Protein Science (1999), *8*:1515–1522. Cambridge University Press. Printed in the USA.
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Structural characterization of the membrane-associated regulatory subunit of type I cAMP-dependent protein kinase by mass spectrometry: Identification of Ser81 as the in vivo phosphorylation site of RIα

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(RECEIVED August 5, 1998; ACCEPTED March 31, 1999)

Abstract

The mechanism by which the type Iα regulatory subunit (RIα) of cAMP-dependent protein kinase is localized to cell membranes is unknown. To determine if structural modification of RIα is important for membrane association, both beef skeletal muscle cytosolic RI and beef heart membrane-associated RI were characterized by electrospray ionization mass spectrometry. Total sequence coverage was 98% for both the membrane-associated and cytosolic forms of RI after digestion with AspN protease or trypsin. Sequence data indicated that membrane-associated and cytosolic forms of RI were the same RIα gene product. A single RIα phosphorylation site was identified at Ser81 located near the autoinhibitory domain of both membrane-associated and cytosolic RIα. Because both R subunit preparations were 30–40% phosphorylated, this post-translational modification could not be responsible for the membrane compartmentation of the majority of RIα. Mass spectrometry also indicated that membrane-associated RIα had a higher extent of disulfide bond formation in the amino-terminal dimerization domain. No other structural differences between cytosolic and membrane-associated RIα were detected. Consistent with these data, masses of the intact proteins were identical by LCQ mass spectrometry. Lack of detectable structural differences between membrane-associated and cytosolic RIα strongly suggests an interaction between RIα and anchoring proteins or membrane lipids as more likely mechanisms for explaining RIα membrane association in the heart.

Keywords: compartmentation; mass spectrometry; phosphorylation; post-translational modification; type Iα cAMP-dependent protein kinase

cAMP-dependent protein kinase (PKA) is a tetrameric enzyme composed of a regulatory subunit dimer and two catalytic subunits. In mammalian tissue it occurs in two major classes, PKA I and PKA II, that are distinguished by the properties of four distinct regulatory subunit gene products (RIα, RIβ, RIIα, and RIIβ) (Døskeland et al., 1993; Brandon et al., 1997). Although physiological roles for PKA isozymes are not fully resolved, differential subcellular localization of the regulatory subunits is one way in which isozyme-selective functions may be conferred. For example, PKA II can associate with multiple subcellular compartments through the interaction of RIIα or RIIβ with A-kinase anchoring proteins (AKAPs) (Rubin, 1994; Dell'Acqua & Scott, 1997).

The PKA Iα isoform has historically been classified as a cytosolic enzyme (Corbin et al., 1977). Exceptions to this rule have included PKA I or RI association with erythrocyte membranes (Rubin, 1979), T lymphocyte plasma membranes (Hasler et al., 1992; Skålhegg et al., 1994), and cardiac myocyte sarcolemma (Robinson et al., 1996; Reinitz et al., 1997). Recently, through the use of the yeast two-hybrid system, RI has been shown to interact with AKAP-like proteins such as D-AKAP1 (Huang et al., 1997a), D-AKAP2 (Huang et al., 1997b), and cytochrome *c* oxidase (Yang et al., 1998), all of which may potentially target the RI subunit to mitochondrial membranes. These anchoring proteins, however, either

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Abbreviations: AKAPs, A kinase anchoring proteins; CID, collision-induced dissociation; HPLC, high-performance liquid chromatography; LC/MS, electrospray mass spectrometry; LC/MS/MS, tandem mass spectrometry; PKA, cAMP-dependent protein kinase; RIα, type Iα regulatory subunit of cAMP-dependent protein kinase.

preferentially bind RII (Huang et al., 1997a, 1997b) or have not been tested for their isozyme specificity (Yang et al., 1998). The RI subunit, but not RII, has been detected in immunoprecipitates of EGF receptors in MCF-10A epithelial cell lysates (Tortora et al., 1997). This interaction is apparently through the SH3 domains of Grb2, suggesting an anchoring mechanism distinct from AKAPs. Finally, a nonclassical AKAP from *Caenorhabditis elegans* has been identified that interacts with the RI-like protein from that organism but not with mammalian RIIα or RIIβ (Angelo & Rubin, 1998). It is not yet clear if any of these RI interacting proteins can account for the extensive membrane association observed in erythrocytes, T lymphocytes, or cardiac myocytes.

Three models are proposed to explain differential compartmentation of RI: (1) The R subunit may interact with membrane-associated RI-specific anchoring proteins in a manner similar to AKAPs, (2) membrane-associated RI may interact directly and noncovalently with the lipid bilayer as does protein kinase C (Orr & Newton, 1992; Zhang et al., 1995), or (3) the membrane-associated RI may be targeted to membranes via post-translational modifications such as palmitoylation (Milligan et al., 1995; Wedegaertner et al., 1995; Fraser et al., 1998; Gray et al., 1998). In this paper, mass spectrometric analysis of the purified membrane-associated and cytosolic forms of RI was undertaken to test the third possibility. In the process, membrane-associated RI was identified as the same gene product as cytosolic RIα, the in vivo phosphorylation site of RIα was defined as Ser81, evidence for variable extents of disulfide bond formation in the amino-terminal dimerization domain was observed, and a tendency for the C-terminus to be proteolytically clipped during purification was noted. No structural differences between the membrane-associated and cytosolic forms of RIα were detected that could explain localization. This strongly suggests that the protein–protein or protein–lipid interaction model accounts for RIα membrane association in the heart.

Results

Purification of membrane-associated RIα

A bovine heart was chosen as the source for purification of detergent-solubilized RI because it has a proportionately high amount of RI relative to RII in the particulate fraction (Reinitz et al., 1997). Purification of cytosolic RI from a bovine heart was not practical, due to the large amount of contaminating RII in this compartment. Instead, bovine skeletal muscle was used. The soluble RIα purified from this tissue was the source for the complete amino acid sequence determination of this protein (Titani et al., 1984). Purification of RI from beef heart particulate fractions required modification from the standard protocol designed for purification of the cytosolic form. This included homogenization of the tissue in high-salt buffer, followed by extensive washing of the pellet and extraction of the pellet with Triton X-100. To get efficient recovery of detergent solubilized RI at the cAMP-affinity chromatography step, it was necessary to incubate the R subunit with an equimolar amount of PKA catalytic (C) subunit. This stimulated the release of cAMP, which allowed subsequent binding of the protein to the affinity column. Figure 1 illustrates the purity of representative preparations of full-length membrane-associated and cytosolic RI. The term membrane-associated RIα will henceforth refer to purified Triton X-100 solubilized RIα from the particulate component of beef heart tissue.

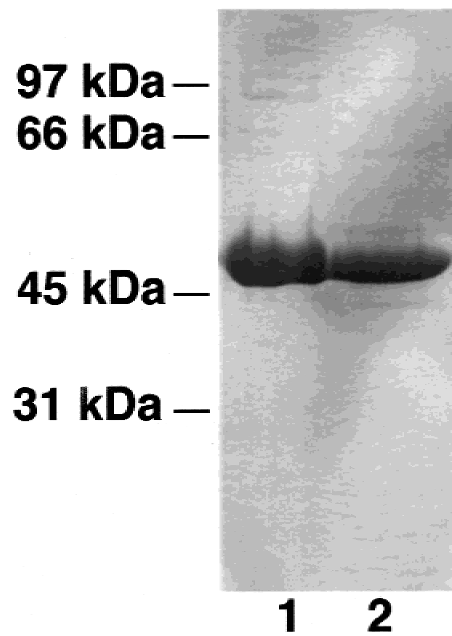

Fig. 1. Polyacrylamide gel electrophoresis of purified cytosolic and membrane-associated RIα. Five micrograms of cytosolic RIα (lane 1) and 5 μg of membrane-associated RIα (lane 2) were incubated in a boiling water bath for 5 min in the presence of 5 μL 10% SDS, 5 μL 2-mercaptoethanol, and 2 μL of a sucrose/bromophenol blue solution. The samples were applied to a 10% SDS-polyacrylamide gel and subjected to electrophoresis according to Laemmli (1970).

Overall sequence coverage

Soluble and membrane-associated forms of RI were analyzed by tandem mass spectrometry (LC/MS/MS) in an attempt to identify unique ions that could represent structural differences between the two proteins. Peptides generated by AspN digestion were separated on capillary HPLC columns directly coupled to the mass spectrometer, and were identified in electrospray mass spectrometry (LC/MS) chromatograms by comparing observed masses to masses of predicted proteolytic fragments. Approximately 94% of the total amino acid sequence was subsequently determined by LC/MS (Table 1). Some short, hydrophilic peptides were not observed in AspN digests. These included peptides D3, D7–D9, D11, D13, and D15. Using different R subunit preparations, the experiment was repeated with trypsin proteolysis (Table 2). Because trypsin digestion resulted in many very small peptides, and one very large poorly ionizable fragment (T24), only 38% of the RI sequence could be determined. Even so, the sequences of the unidentified D3 and D7 AspN fragments and part of D8 were accounted for in the tryptic map. The total sequence coverage was therefore 98% for both the membrane-associated (Fig. 2) and cytosolic forms of the enzyme (not shown). Identities of most peptides were confirmed by collision induced dissociation (CID) at the orifice or by LC/MS/MS. The undetected sequences were SDIF (145–148), DEG (167–169), and DQGEM (176–180).

Because LC/MS sequence coverage was not 100%, the proteins were subjected to electrospray ion trap mass spectrometry to compare the molecular weights of the intact proteins (Table 3). Experimentally determined molecular weights of membrane-associated and cytosolic RI were identical, indicating no other detectable

Table 1. *Fragments of AspN protease-digested cytosolic and membrane-associated RIα detected by LC/MS/MS*

Peptide	Calculated mass	Observed mass cytosolic RIα	Observed mass membrane RIα
Ac-D1	3,446.82[a]	3,446.91[a]	ND
$(Ac\text{-}D1/2)_2$(S-S)	16,903.24[a]	16,902.52[a]	ND
$(Ac\text{-}D1/2)_2(S\text{-}S)_2$	16,901.24[a]	16,900.52[a]	16,900.85[a]
D2	5,023.79[a]	5,024.26[a]	ND
D4	3,220.60[a]	3,221.12[a]	3,220.97[a]
P-D4	3,300.60[a]	3,300.71[a]	3,300.62[a]
D5	1,345.78	1,345.83	1,345.71
D5/6	3,692.40[a]	3,693.02[a]	3,692.97[a]
D6	2,363.82[a]	2,363.99[a]	2,363.98[a]
D10	1,910.19[a]	1,910.52[a]	1,910.34[a]
D12	769.37	769.03	769.45
D14	4,684.30[a]	4,684.62[a]	4,684.72[a]
D14/15	4,955.58[a]	4,956.31[a]	4,956.80[a]
D16	3,750.46[a]	3,751.15[a]	3,751.33[a]
D16b	1,492.81	1,492.98	ND
D17	1,116.60	1,116.51	1,116.68
D18	1,046.50	1,046.21	1,046.29
D19	1,225.64	1,225.74	1,225.62
D20	3,595.00[a]	3,595.82[a]	3,595.53[a]
D21	3,202.90[a]	3,203.59[a]	3,203.40[a]
D22	1,530.78	1,530.75	1,530.80
D23	2,124.43[a]	2,124.69[a]	2,124.80[a]
D23 (-SV)[b]	1,938.23[a]	1,937.98[a]	1,938.28[a]
D23 (-LSV)[c]	1,825.07[a]	1,825.47[a]	1,824.99[a]

[a]Average mass was calculated from ions that are +2 and greater. All other values are monoisotopic masses of +1 ions. ND, not detected.
[b]D23(-SV) indicates the C-terminal peptide minus the last two residues.
[c]23(-LSV) indicates the C-terminal peptide minus the last three residues.

Table 2. *Fragments of trypsin protease-digested cytosolic and membrane-associated RIα detected by LC/MS/MS*

Peptide	Calculated mass	Observed mass cytosolic RIα	Observed mass membrane RIα
T3	1,010.48	1,010.52	1,010.67
T3/5	3,214.80[a]	3,214.90[a]	3,214.30[a]
T4	935.56	935.64	935.63
T5	2,203.63[a]	2,203.65[a]	2,203.65[a]
T6	714.33	714.30	ND
T11/12	1,947.14[a]	1,946.70[a]	1,947.00[a]
T12	1,516.79	1,516.20	1,516.50
P-T12	1,596.79	1,596.90	1,597.20
T17	1,931.05[a]	1,932.00[a]	1,931.70[a]
T23	1,457.70	1,457.70	1,457.70
T31	889.51	889.63	889.54
T34/35	1,173.58	1,173.54	1,173.55
T35	1,045.49	1,045.57	1,045.55
T36	1,002.57	1,002.56	1,002.51
T36/37	1,473.79	1,473.75	1,473.90
T39	2,587.97[a]	2,588.70[a]	2,588.40[a]
T40/41	1,449.69	1,449.60	1,449.60
T41	1,293.59	1,293.45	1,293.60
T42b	1,393.72	1,393.50	1,393.50
T48	1,043.58	1,043.51	1,043.68
T48/49	1,199.68	1,199.62	1,199.60
T50	1,497.75	1,497.75	1,497.90
T50 (-SV)[b]	1,311.65	1,311.60	1,311.75

[a]Average mass was calculated from ions that are +2 and greater. All other values are monoisotopic masses of +1 ions. ND, not detected.
[b]T50(-SV) indicates the C-terminal peptide minus the last two residues.

post-translational modification of the remaining 2% of the sequence not determined by LC/MS.

A clear signal at +80 Da was not detected in either preparation. This was most likely due to a weak signal of the intact protein combined with substoichiometric phosphorylation at Ser81. Based on the identical sequence data obtained from the two tandem mass spectrometry experiments and electrospray ion trap mass spectrometry of the full-length R subunits, it was concluded that the membrane-associated and cytosolic forms of RI were the same RIα gene product. Therefore, there were no striking differences in post-translational modification that could account for membrane association of RIα.

In vivo phosphorylation

Because the degree of phosphorylation can also affect the localization of proteins (Ito et al., 1997; Wartmann et al., 1997), a careful search for Ser/Thr-containing peptide fragments having masses 80 Da greater than expected was made. As indicated in Table 1 and Figure 2, a phosphorylated form of D4 (P-D4) was observed in both the cytosolic and membrane-associated forms of RIα with a mass of 80 Da greater than peptide D4 (residues 78–106). The extents of phosphorylation in the AspN digest experiment were 27% for soluble RIα and 11% for the membrane-associated form as determined by the relative intensities of the phosphorylated and unphosphorylated D4 peptide in the LC/MS chromatogram (not shown). The parent ion exhibited neutral loss of phosphoric acid as determined by LC/MS/MS. This indicated that the phosphorylation was on one or more Ser or Thr residues but not on a Tyr residue (Resing & Ahn, 1997). Further fragmentation, however, was insufficient for sequencing. To precisely locate the phosphorylation site and get a better estimate of the phosphorylation state, soluble and membrane-associated RIα were purified in the presence of the phosphoprotein phosphatase inhibitors fenvalerate, cantharidin, and α-naphthyl acid phosphate. Digestion of these preparations of RIα with trypsin resulted in two smaller more easily sequenced peptide fragments, T12 and T17, that spanned D4, plus the intervening fragments T13–16 (Gly-Arg, Arg, Arg, Arg), which were not recovered. T17 was not phosphorylated, but a phospho-form of T12 was detected (Table 2; Fig. 2). The phosphopeptide P-T12 consisted of residues 77–90 (EDEISP PPPNPVVK). Because Ser81 was the only serine or threonine in the sequence, this was designated as the phosphorylation site. This was confirmed by CID sequencing (Fig. 3). The extents of phosphorylation in membrane-associated and soluble forms of RIα were approximately 30–40% as determined by the relative intensities of T12 to P-T12 (not shown). Therefore, there was no significant difference in phosphate content between membrane-associated and cytosolic forms of RIα.

Cysteine residues

To preserve potential lipid modifications of the four cysteine residues of RIα, all protein was purified in the absence of reducing

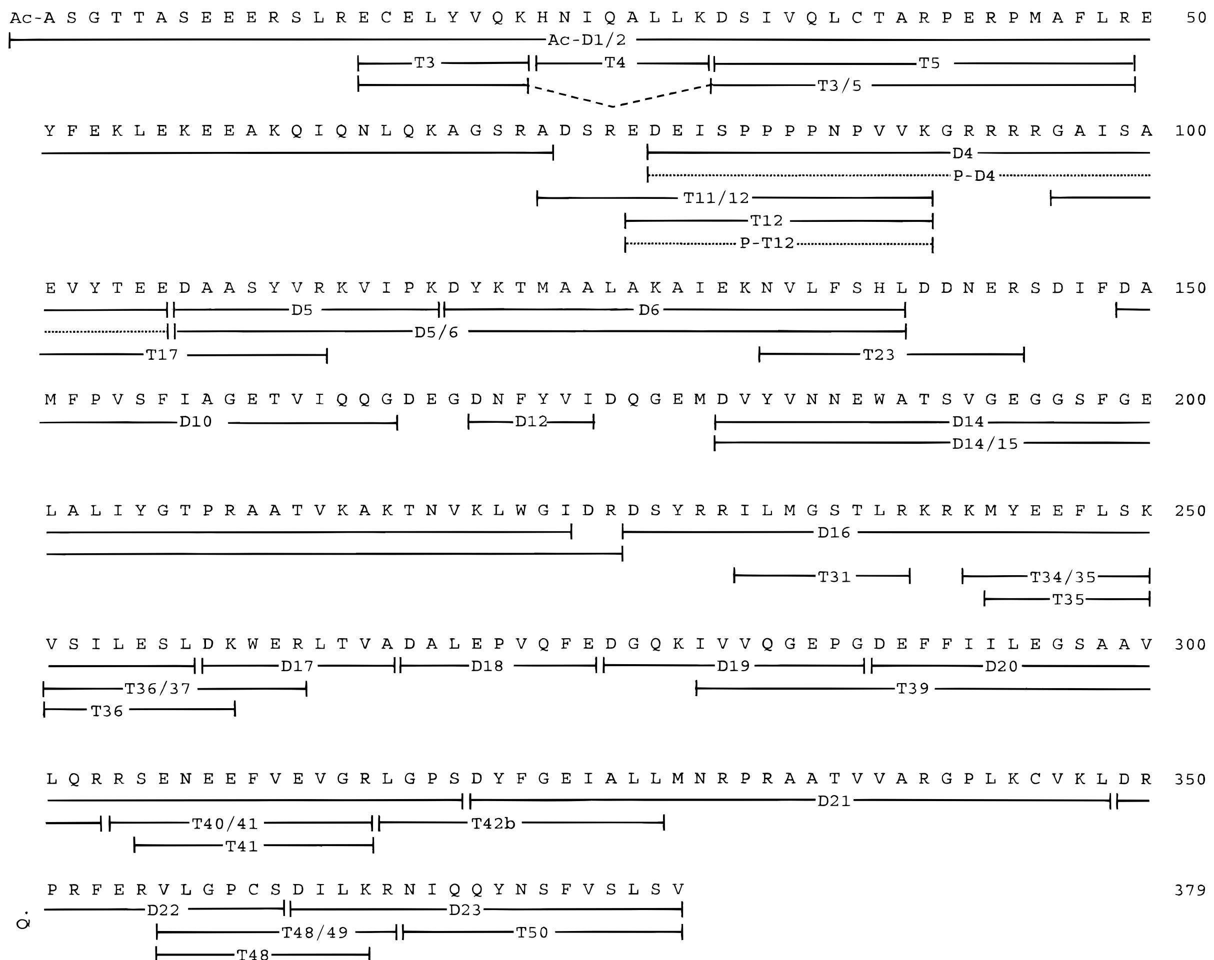

Fig. 2. Coverage map of membrane-associated RIα. Membrane-associated RIα was digested with AspN protease or trypsin and analyzed by LC/MS. Peptides that were identified by comparing their observed masses with calculated masses are indicated (Tables 1, 2). The dashed line in fragment T3/5 indicates intersubunit disulfide bonding between T3 and T5 in the absence of T4. The dotted lines represent phosphopeptides. Ac-D1/2 was detected as the disulfide bonded dimer (Ac-D1/2)$_2$(S-S)$_2$.

agents, and samples were not reduced and carboxymethylated prior to protease digestion and LC/MS. Analysis of AspN digests of preparations indicated that membrane-associated RIα had a higher degree of intersubunit disulfide bonding via Cys16 and Cys37. An ion series with a mass consistent with two amino-terminal peptides cross-linked with two disulfide bonds (Ac-D1/2)$_2$(S-S)$_2$ was recovered after digestion of membrane-associated RIα with AspN (Table 1). HPLC of AspN-digested cytosolic RIα showed two eluting peaks of this peptide complex—one with a mass consistent with one disulfide cross-link (Ac-D1/2)$_2$(S-S) and the other consistent with (Ac-D1/2)$_2$(S-S)$_2$. The relative intensities of the (Ac-D1/2)$_2$(S-S) ions were 5% those of (Ac-D1/2)$_2$(S-S)$_2$ (not shown). A significant amount of uncross-linked Ac-D1 and D2 peptides was also seen in the cytosolic RI chromatogram, but their abundance relative to the cross-linked Ac-D1/D2 fragment could not be quantified. Likewise, the abundance of T3 and T5 relative to T3/T5 in the trypsin data (Table 2) could not be quantified for either the membrane-associated or cytosolic RI. The reason for the qualitatively higher degree of disulfide bonding in membrane-associated RIα relative to cytosolic RIα is not certain, but may be attributed in part to different purification schemes. For example, unlike the cytosolic RIα, membrane-associated RIα was exposed to air during multiple homogenization steps after removal from the reducing environment provided by the cytosol. The antiparallel association of the RIα amino-termini, demonstrated by intersubunit disulfide bond formation between Cys16 and Cys37 (Bubis et al., 1987), was confirmed by the observation of a trypsin-generated fragment T3/5 (Table 2; Fig. 2) but not T3/3 or T5/5. The remaining cys-

Table 3. *Masses of full-length cytosolic and membrane-associated RIα as determined by electrospray ion trap mass spectrometry*[a]

	Observed mass[a]
Cytosolic RIα	42,804
Membrane-associated RIα	42,805

[a]Observed mass was calculated from +2 ions. Units are in Daltons (Da). The calculated average mass of monomeric acetylated RIα is 42,804 Da.

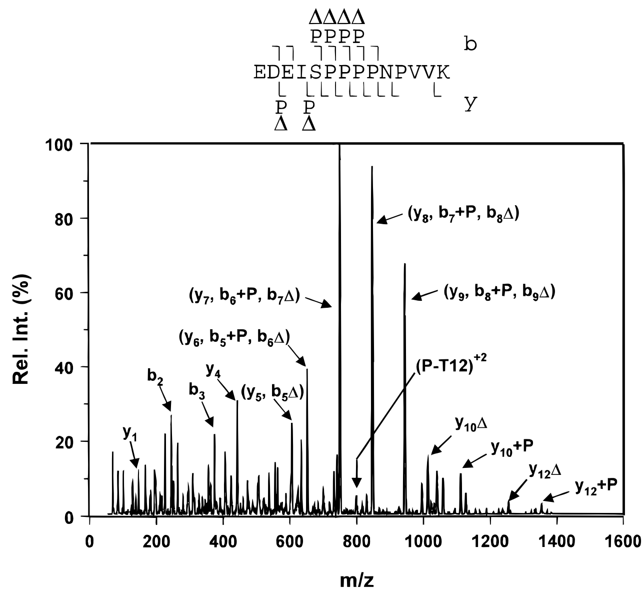

Fig. 3. Identification of Ser81 as the in vivo phosphorylation site of RIα. The +2 ion of the phosphopeptide P-T12 from membrane-associated RIα (Table 2) was subjected to LC/MS/MS as described in Materials and methods. Amino-terminal "b" ions and carboxyl-terminal "y" ions were produced by cleavage of P-T12 between amide nitrogens and carbonyls of its peptide backbone. Δ indicates neutral loss of phosphoric acid. Ambiguities in the designation of several ions are due to the similarities in average masses of y_6 and b_5 + P ions that are propagated through y_9 and b_8 + P by the series of four prolines in the middle of P-T12. Further complicating the interpretation is the similarity between proline (97 Da) and the difference between phosphoserine and the dehydroalanine formed from it upon neutral loss of phosphoric acid (98 Da). For example, the average masses of $b_8\Delta$ (847.90 Da), y_8 (848.04 Da), and b_7 + P (848.78 Da) differ by less than 1 Da. Experimental resolution of these masses was not achieved.

teine residues, Cys345 and Cys360, were unmodified in the AspN-digested preparation (D21, D22, Table 1) for both cytosolic and membrane-associated RIα. The trypsin experiment also showed that Cys360 was unmodified in both forms (T48), but T45, which contained Cys345, was not detected.

N- and C-termini

The N-termini of both soluble and membrane-associated RIα were stoichiometrically acetylated (Table 1, Ac-D1 containing peptides) as has been observed by others (Titani et al., 1984). Mass spectrometric analysis of peptide fragments from initial preparations of RIα showed differing degrees of C-terminal proteolysis of cytosolic and membrane-associated RIα [Table 1, fragments D23, D23(-SV), D23(-LSV); Table 2, fragments T50, T50(-SV)]. For example, the C-terminal peptide fragment D23 was detected more strongly with the membrane-associated RIα. In contrast, D23(-LSV), which lacked the last three residues, was the predominant C-terminal AspN fragment for cytosolic RIα. Subsequent preparations of RIα purified in the presence of the carboxypeptidase inhibitor, 1,10-phenanthroline, yielded mostly the intact C-terminal peptide T50 for both the soluble and membrane-associated forms of RIα. Other difference ions were detected, but in every case were attributed to contaminating proteins in the RIα preparation. No other reproducible structural differences between cytosolic and membrane-associated RIα were observed.

Discussion

Extensive mass spectrometric analysis of membrane-associated RIα and cytosolic RIα showed that the two were identical gene products and that no unique post-translational modifications correlated with membrane binding. Special care was taken to prevent the loss of modifications such as thio-acylations, which are sensitive to reducing agents (Pepperberg et al., 1995). Testing for this type of modification was of particular concern because other important signaling molecules have been shown to be targeted to the plasma membrane via palmitoylation of cysteine residues (Milligan et al., 1995; Wedegaertner et al., 1995; Fraser et al., 1998; Gray et al., 1998). All four cysteine residues were accounted for, including the carboxyl-terminal Cys345 and Cys360, which were unmodified. Although membrane-associated RIα had a higher extent of disulfide bond formation in the amino-terminal dimerization domain, this was not likely the cause for membrane localization. The absence of structural differences between membrane-associated and soluble RIα strongly suggests that RIα is localized to membranes in the heart either through protein–protein interactions or noncovalent protein–lipid interactions.

If membrane localization of RIα is mediated through protein–protein interactions, it is unlikely that the large amount of plasma membrane-associated RI found in erythrocytes (Rubin, 1979), T lymphocytes (Hasler et al., 1992), or cardiac myocytes (Robinson et al., 1996) is a result of interaction with any of the known RI anchoring proteins. The D-AKAP1 and D-AKAP2 (Huang et al., 1997a, 1997b) are thought to be targeted to mitochondria as is cytochrome *c* oxidase (Yang et al., 1998). Although Grb2 can potentially localize RI to plasma membrane receptors such as the epidermal growth factor (Tortora et al., 1997), it is unclear whether there is sufficient Grb2 to account for all membrane-associated RI that is present in the erythrocytes, lymphocytes, and myocytes. In the pig cardiac sarcolemma, for example, such an anchoring protein would need to be present in sufficient abundance to account for the association of 40 pmol of RI per mg of plasma membrane protein (Robinson et al., 1996) in a cellular environment in which 75–80% of the total cellular cAMP-binding activity is contributed by RII (Reinitz et al., 1997). It is therefore likely that the protein(s) responsible for the majority of RI membrane association has yet to be found.

The possibility that RIα may associate with membranes through direct interaction with phospholipids has yet to be adequately tested. Because RIα is predominantly a cytosolic protein, a mechanism would need to be invoked that would limit the number of available membrane sites. The studies described here rule out post-translational modification as one possibility. Ligand-induced conformational change leading to membrane insertion is one option that could be explored. A precedence for this latter scheme is the calcium and diacylglycerol-dependent membrane association of protein kinase C (Orr & Newton, 1992; Zhang et al., 1995).

The phosphorylation site at Ser81 was detected in both membrane and cytosolic RI, suggesting that this modification by itself cannot account for membrane localization. The in vivo phosphorylation of RIα was originally observed as a charge variant of RI in S49 lymphoma cells (Steinberg et al., 1978). Neither the function of this modification nor the protein kinase responsible for the phosphorylation have been determined. Until this paper, attempts to identify the in vivo phosphorylation site in RIα have been unsuccessful (Hashimoto et al., 1981; Steinberg, 1983). Modulation of the phosphorylation state of RIα in response to cAMP

elevating agents (Steinberg et al., 1978; Steinberg & Agard, 1981b; Russell & Steinberg, 1987) suggests, however, that the phosphorylation may serve some physiological purpose.

The location of Ser81 in the primary sequence of RIα suggests potential novel functions (Fig. 4). Its proximity to the autoinhibitory site (RRGA; residues 94–97) suggests a possible influence on the interaction between regulatory and catalytic subunits, though earlier studies on the RIα in vivo phosphorylation site discount this (Geahlen & Krebs, 1980; Geahlen et al., 1981). It is interesting to note that Ser81 is approximately the same distance from the C subunit autoinhibitory site as the heterologous in vivo phosphorylation site in RIIα is from its autoinhibitory site (Carmichael et al., 1982; Hemmings et al., 1982).

Ser81 is also adjacent to the sequence PPPP (residues 82–85), which is the only consensus SH3 domain binding site, Pro-x-x-Pro (Feller et al., 1994; Pawson, 1995), in RIα. This suggests that if RIα interacts with this class of proteins, the interaction could be modulated by phosphorylation of Ser81. The qualitative in vitro interaction observed by others between the Grb2 SH3 domains and RI (Tortora et al., 1997) makes this an attractive possibility.

Both of the heterologous phosphorylation sites in RIα and RIIα reside in potential PEST sequences (Fig. 4) defined as a hydrophilic stretch of 10 or more residues enriched with Pro, Glu/Asp, and Ser/Thr residues, and bounded by positively charged residues (Rogers et al., 1986; Rechsteiner & Rogers, 1996). This sequence motif is present in a number of proteins that have high rates of turnover mediated through the ubiquitin-26S proteasome pathway. Degradation rates of some PEST-containing proteins have been observed to modulate in response to phosphorylation of their PEST sequences (Yaglom et al., 1995; Lin et al., 1996; Schwarz et al., 1996). Because RIα has been demonstrated to undergo ubiquitin-dependent proteolysis (Hegde et al., 1993), there could be a relationship between Ser81 phosphorylation and RIα degradation.

Because of the proximity of the PEST sequence to the autoinhibitory site in RI, Rechsteiner and colleagues have suggested that the rate of RI degradation may be cAMP-dependent (Rogers et al., 1986; Rechsteiner & Rogers, 1996). They suggest that only when cAMP levels are elevated is the PEST sequence revealed upon dissociation of the catalytic subunit. This hypothesis is consistent with the observation by Steinberg and Agard that the 8.4 h half-life of RI in S49 lymphoma cells is reduced 10-fold upon elevation of intracellular cAMP (Steinberg & Agard, 1981a). These investigators also demonstrated that manipulation of cAMP levels in S49 lymphoma cells could affect the phosphorylation state of RI (Steinberg & Agard, 1981b; Russell & Steinberg, 1987), although a direct connection between phosphorylation state and decreased RI half-life could not be made (Steinberg & Agard, 1981b).

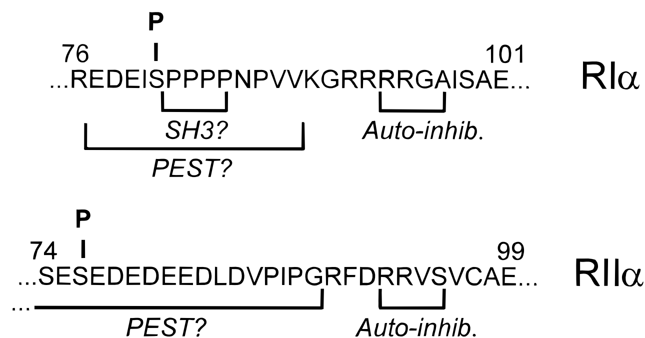

Fig. 4. Features of RIα and RIIα primary sequences near their heterologous in vivo phosphorylation sites. SH3, potential SH3 domain binding site consensus sequence (PXXP); Auto-inhib, C subunit autoinhibitory site (RRX(A/S)); PEST, potential PEST sequence.

On a technical note, the C-terminal truncation of cytosolic RIα that was consistently observed in four independent preparations of bovine skeletal muscle of RIα has implications for the in vitro characterization of RIα. Dissociation assays of the truncated cytosolic RIα show that the B-domain dissociation rate is faster than that of the intact membrane-associated RIα (K.M. Boeshans & J.B. Shabb, unpubl. obs.). This is consistent with the finding that a recombinant form of RIα truncated at the last five C-terminal residues has a B-domain dissociation rate three times faster than the full-length protein (Kapphahn & Shabb, 1997). The addition of 1,10-phenanthroline to the purification protocol is therefore recommended to avoid this kinetic heterogeneity.

Materials and methods

Materials

[2,8-^{3}H]cAMP (35 Ci/mmol) was from Amersham (Arlington Heights, Illinois); Triton X-100, fenvalerate, cantharidin, and α-naphthyl acid phosphate were purchased from Calbiochem (San Diego, California); 1,10-phenanthroline was from Sigma (St. Louis, Missouri); DE52 resin was from Whatman (Clifton, New Jersey); Ecoscint O was from National Diagnostics (Atlanta, Georgia). N_6-$H_2N(CH_2)_2$-cAMP Sepharose was prepared according to the method of Dills et al. (1979). Beef heart and beef skeletal muscle were obtained from a local processing plant and transported to the laboratory on ice. Soluble beef skeletal muscle RIα was purified from the supernatant fraction of 8 kg of beef skeletal muscle as in Rannels et al. (1983), except that following sample application, the N_6-$H_2N(CH_2)_2$-cAMP Sepharose affinity column was washed with 10 column volumes of KPE (10 mM potassium phosphate, 1 mM EDTA, pH 6.8) containing 0.75 M sodium chloride. The affinity column was equilibrated with 10 column volumes of KPE and RI was eluted after a 2 h incubation at room temperature with KPE containing 10 mM cAMP. The addition of protease and phosphatase inhibitors is as indicated below. A beef heart PKA catalytic subunit was purified according to Flockhart and Corbin (1984). All other chemicals were of reagent grade and were purchased from Sigma or Fisher (Pittsburgh, Pennsylvania).

Purification of membrane-associated RIα

Unless otherwise indicated, all steps were done at 4 °C. Ventricular muscle from beef hearts was trimmed of fat, major vessels, pericardium, and endocardium, cut into strips, and ground in a meat grinder. Tissue was divided into four 500-g portions. Each 500 g portion of the beef heart was homogenized in 1.5 L of KPE containing 0.75 M NaCl by three 30 s bursts at high speed with a Waring blender. The homogenate was centrifuged at $10{,}000 \times g_{max}$ for 30 min at 4 °C. Pellets were homogenized with 1.5 L of 0.75 M NaCl in KPE and the centrifugation was repeated. The pellets were washed twice more in 1.5 L of KPE by homogenization and centrifugation as above. The final washed pellets were resuspended in 1.5 L of KPE as above, and Triton X-100 was added to make a 1% solution that was incubated with stirring for 1 h. After centrifugation, the supernatant was collected and loaded batchwise onto 2 L

of DE52 Cellulose resin for 2 h, stirring every 10 min. The resin was washed with 15 L of KPET (KPE plus 0.01% Triton X-100) through a 3 L fritted glass funnel. The resin was poured into an 8 × 90 cm column, and the R subunit was eluted with 5 L of 0.35 M NaCl in KPET collecting 500 mL fractions. The R subunit was detected by assaying for $[^3H]$-cAMP binding activity (Sugden & Corbin, 1976). Fractions containing cAMP binding activity were incubated for 1 h with a one-to-one molar ratio of a beef heart PKA C subunit to an R subunit to release bound cAMP. The Triton X-100 solubilized membrane-associated R subunits were purified by N_6-$H_2N(CH_2)_2$-cAMP Sepharose affinity chromatography as described by Rannels et al. (1983) for cytosolic RIα. Separation of RIα from RII was achieved by chromatography of the cAMP-eluted fraction on a 3 mL DEAE-Sephacel column equilibrated with 30 mL KPET and eluted with a 60 mL linear 0–0.35 M NaCl gradient with KPET collecting 1.5 mL fractions. The R subunits were again detected by assaying for $[^3H]$-cAMP binding activity. Immediately after purification, aliquots were stored in the presence of 0.01% Triton X-100, frozen with liquid nitrogen, and stored at −70 °C to prevent its rapid degradation to a 37 K fragment. Some membrane-associated RIα preparations were purified in the presence of protease and phosphatase inhibitors. Yield was approximately 0.2 mg/2 kg of tissue, which is approximately 13% of the calculated amount of membrane-associated RI in beef heart (Reinitz et al., 1997).

Protease and phosphatase inhibitors

Where indicated in Results, some soluble and membrane-associated RIα preparations were purified with buffers containing 5 μM 1,10-phenanthroline, 400 nM cantharidin, 0.5 mM α-naphthyl acid phosphate, and 40 nM fenvalerate. The chelating agent 1,10-phenanthroline is an effective carboxypeptidase inhibitor (Vallee et al., 1960). Cantharidin is reported to inhibit PP2A (IC_{50} = 40 nM) and PP1 (IC_{50} = 400 nM) (Li et al., 1993). The phosphoprotein phosphatase substrate α-naphthyl acid phosphate (Li, 1984) is used as a broad spectrum phosphatase inhibitor at 5 mM (Pondaven & Meijer, 1986). Fenvalerate has been identified as a PP2B inhibitor (IC_{50} = 4 nM) (Enan & Matsumura, 1992), although this has recently been contested (Fakata et al., 1998).

Electrospray mass spectrometry

All analyses were done by reversed-phase HPLC (Applied Biosystems Model 140B) directly coupled to an API-III (Perkin-Elmer Sciex, Norwalk, Connecticut) triple quadrupole mass spectrometer (LC/MS) as described previously (Resing et al., 1995). Samples were frozen with liquid nitrogen in 0.01% Triton X-100 and stored at −70 °C for up to 18 months before use. Repeated freezing caused loss of phosphate and increased oxidation of Cys residues, and was avoided. Cytosolic and membrane-associated RIα were acetone precipitated on ice (acetone:aqueous 9:1 v/v) and collected by centrifugation at 12,000 rpm for 10 min. Supernatants were decanted and pellets were resolubilized by addition of 30 μL 8 M urea, followed by 120 μL 0.2 M Tris pH 8, 2 mM $CaCl_2$. Digestions were carried out for 3 h at 37 °C by addition of sequencing grade proteases, AspN (Boehringer Mannheim, Indianapolis, Indiana) or trypsin (Worthington, Lakewood, New Jersey), at final concentrations of 10% (w/w). Peptide separations were carried out on a fused silica capillary column containing Poros R120 resin (PerSeptive, Framingham, Massachusetts) directly coupled to the mass spectrometer (LC/MS). In many cases, peptide identities were confirmed by CID at the orifice. In addition, LC/MS/MS was performed on-line as peptides eluted from the capillary column, selecting individual peptide ions for CID in the high-pressure collision cell. Analysis of LC/MS and LC/MS/MS was carried out using software provided by PE Sciex.

Electrospray ion trap mass spectrometry

LC/MS analysis was carried out on intact proteins using a Finnigan LCQ mass spectrometer connected in-line to a Microtech Ultra Plus Micro LC pump. Two micrograms of cytosolic or membrane-associated RI was analyzed on a 0.5 mm × 15 cm Vydac C4 capillary column equilibrated in 0.1% formic acid and eluted with an 80% acetonitrile gradient with 0.1% formic acid at 1.6%/min, with a flow rate of 20 μL/min. The LCQ was operated with a N_2 sheath gas flow rate of 0.45 L/min, 5 kV spray voltage, 47 V capillary voltage, and 200 °C capillary temperature. Data analysis was performed using Bioworks Explore software.

Acknowledgments

The authors thank Dr. Catharine Reinitz for initial development of some of the purification steps of the membrane-associated RIα. This work was supported by an American Heart Association Grant-in-Aid and NSF OSR-9452892.

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