Determination of the Structure of the N-terminal Splice Region of the Cyclic AMP-specific Phosphodiesterase RD1 (RNPDE4A1) by $^1$H NMR and Identification of the Membrane Association Domain Using Chimeric Constructs*

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A 25-residue peptide representing the membrane targeting N-terminal splice region of the cyclic AMP phosphodiesterase RD1 (RNPDE4A1) was synthesized, and its structure was determined by $^1$H NMR. Two independently folding helical regions were identified, separated by a highly mobile “hinge” region. The first helical region was formed by an N-terminal amphipathic $\alpha$-helix, and the second consisted of multiple overlapping turns and contained a distinct compact, hydrophobic, tryptophan-rich domain (residues 14–20). Chimeric molecules, formed between the N-terminal region of RD1 and the soluble bacterial protein chloramphenicol acetyltransferase, were used in an in vitro system to determine the features within the splice region that were required for membrane association. The ability of RD1-chloramphenicol acetyltransferase chimera to become membrane-associated was not affected by deletion of any of the following regions: the apolar section (residues 2–7) of the first helical region, the polar part of this region together with the hinge region (residues 8–13), or the polar end of the C-terminal helical region (residues 21–25). In marked contrast, deletion of the compact, hydrophobic tryptophan-rich domain (residues 14–20) found in the second helical region obliterated membrane association. Replacement of this domain with a hydrophobic cassette of seven alanine residues also abolished membrane association, indicating that membrane association occurs by virtue of specific hydrophobic interactions with residues within the compact, tryptophan-rich domain. The structure of this domain is well defined in the peptide, and although the region is helical, both the backbone and the distribution of side chains are somewhat distorted as compared with an ideal $\alpha$-helix. Hydrophobic interactions, such as the “stacked” rings of residues Pro$^{14}$ and Trp$^{15}$, stabilize this domain with the side chain of residue Leu$^{16}$ adopting a central position, interacting with the side chains of all three tryptophan residues 15, 19, and 20. These bulky side chains thus form a hydrophobic cluster. In contrast, the side chain of residue Val$^{17}$ is relatively exposed, pointing out from the opposite “face” of the peptide. Although it appears that this compact, tryptophan-rich domain is responsible for membrane association, at present the target site and hence the specific interactions involved in membrane targeting by the RD1 splice region remain unidentified.

cAMP serves to influence a variety of cellular processes (1). The sole means of degrading this key second messenger within cells is through hydrolysis to 5'-AMP, which is achieved by a family of CAMP phosphodiesterases (PDE) (2–4). At least five distinct families encode enzymes that display this activity, each of which exhibits distinct regulatory properties (2–4). Indeed, PDEs appear to provide a point of “cross-talk” between the CAMP signaling system and most, if not all, other signaling systems, suggesting that these enzymes may play a key role in signal integration. There is also considerable evidence to suggest that the functioning of CAMP within various cells may be compartmentalized (5–7). The molecular machinery that might allow for this includes the functional targeting of type II CAMP-dependent protein kinase (protein kinase A) to specific cellular membranes through interaction with a family of membrane-associated protein kinase A binding proteins (5–7). Coupled to this are the observations that degradation of cAMP can be compartmentalized, as specific PDEs are also membrane-associated (8–10). The PDE4 family-specific PDEs provide clear examples of both cytosolic and membrane-associated PDEs (11, 12). This family is encoded by four separate genes found on three different chromosomes (13–15) with additional complexity derived from alternative splicing (16–18). The PDE4A family in rat has at least three members (17, 18), which are RD1 (RNPDE4A1), RPDE-6 (RNPDE4A), and RPDE-39 (RNPDE4A6). These enzymes are differentially expressed in various tissues and are formed by 5’ alternative splicing to yield proteins with distinct N-terminal regions (11, 12, 17). The engineered expression of the common “core” PDE4A gene product produces a highly active cytosolic protein (9, 12, 19). In contrast, the splice variant RD1 is found exclusively associated

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The atomic coordinates and structure factors (codes 1LOI and R1LOIMR) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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‡ These abbreviations used are: PDE, cyclic 3',5'-AMP phosphodiesterase; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; TFE, 2,2,2-trifluoroethanol; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; NOE, nuclear Overhauser effect; $d_3$ fully deuterated.
with membranes (12, 19), whereas both RPDE6 (12) and RPDE39 (11) are expressed as both soluble and particulate-associated species. These unique N-terminal regions not only define intracellular targeting, but they also profoundly reduce catalytic activity (V \text{"max\}) and alter thermostability (9, 12, 19).

That the N-terminal splice region of RD1 (18) encodes a structural domain, identified using 1H NMR analysis of a deletion analysis of RD1-CAT chimera to identify which of the distinct domains, identified using 1H NMR analysis of a 25-residue peptide encompassing the N-terminal splice region of RD1, is responsible for determining membrane association.

### MATERIALS AND METHODS

GeneAmp® PCR Reagent kit was purchased from Perkin-Elmer. Restriction and modifying enzymes, T7 promoter Sp6 coupled Reticulocyte Lysate System, Wizard® PCR, and MiniPrep and Maxiprep systems were from Promega. Qiaex PCR purification kit was from Hybaid Ltd. Taq DyDeoxy terminator Cycle Sequencing kits were from Applied Biosystems. 1H Chloramphenicol (54 mCi/mmol), 3H methionine (50 mCi/mmol), H2O-labeled protein molecular weight markers, and ECL Western blot detection reagents were from Amersham International plc. All other chemicals and reagents were of the highest quality available (Sigma). Plasmid pSVL-RD1 containing RD1 has been described previously (9). Plasmid pSVLSPORT was from Life Technologies Inc. PCR and sequencing primers were designed with the Gene Jockey program (Bioisoft) (21) and synthesized on an Applied Biosystems model 381A.

#### Peptide Synthesis

A peptide representing the N-terminal first 25 amino acids of RD1 (MPLVDFFCETCSKPWLVGWWDQFKR) was synthesized on a Rink Amide MBHA resin (Nova Biochem; 01-64-40-24 and 25). This peptide was cleaved from the resin, with simultaneous deprotection of side chains, was done with 10 ml of trifluoroacetic acid containing 0.5 ml of H2O, 0.5 ml of thioanisole, and 0.25 ml of ethanol/1. Crude peptide was precipitated in ice-cold diethyl ether, dried in a stream of N2, and lyophilized.

#### NMR Spectroscopy

1H NMR experiments were carried out on a Bruker AMX500 spectrometer using standard pulse sequence two-dimensional pulse sequences. Two-dimensional experiments were acquired with 2048/2K data points in F2, using a sweep width of 5000 Hz, and with 448 rows in F1. For TOCSY and COSY experiments 16 transients were acquired, and for NOE experiments 80 transients were acquired. TOCSY experiments used an MLEV-17 mixing pulse of 60-ms duration (10 kHz spin locking field).

The NOE experiments were collected with mixing times of 50, 100, and 200 ms. Very weak presaturation was applied to the water resonance both during the relaxation delay (1.5 s) and also during the mixing time of the NOE experiments.

#### TOCSY, NOESY, and COSY spectra for assignment were collected on 7 mW solutions of peptide in the presence of 6–8 m dithiothreitol, at both pH 6.0 in 50% D2O, TFE/50% H2O (v/v, 300 K) and at pH 3.95 ± 0.5% D2O, TFE/50% H2O (v/v, 304 K). The addition of dithiothreitol eliminated the possibility of inter- and intramolecular disulfide bridge formation. Sequential NOEs were identified along the whole of peptide backbone, enabling the protein to be assigned in a high degree of detail for fashion. The unambiguous identity of certain NOE cross-peaks was confirmed taking advantage of pH- and temperature-dependent shifts between different spectra.

In order to detect backbone hydrogen bonding in the peptide, the rates of movement of chemical shifts with changes in temperature were followed in TOCSY spectra at temperatures between 285 and 311 K at both pH 4.1 and 6.0 with essentially no difference seen between the two data sets. Temperature shift coefficients were determined from the slope of a linear plot of temperature against chemical shift. Inter-proton distances were estimated from NOEY experiments by integration to yield cross-peak volumes in the 200-ms NOEY spectrum (all cross-peaks were also seen at 100 ms of mixing time). Cross-peaks were grouped into five classes between strong and very weak (strong and medium NOE's were allocated the same distance constraint values in calculations).

The NOE experiment was determined using XPLOR 3.1 (22) using a protocol in which certain atoms of each residue were embedded using the distance geometry routine (atoms CA, HA, N, HN, C, CB*, CG*, and Cg, sub_embed protocol). The remaining atoms were placed by template fitting, and then the atomic co-ordinates were allowed to evolve under the applied NOE distance constraint. A series of high temperature factor refinements (dp3 and refined protocols) from the 100 calculated co-ordinate sets started, half were discarded (wrong handedness in the distance geometry routine). The final structures were produced by minimizing the averaged co-ordinates produced over the last 4 ps of a 5-ps low temperature simulated annealing trajectory. 25 structures were then chosen with no violations of the applied NOE constraints greater than 0.4 Å for analysis. To aid analysis, a mean structure was calculated by simultaneously refining these 25 structures in a high temperature simulated annealing protocol and applying an additional constraint tending to minimize the RMS difference between the aligned co-ordinates (NCS restraints applied individually to the regions Met4–Cys3, Cys13–Pro18, and Pro15–Arg58). It was not found necessary to include constraints for hydrogen bonds in any calculation. Two patterns of hydrogen bond donors, indicated by changes in the rate of change in chemical shift of the backbone amides with changes in pH, was consistent with the calculated structures. There were no significant differences between the NOE observed at pH 3.9 and 6.0, indicating that the structures were very similar.

NMR data collected in water alone were of poor quality because of limited solubility of the peptide (less than 0.5 mg at pH 4 and lower at pH 6). The data implied that the problem was nonpolar aggregation at residues Leu1, Phe1, and Phe2 in the N-terminal amphipathic helix. However, in the water/TFE mixture intermolecular interactions were eliminated (e.g., there was no change in line width or chemical shift as concentrations were decreased). Trifluorothanol was chosen as a co-solvent because of its ability to replace hydrophobic interactions between peptides (23) and because of the enhancement of secondary structure for small peptides in solution, see e.g., Refs. 24 and 25.

Construction of Deletion Mutations—Various mutations were generated within the 25-amino acid N-terminal region of RD1 by a modification of the method of overlapping extension mutagenesis described by Ho et al. (26). Primer pairs used were GSOL17, 5′-GGTGCAGCTCTGCTTAATTGAAGG-3′, and GSOL18, 5′-CCAGGCTGCTCAGACACTGACC-3′ to generate deletion ΔP2-F7 (inclusive); GSOL27, 5′-GACCTTTCTCGTCGTCGTGTCGCTCCAGGCTCCAGTGGTCG-3′, and GSOL28, 5′-GGCACTGCGCATGGATGCTGAACC-3′ to generate deletion Δ8K-13 (inclusive); GSOL29, 5′-GACTTCTTCCCCTGGCTGG-3′, and GSOL30, 5′-GCCAGTCGCTCCAGAAGG-3′ to generate deletion Δ21-25 (inclusive); GSOL39, 5′-GGTCGACTCTACGGCAAAGG-3′, and GSOL40, 5′-GGTCGACTCTACGGCAAAGG-3′ to generate deletion ΔD21-25 (inclusive); and GSOL62, 5′-CCTGCTCAGAAAGCCAGCAAGC-3′, and GSOL62, 5′-CCTGCTCAGAAAGCCAGCAAGC-3′ to generate deletion ΔD21-25 (inclusive). These oligonucleotides were used to generate P1A4-W20 (inclusive) of pGS7 with a cassette of seven alanine residues through the generation of plasmid pGS22. Primers GSOL10 (5′-CAAGGCAGAGCTGGTGGTAATAATG-3′) and GSOL15 (5′-GGTTCGACTCTACGGCAAAGG-3′) were used as the sense and antisense primers, respectively, for each mutation-generating PCR reaction.

Using plasmid pGS4 (20) as template DNA, the following reaction conditions were used: 1 µl of template DNA, 25 pmol of each primer, 0.2 µM 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 in 1 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 15 sec at 94°C, annealing for 30 sec at 55°C, and extension for 30 sec at 72°C for 30 cycles. Upon completion of the primary PCR reactions, samples were extracted once with an equal volume of chloroform to remove mineral oil before electrophoresis through a 1.75% low gelling temperature agarose gel. Fragments were identified by ultraviolet transilluminator and excised, and a small fraction (~5 µl of each band) was mixed together and subjected to the same reaction conditions as was done previously but in the absence of the complementary mutagenic primers. When a second round of PCR
was carried out under the same conditions as done initially, but in the presence only of primers GSO10L and GSO15L, a hybrid PCR product was generated containing the mutation of interest. Annealing of the two primary PCR products was facilitated by the complementary nature of the 3’ ends of the fragments generated during the primary reaction. Upon completion the reactions were extracted with an equal volume of chloroform before and digested with restriction enzymes XbaI (12 units) and Xhol (8 units) 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. Digested fragments were recovered from 1.75% low gelling temperature agarose gels as before and substitute for the wild type RD1 N-terminal fragment of pGS7 (20) to create the plasmids pGS10L, pGS15L, pG517, pGS15, and pG522 containing the deletions/substrate substitution ΔPFT-7, ΔC8-K13ΔR25, ΔP1P1H20, and the alanine cassette substitution Ala14 (14–20), respectively.

Membrane Association Assay—This was carried out as described previously by us in some detail (20). Here we used a chimera formed between the N-terminal first 100 amino acids of RD1 fused, in frame, to the N terminus of CAT (20). This has been shown (20) to become coupled transcription/translation system (Promega) was used to generate mature proteins from plasmid DNA. Post-translation duplicate aliquots were taken, and either TEN buffer or TEN buffer containing a reduced rate of backbone amide exchange in residues Cys8, Glu9, and Thr10 (−2 ppb/K). The α helical region appears to end with a 3α helical hydrogen bond, Cys8N–C13H, with the side chain of Asp5 (the NOE dN(i,i+3), for residues Met1–Val8 providing supporting evidence for this ion pair). This interaction may offset the destabilizing effect of the charged N terminus upon the helical dipole (28, 29).

Examination of the distribution of NOEs shown in Fig. 2 indicates that very few restraint were identified in the region Cys11–Pro14. This area has the most poorly defined backbone and side chain conformation in the calculated structure, and it may correspond to region of high mobility, forming a “hinge” in the splice region of RD1. This point is illustrated by the aligned calculated structures shown in Fig. 3 in which either the C terminus or the N terminus needs to be aligned independently. The backbone NOE plot is particularly poorly defined at residues Cys13 and Ser15. The reason for termination of the α helix following residue Cys11 is not clear, although the presence in this area of so many consecutive residues with side chains capable of hydrogen bonding with the peptide backbone (e.g., Thr10, Cys11, or Ser15) might be expected to result in the interruption of the α helix by competition with the helical backbone hydrogen bonds (30). Among the few NOEs observed over this region, there are indications of an irregular β-turn between residues Cys11–Pro14 (e.g., located by the medium strength NOE Ser12–Thr10). The absence of other NOEs, there are few positive clues as to the nature of the side chain backbone hydrogen bonding patterns, and so a rather poorly ordered arrangement of the side chains is indicated.

A second distinct helical region is seen in the C-terminal portion of the peptide, extending from Pro14 to Lys24. This helical domain is formed independently of the other structures seen in the peptide. The characteristic NOEs present are: dN(i,i+3) for residues Pro14–Val17, Trp15–Glu18 (present but weak in the NOEY spectra with 200 ms of mixing time, absent at 100 ms), Val17–Trp20, Glu18–Asp21 (weaker), Trp19–Glu22, and Asp25–Lys24, and dN(i,i+3) NOEs are seen for residues Pro14–Val17, Val17–Trp20, Glu18–Asp21, and Asp25–Lys24. Although a significant number of the NOEs expected in a classical α helix were observed, the pattern of connectivities over this region was incomplete. For example, for residues Trp15 and Leu16, the α helical backbone NOEs are either absent or very weak. In addition, from a comparison of the strengths of dN(i,i+1) NOEs with those in the N-terminal domain, it is apparent that the α helical tendency is weaker in this C-terminal region. In a similar fashion, the reduction in backbone amide exchange rates was much less pronounced than in the N-terminal helix, so that although hydrogen bonding is present (e.g., Leu16–Cδ-O–Trp20N and Asp21–Cδ-O–Lys24H), it is relatively weak in the C-terminal region. As a consequence, the helical structure calculated in this area has a somewhat distorted backbone. However, it is not to say that the conformation in this area is poorly defined. The segment of the peptide between residues Pro14–Trp20 is composed principally of large hydrophobic residues forming a distinct, compact hydrophobic
cluster (see Fig. 4). The conformation of this region is defined largely by the numerous NOEs between these side chains. Because the region is helical, many of these interactions are of the type \(i, i+3\) and \(i, i+4\) (summarized in Fig. 2; Refs. 31 and 32). It appears that the overcrowding of the bulky side chains in the cluster is a principal contributor to the distortion of this region away from regular \(\alpha\)-helix. Thus the best description of the conformation over this section of the peptide is an irregular helix formed of multiple overlapping turns, defined in large part by side chain interactions. It should be noted that from the NOEs observed, the calculated structure may reflect a time averaged conformation for the peptide undergoing a dynamic transition between, for example, \(\alpha\) and 3\(10^\circ\)-helix (e.g., Refs. 33–35). Towards the C terminus of the peptide (residues 21–25), the regular pattern of \(\alpha\)-helical NOEs is resumed with the calculated structure adopting that of a regular \(\alpha\)-helix.

There are several noteworthy features in this C-terminal helix. Firstly, the presence of so many large hydrophobic residues over the region Pro\(^{14}\)-Trp\(^{20}\) in a helical conformation is relatively rare. However, other examples are known as can be
seen, for example, in the helix 77–94 of actin (36). The initiation of the C-terminal helix we describe in this study appears to be associated with Pro14, and in this regard, the involvement of proline in the first turn of an \( \alpha \)-helix is well known (37). However, the orientation of the side chains of residues Pro14 and Trp15 is unusual. There are strong inter-residue NOEs, indicating numerous close contacts between the "stacked" flat surfaces of the rings. Over the rest of the hydrophobic cluster the distribution of the side chains is also distorted, as compared with the ideal \( \alpha \)-helix, so that the large tryptophan rings, in particular, spread out more uniformly around the helical cylinder than might normally be expected and, in addition, lie closer to the peptide backbone in a position in which they become more protected from the solvent. As a consequence, there is an especially large number of \( \text{i, i+3} \) NOEs in this hydrophobic domain, as compared with the \( \text{i, i+4} \) side chain connectivities, which are most common in regular \( \alpha \)-helix (see Fig. 2). There are numerous NOEs to the side chain of Leu16, which forms interactions with the side chains of all three tryptophan residues 15, 19, and 20, making it the central residue in a highly distinctive hydrophobic patch. In contrast, the side chain of residue Val17 has many fewer connectivities and lies relatively exposed, pointing out from the opposite "face" of the peptide, forming a second possible site for specific interactions. The \( \alpha \)-helical backbone NOEs are disrupted for residues Trp15 and Leu16, and the helical NOEs \( \text{d}_{\text{a}}(\text{i, i+2}), \text{d}_{\text{a}}(\text{i, i+3}) \) are absent for residues Leu16-Trp19. This, together with the protection of the amide protons in this area from solvent exchange, means that the hydrogen bonding is weak. Consequently, there is a considerable disruption of the helical backbone in this domain and, in particular, the backbone dihedral angles for both Val17 and Gly18 lie outside the most favored region for regular \( \alpha \)-helix in the Ramachandran plot. This disruption corresponds to a degree of conformational freedom around residues Val17–Gly18 presumably arising from the cumulative difficulty of accommodating the tightly packed side chains (especially that of Val17, which is branched close to the backbone at C\( \beta \)), and also the small size of Gly18, which provides some space for movement. This conformational freedom around Val17–Gly18 serves to separate residues of this compact, hydrophobic domain from the residues C-terminal to Glu22.

In small helices, interactions at the termini are a substantial source of stability, and for the C-terminal helix, there appear to be several stabilizing interactions with residues Lys24 and Arg25. Firstly, there will be a favorable interaction of the positively charged side chains with the helix dipole. In addition, an ion pair interaction Asp21–Lys24 is indicated by the NOEs Asp21\( \text{H}_{\alpha} \)-Lys24(HN, H\( \gamma \), and H\( \beta \)). It also appears that the
residue Arg$^{25}$ adopts the role of a C-cap, stabilizing the final turn of the helix by the formation of a hydrogen bond between the arginine side chain and the C$^\alpha$O of Gln$^{22}$ (NOEs: Gln$^{22}$ H$^\alpha$–Arg$^{25}$ H$^d$ and a very weak Gln$^{22}$HN–Arg$^{25}$H$^d$; see Ref. 35).

Deletion Analysis of RD1-CAT Chimera Used to Identify the Membrane Association Domain—

The structural analysis presented here, however, identifies distinct structural domains within this splice region, which encompasses two separate helical regions separated by a highly mobile hinge region. The first helical region comprises a well formed, amphipathic $\alpha$-helix, whereas the second helical region contains a distinct, compact, highly hydrophobic domain (residues 14–20) having a distorted helical structure. This domain is rich in tryptophan residues and is highly ordered with many interactions occurring between the side chains. In order to address whether membrane association was attributable to one of these distinct structural domains, we undertook a deletion analysis. This was done using a chimera formed between an N-terminal region of RD1 fused, in-frame, to the N terminus of CAT (20). In this assay we generated mature radiolabeled chimera using an in vitro transcription/translation system. Such chimera were then incubated with membranes, and subsequently, a membrane pellet and supernatant fraction were isolated. From this it was possible to identify whether specific chimera became membrane-associated.

We have shown previously (20) that although CAT itself did not associate with membranes, 1-25 RD1-CAT did. Similarly, the chimera 1–100 RD1-CAT was membrane-associated, whereas the N-terminal truncated species, 26–100 RD1-CAT, which reflected deletion of the splice region, remained fully soluble (20). This exemplifies the importance of the first 25 amino acids of RD1 in conferring membrane association (9, 19, 20) and showed that the mere addition of extra N-terminal sequence to CAT, as in 26–100 RD1-CAT, was insufficient to elicit membrane association. The presence of sub-optimal Kozak sequences, preceding Met$^26$ and Met$^{37}$, also allowed for the generation of the N-terminal truncated species 26–100 RD1 and, to a lesser extent, 37–100 RD1 in the in vitro synthesis system (20). These N-terminal truncated species did not become associated with membranes, thus providing internal controls in the association assay (20).

Four truncations were selected to probe functional attributes. These were (i) deletion of the hydrophobic region of the N-terminal, amphipathic $\alpha$-helical domain (residues 2–7), (ii) deletion of the polar region of this amphipathic $\alpha$-helical domain together with the hinge region (residues 8–13), (iii) deletion of the compact, hydrophobic, tryptophan-rich domain formed within the second helical region (residues 14–20), and (iv) deletion of the polar end of the second helical region (resi-
dues 21–25). Using this system we showed that as before (20), the 1–100 RD1-CAT expressing plasmid generated two major species representing 1–100 RD1-CAT and 26–100 RD1-CAT, of which only the full-length 1–100 RD1-CAT became membrane-associated (Fig. 5).

Deletions in the splice region of RD1 were then made using the primer pairs detailed in the Methods section. Analysis (Fig. 5a) of mature chimeric proteins containing the deletions ΔPro2- Phe7 (ΔP2-F7), ΔCys8–Lys13 (ΔC8-K13), and ΔAsp21–Arg25 (ΔD21-R25) showed that these all became membrane-associated. In marked contrast to this, the mutant chimera expressing the deletion ΔPro14–Trp20 (ΔP14-W20) was unable to become membrane-associated (Fig. 5). This suggests that the information that is critical for membrane association lies within this compact, highly hydrophobic, tryptophan-rich domain.

In all these constructs, save that for the ΔAsp21–Arg25 deletion, an additional species, Met26–100 RD1-CAT, was formed and observed as a faster migrating species upon electrophoresis (Fig. 5a). We have postulated (9, 12, 20) that Met26–100 RD1-CAT was generated as a result of the presence of a sub-optimal Kozak sequence (39) preceding Met26, producing an N-terminal truncated species. This sub-optimal Kozak sequence (AGGATGG) (39) preceded Met26 in all of these RD1-CAT chimeras save that of the ΔAsp21–Arg25 deletion where it was destroyed upon engineering this particular deletion through the removal of the 15 nucleotides directly upstream of Met26. However, there is a further sub-optimal Kozak sequence found in these chimeric species, namely one that would be predicted to generate Met37–100 RD1-CAT and whose production we have noted previously, albeit to a lesser extent than for Met26–100 RD1-CAT (20). In our studies on the ΔAsp21–Arg25 deletion, we also observed the production of soluble species (Fig. 5a), which migrated on SDS-polyacrylamide gel electrophoresis at a position consistent (20) with that expected for Met37–100 RD1-CAT. Presumably the production of the N-terminal truncate occurred through transcriptional starts occurring at Met37 in RD1.

1H NMR analysis showed that the stretch of amino acids given by Pro14–Trp19 forms a discrete, compact domain that is stabilized by hydrogen bonding and Van der Waals interactions. This is, however, an intensely hydrophobic domain. Thus, in order to investigate whether membrane association was a function of the hydrophobicity of this domain per se, we replaced residues Pro14–Trp20 with a “cassette” of seven alanine residues. Expression of this mutant chimera showed, however, that it failed to become membrane-associated (Fig. 5b). Thus the ability of RD1 to become membrane-associated is not due to a nonspecific hydrophobic interaction at this point within the splice region. Rather, it would appear to be determined by the unique structural properties of this tryptophan-rich domain.

Conclusions—All four PDE4 genes encode multiple splice variants, due to 5' domain swapping, yielding proteins with distinct N-terminal regions (4, 11, 17, 18, 40). This study provides the first structural analysis of a splice region of a cyclic nucleotide phosphodiesterase. We show here that the N-terminal splice region of RD1 forms a distinct structure provided by two independently folding, helical regions separated by a mobile hinge region. That such a relatively small region should have such a distinct structure is consistent with it exhibiting particular biological properties, namely conferring absolute membrane association on RD1 and attenuating the catalytic activity of this enzyme (9, 12, 19). Structural studies, coupled with deletion analyses, have now allowed us to identify the domain with this N-terminal splice region, which confers membrane targeting upon RD1. This is a well defined, compact,
intensely hydrophobic domain encompassing residues Pro14–Trp20. It is, however, difficult to see how this hydrophobic region could insert directly into a membrane bilayer, and thus we consider it much more likely that membrane association is conferred through protein-protein interaction between RD1 and a membrane “anchor” protein. Such an interaction might offer an explanation for our observations that RD1 exhibits a distinct subcellular distribution in both brain and transiently transfected COS cells (9, 19), where it is found associated with both plasma membrane and Golgi fractions, and also in stably transfected human thyroid carcinoma cells, where it is expressed uniquely in the Golgi. For, if RD1 was able to insert generally into lipid bilayers, then one might expect this PDE to have been distributed nonspecifically among all intracellular membranes. The presumptive membrane protein anchor that interacts with this domain of RD1 is unknown. However, the means of interaction, although likely to be intensely hydrophobic in nature, is clearly not nonspecific as replacement of the targeting domain formed by Pro14–Trp20 with a cassette of seven alanine residues failed to allow for membrane association. Thus distinct structural features of this domain of RD1 appear to be required for membrane association to occur. Nevertheless, the underlying hydrophobic basis of such an interaction may explain why it seemingly can be disrupted by very low levels of detergent (9, 19, 20), and, because it is likely to be of high affinity, would explain why all observable RD1 is membrane-associated in brain and in transfected COS cells (12) and transfected thyroid cells.2

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