Identification and characterization of the type-IVA cyclic AMP-specific phosphodiesterase RD1 as a membrane-bound protein expressed in cerebellum

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An antiserum was generated against a dodecapeptide whose sequence is found at the C-terminus of a cyclic AMP (cAMP)-specific, type-IVA phosphodiesterase encoded by the rat '*dunc*-like' cyclic AMP phosphodiesterase (RD1) cDNA. This antiserum identified a single ~ 73 kDa protein species upon immunoblotting of cerebellum homogenates. This species co-migrated upon SDS/PAGE with a single immunoreactive species observed in COS cells transfected with the cDNA for RD1. Native RD1 in cerebellum was found to be predominantly (~ 93%) membrane-associated and could be found in isolated synaptosome populations, in particular those enriched in post-synaptic densities. Fractionation of lysed synaptosomes on sucrose density gradients identified RD1 as co-migrating with the plasma membrane marker 5'-nucleotidase. Laser scanning confocal and digital deconvolution immunofluorescence studies done on intact COS cells transfected with RD1 cDNA showed RD1 to be predominantly localized to plasma membranes but also associated with the Golgi apparatus and intracellular vesicles. RD1-specific antisera immunoprecipitated phosphodiesterase activity from solubilized cerebellum membranes. This activity had the characteristics expected of the type-IV cAMP phosphodiesterase RD1 in that it was cAMP specific, exhibited a low K_m^{cAMP} of 2.3 μM, high sensitivity to inhibition by 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone (rolipram) (K_i ~ 0.7 μM) and was unaffected by Ca^{2+}/calmodulin and low concentrations of cyclic GMP. The phosphodiesterase activities of RD1 solubilized from both cerebellum and transfected COS cell membranes showed identical first-order thermal denaturation kinetics at 50 °C. Native RD1 from cerebellum was shown to be an integral protein in that it was solubilized using the non-ionic detergent Triton X-100 but not by either re-homogenization or high NaCl concentrations. The observation that hydroxylamine was unable to cause the release of RD1 from either cerebellum or COS membranes and that [^{3}H]palmitate was not incorporated into theRD1protein immunoprecipitated from COS cells transfected with RD1 cDNA, indicated that RD1 was not anchored by N-terminal acylation. The engineered deletion of the 25 residues forming the unique N-terminal domain of RD1 caused both a profound increase in its activity (~ 2-fold increase in $V_{max.}$) and a profound change in intracellular distribution. Thus, immunofluorescence studies identified the N-terminal truncated species as occurring exclusively in the cytosol of transfected COS cells. The cDNA for RD1 thus appears to encode a native full-length type-IVA phosphodiesterase that is expressed in cerebellum. The domain formed from the unique N-terminal 25 amino acids of RD1 serves both to constrain its activity and to act as a membrane anchor.

INTRODUCTION

The second messenger cyclic AMP (cAMP) is inactivated by hydrolysis to 5'-AMP by members of a family of cAMP phosphodiesterases (PDE) [1–3]. This diverse family of enzymes results from the presence of both multiple genes together with multiple splicing [2,4–7]. cAMP-hydrolysing PDEs exhibit distinct biochemical characteristics and can be divided into at least five functional classes: a type-I PDE whose activity is stimulated by Ca^{2+}/calmodulin (CaM), a type-II PDE whose hydrolysis of cAMP is stimulated by micromolar concentrations of cGMP, a type-III PDE which specifically hydrolyses cAMP in a fashion which is inhibited by micromolar concentrations of cGMP, a type-IV PDE that is cAMP specific and insensitive to cGMP and a type-VII enzyme that specifically hydrolyses cAMP and is insensitive not only to cGMP but also to inhibitors such as 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone (rolipram) and isobutylmethylxanthine (IBMX) [1,2,8]. Members of this diverse enzyme family are thus placed in a unique position where they are poised to tailor cAMP metabolism to the specific requirements of different cell types and are also able to integrate regulatory signals from the lipid, tyrosyl and NO signalling systems. In a number of cell types evidence has been presented which would imply the existence of compartmentalization or 'pools' of cAMP able to activate certain isoforms of protein kinase A selectively [9–15]. It has been suggested that a key feature involved in creating such functional compartments is the restriction of specific PDE activities within the cell through membrane as-

Abbreviations used: RD1, rat '*dunc*-like' cyclic AMP phosphodiesterase also referred to as either RPDE4A1B or rPDE-IV$_{A1}$ representing rat, type-IV cyclic AMP specific phosphodiesterase, isoform subfamily A, splice variant 1; PDE, cyclic AMP phosphodiesterase; IBMX, isobutylmethylxanthine; rolipram, 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone; met^{26}-RD1, is RD1 with the N-terminal 25 amino acids deleted; G_i, inhibitory G-protein controlling adenylate cyclase activity; cAMP, cyclic AMP; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; TCA, trichloroacetic acid; DMSO, dimethyl sulphoxide; KLH, keyhole limpet haemocyanin; ECL, enhanced chemiluminescence; LDH, lactate dehydrogenase; CaM, calmodulin.

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sociation [1,16]. Whilst both cytosolic and membrane-bound forms of type-II [17], -III [18] and -IV PDEs [16] have been identified (see also [1]), little is known concerning the molecular basis and significance that underlies the attachment and targeting of PDEs to intracellular membranes.

Rat '*dunc*-like' PDE (RD1) is a type-IVA PDE that was cloned by homology from a rat brain cDNA library using the drosophila *dunc* PDE cDNA as a probe [19]. Recently, we [16] have shown that upon transfection of COS-1 cells with RD1 cDNA a major fraction of the activity of this PDE was found to be membrane-associated. Whilst the product of this cDNA has been characterized upon transient expression in COS cells, there is no evidence to indicate whether such a form of PDE is expressed in brain. To address this we have generated a specific polyclonal anti-peptide antiserum against a dodecapeptide that reflects the C-terminus of RD1. We have used this antiserum to detect and characterize native RD1 in cerebellum as a type-IV PDE that is predominantly membrane-associated and can be found associated with synaptosomes. Additionally we show that the unique N-terminal domain of RD1 serves not only to allow association of this PDE to the plasma membrane but also to constrain its activity.

MATERIALS AND METHODS

The rat *dunc* cDNA for RD1 was kindly provided by Dr R. L. Davis (Baylor College of Medicine, Houston, TX, U.S.A.) Restriction enzymes, Dulbecco's modified Eagle's medium and fetal calf serum were from Gibco/BRL (Paisley, U.K.). Tris, Hepes, DEAE-dextran (500 kDa), cytochalasin B, benzamidine hydrochloride, phenylmethanesulphonyl fluoride (PMSF), aprotinin, pepstatin A, antipain, EDTA, EGTA, cAMP, cGMP, Dowex 1X8-400 (chloride form, 200–400 mesh), IBMX, snake venom (*Ophiophagus hannah*) and bovine brain CaM were from Sigma Chemical Co. (Poole, U.K.). [^{3}H]cAMP and [^{3}H]cGMP were from Amersham International (Amersham, U.K.). Leupeptin was from Peptide Research Foundation (distributed by Scientific Marketing Associates, London, U.K.). Dithiothreitol (DTT), Triton X-100 and Triton X-114 were from Boehringer (U.K.), Ltd. (Lewes, U.K.). Triethanolamine was from BDH (Glasgow, U.K.). Glycerol was from Fisons (Leicestershire, U.K.). Bradford reagent was from Bio-Rad (Hertfordshire, U.K.). Dimethyl sulphoxide (DMSO) was from Koch-Light Ltd. (Haverhill, U.K.). Rolipram was a gift from Schering Aktiengesellschaft (Berlin, Germany).

Generation of anti-RD1 antisera

A rabbit polyclonal antiserum was generated against a peptide corresponding to the last 12 amino acids of the RD1 protein [(C)-T-P-G-R-W-G-S-G-G-D-P-A]. The peptide was synthesized with a cysteine at the N-terminus to facilitate conjugation of the peptide to keyhole limpet haemocyanin (KLH). Conjugation was performed according to the procedure of Lerner [20].

Brain cerebellum homogenates

Intact brains were removed from 200–250 g male Sprague–Dawley rats immediately after sacrifice and the cerebellum was rapidly dissected. This was, immediately upon isolation, placed into ice-cold homogenization buffer and homogenized within a few minutes using eight strokes at full speed on a rotary homogenizer using a Teflon pestle and glass vessel. This procedure was done on ice using a 1:10 (w/v) dilution of sectioned brain and homogenization buffer. The homogenization buffer contained 20 mM Tris/HCl (final pH 7.2), 1 mM EDTA and 0.25 M sucrose together with a protease inhibitor cocktail of 0.1 mM PMSF, 2 mM benzamidine, 2 mM aprotinin, 2 mM pepstatin A, 2 mM leupeptin and 2 mM antipain. Each homogenate was centrifuged at 1000 $g_{av.}$ for 10 min. The supernatant was decanted and centrifuged at 100000 $g_{av.}$ for 1 h. This yielded a soluble fraction and a membrane pellet. The latter was resuspended in ice-cold homogenization buffer to the same volume as the soluble fraction. These fractions were either analysed immediately or 'snap-frozen' in 0.5–1.0 ml aliquots, using liquid N_2, and stored at −80 °C. Similar results were obtained in both instances. Frozen aliquots were not subject to re-freezing and frozen samples were not stored beyond 7 days.

Simple fractionation studies were also done with the homogenate being centrifuged at 1000 $g_{av.}$ for 10 min to yield a P1 pellet. The supernatant was decanted and re-centrifuged at 100000 $g_{av.}$ for 1 h to yield a P2 pellet together with a supernatant (S) fraction. The membrane pellets were each individually resuspended in ice-cold homogenization buffer and then analysed.

Treatment with high salt concentrations

Membranes (0.2 mg) from rat brain cerebellum (prepared as described above) were treated with KHEM buffer (50 mM KCl/50 mM Hepes KOH (pH 7.2)/10 mM EGTA/1.92 mM $MgCl_2$) containing a range of NaCl concentrations. The membranes were left on ice for 30 min at 4 °C before centrifugation at 100000 *g* for 1 h at 4 °C. The resulting pellet was resuspended in KHEM buffer containing the appropriate NaCl concentration and the pellet and supernatant fractions were analysed by Western blotting.

Solubilization with Triton X-100

Membranes (0.2 mg) from rat brain cerebellum were treated with KHEM buffer containing a range of Triton X-100 concentrations. The membranes were left on ice for 30 min at 4 °C before centrifugation at 100000 *g* for 1 h at 4 °C. The resulting pellet was resuspended in KHEM buffer containing the appropriate Triton X-100 concentration and the pellet and supernatant fractions were analysed by Western blotting.

Synaptosome preparation

Synaptosomes were prepared as described in Dunkley et al. [21] and Thorne et al. [22]. Briefly, cerebellum tissue (~ 1 g) was rapidly removed from freshly sacrificed adult male Sprague–Dawley rats and placed in ice-cold gradient buffer containing 0.32 M sucrose, 1 mM EDTA and 0.25 mM DTT, pH 7.4. The tissue was then roughly chopped and homogenized in ten volumes of gradient buffer using ten passes at 700 rev. min^{-1} (Janke & Kunkel IKA-Labortechnik RW20). The homogenate was then filtered through two layers of gauze before centrifugation at 1000 *g* for 10 min in a Beckman J21 centrifuge with a JA20 rotor. The post nuclear supernatant (S1) from this step was then loaded onto the step gradient as described [21]. After centrifugation, as described by Dunkley et al. [21], the opalescent material that collected at each of the four interfaces (F1–F4 inclusive), together with the pellet (F5) were collected and analysed. Occluded lactate dehydrogenase (LDH) activity was assessed in these fractions using Triton X-100 (2%) to cause synaptosome rupture with results similar to those described by Thorne et al. [22] being obtained. Thus fractions F1, F2 , F3 and F4 contain synaptosomes, as indicated by the presence of synapsin, receptors for nicotinic and muscarinic ligands and also the presence of the

enzyme acetylcholine esterase [21,22]. Thorne et al. [22] also showed that fraction F5 contained primarily mitochondria and nuclei and, consistent with this, we found no exposed or latent LDH activity in it.

Membranes from lysed synaptosomes were subjected to differential gradient centrifugation as described in [16] in order to resolve a plasma-membrane fraction. Lysis was done by first combining fractions 2, 3 and 4. The combined fractions were then diluted five times in lysis buffer (pH 8.1) containing 5 mM Tris/HCl and 50 μM $CaCl_2$. They were then left on ice for 45 min before homogenization and centrifugation at 500 $g_{av.}$ for 5 min.

The activity of the plasma membrane marker 5'-nucleotidase was assayed as described by the method of Newby et al. [23]. LDH activity was measured in 0.1 ml samples of the gradient fractions as described by Johnson [24] and modified by Marchbanks [25]. Other marker enzyme activities were assayed as described by Shakur et al. [16].

Immunoprecipitation

Membranes (1 mg of protein) were resuspended in 'immunoprecipitation buffer' (0.5% Triton X-100/10 mM EDTA/100 mM $NaH_2PO_4,2H_2O$/50 mM Hepes, pH 7.2) containing protease inhibitors (as added to the KHEM buffer). Antibody (25 μl) was added and the sample was mixed overnight at 4 °C. A 100 μl volume of 10% Pansorbin (Calbiochem) was added and the sample was mixed at 4 °C for 2 h. After this, the sample was spun on a microfuge at 12000 *g* for 2 min, the pellet was washed twice in immunoprecipitation buffer and resuspended in KHEM buffer (containing the protease inhibitor 'cocktail'), for PDE assay, or Laemmli buffer [26] for SDS/PAGE. Samples treated with Laemmli buffer were immediately boiled for 3 min. The addition of antiserum to solubilized membrane extracts from either brain or COS cells transfected with pSVL-RD1 did not appear to alter (< 5%) the PDE activity.

Thermal denaturation studies

These studies were done at 50 °C exactly as described by Shakur et al. [16]. In this instance, however, RD1 activity was analysed from either cerebellum membranes or from RD1-transfected COS cells as source. Before such analyses, RD1 from both membrane sources was solubilized in the 'immunoprecipitation' buffer and then specifically immunoprecipitated as described above. Thus thermal denaturation studies were done on immunoabsorbed RD1 which had been solubilized from both membrane sources.

Relative $V_{max.}$ determinations

Increasing concentrations of membrane protein (25–200 μg) from pSVL-RD1-transfected COS-1 cells or pSVL-met^{26}-RD1-transfected COS cells (met^{26}-RD1, is RD1 with the N-terminal 25 amino acids deleted) were analysed by Western blotting. The resultant blots were scanned and the absorbance (*A*) versus amount of sample protein (μg) plotted in order to gauge the relative concentrations of RD1 in each of the preparations. For the $V_{max.}$ determinations, amounts of membrane protein, from pSVL-RD1- and pSVL-met^{26}-RD1-transfected COS-1 cells, which would provide equal amounts of transfected PDE (either RDI or Met^{26}–RD1), were taken for immunoprecipitation. They were then assayed for PDE activity over a range of cAMP concentrations and the data plotted as a Lineweaver–Burk [27] plot in order to define the relative $V_{max.}$ values.

Treatment of RD1-transfected COS cell membranes with hydroxylamine

This was done by the method of Magee and Courtneidge [28]. Briefly, RD1-transfected COS cell membranes (100 μg/tube) were pelleted and resuspended in either 100 μl of ice-cold 1 M Tris/HCl, pH 8.0, or 100 μl of ice-cold hydroxylamine, pH 8.0, before being incubated at 25 °C for 4 h. After incubation, 100 μl of ice-cold PBS, pH 7.4, was added to each tube and the samples were centrifuged at 130000 *g* for 30 min at 4 °C. After this, the supernatant fractions were removed and added to tubes containing 10 μl of 1 mg/ml BSA. Ice-cold 100% (w/v) trichloroacetic acid (TCA) was then added to a final concentration of 20% with vortexing, before being left on ice for 10 min. The TCA precipitate was then harvested after centrifugation for 5 min at 12000 *g*. The pellets obtained were resuspended in 20 μl of ice-cold 1 M Tris base and Laemmli sample buffer. Native membranes were also resuspended in Laemmli sample buffer and all samples were boiled immediately upon addition of sample buffer for 3 min before being loaded onto an 8% SDS gel with subsequent Western blotting.

Labelling transfected COS cells with [^{3}H]palmitate

This labelling was done by the method of Magee and Courtneidge [28]. Briefly, 48 h after transfection of COS cells with pSVL-RD1, the culture medium was removed from the cell monolayer and replaced with 2 ml of fresh culture medium containing 200 μCi of [^{3}H]palmitic acid (1 mCi/ml). The cells were then incubated at 37 °C for 4 h. After this time, the radioactive medium was removed and the monolayer was washed with fresh culture medium. The cells were then scraped into 200 μl of immunoprecipitation buffer (see above) before being sonicated for 30 s and left on ice for 30 min. RD1 was then immunoprecipitated, as described above, and analysed by SDS/PAGE and autoradiography.

Indirect immunofluorescence: confocal and digital deconvolution immunofluorescence microscopy

COS cells were plated out on coverslips 18 × 18 mm (No.1) and 24 h later were transfected with 5 μg of pSVL-RD1 or 5 μg of pSVL-met^{26}-RD1, as described previously [16]. At 48 h post-transfection the cells were washed three times with PBS and fixed in paraformaldehyde [3% (w/v)] in PBS containing 0.5 M Ca^{2+} and Mg^{2+} for 20 min. The cells were washed three times with PBS and quenched with 50 mM NH_4Cl for 10 min. The PBS washes were repeated and the coverslips placed in PBS containing 0.1% (w/v) Triton X-100 for 4 min. The cells were washed three times with PBS, three times with PBS/gelatine/serum [0.2% fish skin gelatine (Sigma) and 0.1% goat serum (Scottish Antibody Production Unit) in PBS] and incubated with anti-RD1 antisera, diluted in PBS/gelatine/serum for 2 h. The coverslips were washed three times with PBS/gelatine/serum, three times with PBS and stained with second antibody [goat anti-(rabbit IgG) rhodamine-B conjugate; TCS Biologicals] for 2 h. Following this, the cells were washed three times with PBS/gelatine/serum and three times with PBS.

The coverslips were mounted on glass slides with Mowoil (Cal Biochem) and viewed on a Nikon Optiphot 2 using a ×60 oil immersion objective with a high numerical aperture (NA 1.4). Two methods were used for image collection and analysis. One utilized laser scanning confocal microscopy, which was done using an Odyssey system (Noran Instruments Inc., Middleton, WI, U.S.A.) with, routinely, cells being analysed to yield around 25–33 'optical slices' collected as 64 frame averages. For com-

parison, samples were viewed at identical levels of laser intensity, brightness and contrast using the 488 nm (515 nm bandpass filter) line of the argon laser using an upright Nikon Optiphot microscope. The capture software was MetaMorph (Universal Imaging Corporation, West Chester, PA, U.S.A.). Deconvolution analyses were performed on images collected from a Nikon optiphot upright immunofluorescence microscope. Again, cells were analysed to yield around 25–33 'optical slices'. In the deconvolution studies, 'optical slices' were collected using a Monoscan system (Fairfield Imaging, Sussex, U.K.) controlling a Kodak Megaplus camera and Prior *z*-stage driver. Deconvolution of the images obtained using this system was done using 'Hazebuster for Windows' software (v2.0 Vaytek Inc. Fairfield, CT, U.S.A.; supplied by Fairfield Imaging) to remove out of focus haze. In some instances data from the laser scanning confocal microscopy system were also subjected to susequent deconvolution analysis in order to enhance clarity. 3-D reconstruction of images was done using Voxblast software running on a Gateway 2000 PC 486 at 66 MHz (v1.2.0; Vaytek Inc.; supplied by Fairfield Imaging).

As well as being treated with anti-RD1 controls were also done with cells treated with pre-immune antisera, and controls containing both detergent-permeabilized and non-permeabilized cells were used. In contrast to studies done using anti-RD1 antisera and permeabilized cells, where of the order of 26–38% of the cells showed strong immunofluorescence, only an extremely weak, general background immunofluorescence was noted in these various control situations. These studies indicated that the fluoresence detection of RD1 by the antiserum was specific and that the C-terminus of RD1, which provided the basis for antiserum generation, faced the cell interior.

In order to identify Golgi, lysosomes, peroxisomes and plasma membranes, fixed and permeabilized COS cells were labelled with antisera specific to either endogenous or transfected proteins within a given compartment. For identification of Golgi, COS cells were transfected with the plasmid GSS (a kind gift from Dr Munro, LMB, Cambridge; [29]). This encodes a fusion protein consisting of the first 44 residues of sialylyl transferase, a Golgi-specific protein (see [29]), fused to chicken lysozyme. The protein was visualized using a monoclonal antibody specific to lysozyme (again a gift from Dr. S. Munro) together with an anti-mouse IgG conjugated to rhodamine. For identification of lysosomes, COS cells were grown on cover slips and then incubated with BSA conjugated to fluorescein isothiocyanate [30]. Peroxisomes were visualized using a polyclonal antibody raised against a peptide representing a region of the C-terminus of the protein PMP70 [31,32] which is exposed to the cytosol (this was a gift from Professor S. Subramani, UCSD, CA, U.S.A.), followed by visualization with anti-rabbit conjugated IgG conjugated to rhodamine. Plasma membranes were visualized by transfecting COS cells with a cDNA encoding the human insulin receptor (a gift from Dr. L. Ellis, Southwestern University, Dallas, TX, U.S.A.) [33] and the cells then treated with a mouse monoclonal antibody (IR83-14) which reacts with an epitope on the receptor α-subunit located at the external surface of the membrane (a gift from Professor K. Siddle, University of Cambridge, U.K.) [34]. This was followed by visualization using an anti-mouse IgG conjugated to rhodamine.

SDS/PAGE and Western blotting

Acrylamide gels (8 or 10%) were used and the samples were immediately boiled for 3 min after being resuspended in Laemmli buffer [26]. Gels were run at 8 mA/gel overnight or 50 mA/gel for 4–5 h with cooling. For detection of RD1 by Western blotting, 100 μg of protein samples were separated by SDS/PAGE and then transferred to nitrocellulose before being immunoblotted using RD1-specific antisera. Labelled bands were identified by using anti-rabbit peroxidase-linked IgG and the enhanced chemiluminescence (ECL) Western blotting visualization protocol (Amersham).

Detection of the G-protein, α-G_i-2, was done as described before [35,36] using the antiserum SG1 which was raised against the C-terminal decapeptide of the α-subunit of transducin (conjugated to KLH).

PDE assay

PDE activity using 1 μM cAMP as substrate was assayed by a modification of the two-step procedure of Thompson and Appleman [37] and Rutten et al. [38] as described previously by Marchmont and Houslay [39]. All assays were conducted at 30 °C and in all experiments a freshly prepared slurry of Dowex:H_2O:ethanol (1:1:1, by volume) was used for determination of activities. In all the experiments described, initial rates were taken from linear time-courses of activity. Rolipram was dissolved in 100% DMSO as a 10 mM stock solution and diluted in 20 mM Tris/HCl/10 mM $MgCl_2$ buffer at a final pH of 7.4 to provide a range of concentrations for use in the assay. The residual levels of DMSO were shown not to affect PDE activity over the ranges used in this study.

Protein determination

Protein was routinely measured by the method of Bradford [40] using BSA as a standard.

RESULTS AND DISCUSSION

The first cAMP-specific type-IV phosphodiesterase to be cloned was a species from drosophila [41]. The cDNA for this drosophila *dunc* species was then used to isolate a cognate cDNA, called RD1, from rat brain [17]. In addition, two further partial clones were identified, but not characterized, and the suggestion was made that splice variants of RD1 could exist [5,17]. The possibility was also mooted [5] that RD1 might not reflect a full-length PDE expressed *in vivo*. Nevertheless, when COS cells were transfected with the cDNA for RD1, both ourselves [16] and others [17] have observed a profound increase in PDE activity whose characteristics are typical of the type-IV PDE family. Furthermore, such PDE activity was shown to be predominantly, but not exclusively, membrane associated [16]. In order to address questions relating to whether native RD1 is expressed in brain, and what its properties might be, we raised antibodies to a dodecapeptide whose sequence reflects that seen at the C-terminus of RD1. As Northern blot analyses of various rat tissues with a specific cDNA probe for RD1 showed that transcripts were essentially confined to brain [17,42–44], we focused on this tissue and undertook an analysis of a specific brain region, namely cerebellum.

Rat cerebellum homogenates were subjected to SDS/PAGE with subsequent Western blotting using our anti-RD1 antiserum. This identified a single immunoreactive species of molecular size 73 ± 3 kDa (Figure 1a). The presence of an excess (competing) concentration of the peptide used to raise the antiserum blocked such recognition, whereas the use of an 'irrelevant' peptide did not (Figure 1b). The size of this immunoreactive species is similar to that predicted ($\sim$ 68 kDa) from the sequence of the cDNA for RD1 [19] but, importantly, it is identical with that which can be seen upon transient expression of RD1 in COS cells transfected

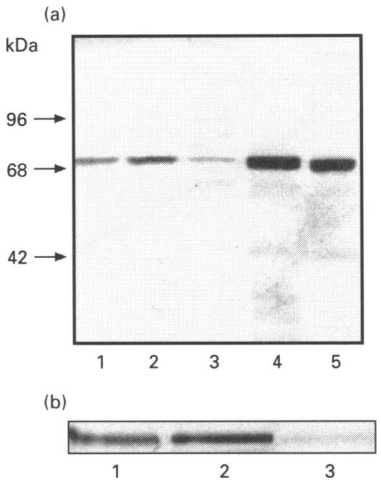

Figure 1 Immunoblot analyses done with an antiserum raised against a dodecapeptide reflecting the C-terminus of RD1

SDS/PAGE gels of proteins were subjected to Western blotting using an anti-peptide antiserum generated against a dodecapeptide which reflects the C-terminus of RD1 (see Materials and methods section). (**a**) Comparative analyses done on the same gel for cerebellum homogenate (lane 1), cerebellum membrane (lane 2) and cerebellum cytosol (lane 3) fractions with COS cell homogenates from cells which had been transfected with pSVL-RD1 (lane 4) and COS cells transfected with pSVL-met^{26}-RD1 (lane 5). No immunoreactive species were evident (results not shown) in COS cells which had either been mock-transfected with vector alone (pSVL) or with native (untreated) cells. Studies done with pre-immune antiserum failed to identify any immunoreactive species (results not shown). (**b**) Cerebellum homogenates immunoblotted in the absence (lane 1) and presence (lane 3) of the competing peptide (0.5 mg/ml) used to generate the antiserum [(C)-T-P-G-R-W-G-S-G-G-D-P-A]. Use of a non-specific peptide to the type-IVB PDE [49] failed to block the identification of the immunoreactive species using anti-RD1 antiserum (lane 2). Gels are typical of experiments done at least three times.

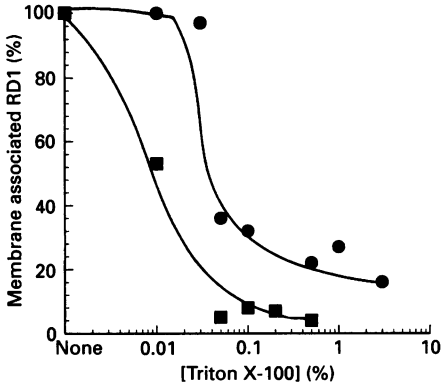

Figure 2 Triton X-100 mediated solubilization of brain RD1

Cerebellum membranes were treated with various concentrations of the non-ionic detergent Triton X-100. After incubation and centrifugation, as described in the Materials and methods section, the residual pellet and supernatant fractions were subjected to SDS/PAGE and Western blotting with anti-RD1 antiserum. The resultant ECL/autoradiographs were scanned and absorbance changes quantified. These data show the amount of RD1 immunoreactive material remaining in the membrane fraction for cerebellum (●) and for COS cells (■) which had been transfected with the cDNA for RD1. This data is representative of experiments done at least three times.

with the cDNA for RD1, where the immunoreactive species from both sources co-migrated (Figure 1a). It would seem then that cerebellum expresses a protein of identical size and, as we shall demonstrate, properties with RD1.

The cerebellum homogenate was subjected to differential centrifugation to provide a low-speed pellet (P1; 1000 *g* for 10 min) whose supernatant was then subjected to further centrifugation to yield a high-speed pellet (P2; 100000 *g* for 1 h) together with a membrane-free supernatant (SN; cytosol) fraction. Analysis by Western blotting with the anti-RD1 antiserum showed that some $92 \pm 3\%$ ($n = 12$) of the immunoreactivity was found in the high-speed (P2) membrane fraction (Figure 1a) with the rest occurring in the cytosol fraction. This is consistent with our previous studies [16] which showed that upon transient expression of RD1 in COS cells around 85% of the activity of this enzyme was membrane bound and the remainder occurred as a soluble species in the cytosol. The slightly higher proportion of RD1 that was associated with membranes in cerebellum may be either because COS cell transfections can lead to substantial overexpression, where membrane integration may be rate-limiting, or may be due to some process which increases the efficiency of membrane association in cerebellum.

As with COS cell-expressed RD1 [16], that from cerebellum was neither solubilized by re-homogenization with membrane washing (< 5%) nor released (< 5%) by using high concentrations of NaCl (0.5–1.5 M), both of which treatments are able to elute peripheral proteins [39]. However, as in COS cells transfected with RD1 cDNA [16], cerebellum RD1 was released using low concentrations of the non-ionic detergent Triton X-100 (Figure 2). Intriguingly however, slightly higher concentrations of the detergent Triton X-100 were needed to effect the solubilization of native brain RD1 compared with those needed to release RD1 from transfected COS cells. Such data also indicate that the fidelity of membrane association in cerebellum is greater than that found in transfected COS cells.

We have previously suggested [16] that RD1 might be palmitoylated by modification at one or both of the two cysteine residues found within the putative N-terminal targeting domain and that this could lead to membrane association as has been found for other proteins [45,46]. Treatment of acylated proteins with hydroxylamine has been shown to cause the release of covalently associated palmitate [28,47] and the solubilization of at least a fraction of such proteins. However, hydroxylamine failed to release RD1 (< 4%) from membranes from either cerebellum or transfected COS cells under conditions that led to the release of 20–30% (range $n = 3$) of the G-protein α-G_i-2 from cerebellum membranes. Furthermore, we were unable to show the incorporation of [^{3}H]palmitate into membrane-bound RD1 that had been immunoprecipitated from pSVL-RD1-transfected COS cells. These data suggest that RD1 is unlikely to be anchored to membranes through palmitoylation.

In order to try and immunoprecipitate RD1 specifically as an active enzyme for analysis, cerebellum membranes were solubilized using Triton X-100 and then treated with RD1-specific antiserum (see Materials and methods section). Such an addition of antiserum did not alter the total PDE activity of the mixture (< 5% change). This strategy allowed RD1 to be immunoprecipitated from this solubilized extract where it formed some 8–12% (range $n = 6$) of the total cAMP-PDE activity and some 19–29% of the type-IV PDE activity, defined as that fraction which could be inhibited by 10 μM rolipram (with 1 μM cAMP as substrate). Such immunoprecipitated PDE activity was cAMP specific, insensitive to the addition of either Ca^{2+}/CaM (100 μM; 20 ng/ml) or low cGMP concentrations (1 μM) and was inhibited by low concentrations of the selective type-IV PDE inhibitor rolipram in a dose-dependent fashion (Figure 3a).

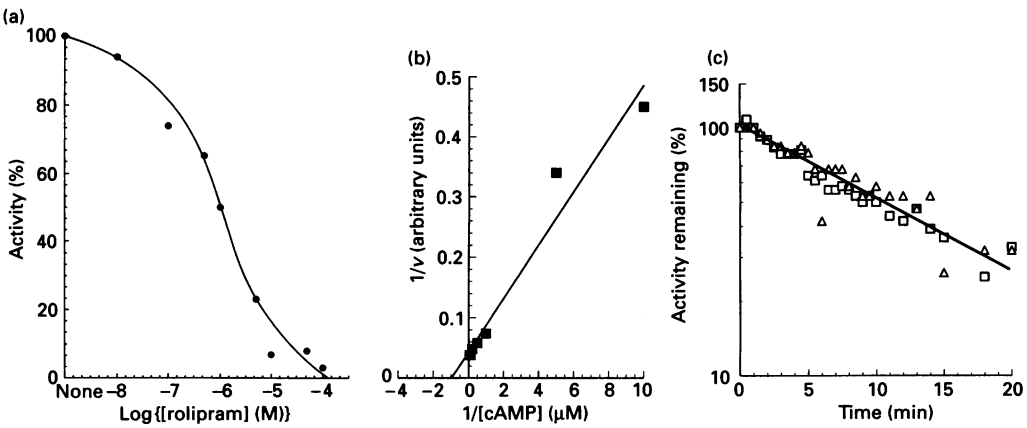

Figure 3 The activity of native RD1 immunoprecipitated from solubilized cerebellum membranes

Cerebellum membranes were solubilized using the detergent Triton X-100 and then subjected to immunoprecipitation using the anti-RD1 C-peptide antiserum (see Materials and methods section). The immunoprecipitate was resuspended for enzyme assay. In (**a**) increasing concentrations of rolipram are shown to inhibit native RD1 dose-dependently. Activity was analysed in the presence of 3 μM cAMP. In (**b**) is shown a Lineweaver-Burk [27] plot for substrate utilization. This was linear in all instances. In (**c**) is shown a semi-log plot which describes the thermal stability of RD1 activity at 50 °C. Data are for RD1 which had been solubilized from membranes from either COS cells transfected with RD1 cDNA (△) or from cerebellum (□). The solubilized species were then specifically immunoprecipitated and the immunoabsorbed enzyme activity analysed after treatment at 50 °C for the indicated time. These data are an aggregate of three experiments done with different preparations in each instance.

Indeed, rolipram inhibited the immunoprecipitated cerebellum RD1 activity with an EC_{50} of 0.7 ± 0.3 μM, which compared well with the value of 0.4 ± 0.1 μM seen for RD1 expressed in COS cells [16]. Certainly, the fact that all the immunoprecipitated PDE activity was inhibited by low concentrations of rolipram (Figure 3a) was consistent with the presence of a type-IV PDE that was subject to competitive inhibition by this compound. Lineweaver–Burk [27] analyses of cAMP hydrolysis were linear over a range of substrate concentrations from 0.1–10 μM (Figure 3b), yielding a similar value for the K_m (2.3 ± 0.4 μM; $n = 4$) to that seen (4.1 ± 2.3 μM) for COS cell-expressed RD1 [16]. We also determined the thermostability of RD1 which had been solubilized and then immunoabsorbed from both cerebellum and RD1-transfected COS cell membranes (Figure 3c). We noted that the PDE activities from both sources decayed with apparently identical half-lives ($t_{1/2}$ at 50 °C) of 10 ± 1 min and 11 ± 1 min, for the activities from cerebellum and transfected COS cells respectively (S.D.; $n = 3$ separate experiments); these values being determined from linear semilog plots (Figure 3c). In a previous study we showed [16] that membrane-bound RD1 in transfected COS cells decayed with a $t_{1/2}$ of $\sim$ 11 min at 50 °C and that this was decreased to $\sim$ 5 min upon solubilization of RD1. It would thus appear that immunoabsorption of RD1, through antibody binding to its C-terminal tail, engenders a similar increase in thermostability as was seen for membrane association achieved through its N-terminal domain [16]. These data, coupled with observations on the size of the immunoreactive species identified by our antiserum and its membrane association, indicate that our antiserum does indeed recognise native RD1 in cerebellum and is able to immunoprecipitate this enzyme specifically in an active state.

We next undertook a study to determine whether RD1 in cerebellum was associated with synaptosomes. To do this we employed the Percoll gradient system developed by Thorne et al. [22] for resolving synaptosome sub-populations from a low-speed supernatant (S1) fraction (see Materials and methods section). Of the five fractions obtained by this procedure, they showed [22] that synaptosomes were highly enriched in fractions F2, F3 and F4 and present to a lesser extent in the F1 fraction and minimally in the F5 fraction. We were able to identify a comparable distribution of LDH activity in these fractions to that described by Thorne et al. [22] (results not shown; see Materials and methods section). Analysis (Figure 4a, b) of the distribution of RD1 immunoreactivity showed it to be highest in the F2 and F3 fractions and present to a lesser extent in the F1 and F4 fractions. Negligible immunoreactivity was observed (Figure 4a, b) in the F5 fraction which has been shown to be enriched in mitochondria and nuclei and to have a minimal presence of synaptosomes ([22]; see Materials and methods section). It was of interest that RD1 immunoreactivity was lower in the F4 fraction compared with the F3 fraction (Figure 4a, b). This is because, although Thorne et al. [22] showed these fractions to be the most highly enriched in intact synaptosomes, they found that the synaptosomes in the F4 fraction were predominantly pre-synaptic in origin whereas those in fractions F2 and F3 were enriched in synaptosomes that had post-synaptic densities attached. This might indicate a preferential association of RD1 at the post-synaptic junctions that contain receptors and associated signalling systems.

The combined F2 and F3 fractions were lysed (see Materials and methods section) and then sub-fractionated on a sucrose density gradient to resolve membrane populations. The peak of RD1 immunoreactivity coincided with that seen for the plasma membrane enzyme marker enzyme 5′-nucleotidase (Figure 4c). On this basis it would seem likely that RD1 is associated with plasma membranes in cerebellum. Indeed, Thorne et al. [22] showed that the F1 fraction from the Percoll gradient procedure primarily contains the plasma membranes accruing from lysed synaptosomes. We found significant RD1 immunoreactivity associated with this fraction (Figure 4a, b), which would be in accord with a predominantly plasma membrane localization for this enzyme in cerebellum. Such a conclusion is also in agreement with our earlier suggestion [16] that RD1 appeared to be primarily associated with the plasma membrane fraction isolated from COS cells transfected with RD1 cDNA. However, at that time, we were tentative as to such a conclusion in that we found COS cells to be extremely difficult to disrupt and fractionate successfully. We did, however, develop a novel subcellular frac-

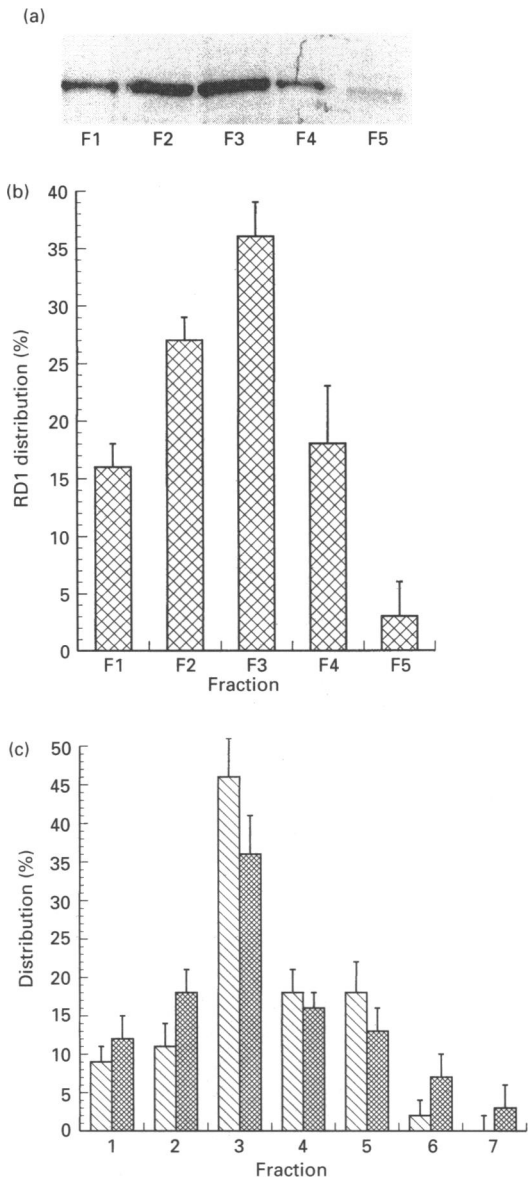

Figure 4 Analyses of RD1 in brain synaptosomes

(**a**) These data show a typical immunoblot study done with the anti-RD1 C-terminus antiserum on the fractions obtained (F1–F5 inclusive) from the Percoll gradient synaptosome isolation procedure described by Dunkley et al. [21] and Thorne et al. [22] (see Materials and methods section). Equal amounts (100 μg) of protein were analysed on each gel track. The distribution (%) of RD1 immunoreactivity between these various fractions is shown for data aggregated from three experiments using different preparations with means $\pm$ S.D. (**b**) Shown is the distribution (%) of both immunoreactive RD1 (▨) and the activity of the plasma membrane marker 5′-nucleotidase (⊠) on sucrose density gradient analysis of lysed synaptosomes (see Materials and methods section). Fraction 6 contained the peak of the mitochondrial marker, cytochrome oxidase and fraction 7 the peak of the lysosomal marker β-hexosaminidase. These data are an aggregate of three experiments done with different preparations.

tionation procedure [16] utilizing cytochalasin B treatment as an essential pre-requisite to achieve centrifugal resolution of membranes. The availability of RD1-specific antisera has allowed us in the present work to determine *in situ* the location of RD1 in transfected COS 1 cells. We have done this using fluorescence labelled anti-rabbit antisera to treat fixed and permeabilized cells that have been exposed to anti-RD1 antiserum. Analyses were then done using both fluorescence laser scanning confocal microscopy and deconvolution fluorescence microscopy, yielding similar results (Figure 5). Such strategies obviate any potential problems arising from cellular disruption and subsequent subcellular fractionation where redistribution of proteins, abnormal fractionation of membranes and selective fractionation of membrane fragments showing lateral heterogeneity may occur. Using these procedures we were able to show that, in COS cells transfected with RD1, a strong immunofluorescence signal was found to occur which was predominantly associated with the cell plasma membrane (Figure 5 i and ii). That this was not seen in cells that had not been permeabilized (results not shown) indicates that the C-terminal domain of RD1, where the antisera can be expected to bind, was located within the cell interior. 3-D reconstruction analyses were done for optical sections obtained from cells which had been transfected with RD1. These gave results that were consistent with the location of RD1 at the plasma membrane. Thus, strong fluorescence was observed spreading over the cell surface (Figure 5 v and vi). From both optical sections and from 3-D reconstructions we noted (Figure 5) that plasma membrane immunofluorescence of RD1 was not evenly distributed, with, for example, areas above and below the nucleus exhibiting much lower levels of fluorescence and other areas showing punctate staining. This may imply the association of RD1 with specific acceptor proteins or lipids that are themselves disposed asymmetrically within the lateral plane of the plasma membrane or that RD1 is either aggregated or specifically excluded from certain domains within the plasma membrane. Indeed, a punctate staining in transfected COS cells has also been noted for plasma membrane associated G-protein $\beta\gamma$-subunits [48]. We also noted in a small number of cells a proportion of immunofluorescence associated with the Golgi apparatus as well as small vesicles underlying the plasma membrane (Figure 5 ix and x). This would be consistent with our biochemical studies [16] where a minor fraction of RD1 appeared to be Golgi associated. Thus, in intact COS cells, the major fraction of RD1 is indeed membrane bound and is associated primarily with the cytosol surface of the cell plasma membrane rather than distributed non-specifically between various membrane fractions. RD1 must therefore contain information that not only allows it to become membrane associated but also allows for its targeting, presumably through association with a particular species of membrane protein.

Our previous studies [16] have suggested that the information required for membrane association resides within the first 25 amino acids of RD1. This was based upon the observation that engineering the cDNA of RD1 to achieve a truncated species that lacks these residues, met^{26}-RD1, yielded an enzyme whose activity was found exclusively in the cytosol of transfected COS cells. Here we can confirm that met^{26}-RD1 is indeed a soluble enzyme, as a uniform immunofluorescence was found throughout the cytosol (Figure 5 iii and iv) of COS cells transfected with the cDNA for this truncated enzyme. This was noted when analyses were done on discrete optical sections made through the cells (Figure 5 iii and iv). Strong uniform fluorescence was also noted in 3-D reconstructions of such data (Figure 5 vii and viii).

We [16] previously showed that the engineered deletion of this unique 25 amino acid N-terminal domain of RD1 did not alter either the K_m for cAMP or the inhibitor selectivity/sensitivity of the enzyme. However, we were unable to determine whether such N-terminal truncation affected the $V_{max.}$ of the reaction. Here, using antisera able to interact with the common C-terminal region of both RD1 and met^{26}-RD1, we were able to determine the relative amounts of these enzymes in transfected COS cells. This allowed us to determine that deletion of this domain led to

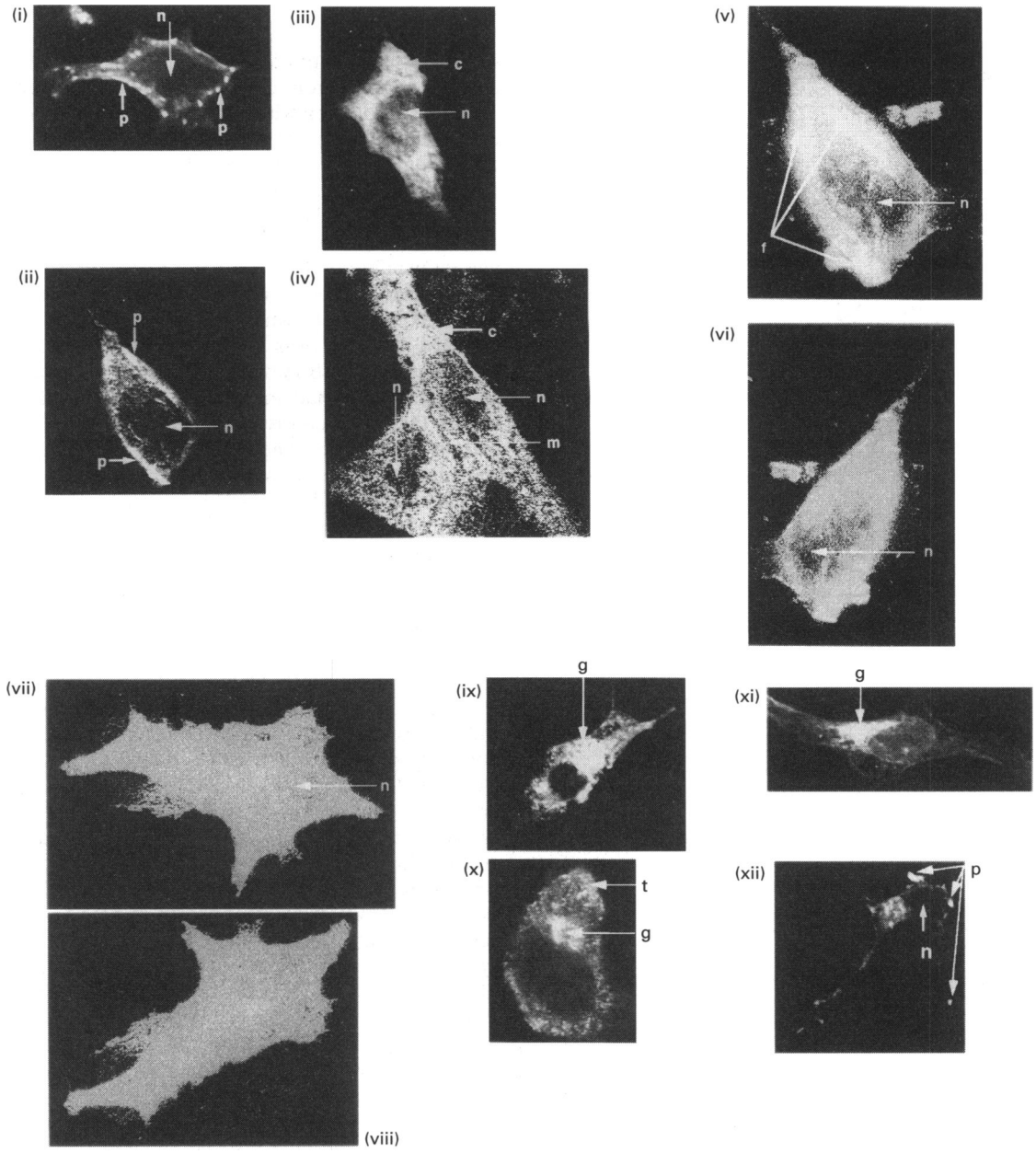

Figure 5 Immunofluorescence microscopy analyses of COS cells transfected with RD1 and met^{26}-RD1 and analysed using anti-RD1 C-terminal anti-peptide antiserum

Experiments were performed as described in the Materials and methods section. Analyses were done with either laser scanning confocal microscopy (i, iii, vii, viii ix, x, xi, xii) or deconvolution immunofluorescence microscopy (ii, iv, v, vi). Similar results were obtained using both laser scanning confocal microscopy and fluorescence deconvolution microscopy for analyses. These photographs show COS cells transfected with either pSVL-RD1 (i and ii) or pSVL-met^{26}-RD1 (iii, iv). Shown are typical 2-D 'slices' (optical sections through distinct focal planes) through a region close to the centre of the cell. 'White' represents the highest immunofluorescence and hence the highest concentration of either RD1 or met^{26}-RD1. Note in (i) and (ii) that immunofluorescence is highly localized at and underlying the cell periphery (see arrows marked 'p'), indicating a location at the cytosol surface of the plasma membrane. However, staining is punctate and indicates segregation within the lateral plane of the membrane. The nucleus of each of these cells is indicated (n). Weak fluorescence was also noted within the cytosol which would be consistent with a fraction of the enzyme being found there also (see [16]). In (iii and iv) the immunofluorescence due to met^{26}-RD1 detection is shown as dispersed throughout the cell cytosol (c). These examples are typical for various transfected cells where immunofluorescence extended completely between the cell margins at any particular focal plane (optical 'slices' made through them). The fluorescence is concentrated in the cytosol domain (c). The nucleus is indicated (n). In (iv) two juxtaposed cells are shown with their adjacent plasma membrane indicated (m). 3-D reconstructions were done of the optical slices generated upon deconvolution immunofluorescence microscopy. Shown are the top (v) and underneath (vi) views of a COS cell which had been transfected with the cDNA for RD1. This identifies the coating of RD1 which is found to underlie the cell surface plasma membrane, with particularly intense areas of fluorescence indicated (f). This was not uniform over the surface of the cell, with an area of membrane which overlay the position of the nucleus (n) showing much reduced fluorescence, indicative of the lateral segregation of RD1. Similar conclusions can be drawn from perspectives taken both above and below the cell. In contrast to this, in COS cells transfected with the cDNA for met^{26}-RD1 a dense uniform fluorescence was observed whether viewing from above (vii) or below (viii) the cell. The position of the nucleus (n) is shown. In $\sim$ 15% of the COS cells transfected with the cDNA for RD1, localization within the Golgi was evident (ix and x), indicated by 'g', as was punctate immunofluorescence (t) associated with an intracellular vesicle system (x). Identification of Golgi apparatus was achieved using cells transfected with a fusion protein formed from the Golgi-specific enzyme sialylyl transferase together with chicken lysozyme. This is shown in (xi), with Golgi indicated as 'g'. Transfection of cells with the cDNA for the plasma-membrane-localized insulin receptor led to labelling at the cell surface in a highly localized, punctate pattern (p) as well as labelling within the Golgi, where this integral enzyme is synthesized (xii). Transfection experiments were done on six separate occasions with duplicate analyses being carried out for both RD1 and met^{26}-RD1. Control experiments were performed as described in the Materials and methods section, as were experiments done with markers to identify cellular membrane systems. In this study, of the order of 26–38% of COS cells appeared to be successfully expressing either RD1 or met^{26}-RD1 after transfection with the appropriate plasmid, as indicated by positive labelling with the anti-peptide antiserum.

a marked (2.1 ± 0.2-fold; $n = 3$ separate experiments using different preparations) increase in the $V_{max.}$ value. Thus the unique N-terminal domain of RD1 appears not only to confer membrane association but also exerts an inhibitory function. We believe that it also may be possible that membrane-induced changes in this domain could affect the structure and activity of RD1. We have shown [16] that whilst detergent-solubilized RD1 was more thermostable than met^{26}-RD1, a further marked increase in thermostability was seen when RD1 was membrane associated. Thus, interaction with plasma membrane components appears to engender a conformational change in RD1 that is transduced through this N-terminal domain. It is therefore possible that RD1 could form part of a signal transduction pathway where changes in the conformation of an associated membrane protein could regulate its activity.

Our data therefore show that the RD1 cDNA isolated by Davis et al. [19] does indeed reflect that of a full-length protein that is functionally expressed in cerebellum. The N-terminal domain of RD1 is shown not only as conferring membrane association on this enzyme, which is preferentially localized to the plasma membrane, but also serves to inhibit or constrain the activity of RD1.

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