correlate with the presence of outwardly rectifying Cl^- channels[14]. Second, CFTR introduced into experimental cell systems such as COS cells, SF9 cells and *Xenopus* oocytes[6–8] expresses a small, linear conductance Cl^- with blocker sensitivities and ionic selectivities very different from the larger channel. Another compelling possibility is that CFTR can have more than one function[6]. For example, CF is associated with defective acidification of intracellular organelles[4]. Thus, it is possible that CFTR may induce PKA regulation by affecting outwardly rectifying Cl^- channels as they are processed in the intracellular organelles. Alternatively, CFTR may associate with or transport a substance that is necessary for PKA activation of outwardly rectifying Cl^- channels[4]. □

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High brain densities of the immunophilin FKBP colocalized with calcineurin

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THE immunophilins cyclophilin and FK506 binding protein (FKBP) are small, predominantly soluble proteins that bind the immunosuppressant drugs cyclosporin A and FK506, respectively, with high affinity, and which seem to mediate their pharmacological actions[1,2]. The Ca^{2+}-dependent protein phosphatase, calcineurin, binds the cyclophilin–cyclosporin A and FKBP–FK506 complexes, indicating that calcineurin might mediate the actions of these drugs[3]. A physiological role for the immunophilins in the nervous system is implied by a close homology between the structure of NINA A, a protein in the neural retina of *Drosophila*, and cyclophilin[4,5], as well as by the high density of FKBP messenger RNA in brain tissue[6]. Here we report that the levels of FKBP and mRNA in rat brain are extraordinarily high and that their regional localization is virtually identical to that of calcineurin, indicating that there may be a physiological link between calcineurin and the immunophilins. We also show that at low concentrations FK506 and cyclosporin A enhance the phosphorylation of endogenous protein substrates in brain tissue and in intact PC12 cells, indicating that these drugs may inhibit phosphatase activity by interacting with the immunophilin–calcineurin complexes.

[^{3}H]FK506 binds saturably and with high affinity to soluble and particulate fractions of rat brain (data not shown). In peripheral tissues in both membrane and soluble brain fractions, a high-affinity binding site has a K_D of about 0.6 nM. We also observe a lower-affinity site in both soluble and particulate fractions with a K_D of 30 nM. The immunosuppressant rapamycin has a K_i value of about 1 nM, whereas cyclosporin A fails to inhibit binding at concentrations as high as 100 μM. The density of [^{3}H]FK506 binding sites in various brain regions greatly exceeds levels in peripheral tissues (Table 1). Regions such as the striatum and hippocampus have binding levels about 15 times greater than the highest levels of any of the peripheral tissues, including organs involved in immune responses such as thymus and spleen. Marked regional differences are evident in the brain. Soluble binding in the cerebellum is less than one-tenth the levels in the striatum. Binding is also low in the brain stem and hypothalamus. Although FKBP has been reported as being primarily a soluble protein, we find comparable levels of [^{3}H]FK506 binding in soluble and membrane fractions in the brain. By contrast, in peripheral tissues soluble levels of binding are always higher than membrane values, although in the thymus and spleen particulate binding is about half the soluble levels. Scatchard analysis and drug specificity studies indicate that the properties of [^{3}H]FK506 binding sites are closely similar in membrane and soluble fractions in all brain regions and peripheral tissues (data not shown). Interestingly, in soluble fractions from all brain regions and in membrane fractions from most brain areas, only a single high-affinity binding site with a K_D of about 0.6 nM is detected. But the cerebral cortex, corpus striatum, hippocampus, thymus and spleen membrane fractions give biphasic Scatchard plots with the second low affinity site having a K_D of about 30 nM.

Autoradiograms of [^{3}H]FK506 binding sites and *in situ* hybridization of mRNA for FKBP highlight striking regional variations (Fig. 1). The regional patterns for *in situ* hybridization and autoradiography are essentially the same, ensuring that [^{3}H]FK506 labels FKBP and that the FKBP distribution is essentially the same as that of its mRNA. Highest numbers of [^{3}H]FK506 binding sites are evident in the corpus striatum and the associated nucleus accumbens, and comparable levels are evident in the substantia nigra zona reticulata. The entopeduncular nucleus and the striatonigral pathway are labelled, suggesting that labelling in the nigra reflects the descending striatonigral pathway. We have confirmed this by quinolinate lesions of the corpus striatum, which reduce binding in the striatum and abolish it in the substantia nigra (Fig. 1). High [^{3}H]FK506 binding and FKBP mRNA are also apparent in the hippocampus, with particular enrichment in CA1, and lower amounts in CA3 and the dentate gyrus. [^{3}H]FK506 binding in the cerebral cortex is most prominent in superficial layers. Very high densities of [^{3}H]FK506 binding are also evident in the olfactory tubercle and olfactory tract. In the cerebellum, FKBP mRNA is most enriched in the granule cell layer and [^{3}H]FK506 binding sites are most abundant in the molecular layer, indicating that FKBP may occur in parallel fibres arising from granule cells.

Calcineurin immunoreactivity and mRNA are very similar in their localizations to FKBP. High densities are evident in the corpus striatum, striatonigral pathway and the substantia nigra zona reticulata. In the hippocampus, high densities are most apparent in the CA1 region. Within the cerebral cortex, calcineurin is also enriched in superficial layers. The olfactory tubercle as well as the olfactory tract display very high levels of calcineurin. As with FKBP, cerebellar calcineurin mRNA is most concentrated in the granule cell layer and the protein is

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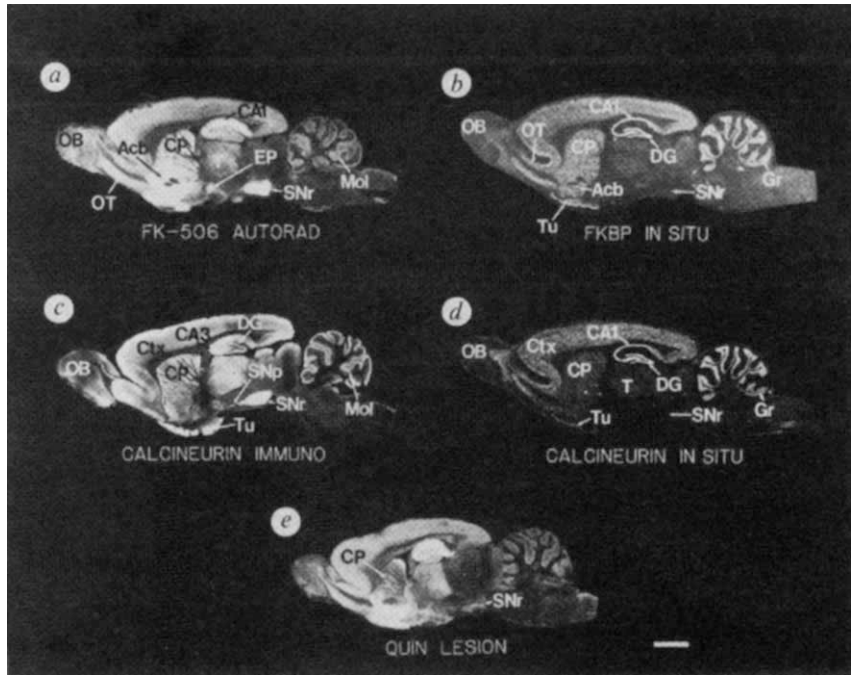

FIG. 1 Dark-field photomicrographs illustrating the dramatic colocalization of FKBP, as labelled by [^{3}H]FK506 autoradiography (*a*) with calcineurin immunoreactivity (*c*). Serial sagittal sections of rat brain, 20-μm thick, were labelled with [^{3}H]FK506 by preincubating (60 mins) slide-mounted tissue sections in a buffer consisting of 50 mM HEPES, 2 mg ml^{-1} BSA, 0.02% Tween-20, pH 7.4. This was followed by incubating (60 mins) the tissue sections in 50 mM HEPES, 2 mg ml^{-1} BSA, 0.02% Tween-20, pH 7.4, buffer containing 1 nM [^{3}H]FK506 (86.5 Ci mmol^{-1}; DuPont-NEN, Boston). Nonspecific binding was defined by the addition of 1 μm FK506. Tissue was then rinsed for 4 × 5 min in fresh, ice-cold buffer before drying. Radiolabelled sections were then juxtaposed to tritium-sensitive film. Under our conditions, non-specific binding was routinely at background levels. Immunohistochemistry was as follows: free-floating 40-μm thick tissue sections from adult male Sprague–Dawley rats perfused with 4% freshly depolymerized paraformaldehyde in 0.1 M phosphate buffer were incubated overnight in Tris-buffered saline (50 mM Tris-HCl) containing affinity purified calcineurin antiserum (1:50) (gift from C. Klee). Sections were stained with an avidin–biotin–peroxidase system (Vector Labs) with diaminobenzidine as the chromogen. In addition, the mRNA for both FKBP (*b*) and calcineurin (*d*) have the same distribution. *In situ* hybridization was as described[25] using serial sections to those in *a*. Antisense oligonucleotides were end-labelled with [^{35}S]dATP. For FKBP, the following antisense oligonucleotides, complementary to nucleotides 70–114, 214–258 and 441–485 of the cloned cDNA[6,26] were used. For calcineurin, the following antisense oligonucleotides, complementary to the nucleotides of the cloned cDNA (1,363–1,410, 1,711–1,758) (ref. 27) and 1,339–1,386, 1,569–1,616 (ref. 28) were used. Quinolinic acid lesions within the striatum (*e*) reduce [^{3}H]FK506 binding, demonstrating that FKBP is localized to the striatonigral pathway. Quinolinic acid (150 nmol in 0.5 μl of phosphate-buffered saline) (Sigma) was injected stereotaxically into the caudate–putamen to produce axon-sparing lesions[29]. ^{3}H-FK506 autoradiography was dramatically reduced in the striatum and substantia nigra reticulata ($n = 3$). These localization patterns have been replicated 6 to 8 times. Abbreviations: Acb, nucleus accumbens; CA1–CA3, fields of Ammon's horn; CP, caudate putamen; Ctx, cortex; DG, dentate gyrus; Gr, granule cell layer of cerebellum, EP, entopeduncular nucleus; OB, olfactory bulb; OT, olfactory tract; SNr, substantia nigra reticulata; SNp, striatonigral pathway; Tu, olfactory tubercle; T, thalamus; Mol, molecular layers of cerebellum. Scale bar, 2.5 mm.

most evident in the molecular layer. Interestingly, the superior and inferior colliculi are more enriched in calcineurin than FKBP, whereas for mRNA, enrichment is greater for FKBP.

As 300 nM FK506 or cyclosporin A inhibits the phosphatase activity of calcineurin towards a synthetic phosphopeptide substrate[3], the immunophilins might regulate phosphorylation physiologically. We have directly observed enhanced phosphorylation of physiological protein substrates in the presence of very low concentrations of FK506 and cyclosporin A (Fig. 2). We incubated soluble rat-brain extracts with [γ-^{32}P]ATP in the presence or absence of FK506, cyclosporin A and *O*-tetradecanoylphorbol 13-acetate (TPA) to activate protein kinase (PKC). As little as 1 nM FK506 enhances the phosphorylation of several protein bands. Cyclosporin A shows a similar activity, although it is 10–100 times less potent than FK506. The most prominent band corresponds to an M_r of ~45,000 (45K) when

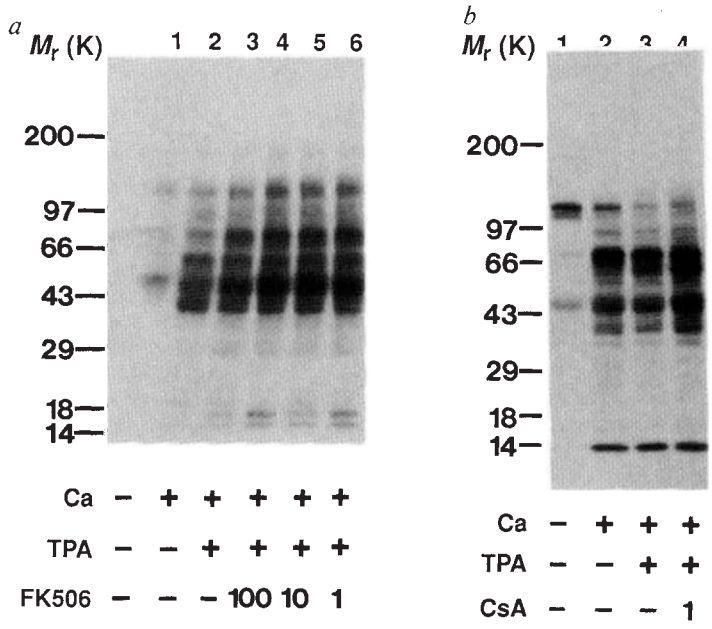

FIG. 2 FK506 and cyclosporin A mediate phosphorylation of substrate protein *in vitro*. *a*, Whole rat-brain soluble extracts from 200 mg ml^{-1} wet-weight brain homogenates, were incubated with 50 μg ml^{-1} phosphatidylserine, 20 μM [γ-^{32}P]ATP in 50 mM HEPES, 1 mM Na EGTA, 2 mM dithiothreitol, pH 7.4, buffer in the absence or presence of 10 μM free Ca^{2+}, 200 nM TPA and 1, 10 or 100 nM FK506 for 20 min at 25 °C in a final vol of 0.2 ml. Reactions were stopped by addition of PAGE sample buffer, proteins were resolved on 3.5–17% linear gradient polyacrylamide gels using the buffers of Laemmli[30], gels were dried down and exposed to X-ray film (Kodak XR5 film) and autoradiograms developed. Lane 1, control (no Ca^{2+}, no TPA); lane 2, Ca^{2+}-stimulated phosphorylation; lane 3, Ca^{2+} and TPA-stimulated phosphorylation; lanes 4–6 Ca^{2+} and TPA-stimulated phosphorylation in the presence of 100 nM, 10 nM and 1 nM FK506, respectively. Molecular weight markers are indicated. *b*, Whole rat-brain soluble extracts were phosphorylated and processed as in *a*, except that 1 μM cyclosporin A was included instead of FK506. Lane 1, control (no Ca^{2+}, no TPA); lane 2, Ca^{2+}-stimulated phosphorylation; lane 3, Ca^{2+} and TPA-stimulated phosphorylation; lane 4, Ca^{2+} and TPA-stimulated phosphorylation in the presence of 1 μM cyclosporin A. Molecular weight markers are indicated.

TABLE 1 Distribution of [^{3}H]FK506 binding sites in brain and peripheral tissues

Tissue	Membrane	Soluble
Cortex	24.5±2.2	17.5±1.0
Cerebellum	4.0±0.3	9.6±0.7
Striatum	45.8±3.9	33.8±2.5
Hippocampus	34.7±1.5	42.6±3.2
Brainstem	5.9±0.6	9.7±0.4
Hypothalamus	10.3±0.9	7.5±0.5
Midbrain	12.8±1.0	29.9±1.0
Pituitary	*	11.3±1.2
Thymus	1.8±0.2	3.1±0.4
Spleen	1.4±0.2	2.0±0.1
Heart	*	3.6±0.4
Kidney	0.5±0.02	3.4±0.4
Liver	*	3.7±0.5
Lung	0.7±0.04	2.6±0.3

[^{3}H]FK506 bound is expressed as pmol per mg protein. Regions of rat brain were dissected, homogenized at 100 mg ml^{-1} wet weight in 50 mM Tris-HCl, pH 7.4, 1 mM sodium EDTA, 100 μg ml^{-1} phenylmethylsulfonylfluoride and centrifuged at 40,000*g* for 20 min at 4 °C. Membranes were resuspended in 20 volumes of homogenization buffer, repelleted at 40,000*g* for 20 min, and resuspended in homogenization buffer to the original volume. Protein concentration was determined by the Coomassie blue dye binding assay[23] using bovine serum albumin as a standard. Binding of 250 pM [^{3}H]dihydroFK506 (86.5 Ci mmol^{-1}; NEN/DuPont) to membrane protein prepared from the indicated rat brain regions was performed in a final volume of 0.5 ml composed of 50 mM Tris-HCl, pH 7.4, 2 mg ml^{-1} BSA buffer (assay buffer) and various concentrations of unlabelled FK506. After 60 min at room temperature incubation, the membrane-bound [^{3}H]FK506 was recovered by filtration on 0.2% TX100-soaked glass fibre filter strips in a cell harvester (Brandel, Gaithersburg, MD) followed by three 4-ml washes in 50 ml NaCl. The glass filter disk was mixed with 5 ml of Formula 963 scintillation cocktail (NEN/DuPont) and counted in a Beckman scintillation counter. Nonspecific binding in the presence of 1 μM FK506 is subtracted from total binding to yield specific binding. Binding of [^{3}H]FK506 to peripheral tissue membrane protein was assayed with 300–400 μg membrane protein in assay buffer and 250 pM [^{3}H]FK506 in a final volume of 0.5 ml. Membrane bound [^{3}H]FK506 was separated from unbound ligand by centrifugation at 20,000*g* for 15 min. Nonspecific binding was determined in the presence of 1 μM unlabelled FK506. [^{3}H]FK506 binding to soluble protein was done as described for cyclosporin A (ref. 24), using 250 pM [^{3}H]FK506 and 10–50 μg protein in assay buffer to a final volume of 0.4 ml. After 60 min incubation 25 °C, 0.35 ml was layered over a 0.8-ml column of LH-20 Sephadex (Pharmacia LKB), pre-equilibrated with assay buffer. The column was further washed with 0.4 ml of assay buffer, the eluates collected, mixed with Formula 963 cocktail and counted. Specific binding was determined by subtracting binding in the presence of 1 μM unlabelled FK506 from total [^{3}H]FK506 bound. All binding experiments were done in duplicate, with an average range of 7–10%. Similar results have been obtained from three separate determinations of [^{3}H]FK506 binding levels.

* Binding detected, but at extremely low level (<0.05 pmol mg^{-1}).

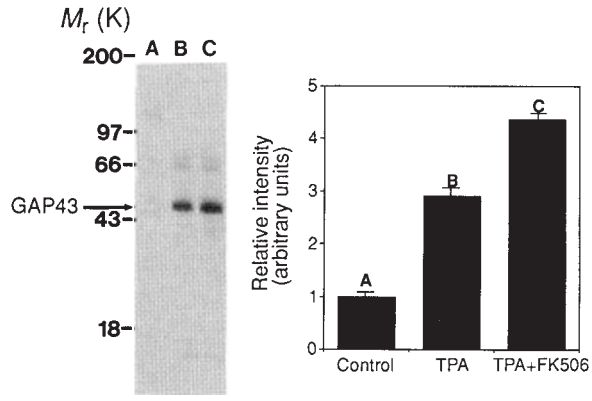

FIG. 3 GAP-43, purified from bovine brain[31], was incubated with rat brain cytosol (lane A) in the presence of Ca^{2+}, TPA (lane B), and Ca^{2+}, TPA and 10 nM FK506 (lane C) as described in Fig. 2 legend. The level of phosphorylation of purified GAP-43 was quantitated by densitometry using the Amersham/LOATS RAS3000 imaging system. This experiment has been replicated three times with similar results.

visualized on 3.5–17% gradient gels, but migrates from 43K to 60K on 7% and 12% polyacrylamide gels, respectively. The mobility changes in different percentage polyacrylamide gels of a PKC-phosphorylated protein of this size are characteristic of GAP43, a prominent calcineurin substrate; indeed, phosphorylation of purified GAP43 by PKC is enhanced by FK506 (Fig. 3) and cyclosporin A (data not shown). We have not identified definitively the protein bands at 27, 60, 80 and 120K whose phosphorylation is augmented by FK506 and cyclosporin A.

To investigate this regulated phosphorylation further, we used intact PC12 cells, a neuronal-like cell line, labelling cellular ATP pools with ^{32}P. At 1 and 10 nM, FK506 enhances phosphorylation of a 27K and a 50K protein, which are also labelled in brain preparations *in vivo* (data not shown).

The extraordinarily high concentrations of FKBP and its mRNA in discrete neuronal populations in the brain suggests that neural functions of the immunophilins may be as important as their role in mediating responses to immunosuppressant drugs. We have not measured brain levels of cyclophilin here, but regional variations in mRNA levels of cyclophilin have been reported, with highest densities in neuronal tissue in the hippocampus and cerebellum[7].

The immunophilins have peptidyl prolyl isomerase activity which is inhibited by FK506 and cyclosporin A (refs 1, 2, 8–11), but the ability of immunosuppressant drugs to inhibit this activity does not correlate with their immunosuppressant function. The binding of the immunophilins to calcineurin in the presence of their drug ligands may be a more likely mechanism of action as the immune functions regulated by these drugs are invariably Ca^{2+}-dependent processes[12–15]. The immunophilins regulate exocytosis of secretory granules in mast cells, which is also a Ca^{2+}-dependent process[16–18], and regulation of synaptic functions, especially neurotransmitter release, may be a function for immunophilins working with calcineurin in the brain. GAP43, a prominent substrate of calcineurin whose phosphorylation is regulated by immunophilins, is a 'growth-associated protein' for neuronal growth cones[19–21] but is probably also involved in neurotransmitter release, because antibodies against GAP43 selectively inhibit neurotransmitter release[22].

The colocalization of FKBP and calcineurin implies that they interact in normal brain function. As FKBP alters calcineurin activity only when bound to a ligand such as FK506, we propose that endogenous ligands exist for FKBP and cyclophilin that modulate calcineurin activity and the phosphorylation of its substrate proteins. □

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Homodimer formation of retinoid X receptor induced by 9-*cis* retinoic acid

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RETINOID response pathways are mediated by two classes of receptors, the retinoic acid receptors (RARs)[1–6] and the retinoid X receptors (RXRs)[7–11]. A central question is whether distinct response pathways are regulated by these two classes of receptors. The observation that the stereoisomer 9-*cis*-retinoic acid binds with high affinity to RXRs[12,13] suggested that this retinoid has a distinct role in controlling RXR activity, but it was almost simultaneously discovered that RXRs function as auxiliary receptors for RARs and related receptors, and are essential for DNA binding and function of those receptors[9,10,14–17]. Hence, although RARs seem to operate effectively only as heterodimeric RAR/RXR complexes, RXRs themselves apparently function predominantly, if not exclusively, as auxiliary receptors. Here we report that 9-*cis*-retinoic acid induces RXR homodimer formation. Our results demonstrate a new mechanism for retinoid action by which a ligand-induced homodimer mediates a distinct retinoid response pathway.

We investigated the effect of 9-*cis*-retinoic acid on RXRα binding to the palindromic thyroid hormone response element (TREp), an RXR responsive element[8,14]. In the absence of ligand, RXR alone did not bind effectively to the TREp but required RAR or thyroid hormone receptor (TR) for response element interaction. But in the presence of 9-*cis*-retinoic acid, DNA binding of RXR was dramatically increased (Fig. 1*a*) and did not require thyroid hormone receptors or RARs. The prominent RXR-specific band observed in the presence of 9-*cis*-retinoic acid migrated more slowly than the TR–RXR complex at a position indistinguishable from that of the RAR–RXR heterodimer complex (Fig. 1*a*). These data therefore suggested that 9-*cis*-retinoic acid induced RXR homodimer binding to the TREp. Interestingly, the retinoid did not prevent heterodimer interaction because both TR–RXR heterodimers and RXR homodimers were observed when thyroid hormone receptor was mixed with RXR in the presence of 9-*cis*-retinoic acid. To determine whether RXR homodimers could also form in the presence of RARs, we used RAR or RXR protein derivatives that carried the Flag epitope[14,18]. When Flag–RXR was incubated with RAR in the presence of 9-*cis*-retinoic acid and the anti-Flag antibody, a complete supershift was observed. But when Flag–RAR was used under the same condictions, there was only a partial supershift, in support of the presence of RXR homodimers that could not react with the antibody (Fig. 1*b*).

Remarkably, all-*trans*-retinoic acid (10^{-6} M) also induced RXRα homodimer binding to some degree, whereas thyroid hormone T3 did not. RAR homodimer DNA binding was not induced by 9-*cis*-retinoic acid (Fig. 1*a*). Formation of the 9-*cis*-retinoic acid-induced complex was dependent on the concentration of RXR protein and was strongly cooperative (Fig. 1*c*). RXR complex formation was enhanced by 9-*cis*-retinoic acid at all receptor concentrations tested, being already effective at 10^{-9} M, with optimal binding at 10^{-8} M (Fig. 1*d*).

We next tested RXR interaction with several natural and synthetic response elements (Fig. 2*a*). In the case of the ApoAI-retinoic acid response element (RARE)[18], a strong RXR complex was observed in the presence of 9-*cis*-retinoic acid and a weaker one with retinoic acid (10^{-6} M). The RAR–RXR heterodimer also bound effectively to this response element (Fig. 2*b*). Homodimer complexes were also induced by 9-*cis*-retinoic acid with the CRBPII–RARE (ref. 19) and the β-RARE (refs. 20, 21; Fig. 2*c*) elements that contain 1-base-pair (bp) or 5-bp spacers, respectively. In this latter case, however, the RXR homodimer band was considerably weaker than the RAR–RXR heterodimer band. Interestingly, another natural RARE derived from the rat CRBPI promoter (M. Husmann and M. P., unpublished results), which is similar to the ApoAI–RARE in that it contains a 2-bp spacer, did not bind RXR at all in the presence of 9-*cis*-retinoic acid (Fig. 2*c*), indicating that the actual sequence of the motif is critical for RXR homodimer binding and not simply the spacing separating the two half-sites. Similarly, the DR5-RARE (ref. 22), a perfect repeat element derived from the β-RARE, did not interact with RXR in the presence of 9-*cis*-retinoic acid, although it interacted strongly with the RAR–RXR heterodimer (Fig. 2*c*).

None of the T3 response elements shown here (Fig. 1*a*), the rat α-myosin heavy chain TRE (ref. 23), the rat malic enzyme TRE (ref. 24) and the perfect repeat DR4 (ref. 22), bound the RXR in the presence of 9-*cis*-retinoic acid, but all three response elements interacted effectively with TR–RXR heterodimers. Similarly, the perfect palindromic oestrogen response element (ERE; ref. 25) did not measurably bind RXR homodimers (data not shown).

To determine whether 9-*cis*-retinoic acid can induce RXR homodimer formation in solution, we again used the Flag–RXR derivative. When we mixed Flag–RXR with *in vitro*-labelled ^{35}S-RXR protein in the presence of 9-*cis*-retinoic acid, the labelled RXR could be coprecipitated by anti-Flag antibody but not by nonspecific serum (Fig. 3). Coprecipitation efficiency was slightly increased in the presence of the ApoAI–RARE but not in the presence of the α-myosin heavy chain TRE. In addition, incubation with the protein crosslinker DSP (dithiobis-succinimidyl propionate) enhanced coprecipitation of labelled RXR. Specific coprecipitation in all cases, was observed only in the presence of 9-*cis*-retinoic acid. Thus 9-*cis*-retinoic acid induces RXR homodimer formation in solution in the absence of DNA.

In transient transfection assays with a TREp-containing reporter, we found activation by RXRα was strong in the presence of 9-*cis*-retinoic acid and weak when retinoic acid was added. Activation could be enhanced by cotransfection of RARα. In this case, however, retinoic acid was also an effective activator, although not as efficient as 9-*cis*-retinoic acid (Fig. 4*a*). The β-RARE (Fig. 4*b*) was highly activated by endogenous CV-1

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