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Regulation of the Nur77 Orphan Steroid Receptor in Activation-Induced Apoptosis

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T-cell receptor (TCR)-mediated apoptosis in immature thymocytes and T-cell hybridomas is calcium dependent and can be inhibited by cyclosporin A (CsA). Induction of the orphan steroid receptor Nur77 (NGFI-B) is required for activation-induced apoptosis. Here, we examined the regulation of Nur77 expression, in response to apoptotic TCR signals, which consists of kinase C and calcium pathways. We show that the major control of Nur77 induction is mediated by the calcium signaling pathway. In contrast, protein kinase C signals induce only a low level of Nur77 activity. Nur77 promoter activity parallels its protein levels. CsA decreases both Nur77 protein levels and promoter activity, and the kinetics of CsA inhibition of apoptosis correlates with a decrease in Nur77 protein levels. TCR signals and kinase C signals result in a similar level of Nur77 protein phosphorylation but mediate differential transactivation activity of Nur77. In addition, Nur77 promoter deletion analysis revealed two RSRF (related to serum-responsive factor) binding sites, which can confer calcium and CsA sensitivity on a heterologous promoter. Taken together, our data suggest that the levels of transcriptional induction of Nur77 play an important role during activation-induced apoptosis and that calcium signals regulate a novel CsA-sensitive nuclear factor required for Nur77 transcription in T cells.

During development in the thymus, immature thymocytes which express self-reactive T-cell antigen receptors (TCR) are eliminated from the developing T-cell repertoire (for a review, see reference 38). This process of clonal deletion, or negative selection, is thought to be mediated by apoptotic signals delivered to thymocytes whose TCR have a high affinity for self-antigen/major histocompatibility complexes (for a review, see reference 1). This mechanism is crucial for generating a peripheral T-lymphocyte population which will recognize only foreign pathogens and helps prevent unwanted immune responses which could lead to autoimmunity. Negative selection therefore represents a first line of defense in a battle for immunological tolerance of self.

Apoptosis can be induced in immature thymocytes and many T-cell hybridomas by using antibodies directed against the TCR-CD3 complex (anti-CD3) (51, 53). T-cell hybridomas are a convenient in vitro model for studying the transcription and biochemical signaling pathways involved in apoptosis of T cells and may mimic some aspects of negative selection. Activation-induced apoptosis in thymocytes and T-cell hybridomas requires an increase in intracellular Ca^{2+} and de novo protein synthesis (34, 36, 55). The immunosuppressive drug cyclosporin A (CsA) blocks the calcium-mediated signaling pathway of the TCR-CD3 complex, presumably by inhibiting the calcium/calmodulin-dependent phosphatase calcineurin (49). CsA is therefore, a valuable tool for studying T-cell responses dependent upon increases in intracellular calcium, and several groups have shown that CsA is a potent inhibitor of anti-CD3-mediated apoptosis (52). The effect of CsA on negative selection, however, remains controversial (2, 17, 23, 56, 57, 59).

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To date, several genes which are required for activation-induced apoptosis in T-cell hybridomas have been identified. These are c-*myc* (50), the Fas (Apo-1)/Fas ligand receptor pair (5, 13, 24), and Nur77 (29, 65). Nur77 (NGFI-B) (19, 37, 46) is an immediate-early gene product encoding an orphan steroid receptor. Expression of dominant negative or antisense Nur77 mutations can inhibit apoptosis (29, 65). The DNA-binding activity of Nur77 is sensitive to CsA, suggesting a mechanism for CsA inhibition of activation-induced cell death (64, 67). In addition to its role in apoptosis, Nur77 is also induced in a variety of cells in response to signals for growth and differentiation. For instance, Nur77 is activated transiently by mitogenic serum growth factors in fibroblasts (19, 46) and by nerve growth factor (NGF) during neuronal differentiation of the pheochromocytoma cell line PC12 (37). It is not entirely clear how Nur77 functions during such diverse cellular responses.

Nur77 is classified as a member of the superfamily of nuclear steroid receptors because of sequence homology in the DNA-binding domain and is an "orphan" member because the ligand for Nur77 is unknown. However, it is not clear if a ligand exists, because Nur77 is transcriptionally active in many cell types (12, 43) and does not require any component of tissue culture medium for its activity (43). The Nur77 protein has the typical steroid receptor structure, composed of an N-terminal transactivation domain, a central DNA-binding domain containing two zinc fingers, and a C terminus with homology to hormone-binding domains (7). Unlike most hormone receptors, which bind as dimers to either inverted or direct repeats of hormone response elements, Nur77 can bind as a monomer to an estrogen receptor half-site DNA element containing two additional adenine nucleotides at the 5′ end (Nur77/NGFI-B DNA-binding element [NBRE]; 5′-AAAGGTCA-3′) (61, 62). A region immediately C-terminal to the DNA-binding domain, the A box, is required for recognition of the two A · T base pairs at the 5′ end of the NBRE (63). In the presence of

9-*cis*-retinoic acid, Nur77 can also heterodimerize with the 9-*cis*-retinoic acid receptor (RXR) to bind to a retinoic acid response element composed of direct repeats separated by 5 nucleotides (16, 44).

To investigate the regulation of Nur77 expression during activation-induced apoptosis of T-cell hybridomas, we characterized the Nur77 protein and promoter induction in response to apoptotic TCR signals. The TCR signals, which can be activated by anti-CD3 antibody, consist of two intracellular signaling pathways: the kinase C pathway, which can be mimicked by addition of phorbol ester (phorbol myristyl acetate [PMA]), and the calcium pathway, which can be mimicked by addition of calcium ionophore (ionomycin) (60). We report here that PMA induces only a low level of Nur77 protein, which is barely detectable by Western (immunoblot), gel shift, or reporter gene assays. In contrast, the calcium signaling pathway mediates a high level of Nur77 expression during anti-CD3-induced apoptosis, which correlates with a high degree of NBRE-mediated transactivation activity. Nur77 promoter activity is also weakly induced by PMA alone but becomes highly induced by the addition of calcium ionophore. The calcium-responsive promoter elements were delineated to lie between −336 and −163 and were found to be CsA sensitive. This region contains two RSRF (related to serum response factor) binding sites which can confer calcium inducibility and CsA sensitivity in T cells. The Nur77 protein induced by PMA versus that induced by PMA plus ionomycin is phosphorylated to a similar level, and the kinetics of CsA inhibition of apoptosis correlates with the decrease in Nur77 protein levels. Although the role of phosphorylation in Nur77 function is still not entirely clear, these results suggest that the transcriptional level of Nur77 induction is important during activation-induced apoptosis in T-cell hybridomas and identify the RSRF transcription factor as a novel target of cyclosporin inhibition.

MATERIALS AND METHODS

Cell culture and IL-2 assay. The murine T-cell hybridomas AO4H5.3 and DO11.10 were grown in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. Where indicated, the hybridomas were stimulated with immobilized anti-CD3 monoclonal antibody 500A2 or PMA (10 ng/ml) and ionomycin (A23187; 0.5 μM) in the presence or absence of CsA (100 ng/ml) (Sandoz Pharmaceuticals). Thymocytes were freshly isolated from 4- to 6-week-old C57BL/6 mice and cultured in RPMI–10% FCS. The interleukin-2 (IL-2) assay was performed with 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously (65) in the IL-2-dependent cell line CTLL-2. The apoptotic DNA ladder assay was performed as described before (65).

Gel shift analysis. In vitro transcription and translation of Nur77 and Nurr1 were performed as follows. The Nur77 cDNA in pBSKS(+) was linearized with *Bam*HI, and the Nurr1 cDNA in pBSKS(−) was linearized with *Xho*I followed by in vitro transcription with T_3 RNA polymerase at 37°C for 1 h. The in vitro translation was performed with rabbit reticulocyte lysate (Promega) as described by the manufacturer for 1 h at 30°C. All of the gel shifts were performed as described before (12) in 90 mM NaCl–20 mM NaF–1 mM sodium orthovanadate–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5)–1.5 mM $MgCl_2$–10 μM $ZnSO_4$–10 mM dithiothreitol–0.02% Triton X-100–10% glycerol–1% aprotinin–10 μg of pepstatin per ml–10 μg of leupeptin per ml. The nuclear extracts and whole-cell extracts were prepared as described before (48, 58). The NBRE gel shift analysis was performed with an oligonucleotide containing one Nur77 DNA-binding site (61, 65), TCGAGTTTTAAAAGGT CATGCTCAATTTG. The RSRF gel shifts were performed with either purified Nur77 promoter fragments spanning −336 to −163, −336 to −287, or −287 to −163 or a synthetic oligonucleotide corresponding to −307 to −242 (see Fig. 7d) of the Nur77 promoter. The GT box oligonucleotide sequence was described previously (25). The proximal NF-AT IL-2 promoter sequence is described in reference 22. The gel shift reaction mixes (20 μl) contained 10 μg of nuclear extract, 3×10^4 cpm of ^{32}P-labeled oligonucleotide, and 2 μg of poly(dIdC) and were incubated at room temperature for 20 to 25 min and then separated on 0.5× TBE–4% native acrylamide gels. The enhanced gel shift analysis mix also contained 1 μl of rabbit preimmune serum. To block the Nur77 DNA-binding complex, 0.9 μg of affinity-purified anti-Nur77 antiserum was added to the gel shift reaction mix and incubated on ice for 1 h before addition of the probe. The control antiserum (0.1 μl of preimmune serum or 0.9 μg of affinity-purified anti-SP3) was also incubated for 1 h before addition of the probe. To supershift the RSRF complex, 1 μl of anti-RSRF antibody was added to the gel shift reaction mix 10 min before addition of the probe.

Plasmids and transient transfections. The luciferase reporter plasmids were constructed as follows. The −56 c-*fos* promoter containing one to four NBRE sites was cloned from the −56 ced 1-4 CAT constructs (65) by digestion with *Hin*dIII; the 5′ overhang was filled in with Klenow fragment, and then the plasmid was digested with *Bam*HI. The promoter fragments were isolated and cloned into the *Sma*I and *Bgl*II sites of pGL2 Basic (Promega). The mutant NBRE sequence is TCGAGTTTTGCGAGGTCATGCTCAATTTG (the base pairs mutated are underlined). The Nur77 promoter-CAT plasmids were constructed as follows. The Nur77 promoter was cloned from a mouse genomic C57BL/6 cosmid library. A 3.8-kb *Tth* III I promoter fragment was blunt ended with Klenow enzyme and cloned into the *Sma*I site of pCAT 3′L. The 5′ promoter deletion constructs were made by digesting the 3.8-kb Nur77 promoter-CAT plasmid with *Hin*dIII (which cuts in the pCAT 3′L polylinker 5′ of the promoter fragment) and either *Eco*NI to generate the −480 Nur77, *Xma*I to make the −336 Nur77, *Ngo*MI to make the −287 Nur77, or *Ava*I to make the −163 Nur77 promoter deletions, followed by blunting the ends with Klenow and religating the plasmids. The RSRF luciferase reporter plasmids were constructed as follows. The pGL2 Basic plasmid was digested with *Sal*I, and the site was destroyed by filling in the overhang with Klenow and religating the blunt ends. The −56 c-*fos* promoter was isolated from −56 CAT with *Hin*dIII and *Bam*HI as described above. The −56 LUC construct was created by ligating the minimal c-*fos* promoter fragment into the *Sma*I and *Bgl*II sites of pGL2 Basic (−Sal I). A synthetic oligonucleotide corresponding to the wild-type or mutated RSRF sequence (see Fig. 7d) of the −307 to −242 Nur77 promoter region was cloned into the *Sal*I site of −56 LUC. The transient transfections were performed in duplicate or triplicate on 10^7 cells with DEAE-dextran (30-min incubation) and 5 μg of plasmid DNA and are representative of at least four independent transfections.

Antibody production and Western blot analysis. For the production of antibodies, a glutathione *S*-transferase (GST)–Nur77 (212 to 601) fusion protein was constructed by inserting a 1.35-kb *Xmn*I-*Sma*I fragment of the Nur77 cDNA into the *Sma*I site of pGex1N. The GST-Nur77 fusion protein was purified from *Escherichia coli* by the standard protocol and injected every 2 weeks into New Zealand White rabbits for a total of six injections. The rabbit antiserum was purified sequentially on GST and GST-Nur77 fusion protein columns, made by cross-linking the fusion proteins to Affi-gel A10 resin (Bio-Rad) according to the manufacturer's instructions. For Western blot analysis, 50 μg of nuclear extracts was electrophoresed on 8% denaturing gels and electroblotted onto a nitrocellulose membrane. The membrane was probed with affinity-purified anti-Nur77 rabbit antiserum followed by a donkey anti-rabbit immunoglobulin-horseradish peroxidase conjugate (Amersham). The antibody reactivity was detected with an Amersham ECL kit according to the manufacturer's instruction. For the gel shift-Western blot, the gel shift was performed as described above and then electroblotted onto nitrocellulose. There were no ^{32}P counts on the nitrocellulose, which was probed with the affinity-purified Nur77 antiserum as described. The positions of the Nur77 bands identified on the Western blot lined up with duplicate Nur77 marker lanes from the same gel shift. For the cytoplasmic and nuclear fractionation, the cytoplasmic fraction was retained from the nuclear extract procedure, spun for 10 min at 14,300 × *g*, and then adjusted to 150 mM NaCl. The monoclonal antibody 2E1, specific for Nur77, has been described previously (15). The rabbit polyclonal antiserum specific for RSRF (MEF2) was provided by Dr. Treisman (45).

Phosphoamino acid and phosphopeptide analysis. Phosphoamino acid and tryptic peptide analyses were performed as described before (3, 14). The DO11.10 T-cell hybridoma or 293^+ cells were washed once and then incubated in phosphate-free medium containing 10% dialyzed FCS for 40 min, followed by labeling with 0.75 mCi of $^{32}P_i$ (Dupont/NEN) per ml for 2 to 3 more hours. The cells were stimulated by addition of PMA (10 ng/ml), ionomycin (0.5 μM), or PMA plus ionomycin in the presence or absence of CsA (200 ng/ml) for 1.5 h. The cells were washed once with 10 ml of ice-cold 1× phosphate-buffered saline (PBS), lysed in 0.9 ml of RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl [pH 8.0], 1 mM phenylmethylsulfonyl fluoride [PMSF], 50 mM NaF, 0.1 mM Na_3VO_4), and Nur77 was immunoprecipitated with a monoclonal antibody (2E1) specific for Nur77, followed by protein A-Sepharose beads. The immunoprecipitate was run on a 10% denaturing acrylamide gel and transferred to a nitrocellulose membrane. For tryptic peptide analysis, the corresponding Nur77 proteins were excised and digested with 10 μg of sequencing-grade trypsin for 18 h at 37°C. The peptides were repeatedly lyophilized, and then 200 to 300 cpm were electrophoresed on two-dimensional cellulose thin-layer chromatography (TLC) plates in pH 1.9 buffer in the first dimension and chromatographed in phosphochromatography buffer in the second dimension.

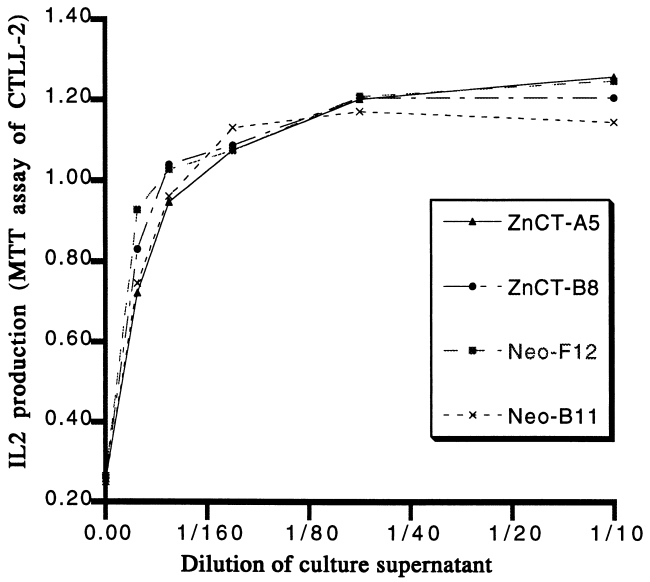

FIG. 1. Nur77 dominant negative mutation does not affect IL-2 production. The AO4H5.3 T-cell hybridoma stably transfected with a Nur77 dominant negative mutant (ZnCT-A5 and -B8) or vector control (Neo-F12 and -B11) was stimulated on anti-CD3-coated 96-well plates overnight. Culture supernatant was collected after 18 h and assayed for the presence of IL-2 with the IL-2-dependent cell line CTLL-2. The viability of the CTLL-2 cells was quantitated by using MTT. A value of 1.00 corresponds to 12.5 U of IL-2 per ml.

RESULTS

A Nur77 dominant negative mutation does not affect IL-2 production during T-cell activation. In T-cell hybridomas, Nur77 was shown to function in a pathway leading to apoptosis. Since Nur77 is induced by TCR signals and the same signals (anti-CD3) can also lead to IL-2 production, we analyzed the effect of a Nur77 dominant negative mutation on IL-2 synthesis to determine if it inhibits T-cell activation as well as apoptosis. As reported previously, several stable clones were generated in the T-cell hybridomas AO4H5.3 and DO11.10, which overexpress a dominant negative form of the Nur77 orphan steroid receptor (65). These stable clones exhibited increased viability after receiving apoptotic signals through their TCR, whereas control neomycin gene transfectants showed a high level of apoptosis (see Fig. 4 in reference 65). As shown in Fig. 1, the same stable clones expressing a dominant negative Nur77 (ZnCT-A5 and -B8) produced as much IL-2 as the neomycin gene-transfected controls. These data suggest that the Nur77 protein induced by anti-CD3 signals functions in a pathway leading to apoptosis that is distinct from the NF-AT transcription factor pathway leading to IL-2 synthesis.

Induction of the Nur77 DNA-binding activity requires calcium signals. Since Nur77 is required for activation-induced apoptosis but is also stimulated by nonapoptotic signals, we analyzed the Nur77 protein induced by PMA, ionomycin, and PMA plus ionomycin, which mimics signaling by the TCR-CD3 complex. DO11.10 and AO4H5.3 T-cell hybridomas were stimulated with the various agents, and the Nur77 protein activity in nuclear extracts was examined by gel shift analysis with the NBRE as a probe (61, 63). The Nur77 protein is not expressed in unstimulated T-cell hybridomas (65). We found that stimulation with PMA resulted in expression of a small amount of the NBRE DNA-binding complex (Fig. 2a), which could be blocked completely by Nur77-specific antiserum (see below and data not shown). In contrast, the addition of ionomycin alone or together with PMA resulted in the appearance of a high level of NBRE DNA-binding complex (Fig. 2a, last two lanes). The majority of the protein in this binding complex is Nur77 (see below).

To confirm that calcium signals mediate the high level of Nur77 expression, we stimulated DO11.10 cells and freshly isolated thymocytes with anti-CD3 antibody in the presence and absence of CsA. CsA has been shown to inhibit the Ca^{2+} signaling pathway of the TCR by blocking activation of the calcium-dependent phosphatase calcineurin (for a review, see reference 49). The NBRE DNA-binding activity can be detected as early as 30 min to 1 h after stimulation and is present up to 10 h poststimulation (Fig. 2b) (65). That calcium signals are responsible for the major NBRE binding protein induction in T-cell hybridomas and thymocytes is clearly shown by the CsA inhibition of the binding complex at all time points (Fig. 2b). As a control, CsA did not affect the activity of the GT box-binding proteins (Sp1 family). It was previously reported that CsA completely inhibits the Nur77 DNA-binding activity induced by anti-TCR cross-linking (67). However, we find that the inhibition is not complete, since a small amount of NBRE DNA-binding complex is still detected in nuclear extracts of anti-CD3- plus CsA-stimulated cells (Fig. 2b). These data show that PMA induces a low level of NBRE DNA-binding activity, whereas PMA plus ionomycin induces a high level of NBRE DNA-binding activity.

To confirm that Nur77 protein is indeed present in the NBRE DNA-binding complex, we performed an antibody blocking experiment and a Western blot analysis of the gel shift complex. Affinity-purified rabbit antiserum generated against a GST-Nur77 fusion protein was used in these experiments. This antiserum is specific for Nur77 and does not recognize the related protein Nurr1 (26, 47) in gel shift analysis of in vitro-translated Nur77 and Nurr1 proteins (Fig. 2a). In vitro-translated Nur77 binds poorly to the NBRE but is clearly blocked by the specific antiserum. A control affinity-purified rabbit antiserum to Sp3 did not recognize either Nur77 or Nurr1 (Fig. 2a). Western blot analysis with the purified anti-Nur77 antibodies indicated that the CsA-sensitive protein-DNA complex detected by the NBRE DNA-binding element indeed contains Nur77 (Fig. 2c). Similarly, addition of purified anti-Nur77 antiserum in the gel shift reaction mix resulted in blockage of most of the original NBRE DNA-binding activity from the T-cell hybridoma extracts and all of the NBRE binding activity from the stimulated thymus extracts (Fig. 2d). Together, these data demonstrate that calcium signals induce a high level of Nur77, which correlates with the role of calcium in activation-induced apoptosis of T-cell hybridomas and thymocytes.

Analysis of Nur77 protein levels by Western blot. To investigate the levels of Nur77 protein induced by the different stimuli, we performed Western blot analyses of nuclear extracts with the purified Nur77-specific antiserum. We found that PMA induces a low level of Nur77 protein, whereas PMA plus ionomycin or ionomycin alone induces a high level of Nur77 protein, starting at 1 h poststimulation (Fig. 3a). Hardly any Nur77 can be detected in unstimulated or 0.5-h-stimulated T-cell extracts. Nur77 from PMA-stimulated T cells can be seen as a protein species of 70 kDa, whereas ionomycin and PMA-ionomycin-stimulated Nur77 ranges in size from approximately 70 up to 90 kDa. This wide range of Nur77 protein species is due to heavy phosphorylation of the Nur77 protein, as demonstrated previously in NGF- and KCl-treated PC12 cells (15, 18). Phosphatase treatment of the anti-Nur77-reactive proteins resulted in a faster-migrating protein species of 65 kDa (15, 64a). Two-dimensional phosphopeptide mapping analysis also showed that both smaller (70 kDa) and larger (90

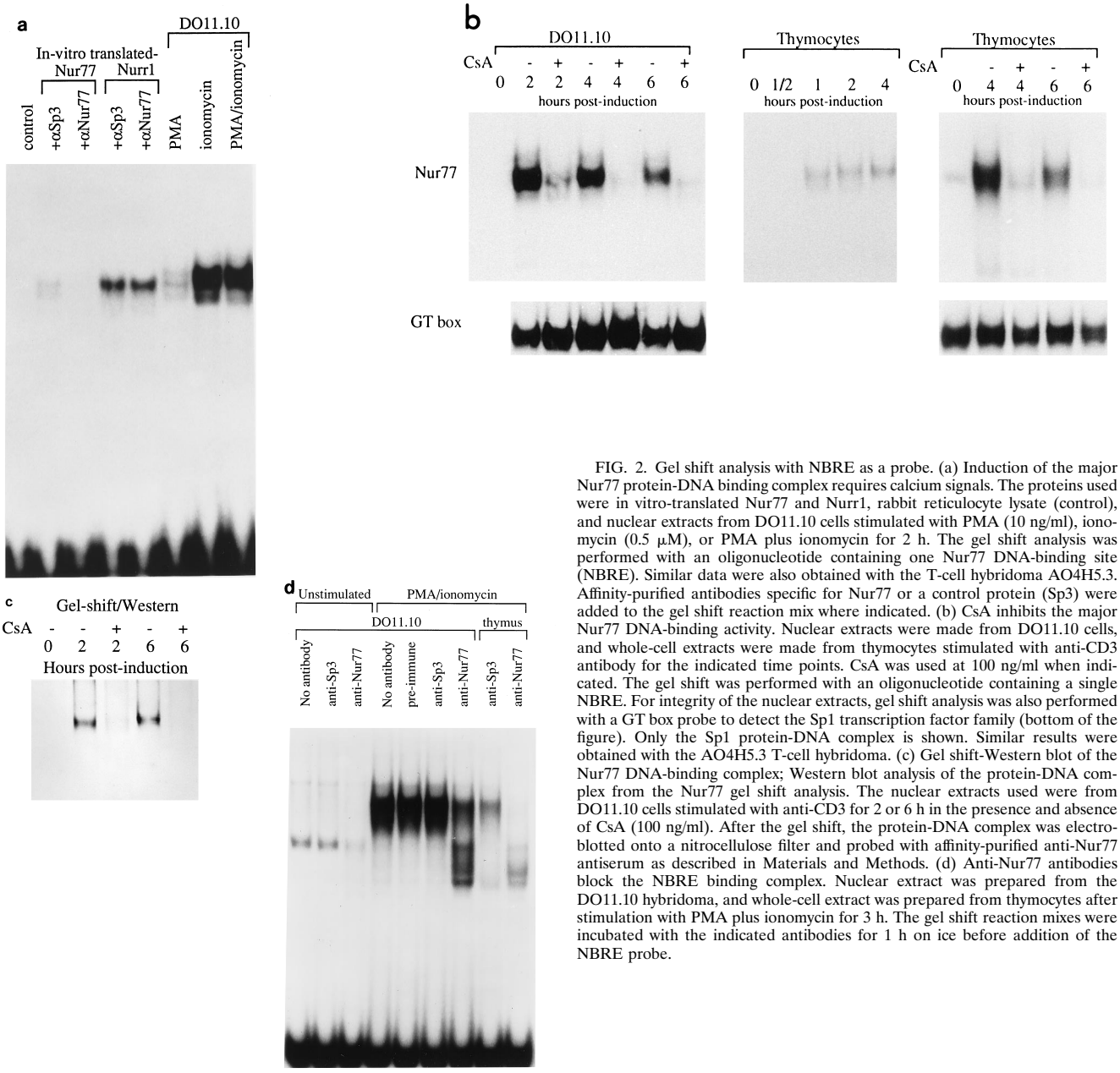

FIG. 2. Gel shift analysis with NBRE as a probe. (a) Induction of the major Nur77 protein-DNA binding complex requires calcium signals. The proteins used were in vitro-translated Nur77 and Nurr1, rabbit reticulocyte lysate (control), and nuclear extracts from DO11.10 cells stimulated with PMA (10 ng/ml), ionomycin (0.5 μM), or PMA plus ionomycin for 2 h. The gel shift analysis was performed with an oligonucleotide containing one Nur77 DNA-binding site (NBRE). Similar data were also obtained with the T-cell hybridoma AO4H5.3. Affinity-purified antibodies specific for Nur77 or a control protein (Sp3) were added to the gel shift reaction mix where indicated. (b) CsA inhibits the major Nur77 DNA-binding activity. Nuclear extracts were made from DO11.10 cells, and whole-cell extracts were made from thymocytes stimulated with anti-CD3 antibody for the indicated time points. CsA was used at 100 ng/ml when indicated. The gel shift was performed with an oligonucleotide containing a single NBRE. For integrity of the nuclear extracts, gel shift analysis was also performed with a GT box probe to detect the Sp1 transcription factor family (bottom of the figure). Only the Sp1 protein-DNA complex is shown. Similar results were obtained with the AO4H5.3 T-cell hybridoma. (c) Gel shift-Western blot of the Nur77 DNA-binding complex; Western blot analysis of the protein-DNA complex from the Nur77 gel shift analysis. The nuclear extracts used were from DO11.10 cells stimulated with anti-CD3 for 2 or 6 h in the presence and absence of CsA (100 ng/ml). After the gel shift, the protein-DNA complex was electroblotted onto a nitrocellulose filter and probed with affinity-purified anti-Nur77 antiserum as described in Materials and Methods. (d) Anti-Nur77 antibodies block the NBRE binding complex. Nuclear extract was prepared from the DO11.10 hybridoma, and whole-cell extract was prepared from thymocytes after stimulation with PMA plus ionomycin for 3 h. The gel shift reaction mixes were incubated with the indicated antibodies for 1 h on ice before addition of the NBRE probe.

kDa) forms of Nur77 protein are highly phosphorylated (data not shown).

Western blot analysis of the anti-CD3-stimulated cells also showed that the Nur77 protein is highly expressed and is maintained throughout activation-induced apoptosis (Fig. 3b). Strikingly, in anti-CD3- and CsA-stimulated T-cell hybridomas, in which the NBRE has lost its major DNA-binding activity, the level of Nur77 protein is dramatically reduced at all time points examined (Fig. 3b). The decrease in Nur77 protein levels in the presence of CsA was not due to rapid protein degradation, since pulse-chase analysis revealed that the half-life is comparable (30 to 40 min) with and without the addition of CsA (data not shown). Most of the Nur77 detected in the CsA- plus anti-CD3-treated extracts is of the smaller size (70 kDa).

To see if CsA affects the cellular distribution of Nur77, we performed Western blot analyses of cytoplasmic and nuclear extracts from T-cell hybridomas. In the presence of PMA plus ionomycin, most of the Nur77 protein species are located in the nuclear fraction, with only a small amount of Nur77 in the cytoplasmic fraction (Fig. 3c). Addition of CsA to the PMA-ionomycin-stimulated cells did not result in accumulation of Nur77 protein in the cytoplasmic fraction (Fig. 3c). Thus, CsA did not inhibit the ability of Nur77 protein to translocate to the nucleus.

Nur77 is phosphorylated to a similar level by phorbol ester and TCR signals. Since Nur77 is known to be highly phosphorylated, like other members of the steroid receptor family (42), we investigated the phosphorylation status of the Nur77 protein induced by PMA alone, ionomycin alone, and PMA plus ionomycin in the presence and absence of CsA. For these studies, DO11.10 and AO4H5.3 cells were labeled in vivo with

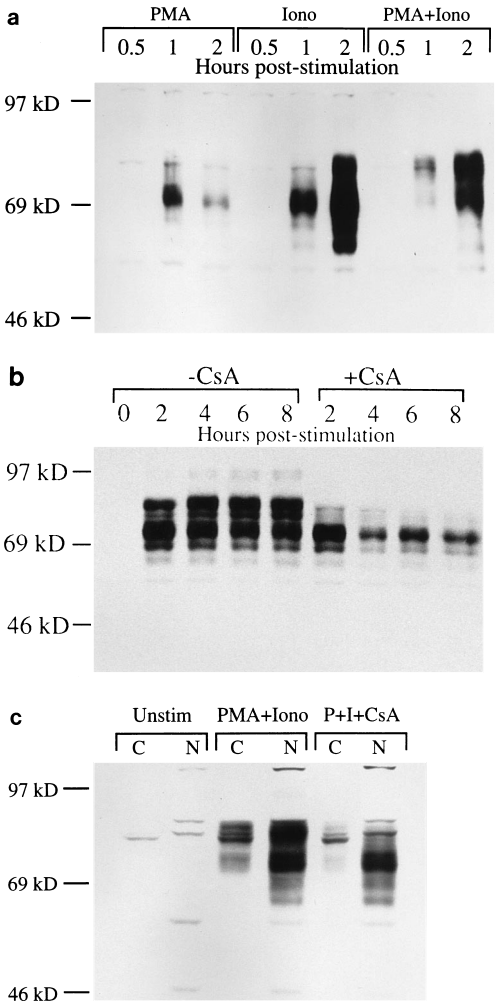

FIG. 3. Western blot analysis of the Nur77 protein induced by PMA, ionomycin, or TCR signals. (a) Western blot analysis of Nur77 from T-cell hybridomas. Nuclear extracts were made from the DO11.10 T-cell hybridoma after stimulation with PMA (10 ng/ml), ionomycin (0.5 μM), or PMA plus ionomycin for 0.5, 1, and 2 h. (b) Western blot analysis of Nur77 from T-cell hybridomas stimulated with anti-CD3 in the presence and absence of CsA. Nuclear extracts were made from the DO11.10 T-cell hybridoma after stimulation on anti-CD3 antibody-coated plates at various time points (0, 2, 4, 6, and 8 h) in the presence and absence of CsA (100 ng/ml). (c) Western blot analysis of cytoplasmic (C) and nuclear (N) fractions from T-cell hybridomas. Nuclear and cytoplasmic fractions were prepared from DO11.10 cells after stimulation with PMA plus ionomycin for 2 h in the presence (P+I+CsA) or absence (PMA + Iono and Unstim) of CsA.

$^{32}P_i$. The Nur77 protein was immunoprecipitated with a monoclonal antibody (2E1) specific for Nur77, followed by phosphoamino acid and tryptic peptide analysis. As found by phosphoamino acid analysis in other cell types (18), Nur77 is highly phosphorylated on serine and has a low level of threonine phosphorylation in apoptotic T cells (data not shown). Tryptic peptide analysis revealed that all of the different activation conditions produced very similar two-dimensional phosphopeptide maps, indicating that PMA and PMA plus ionomycin induce a similar level of Nur77 phosphorylation and that there is no inhibition of phosphorylation by CsA (Fig. 4a). Identical results were seen when V8 protease was used to generate phosphopeptide maps (data not shown). Although there are slight differences in the intensities of several tryptic phosphopeptides, these differences vary between experiments and are not reproducible.

It has previously been shown that Nur77 is transcriptionally active when transfected into many cell types in the absence of any stimuli (12, 43, 64a, 65). To see if the phosphorylation status of Nur77 would be different in the presence and absence of activation signals, we transiently transfected the 293^+ kidney cell line with a Nur77 expression plasmid followed by in vivo $^{32}P_i$ labeling. Tryptic phosphopeptide analysis of the exogenously expressed Nur77 from 293^+ cells showed that unstimulated cells produce a fairly complex peptide map (Fig. 4b). This analysis shows that newly synthesized Nur77 protein becomes highly phosphorylated in the absence of extracellular signals and that phosphorylation increases slightly after PMA or PMA-ionomycin stimulation. However, the transactivation activity of cotransfected Nur77 cannot be increased further by addition of PMA plus ionomycin (data not shown), suggesting that calcineurin- or kinase C-dependent phosphorylation might not be essential for Nur77 activity.

Differential NBRE-mediated transactivation activity correlates with the levels of Nur77 protein expression. Since phorbol ester and anti-CD3 signals induce different levels of Nur77 protein, we checked for NBRE-mediated transactivation activity in a transient reporter gene assay. To increase the sensitivity, we used the luciferase reporter constructs under the control of one to four NBRE response elements. The T-cell hybridoma DO11.10 was transiently transfected and stimulated with either PMA or PMA plus ionomycin. When reporter genes containing one or two copies of the NBRE were used, hardly any transactivation activity could be detected in response to PMA stimulation (Fig. 5a). A modest transactivation activity by PMA can only be seen with reporter constructs containing three or four copies of NBRE. In comparison, PMA plus ionomycin or ionomycin alone resulted in a high level of transactivation activity through one or two NBRE sites, which can be inhibited by CsA (Fig 5b and c). The PMA ionomycin-stimulated transactivation activity mediated by three or four copies of NBRE, however, is not CsA sensitive. Thus, one or two NBRE are sufficiently sensitive to study Nur77-specific transactivation, and large numbers of multimerized sites can become relatively insensitive to fluctuations in the protein level of a transcription factor. Mutations at NBRE which abolish Nur77 binding activity eliminate the transactivation activity (Fig. 5a and c). Together, these data show that phorbol ester and calcium signals mediate differential NBRE-mediated transactivation activity, which correlates with the differential regulation of the Nur77 protein levels.

Kinetics of cyclosporin inhibition of apoptosis parallels the inhibition of Nur77 protein. To correlate further the role of Nur77 in activation-induced apoptosis of T-cell hybridomas, we compared the kinetics of CsA inhibition of Nur77 with the kinetics of CsA inhibition of cell death. To do this, we slightly modified the gel shift assay to enhance the sensitivity. Normally, we do not detect NBRE DNA-binding complex at 5 h poststimulation in the presence of CsA because of the decreased protein levels. However, we found that by adding 1 μl of preimmune rabbit antiserum to the gel shift reaction mix, the sensitivity was increased, enabling us to detect a low level of protein-DNA binding complex at later time points poststimulation (the enhanced binding by preimmune serum is not restricted to Nur77). The addition of preimmune serum does not affect the specificity of the binding, as competition with an unlabeled NBRE oligonucleotide abolishes the binding complex (data not shown).

In this kinetics experiment, we added CsA at various time points before and after PMA-ionomycin stimulation of the

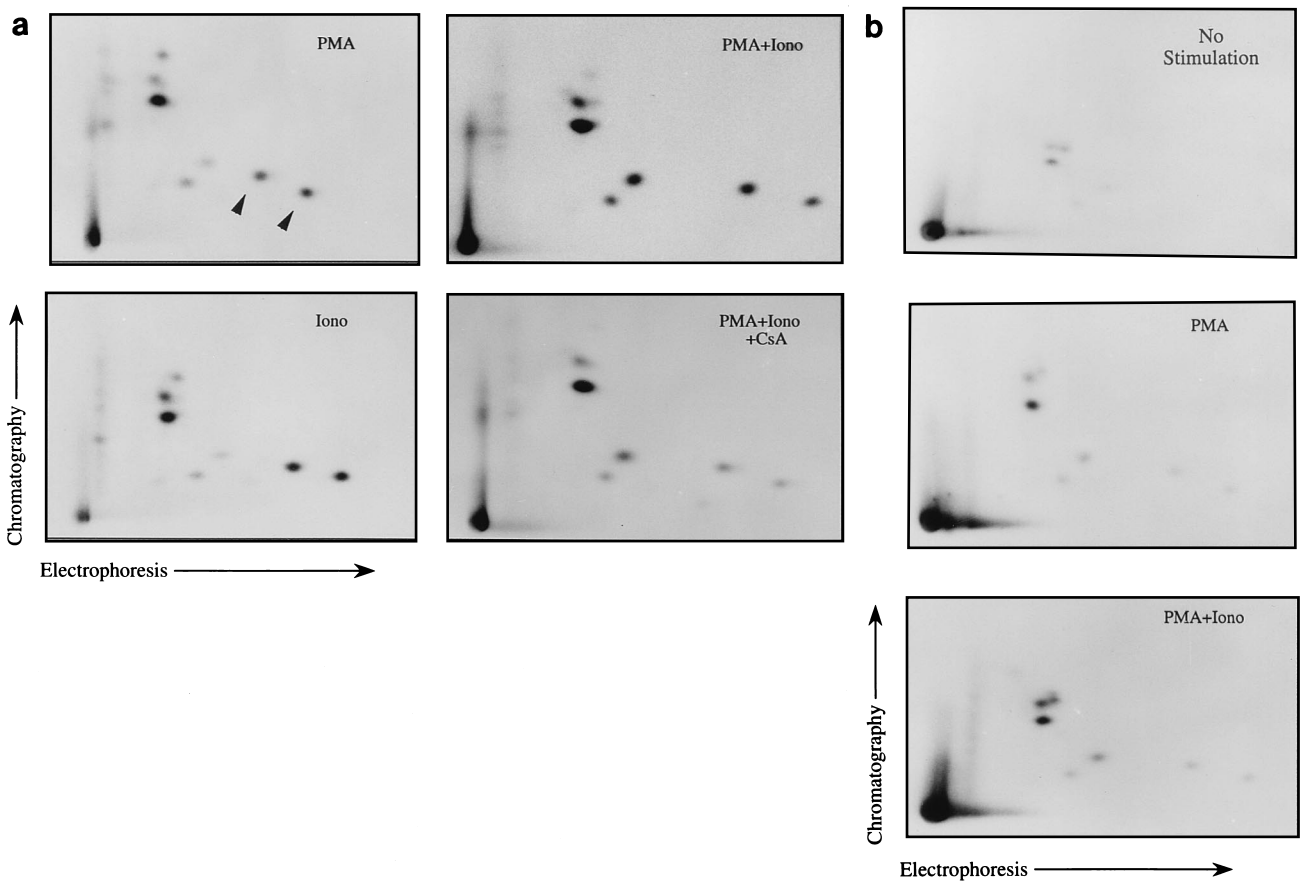

FIG. 4. Tryptic phosphopeptide analysis of endogenous and exogenously expressed Nur77 protein stimulated with either PMA, ionomycin, or PMA plus ionomycin in the presence or absence of CsA. (a) Tryptic phosphopeptide analysis of the Nur77 protein from T-cell hybridomas. The DO11.10 T-cell hybridoma was in vivo labeled with $^{32}P_i$ followed by stimulation for 1.5 h with PMA, ionomycin, or PMA plus ionomycin in the presence or absence of CsA. Immunoprecipitated Nur77 protein was digested with trypsin, and the resulting phosphopeptides were separated in two dimension on cellulose TLC plates. Similar results were observed with the AO4H5.3 cell line. Arrowheads indicate the tryptic peptides containing serine 354. (b) Tryptic phosphopeptide analysis of exogenously expressed Nur77 protein from 293^+ cells. 293^+ cells were transiently transfected with a Nur77 expression plasmid. After 24 h, the cells were ^{32}P labeled and either left unstimulated or stimulated for 15 min with PMA or PMA plus ionomycin. The immunoprecipitated Nur77 protein was analyzed as described above.

AO4H5.3 T-cell hybridoma. After a total of five h of stimulation, the onset of apoptotic DNA ladders was compared with the appearance of the major NBRE DNA-binding activity in nuclear extracts (Fig. 6a). We found that CsA can inhibit the major NBRE binding complex when it is added within 2 h of PMA-ionomycin stimulation (Fig. 6a). Western blot analysis of the same nuclear extracts shows that CsA treatment results in a significant reduction in Nur77 protein levels when administered at earlier time points. At similar time points of CsA inhibition, apoptotic DNA ladders were not detected, indicating that CsA can block the apoptotic process even after the death program is initiated (Fig. 6b). This was also confirmed by a cell viability assay with propidium iodide. Addition of CsA within 2 h of stimulation can still rescue up to 80% of the T-cell hybridoma cells from apoptosis (Fig. 6c). When CsA is added 2 or more h poststimulation, the appearance of the major Nur77 protein species is detected by Western blot analysis (Fig. 6a) and correlates with the onset of apoptotic DNA ladders at these time points (Fig. 6b). Thus, the increase in the Nur77 protein level correlates with the appearance of the major NBRE DNA-binding complex as well as the induction of apoptotic DNA ladders and the decrease in cell viability.

Deletion analysis of the Nur77 promoter reveals two calcium-responsive regions. To examine Nur77 transcriptional activity, the Nur77 promoter was isolated from a C57BL/6 genomic library, and a fragment of the promoter from −3800 to +87 or −480 to +87 was cloned into a chloramphenicol acetyltransferase (CAT) reporter plasmid. These plasmids were transfected individually into the T-cell hybridomas AO4H5.3 and DO11.10, and CAT activity was measured after stimulation with either PMA alone, ionomycin alone, or a combination of PMA and ionomycin. Similar results were obtained with both the −3800 and −480 Nur77 promoter constructs, and therefore subsequent analysis was performed with the −480 promoter construct. The Nur77 reporter plasmids showed no CAT activity in unstimulated cells. When the transfected cells were stimulated with PMA plus ionomycin, however, a several hundredfold increase in CAT activity was detected (Fig. 7a). The addition of ionomycin alone also led to a substantial increase in CAT activity (80 to 160-fold). In contrast, PMA alone weakly activated the Nur77 promoter (10- to 17-fold). These data are consistent with the observed levels of Nur77 protein induced by these agents. This analysis shows that maximal Nur77 promoter activity requires both the protein kinase C and calcium signaling pathways but that calcium mediates the major Nur77 transcriptional activity.

To identify the calcium-sensitive regions of the Nur77 promoter, we generated a series of 5′ promoter deletions and

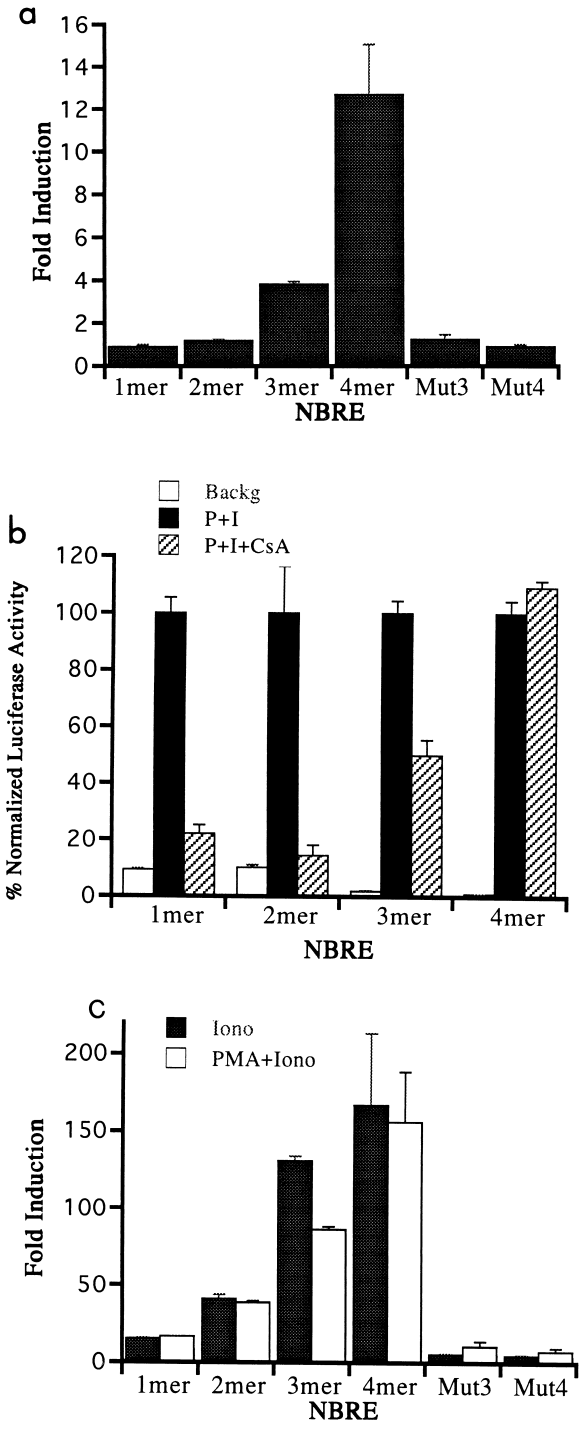

FIG. 5. Phorbol ester and TCR signals mediate differential Nur77 transactivation activity. (a) DO11.10 T cells were transfected with luciferase reporter constructs containing one, two, three, or four copies of the NBRE or NBRE mutant, followed by PMA stimulation. Twenty-four hours after transfection, PMA was added for 6 h, and cells were harvested for the luciferase assay. The values shown are the averages of three independent experiments plus the standard deviation. (b) DO11.10 T cells were transfected with luciferase reporter constructs containing one, two, three, or four copies of the NBRE, followed by PMA plus ionomycin (P+I) stimulation. Twenty-four hours after transfection, the cells were stimulated with PMA (10 ng/ml) and 0.5 μM ionomycin for 6 h, and cells were harvested for the luciferase assay. CsA (100 ng/ml) was also added where indicated (P+I+CsA). Nonstimulated (Backg) transfected cells were included as controls. The luciferase activity in the PMA-ionomycin samples was normalized to 100%. (c) Experiments similar to those described in the legend to panel a were performed except that ionomycin or a combination of PMA plus ionomycin was used for stimulation. Mut3 and Mut4 are luciferase constructs with three or four copies of the mutant NBRE.

analyzed them by transient transfection. The deletion of the Nur77 promoter to −336 had no effect on its calcium sensitivity. Further deletion to −287 resulted in a threefold drop in Nur77 promoter activity, indicating the presence of an essential calcium-responsive element between −336 and −287 (Fig. 7a). A dramatic drop in activity was also observed when the Nur77 promoter was deleted to −163 (Fig. 7a). Thus, another crucial calcium-responsive element is located between −287 and −163 of the Nur77 promoter. In contrast to a previous report (29), which mapped the PMA response of the Nur77 promoter to between −378 and −332, we did not find any significant differences in the response to PMA between the −480 and −336 Nur77 promoter constructs (Fig. 7a). These results identify two regions of the Nur77 promoter that mediate calcium inducibility in T cells and demonstrate that there is a dramatic difference in Nur77 promoter activity (>20-fold) between the kinase C and calcium pathways.

To see if CsA affects Nur77 promoter activity, the various promoter plasmids were transfected into the AO4H5.3 or DO11.101 T-cell hybridomas and stimulated with PMA plus ionomycin in the presence and absence of CsA. We found that CsA inhibits the calcium-inducible component of the Nur77 promoter four- to fivefold. It consistently reduced the Nur77 promoter activity stimulated by either ionomycin alone or a combination of PMA and ionomycin (Fig. 7b and c). As expected, CsA had no effect on the Nur77 promoter activity that is regulated by PMA (Fig. 7b and c). A similar response was seen for the activities of all of the measurable Nur77 promoter constructs (−480, −336, and −287). We also tested the effect of dexamethasone on the Nur77 promoter because we have observed that dexamethasone treatment together with T-cell activation signals can slightly reduce the Nur77 protein-DNA complex levels seen in gel shift assays. The effect was not very dramatic, with at most a twofold reduction in CAT activity in either ionomycin- or PMA-stimulated cells. These results show that the Nur77 promoter is calcium inducible and CsA sensitive.

RSRF elements in the Nur77 promoter are calcium inducible and CsA sensitive. To identify the elements controlling the calcium inducibility of the Nur77 promoter, DNase I footprinting and gel shift analyses were performed on the DNA region between −336 and −163. Several potential transcription factor-binding sites can be found in this region, including ets, NF-AT, and two RSRF elements (Fig. 7d). The footprint analysis revealed a prominent protected region around −260, corresponding to an RSRF consensus binding site (data not shown). Gel shift analysis with promoter fragments corresponding to −336 to −163 or −336 to −287 showed that protein-binding complexes were generated with either of the DNA fragments (Fig. 8a and b). The major protein complexes detected by either fragment can be seen in nonstimulated cells and are specific, as they can be eliminated with excess unlabeled Nur77 promoter DNA but not by a nonspecific fragment (GT box oligonucleotide). Also, competition was not observed when an oligonucleotide corresponding to the IL-2 NF-AT element was used (Fig. 8a and b), indicating that the NF-AT protein does not bind to the Nur77 promoter. The role of the ets binding site has not been examined, although ets family proteins are not known to be CsA sensitive.

To see if RSRF protein actually binds to the Nur77 promoter, we used an antibody specific for mouse RSRF (45) in the gel shift analysis to see if we could supershift or block the protein-DNA complex. RSRF has been shown to bind to the consensus sequence $CTA(A/T)_4TAG$ (45), and there are two putative RSRF binding sites in the Nur77 promoter, one at −297 and another at −262. Indeed, the RSRF-specific anti-

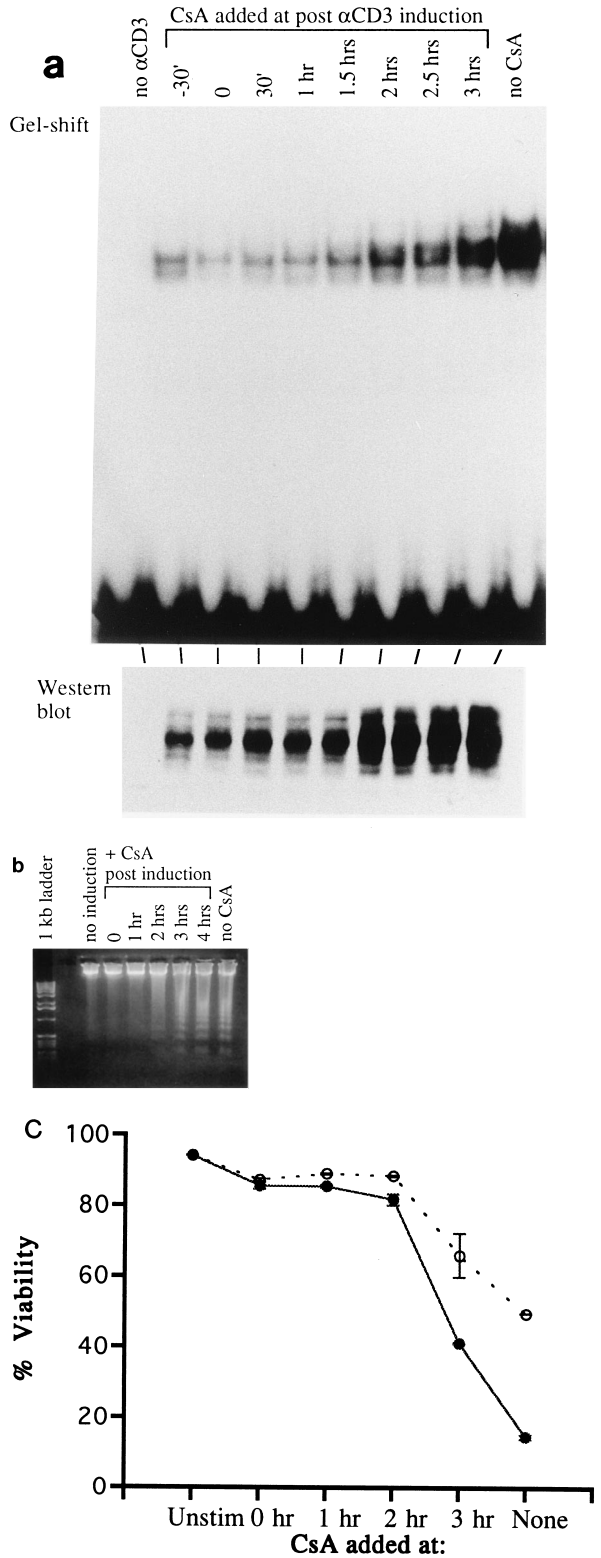

FIG. 6. Kinetics of apoptosis inhibition by CsA correlates with the inhibition of Nur77 protein levels. (a) Gel shift and Western blot analyses of Nur77 during CsA inhibition of apoptosis. The AO4H5.3 T-cell hybridoma was stimulated with PMA plus ionomycin for a total of 5 h. CsA was added 30 min prior to stimulation (−30′), at the time of stimulation (0), or after PMA-ionomycin stimulation (30 min or 1, 1.5, 2, 2.5, or 3 h). Gel shift and Western blot analyses of the corresponding nuclear extracts were performed as described above. (b) Apoptotic DNA ladder analysis of the kinetics of CsA inhibition of activation-induced apoptosis. An experiment similar to that described for panel a was performed, except that total DNA was isolated at the end of the 5-h stimulation and run on a 1.5% agarose gel. (c) Cell viability analysis of the kinetics of CsA inhibition of activation-induced apoptosis. An experiment similar to that described in the legend to panel a was performed, except that cell viability was determined by propidium iodide staining, as described before (33). Cross-linked anti-CD3 antibodies were used to initiate apoptosis at a 1:10,000 dilution (dotted line) or 1:5,000 dilution (solid line) of the anti-CD3 ascites fluid. Similar results were found when the T-cell hybridomas were stimulated with PMA and ionomycin.

body but not the preimmune serum can supershift the protein-DNA complex detected by either the −336 to −163 or −336 to −287 Nur77 promoter fragment (Fig 8a and b). Similar results were also obtained with a fragment spanning −287 to −163 (data not shown). The RSRF DNA-binding activity present in unstimulated cells did not change upon T-cell activation. In addition, no changes were observed when extracts from CsA- or dexamethasone-treated cells were used. Thus, RSRF protein binds at two different sites in the Nur77 promoter.

To see if the Nur77 promoter fragment containing both RSRF sites was sufficient to mediate the calcium- and CsA-sensitive activity, the −307 to −242 Nur77 promoter fragment (Fig. 7d) was cloned into a luciferase reporter plasmid upstream of a minimal −56 c-*fos* promoter. Luciferase activity was measured after transfection into the DO11.10 T-cell hybridoma followed by stimulation in the presence and absence of CsA. We found that one copy of the −307 to −242 promoter fragment could mediate calcium- but not PMA-inducible transcriptional activity (Fig. 9a). Interestingly, the addition of CsA leads to a complete inhibition of the calcium-sensitive promoter fragment (Fig. 9a). Also, 2-bp mutations at each RSRF site which are known to abolish RSRF binding (45) blocked the calcium-mediated luciferase activity (Fig. 9a). The −307 to −242 promoter fragment containing the RSRF mutations also failed to compete for the RSRF protein-DNA complex seen in the gel shift assay (Fig. 9b). These data show that the RSRF transcription factor plays a crucial role in the calcium-inducible and CsA-sensitive nature of the Nur77 promoter.

DISCUSSION

The Nur77 orphan steroid receptor is required for activation-induced apoptosis of T-cell hybridomas (29, 65). To gain insight into its function during apoptosis, we characterized Nur77 protein phosphorylation, transactivation activity, and promoter induction in T-cell hybridomas. TCR signals can be mimicked by addition of anti-CD3 antibody or by a combination of phorbol ester and ionomycin and can be inhibited by addition of CsA. We find that phorbol ester induces a transient, low level of Nur77 protein that is barely detectable by the gel shift assay. This protein is functional, because it transactivates reporter genes containing three or more NBRE binding sites. On the other hand, TCR signals induce a high level of Nur77 protein that is maintained throughout apoptosis. The high level of Nur77 expression correlates with a high degree of transactivation activity through one or two NBREs. Induction of the major NBRE DNA-binding activity requires the calcium-mediated signaling pathway of the TCR, and as such, CsA blocks its induction down to a level similar to induction by phorbol ester (PMA) alone. Western blot analysis showed that PMA plus ionomycin induces hyper- and hypophosphorylated forms of Nur77 (70 and 90 kDa, respectively). Addition of CsA inhibits the hyperphosphorylated form of Nur77, suggesting that a calcium-mediated phosphorylation might regulate Nur77 function. However, the tryptic phosphopeptide analysis shows that the overall patterns of Nur77 phosphorylation are

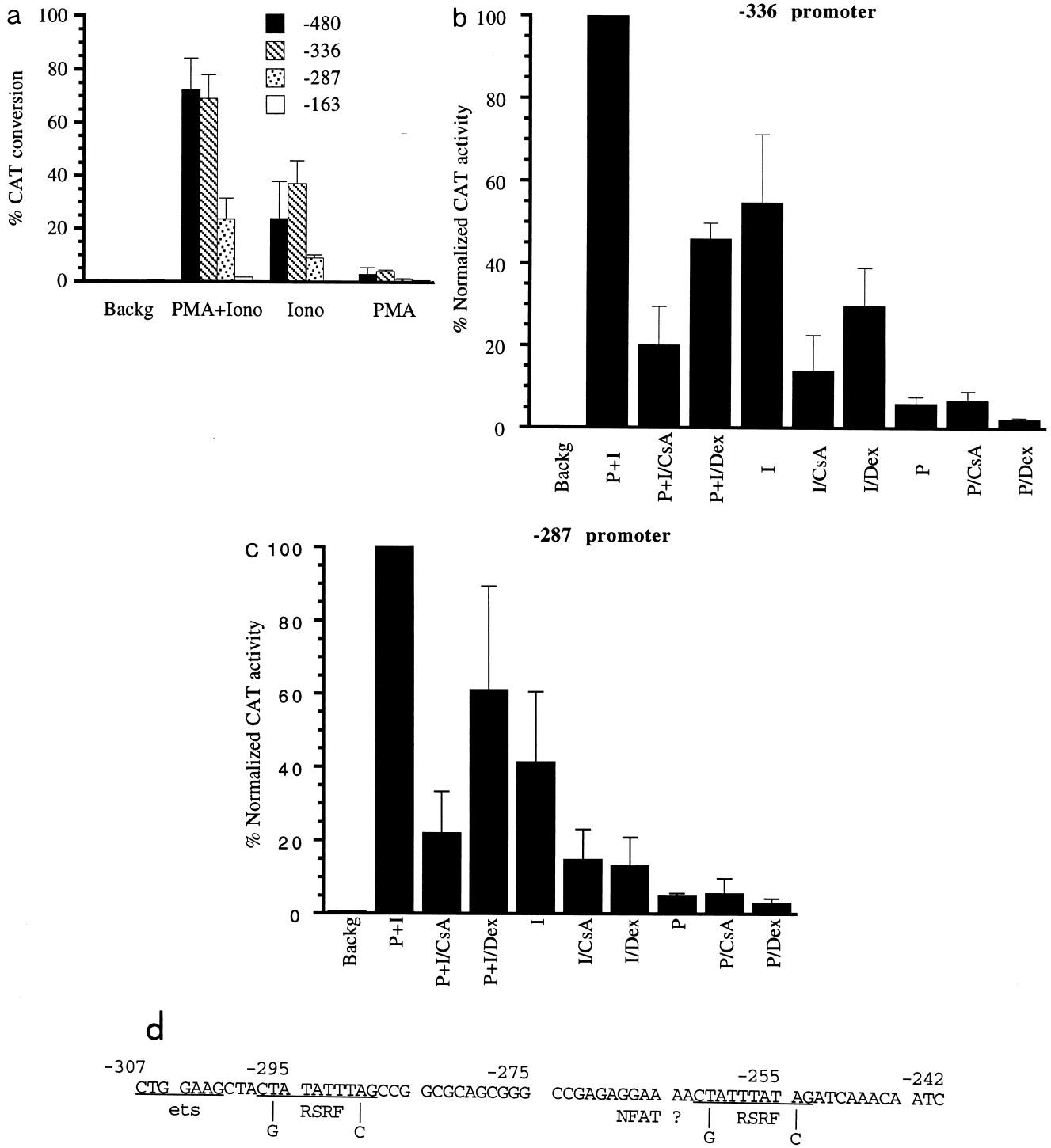

FIG. 7. Deletion analysis of the Nur77 promoter. (a) Progressive 5′ deletion of the Nur77 promoter. CAT constructs containing Nur77 promoter regions from −480 to +87, −336 to +87, −287 to +87, or −163 to +87 were transfected into T-cell hybridomas. The transfected cells were stimulated with the indicated reagents for 2 h before harvesting. CAT activity was measured as described before (25). Backg, background. (b) CsA sensitivity of the −336 Nur77 promoter. A CAT construct containing bases −336 to +87 of the Nur77 promoter was transfected into T-cell hybridomas and stimulated with the various reagents (Dex, dexamethasone; P, PMA; I, ionomycin). The CAT value for the Nur77 promoter activity stimulated by PMA plus ionomycin was normalized to 100%. (c) CsA sensitivity of the −287 Nur77 promoter. Experiments were performed with the −287 to +87 Nur77 promoter construct. (d) The Nur77 promoter sequence (−307 to −242) was shown to indicate potential protein-binding sites. The promoter sequence was taken from reference 46. Mutations at the RSRF sites that abolish RSRF DNA-binding and transactivation activity are indicated.

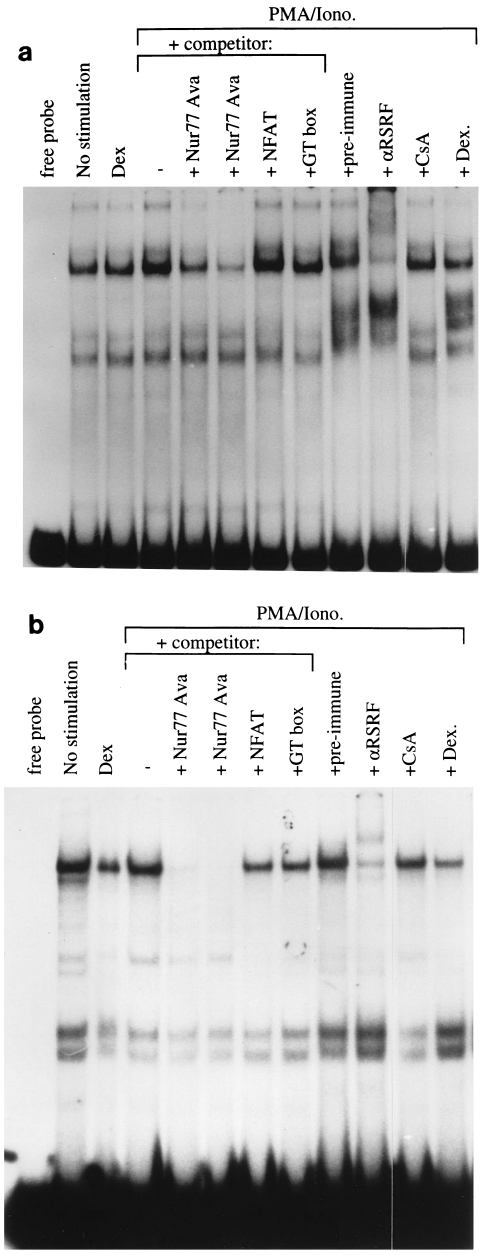

FIG. 8. RSRF binds to the Nur77 promoter. (a) Gel shift DNA-binding assays were performed with nuclear extracts from the DO11.10 T-cell hybridoma after treatment with the indicated reagents. The probe used was the Nur77 −336 to −163 promoter fragment. Competition was performed with unlabeled DNA fragment where indicated. Nur77 Ava, Nur77 −336 to −163 promoter fragment. NFAT, competition with oligonucleotides containing the NF-AT IL-2 proximal sequence (22). GT, competition with oligonucleotides containing the GT box Sp1 family binding site (25). Antiserum (preimmune or anti-RSRF) was added where indicated. (b) Gel shift analysis of the Nur77 −336 to −287 promoter fragment. Experiments were performed with the −336 to −287 Nur77 promoter fragment as a probe.

similar after PMA, ionomycin, and PMA-ionomycin stimulation in the presence and absence of CsA. Although we cannot rule out the possibility that there are subtle differences in the phosphorylation state of Nur77 induced by these different stimuli, calcium- or kinase C-mediated phosphorylation might not be important for regulation of Nur77 function, as the transactivation activity of the cotransfected Nur77 cannot be increased further by addition of PMA plus ionomycin.

We have recently generated transgenic mice expressing the Nur77 dominant negative mutant protein in T cells. In addition to a slight increase in the number of $CD4^+$ $CD8^-$ mature T cells in these transgenic mice, we also observed inhibition of antigen negative selection by the Nur77 dominant negative protein (6). In contrast, antigen-induced negative selection is not affected in mutant mice lacking Nur77 (27). Indeed, $Nur77^{-/-}$ mice are normal in all aspects examined so far (11, 27). However, Nur77 has two other closely related family members, Nurr1 (also called Rnr-1, NOT, and TINUR) and Nor-1 (26, 30, 39, 40, 47) with highly homologous DNA-binding domains (91 to 95%). Both the Nurr1 and Nor-1 proteins can also bind to the NBRE DNA-binding site (26, 39). Compensation by these Nur77-related proteins in the $Nur77^{-/-}$ mice may account for the lack of any observable phenotype in these mice. On the other hand, the dominant negative Nur77 protein can bind to the NBRE and inhibit the activities of all the Nur77 family proteins. Nur77 constitutes the majority of the NBRE binding complex in the thymus extract (Fig. 2d), and thus it is the most likely effector of apoptosis accompanying negative selection in the normal context.

The role of CsA in negative selection remains controversial. Several groups have previously shown that CsA can inhibit anti-CD3- and superantigen-induced apoptosis (17, 23). CsA can also delay the onset of negative selection in H-Y TCR transgenic mice (56). However, others have shown that CsA has no effect on negative selection (2, 57, 59). As we have demonstrated above, the effect of CsA on Nur77 transactivation function is limited to a reporter gene under the control of one or two copies of NBRE. Thus, any downstream target gene regulated by three or more copies of the Nur77 binding site will be minimally affected by CsA.

It is interesting to speculate that a high level of Nur77 protein expression would be sufficient to induce apoptosis in T cells. Indeed, constitutive expression of wild-type Nur77 in transgenic mice leads to massive apoptosis in the thymus (6). However, in T-cell hybridomas, ionomycin alone induces a high level of Nur77 DNA binding and transactivation activity but does not induce apoptosis. This suggests that a signal is delivered by the protein kinase C pathway that is required, in conjunction with ionomycin, to induce apoptosis in T-cell hybridomas. In contrast, ionomycin alone is able to induce apoptosis of immature thymocytes in fetal thymic organ cultures (54). It is possible that the thymic environment provides a signal that can lead to apoptosis in the presence of increased intracellular calcium and that this signal is absent in tissue culture medium. Also, this difference in the susceptibility to apoptosis may reflect the fact that T-cell hybrids are a fusion of an immature thymoma with a mature antigen-specific T cell. The susceptibility to activation-induced cell death is contributed by the thymoma, and the antigen-specific T-cell activation is contributed by the mature T-cell fusion partner. However, mature T cells do not undergo apoptosis when they encounter an antigen for the first time. Therefore, there may be some aspect of the mature T-cell phenotype that makes T-cell hybridomas resistant to apoptosis in response to ionomycin alone. Thus, in addition to the high level of Nur77 gene expression, other factors in the kinase C pathway may also contribute to the apoptotic machinery of activation-induced cell death in T-cell hybridomas.

The transcriptional activity of the Nur77 promoter seems to play a crucial role in the high level of Nur77 protein expression during activation-induced apoptosis. Signals through the TCR but not the kinase C pathway induce a high level of Nur77

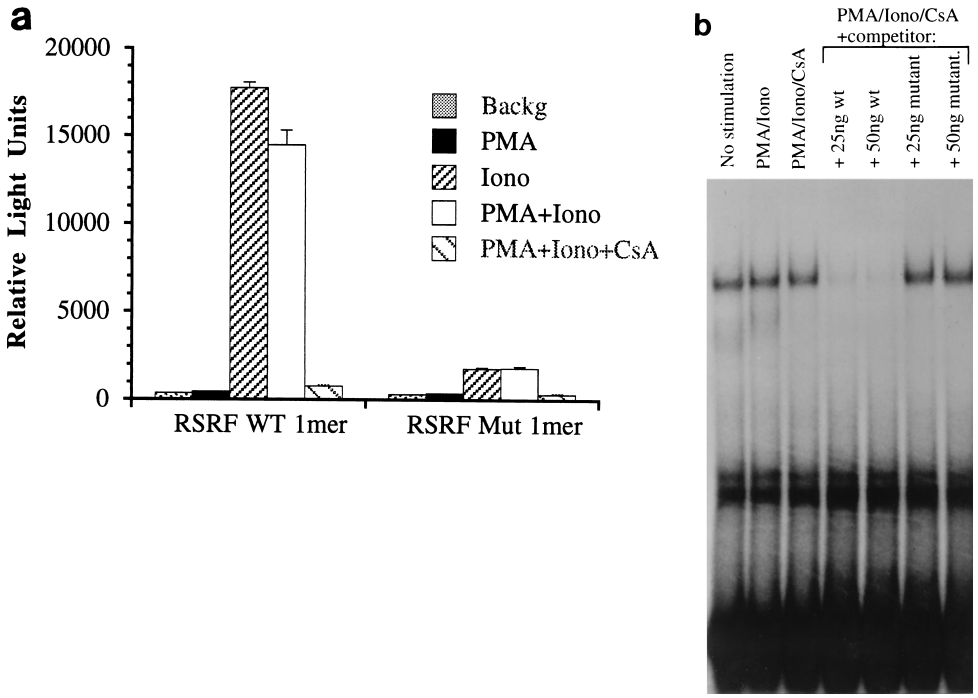

FIG. 9. RSRF mediates the calcium- and CsA-sensitive nature of the Nur77 promoter. (a) RSRF elements are calcium inducible and CsA sensitive. One copy of the Nur77 −307 to −242 promoter fragment with and without the mutations at both RSRF binding sites (see Fig. 7d) was placed upstream of a minimal c-*fos* promoter (−56 to +109) construct driving the luciferase reporter gene. The constructs were transfected into the DO11.10 T-cell hybridoma and stimulated with the various reagents for 4 h prior to harvesting to assay for luciferase activity. (b) Gel shift analysis with the Nur77 −307 to −242 promoter fragment as a probe. Experiments were performed with the Nur77 −307 to −242 promoter fragment as a probe. Competition was performed with either unlabeled oligonucleotides corresponding to the Nur77 −307 to −242 promoter sequence (wt) or similar oligonucleotides with mutations at the two RSRF sites (mutant [see Fig. 7d]).

promoter activity. CsA reduces the maximal Nur77 promoter activity by four- to fivefold. This is consistent with the analysis of the Nur77 protein expression. Promoter deletion analysis identified two calcium-responsive regions which contain binding sites for the RSRF (MEF2) family of transcription factors. A region of the Nur77 promoter spanning these two sites (−307 to −242) was sufficient to confer calcium inducibility and CsA sensitivity on T cells. The two RSRF (MEF2) sites at −262 and −297 in the Nur77 promoter were previously shown to bind RSRFC4 (MEF2A) in vitro (45), and we show that RSRF protein is bound to both of these sites in a gel shift analysis of nuclear extracts from T-cell hybridomas. The RSRF gene family was initially identified by low-stringency hybridization with the serum response factor (SRF) DNA-binding domain as a probe (45). RSRF is similar to SRF only in the DNA-binding and dimerization domains and binds to a different sequence, $CTA(A/T)_4TAG$, which is found in both growth factor-regulated and muscle-specific promoters (45, 68). Four different MEF2 (RSRF) genes, MEF2A to -D, have now been identified (4, 28, 31, 32, 35, 45, 68). The mRNA for MEF2A -B, and -D is expressed in a wide range of cell types, whereas MEP2C mRNA is primarily expressed in skeletal muscle and brain. All of these family members can be alternatively spliced, and multiple isoforms exist, some of which show tissue-specific expression. It is interesting that the DNA-binding activity that we detect in gel shifts is constitutive, whereas the transactivation activity requires calcium signals. This implies that calcium signals must modify the RSRF activity by inducing a cofactor or a posttranslational modification of RSRF that stimulates transactivation. Since the RSRF (MEF2)-specific antiserum used to supershift the protein-DNA complex recognizes MEF2A, -C, and -D, we presently do not know which family member(s) mediates Nur77 transcriptional activity during activation-induced cell death of T cells.

Our studies show that a member(s) of the RSRF (MEF2) family is a novel CsA-sensitive nuclear factor, and this suggests another calcineurin pathway that diverges from the NF-AT transcription factor pathway required for T-cell activation. Calcineurin is a cytoplasmic, calcium/calmodulin-dependent phosphatase which is an important intermediate in the calcium signaling pathway of the TCR complex (for a review, see reference 10). Upon T-cell activation, an increase in intracellular calcium leads to the dephosphorylation and translocation of the NF-AT transcription factor from the cytoplasm to the nucleus, where it combines with AP-1 (Fos/Jun) components to participate in transcription of the IL-2 gene. The immunosuppressive drugs CsA and FK506 block T-cell activation, and subsequent IL-2 production, by inhibiting the activity of calcineurin (9, 41). The direct substrates for calcineurin are not known, but one of the NF-AT family members (NFATp) is a likely candidate and has been shown to be a substrate for calcineurin in vitro (21). Since NF-AT is sequestered in the cytoplasm and translocates upon T-cell activation, it either is a direct substrate for calcineurin or acts downstream of a cytoplasmic substrate for calcineurin. In contrast, the RSRF (MEF2) factor which binds to the two Nur77 promoter sites is a nuclear factor that can bind to DNA in a transcriptionally inactive state. It requires calcium signals for its activity and is CsA sensitive, suggesting the existence of a calcineurin pathway that extends into the nucleus to modulate the activity of transcription factors in a manner distinct from that of NF-AT.

In addition to its expression in T cells, Nur77 (NGFI-B) is induced transiently in response to NGF (differentiative), epidermal growth factor (proliferative), or KCl-mediated mem-

brane depolarization of the neuronal cell line PC12 (15, 18). These different stimuli result in differential phosphorylation of the Nur77 protein, suggesting that in PC12 cells, phosphorylation modulates the activity of Nur77 during these various biological responses (15, 18). It is interesting that in T cells, we see no obvious differences in the phosphorylation state of Nur77 induced by PMA alone, ionomycin alone, or both agents together. It may be that in PC12 cells, Nur77 activity is controlled to a larger extent by phosphorylation, whereas in T cells, the level of protein induction is critical. Interestingly, Fahrner et al. (15) have shown that in NGF-treated PC12 cells, the nuclear forms of Nur77 are predominantly hyperphosphorylated, whereas the cytoplasmic forms are more hypophosphorylated. Also, phosphorylation may affect protein-protein interactions that could modulate the activity of Nur77. A novel G_1 cyclin-dependent kinase inhibitor, p19, that associates with Nur77 in a yeast two-hybrid screen was recently identified (8). This finding suggests that Nur77 may also be regulated in a cell cycle-restricted fashion, and this could be important during the growth arrest that accompanies both neuronal differentiation of PC12 cells and activation-induced cell death of T-cell hybridomas. Alternatively, Nur77 may activate different sets of genes in a developmentally restricted fashion or in disparate cell types, depending on their chromosomal accessibility.

It was previously reported that PMA induces a nonpolyadenylated form of Nur77 mRNA, whereas apoptotic signals (PMA plus ionomycin) induce polyadenylated Nur77 message and subsequent Nur77 protein in T-cell hybridomas (29). While we did not directly examine the poly(A) tail of PMA-induced Nur77 mRNA, low levels of functional mRNA and protein are clearly detected after PMA treatment. This also correlates with the level of Nur77 promoter activity induced by PMA. Therefore, the reported absence of a polyadenylated mRNA in response to PMA stimulation does not lead to a loss of functional Nur77 protein.

In vitro phosphorylation of Ser-354 in the A box of a bacterially expressed Nur77 DNA-binding domain was reported to inhibit the ability of this domain to bind the NBRE (20). However, we find that endogenous Nur77 is highly phosphorylated at Ser-354 by either PMA or ionomycin stimulation (Ser-354 is indicated by arrows in Fig. 4a). Since PMA alone, ionomycin alone, and PMA plus ionomycin all induce Nur77 protein-DNA binding complexes in T-cell hybridomas (Fig. 2a), and since exogenously expressed Nur77 is known to be transcriptionally active but is not phosphorylated on Ser-354 (see Fig. 4b, top panel), phosphorylation at this site does not seem to correlate with an inhibition of DNA binding in T cells.

During the preparation of the manuscript, two papers were published showing that Nur77 and the related orphan steroid receptor Nurr1, which both bind DNA as a monomer, can also heterodimerize with the 9-*cis*-retinoic acid receptor, RXR (16, 44). These heterodimers allow RXR to activate gene transcription in a ligand-dependent manner at retinoic acid response elements composed of direct repeats separated by 5 nucleotides. This finding reveals a novel mechanism for cross-talk between retinoid and growth factor or apoptotic signals and is interesting because 9-*cis*-retinoic acid is known to inhibit anti-CD3-induced apoptosis (66). It will be interesting to investigate the possibility of Nur77-RXR association during retinoic acid inhibition of anti-CD3-induced cell death. In addition, the identification of downstream target genes regulated by Nur77 versus Nur77-RXR will help to elucidate the role of Nur77 in such diverse cellular responses as proliferation, differentiation, and cell death.

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The first two authors contributed equally to this work.

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