Attenuation of the Activity of the cAMP-specific Phosphodiesterase PDE4A5 by Interaction with the Immunophilin XAP2*

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The cyclic AMP-specific phosphodiesterase (PDE4) isoform PDE4A5 interacted with the immunophilin XAP2 in a yeast two-hybrid assay. The interaction was confirmed in biochemical pull-down analyses. The interaction was specific, in that PDE4A5 did not interact with the closely related immunophilins AIP/L1, FKBP51, or FKBP52. XAP2 also did not interact with other PDE4A isoforms or typical isoforms from the three other PDE4 subfamilies. Functionally, XAP2 reversibly inhibited the enzymatic activity of PDE4A5, increased the sensitivity of PDE4A5 to inhibition by the prototypical PDE4 inhibitor 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone (rolipram) and attenuated the ability of cAMP-dependent protein kinase to phosphorylate PDE4A5 in intact cells. XAP2 maximally inhibited PDE4A5 by ~60%, with an IC50 of 120 nM, and reduced the IC50 for rolipram from 390 nM to 70–90 nM. Co-expression of XAP2 and PDE4A5 in COS7 cells showed that they could be co-immunoprecipitated and also reduced both the enzymatic activity of PDE4A5 and its IC50 for rolipram. Native XAP2 and PDE4A5 could be co-immunoprecipitated from the brain. The isolated COOH-terminal half of XAP2 (amino acids 170–330), containing its tetraoctapeptide repeat domain, but not the isolated NH2-terminal half of XAP2 (amino acids 1–169), containing the tetratricopeptide repeat domain, decreased PDE4A5 activity and its IC50 for rolipram. Mutation of Arg271 to alanine, in the XAP2 tetraoctapeptide repeat region, attenuated its ability to both interact with PDE4A5 and to inhibit PDE4A5 activity. Either the deletion of a specific portion of the unique amino-terminal region or specific mutations in the regulatory UCR2 domain of PDE4A5 attenuated its ability to be inhibited by XAP2. We suggest that XAP2 functionally interacts with PDE4A5 in cells.

Signal transduction mediated by the second messenger cAMP is pivotal in a multitude of cellular processes, including the action of numerous hormones, neurotransmitters, and growth factors (1). The PDE4 family of cAMP-specific phosphodiesterases modulate cAMP signaling by their ability to hydrolyze cAMP and thereby contribute to the regulation of its levels in cells (2, 3). PDE4 enzymes can be differentiated from other cyclic nucleotide phosphodiesterases by sequence homology in the catalytic region of the proteins and by the presence of unique regions of amino acid sequence in the amino-terminal half of the proteins, outside the catalytic region, which are called upstream conserved regions 1 and 2 (UCR1 and UCR2) (2, 4). In addition, PDE4s are also characterized by their ability to be inhibited by a specific class of drugs, such as rolipram, which have anti-depressant, anti-inflammatory, and smooth muscle relaxant activity in humans (2, 3). At least 15 different PDE4 isoforms have been described in mammals, which are encoded by four different genes (PDE4A, PDE4B, PDE4C, and PDE4D), with additional diversity being generated by the generation of alternatively spliced mRNAs from each gene (2, 3).

PDE4A5 is a cAMP-specific phosphodiesterase isoform encoded by the PDE4A4 gene that is expressed in a wide variety of tissues, including the lung and various regions of the brain (4–7). PDE4A5 contains UCR1 and UCR2 as well as a unique amino-terminal region (Fig. 1). The PDE4A5 unique amino-terminal region is highly conserved in mammals; of the 107 amino acids in the human PDE4A5 unique amino-terminal region, 94 (88%) are identical to those in the amino-terminal regions of both the rat and murine PDE4A5 proteins (5). This high degree of conservation among species suggests that the PDE4A5 unique amino-terminal region has specific functions, which are now being identified. Truncation of the PDE4A5 amino-terminal region alters the enzymatic activity and intracellular targeting of the protein (8, 9), and pull-down assays have demonstrated that it can interact with the SH3 domains of SRC family tyrosyl kinases, such as LYN, FYN, and SRC (8, 10).

We now report that PDE4A5 selectively associates with the immunophilin XAP2 (also called AIP and ARA9) (11–13). Immunophilins are a large class of proteins, all of which are characterized by the presence of a region of amino acid sequence called the immunophilin domain (14). In many immunophilins, the immunophilin domain has cis-trans peptidyl-

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The abbreviations used are: PDE4, cAMP-specific cyclic nucleotide phosphodiesterase; PDE, cyclic nucleotide phosphodiesterase; PRA, cAMP-dependent protein kinase; TPR, tetratricopeptide repeat; SH3, SRC homology domain 3; GST, glutathione S-transferase; MBP, maltose-binding protein; ORF, open reading frame; rolipram, 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone; UCR, upstream conserved region; PBS, phosphate-buffered saline; IBMX, isobutylmethylxanthine; Hsp90, heat-shock protein of 90 kDa; CREB, cAMP-response element-binding protein.
prolyl isomerase (rotamase) activity and is the target for immunosuppressive drugs such as cyclosporin, FK506, or rapamycin (14). However, XAP2 does not appear to have either of these functions (11, 15). XAP2 was first identified as a protein that interacts with the X protein of hepatitis B virus (16). Subsequently, XAP2 has been shown to be capable of interacting with Hsp90 as part of a complex containing the aryl hydrocarbon receptor (11–13, 17). In this study, we identify a novel function of XAP2; it binds specifically to PDE4A5. This interaction requires a tetratricopeptide repeat (TPR) within the carboxyl-terminal region of XAP2 (Fig. 15) and both the unique amino-terminal region of PDE4A5 and a motif located within UCR2. We also show that XAP2 inhibits the enzymatic activity of PDE4A5, increases its sensitivity to inhibition by rifampin, and attenuates its ability to be phosphorylated by the cAMP-dependent protein kinase (PKA).

**EXPERIMENTAL PROCEDURES**

**Materials**—cDNAs for human AIPL1 (GenBankTM accession number AF148864) (18) and FBKP51 (GenBankTM accession numbers U71321 and U42031) (19) were obtained from M. M. Soghoki and D. P. Smith, respectively.

**Generation of Bacterial Expression Constructs**—To express XAP2, a NotI site was inserted into the polylinker.

**Generation of COS7 Cell Expression Constructs**—To express PDE4A5, the full ORF of its cDNA was cloned into the pcDNA3 (from Invitrogen) site, resulting in a carboxyl-terminal fusion, as described by us previously for PDE4D5 (21).

**Preparation of Antibodies against XAP2**—Rabbits were immunized with a synthetic peptide of sequence NH2-CIKDEEDKARFGRIFSHCOOH, corresponding to the carboxyl-terminal region of XAP2, conjugated to KLH. The initial immunization was performed in complete Freund's adjuvant, and monthly booster immunizations were performed in complete Freund's adjuvant and then Freund's incomplete adjuvant. The animals were bled 4 weeks after the last immunization.

**Preparation of Antibodies against PDE4**—Rabbits were immunized with the Immunophilin PDE4

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all rat PDE4A5 isoforms as described in detail by us previously (6, 8, 23, 29). Although this antibody detects all PDE4A5 isoforms, the individual isoforms can in turn be distinguished by their different mobilities on SDS-PAGE. The PDE4A5-specific antibody was generated against the unique PDE4A5 amino-terminal region, as we have described previously (9).

**PDE Assay**—For determination of PDE activity, COS7 cells were transfected as described above and then homogenized in KHEM buffer (50 mM KCl, 10 mM EGTA, 1.92 mM MgCl₂, 1 mM dithiothreitol, 50 mM HEPES, pH 7.2) containing Complete™ protease inhibitor mixture to produce final concentrations of 40 μg/ml phenylmethylsulfonyl fluoride, 156 μg/ml benzamine, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml antipain. As described previously (23), in the transiently transfected cells, greater than 98% of the total PDE activity was due to the recombinant PDE4A5 enzyme. In some instances, the transfected COS7 cells were plated into six-well plates and then serum-starved overnight before being treated with the indicated ligands. Cyclic nucleotide phosphodiesterase activity was assayed using 1 μM cAMP as substrate, as described previously (28). Reactions (total volume of 100 μl) were initiated by the addition of 20 μl of 5 μM [³²P]cAMP. All assays were performed using a modification of a procedure we have described previously (6, 23, 28). In brief, a volume of slurry containing 400 μg of protein was taken for assay. Protein concentration was determined by the method of Bradford (24), using bovine serum albumin as a standard.

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**PKA Phosphorylation**—PKA-mediated phosphorylation of S140 in PDE4A5 was performed using a modification of a procedure that we have described previously (27). In brief, COS7 cells were either transfected with plasmids encoding PDE4A5 or co-transfected with plasmids encoding both PDE4A5 and XAP2. The cells were pretreated with 100 μM IBMX for 15 min prior to the addition of 100 μM forskolin. At the indicated times, the cells were harvested in ice-cold KHEM buffer and homogenized. Cell lysates (25 μg of protein) were run on NuPAGE gradient gels (4–12% Bis-Tris; Invitrogen), blotted to nitrocellulose, and then probed with antibodies specific for PKA substrate XAP2 in a PKA-dependent manner. Antibody binding was detected by chemiluminescence. Protein immunoblotting. For co-immunoprecipitations of proteins expressed endogenously in brain, rat brain tissue was homogenized in a glass Dounce homogenizer in 0.25 ml of buffer on ice. Subcellular fractions were prepared as described above. Then 3 μl of PDE4A5 antibody (9) or, as a control, preimmune serum was added to 250 μg of S2 fraction protein in a 500-μl volume and incubated for 1 h at 4 °C. Washed protein A-agarose beads (50 μl) were added, incubated end-over-end for an additional 1 h, centrifuged at 5000 g for 1 min, and then washed three times with 500 μl of KHEM buffer. The beads were boiled in Laemmli buffer for 2 min and then loaded on NuPAGE gel. Samples of the supernatant from the immunoprecipitations (50 μg of protein) were run as controls. The samples were then immunoblotted with antibodies specific for XAP2 and PDE4A5.

**RESULTS**

**Isolation of XAP2 as a Protein Interacting with PDE4A5 in a Two-hybrid Screen**—PDE4A5 has a unique amino-terminal region linked to the catalytic region via the UCR1 and UCR2 regulatory regions (Fig. 1a). We used the yeast two-hybrid system to identify proteins that might interact with PDE4A5. Rat PDE4A5 was used as a “bait” in two independent screens of a two-hybrid rat brain cDNA library, with identical results. The results of one screen are shown (Table I). Both screens identified XAP2, a 330-amino acid protein of the immunophilin family (11–13) (Fig. 1b, schematic), as a novel protein “partner” for PDE4A5. XAP2 contains an immunophilin homology domain in its amino-terminal half and at least one TPR in its carboxy-terminal half (13). The TPR domains of a variety of proteins have been shown to mediate the interactions of these proteins with other proteins (29–33).

**Specificity of the PDE4A5-XAP2 Interaction**—To obtain preliminary evidence that the PDE4A5-XAP2 interaction is specific, we used yeast two-hybrid filter β-galactosidase assays to test for the interaction of XAP2 as a GAL4 fusion with a variety of “baits” expressed as LexA fusions. These included lamin C, casein kinase II, Raf, Ras, several transcription factors, and LexA itself (i.e. not as a fusion). We also tested LexA-PDE4A5 for its ability to interact with these proteins as GAL4 fusions and also to the GAL4 activation domain itself (i.e. not as a fusion). No interaction was detected with these “baits” under conditions where we could demonstrate an interaction between PDE4A5 and XAP2 (data not shown). Using identical assays, we detected no interaction between PDE4A5 and the closest known relatives of XAP2 (Fig. 2, top row), specifically the AIP1 protein (18), FKBP51, and FKBP52 (Fig. 2, top row). Like XAP2, all of these proteins contain immunophilin domain(s) in the amino-terminal half of the protein and one or more TPRs in their carboxy-terminal half. These homologs of XAP2 were identified in a BLAST search of the GenBank™ database, using XAP2 as a query. Essentially identical results were obtained on BLAST searching, whether the full-length XAP2 protein, or just the immunophilin homology domain, or just the TPRs were used as a query. These data suggest that PDE4A5 interacts specifically with XAP2 and not with immunophilins or TPR-containing proteins generally.

**Pull-down Assays Demonstrate That PDE4A5 Interacts with XAP2**—We obtained independent confirmation of the interaction between PDE4A5 and XAP2 with a GST pulldown assay. For this purpose, we expressed and affinity-purified XAP2 in
E. coli either as a GST fusion protein (GST-XAP2) (Fig. 3a) or as an MBP fusion protein (MBP-XAP2) (Fig. 3b). Using the high speed supernatant (S2; cytosolic) fraction of lysates from COS7 cells transfected with cDNA constructs expressing PDE4A5 (6, 23), we demonstrated that GST-XAP2 interacted with cytosolic PDE4A5 in “pull-down” assays (Fig. 3c). Similar data were obtained with MBP-XAP2 (not shown).

The Carboxyl-terminal Half of XAP2 Is Necessary for It to Interact with PDE4A5—To determine which portion of XAP2 was involved in its interaction with PDE4A5, we expressed its amino-terminal and carboxyl-terminal halves (shown schematically in Fig. 1b) as separate GST fusion proteins (Fig. 3a) and tested them for their ability to interact with recombinant PDE4A5 in pull-down assays (Fig. 3c). These assays showed that the TPR-containing half of XAP2 (GST-XAP2170–330) was able to pull down PDE4A5, whereas the amino-terminal half, containing the immunophilin homology domain (GST-XAP21–169), was ineffective (Fig. 3c).

XAP2 Selectively Inhibits the Enzymatic Activity of PDE4A5—We next examined whether the physical interaction between XAP2 and PDE4A5 had any functional effect, as reflected by changes in the enzymatic activity of PDE4A5. The addition of GST-XAP2 to PDE4A5 assays produced a striking dose-dependent inhibition of cytosolic recombinant PDE4A5 (Fig. 4a). Half-maximal inhibition (IC50) occurred at 125 ± 11 nM XAP2 (mean ± S.D., n = 6 separate experiments). Intriguingly, whereas XAP2 exerted a saturable inhibitory effect, it failed to inhibit PDE4A5 activity completely, achieving a maximal inhibition of 62 ± 9% (mean ± S.D., n = 6 separate experiments). The inhibitory action of XAP2 was reversible upon dilution and showed no time dependence, in that pretreatment for 1 h showed no increase in inhibition over that seen by adding...
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**Fig. 2. Specificity of interaction between PDE4A5 and XAP2.** Various PDE4 isoforms, cloned into pLEXAN to be expressed as LexA fusions, were tested for their ability to interact with various immunophilins, cloned into pGADN to be expressed as GAL4 fusions. The interactions were assayed by yeast two-hybrid filter β-galactosidase assays, as described previously (20, 21). Also included are internal positive and negative standards (21), respectively (the oncoproteins RAS92 and Raf and the vectors without inserts). The GenBank\textsuperscript{TM} accession numbers of the PDE4 clones are as follows: PDE4A5, L27057; PDE4A1, L27062; PDE4A4/8, L36467; PDE4B2, L27058; PDE4B3, U95748; PDE4D3, L29970; PDE4D4, L29969 (see Ref. 2 for a review).

![Diagram of PDE4A5 and XAP2 interactions](Image)

GST-XAP2 immediately to the assay (data not shown). This reversibility and lack of time dependence indicates that XAP2 is not affecting the stability of PDE4A5. No inhibition of PDE4A5 was seen with GST alone, over the dose range examined (Fig. 4a), showing that the inhibitory action was due to XAP2 rather than any effect of GST. We also showed that the different XAP2 fusion, MBP-XAP2 (Fig. 3b), caused similar dose-dependent inhibition of PDE4A5 (Fig. 4b), whereas no inhibition occurred with MBP alone. MBP-XAP2 produced dose-dependent inhibition of PDE4A5, with an IC$_{50}$ of 103 ± 10 nM MBP-XAP2 and a maximal inhibitory effect of 58 ± 8% ($n = 3$). Since both MBP-XAP2 and GST-XAP2 inhibit PDE4A5 similarly, it is likely that XAP2 inhibits PDE4A5 independently of its fusion status.

We then demonstrated that the inhibition of PDE4A5 activity by XAP2 does not require any intermediate proteins. For these experiments, we expressed PDE4A5 in E. coli as a GST fusion (GST-PDE4A5) and purified it to apparent homogeneity on SDS-PAGE. We then assayed its enzymatic activity in the presence of GST-XAP2 and GST alone (Fig. 4c). These experiments showed that GST-XAP2 could inhibit GST-PDE4A5 with an IC$_{50}$ of 99 ± 15 nM and a maximal inhibitory activity of 61 ± 8% ($n = 3$). Since the kinetics of inhibition of E. coli-expressed PDE4A5 by XAP2 in these assays are similar to those when PDE4A5 was expressed in mammalian cells, then it is likely that XAP2 inhibits PDE4A5 directly, without the necessity of intermediate proteins.

The Carboxyl-terminal Half of XAP2 Is Necessary for It to Inhibit PDE4A5—We then wished to determine which portion of XAP2 (shown schematically in Fig. 1b) was needed to inhibit PDE4A5. We found that the TPR-containing GST-XAP2170–330 produced dose-dependent inhibition of PDE4A5 activity, whereas GST-XAP21–169, containing the immunophilin homology domain, had no effect on PDE4A5 activity (Fig. 5a). The inhibition produced with GST-XAP2170–330 was similar to that observed with GST-XAP2, with half-maximal inhibition occurring at 155 ± 15 nM GST-XAP2170–330 and a maximal effect of 58 ± 8% inhibition ($n = 3$; Fig. 5a).

**Specific Amino Acids in the XAP2 TPR Are Essential for It to Inhibit PDE4A5**—The carboxyl-terminal half of XAP2 contains a TPR domain, located between amino acids 265 and 298 (13) (shown schematically in Fig. 1b). The TPR domains of a variety of proteins have been shown to mediate the interactions of these proteins with other proteins (29, 30). The crystallographic structures of three different TPRs, those of PP5A (31), Hop1 (32), and Cyp40 (33), have been reported. Structural and mutagenesis studies have shown that a number of conserved positively charged amino acids within these TPRs play a key role in determining their protein-protein interactions (31, 32, 34, 35). The TPRs of these three proteins interact with Hsp90. In particular, a specific stretch of negatively charged amino acids, of the sequence EEVD, at the carboxyl terminus of Hsp90, is essential for these interactions, with other regions of Hsp90 required for conferring specificity (36–38).

The structural study of the Hop1 TPR demonstrates that there is a direct interaction, involving mostly electrostatic forces, between the conserved amino acids in the Hop1 TPR and the EEVD sequence in Hsp90 (32). Using these prior studies as a guide, we wished to identify specific amino acids in the XAP2 TPR that might mediate its interaction with PDE4A5 (this section) and, conversely, to identify specific amino acids in PDE4A5 that might have properties similar to the EEVD motif of Hsp90 (see below).

For study of XAP2, we tested whether mutations of specific conserved amino acids in its TPR would block its interaction with PDE4A5. Specifically, we analyzed the amino acids Asn236, Lys266, and Arg271 in XAP2 (Fig. 2b). Asn236 of XAP2 corresponds to Asn67 in the TPR of PP5A (31, 34) and to Asn264 in the two different TPRs in Hop1 (32). Lys266 in XAP2 corresponds to Lys67 in the PP5A TPR (31, 34) and to Lys73 and Lys301 in the two different Hop1 TPRs (32). Arg271 in XAP2 corresponds to Arg101 in PP5A (31, 34) and Arg77 and Arg305 of the two different Hop1 TPRs (32). Therefore, we generated separate constructs encoding the individual mutations N236A, K266A, or R271A in XAP2. Using two-hybrid assays, we found that the R271A mutant significantly attenuated the interaction of XAP2 with PDE4A5, whereas the K266A and N236A mutants interacted with PDE4A5 with avidities that were very similar to that of wild-type XAP2 (Fig. 6a).

We then generated GST fusions with the individual N236A, K266A, and R271A mutants in XAP2 and evaluated their ability to inhibit the activity of cytosolic recombinant PDE4A5 (Fig. 5b). Consistent with the two-hybrid data, we found that the R271A mutant markedly reduced the ability of XAP2 to inhibit PDE4A5 (Fig. 5b). Again, consistent with the two-hybrid data, the N236A and K266A mutants were similarly as effective as wild-type XAP2 in inhibiting PDE4A5 (Fig. 5b). These results highlight the importance of Arg271 in the XAP2 TPR in mediating a functional interaction with PDE4A5 and suggest that the XAP2-PDE4A5 interaction may occur in a manner similar to that of the interaction of TPRs with Hsp90.

The Sequence EEVD in the UCR2 Domain of PDE4A5 Is Essential for It to Interact with XAP2—Next, we wished to identify amino acids in PDE4A5 that might resemble the EEVD motif in Hsp90 that is involved, at least in part, in mediating the interaction of Hsp90 with various TPRs (36–38). There is a similar sequence, EELD, located within the UCR2 domain of PDE4A5 that is conserved among all PDE4 isoforms (Fig. 1a, inset) (2). We have shown previously that this sequence is necessary for the interaction between the UCR1 and UCR2 domains of PDE4D3 (39). This sequence region has also been implicated in the dimerization of PDE4D isoforms (40), again suggesting that it can mediate protein-protein interactions. Therefore, we created mutations in the EEVD sequence of...
PDE4A5 and XAP2 interact in pull-down assays. a, SDS-PAGE analysis of GST fusion proteins. The various lanes show Coomassie Brilliant Blue protein staining of 10 µg of protein/lane of 27-kDa GST, 65-kDa GST-XAP2, 47-kDa GST-XAP2170–169, and 46-kDa GST-XAP2170–330. A single protein-staining band was observed in each instance. b, SDS-PAGE analysis of 52-kDa MBP and the 89-kDa MBP-XAP2 fusion proteins. Coomassie Brilliant Blue staining was performed of 10 µg of protein/lane. c, pull-down assays using either GST or the indicated GST-XAP2 fusion protein with various preparations from COS7 cells transfected with PDE4A5, followed by immunoblotting with a PDE4A-specific antibody. PDE4A5 migrates as a 109-kDa protein. Bound fractions were resuspended in the same volume as unbound fractions so that direct comparison can be made. Loading was equivalent to the application of 10 µg of protein of the S2 fraction. No immunoreactive material was evident in extracts from untransfected or mock-transfected (vector-only) COS7 cells. These experiments are typical of one done at least six times.

PDE4A5 and tested their effect on its interaction with XAP2, using our two-hybrid assay (Fig. 6b). These studies showed that the mutation of the EELD motif to LLLL significantly attenuated the interaction with XAP2. In contrast, mutation of an adjacent portion of UCR2 that also showed some sequence similarity between XAP2 and Hsp90 (i.e. changing the LSEET motif (Fig. 1a (inset) to DSLLT) had no effect on the interaction (Fig. 6b), suggesting that the EEVD to LLLL mutation had a specific effect on the interaction rather than just acting to destabilize the PDE4A5 protein. We then expressed the EELD to LLLL mutant of PDE4A5 in COS7 cells and showed that it had a profoundly reduced ability to be inhibited by XAP2 (Fig. 7a). We also expressed in COS7 cells a construct of PDE4A5 where the entire amino-terminal portion of UCR2, containing the EELD motif, had been deleted and found that this construct also could not be inhibited by XAP2 (Fig. 7a).

XAP2 Interacts with PDE4A5 but Not with Other PDE4 Isoforms—Approximately 15 different PDE4 isoforms have been identified to date. The PDE4A gene encodes four of these isoforms, PDE4A1, PDE4A5, PDE4A8, and PDE4A10 (2, 41).
To determine whether XAP2 binds to any of a number of other PDE4 isoforms, the isoforms were expressed as LexA fusions and tested for their interaction with GAL4-XAP2, using yeast two-hybrid filter-galactosidase assays. No interactions were detected (Fig. 2), indicating that XAP2 interacts specifically with PDE4A5.

**XAP2 Inhibits Specifically PDE4A5 and Not Other PDE4 Isoforms**—We confirmed and extended our two-hybrid data on the specificity of XAP2 for PDE4A5 by using biochemical approaches. We showed that GST-XAP2\textsubscript{170−330} fails to inhibit the activity of the “long” (i.e. UCR1-containing) isoforms PDE4B1, PDE4C2, and PDE4D3 (Fig. 7, b and c). Additionally, it failed to inhibit another long PDE4A isoform, specifically PDE4A8 (Fig. 7b) and also the “short” isoform PDE4A1 (range 0.01−5 μM GST-XAP2\textsubscript{170−330}, data not shown). We also were unable to detect any interaction between these isoforms and GST-XAP2\textsubscript{170−330} in pull-down assays (data not shown). This suggests that XAP2 interacts specifically with PDE4A5 and not with other PDE4 isoforms.

**The Unique Amino-terminal Region of PDE4A5 Is Essential for It to Interact with XAP2**—The specificity of XAP2 for PDE4A5 suggests that the unique amino-terminal region of PDE4A5 is essential for it to interact with, and be inhibited by, XAP2. To explore the role of this region, we expressed in COS7 cells a panel of progressive amino-terminal truncations of PDE4A5 (schematic in Fig. 1a) that we have used previously to define functional areas within this region (8). We found that deletion of amino acids 1–10 had no detectable effect on the ability of XAP2 to inhibit PDE4A5 but that removal of amino acids 1–42 blocked the ability of GST-XAP2 to inhibit PDE4A5 activity (Fig. 7a). The various truncations used in these experiments exhibited similar or slightly higher activities than the full-length enzyme and are still capable of showing defined aspects of intracellular targeting (8). Therefore, amino acids 11–42 in the unique amino-terminal region of PDE4A5 are necessary for it to be functionally inhibited by XAP2.

It has been shown that the SH3 domain of the LYN tyrosyl kinase can interact with the amino-terminal 10 amino acids of PDE4A5 (8). In contrast to the inhibition of PDE4A5 by XAP2 that we report here, the binding of LYN-SH3 did not alter markedly the activity of PDE4A5. Since the binding site for LYN-SH3, like one of those for XAP2, is located in the amino-terminal region of PDE4A5, we wished to determine whether the addition of LYN-SH3 might alter the ability of XAP2 to inhibit PDE4A5. We demonstrated that GST-XAP2\textsubscript{170−330} is capable of inhibiting PDE4A5 even when PDE4A5 is bound to GST-LYN-SH3 (Fig. 7d). Additionally, the addition of GST-LYN-SH3 to an enzymatic assay of PDE4A5, even at a concentration known to allow interaction with PDE4A5 (10) did not affect the ability of GST-XAP2\textsubscript{170−330} to inhibit PDE4A5 (Fig. 7e). These data imply that the binding of LYN-SH3 to PDE4A5
FIG. 7. XAP2 shows specificity for inhibition of PDE4A5. a, the action of GST-XAP2\textsuperscript{170–330} on the indicated PDE4A5 truncates, as diagrammed schematically in Fig. 1a; on wild-type PDE4A5; on the EELD to LLLL mutant of PDE4A5; and on a deletion construct (Δ loop7) of PDE4A5 that removes the amino-terminal portion of UCR2 containing the EELD motif (Fig. 1a). Equal (immunoreactive) amounts of the various COS7-expressed PDE4A5-truncated proteins were added to assays together with 1 μM GST-XAP2\textsuperscript{170–330}. Recombinant PDE4A8 (b), PDE4B1 (b), PDE4C2 (c), and PDE4D3 (c) were expressed in COS7 cells. Samples were taken and assayed for PDE activity using 1 μM cAMP as substrate in the presence of increasing levels of GST-XAP2\textsuperscript{170–330}. In each assay, an equal amount of enzyme activity was added, to achieve about 1 nmol/min/100 μl assay. d, the dose-dependent inhibition of COS7 cell-expressed PDE4A5 activity by GST-XAP2\textsuperscript{170–330} when PDE4A5 is bound to GST-LYN-SH3 (see “PDE Assay,” first approach). Activity is expressed as a percentage of that determined in the absence of GST-XAP2\textsuperscript{170–330}. e shows the effect of 1 μM GST-XAP2\textsuperscript{170–330} on the activity of PDE4A5 in the presence or absence of 1 μM GST-LYN-SH3 (see “PDE Assay,” second approach). Activity is expressed as a percentage of that determined in the absence of GST-XAP2\textsuperscript{170–330}. The data show the means ± S.D. (n = 3).
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Fig. 8. XAP2 alters the sensitivity of PDE4A5 to inhibition by rolipram. Dose-response curves for the effect of rolipram on PDE4A5 activity were performed with 1 μM cAMP as substrate. As indicated in the box at the top, recombinant PDE4A5 expressed in COS7 cells was used either alone or together with E. coli-expressed GST-XAP2 added at a final concentration of 1 μM to the assays or using an enzyme source derived from co-expression of PDE4A5 and XAP2 in COS7 cells. The data show the means ± S.D. (n = 3).

does not interfere with the interaction of XAP2 with PDE4A5 and, when interpreted in light of our prior data on LYN-SH3 (8), are consistent with LYN-SH3 and XAP2 binding to different regions within the unique PDE4A5 amino-terminal region.

FK506 or Rapamycin Do Not Detectably Alter the Binding or Inhibitory Effects of XAP2—The immunophilin domain of some members of the immunophilin family can bind to specific immunosuppressive drugs, such as cyclosporin, FK506, or rapamycin. For example, cyclosporin binds specifically to Cyp40, whereas FK506 binds specifically to several members of the FKBP family, most avidly to FKBP12 (K_i ~ 1 nM) and more weakly to FKBP25 (K_i ~ 180 nM) (14, 42). Although the immunophilin homology domain of XAP2 has significant similarity to the immunophilin binding domains of FKBP12, FKBP52, and FKBP25, at least one group has demonstrated that FK506 does not detectably bind to XAP2 (15). Nonetheless, because XAP2 has immunophilin homology domains, we wished to determine whether FK506 has any effect on the ability of XAP2 to inhibit PDE4A5. We therefore tested the ability of FK506, at a range of concentrations from 1 nM to 1 μM, to inhibit PDE4A5 activity, at a concentration of 500 nM GST-XAP2. We evaluated inhibition either when FK506 was added directly to the PDE assay or when it was preincubated with GST-XAP2 for 1 h. In all cases, FK506 failed (<7% change) to affect the degree of inhibition of PDE4A5 produced by XAP2.

We also tested the effect of rapamycin, which binds most avidly to FKBP12 (K_i ~ 0.2 nM) and more weakly to FKBP52 (K_i ~ 8 nM) (43, 44), to inhibit PDE4A5 activity. In experiments otherwise identical to those described for FK506, rapamycin failed to affect (<6% change) the degree of inhibition of PDE4A5 produced by XAP2 (1 μM).

XAP2 Enhances the Sensitivity of PDE4A5 to Inhibition by Rolipram—Recombinant cytosolic PDE4A5 was inhibited by the prototypical PDE4 inhibitor rolipram in a dose-dependent fashion, with an IC50 of 389 ± 39 nM (n = 3; Fig. 8). However, the IC50 was reduced by ~4-fold, to a value of 92 ± 10 nM (n = 3), in the presence of a functionally saturating concentration (1 μM) of GST-XAP2, chosen to ensure a maximally inhibitory effect (Fig. 8). We noted similar inhibitory action of rolipram on PDE4A5 using GST-XAP2 (IC50 = 89 ± 12 nM; n = 3). In addition to this, GST-XAP2 (38 kDa) changed the slope of the rolipram inhibition curve (Fig. 8). Thus, when XAP2 interacts with PDE4A5, it appears to affect the conformation of the catalytic site of the enzyme, as demonstrated by both its intrinsic ability to inhibit PDE4A5 and its ability to alter the sensitivity of PDE4A5 to inhibition by rolipram. These experiments further demonstrate that the functional effect of XAP2 on the catalytic function of PDE4A5 requires only the TPR-containing half of XAP2.

XAP2 Can Be Co-immunoprecipitated with PDE4A5 in Transfected COS7 Cells—We wished to ascertain whether PDE4A5 and XAP2 were capable of associating in mammalian cells. We transiently expressed recombinant XAP2 in COS7 cells and detected a band of ~37 ± 2 kDa on immunoblotting of cellular extracts with a XAP2-specific antibody (Fig. 9a), which is compatible with the migration of XAP2 under denaturing conditions (36 kDa) observed previously by other groups (11, 13, 15, 16). Pretreatment of the XAP2 antiserum with recombinant XAP2 blocked the detection of this band, indicating the specificity of the antibody (Fig. 9a). XAP2 has been shown previously to be a soluble protein (11, 13, 15, 16), and, consistent with this, we found recombinant COS7-expressed XAP2 to be present predominantly in the high speed supernatant (S2; cytosolic) fraction (Fig. 9c). This fraction has also been shown to contain the predominant amount of PDE4A5 (8, 10, 23). When XAP2 and PDE4A5 were co-transfected into COS7 cells, PDE4A5 and XAP2 could be co-immunoprecipitated (Fig. 9b), demonstrating that they were capable of interacting in mammalian cells.

We also demonstrated that XAP2 and PDE4A5 can be co-immunoprecipitated from native (i.e. untransfected) cells. We prepared cytosolic (S2) fractions (6, 23, 28) (see “Experimental Procedures”) from rat brain and demonstrated by immunoblotting that they contained native PDE4A5 (108 kDa) and XAP2 (38 kDa) that co-migrated with recombinant forms of the two proteins (Fig. 9c). When we immunoprecipitated PDE4A5 from these preparations, we detected both PDE4A5 and XAP2 in the immunoprecipitates (Fig. 9d). In these experiments, we purposely used a low concentration of the PDE4A5-specific antibody to minimize nonspecific binding of the antibody to other proteins. This provided a clear signal when PDE4A5 and XAP2 were co-immunoprecipitated (Fig. 9d). The co-immunoprecipitate contained ~15% of the PDE4A5 present in the lysates and ~6% of the XAP2, suggesting that approximately one-third of the total XAP2 might be complexed with PDE4A5. These data strongly suggest that PDE4A5 and XAP2 can interact in intact cells and tissues.

If XAP2 and PDE4A5 interact when co-transfected into COS7 cells, then XAP2 should alter the functional properties of PDE4A5 in those cells. Therefore, we determined whether XAP2 modified the activity and sensitivity to inhibition by rolipram of PDE4A5 when they were co-transfected in COS7 cells. To correct for any differences in the level of expression of PDE4A5 in the transfections, assays were performed on equal amounts of immunoreactive PDE4A5, as determined by immunoblotting of the cytosolic (S2) fraction (6, 8, 23, 25). These experiments demonstrated that co-transfection of cells with XAP2 and PDE4A5 reduced the activity of PDE4A5 by 61 ± 6% (n = 3). Additionally, we assessed the sensitivity of PDE4A5 to inhibition by rolipram in cells co-transfected with XAP2 and PDE4A5 (Fig. 8). As seen with the addition of exogenous XAP2 to PDE4A5, co-expression of XAP2 and PDE4A5 heightened
transfected COS7 cells, generated as described under "the FLAG antibody. C immunoprecipitate (cell-expressed PDE4A5 (P2 pellet; added at 0.1 mg/ml to the antibody. A signal due to native XAP2 is also evident in lysates from the untransfected cells. b nontransfected cells.

immunoprecipitate (lower panel migrates as a 109-kDa band. The functionally inhibited by XAP2 (Fig. 7a mutant that is missing amino acids 1–42, which cannot be functionally inhibited by XAP2 (Fig. 7a), we were unable to observe co-immunoprecipitation of the PDE4A5 mutant with XAP2 (data not shown) or any change (<5%) in the IC50 for rolipram exhibited by this construct.

XAP2 Attenuates the Ability of PKA to Phosphorylate PDE4A5—The long PDE4 isoforms can be phosphorylated by PKA at a conserved serine that is located at the beginning of UCR1 (Ser140 in PDE4A5) (27, 28, 39, 45–50). PKA phosphorylation increases the activity of the enzyme (about 2-fold for the PDE4D3 isoform (39, 45–50) and about 50% for other PDE4 isoforms (27, 28)). Treatment of COS7 cells transfected with PDE4A5 alone with forskolin and IBMX elevates cAMP levels and activates PKA, which in turn causes the rapid phosphorylation of PDE4A5 (27). PKA phosphorylation of PDE4A5 can be detected by an antibody (P-UCR1) that recognizes phospho-Ser140 (27). To determine the effect of XAP2 on the phosphorylation of PDE4A5 by PKA, we co-transfected cDNAs encoding PDE4A4 and XAP2 into COS7 cells. When we transfected the cells with PDE4A5 alone, IBMX and forskolin treatment produced rapid and maximal phosphorylation of PDE4A5 (Fig. 10, a and b), similar to results that we have reported previously (27). Treatment of these cells with the PKA inhibitor H89 (0.5 μM) blocked the phosphorylation of Ser140 (data not shown).

Fig. 9. Co-immunoprecipitation of PDE4A5 and XAP2. a, an immunoblot with the XAP2 antibody. This detects a single 37-kDa band in cells transfected with FLAG-XAP2. This signal is blocked if the antibody is pretreated with added GST-XAP2 (Tr+Ag), but not by added GST (Tr+Gst), added at 0.1 mg/ml to the antibody. A signal due to native XAP2 is also evident in lysates from the untransfected cells. Tr, transfected cells; NTr, nontransfected cells. b, an immunoblot with the FLAG antibody of lysates (Lys) of COS7 cells co-transfected with PDE4A5 and FLAG-XAP2 or an immunoprecipitate (4A5 IP) from the same cells. Immunoprecipitations were performed with the PDE4A antibody and then immunoblotted with the FLAG antibody. C, an identical immunoprecipitate, but from cells not transfected with XAP2. c, subcellular fractions of FLAG-XAP2-transfected COS7 cells, generated as described under “Experimental Procedures,” were immunoblotted with the XAP2 antibody. P1, low speed pellet; P2, high speed pellet; S2, high speed supernatant. d, immunoblotting of immunoprecipitates from rat brain. Shown are standards of cost cell-expressed PDE4A5 (4A5 Std) and XAP2 (XAP2 Std), a cytosolic (S2) fraction from rat brain (Lys), and immunoprecipitations performed with the PDE4A5 antibody (4A5 IP) or with preimmune serum (C). The upper panel shows an immunoblot performed with a PDE4A5 antibody; PDE4A5 migrates as a 109-kDa band. The PDE4A5 immunoprecipitate (4A5 IP) was from 250 μg of lysate protein, whereas the lysate (Lys) blot was from 50 μg of lysate protein. All experiments are typical of ones done three times.

the sensitivity of PDE4A5 to inhibition by rolipram, with an IC50 of 74 ± 11 μM rolipram (n = 3; Fig. 8). These functional data are consistent with the co-immunoprecipitation experiments, demonstrating that PDE4A5 and XAP2 are capable of interacting when co-expressed in cells. In contrast, when COS7 cells were co-transfected with XAP2 and a PDE4A5 truncation mutant that is missing amino acids 1–42, which cannot be functionally inhibited by XAP2 (Fig. 7a), we were unable to observe co-immunoprecipitation of the PDE4A5 mutant with XAP2 (data not shown) or any change (<5%) in the IC50 for rolipram exhibited by this construct.

Forskolin and IBMX treatment of cells was accompanied by an increase in the activity of PDE4A5 (IC50 of 74 ± 11 μM rolipram; mean ± S.D., n = 3), similar to that reported previously (27). We estimate that any carryover of IBMX from the cells into the PDE assays had no effect on PDE4A5 enzymatic activity, since it would be diluted to less than 1 nM in the assay.

When we co-transfected XAP2 and PDE4A5 into the cells, both the rate and magnitude of PDE4A5 phosphorylation were attenuated significantly (Fig. 10, a and b). This effect was not caused by any alteration in PKA function, since co-transfection with XAP2 did not cause any alteration in PKA phosphorylation of CREB in these cells upon treatment with forskolin and IBMX (Fig. 10c). In addition, we did not detect any difference in the expression of PDE4A5 in cells upon co-transfection of XAP2 (95 ± 3% of the levels seen in cells transfected to express PDE4A5 alone; n = 3).

We also evaluated whether PKA-phosphorylated PDE4A5, as opposed to non-PKA-phosphorylated PDE4A5, might be differentially inhibited by XAP2. We therefore treated PDE4A5-transfected cells with IBMX and forskolin as above, prepared extracts, and examined the effect of the addition of recombinant GST-XAP2170–330 to the assay on the enzymatic activity of PDE4A5. In order to compare the action of XAP2 on the phosphorylated and nonphosphorylated enzyme, we assessed the fractional response, as expressed as the percentage of activity produced by XAP2 in each case. This analysis showed (Fig. 10d) that the percentage of the activity of phosphorylated PDE4A5 that was inhibited by XAP2 is the same as the percentage of activity of nonphosphorylated PDE4A5 that was inhibited by XAP2. Therefore, the same fraction of PDE4A5 activity was
inhibited in each case, although the actual magnitude of activity change produced by XAP2 was greater for the phosphorylated enzyme. Inhibition by XAP2 was noncompetitive in both cases (Fig. 10d). These data show that PKA phosphorylation of PDE4A5 had little effect on the ability of XAP2 to inhibit PDE4A5. These experiments suggest that XAP2 and PKA phosphorylation are independent “inputs” into the regulation of PDE4A5.

**DISCUSSION**

The four PDE4 cAMP-specific phosphodiesterase genes encode a large family of distinct isoforms (2, 3). Each isoform is defined by its regulatory regions, located in the amino-terminal half of the protein, whose functional roles include intracellular targeting, phosphorylation by PKA and extracellular signal-regulated kinase (46, 50), regulation of enzyme activity (2, 8), and association with signaling scaffold complexes (2, 3, 21, 51). In the present study, we have identified a novel interaction between the PDE4 isoform PDE4A5 and the immunophilin XAP2.

XAP2 is a member of the immunophilin protein superfamily, which is a diverse group of proteins characterized by the presence of a region of amino acid sequence called the immunophilin domain (14). In many immunophilins, the immunophilin domain has cis-trans peptidyl-prolyl isomerase (rotamase) activity and is the target for immunosuppressive drugs such as cyclosporin, FK506, or rapamycin (14). However, XAP2 does not appear to have either of these functions (11, 15). Various members of the immunophilin family have also been shown to have protein chaperone function and to regulate the activity of numerous enzymes and other proteins (14). Genetic studies have implicated AIPL1, the closest human ortholog of XAP2, in retinal function, since loss of function AIPL1 mutations cause inherited retinal diseases in humans (18). XAP2 has been shown previously to be capable of interacting with Hsp90 as part of a complex containing the aryl hydrocarbon (dioxin) receptor and to modulate aryl hydrocarbon (dioxin) receptor function (11–13, 15, 17). However, since the various members of the immunophilin superfamily can each exhibit a diversity of functions, it is highly possible that XAP2 also has multiple functions.

We demonstrate that XAP2 interacts specifically with PDE4A5 but not other PDE4 isoforms and that this selectivity requires amino acids 11–42 of the unique amino-terminal region of PDE4A5. The interaction between XAP2 and PDE4A5 is mediated through the XAP2 TPR and, specifically, requires Arg271 within the XAP2 TPR. In other TPRs, this arginine is conserved and participates directly in electrostatic interactions with a conserved TPR-binding motif of sequence EEVD to produce a dicarboxylate clamp (31, 32). We have shown that a very similar sequence, EELD, located in the UCR2 of PDE4A5, is essential for its interaction with XAP2 and presumably interacts directly with Arg271. Although this UCR2 motif is con-
PDE4A5 may provide an additional mechanism of changing the binding of SRC family tyrosyl kinases, through its SH3 domain, which is poorly understood. One important regulator of rolipram sensitivity is heightened sensitivity to inhibition by rolipram. However, the mechanism(s) are attributable to actions on PDE4 isoforms exhibiting dimerization sites on PDE4A5, which is also involved in its interaction mediated by UCR2. For instance, since UCR1 and UCR2 bind to similar regions of UCR2 (39), then XAP2 could prevent UCR1 from interacting with UCR2 and thereby induce a conformational change within UCR1 that affects the ability of Ser140 to be phosphorylated.

Further investigation will be necessary to determine the functional significance of the PDE4A5-XAP2 interaction. In particular, mouse knockout of one or both genes should produce valuable insights, particularly if these knockouts are confined to specific tissues, such as either the lung or regions of the brain. An additional issue is whether the XAP2 immunophilin domain has any functional role in the PDE4A5-XAP2 interaction in cells. The immunophilin domains of other immunophilins have been shown to interact with various signal transduction components; for example, the immunophilin domain of FKBP12 interacts with the transforming growth factor β receptor (57, 58) and cardiac ryانodine receptors (14, 59) and regulates their functions in cells. It is possible that the immunophilin domain of XAP2 could be involved in recruiting PDE4A5-XAP2 into specific signaling complexes; this remains to be determined.

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