6. C. A. Mannella, *Science* **224**, 165 (1984).
7. C. J. Jones, R. M. Macnab, H. Okino, S.-I. Aizawa, *J. Mol. Biol.* **212**, 377 (1990).
8. H. Nikaido, and H. Vaara, in *Escherichia coli and Salmonella typhimurium*, F. C. Neidhardt, Ed. (American Society for Microbiology, Washington, DC, 1987), pp. 7–22.
9. C. J. Jones, M. Homma, R. M. Macnab, *J. Bacteriol.* **171**, 3890 (1989).
10. B. Kleffel, R. M. Garavito, W. Baumeister, J. P. Rosenbusch, *EMBO J.* **4**, 1589 (1985).
11. H. Yoshimura, M. Matsumoto, S. Endo, K. Nagayama, *Ultramicroscopy* **32**, 265 (1990).
12. After the addition of sucrose to a final concentration of 10%, 5 μl of the sample solution was spread on a clean liquid mercury surface kept at 30°C. The spread sample was transferred to a carbon-coated grid by pressing the grid against the mercury surface. For these samples, this method was effective for spreading the lipid membrane and resulted in planar sheets, but it did not facilitate the growth of the arrays in the membrane.
13. The L-P ring solution (10 μl) was mixed with an equal volume of 4% (w/v) DMPC dissolved in TEC solution (*4*) in a 0.5-ml Eppendorf tube. The tube was tightly sealed with Parafilm and the solution was incubated overnight at 37°C in a humid chamber to minimize the evaporation of the liquid. Then 2 μl of a solution containing phospholipase A_2 (50 U/ml) and 10 mM $CaCl_2$ was added and the resulting solution was incubated overnight at room temperature. Monolayer crystals were adsorbed onto a carbon-coated grid for electron microscopy.
14. We thank H. Hotani, F. Oosawa, and K. Nagayama for support and discussion, S.-I. Aizawa for advice in sample preparation, and C. Jones for critically reading the manuscript.

14 January 1991; accepted 1 April 1991

Dopamine Activation of an Orphan of the Steroid Receptor Superfamily

RONAN F. POWER, JOHN P. LYDON, ORLA M. CONNEELY, BERT W. O'MALLEY*

The chicken ovalbumin upstream promoter transcription factor (COUP-TF) is a member of the steroid receptor superfamily and participates in the regulation of several genes. While a number of functions have been ascribed to COUP-TF, no ligand or activator molecule has been identified, and thus it is classified as one of a group of orphan receptors. Activation of COUP-TF by physiological concentrations of the neurotransmitter dopamine was observed in transient transfection assays. Treatment of transfected cells with the dopamine receptor agonist α-ergocryptine also activated COUP-dependent expression of a reporter gene. COUP-TF that contained a deletion in the COOH-terminal domain was not activated by these compounds. These observations suggest that dopamine may be a physiological activator of COUP-TF.

THE COUP (CHICKEN OVALBUMIN upstream promoter) transcription factor is a member of the steroid-thyroid hormone receptor superfamily (*1*). COUP-TF participates in the regulation of the chicken ovalbumin gene (*2*), the rat insulin II gene (*3*), the proopiomelanocortin (POMC) gene (*4*) and the apo–very low density lipoprotein II (apo VLDLII) gene (*5*). A *Drosophila* protein related to COUP-TF termed Seven Up (*6*) regulates retinal cell differentiation and shares 93% sequence identity with human COUP-TF in the COOH-terminal or putative ligand binding domain. Because an activator ligand for COUP-TF has not been identified, it is classified as one of a list of orphan receptors in search of either a ligand or an activator. Dopamine is the predominant catecholamine in the retina (*7*), yet the function of dopamine in this tissue is unclear. Dopamine action is mediated by its interaction with D1 and D2 G protein–coupled receptors that stimulate and inhibit adenylyl cyclase, respectively (*8*). Here we report the activation of human COUP-TF by dopamine and a dopamine receptor agonist α-ergocryptine.

To search for potential COUP-TF ligands we constructed a chimeric COUP receptor that contained the NH_2- and COOH-terminal regions of human COUP-TF and the DNA binding domain of the chicken progesterone receptor (cPR) (Fig. 1A). This strategy was implemented to prevent competition from endogenous COUP-TF in transfected cells. Transient transfection assays were conducted in a PR-negative monkey kidney cell line (CV1) transfected with the chimeric COUP construct and a reporter plasmid (PRETKCAT) (*9*); the reporter contained two copies of a progesterone response element (PRE) located upstream of the herpes simplex virus thymidine kinase promoter linked to a chloramphenicol acetyl transferase (CAT) gene. Candidate compounds were tested for their ability to activate COUP-dependent CAT gene expression.

Because of the possible function of COUP-TF in human retinal development, dopamine and related compounds were tested. Concentrations of dopamine as low as 3 μM stimulated CAT gene activity five- to tenfold (Fig. 2A). Dopamine (100 μM) did not stimulate CAT activity in cells transfected with the parent expression vector P91023 (B), which lacked COUP-TF coding sequences. This demonstrates that dopamine stimulation of transcription required COUP-TF and did not result from a general increase in transcriptional activity. The dopamine receptor agonist α-ergocryptine also stimulated COUP-dependent CAT gene expression at concentrations as low as 300 nM. Concentrations of α-ergocryptine greater than 8.5 μM inhibited the response. More than 150 other candidate compounds did not activate gene expression in this assay system (*10, 11*). Of the other catecholamines tested, only L-dopa elicited a weak transcriptional response (*10, 12*). Activation of COUP-TF therefore appears to be relatively specific for dopamine.

Another chimeric COUP-TF (COUPΔ, Fig. 1B), which contained a deletion within the COOH-terminal domain, was tested in this assay system. Translation in vitro of RNA derived from this mutant COUP cDNA produced a stable protein of the correct size, which did not bind to a PRE oligonucleotide in gel retardation assays (*10*). This mutant protein is not capable of transcriptional activation. The inability of dopamine and α-ergocryptine to stimulate CAT gene expression in cells transfected with this mutant COUP-TF (Fig. 2B) demonstrates that an intact COUP-TF capable of transactivation is essential for the dopamine response to be observed. This result argues against the hypothesis that the effect on transcription is brought about by dopamine-mediated stimulation of transcription factors unrelated to COUP-TF.

We performed immunoblot analysis to

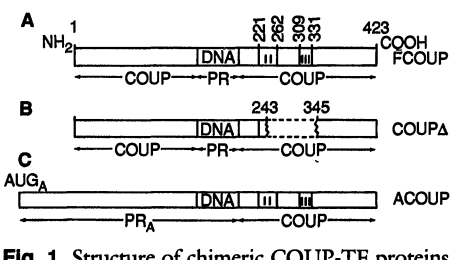

Fig. 1. Structure of chimeric COUP-TF proteins (*22*). (**A**) The chimeric COUP-TF (FCOUP) with the NH_2- and COOH-terminal domains of human COUP-TF fused to the DNA binding domain of cPR. The boundaries of domains II and III, which are conserved among the steroid receptor family (*1*), are indicated by amino acid number. (**B**) The COOH-terminal deletion mutant of (A) (COUPΔ). (**C**) The PR_A-COUP chimera (ACOUP) with the NH_2-terminal and DNA binding domains of cPR_A fused to the COOH-terminal domain of COUP-TF.

Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030.

*To whom correspondence should be addressed.

evaluate the effect of dopamine and α-ergocryptine on the production and stability of the COUP-TF protein. Because the concentration of COUP-TF produced in transfected cells is below the limit of detection by immunoblot analysis with COUP-specific antibodies, we produced a chimeric COUP-TF protein that contained the NH_2-terminal and DNA binding domains of the cPR_A (PR, A form) receptor (*13*) fused to the COOH-terminal domain of COUP-TF. This chimeric COUP-TF protein (Fig. 1C) can be readily detected in transfected cells with PR-specific antibodies (*14*) and was activated by the same concentrations of dopamine and α-ergocryptine needed to activate the COUP-PR-COUP chimera (Fig. 1A). Likewise, a deletion within the COOH-terminal domain of this PR_A-COUP chimera renders it unresponsive to dopamine and α-ergocryptine (*10*). Immunoblot analysis (Fig. 3) showed that intact, chimeric COUP-TF was produced in approximately equal amounts in both untreated transfected cells and in cells treated with dopamine and α-ergocryptine. Thus transcriptional stimulation by dopamine is not due to increased cellular concentrations of COUP-TF or changes in COUP-TF integrity.

We have been unable to demonstrate high affinity binding of either [^{3}H]dopamine or [^{3}H]ergocryptine to human COUP-TF extracted from transfected cells or to COUP-TF produced by in vitro translation in rabbit reticulocyte lysates (*10*). We conclude that dopamine does not bind directly to COUP-TF. In support of this, there are no notable sequence similarities between COUP-TF and dopamine receptor sequences (*15, 16*). It is likely that the activation of COUP-TF by dopamine is a phosphorylation-mediated event brought about by dopamine stimulation of cell surface receptors. To support this hypothesis we found that 8-bromo–cyclic adenosine monophosphate (8-Br–cAMP), a stimulator of cAMP-dependent protein kinase A, and okadaic acid, an inhibitor of protein phosphatases 1 and 2A, also stimulate COUP-TF–dependent transcription in our assay system (Fig. 2C). It has been demonstrated that these compounds can regulate PR-mediated transcription by phosphorylation (*17*). Neither compound stimulated transcription in cells transfected with P91023 (B), which lacks COUP-TF sequences, and PRETKCAT (*10*).

A variety of kidney cell types contain dopamine receptors linked to adenylyl cyclase (*18, 19*). We have found that the CV1 monkey kidney cells used in this study possess a dopamine-sensitive adenylyl cyclase that is maximally activated by dopamine (100 μM) three to four times over basal levels (*10*). The dopamine concentration needed to maximally activate retinal adenylyl cyclase in a number of species varies from 20 to 200 μM (*20*), which is similar to the concentrations needed to activate COUP-TF in our assay system. Furthermore, in carp retinal homogenates, ergocryptine stimulates adenylyl cyclase activity at concentrations between 0.1 and 10 μM (*21*). At higher concentrations ergocryptine produces an inhibitory response, which also agrees with our findings (Fig. 2A).

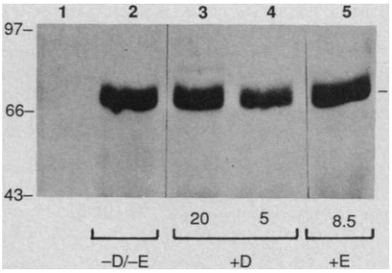

Fig. 3. Immunoblot analysis of chimeric COUP-TF protein in dopamine-treated and α-ergocryptine–treated cells. COSM6 monkey kidney cells cultured in nutridoma-supplemented media were transfected with the chimeric COUP-TF expression construct pADACOUP (5 μg) as described for Fig. 2. After 48 hours of treatment with either dopamine (D) of α-ergocryptine (E), high-salt extracts were prepared and analyzed by immunoblotting as described (*24*) with PR22 (*14*), an antibody to PR. Lane 1, protein (200 μg) from mock-transfected cells; lane 2, protein (200 μg) from transfected cells cultured in the absence of agonists; lanes 3 to 5, protein (200 μg) from transfected cells treated with various concentrations of dopamine or α-ergocryptine. Molecular size markers in kilodaltons are indicated at the left. The arrow indicates the position of chimeric COUP-TF protein.

The ability of a catecholamine neurotransmitter to activate an orphan member of the steroid receptor superfamily provides direct evidence of "cross-talk" between two signal transduction pathways that are not usually thought to be linked. Activation of an intracellular receptor by a membrane receptor agonist prompts a closer look at whether classical steroid hormone receptors can be activated by compounds other than their cognate ligands.

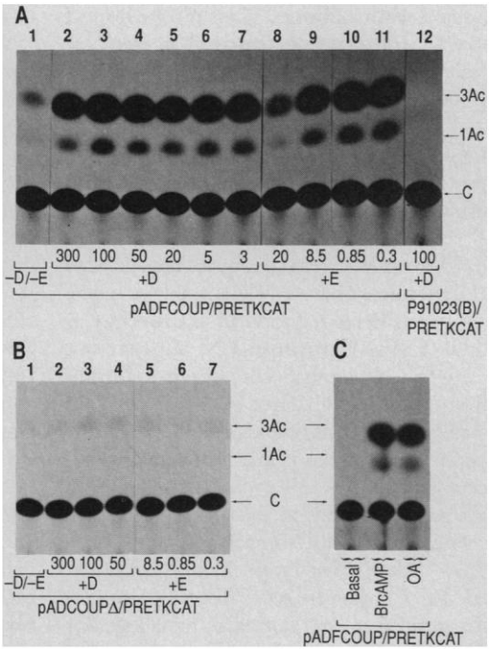

Fig. 2. Induction of CAT gene expression by dopamine (D), α-ergocryptine (E), 8-Br–cAMP, and okadaic acid (OA). (**A**) pADFCOUP (5 μg) and PRETKCAT (5 μg) were cotransfected into CV1 cells as described (*17*). Cells were cultured for 2 days in serum-free media supplemented with Nutridoma (Boehringer Mannheim; Indianapolis, Indiana) in the absence (lane 1) or presence of varying concentrations of dopamine (lanes 2 to 7) and α-ergocryptine (lanes 8 to 11). The media was replaced after 24 hours and fresh compounds were added. As a control, cells were transfected with P91023 (B), which lacked COUP-TF coding sequences, and PRETKCAT and cultured in the presence of 100 μM dopamine (lane 12). CV1 cells were prepared for CAT assays as described (*23*) and the assays were performed for 12 hours with 50 μg of protein extract. The results shown are representative of at least six separate experiments where duplicate points were performed. The variation in signals between duplicate points in any one experiment was not more than 5%. (**B**) pADCOUPΔ (5 μg) and PRETKCAT (5 μg) were cotransfected into CV1 cells cultured as above in the absence (lane 1) or presence of varying concentrations of dopamine (lanes 2 to 4) and α-ergocryptine (lanes 5 to 7). (**C**) Cells transfected with pADFCOUP (5 μg) and PRETKCAT (5 μg) were untreated (Basal) or treated with 8-Br–cAMP (10^{-3} M) or okadaic acid (OA) (5×10^{-8} M) as described (*17*). In all experiments the positions of [^{14}C]chloramphenicol (C) and the 1- and 3-acetylated forms of [^{14}C]chloramphenicol are indicated (1AC and 3AC, respectively).

REFERENCES AND NOTES

1. L.-H. Wang *et al., Nature* **340**, 163 (1989).
2. L.-H. Wang, S. Y. Tsai, I. Sagami, M.-J. Tsai, B. W. O'Malley, *J. Biol. Chem.* **262**, 16080 (1987).
3. Y.-P. Hwung, D. T. Crowe, L.-H. Wang, S. Y. Tsai, M.-J. Tsai, *Mol. Cell. Biol.* **8**, 2070 (1988).
4. J. Drouin *et al., Genome* **31**, 510 (1989).
5. J. Wijnholds and J. N. Philipsen, *EMBO J.* **7**, 2757 (1988).
6. M. Mlodzik, Y. Hiromi, U. Weber, C. S. Goodman, G. M. Rubin, *Cell* **60**, 211 (1990).
7. B. Ehinger, *Prog. Retinal Res.* **2**, 213 (1983).
8. J. Kebabian and D. Calne, *Nature* **277**, 93 (1979).
9. H. N. Jantzen *et al., Cell* **49**, 29 (1987).
10. R. F. Power, J. P. Lydon, O. M. Conneely, B. W. O'Malley, unpublished data.
11. Compounds included adrenal and gonadal steroids and their major metabolites. All fat soluble and water soluble vitamins were tested as were retinoic acid and derived compounds. Forskolin, an adenylyl cyclase ac-

tivator, had no stimulatory effect on transcription. A protein kinase C activator (phorbol 12-myristate 13-acetate) also failed to stimulate CAT gene expression.
12. Epinephrine, norepinephrine and L-tyrosine, the catecholamine precursor, failed to stimulate transcription.
13. O. M. Conneely *et al.*, *J. Biol. Chem.* **264**, 14062 (1989).
14. W. P. Sullivan, T. G. Beito, J. Proper, C. J. Krco, D. O. Toft, *Endocrinology* **119**, 1549 (1986).
15. A. Dearry *et al.*, *Nature* **347**, 72 (1990).
16. D. K. Grandy *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9762 (1989).
17. L. A. Denner, N. L. Weigel, B. L. Maxwell, W. T. Schrader, B. W. O'Malley, *Science* **250**, 1740 (1990).
18. R. A. Felder, M. Blecher, P. L. Calcagno, P. A. Jose, *Am. J. Physiol.* **247**, F499 (1984).
19. C. C. Felder, M. Blecher, P. A. Jose, *J. Biol. Chem.* **264**, 8739 (1989).
20. M. H. Makman, J. H. Brown, R. K. Mishra, in *Advances in Cyclic Nucleotide Research*, G. I. Drummond, P. Greengard, G. A. Robison, Eds. (Raven Press, New York, 1975), vol. 5, pp. 661–679.
21. K. J. Watling and J. E. Dowling, *J. Neurochem.* **36**, 559 (1981).
22. The PR cDNA was digested with Hind III at nucleotide 1801, repaired, and ligated to BSM1-digested, blunt-ended, human COUP-TF cDNA to fuse the PR DNA binding domain to the COOH-terminus of COUP-TF. A polymerase chain reaction [R. K. Saiki *et al.*, *Science* **239**, 487 (1988)] was used to generate a Sac 1 site in the COUP-TF cDNA immediately 5′ to the DNA binding domain. Sac 1–digested COUP-TF cDNA was then ligated to Sac 1–digested PR cDNA to fuse the NH_2-terminus of COUP-TF to the PR DNA binding domain. The COOH-terminal deletion mutant was made with two in-frame Pst 1 sites in the COUP-TF cDNA. The PR_A-COUP chimera was constructed as described above except that the NH_2-terminus of PR was left intact. The expression constructs pADF-COUP, pADCOUPΔ, and pADACOUP were made by inserting the respective chimeric cDNAs into the Eco R1 site of the eukaryotic expression vector P91023 (B) [G. G. Wong *et al.*, *ibid.* **228**, 810 (1985)].
23. C. M. Gorman, L. F. Moffat, B. M. Marvard, *Mol. Cell. Biol.* **2**, 1044 (1982).
24. L. A. Denner *et al.*, *Endocrinology* **125**, 3051 (1989).
25. We thank D. O. Toft for antibody PR22. We also thank D. Gallup, K. Jackson, and D. Scott for technical assistance and L. Gamble and D. Scarff for help in preparing this manuscript.

26 December 1990; accepted 26 March 1991

Recognition by Class II Alloreactive T Cells of Processed Determinants from Human Serum Proteins

PAOLA PANINA-BORDIGNON, GIAMPIETRO CORRADIN, EDDY ROOSNEK, ALESSANDRO SETTE, ANTONIO LANZAVECCHIA*

Alloreactive T cells recognize a complex composed of an allogeneic major histocompatibility complex (MHC) molecule and a peptide derived from the processing of nonpolymorphic proteins. A sizable fraction of MHC class II alloreactive T cells is shown to recognize peptides derived from constitutive processing of human serum proteins. One such epitope is a fragment of human serum albumin. This epitope bound selectively to the human class II molecule DRw11 and was constitutively present on antigen-presenting cells in vivo. These data indicate that, in the case of MHC class II, peptides involved in allorecognition may originate from exogenous proteins.

IN ORDER TO TRIGGER HELPER T cells, exogenous protein antigens must be taken up by antigen-presenting cells (APC) and processed into peptides that bind to MHC class II molecules. Since APC cannot distinguish between self and foreign proteins, it is likely that self peptides are continuously generated and presented in association with class II molecules. Peptides have been found to constitutively occupy the antigen-binding groove of class II molecules (*1*), but the origin and nature of these peptides and their functions in alloreactivity (*2*) have not been established.

Human serum (HS) is the most abundant source of soluble proteins in vivo. If HS proteins are constitutively processed and presented by APC, a fraction of alloreactive T cells should be specific for peptides derived from HS presented by allogeneic class II molecules. These T cells should recognize allogeneic Epstein Barr virus–transformed B cells (EBV-B) grown in HS, but may not recognize the same cells grown in serum from a different source such as fetal calf serum (FCS), that provides a different set of peptides. Nineteen primary mixed lymphocyte reactions (MLRs) were set up with allogeneic peripheral blood mononuclear cells (PBMC) as stimulators, and 1489 $CD4^+$ alloreactive T cell clones were isolated. The clones were tested for their capacity to proliferate in response to allogeneic EBV-B cells that had been exposed to either HS or to FCS. Of these clones, 93.4% did not discriminate between HS-treated and FCS-treated B cells. However, 6.6% of the clones (from 2.2 to 10.1% in different MLRs) proliferated only in response to EBV-B cells that had been exposed to HS (Table 1). None of the alloreactive clones proliferated only in response to FCS-treated B cells.

The antigen-specificity and restriction of the HS-specific alloreactive clones were characterized. (i) All clones proliferated only in the presence of allogeneic EBV-B cells from the original allogeneic stimulator used in the primary MLR and 1 to 5% HS. (ii) The allodeterminants recognized were present on APC in vivo, since all T cell clones proliferated in response to allogeneic PBMC from the original stimulator. (iii) The stimulatory activity of HS was stable to heating for 30 min at 60°C and represented a protein because it was destroyed by treatment of HS with Pronase. (iv) The HS proteins recognized were not polymorphic, since serum autologous to the T cell clones was as effective as serum taken from other donors. (v) The clones displayed expected allospecificities toward MHC class II DR or DQ allelic products, as shown by inhibition of alloreactivity with monoclonal antibodies to DR or DQ molecules and by the use of homozygous EBV-B cells as APC. (vi) Different proteins were recognized by different alloreactive T cells. The proteins were separated by size and identified as distinct peaks by gel filtration chromatography of HS (*3*). These results indicate that approximately 7% of class II alloreactive T cells recognize determinants of proteins from HS presented by allogeneic class II molecules.

We tested the alloreactive clones for recognition of human serum albumin (HSA), a protein of known sequence that is available in sufficient amounts to allow epitope mapping. Two clones of the 98 tested recognized HSA (Table 2). These clones were isolated from two independent MLR's and were shown to be specific for HSA and restricted to DRw11. They proliferated in response to both purified and recombinant HSA (*4*), only in the presence of allogeneic DRw11 EBV-B cells or DRw11 L cell transfectants, but not in the presence of autologous EBV-B cells. In addition, these clones proliferated in response to DRw11 PBMC from the original stimulator that had been isolated and cultured in FCS, indicating that the HSA-derived allodeterminant was present on APC in vivo. HSA, like conventional antigens, had to be processed for at least 60 min at 37°C, and processing was inhibited by leupeptin or chloroquine (Table 2).

The HSA epitope recognized by clone AK42 was identified by proteolytic digestion of HSA that had been cleaved with cyanogen bromide (*5*). The epitope corre-

P. Panina-Bordignon, E. Roosnek, A. Lanzavecchia, Basel Institute for Immunology, CH 4005, Basel, Switzerland.
G. Corradin, Institute of Biochemistry, University of Lausanne, 1066 Epalinges, Switzerland.
A. Sette, Cytel, La Jolla, CA 92037.

*To whom correspondence should be addressed.