Chimeric constructs show that the unique N-terminal domain of the cyclic AMP phosphodiesterase RD1 (RNPDE4A1A; rPDE-IV_{A1}) can confer membrane association upon the normally cytosolic protein chloramphenicol acetyltransferase

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A novel plasmid was generated which allowed the expression of the cytosolic bacterial enzyme chloramphenicol acetyl transferase (CAT) in COS-7 cells. Upon transfection, the majority of the novel CAT activity was found in the cytosol fraction of COS cells. Chimeric molecules were made between N-terminal portions of the type IV_A cyclic AMP-specific rat 'dunce-like' phosphodiesterase (RD1) (RNPDE4A1A; rPDE-IV_{A1}) fused to CAT at its N-terminus. Expression in COS-7 cells of chimeras formed from 1–100RD1–CAT and 1–25RD1–CAT now showed CAT activity associated with the membrane fraction. In contrast, a chimera formed from 26–100RD1–CAT showed an identical expression pattern to native CAT, with the major fraction of CAT activity occurring in the cytosol fraction. Membrane-bound CAT activity provided by 1–100RD1–CAT and 1–25RD1–CAT was not released by either high-salt or washing

treatments but was solubilized in a dose-dependent fashion by the non-ionic detergent Triton X-100. Subcellular fractionation of COS-7 cells showed that, as with RD1, the membrane-bound activity of the RD1–CAT chimera followed that of the plasma membrane marker 5'-nucleotidase. Plasmids containing chimeric cDNAs were exposed to a coupled transcription–translation system that, in addition to the full-length chimeras, was found to generate a range of N-terminal truncated species due to initiation at different methionine residues. Incubation of the mature protein products formed in this system with a COS cell membrane fraction showed that only those chimeric CAT constructs containing the first 25 amino acids of RD1 became membrane-associated. The unique 25 amino acid N-terminal domain of RD1 contains structural information that can confer membrane association upon an essentially soluble protein.

INTRODUCTION

A diverse family of cyclic AMP (cAMP) phosphodiesterases (PDE) is responsible for inactivating the second messenger cAMP [1–4]. The type-IV PDE sub-family is formed from a group of enzymes that specifically hydrolyse cAMP in a fashion which is insensitive to cGMP and is inhibited selectively by the compound 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone (rolipram) [3–6]. The diversity of this family arises by virtue of there being both multiple genes coupled with, in certain instances, multiple splicing [3,4,7,8]. It has been suggested that there are four genes encoding the type-IV isoform family [3,4,7,9], with multiplicity increased through the production of various splice variants [3,8–11]. There is considerable similarity between these forms in their central region [3,4,9,11], which is believed to provide the catalytic domain [12]. However, at their extreme N- and C-termini there is little if any similarity. To date, however, little is known about the function, regulation and distribution of these various isoforms. However, it has been suggested that such domains may be involved in conferring such properties as membrane association [13–17] and susceptibility to phosphorylation on specific isoforms [18].

Both cytosolic and membrane-bound forms of various type-II [19], -III [1,20–22] and -IV [1,13–17,23,24] PDEs have been identified. The mechanisms whereby membrane association is conferred upon these various species are likely to be rather

different. For example, a membrane-associated type-IV PDE has been characterized as a peripheral protein associated with hepatocyte plasma membranes [23,24]. In contrast, also associated with hepatocyte plasma membranes is a type-II PDE that requires detergents to effect its solubilization [19]. Its very close relatedness to a soluble type-II enzyme that is also found in hepatocytes suggests that these two species are most likely to result from multiple splicing with membrane association conferred by an additional targeting domain [19]. A membrane-bound type-III activity has also been characterized in hepatocytes where mild proteolysis released a soluble protein some 5 kDa smaller [1,25,26], indicating that, as with these other species, membrane-association may occur through a small anchoring domain.

Screening a rat brain cDNA library with the gene for *Drosophila dunce* PDE allowed the first type-IV mammalian PDE to be isolated [27]. This was called RD1 (RNPDE4A1A ; rPDE-IV_{A1}). Shakur et al. [13] have shown that transfection of COS cells with the cDNA for RD1 allowed for the expression of a novel type-IV PDE activity. However, a major fraction of its activity was associated with the membrane fraction [13]. Intriguingly, the engineered deletion of the unique N-terminal 25 amino acid domain of RD1 led to the expression of RD1 activity now being found exclusively in the cytosol fraction [13]. It was suggested [13] that this unique domain did not form a signal sequence but, rather, might serve as a membrane anchor for what was an

Abbreviations used: CAT, chloramphenicol acetyltransferase; PDE, cyclic 3',5'-AMP phosphodiesterase; RD1, rat 'dunce-like' phosphodiesterase 1 (also referred to as either RNPDE4A1A or rPDE-IV_{A1}) representing rat, type-IV cyclic AMP-specific phosphodiesterase, isoform subfamily A, splice variant 1; cAMP, cyclic AMP; LDH, lactate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; TM buffer, 10 mM Tris-HCl (pH 7.8)/0.2 mM MgCl₂; TEN buffer, 40 mM Tris-HCl/10 mM EDTA/120 mM NaCl, pH 8.0.

The nucleotide sequence of RNPDE4A1A appears in the GenBank Database under accession number M26715.

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essentially 'soluble' protein. To test this hypothesis we have constructed various chimeras formed between N-terminal segments of RD1 fused to the N-terminus of the soluble bacterial enzyme chloramphenicol acetyltransferase (CAT). Using such a strategy we show that the unique N-terminal 25 amino acid domain of RD1 can indeed confer membrane association on a normally soluble cytosolic enzyme such as CAT.

MATERIALS AND METHODS

Plasmid pBLCAT2 [28] containing the CAT gene of Tn9 was a gift from Dr. M. L. M. Anderson (University of Glasgow, U.K.). Plasmid pSVL-RD1 containing the RD1 type IV_{A1}PDE was constructed as described previously [13]. Plasmid pSV.SPORT was purchased from Life Technologies Ltd. PCR and sequencing primers were designed with the Gene Jockey programme (Biosoft) according to Sheffield et al. [29] and synthesized on an Applied Biosystems model 381A oligonucleotide synthesizer. The codes (GSOLxx) and sequences for these were:

GSOL12, 5'-GCGAGGGAATTCTAGAATGCCTCTGGTT-3';

GSOL13, 5'-GCATATCTCGAGCGCCTTTTGAAGTGGT-3';

GSOL15, 5'-GGCTCCTCGAGCTTCCAGTGTGT-3';

GSOL24, 5'-GCTTATCTAGAATGCTGAACCGTGAGCTC-3'

GeneAmp PCR reagent kit was purchased from Perkin-Elmer. Restriction and modifying enzymes, TnT SP6 Coupled Reticulocyte Lysate System, Wizard PCR, Miniprep and Maxiprep Systems were supplied by Promega U.K. Qiaex PCR purification kit was purchased from Hybaid. Taq DyeDeoxy terminator cycle sequencing kits were purchased from Applied Biosystems. [¹⁴C]Chloramphenicol (54 mCi/mmol), [³⁵S]methionine (50 mCi/mmol), ¹⁴C-labelled protein molecular mass markers and enhanced chemiluminescence (ECL) Western blot detection reagents were purchased from Amersham International. All other chemicals and reagents were of the highest quality available and purchased from Sigma Chemical Co.

Construction of pGS4

A 321 bp DNA fragment encoding amino acids 1–100 of RD1 was generated by using primers GSOL12 and GSOL15 which were designed to contain recognition sites for the restriction enzymes *Xba*I and *Xho*I respectively. pSVL-RD1 formed the template in the following reaction mixture: 1 µg of template DNA, 25 pmol of each primer, 0.2 mM of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100 (v/v), 1.5 mM MgCl₂ and 2.5 units of Taq polymerase 1 in a final volume of 50 µl with a mineral oil overlay. The reaction was subjected to the following conditions in a Techne PHC-3 thermocycler: denaturation for 1 min at 94 °C, annealing for 2 min at 37 °C, extension for 3 min at 72 °C for one cycle followed by denaturation for 1 min at 94 °C, annealing for 2 min at 60 °C, extension for 3 min at 72 °C for 30 cycles. Upon completion, the reaction was extracted with an equal volume of chloroform and digested using 12 units of *Xba*I and 8 units of *Xho*I in 6 mM Tris-HCl (final pH 7.9), 150 mM NaCl, 6 mM MgCl₂ and 1 mM dithiothreitol for 2 h at 37 °C. The digested fragment was analysed by electrophoresis through a 1.75% low gelling-temperature agarose gel against *Hae*III-digested *phi* X174 DNA molecular mass markers and purified directly from the gel by Wizard PCR Prep kit (Promega).

Figure 1 Generation of the eukaryotic expression vector pGS7

The fragment encoding the first 100 amino acids of RD1 was generated by PCR. This was then ligated into the plasmid pBLCAT2 after digestion with restriction enzymes *Xba*I and *Xho*I to generate pGS4. The SV40 early promoter and SP6 promoter were obtained by *Cla*I/*Xba*I digestion of pSV.SPORT1 and subsequently ligated into *Nar*I/*Xba*I-digested pGS4. Both the *Nar*I and *Cla*I sites were lost due to the cloning strategy. The full sequences of the generated plasmids are available on request.

An 'in-frame' fusion of the RD1 N-terminal fragment with the CAT gene of pBLCAT2 [28] was created by ligation of the digested PCR fragment into *Xba*I/*Xho*I-digested plasmid following gel purification of the vector to remove the 174 bp TK promoter region. The fidelity of the inserts generated by this method was confirmed by Taq cycle sequencing and electrophoresis on an Applied Biosystems model 373A automated sequencer according to the manufacturer's instructions. The resulting construct, named pGS4 (Figure 1), was used as a template for subsequent manipulations including mutagenesis and construction of an SV40 vector (pGS7) to facilitate expression in COS-7 cells [30].

Generation of pGS7, pGS8, pGS11, pGS13

In order to express RD1–CAT chimeras in COS cells, the SV40 early promoter and SP6 promoter region were both isolated from the plasmid pSV.SPORT by digestion of the plasmid DNA with the restriction enzymes *Cla*I and *Xba*I. The 433 bp fragment generated was purified from a 1% low melting-point agarose gel and ligated into pGS4, which had been digested with the restriction enzymes *Nar*I and *Xba*I after removal of the 172 bp digestion product by electrophoresis. The resultant construct was called pGS7 (Figure 1). Two further plasmids encoding 'in frame' chimeras between RD1 fragments placed at the 5'-end of

CAT were generated. For these, distinct PCR primer pairs were used instead of GSOL12 and GSOL15, in the procedure described above. Thus the plasmid pGS11, containing the fragment 26–100RD1, was generated using the primers GSOL24 and GSOL15, which yielded a 241 bp PCR fragment. The plasmid pGS13, containing the fragment 1–25RD1, was generated using the primers GSOL12 and GSOL13, which yielded a 79 bp PCR fragment. A control plasmid that contained the CAT gene, but without any RD1 sequences, was constructed by digestion of pGS7 with the restriction enzymes *Sal*I and *Xho*I to remove the RD1 fragment. Following gel purification to remove the 330 bp digestion product, the *Sal*I/*Xho*I-cut vector was religated to generate the vector pGS8 (Figure 1).

Expression in COS-7 cells

The SV40 transformed monkey kidney cell line COS-7 was propagated in an atmosphere of 5% CO₂ at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% v/v penicillin/streptomycin (100 units/ml), 20 mM L-glutamine and 10% v/v fetal calf serum. Cells were plated on 10 cm plates and grown to 75% confluency before transfection. DNA (7 µg) was added to the cells in serum-free DMEM containing 50 mM Tris-HCl (pH 7.3), 20 mM Hepes (pH 7.2) and 750 µg/ml DEAE-dextran. The plates were incubated at 37 °C for 4 h. After aspiration of the transfection medium the cells were incubated in 25% glycerol/serum-free DMEM for 1 min at 25 °C. After rinsing the plates with serum-free DMEM, the cells were cultured at 37 °C for 48 h. Mock-transfected cells were treated as above but without DNA.

Assay of CAT activity

CAT activity was monitored essentially by the method of Seed and Sheen [31]. Briefly, after aspiration of the growth medium, the plates were washed with PBS before incubation in 1.5 ml of TEN buffer [40 mM Tris-HCl (pH 7.8)/10 mM EDTA (pH 8.0)/120 mM NaCl], at 25 °C for 5 min. The cells were scraped from the plate and harvested in an Eppendorf tube by centrifugation at 6000 *g* for 5 min at 4 °C. The pellets were resuspended in 100 µl of 10 mM Tris-HCl (pH 7.8)/0.2 mM MgCl₂ (TM buffer) and lysed by three cycles of liquid nitrogen (2 min)/37 °C (2 min). The lysate was spun at 350 *g* for 5 min at 4 °C and the supernatants aspirated to fresh tubes. The pellets were washed by resuspending in 100 µl of 40 mM Tris-HCl (pH 7.8)/10 mM EDTA (pH 8.0)/120 mM NaCl and centrifuging as above. Supernatants were pooled and re-centrifuged at 150000 *g* in a Beckman Airfuge for 20 min with the supernatants designated as S100. Both the low-speed (P1) and the high-speed (P100) pellets were resuspended in 25 mM Tris-HCl (pH 7.8)/5 mM EDTA (pH 8.0)/0.1 mM MgCl₂/60 mM NaCl. Duplicate samples of each fraction were assayed in 0.1 mM Tris-HCl (pH 7.8), 0.25 mM butyryl CoA and 0.2 µCi [¹⁴C]chloramphenicol in a final volume of 100 µl by incubating at 37 °C for 1 h. Reactions were stopped by the addition of 200 µl of tetramethylpentadecane/xylene (2:1, v/v) and vortexing for 15 s. The organic and aqueous phases were separated by centrifugation at 13000 *g* in a microfuge before the upper organic phase was transferred to scintillation vials. After addition of scintillation fluid, the samples were counted for 60 s in an LKB Wallac model 1409 liquid scintillation counter.

Since it was feasible that some transfected cells may not have been lysed during the freeze/thaw procedure, we assayed, using the method described by Houslay and Tipton [32], the pellet (P1) and high-speed supernatant (S100) fractions for the soluble enzyme lactate dehydrogenase (LDH). We observed that > 95%

of activity for this enzyme was found in the S100 fraction while the remaining activity was associated with the P1 fraction. Upon addition of Triton X-100 to the P1 fraction there was no increase in LDH activity, which would have indicated incomplete cell lysis, suggesting that our homogenization procedure had led to complete cell breakage.

Western-blot analysis of transfected COS cells

Cells were transfected as described above, harvested and the pellet and supernatant fractions containing 100 µg of protein were subjected to SDS/PAGE on a 10% gel [33]. Proteins were transferred to nitrocellulose membrane by incubation in 25 mM Tris-HCl/192 mM glycine/0.05% SDS/20% methanol at 1 A for 2 h. After blocking with 5% dried skimmed milk reconstituted in PBS for 45 min, the membrane was incubated overnight with a 1:500 dilution of a commercially available anti-CAT polyclonal antibody (5 Prime-3 Prime inc.) in PBS containing 1% dried skimmed milk/0.2% Nonidet P40/0.05% thimerosal. Proteins were detected by subsequent incubation of the blot with a 1:200 dilution of a horseradish peroxidase-conjugated secondary IgG antibody (SAPU) and visualization by incubation in ECL reagents (Amersham) for 1 min at room temperature, followed by exposure to either, or both, Hyperpaper-ECL (Amersham) and X-ray film.

Subcellular fractionation

This was done by a modification of the procedure described previously [13]. The COS-7 cells were harvested 48 h post-transfection, resuspended in a final volume of TEN buffer and disrupted by 40 strokes of the pestle in a Dounce homogenizer. A post-nuclear supernatant was prepared as before [13]. This was layered on top of a non-linear 0.5 M/1.6 M sucrose step-gradient and centrifuged at 50000 *g*_{av} for 1 h. Membranes were harvested from the interface and solid sucrose added to yield a concentration of 1.6 M. These were layered under a 0.6–1.6 M continuous sucrose gradient before centrifugation at 50000 *g* for 18 h. After this the gradient was separated into fractions of 180 µl which were taken for immediate analysis of CAT activity and the plasma membrane marker enzyme 5'-nucleotidase. The method of Newby et al. [34] was used to determine 5'-nucleotidase activity.

Cell-free expression of chimeras

A coupled transcription/translation system (Promega U.K.) was used essentially according to the manufacturer's instructions. Reaction mixtures were programmed with 1 µg of either pGS8, pGS7, pGS11 or pGS13 in the presence of [³⁵S]methionine and incubated at 30 °C for 90 min unless specified otherwise (see time-course experiments). Post-translation, duplicate 10 µl aliquots were treated by the addition of either 10 µl of TEN buffer (no added membranes) or 10 µl of TEN containing 0.27 mg/ml COS cell membranes (added membranes). Samples were incubated at 37 °C for 30 min and then centrifuged at 150000 *g* in a Beckman Airfuge for 20 min. Supernatants were removed to fresh tubes and pellets were washed by resuspension in 20 µl of TEN and re-centrifuged as above. The supernatants were removed and pooled with those from the first centrifugation and the pellets were finally resuspended in TEN containing 0.5% Triton X-100. All samples were subsequently adjusted to give equal volumes, salt and detergent concentrations before running on a 10% SDS-polyacrylamide gel against ¹⁴C-labelled molecular mass markers. Gels were visualized on a Fuji Bas 1000 phosphor-imager with MacBas software.

In experiments done to determine the effect of protease inhibitors on the coupled transcription/translation system, either 1 mg/ml soya bean trypsin inhibitor was added or a 'cocktail' of protease inhibitors (as added routinely by us to brain homogenates when determining PDE activity [14,15]); these were final concentrations of 0.1 mM phenylmethanesulphonyl fluoride, 2 mM benzamidine, 2 mM aprotinin, 2 mM pepstatin A, 2 mM leupeptin and 2 mM antipain.

The COS cell membranes used in this study were provided by those isolated from the post-nuclear supernatant as described by Shakur et al. [13]. Briefly, COS-7 cells were seeded onto 10 cm culture plates and grown to near confluency (> 75%). These were harvested by scraping and resuspending in TEN at room temperature for 5 min after which they were re-harvested by centrifugation at 6000 g_{av} for 5 min at 5 °C. They were then disrupted by three cycles of freeze/thawing in liquid nitrogen involving 2 min of freezing alternating with 2 min of incubation at room temperature. After this, a post-nuclear supernatant was generated by centrifugation at 350 g for 5 min. This post-nuclear supernatant was then centrifuged at 150000 g_{av} for 20 min on a cooled Beckman airfuge. The pellet was resuspended in TEN buffer. Similar results were obtained using membranes found at the interface of the 0.5 M/1.6 M sucrose step-gradient using the method described for subcellular fractionation (see above). Both sets of membranes contained plasma membranes as indicated by the presence of 5'-nucleotidase activity (this study and see [13]). In excess of 85% of cellular 5'-nucleotidase activity was found in these two membrane preparations.

RESULTS AND DISCUSSION

Upon transfection of COS cells with a plasmid encoding the type-IVA PDE RD1, around 85% of the enzyme was found to be membrane associated and requiring detergent to effect its

Figure 2 N-terminal domain of RD1

(a) The sequence of the first 100 amino acids of RD1. The plasmid pGS7 allowed for the expression, in COS-7 cells, of a chimera where these 100 amino acids were fused to the N-terminus of CAT, namely 1–100RD1–CAT. Also indicated is Met²⁶, where the N-terminal truncated species 26–100RD1–CAT was generated through the expression of plasmid pGS11, and Met³⁷, which, like Met²⁶, forms another potential site for the initiation of translation due to their being within sub-optimal Kozak sequences [37]. (b) The secondary structure of the membrane targeting 1–25 amino acid region of RD1 as predicted by the analytical procedures of Chou and Fasman [46]. These indicate two regions of putative α -helix, the first (α 2) extending from Leu³ to Thr¹⁰ and the second (α 2) extending from Gln²² to Arg²⁵. In full-length RD1, α 2 forms part of a much larger predicted α -helix. Sandwiched between these two putative α -helical domains are a putative region of turn (Cys¹¹–Pro¹⁴) and a putative region of β -sheet (Trp¹⁵–Asp²¹).

Table 1 Distribution of CAT activity in native- and chimeric-CAT transfected COS cells

COS cells were transfected with the indicated plasmid and then, after 48 h, membrane and high-speed supernatant fractions isolated as described in the Materials and methods section. All CAT assays were performed in duplicate as described in the Materials and methods section. The distribution between the membrane (pellet) and cytosol (high-speed supernatant) fractions are given as percentages. These data provide the mean of three separate transfection experiments done on different occasions with errors as S.E.M. No CAT activity was evident in COS cells which were mock-transfected with either the plasmids pSVL or pSPORT.

Plasmid	Construct	Membrane (%)	Cytosol (%)
pGS8	CAT	13 ± 2	87 ± 2
pGS7	1–100RD1–CAT	64 ± 3	36 ± 3
pGS11	26–100RD1–CAT	17 ± 2	83 ± 2
pGS13	1–25RD1–CAT	51 ± 3	49 ± 3

release [13]. The residual 'soluble' enzyme is now known to be due to an N-terminal truncated form, Met²⁶ RD1, formed by initiation occurring at Met²⁶ (I. McPhee and M. D. Houslay, unpublished work). The conferment of membrane association upon RD1 has been suggested [13] to be determined by a 25 amino acid N-terminal domain that is unique to this type IVA PDE (Figure 2a). To assess whether such a domain can confer membrane association upon a normally soluble enzyme we have generated various chimeras where this domain was fused to the N-terminus of the cytosolic bacterial enzyme, CAT.

The plasmid pBLCAT2 [26] contains the CAT gene, from the bacterial transposon Tn9, as well as the SV40 polyadenylation site in the high copy number vector pUC18 (Figure 1). We used this vector as a source of the CAT gene in our construction of novel plasmids. Our aim was to generate a variety of chimeric CAT molecules with additions fused to the N-terminus of this normally soluble enzyme and for these to be expressed in COS-7 cells. To do this we first replaced (Figure 1) the *tk* promoter with a 321 bp PCR fragment encoding amino acids 1–100 of the type IV cAMP phosphodiesterase RD1 (Figure 2a). The resultant plasmid, called pGS4, contained the 1–100RD–CAT construct as an in-frame fusion. The fidelity of this fragment was confirmed by Taq cycle sequencing. This vector was then modified further by subcloning into it, from the eukaryotic expression vector pSV.SPORT1, the SV40 early promoter so as to enable expression in COS-7 cells, and also the SP6 promoter, so as to allow use in an *in vitro* transcription/translation system. The resultant plasmid was designated pGS7 (Figure 1).

It has been shown [13] that deletion of the first 25 amino acids from RD1 produced an active enzyme (Met²⁶ RD1) which was expressed exclusively in the cytosol of transfected COS cells. We also generated the equivalent chimera with amino acids 26–100 of RD1 fused to the N-terminus of CAT, using the construct pGS11. Furthermore, to determine whether the first 25 amino acids were themselves sufficient to confer membrane association upon CAT, we generated a chimera with amino acids 1–25 of RD1 fused to CAT, designating this construct pGS13. We also constructed a plasmid, called pGS8, in which native CAT could be expressed in COS-7 cells.

Expression of RD–CAT fusion chimeras in COS-7 cells

Upon transfection of COS-7 cells with our novel plasmids, we observed the expression of CAT activity (Table 1). In typical transfection experiments we observed of the order of 1–10 units of CAT activity per μ l of cell homogenate (1.5–2.0 c.p.m. $\times 10^{-4}$; range $n = 5$ separate transfections).

Figure 3 Effect of Triton X-100 concentration on membrane association of 1-100RD1-CAT

Membranes were used from COS-7 cells which had been transfected with the plasmid pGS7 in order to express the chimera 1-100RD1-CAT. These were incubated at 0 °C for 30 min in the presence of the indicated concentrations of the non-ionic detergent Triton X-100. High-speed pellet and supernatant fractions were prepared by centrifugation (see Materials and methods section) and CAT activity determined in each of them. The data shown are typical of one experiment done three times using membranes from separate transfection experiments.

For native CAT, expressed using the plasmid pGS8, the majority of CAT activity was clearly found in the high-speed supernatant, cytosol fraction (Table 1). This is consistent with data reported by Seed and Sheen [31] using a different CAT plasmid. In marked contrast to this, when COS cells were transfected with the plasmid pGS7, to produce the chimera 1-100RD1-CAT, a profound shift in the distribution of activity was seen such that the major fraction of CAT activity now occurred in the membrane fraction (Table 1). This distribution reflects that of the type-IVA PDE RD1 where, in various transfection experiments, around 85 % of this enzyme was found to be membrane associated [13]. However, using pGS11, which encoded the chimera 26-100RD1-CAT, we found that the distribution of CAT activity paralleled that of native CAT itself in that CAT activity now predominated in the cytosol fraction (Table 1). Such data show that fusion of the N-terminal 100 amino acids of RD1 to CAT can confer membrane association on a normally soluble enzyme, in this instance CAT. That 26-100RD1-CAT demonstrated an identical distribution with soluble native CAT is consistent with previous studies [13] done on the analogous truncation for RD1 (Met²⁶RD1). Thus the domain formed from the first 25 amino acids of RD1 appears to provide the information necessary for conferring membrane association on a soluble protein.

In order then to assess whether the 25 amino acid domain of RD1 itself would suffice to confer membrane association on CAT, we analysed the distribution of the chimera 1-25RD1-CAT in COS cells transfected with the plasmid pGS13. This chimera also showed a marked increase in membrane association when compared with either native CAT or the 26-100RD1-CAT chimera (Table 1). However, from these analyses, where distribution was assessed using CAT activity, this 25 amino acid segment of RD1 appeared to be less efficient in conferring membrane association on CAT than was seen for the longer extension found in the 1-100RD1-CAT chimera (Table 1).

As with RD1 [13], we were able to show that treatment of membranes with either high NaCl concentrations (200 mM–1 M) or the chelating agent EDTA (10 mM) failed to effect the

Figure 4 Subcellular analysis

A post-nuclear membrane preparation of COS-7 cells which had been transfected so as to express the 1-25RD1-CAT chimera was subjected to centrifugation on a continuous sucrose density gradient (see Materials and methods section) under conditions shown previously to resolve cellular membranes [13]. This showed that the CAT activity of the chimera (□) co-migrated with that of the plasma membrane marker 5'-nucleotidase (●). This shows results from a typical experiment done twice.

solubilization of CAT activity provided by either the 1-100RD1-CAT or the 1-25RD1-CAT chimera (< 5 % release; $n = 3$ separate experiments). However, we noted that the non-ionic detergent Triton X-100 did indeed render the membrane-associated CAT activity of these chimeras soluble. This is shown in Figure 3 for the chimera 1-100RD1-CAT where Triton X-100 solubilizes the enzyme in a dose-dependent fashion. As with RD1 [13], only very low concentrations of this detergent were needed to achieve solubilization (Figure 3). In a similar fashion also to RD1 [13], subcellular fractionation of COS-7 cells, transfected so as to express the 1-25RD1-CAT chimera (Figure 4), showed that the membrane-bound CAT activity co-migrated with that of the plasma membrane marker 5'-nucleotidase.

Western-blot analysis of chimeras expressed in COS cells

An independent assessment of the ability of CAT and the various chimeras to distribute selectively between the cytosol and membrane fractions was obtained from Western blotting experiments (Figure 5). In these experiments both the pellet and high-speed supernatant fractions from transfected cells were subjected to SDS/PAGE with subsequent analysis by immunoblotting using an anti-CAT antibody. In cells transfected with pGS8 we were able to identify (Figure 5, lanes 1 and 2) an immunoreactive species of ~ 26 kDa, which is as expected for native CAT [31]. This species was found predominantly (85–91 %, range; $n = 3$ separate experiments) in the supernatant fraction (Figure 5, lane 2) thus showing that native CAT is expressed in cells as an essentially soluble protein, as indicated from the experiments done using CAT assays (Table 1) and of the size reported previously by others [31]. In contrast to this, with cells that had been transfected with the plasmid pGS13 to generate the 1-25RD1-CAT chimera, we noted (Figure 5, lane 3) two major protein species of ~ 30 and ~ 26 kDa being produced. Whilst the ~ 30 kDa component is of the size predicted for the chimera 1-25RD1-CAT, the species at ~ 26 kDa is as expected for native CAT. This suggests to us that both the 1-25RD1-CAT chimera

Figure 5 Western-blot analysis of COS cells transfected with CAT-containing plasmids

After SDS/PAGE of samples, they were transferred to nitrocellulose and then probed with an anti-CAT antibody. Here, immunoblots were visualized by enhanced chemiluminescence (ECL, Amersham, U.K.). Native CAT is shown in lanes 1 and 2 and 1-25RD1-CAT in lanes 3 and 4. Samples in lanes 2 and 4 are from membranes and those in lanes 1 and 3 are cytosolic high-speed supernatant fractions. The size of the single band found after transfection with the plasmid pGS8 was 25.6 ± 0.6 kDa (lanes 1 and 2). The size of the upper band (1-25RD1-CAT) found in cells transfected with the plasmid pGS13 was 30.3 ± 0.9 kDa and the lower band, presumably native CAT, was 25.8 ± 0.8 kDa. These results are typical of one experiment done three times.

and native CAT were simultaneously expressed in COS cells from this plasmid. Consistent with the ~ 26 kDa band being native CAT, we observed that it migrated identically with the single species found in COS cells transfected with pGS8 and was primarily associated with the cytosol fraction (Figure 5, lane 4). In profound contrast to this, we observed (Figure 5, lane 3) that the ~ 30 kDa band, corresponding to 1-25RD1-CAT, was exclusively associated with the membrane fraction. This shows that fusion of the N-terminal 25 amino acid domain of RD1 does indeed confer membrane association on CAT with high efficiency.

These experiments also highlight potential problems which can arise from using CAT activity as the sole detection mechanism for analysing the chimeric species. Our experiments indicate that initiation within the plasmid pGS13 occurred at two sites, one of which produced the chimera 1-25RD1-CAT and the other native CAT. This explains why the distribution of CAT activity observed in cells transfected with the plasmid for this chimera (Table 1) showed considerable soluble, cytosolic CAT activity. From the Western-blot experiments it would seem that this soluble activity is due essentially to native CAT as we could identify little if any evidence of the occurrence of 1-25RD1-CAT in the cytosol. Thus analysis of the CAT activity data given in Table 1 will give a considerable underestimate of the fraction of the chimera 1-25RD1-CAT that is membrane associated.

Cell-free expression of chimeras

To determine whether mature chimeras of 1-100RD1-CAT and 1-25RD1-CAT would be capable of subsequently associating with membranes, we utilized a cell-free, coupled transcription/translation system. Such systems use plasmid DNA, rather than RNA, as a template for protein production and have the feature that they are prone to multiple initiation events [35,36]. We have exploited this characteristic promiscuity to generate, in the same experiment, both full-length and N-terminal-truncated chimeric proteins from our plasmid constructs. The production of multiple chimeric proteins with various N-terminal truncations can thus serve as internal controls for determining which species allow membrane association in the same experimental mixture. Figure 6(a) shows a typical experiment where, in lane 1, we used the plasmid pGS8 containing the native CAT gene, which yielded in

Figure 6 Cell-free expression of various RD1-CAT chimeras

The plasmids generated in this study were used to prime a coupled transcription/translation system to produce mature chimeric CAT species in an system *in vitro*. This was done in the presence of [35 S]methionine to produce radioactive products. In (a), after transcription/translation a portion of the products of the reaction was kept for analysis and the rest incubated with COS cell membranes as described in the Materials and methods section. After incubation a high-speed supernatant (soluble) fraction and a membrane fraction were isolated. These two fractions, and that of the original product of the transcription/translation reaction, were subjected to SDS/PAGE. Identification of the radioactive species was done using a phosphorimager. Gel lanes were loaded such that the amount of the original transcription/translation product analysed was identical with that found in the combined membrane and soluble lanes. Thus lanes analysing particular plasmids can be compared directly. Lanes 1-3 represent native CAT provided by the plasmid pGS8; lanes 4-6 represent 1-100RD1-CAT provided by the plasmid pGS7; lanes 7-9 represent 26-100RD1-CAT provided by the plasmid pGS11; lanes 10-12 represent 1-25RD1-CAT provided by the plasmid pGS13. The lanes are in sets of three, where the first track shows the original products of the transcription/translation reaction, the second the membrane (high-speed pellet) fraction and the third the soluble (high-speed supernatant) fraction. Thus lanes 1, 4, 7 and 10 show the original material emanating from the transcription/translation experiment; lanes 2, 5, 8 and 11 the high-speed pellets after incubation in the presence of COS membranes (0.27 mg/ml protein) and lanes 3, 6, 9 and 12 the high-speed supernatants obtained after centrifugation of the incubation mixture done in the presence of COS membranes. The predicted products with initiation beginning at the indicated Met residues are shown. The sizes of these species are given in the text. These data are typical of an experiment done three times. In (b) the transcription/translation reaction was performed for the various times indicated using the plasmid pGS7 (1-100RD1-CAT) as a template. After the designated time aliquots of the reaction mixture were immediately boiled in Laemmli [33] sample buffer. These were then subjected to SDS/PAGE and the radioactive species identified using a phosphorimager. Gel lanes were loaded such that equal amounts of the transcription/translation reaction mixture were analysed. The levels of radioactivity associated with the three predominant bands which were observed (see a, lane 4) are shown. These species were: at ~ 38 kDa (■), presumed to be 1-100RD1-CAT; at ~ 35 kDa (○), presumed to be 26-100RD1-CAT; and at ~ 33 kDa (▲), presumed to be 37-100RD1-CAT. These data are typical of an experiment done twice.

this system a single species of ~ 26 kDa, corresponding to that expected for native CAT (25.5 kDa; [31]). Subsequent incubation of this product with COS cell membranes, followed by separation of pellet (Figure 6a, lane 2) and supernatant (Figure 6a, lane 3) fractions, failed to show membrane association of native CAT. However, when the plasmid pGS7 was expressed in the system *in vitro*, three predominant bands were observed of ~ 38 , ~ 35 and ~ 33 kDa (Figure 6a, lane 4). These are of the calculated sizes

for initiation events occurring not only at the first Met start codon (38.6 kDa) but also at Met²⁶ (35.5 kDa) and Met³⁷ (34.2 kDa) which provide the next two available Met residues. Initiation at these three sites would be expected to yield the full-length 1–100RD1–CAT chimera together with 26–100RD1–CAT and 37–100RD1–CAT. Additionally, several other minor bands were noted which might reflect other initiation events in the reaction (Figure 6a, lane 4). However, using the mixture of mature chimeric CAT species formed in this synthetic system, we noted that only the 1–100RD1–CAT chimera became membrane associated (Figure 6a, lane 5) and thus depleted from the soluble fraction (Figure 6a, lane 6). In marked contrast to this, the intensity of the ~35 and ~33 kDa bands (Figure 6a, lanes 4 and 6), due to 26–100RD1–CAT and 37–100RD1–CAT, respectively, remained identical in both the initial synthesis mix and the supernatant from membrane incubation. Thus these truncated species showed no evidence of membrane association. In confirmation that these two truncated species did not associate with membranes, we used the plasmid pGS11, which not only produced in this system a ~35 kDa species reflecting 26–100RD1–CAT but also a ~33 kDa species that is presumably the chimera 37–100RD1–CAT and also native CAT itself at ~26 kDa (Figure 6a, lane 7). None of these species became associated with the membrane fraction (Figure 6, lane 8) with both remaining exclusively in the supernatant fraction (Figure 6a, lane 9). The failure of both the 26–100RD1–CAT chimera and the 37–100RD1–CAT chimera to become membrane associated also indicates that merely adding additional amino acid residues to the N-terminus of CAT does not confer upon this protein the ability to become membrane associated.

Using the plasmid pGS13, which encodes a chimera formed from the first 25 amino acids of RD1 fused to CAT, we saw two major bands. These were a species of ~30 kDa, which would be predicted to reflect the chimera 1–25RD1–CAT, and a further band of ~26 kDa, which would be predicted to be native CAT, as this provides the next available Met codon (Figure 6a, lane 10). Lane 11 (Figure 6a) shows that only the 1–25RD1–CAT chimera became associated with the membrane pellet fraction and depleted in the soluble fraction (Figure 6a, lane 12) whereas the native CAT species remained exclusively in the soluble fraction (Figure 6a, lane 12).

Multiple transcription can occur in the combined transcription/translation system [35,36] and we believe that such an event provides the most plausible explanation for the production of multiple products by this system. Certainly the species observed upon SDS/PAGE migrate with sizes consistent with the start of transcription occurring at the indicated Met residues. This is exemplified by, for example, the observed co-migration of the first product (Figure 6a, lane 7) from the plasmid pGS11, namely 26–100RD1–CAT, with the second product (Figure 6a, lane 4) from the 1–100RD1–CAT-containing plasmid pGS7, which would be predicted to produce 26–100RD1–CAT due to a second start occurring at Met²⁶. Indeed, analysis of the nucleotide sequence surrounding these Met residues indicates that they do in fact provide sub-optimal Kozak sequences [37]. Thus, for the initiation of transcription, Kozak [37] has identified an optimal sequence of ACCATGG, with sub-optimal sequences being those with a purine at position –3 to the ATG codon. From inspection of the sequence of RD1 [27], an optimal start can be found at Met¹ (AGAATGC), which leads to the production of the predicted full-length species in both COS cells transfected so as to express RD1 [13]. By immunoblotting using a specific anti-serum, a species of this size can also be found in brain membranes and has been shown to reflect native RD1 [14]. However, sub-optimal Kozak sequences [37] are also evident at both Met²⁶

(AGGATGC) and at Met³⁷ (GAAATGA), for example. This can be expected to heighten the chances of translation products occurring at these points in the transcription/translation system *in vitro* used here. Additionally, it may also explain why multiple species were seen in the COS cell expression studies using our RD1–CAT chimera (see Figure 5). For example, analysis of the products of the plasmid pGS13 showed that in both COS cells (Figure 5) and the transcription/translation system *in vitro* (Figure 6a, lane 10), we observed two products, namely the 1–25RD1–CAT chimera and also native CAT.

We thus believe that it is extremely unlikely that proteolysis leads to the production of the multiple species formed in this system. However, in order to try and address the question of possible proteolysis we employed various strategies. These included employing a cocktail of protease inhibitors, as used routinely by us when isolating PDEs (see for example [13]) in the reaction using the plasmid pGS7. However, these compounds prevented product formation by the transcription/translation system *in vitro* (results not shown). In contrast, addition of soybean trypsin inhibitor, at concentrations of up to 1 mg/ml, allowed for a pattern of product formation (results not shown) which was identical with that seen in its absence (Figure 6a, lane 4). However, as the targets for action of trypsin inhibitor are proscribed, this observation cannot be used to eliminate protease action. If, however, the smaller species found using, for example, the plasmid pGS7 were due to proteolysis then a time-course of the production of these species might be expected to follow a product precursor relationship, with 1–100RD1–CAT being produced initially. In contrast, we observed (Figure 6b) that the time-course of production of the three products examined showed great similarity in both the rate of production and the time at which a maximum yield was produced. Routinely we performed analyses after 90 min of reaction (see Materials and methods section). However, here we can see (Figure 6b) that the absolute levels and the proportions of all three products remained stable after even some 240 min of reaction. Additionally, it is evident that the addition of the crude membrane fraction did not alter the relative proportions of the species produced in the transcription/translation system *in vitro*. For example, the relative amounts of three products of plasmid pGS11, presumed to be 26–100RD1–CAT, 37–100RD1–CAT and CAT itself, appear identical upon analysis of both the products of the transcription/translation system *in vitro* (Figure 6a, lane 7) and also in the supernatant fraction after incubation with membranes (Figure 6a, lane 9). Furthermore, any proteolysis occurring exclusively at the C-terminal end of the 1–100RD1–CAT chimera, whilst producing smaller species, would not be expected to prevent membrane association as it is the N-terminal domain which is essential for membrane binding. However, in no instance did we see species smaller than that of the mature 1–100RD1–CAT chimera associating with membranes (Figure 6, lane 5). Of course proteolysis at the N-terminus might be expected to disrupt or delete such a membrane targeting domain and lead to the production of soluble species.

We believe, however, that such time-course data, together with the observations that Met²⁶ and Met³⁷ in RD1 provide sub-optimal Kozak sequences [37] and that the transcription/translation system *in vitro* is susceptible to multiple initiation events [35,36], make multiple initiation the most probable explanation for the multiple products produced by the transcription/translation system *in vitro* in this study (Figure 6).

CONCLUSION

cAMP phosphodiesterases show high similarity within a central

domain that is believed to encompass their active site. This degree of homology is particularly high within specific sub-families. However, there is little or no similarity with sequences found at their extreme N- and C-termini. It has been suggested, in the case of the type-IVA PDE, RD1 [13] and in the case of the type-IVB enzyme, PDE-4 [15], that their respective N-terminal domains confer membrane association.

The extreme N-terminal domain of RD1 comprises a sequence of 25 amino acids that is unique amongst all PDEs that have been cloned to date. Deletion of this domain by engineered truncation has been shown to render truncated RD1 (Met²⁶ RD1) a fully soluble enzyme that is found as an active species in the cytosol of transfected COS cells [13,14]. The contention that this domain can confer membrane association upon an essentially soluble protein [13] has been tested here using chimeric constructs formed with the soluble, bacterial enzyme CAT. Expression in COS cells clearly shows that this domain is sufficient to confer membrane-association upon CAT. Not only that, but by using a transcription/translation system *in vitro* we have also demonstrated that mature chimeras having the N-terminal 25 amino acids of RD1 fused to CAT are capable of subsequent association with membranes. Thus the N-terminal 25 amino acids of RD1 contain structural information that allows for membrane association even after biosynthesis of the mature protein.

How then might this domain confer membrane association upon a soluble protein? Certainly in the case of RD1 we do not consider it likely that it serves as a cleavable N-terminal signal sequence. As discussed before [13] there are a number of reasons for this. These include: (1) the lack of evidence for a putative hydrophobic transmembrane domain occurring in RD1; (2) that the engineered deletion of amino acids 1–25 produces a fully soluble enzyme [13] whose activity is some 2-fold greater than that of native RD1 [14], rather than an inactive enzyme which might be expected to ensue due to incorrect folding of a transmembrane integral protein with a cleaved signal sequence; (3) that membrane-bound RD1 in both transfected COS cells [13] and in brain [14] is greater in size than Met²⁶RD1; (4) that the membrane-associating 1–25RD1–CAT and 1–100RD1–CAT chimeras are of the predicted size; and (5) that these two chimeras can be synthesized *in vitro* to yield mature products which associate selectively with membranes. However, in some instances N-terminal signal sequences are not cleaved but can themselves provide membrane anchors [38]. Unfortunately there is a low degree of sequence conservation between signal sequences [38]. However, certain features of secondary structure appear to be common. These take the form of three distinct domains: at the extreme N-terminus is a positively charged region of 1–5 residues, followed by a central hydrophobic domain of 7–15 residues, and then a more polar C-terminal domain of 3–7 residues which, if applicable, contains the site for cleavage by the signal peptidase. Inspection, however, of the 1–25 amino acid N-terminal domain of RD1 (Figure 2b) shows an absence of the characteristic positively charged polar sequence at the extreme N-terminus. Furthermore, although there are distinct hydrophobic regions within this domain, such as from Leu³ to Phe⁷ and from Trp¹⁵ to Trp²⁰, neither is of a sufficient size to form a membrane-spanning anchor (Figure 2b). The N-terminal 1–25 amino acid domain is an amphipathic structure and it is possible that it could fold to yield a hydrophobic loop able to partition into the lipid bilayer in a similar fashion to that proposed [38] for the initial insertion of the hydrophobic domain of anchoring signal peptides. Certainly such a mode of attachment would be consistent with our observations that membrane-bound RD1–CAT chimeras, and RD1 itself [13,14], are solubilized only with Triton X-100 and not by high ionic strength, implying that ionic interactions

do not solely, if at all, determine the anchoring reaction. However, that both RD1 [13,14] and the RD1–CAT chimeras appear to show specificity as regards membrane association, with preferential association with the plasma membrane, militates against a simple partition of this anchor into the lipid milieu as either part of, or the sole means of, membrane association. Rather it suggests that this N-terminal domain associates with a specific membrane protein whose cellular location, in this case the plasma membrane, is then conferred upon RD1. Thus association of the N-terminal domain of RD1 with such a protein may provide either the sole means of membrane attachment or a means of targeting to a particular cellular membrane in association with any integration of the anchor into the bilayer lipid. For example, this domain might form a hairpin structure with the extreme C-terminal Lys²⁴–Arg²⁵ interacting with acidic phospholipids at the cytosol surface of the plasma membrane, the major hydrophobic domain between Trp¹⁵ and Phe²³ interfacing with the apolar core of the bilayer, and the Met¹–Lys¹³ interfacing with an integral membrane protein (Figure 2b). Certainly the 1–25 amino acid domain of RD1 appears to have the potential for forming distinct secondary structural features (Figure 2b) which could interact with an appropriate anchoring protein. These include a putative α -helical domain which is circumscribed by prolines (Pro², Pro¹⁴; Figure 2b), a predicted region of turn and a hydrophobic, tryptophan-rich (Trp¹⁵, Trp¹⁹, Trp²⁰) region which is predicted to lie within a β -sheet. In this regard, it is of interest that the Trp–Asp motif is thought to define regions involved in determining protein–protein interaction [39] and such a motif is found at the C-terminal end of this tryptophan-rich region. Indeed such ‘WD’ domains are found in G-protein β -subunits, which are also essentially soluble proteins found anchored to plasma membranes and which show similar punctate patterns of expression [40] in the plasma membrane to RD1 [14]. However, in G-protein β -subunits, and related proteins, such domains are often found in repeated units [39]. Whilst interaction with the membrane may involve domains with major secondary structural features, protein–protein interactions can occur through relatively small epitopes. Thus, for example, targeting to peroxisomes and Golgi retention can be achieved by extremely small epitopes of just a few amino acids [41,42]. It is thus possible that anchoring could be achieved through a small epitope within the 1–25 amino acid domain of RD1. The fact that solubilization is dependent upon low concentrations of detergent, is insensitive to high ionic strength and that association is clearly of extremely high affinity, as neither RD1 nor these CAT chimeras are released by repeated washing, suggests that attachment is likely to be determined primarily through hydrophobic interactions.

One means of attachment of essentially soluble proteins to membranes using hydrophobic interaction is via N-terminal acylation using either myristoyl [43] or palmitoyl [44] residues. However, as there is no putative myristoylation site (glycine) within this N-terminal region, the only other possibility is palmitoylation of cysteine residues. However, this mode of attachment is extremely unlikely as efficient membrane association occurred using both the 1–25RD1–CAT and 1–100RD1–CAT chimeras that had been synthesized in the cell-free system described here. Furthermore, not only was labelled palmitate not incorporated into COS cell-expressed RD1, but hydroxylamine, which causes the release of covalently associated palmitate [45], was unable to cause the release of RD1 from either cerebellum or COS membranes [14].

The type IVA PDE RD1 appears then to possess information contained within the unique domain formed from the first 25 amino acids of the protein that allows it to become membrane

associated. That deletion of this domain from RD1 produces a fully active soluble enzyme [13,14] and that this domain can confer membrane association upon CAT suggests that RD1 is formed from a core-soluble protein together with an N-terminal anchoring domain. It is our contention that RD1, through this N-terminal domain, becomes membrane-anchored by interacting with a membrane-associated protein via an interaction which is predominantly hydrophobic in nature. Such a specific targeting may be intimately related to the biological functioning of this particular type-IVA PDE splice variant.

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REFERENCES

- Houslay, M. D. and Kilgour, E. (1990) Wiley Ser. Mol. Pharmacol. Cell Regul. **2**, 185–226
- Beavo, J. A. (1990) Wiley Ser. Mol. Pharmacol. Cell Regul. **2**, 3–15
- Bolger, G. B. (1994) Cell. Signalling **6**, 851–860
- Conti, M. and Swinnen, J. V. (1990) Wiley Ser. Mol. Pharmacol. Cell Regul. **2**, 243–266
- Sullivan, M., Egerton, M., Shakur, Y., Marquardsen, A. and Houslay, M. D. (1994) Cell. Signalling **6**, 793–812
- Wilson, M., Sullivan, M., Brown, N. and Houslay, M. D. (1995) Biochem. J. **304**, 407–415
- Monaco, L., Vicini, E. and Conti, M. (1994) J. Biol. Chem. **269**, 347–357
- Beavo, J. A., Conti, M. and Heaslip, R. J. (1994) Mol. Endocrinol. **46**, 399–405
- Bolger, G. B., Michaeli, T., Martins, T., St. John, T., Steiner, B., Rodgers, L., Riggs, M., Wigler, M. and Ferguson, K. (1993) Mol. Cell. Biol. **13**, 6558–6571
- Houslay, M. D. and Lobban, M. (1994) FASEB J. **8**, A369 (abstr 2134)
- Bolger, G. B., Rodgers, L. and Riggs, M. (1994) Gene **149**, 237–244
- Jin, S. L., Swinnen, J. V. and Conti, M. (1992) J. Biol. Chem. **267**, 18929–18939
- Shakur, Y., Pryde, J. G. and Houslay, M. D. (1993) Biochem. J. **292**, 677–686
- Shakur, Y., Wilson, M., Pooley, L., Lobban, M., Griffiths, S. L., Campbell, A. M., Beattie, J., Daly, C. and Houslay, M. D. (1995) Biochem. J. **306**, 801–809
- Lobban, M., Shakur, Y., Beattie, J. and Houslay, M. D. (1994) Biochem. J. **304**, 399–406
- Houslay, M. D. (1995) Adv. Enzyme. Regul. **35**, in the press
- Houslay, M. D., Scotland, G., Pooley, L. et al. (1995) Biochem. Soc. Trans., **23**, 393–398
- Sette, C., Vicini, E. and Conti, M. (1994) J. Biol. Chem. **269**, 18271–18274
- Pyne, N. J., Cooper, M. E. and Houslay, M. D. (1986) Biochem. J. **234**, 325–334
- Manganiello, V. C., Smith, C. J., Degerman, E. and Belfrage, P. (1990) Wiley Ser. Mol. Pharmacol. Cell Regul. **2**, 87–116
- Marchmont, R. J., Ayad, S. R. and Houslay, M. D. (1981) Biochem. J. **195**, 645–652
- Reeves, M. L., Leigh, B. K. and England, P. J. (1987) Biochem. J. **241**, 535–541
- Marchmont, R. J. and M. D. Houslay (1980) Nature (London) **286**, 904–906
- Meacci, E., Taira, M., Moos, M., Jr., Smith, C. J., Movsesian, M. A., Degerman, E., Belfrage, P. and Manganiello, V. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 3721–3725
- Pyne, N. J., Cooper, M. E. and Houslay, M. D. (1987) Biochem. J. **242**, 33–42
- Kilgour, E., Anderson, N. G. and Houslay, M. D. (1989) Biochem. J. **260**, 27–36
- Davis, R. L., Takayasu, H., Eberwine, M., Myres, J. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3604–3608
- Luckow, B. and Schutz, G. (1987) Nucleic Acids Res. **15**, 5490–5493
- Sheffield, V. C., Cox, D. R., Lerman, L. S. and Myers, R. M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 232–236
- Gluzman, Y. (1981) Cell **23**, 175–182
- Seed, B. and Sheen, J.-Y. (1988) Gene **67**, 271–277
- Houslay, M. D. and Tipton, K. F. (1973) Biochem. J. **135**, 175–186
- Laemmli, U. K. (1970) Nature (London) **227**, 680–685
- Newby, A. C., Luzio, J. P. and Hales, C. N. (1975) Biochem. J. **146**, 625–633
- Craig, D., Howell, M. T., Gibbs, C. L., Hunt, T. and Jackson, R. J. (1992) Nucleic Acids Res. **20**, 4987–4995
- Promega (1993) Protein Guide: Tips and Techniques, Promega Corporation
- Kozak, M. (1986) Cell **44**, 283–292
- von Heijne, G. (1990) J. Membr. Biol. **115**, 195–201
- Neer, E. J., Schmidt, C. J., Nambudripad, R. and Smith, T. F. (1994) Nature (London) **371**, 297–300
- Wang, H., Berrios, M. and Malbon, C. (1989) Biochem. J. **263**, 519–532
- Gould, S. J., Keller, G. A. and Subramani, S. (1989) J. Cell Biol. **109**, 1657–1664
- Janiak, F., Glover, J. R., Leber, B., Rachubinski, R. A. and Andrews, D. W. (1994) Biochem. J. **300**, 191–199
- McIlhinney, R. A. J., Chadwick, J. K. and Pelly, S. J. (1987) Biochem. J. **244**, 109–115
- Spiegel, A. S., Backlund, P. S., Butrynski, J. E., Jones, T. L. Z. and Simonds, W. F. (1991) Trends Biochem. Sci. **16**, 338–341
- Magee, A. I. and Courtneidge, S. A. (1985) EMBO J. **4**, 1137–1144
- Chou, P. Y. and Fasman, G. D. (1978) Annu. Rev. Biochem. **47**, 251–276