorylation of CREB at Ser119 (Schwaninger et al., 1993a,b, 1995; Krüger et al., 1997), suggesting that calcineurin phosphatase activity is required for the transactivation by phosphorylated CREB. The calcineurin substrate involved is unknown. Recent studies performed in vitro or in some cell lines suggest a general model of transactivation by CREB in response to cAMP or other stimuli whereby the phosphorylation of CREB allows the binding of the coactivators CBP/p300; CBP then stimulates transcription through its acetyltransferase activity, through the recruitment of p/CAF, p/CIP, and RNA helicase A, as well as through interactions with the general transcription machinery (Chrivia et al., 1993; Kwok et al., 1994; Lundblad et al., 1995; Nakajima et al., 1997; Korzus et al., 1998; Kurokawa et al., 1998). However, the acetyltransferase activity of CBP can stimulate transcription only from certain promoters (Martínez-Balbás et al., 1998). Furthermore, several transcription factors in addition to CREB bind to CBP/p300 and confer distinct requirements for the composition and function of the multiprotein coactivator complex (Korzus et al., 1998; Kurokawa et al., 1998). Therefore, we speculate that in some promoters, specific interactions between the CRE and other promoter elements may alter the mode of CREB transactivation, thereby bypassing the calcineurin-dependent step.

The CRE-containing promoters, the CREB-mediated activation of which was inhibited by cyclosporin A and FK506 in the present study, include the human *c-fos* gene promoter. This may be noteworthy because AP-1 is an important regulator of T cell activation and interleukin 2 transcription (Ho et al., 1996; Rühlmann and Nordheim, 1997), and the inducible expression of *c-fos* and other AP-1 family members was markedly and specifically decreased in thymocytes expressing a dominant-negative CREB mutant and displaying a profound proliferative defect (Barton et al., 1996). The finding of the present study that cyclosporin A and FK506 inhibit the stimulation of some, but not all, CRE-containing promoters raises the possibility that cyclosporin A and FK506 may alter target tissue function through the regulation of a subset of CRE-containing genes. In CREB-deficient mice, some, but not all, of the genes known to contain a CRE were downregulated in T cells (Barton et al., 1996; Rudolph et al., 1998) indicating that the inhibition of a subset of CRE-containing genes is sufficient to cause immunosuppression.

The use of cyclosporin A and FK506 in human organ transplantation has been associated with diabetes mellitus, and cyclosporin A and FK506 have been reported to decrease insulin secretion and insulin mRNA levels in studies with rat islets or insulin-secreting tumor cell lines (Herold et al., 1993; European FK506 Multicenter Liver Study Group, 1994; U.S. Multicenter FK506 Liver Study Group, 1994; Redmon et al., 1996). However, these effects required long-term exposures to the drugs and, thus, could be secondary effects or toxic effects (Herold et al., 1993; Ebihara et al., 1996; Redmon et al., 1996). Short-term exposure to cyclosporin A has been shown to stimulate insulin secretion from mouse insulinoma cells (Ebihara et al., 1996). In the present study, cyclosporin A and FK506 had no effect on basal and cAMP-induced transcription of the rat insulin I gene promoter. Because the human insulin gene and the rat insulin I gene differ in the sequence of their CREs as well as in the detailed organization of the promoter context (Karlsson et al., 1987; Inagaki et al., 1992; Oetjen et al., 1994; Eggers et al., 1998), the effect of cyclosporin A and FK506 on CREB-mediated activation of the human insulin gene awaits examination.

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