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Cyclosporin A Potentiates Estradiol-Induced Expression of the Cathepsin D Gene in MCF7 Breast Cancer Cells

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Although the physiological role of the immunophilins cyclophilin-40 and FKBP52 is unknown, their identification as components of the unactivated estrogen receptor has raised the possibility that they might influence receptor activity in response to the binding of immunosuppressants cyclosporin A and FK506, respectively. We have used Northern analysis to determine the influence of cyclosporin A on the expression of the estrogen-inducible cathepsin D gene in human MCF7 breast cancer cells. We report that 1–3 μM cyclosporin A can potentiate cathepsin D mRNA expression by up to 2-fold in cells treated with 10^{-12} to 10^{-10} M estradiol. A decreased potentiation effect was noted at higher hormone concentrations. Cyclosporin A alone was unable to induce cathepsin D expression and the increased gene activation observed with combined estradiol/cyclosporin A treatment was negated by the antiestrogen ICI 164,384. Our results suggest that the increased potency of estradiol in the presence of cyclosporin A is associated with an enhanced transcriptional activity of the estrogen receptor and support a role for receptor-associated cyclophilin-40 in the activation process.

The multicomponent complexes of the unactivated steroid receptors for glucocorticoid, progestin, estrogen and androgen include p59 (1) and the abundant heat shock protein, hsp90 (2). The presence of hsp70 together with its cognate isoform, hsc70, has also been noted (3,4). Identification of p59 as a binding protein for the immunosuppressant FK506 suggested that the drug might be capable of modulating steroid receptor activity (5). The human homolog of p59 is termed FKBP52 (6). We have previously identified cyclophilin-40 (CyP-40) as an additional component of the unactivated estrogen receptor (7). CyP-40 displays a similar molecular architecture to FKBP52 (7) giving rise to speculation that these immunophilins may have closely related or competing roles in steroid receptor function (8). There is now compelling evidence for the mutually exclusive association of CyP-40 and FKBP52 with hsp90 (8,9). The formation of separate immunophilin-hsp90-receptor complexes would place steroid receptors under the ontrol of distinct immunophilin signalling pathways.

Using L929 cells stably transfected with the reporter plasmid mouse mammary tumour virus - chloramphenicol acetyltransferase (MMTV-CAT; LMCAT cells) Ning and Sanchez (10) were the first to observe potentiation of glucocorticoid receptor-mediated gene expression in response to both FK506 and rapamycin, in the presence of submaximal concentrations of dexamethasone. A subsequent study by Tai et al. (11) confirmed a similar potentiating effect of FK506 on progesterone receptor-mediated transactivation of a progestin-responsive reporter gene expressed in yeast. Significantly, a recent report (12) describes the increased sensitivity to dexamethasone of MMTV-CAT activity in LMCAT cells pretreated with cyclosporin A. In the present study we show for the first time that cyclosporin A can potentiate the estradiol-induced expression of an endogenous gene,

Abbreviations: CyP-40, cyclophilin-40; FKBP, FK506 binding protein; CsA, cyclosporin A; MMTV-CAT, mouse mammary tumour virus–chloramphenicol acetyltransferase; CATH D, cathepsin D; E_2, estradiol.

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cathepsin D (13), in MCF7 breast cancer cells. Together the results are consistent with a model in which CyP-40 and FKBP52 behave as ligand-dependent modulators of steroid receptor function.

MATERIALS AND METHODS

Estradiol-17β was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The antiestrogen ICI 164,384 was a generous gift from Dr Alan Wakeling, ICI Pharmaceuticals (Macclesfield, UK). Cyclosporin A was provided by Dr D. Römer, Sandoz Pharma Ltd (Basle, Switzerland). Fetal calf serum, new born calf serum and RPMI 1640 culture medium were purchased from Trace Biosciences (Australia). An M13 phage containing human cathepsin D cDNA(14) was obtained from Dr Henri Rochefort and was modified for routine use by inserting the *EcoRI* fragment of the cathepsin D gene into pGEM-3Z. After excision with *HindIII* the cathepsin D probe was labelled with [α-^{32}P] dCTP (Du Pont, Australia; specific activity ~3,000 Ci/mmole) using random-hexamer priming (15). An 18S ribosomal RNA probe of rat origin was provided by Dr George Yeoh.

Cell culture. MCF7 mammary carcinoma cells (16) were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum, 22.7 mM $NaHCO_3$ (Sigma) and 20 mM Hepes (Sigma) at 37°C in a humidified chamber containing 5% CO_2. Routine screening showed the cells to be free of *Mycoplasma* contamination. A standard protocol was used for estradiol/cyclosporin A treatment experiments. Briefly, at 50% confluence cells were washed with 0.9% NaCl/0.02% EDTA and cultured over 3 days in phenol red-free medium containing 5% charcoal-stripped new born calf serum. On days 3 and 5 the medium was replaced with phenol red-free medium containing 0.5% charcoal-stripped new born calf serum. Individual 90 mm diameter plates of MCF7 cells were pretreated for 2 h with cyclosporin A (0–3 μM final concentration) and then exposed to estradiol or ethanol vehicle for 22 h. ICI 164,384 was used in inhibition studies of estradiol-induced cathepsin D gene expression. Cells were washed with ice-cold PBS and RNA was isolated using RNAzol B reagent (Bresatec, Australia) according to the manufacturer's instructions. Unless otherwise indicated, all experiments were conducted in triplicate.

Northern blot analyses. Total RNA (10 μg) was electrophoresed through a 1% denaturing agarose gel and then transferred by overnight capillary blotting to Zeta Probe GT membranes (Bio-Rad, CA, USA). Prehybridization and hybridization with [^{32}P]-labelled cathepsin D cDNA probe was carried out at 42°C in 50% formamide containing 5 × Denhardts, 1% (w/v) SDS, 1.5 × SSPE (225 mM NaCl, 16 mM NaH_2PO_4, 1.5 mM EDTA, pH 7.4), 0.25 mg/ml salmon sperm DNA (Sigma), 0.2 mg/ml tRNA (Boehringer-Mannheim, Germany) and 10% (w/v) dextran sulphate (Sigma). The membranes were washed at high stringency - 0.1 × SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0), 0.1% (w/v) SDS at 60°C and exposed to Kodak X-OMAT film at −70°C. mRNA was quantitated by densitometric scanning using Image Quant (Molecular Dynamics) software.

RESULTS

To determine the influence of cyclosporin A on estradiol receptor-mediated transactivation we used the MCF7 human breast cancer cell line to study estradiol-induced cathepsin D mRNA expression with increasing concentrations of cyclosporin A. The level of estradiol (5×10^{-12} M) chosen for these initial studies was based on previous reports (10,11) with the glucocorticoid and progesterone receptor systems in which a significant effect of FK506 on receptor-mediated gene expression was observed only at submaximal hormone concentrations. Fig. 1 shows that 5×10^{-12} M estradiol increased cathepsin D expression over basal transcription levels in the absence of hormone. This hormonal response was shown to be further enhanced with increasing cyclosporin A concentrations (Fig. 1). The effect was maximal at 1–3 μM cyclosporin A and resulted in a 2–3 fold increase in cathepsin D expression relative to that observed with hormone alone (Fig. 1). Cyclosporin A (3 μM) had no significant influence on expression of the cathepsin D gene in the absence of estradiol (Fig. 1).

We next wanted to determine if cyclosporin A-mediated potentiation was also evident at higher estradiol concentrations, cells were treated with ethanol vehicle or 3 μM cyclosporin A, either without hormone, or with estradiol concentrations ranging from 10^{-12} to 10^{-8} M. Fig. 2 shows again that cyclosporin A treatment alone was unable to induce increased cathepsin D gene expression in MCF7 cells. Enhanced levels of activity were seen with cyclosporin A at all hormone concentrations although optimal cyclosporin A potentiation was noted with 10^{-11} to 10^{-10} M estradiol (Fig. 2). The relative impact of cyclosporin A on cathepsin D expression was reduced with 10^{-8} M estradiol (Fig. 2), a level of hormone known to confer maximal induction of the cathepsin D gene in MCF7 cells (13).

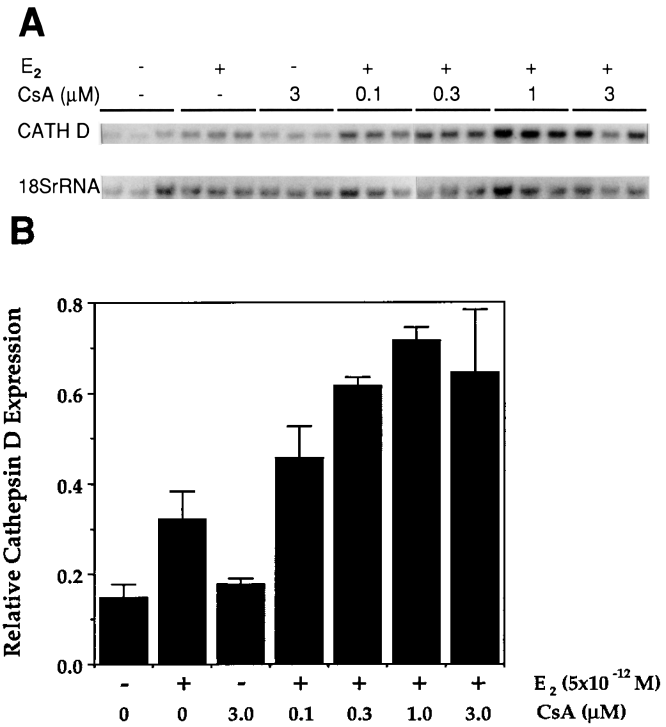

FIG. 1. Cyclosporin A potentiates estradiol-induced expression of cathepsin D. (A) Replicate plates (90 mm) of MCF7 cells were treated for 2 h with ethanol vehicle or with 0.1 to 3 μM cyclosporin A (CsA). Immediately after this pretreatment, estradiol (E_2, 5×10^{-12} M final concentration) or ethanol vehicle were added to the plates as indicated and the cells were cultured for an additional 22 h. RNA was isolated with RNAzol B reagent and Northern analysis was performed to determine cathepsin D (CATH D) expression. After autoradiography the membrane was stripped and reprobed for 18S ribosomal RNA (18SrRNA) to control for loading. (B) Autoradiographs obtained from the experiment in A were submitted to densitometric scanning to quantitate signals for cathepsin D mRNA and 18S ribosomal RNA. Levels of cathepsin D gene expression were determined relative to 18S ribosomal RNA. Values shown are means ± SE (n = 3).

Pretreatment of MCF7 cells with cyclosporin A and the pure estrogen antagonist, ICI 164,384 (17), prior to addition of 10^{-10} M estradiol completely inhibited cyclosporin A-enhanced expression of cathepsin D (Fig. 3). We conclude therefore that the estrogen receptor has a primary role in mediating the observed potentiating effects of cyclosporin A on hormonal activity. In Fig. 3 there was an apparent increase over basal expression of cathepsin D mRNA with cyclosporin A alone. This was not significant ($P<0.05$) by one way analysis of variance, consistent with the results shown in Figs. 1 and 2.

DISCUSSION

We have demonstrated that cyclosporin A treatment of the human MCF7 breast cancer cell line increases the sensitivity of cathepsin D gene induction to estradiol. Optimal potentiation of hormonal activity occurred at 1–3 μM cyclosporin A and was most readily observed with 10^{-12} to 10^{-10} M estradiol concentrations. The increased level of cathepsin D gene expression in the presence of cyclosporin A was blocked by the pure antiestrogen, ICI 164,384, indicating that the potentiating effects of the immunosuppressant with estradiol are mediated through the estrogen receptor.

Our results complement earlier reports of the potentiation of glucocorticoid and progesterone receptor-mediated gene expression by FK506 (10,11) and more recently of the increased transcriptional activity of the glucocorticoid receptor in the presence of cyclosporin A (12). The present

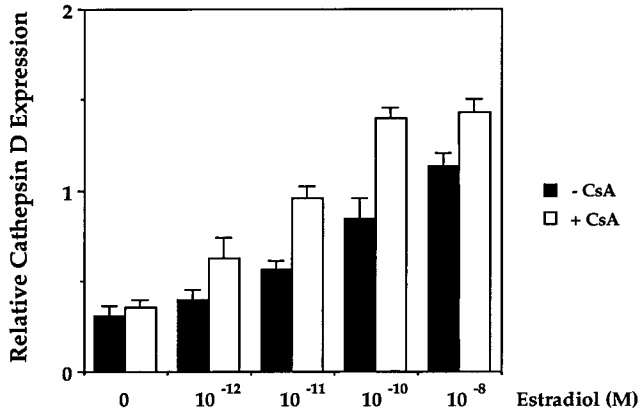

FIG. 2. Estradiol concentration dependence of cyclosporin A potentiation of cathepsin D expression. Following a 2 h pretreatment with ethanol vehicle (black bars) or 3 μM CsA (open bars), MCF7 cells were cultured either without estradiol or with hormone in concentrations ranging from 10^{-12} to 10^{-8} M, for an additional 22 h. After RNA isolation, cathepsin D mRNA expression was determined by Northern analysis. Levels of cathepsin D expression were quantitated and were calculated relative to 18S ribosomal RNA as control. Values shown are means ± SE (n = 3).

study extends the potentiating influence of cyclosporin A on steroid receptor activity to the estrogen receptor system and shows for the first time that this modulating effect can impact on the expression of an endogenous gene. Hitherto, the influence of immunosuppressants on glucocorticoid and progesterone receptor function was determined by monitoring the expression of steroid responsive chloramphenicol acetyltransferase reporter plasmids (10–12). It is noteworthy that similar concentrations of cyclosporin A (1–3 μM) were shown to be effective in modulating both glucocorticoid (12) and estrogen receptor activity. This is compatible with the low binding affinity displayed by CyP-40 for cyclosporin A (18), but contrasts sharply with the high levels of drug (50–100 μM) required to elicit potentiation of the progesterone receptor in T47D breast cancer cells (19). In our own studies with the MCF7 cell line, elevated levels of cyclosporin A (20 μM) resulted in cell toxicity (not shown).

From experiments with L929 cells it has been proposed that FK506 potentiates glucocorticoid

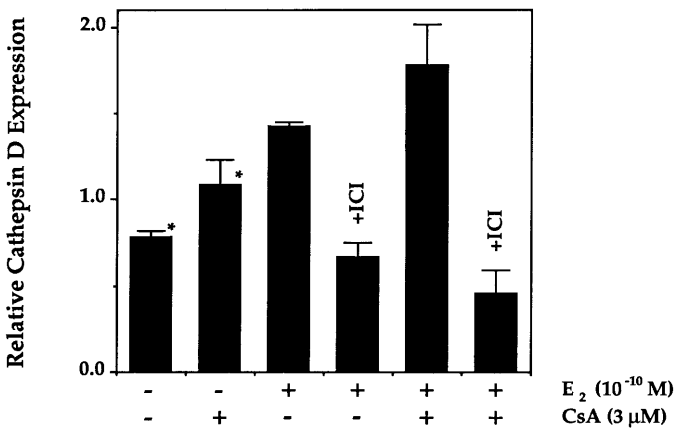

FIG. 3. Cyclosporin A potentiation of estradiol-induced cathepsin D expression is inhibited by ICI 164,384. MCF7 cells were pretreated for 2 h with ethanol vehicle or 3 μM CsA, with or without ICI 164,384 (0.5 μM), and then cultured for an additional 22 h, either without estradiol or in the presence of hormone (10^{-10} M) as indicated. After RNA isolation, cathepsin D mRNA expression was determined by Northern analysis. Levels of cathepsin D expression were quantitated and were calculated relative to 18S ribosomal RNA. Values shown are means with the range (n = 2* or 3).

receptor-mediated gene expression by enhancing receptor transformation and nuclear translocation (10). A separate report (12) has described a 2–3 fold increase in steroid binding affinity for the glucocorticoid receptor in cytosolic extracts derived from the same cell line grown in the presence of cyclosporin A or FK506. The positive effects of these immunosuppressants on glucocorticoid receptor transactivating properties were shown to be additive (12), a result that is consistent with recent evidence for the existence of separate FKBP52- and CyP-40-hsp90-receptor complexes (8,9,12). These observations, together with our own results presented herein, support proposals that implicate the receptor-associated immunophilins in mechanisms leading to the potentiation of steroid-induced receptor activity by immunosuppressant drugs (7–9,12). Two very recent reports (19,20) however, suggest that the mode of action of immunosuppressants on steroid receptor function might be more complex. Differences in the influence of the individual drugs have been noted that appear to be related to receptor type and cell line specificity and instances of no effect, induction and inhibition of receptor activity have been described (19,20). Some of these differences might be attributable to cellular variations in the relative abundance of CyP-40 and FKBP52 (8,21). It is clear that further studies are required to define the signalling pathways through which immunosuppressants modulate steroid receptor function.

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REFERENCES

1. Tai, P. K. K., Maeda, Y., Nakao, K., Wakim, N. G., Duhring, J. L., and Faber, L. E. (1986) *Biochemistry* **25,** 5269–5275.
2. Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M-G., Binart, N., Mester, J., and Baulieu, E-E. (1984) *Nature* **308,** 850–853.
3. Kost, S. L., Smith, D. F., Sullivan, W. P., Welch, W. J., and Toft, D. O. (1989) *Mol. Cell. Biol.* **9,** 3829–3838.
4. Edwards, D. P., Estes, P. A., Fadok, V. A., Bona, B. J., Onate, S., Nordeen, S. K., and Welch, W. J. (1992) *Biochemistry* **31,** 2482–2491.
5. Tai, P. K. K., Albers, M. W., Chang, H., Faber, L. E., and Schreiber, S. L. (1992) *Science* **256,** 1315–1318.
6. Peattie, D. A., Harding, M. W., Fleming, M. A., DeCenzo, M. T., Lippke, J. A., Livingston, D. J., and Benasutti, M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10974–10978.
7. Ratajczak, T., Carrello, A., Mark, P. J., Warner, B. J., Simpson, R. J., Moritz, R. L., and House, A. K. (1993) *J. Biol. Chem.* **268,** 13187–13192.
8. Ratajczak, T., and Carrello, A. (1996) *J. Biol. Chem.* in press.
9. Owens-Grillo, J. K., Hoffman, K., Hutchinson, K. A., Yem, A. W., Deibel, M. R. Jr., Handschumacher, R. E., and Pratt, W. B. (1995) *J. Biol. Chem.* **270,** 20479–20484.
10. Ning, Y-M., and Sanchez, E. R. (1993) *J. Biol. Chem.* **268,** 6073–6076.
11. Tai, P. K. K., Albers, M. W., McDonnell, D. P., Chang, H., Schreiber, S. L., and Faber, L. E. (1994) *Biochemistry* **33,** 10666–10671.
12. Renoir, J-M., Mercier-Bodard, C., Hoffman, K., Le Bihan, S., Ning, Y-N., Sanchez, E. R., Handschumacher, R. E., and Baulieu, E-E. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 4977–4981.
13. Cavailles, V., Augereau, P., Garcia, M., and Rochefort, H. (1988) *Nucleic Acids Res.* **16,** 1903–1919.
14. Augereau, P., Garcia, M., Mattei, M. G., Cavailles, V., Depadova, F., Derocq, D., Capony, F., Ferrara, P., and Rochefort, H. (1988) *Mol. Endocrinol.* **2,** 186–192.
15. Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
16. Soule, H. D., Vazquez, J., Long, A., Albert, S., and Brennan, M. E. (1973) *J. Natl. Cancer Inst.* **51,** 1409–141.
17. Wakeling, A. E., and Bowler, J. (1987) *J. Endocrinol.* **112,** R7–R10.
18. Ratajczak, T., Carrello, A., and Minchin, R. F. (1995) *Biochem. Biophys. Res. Commun.* **209,** 117–125.
19. Milad, M., Sullivan, W., Diehl, E., Altmann, M., Nordeen, S., Edwards, D. P., and Toft, D. O. (1995) *Mol. Endocrinol.* **9,** 838–847.
20. Le Bihan, S., Mercier-Bodard, C., Baulieu, E-E., and Renoir, J-M. (1995) Program of the 5th International Congress on Hormones and Cancer, Quebec City, Canada. [Abstract p105].
21. Johnson, J. L., and Toft, D. O. (1994) *J. Biol. Chem.* **269,** 24989–24993.