The SH3 domain of Src tyrosyl protein kinase interacts with the N-terminal splice region of the PDE4A cAMP-specific phosphodiesterase RPDE-6 (RNPDE4A5)

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The PDE4A (type IV) cAMP-specific, rolipram-inhibited phosphodiesterase RPDE-6 (RNPDE4A5), when transiently expressed in COS7 cells, could be complexed with the v-Src-SH3 domain expressed as a glutathione S-transferase (GST) fusion protein. RPDE-6 did not interact with GST itself. This complex was not disrupted by treatment with high NaCl concentration together with Triton X-100. Interaction was apparently determined by the N-terminal splice region of RPDE-6, as the PDE4A splice variant RPDE-39, which differs from RPDE-6 at the extreme N-terminus, failed to associate with v-Src-SH3; met[26]RD1 (where RD1 is rat '*dunc*-like' PDE), which has the N-terminal splice region deleted, failed to associate with v-Src-SH3, and the association of RPDE-6 and v-Src-SH3 was blocked by a fusion protein formed from the N-terminal splice region. RDPE-6 showed binding to GST fusion proteins of both the intact Src kinase and an SH2–SH3 construct but did not bind to the Src-SH2 domain or to the adaptor protein Grb-2. RPDE-6 could be co-immunoprecipitated from cytosol extracts of transfected cells by using anti-Src antiserum. RPDE-6 exhibited selectivity in binding to the SH3 domains of c-Abl, Crk, Csk, Lck, Lyn, Fyn and v-Src, with binding to the SH3 regions of the Src-related tyrosyl kinases Lyn and Fyn being the most effective. The binding of RPDE-6 to the SH3 domains of Crk, Csk and Lck led to a marked reduction in PDE activity, but no change was apparent in complexes with other species. Endogenous RPDE-6 from brain, but not endogenous RPDE-39 from testis, bound to the Src-SH3 domain. We suggest that the PDE4A splice variant RPDE-6 has a propensity for interaction with selective SH3 domains, in particular those from Src and the Src-related tyrosyl kinases Lyn and Fyn. This interaction seems to be governed by alternative splicing of the PDE4A gene, because RPDE-39, a splice variant that lacks the proline-rich N-terminal splice region of RPDE-6, does not interact with these SH3 domains. It is proposed that the binding site on RPDE-6 for SH3 domains lies within the unique first 102 residues of its N-terminal splice domain, where two motifs representing Class I SH3 binding sites with selectivity for Src kinase SH3 domains can be identified and one motif for a putative Class II SH3 binding site.

INTRODUCTION

cAMP is a key second messenger that regulates a wide variety of cellular functions. It is produced by the action of adenylate cyclase, an enzyme for which there are multiple genes, yielding tissue-specific expression patterns [1,2]. Similarly, a large family of enzymes provide the sole means of inactivating cAMP in cells [3–6]. These cAMP phosphodiesterases (PDEs) show diverse regulatory properties, subcellular distribution and sensitivity to selective inhibitors, which can serve as therapeutic agents. The molecular basis of such diversity is due to multiple genes, coupled with alternative splicing [3–6]. The PDE4 family of enzymes hydrolyse cAMP specifically and are selectively and potently inhibited by the antidepressant drug rolipram [4,5]. Four genes encode PDE4 enzymes in mammals, with PDE4A and PDE4C being located on human chromosome 19, PDE4B on chromosome 1 and PDE4D on chromosome 5 [7,8]. Alternative splicing seems to play a major role in achieving diversity of the PDE4 enzyme family, where it leads, primarily, to 5′ domain swapping, producing various splice variants with distinct N-terminal regions [4,5].

N-terminal domain swapping within the 4A family seems to have a variety of functional roles. These include membrane targeting [9–12], regulation of enzyme activity (V_{max}) [11,12] and conferring susceptibility to phosphorylation by protein kinase A [13]. The 'core' PDE4A enzyme can be considered as that species which is encoded by bases from the 3′ end to the first 5′ splice junction [4,9]. This has been engineered and expressed as a highly active, freely soluble species, which we have called met[26]RD1 (where RD1 is rat '*dunc*-like' PDE) [9,11]. Various N-terminal domains are spliced to this 'core' PDE4A unit, which determines subcellular localization and activity [4,9–12]. In the case of RD1

Abbreviations used: PDE, cAMP phosphodiesterase (the PDE4 enzymes are rolipram-inhibited cAMP-specific phosphodiesterases for which there are four known genes, giving rise to PDE4A, PDE4B, PDE4C and PDE4D); RD1, rat '*dunc*-like' PDE [also referred to as either RNPDE4A1A or rPDE-IV$_{A1}$ representing rat PDE4, isoform subfamily A splice variant 1 (GenBank accession number M26715)]; met[26]RD1, 'core' PDE4A enzyme, encoded from the 3′ end to the first 5′ splice junction; RPDE-6, a splice variant of the PDE4A gene referred to formally as RNPDE4A5 (GenBank accession number L27057); RPDE-39, a splice variant of the PDE4A gene referred to formally as RNPDE4A8 (GenBank accession number L36467); GST, glutathione S-transferase; ECL, enhanced chemiluminescence.

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(PDE4A1), the unique N-terminal 23-amino-acid splice region confers an exclusively membrane-associated location on this protein product of the PDE4A gene [9–12]. In contrast, the splice variant RPDE-6 (RNPDE4A5) is found distributed between both particulate and cytosol fractions, in both brain and transfected COS cells [12]. The N-terminal splice region of RPDE-6 shows no similarity to that of RD1 [4] but, instead, is rich in both proline and arginine residues. This is characteristic of proteins able to interact with SH3 domains [14–17], which are distinct protein domains formed from around 60 amino acids and which have been identified in a variety of cytoplasmic tyrosyl protein kinases as well as in cytoskeletal and adaptor proteins [14–17]. These SH3-domain-containing proteins perform a crucial role in recruiting specific proteins so that they can take part in signalling cascades, and also in conferring distinct subcellular localizations on proteins that bind to them [14–17]. Here we show that RPDE-6 can bind to the SH3 domain of v-Src by virtue of its N-terminal splice region. To our knowledge this provides the first direct connection between an enzyme involved in the cAMP signalling pathway and SH3-domain-expressing proteins and identifies selectivity in this at the level of both the particular SH3 domain species and the particular PDE4A splice variant.

MATERIALS AND METHODS

Materials

The production of the glutathione S-transferase (GST)–v-Src-SH3 fusion protein in pGEX-2T and the v-Src-SH2–SH3 domain GST fusion has been described elsewhere [18]. Fusion proteins of the Csk and Crk SH3 domains in pGEX-2T, as described elsewhere [19,20], were provided by Dr. Siegmund Fischer (Laboratory of Clinical and Molecular Oncology, INSERM, Paris, France), and that for c-Abl by Dr. David Baltimore (Department of Biology, MIT, Cambridge, MA, U.S.A.) [21]. The intact GST–v-Src fusion protein was a gift from Dr. Val Brunton (Beatson Institute, Glasgow, Scotland, U.K.) and the GST fusion proteins for the intact Grb-2 (accession number M96995) were from Onyx (San Francisco, CA, U.S.A.). Glutathione–Sepharose was obtained from Pharmacia (St. Albans, Herts., U.K.). All tissue culture reagents were from Gibco Life Technologies (Paisley, Scotland, U.K.). [8-^{3}H]Adenosine 3′:5′-cyclic monophosphate (24 Ci/mmol) and the enhanced chemiluminescence (ECL) detection system were from Amersham International (Amersham, Bucks., U.K.). All other reagents were from Sigma (Poole, Dorset, U.K.).

Cloning of human SH3 cDNAs

Human SH3-domain cDNAs were cloned from human monocyte-like THP-1, peripheral-blood-cell or brain-cortex cDNA libraries into a pGEX-2T plasmid vector (Pharmacia). The DNA sequences were determined, and agreed with those reported in the deposited sequences (see the accession numbers given below). The pGEX-2T vector is inducible by isopropyl β-D-thiogalactoside and allows for expression of the SH3 proteins as GST–SH3 fusions (see below). SH3 domains were cloned into the *Bam*HI site of pGEX-2T. The PCR primers used in generating these various SH3 domains for insertion into the pGEX-2T vector were: Lck5′, GCGGAGGGATCCATGGACAACCTG-GTTATCGCTCTGC; Lck3′, GCGGAGGGATCCATGGTT-ACTCCAGGCTGTTCGCTTTGG; *Bam*H1hAblSH3-5′, GT-GCGTGGATCCCTTTTCGTTGCACTGTATG; *Bam*H1h-AblSH3-3′, GGTCCTGGATCCTCAGACTGGCGTGATGT-AGTTG; Lyn-5′, GCGGAGGGATCCATGGATCCAGAG-GAACAAGGAG; Lyn-3′, GCGGAGGGATCCATGGTTAT-GTTTCTAAGGTGTTGAG; Fyn-5′, GCGGAGGGATCCA-TGGGAACAGGAGTGACACTC; Fyn-3′; GCGGAGGGA-TCCATGGTTATGCCTGGATAGAGTCAAC.

The accession numbers reporting the sequences of the intact proteins and their SH3 domains are: human: Lck (M36881), Abl (X16416), Lyn (M16038) and Fyn (M14333).

Production of GST fusion protein and incubation with RPDE-6

Escherichia coli cultures (JM109), transformed with either pGEX (for GST production) or the recombinant pGEX-SH3-containing plasmid (for GST–SH3 production), were first grown overnight at 37 °C with agitation in Luria broth containing 60 μg/ml ampicillin, diluted 1:10 in the same medium and incubated at 37 °C for 1.5 h. Fusion protein expression was induced at 37 °C by adding isopropyl β-D-thiogalactoside and growth was continued at 37 °C for 4–6 h. Bacteria were harvested by centrifugation and then lysed by sonication in PBS containing a protease inhibitor cocktail (40 mg/ml PMSF, 156 mg/ml benzamidine, 1 mg/ml apoprotinin, 1 mg/ml antipain, 1 mg/ml leupeptin, 1 mg/ml pepstatin, dissolved in DMSO). The debris was pelleted by centrifuging for 1 min at high speed in a benchtop centrifuge. For every 1 ml of supernatant, 100 μl of glutathione–Sepharose beads equilibrated in PBS were added and incubated end-over-end for 1 h at room temperature. The beads were then washed three times with 200 μl of PBS over 15 min, resuspended as a 50 % (w/v) slurry and assayed for protein concentration.

As a routine, volumes of slurry containing 400 μg of beads were then pelleted and the supernatants removed. To each of the pellets an amount of cytosolic RPDE-6, from COS7 cells transfected with pSV.SPORT-RPDE-6, capable of hydrolysing 2 nmol of cAMP per min at 30 °C, was added and incubated for 10 min at 4 °C. The beads were then washed as before, the washes being retained for assay. The determination of RPDE-6 bound to the immobilized GST fusion proteins was performed by Western blot, with a polyclonal antibody raised against the conserved C-terminal region specific to rat PDE4A (see [11,12]). Bound PDE activity was measured by first releasing the SH3–PDE complex from the agarose beads by incubation with a 100 μl aliquot of 10 mM glutathione in 50 mM Tris/HCl buffer, pH 8.0, for 10 min at room temperature. This treatment was repeated twice more and the supernatants were pooled. Such a treatment had no effect on the total PDE activity.

The relative amounts of RPDE-6 bound to these SH3 domains was determined by both immunoblotting and PDE assay, as detailed before [12]. As a routine, detection was achieved with ECL. However, quantitative results were obtained with two distinct procedures, namely (1) by scanning ECL-developed blots with increasing amounts of protein so as to be able to plot extinction against sample applied, to utilize linear ranges, and (2) with detection with ^{125}I-labelled second antibody yielding similar quantitative data through Phosphorimager analysis.

Construction of RPDE-6 N-terminal biotinylated fusion protein

The polynucleotide sequences GACAGAGGGATCCCCATG-GAGCCTCCGGCC and CAGCATCCTGATATCCTTGTG-TGAGGCCAT were used to construct 5′ and 3′ primers respectively, to amplify a sequence encoding the first 258 amino acids of RPDE6. This amplified fragment was then subcloned, in-frame, into the *Bam*HI and *Eco*RV sites of the pinpoint Xa-1 plasmid (Promega), allowing the production of a biotinylated fusion protein with the RPDE6 peptide when expressed in *E. coli* strain JM109 (see method above for GST fusion protein induction).

Co-immunoprecipitation procedure

Both COS-7 cells, which had either been transfected to express RPDE-6 or had been mock-transfected with vector only, and Rat-1 fibroblasts expressing a temperature-sensitive mutant of v-Src [18] were grown to a confluent monolayer. Expression of v-Src was induced by the culturing of the Rat-1 cells at 35 °C [18]. These were then washed with PBS and stored at −80 °C until required. The monolayers were then lysed in 2 ml of immunoprecipitation buffer [1% (v/v) Triton X-100, 10 mM EDTA, 100 mM NaH_2PO_4,$2H_2O$, 50 mM Hepes, pH 7.2, plus protease inhibitor cocktail] for 15 min before being scraped and split into 1 ml samples in Eppendorf tubes. Samples were then incubated for a further 60 min with constant rotation before Triton X-100-insoluble complexes were removed by centrifugation at full speed in a microcentrifuge for 5 min, and the supernatant was incubated overnight at 4 °C with 7.5 μl of v-Src monoclonal antibody 327 [18] with constant rotation. Protein A–agarose (50 μl) was then added and the samples were incubated for a further 2 h with constant rotation at 4 °C. The Protein A–agarose immune complexes were centrifuged at full speed in a Microfuge (15000 ***g*** for 10 s at 4 °C) and the pellets washed three times with immunoprecipitation buffer and then three times with kinase buffer plus protease inhibitor cocktail. In control experiments preimmune antiserum was used. These samples were then boiled in 100 μl of SDS sample buffer [22] and loaded onto 8% acrylamide gels, blotted and the resulting blots probed with antiserum 651, against the common C-terminus of rat PDE4A [11], at a dilution of 1:250.

Phosphotyrosine detection

COS-7 cells were transfected with RPDE-6, by using DEAE-dextran with 5 μg of DNA in a 90 mm dish, into subconfluent cells and cultured for 48 h before harvesting into the PDE lysis buffer with inhibitors as detailed above. The total lysate from one dish was split into three portions and RPDE-6 was immunoprecipitated with 5 μl of antiserum together with Protein A–Sepharose. This precipitated RPDE-6 was washed twice with PDE lysis buffer, once with wash buffer (400 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl, pH 8, 0.25% deoxycholate and 1% Nonidet P40) and then three times with kinase assay buffer (100 mM Pipes, pH 6.8, 20 mM $MnCl_2$ and 10 μM sodium orthovanadate), to remove all salt and detergent. The immunoprecipitated RPDE-6 was incubated with a similarly treated immunoprecipitate of v-Src immunoprecipitated from chicken embryo fibroblasts infected with an avian retrovirus (RCAN) expressing a temperature-sensitive v-Src mutant LA29 [18]. The cells expressing this form of v-Src had been grown at the permissive temperature of 35 °C. The two immunoprecipitates were mixed and incubated in kinase assay buffer together with 0.1 μM ATP. The immunoprecipitates were back-probed with either 1:1000 of antiserum to RPDE6 or 1:1000 anti-phosphotyrosine (PY 20) and detected with horseradish peroxidase-conjugated second antibody and ECL.

Probing cytosol fractions from brain and testes with an GST–Src-SH3 domain fusion protein

A brain from a 200–250 g male Sprague–Dawley rat was dissected and finely chopped in 20 ml of ice-cold homogenization buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.1 mM dithiothreitol, protease inhibitor cocktail). The chopped brain was then rinsed in a tea-strainer with 50 ml of ice-cold homogenization buffer and homogenized in 5 ml of homogenization buffer with eight strokes at full speed in a rotary homogenizer with a Teflon pestle and a glass vessel. The homogenate was then centrifuged at 100000 ***g*** for 20 min at 4 °C. The supernatant was decanted into 100 μl aliquots, snap-frozen in liquid nitrogen and stored at −80 °C. A similar procedure was used to produce a cytosol extract from testis. A glutathione–Sepharose-immobilized Src-SH3 domain GST fusion protein was generated as described above. In typical experiments, 400 μg of fusion protein was added to 400 μg of cytosol protein in 200 μl of homogenization buffer. This mixture was incubated with end-over-end mixing for 30 min at 4 °C. The beads were then harvested by centrifugation in a Microfuge for 10 s and then washed three times with 1 ml of PBS containing protease inhibitors, each for 5 min with end-over-end mixing. The beads were then resuspended in 100 μl of Laemmli [22] mixture, immediately boiled for 3 min and harvested in a Microfuge as above, and samples were analysed by Western blotting. In control experiments GST was used rather than the fusion protein.

Other methods

All other methods used in this study have been described in some detail previously [9–12]. These include: the cloning of met^{26}RD1 [9], splice variant RPDE-39 (RNPDE 4A8) [23] and RPDE-6 [12] cDNAs into mammalian expression vectors; the transient expression of these species in COS7 cells and the harvesting of a cytosolic fraction, 72 h after transfection, containing enzymically active proteins [9,12]; the generation and characterization of a C-terminal anti-peptide antiserum (Ab1992) able to detect all known splice variants of the rat PDE4A gene [11,12]; the Western blotting procedure and detection with the ECL protocol [11,12], and assays for both protein and cAMP PDE activity with 1 μM cAMP as substrate [9–12].

RESULTS AND DISCUSSION

Interactions between SH3 domains and putative target proteins have been investigated in a number of laboratories by using specific SH3 domains expressed as in-frame fusion proteins with GST (see [18,20,21]). We have adopted this approach to analyse the potential interaction between RPDE-6, generated in the cytosol of COS7 cells transfected with pSV.SPORT-RPDE-6 [12], and various SH3 domains expressed as GST fusion proteins

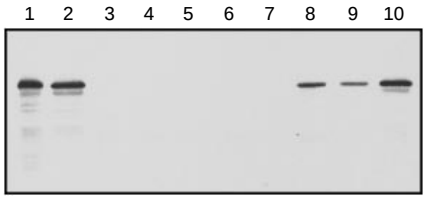

Figure 1 Binding of RPDE-6 to the v-Src-SH3 domain expressed as a GST fusion protein

This shows a typical immunoblot with a rat PDE4A-specific anti-peptide antiserum that recognizes the C-terminus of RPDE-6. It demonstrates the binding of RPDE-6 to the SH3 domain of v-Src expressed as a fusion protein. Track 1, membranes (25 μg) from RPDE-6-expressing COS7 cells; track 2, cytosol (25 μg) from RPDE6-expressing COS7 cells; track 3, washed glutathione–agarose beads after exposure to RPDE-6; track 4, as track 3 but washed with 0.5 M NaCl plus 0.5% Triton X-100; track 5, blank; track 6, washed glutathione–agarose beads with attached GST after exposure to RPDE-6; track 7, as track 6 but washed with 0.5 M NaCl plus 0.5% Triton X-100; track 8, washed glutathione–agarose beads with attached GST–v-Src-SH3 after exposure to RPDE-6; track 9, as track 8 but washed with 0.5 M NaCl plus 0.5% Triton X-100; track 10, supernatant from the GST–v-Src-SH3 glutathione–agarose beads after exposure to RPDE-6.

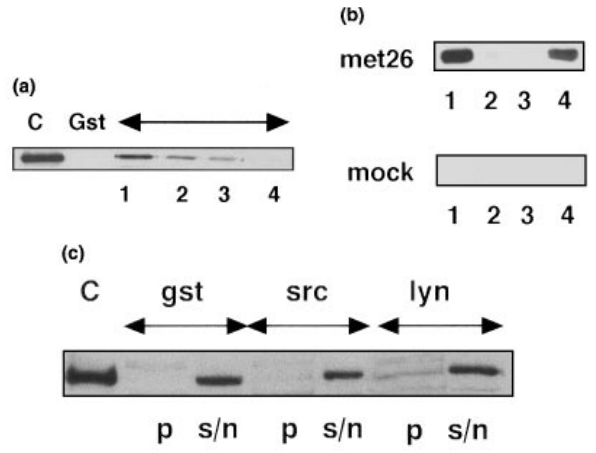

Figure 2 The N-terminal splice domain of RPDE-6 is required for binding to the v-Src-SH3 domain

(**a**) The GST–v-Src-SH3 fusion protein can adsorb all of the RPDE-6. Here are shown immunoblots with a rat PDE4A-specific antiserum: C, soluble COS7-cell expressed RPDE-6; Gst, GST-containing agarose beads exposed to RPDE-6 and then pelleted and washed; tracks 1–3, the results of probing agarose beads with attached GST–v-Src-SH3 domain that had been exposed to soluble RPDE-6, harvested and washed. In this experiment GST–v-Src-SH3 agarose was added to RPDE-6 and the beads were isolated (track 1) as well as a supernatant that was treated again with beads to yield a further pellet (track 2), which was repeated again to yield a pellet (track 3) and a final, RPDE-6-free supernatant (track 4). These results represent one typical experiment of at least three. (**b**) Experiments done with met^{26}RD1 (met26) and mock (vector-only) extracts of COS7 cells (mock) as the enzyme source. The results show immunoblots with the rat PDE4A-specific antiserum. Track 1, the soluble extract of COS7 cells used in the experiments. The pelleted beads harvested after incubation with either GST–agarose (track 2) or with GST–v-Src-SH3 agarose (track 3) were immunoblotted, as was the supernatant fraction from the GST–v-Src-SH3 agarose experiment (track 4). This experiment is typical of one done three times. The lower segment shows a set of experiments identical with those described above for the upper segment except that cytosol from mock (vector-only) transfected COS-7 cells was used. This shows that the immunoreactive species detected by the PDE4A-specific antisera are dependent on transfection and expression of RPDE6. These results represent one typical experiment of at least three. (**c**) Experiments done as described above, but using RPDE-39 with GST alone (gst) and with GST fusion proteins formed with the SH3 domains of Src (src) and Lyn (lyn), as indicated. The immobilized (p) and remaining soluble (s/n) fractions are shown. The results represent one typical experiment of at least three.

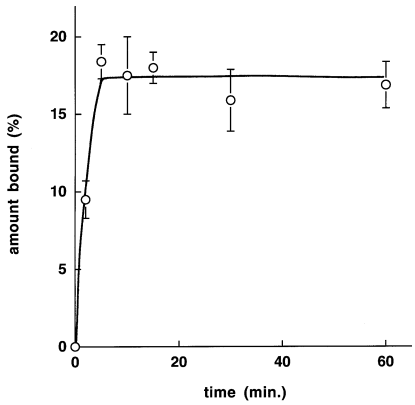

Figure 3 Time course for the binding of RPDE-6 to the v-Src-SH3 domain

In this experiment, conditions were chosen (25 μg of RPDE-6-transfected COS7 cell cytosol; 200 μg of GST–v-Src-SH3 fusion protein) such that not all of the RPDE-6 protein would become bound to the GST–v-Src-SH3 fusion protein, and the time course of binding was followed by measuring PDE activity. Binding was done at 4 °C, This shows one typical experiment of three.

Table 1 Assessment of the binding of RPDE-6 to SH3 domains

This was done as described in the Materials and methods section. Detection of the amount of associated RPDE was done by following immunoreactive protein and also by following PDE activity. For comparison, these results are shown relative to those found for v-src-SH3 (set equal to 1.00). Results are means ± S.D. for the indicated number of experiments (*n*).

PDE4A species	GST fusion protein species	Relative binding	Relative activity	*n*
RPDE-6	Src-SH3	1	1	25
RPDE-6	GST alone	0.05 ± 0.03	0.04 ± 0.03	25
RPDE-6	Src-SH3 + (1–256 NT-RPDE6/biotin)	0.08 ± 0.6	0.1 ± 0.5	3
RPDE-39	GST alone	0.04 ± 0.04	0.07 ± 0.05	4
RPDE-39	Src-SH3	0.05 ± 0.04	0.05 ± 0.03	4
RPDE-39	Lyn-SH3	0.09 ± 0.05	0.08 ± 0.05	4
RPDE-6	Lck-SH3	0.39 ± 0.09	0.07 ± 0.04	6
RPDE-6	Crk-SH3	0.40 ± 0.12	0.05 ± 0.04	6
RPDE-6	Csk-SH3	0.25 ± 0.06	0.06 ± 0.05	6
RPDE-6	Abl-SH3	0.58 ± 0.14	0.48 ± 0.09	6
RPDE-6	Fyn-SH3	2.1 ± 1.1	3.1 ± 1.2	4
RPDE-6	Lyn-SH3	8.8 ± 1.5	9.1 ± 1.7	4
RPDE-6	Grb2-(SH3-SH2-SH3)	0.08 ± 0.05	0.07 ± 0.04	3
RPDE-6	Src kinase	7.8 ± 1.2	7.0 ± 0.9	4
RPDE-6	Src-SH2	0.04 ± 0.04	0.05 ± 0.03	4
RPDE-6	Src-(SH2 + SH3)	8.1 ± 0.8	7.9 ± 0.5	4

that can be immobilized on glutathione–agarose. Here we show (Figure 1, track 8) that soluble RPDE-6 became associated with the immobilized GST–v-Src-SH3 fusion protein. This was readily detected by specific immunoblotting of the washed, harvested beads with the anti-RPDE4A-specific antiserum. This binding seemed to be specific in that soluble RPDE-6 did not become attached to either glutathione–agarose beads alone or native, immobilized GST (Figure 1, tracks 3 and 6). Thus for association to occur there was an absolute requirement for the presence of the v-Src-SH3 domain. Such an interaction between RPDE-6 and the v-Src-SH3 domain was little affected (Figure 1, track 9; 5–22 % decrease, range for four separate experiments) by washing the immobilized species with a mixture of the detergent Triton X-100 (0.5 %) together with 0.5 M NaCl, conditions that have been shown [12] to release minimal amounts of particulate RPDE-6 in brain and transfected COS7 cells [12].

The soluble, cytosolic cell extract containing RPDE-6 can be expected to contain other proteins that might compete for binding to the v-Src-SH3 in such experiments. Nevertheless we were able to demonstrate that all of the RPDE-6 could be extracted from the transfected COS cell cytosol by using the immobilized GST–v-Src-SH3 (Figure 2a). Such binding of RPDE-6 to the SH3 domain of v-Src was a rapid event, with maximal association being evident after approx. 5 min at 4 °C (Figure 3).

The 'core' enzyme, which can be considered to be encoded by the PDE4A gene, would be a form that lacks the N-terminal alternatively spliced regions found in RPDE-6 and RD1. We have engineered such a species previously [9,10,12] and called it met^{26}RD1. This enzyme is expressed exclusively as a soluble, cytosolic and fully active species in COS7 cells transfected with the plasmid pSVL–met^{26}RD1 [9,11]. In contrast with our observations with RPDE-6, met^{26}RD1 did not become associated with immobilized GST–v-Src-SH3 (Figure 2b). Thus the N-terminal splice domain of RPDE-6 was essential for this PDE to be able to interact with the v-Src-SH3 domain. Further confirmation of

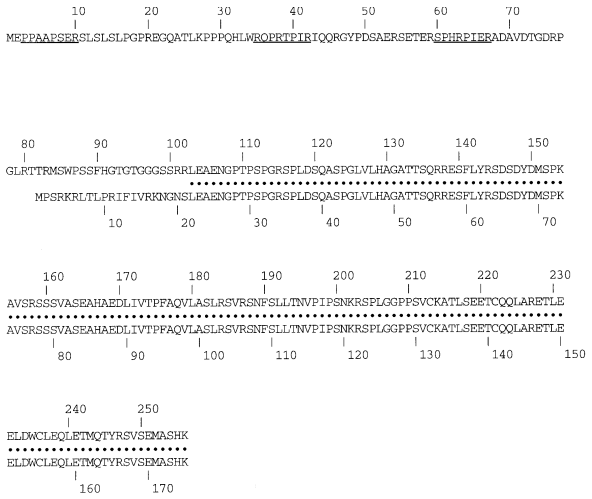

Figure 4 Comparison of the N-terminal regions of the two PDE4A splice variants RPDE-6 and RPDE-39

RD1 is the first and the smallest splice variant found so far for the rat PDE4A gene [4]. The first splice junction is the point at which both RPDE-6 and RPDE-39 diverge from RD1 [4]. This identifies a 256-residue N-terminal splice domain for RPDE-6 and a 175-residue N-terminal splice domain for RPDE-39. However, the splice variants RPDE-6 and RPDE-39 share a common region of 154 amino acids that extends from residues Leu^{103} to Lys^{256} in RPDE-6 and Leu^{22} to Lys^{175} in RPDE-39. This leaves unique extreme N-terminal domains of 102 residues in RPDE-6 and 21 residues in RPDE-39. In the entire N-terminal splice region of RPDE-6 there are 27 proline residues, 26 arginine residues and also 37 serine residues. Only within the unique splice domain of RPDE-6 are there proline/arginine-rich regions of the form that it has been suggested might interact with SH3 domains [14–17,20]. The 'core' motif considered as providing an identifiable SH3 binding site is given by PXXP [20], and the three such features are shown here underlined. All three of these can be classified within the two motifs PPXXPX–R and PXXPXX–R (where hyphens indicate possible inserts of up to two additional residues) which, on the basis of NMR analyses, have been suggested [16] as providing consensus sites for binding to SH3 domains. It is believed that binding at such domains is such that these motifs can orientate in either direction (N-terminus to C-terminus or vice versa). In view of the apparent specificity for binding of RPDE-6 to Src and Src family kinase SH3 domains, the first (residues Pro^3–Arg^{10}) and third (residues Ser^{60}–Arg^{67}) of these indicated motifs fall into the so-called Class I SH3 binding site motifs [20], which show specificity for binding to the SH3 domain of Src. The second motif (residues Arg^{35}–Arg^{42}) falls within the category of a potential Class II binding site, where some preference for interaction with the SH3 domains of Crk and Abl has been indicated in model studies [21].

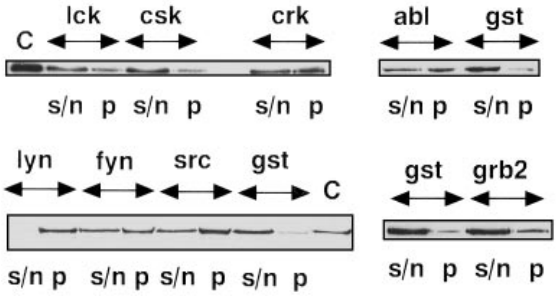

Figure 5 Selectivity for the binding of RPDE-6 to various SH3 domains expressed as GST fusion proteins

Experiments were performed as described in the legend of Figure 2 but with SH3 domains of Lck, Csk, Crk, Abl, Lyn, Fyn and Src expressed as GST fusion proteins. Also shown are results for full-length Grb-2 expressed as a GST fusion protein. The immobilized (p) and remaining soluble (s/n) fractions are shown. The presence of RPDE-6 was detected by immunoblotting with the PDE4A antiserum. The results are typical of experiments done at least three times.

this was obtained when a biotinylated fusion protein expressing the N-terminal domain of RPDE-6 was shown to out-compete the binding of RPDE-6 to immobilized GST-v-Src-SH3 (Table 1).

We have recently described [23] the molecular cloning, distribution and biochemical characterization of an additional splice variant produced by the PDE4A gene. This species, RPDE-39, is identical in sequence with RPDE-6 except for a domain swap at its extreme N-terminus of the first 112 residues of RPDE-6 for a novel sequence of 21 residues (Figure 4). Using RPDE-39 from the cytosol of transfected COS-7 cells we noted that this species failed to become associated with the SH3 domain of v-Src and h-Lyn, with detection by both immunoblotting and activity analyses (Figure 2c and Table 1). This indicates that the site in RPDE-6 that interacts with SH3 domains is located within the N-terminal first 112 residues of its unique N-terminal splice region. In this regard we note (Figure 4) that within this unique region there are two putative Class I SH3 binding sites [20]. In contrast, no putative SH3 binding sites are evident within the unique N-terminal sequence found in RPDE-6 or the common N-terminal region shared between RPDE-6 and RPDE-39, which is not seen in the entirely membrane-bound splice variant RD1 (Figure 4). Thus any propensity for interaction with SH3 domains seems to be determined by differential splicing between PDE4A gene products.

Using the immunoblotting procedure described previously [12] to determine the relative activities of soluble and particulate forms of RPDE-6, we here determined whether the PDE activity of RPDE-6 was altered on binding to the v-Src SH3 domain. Such analyses showed that binding of RPDE-6 had little discernible effect on PDE activity (less than 7% change; $n = 6$; 1 μM cAMP substrate).

Various SH3 domains have been shown to exhibit a selectivity for interaction with 'target' species [16,20]. Here we see that, in experiments monitored on the basis of immunoblotting, RPDE-6 appeared to show a selectivity for binding to the SH3 domains of various proteins (Figure 5). In particular, there seemed to be selectivity (Table 1) for the SH3 domains of Src- and the Src-family-related tyrosyl protein kinases Lyn and Fyn [24,25], with the SH3 domain of Lyn being particularly effective (Table 1 and Figure 5). However, intriguingly, the SH3 domain of the Src-related kinase Lyk [24,25] was unable to interact with RPDE-6 (Table 1 and Figure 5). Note, however, that despite the marked potency for the SH3 domain of Lyn in associating with RPDE-6, this species was still unable to interact with the splice variant RPDE-39 (Figure 2a). Such putative interactions were also analysed on the basis of assessing the immobilized PDE activity (Table 1). Expressing such results relative to the degree of association found between RPDE-6 and the v-Src-SH3 domain, we noted a selectivity with detection by both PDE activity and immunoblotting (Table 1). However, although the levels of RPDE-6 bound to the SH3 domains of Lyn, Fyn and Abl were similar under both procedures, this did not seem to hold true for the binding of RPDE-6 to the SH3 domains of Csk, Crk and Lyk (Table 1). In such instances, the magnitude of immobilized PDE activity was much lower than might have been expected on the basis of the amount of RPDE-6 that became bound as detected by immunoblotting (Table 1). This suggests that although RPDE-6 might not bind so effectively to these other species, the fraction that does so exhibits a marked diminution in activity.

SH3 domains generated as distinct species, including that of Src, have been shown to self-fold into active conformations as evidenced from a variety of structural studies [16,26–30]. Consistent with this, GST fusion proteins formed with the SH3 domains of various proteins have been shown to be effective in functional binding studies and have been used successfully to

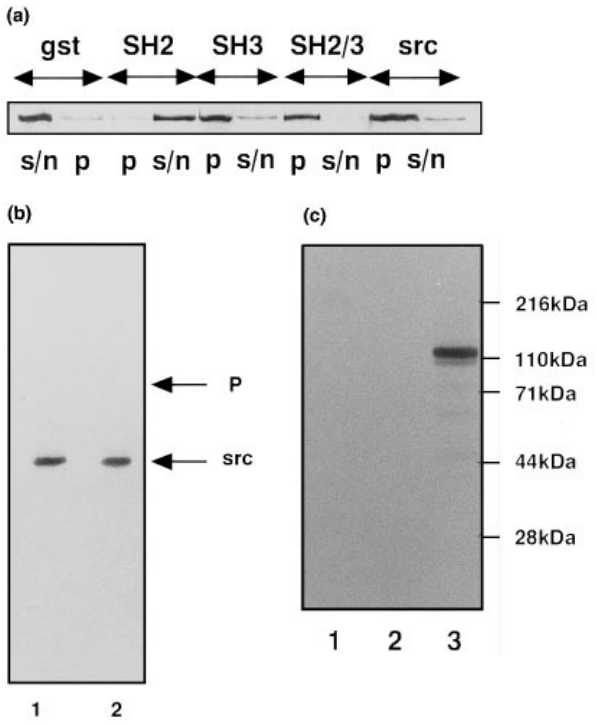

Figure 6 Binding of RPDE-6 occurs to full-length Src and to a Src-(SH3 + SH2) domain complex but not to Src-SH2

(**a**) Experiments were performed as described in the legend of Figure 2 but with GST fusion proteins of Src-SH2 domain, Src-SH3 domain, Src-(SH2 + SH3) domain and full-length Src. The immobilized (p) and remaining soluble (s/n) fractions are shown. The presence of associated RPDE-6 was detected by immunoblotting with the PDE4A antiserum. Experiments are typical of those done at least three times. (**b**) Immunoblots with anti-phosphotyrosine antiserum probing an immunoprecipitate of v-Src incubated in a buffer system that allows the functional tyrosyl kinase activity of Src to be expressed. Track 1, incubation of the Src immunoprecipitate alone, with the single auto-phosphotyrosyl phosphorylated Src protein being identified; track 2, incubations with the addition of immunoprecipitated RPDE-6. No evidence of any phosphotyrosine associated with RPDE-6 was obtained. The position where RPDE-6 migrated on these gels, as detected by stripping the blots and re-probing with antibody for RPDE-6, is shown (P). The immunoprecipitated Src protein kinase was able to exogenously phosphorylate enolase on tyrosine residues (results not shown). Results are typical of experiments done at least three times. (**c**) Cytosol fractions from v-Src-transfected cells mixed with those from COS7 cells transiently expressing RPDE-6. In track 3 anti-Src antibody was added and the immunoprecipitate probed by Western blotting with antibody for RPDE-6. In track 2 a non-specific antibody was used and in track 1 no antibody was added to the immunoprecipitation system. Results are typical of experiments done at least three times.

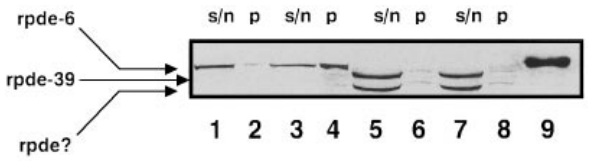

Figure 7 The binding of endogenous brain RPDE-6 to Src-SH3 domain

As described in the Materials and methods section, soluble extracts were prepared from both brain and testis. These were incubated with Src-SH3 domain GST fusion proteins, which were immobilized on glutathione–agarose. After incubation, the Sepharose beads were harvested, washed and subjected to SDS/PAGE with subsequent immunoblotting to detect associated RPDE-6. The immobilized (p) and remaining soluble (s/n) fractions are shown. The experiments done were for brain soluble extracts incubated with GST (tracks 1 and 2), brain soluble extracts incubated with GST–Src-SH3 (tracks 3 and 4), testis soluble extracts incubated with GST (tracks 5 and 6), testis soluble extracts incubated with GST–Src-SH3 (tracks 7 and 8) and COS-cell-expressed RPDE-6 (track 9). Results are typical of experiments done at least three times.

highlight and explore possible physiologically relevant interactions (see [18,31–34]). Here we have demonstrated that RPDE-6 can bind to the SH3 domain of v-Src (Figures 1 and 2 and Table 1). However, RPDE-6 can also bind to the entire Src kinase, shown here by using a GST fusion construct (Figure 6 and Table 1). This is not, however, due to any binding of RPDE-6 to the Src-SH2 domain, as demonstrated with a Src-SH2 domain GST construct (Figure 6a and Table 1). Indeed, to bind to SH2 domains then one might expect that RPDE-6 would have to be phosphorylated on an appropriate tyrosine residue. However, we could find no evidence to suggest that RPDE-6 was tyrosyl phosphorylated or, indeed, could even be so tyrosyl phosphorylated by functional Src kinase (Figure 6b). It has been shown [18] that the binding of phosphatidylinositol-3 kinase to the Src-SH3 domain was enhanced in constructs formed from the Src-SH2 and Src-SH3 domains. This was not due to any interaction of phosphatidylinositol-3 kinase with the SH2 domain [18] but was presumed to be due to a more effective folding of the SH3 domain elicited by the presence of the SH2 domain, which is normally adjacently located in the intact kinase. Similarly, we noted (Table 1 and Figure 6a) that a GST fusion protein that was formed to express both the SH2 and adjacent SH3 domains of Src was considerably more effective than the Src-SH3 domain alone in binding RPDE-6, but similar in effectiveness to the intact kinase (Table 1 and Figure 6). The inability of intact Grb-2, expressed as a GST fusion protein, to bind RPDE-6 demonstrated that not just any combination of SH2 and SH3 domain will allow for interaction with RPDE-6 (Table 1 and Figure 5). Thus RPDE-6 does not seem to interact with this key adaptor protein, again indicating specificity in its putative interaction with SH3-domain-expressing proteins. As well as being able to demonstrate that RPDE-6 could interact with the intact Src kinase expressed as a GST fusion protein, we were also able to show that RPDE-6 could be co-immunoprecipitated with Src, using anti-Src antisera, from cytosol extracts of transfected cells (Figure 6c).

To determine whether endogenously expressed RPDE-6 could interact with SH3 domains, we treated a soluble brain extract with immobilized GST–Src-SH3 fusion protein and then performed immunoblots on this material with antiserum to PDE4A. This showed that the GST–Src-SH3 fusion protein could indeed adsorb an immunoreactive species that co-migrated on SDS/PAGE with COS7-cell-expressed RPDE-6 (Figure 7). A similar approach was used to probe soluble extracts from testis, where we have shown previously [23] that RPDE-39, but not RPDE-6, is expressed. In such experiments the endogenous RPDE-39, and a faster migrating species thought to be another PDE4A splice variant [23], did not become associated with the immobilized GST–Src-SH3 fusion protein (Figure 7). Thus these endogenous forms of RPDE-6 and RPDE-39 seem to exhibit a similar selectivity in their ability to bind to Src-SH3 domains as found with the COS-cell-expressed forms.

Conclusion

Here we have provided evidence that the PDE4A splice variant RPDE-6 can bind to the SH3 domains from various proteins. There seems to be a profound selectivity in this interaction, with a distinct preference indicated for those SH3 domains of Src and certain Src-related kinases. That such an interaction is not seen for the PDE4A splice variant RPDE-39 suggests that alternative splicing of the PDE4A gene might determine the cell-specific expression of protein products, such as RPDE-6, that are able to

interact with (specific) SH3 domain-containing proteins. In this respect, within the unique N-terminal splice region RPDE-6 are two Class I SH3-binding sites [20], which have been shown to confer specificity for association with Src-kinase SH3 domains, together with a Class II binding site, which seems to exhibit some specificity for the binding of SH3 domains of Crk and Abl [21]. The functional implications of such an interaction between RPDE-6 and SH3-domain-containing proteins remains to be determined. Interestingly, however, it has been noted that overexpression of c-Src can lead to a potentiation of the ability of β-adrenoceptor agonists both to stimulate membrane adenylate cyclase activity [35] and to increase intracellular concentrations of cAMP [36,37]. Based on *in vitro* studies, it is possible that under such conditions protein kinase A could elicit the phosphorylation of c-Src on Ser^{17} [38–40], although the functional consequences of such a modification are, as yet, unclear [40,41]. Nevertheless, it is possible that the recruitment of a PDE to the immediate vicinity of Src kinase could serve to militate against any such action of protein kinase A by decreasing local concentrations of cAMP. Thus a possible role for the association of this high-affinity cAMP-specific PDE with such tyrosyl kinases may be to provide a means of reducing the local concentrations of cAMP around both the tyrosyl kinase and other proteins that are recruited around them to form complexes at the plasma membrane. This would provide a local and specific control of actions of cAMP, rather than the tyrosyl kinases and their complexes being solely subject to changes in the activity of adenylate cyclase, which is also located at the plasma membrane [1]. Binding to specific SH3 domains might also offer a means of regulating RPDE-6 activity, as indicated in complexes formed with the SH3 domains of Csk, Crk and Lck. Certainly the fact that the N-terminal splice domains of PDE4A enzymes appear to exert profound effects on enzyme catalytic activity [11,12,23] indicates that changes in the conformation of such domains might be expected to have profound functional consequences. In becoming associated with the SH3 domains of specific proteins, RPDE-6 might thus serve to modulate the effect of cAMP on the functioning of such signalling pathways. Interactions of RPDE-6 with SH3-domain-containing proteins might also offer a means through which a fraction of RPDE-6 can be found associated with membrane/cytoskeleton fractions. Thus the two key features of the distinct N-terminal regions of PDE4A species that arise through alternate splicing appear to be (i) targeting within the cell and (ii) regulation of catalytic activity. As PDE4A splice variants show profoundly different expression patterns, this might indicate that the control of alternative splicing of the PDE4A gene could have an important regulatory role.

This is, to our knowledge, the first demonstration of a linkage between the cAMP signalling pathway and SH3-domain-containing proteins. It might thus open up exciting new avenues of research in defining the controls and interactions that characterize the 'cross-talk' between various intracellular signalling pathways, the recruitment and organization of signalling proteins within the cell and the identification of novel compounds that could disrupt such targeting interactions and thus alter cellular functioning. As PDE4A splice variants show profoundly different expression patterns, this might indicate that the control of alternative splicing of the PDE4A gene could have an important regulatory role.

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