Identification of RII-binding proteins in the mollusc *Mytilus galloprovincialis*

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Abstract Several proteins with $M_r > 70$ kDa from various tissues of the sea mussel *Mytilus galloprovincialis* were specifically recognized in vitro by the regulatory subunit (type RIIα) of cAMP-dependent protein kinase (cAPK) from porcine heart. However, none of these proteins interacted with the regulatory subunit of cAPK from the mollusc itself. The results suggest that, unlike mammalian RII, regulatory subunit from mussel lacks the specific residues responsible for interaction with R-binding proteins. Consequently, the identified molluscan RIIα-binding proteins should play a distinct role from cAPK anchoring.

Key words: cAMP-dependent protein kinase; Regulatory subunit; R-binding protein; Kinase anchor protein; Mollusc

1. Introduction

cAMP-dependent protein kinase (cAPK) is a multifunctional serine/threonine kinase that can phosphorylate a great number of cytosolic and nuclear proteins in response to hormones and neurotransmitters which increase cyclic AMP levels [1,2]. Its broad substrate specificity suggests the need for physiological regulatory mechanism(s) to localize the effects of cyclic AMP and so ensure the selective phosphorylation of protein substrates. One proved mechanism consists of maintaining cAPK in specific cellular compartments close to its target substrates. This compartmentalization occurs by the attachment of type II cAPK to certain cellular structures through the interaction of its regulatory subunit (RII) with specific R-binding proteins also termed AKAPs (*A kinase anchor proteins*) [3–8]. The primary structure of several AKAPs is presently known [9–14]. Although there is little overall sequence homology among anchor proteins, they all contain a RII-binding site that corresponds to a conserved secondary structure motif. This is an amphipatic α-helix, whose hydrophobic face probably interacts with the side chains of hydrophobic residues located at the NH_2 termini of each regulatory subunit protomer [11,12,15,16].

As part of a study to investigate the implications of cAMP cascade in molluscs, we have recently reported the isolation of a 54 kDa cAMP-binding protein from the sea mussel *Mytilus galloprovincialis* that probably acts as the regulatory subunit of cAPK [17]. Since it seems to be homologous to mammalian type II regulatory subunit, we have now investigated its ability to interact with R-binding proteins. The aims of this study were: (1) to prove the existence of cAPK-anchoring proteins in a lower species (the presence and role of R-binding proteins in species different from mammals is practically unknown so far) and (2) to comparatively analyze the ability of a mammalian (porcine heart) and a molluscan (sea mussel) RII type subunit to specifically recognize R-binding proteins.

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Abbreviations: cAPK, cAMP-dependent protein kinase (EC 2.7.1.37); C and R, catalytic and regulatory subunits of cAPK; RII, type II regulatory subunit; $PheMeSO_2F$, phenylmethylsulfonyl fluoride

2. Material and methods

2.1. Tissue preparation and homogenization

Sea mussels (*Mytilus galloprovincialis* Lmk.) were supplied by a purification plant of molluscs located at the Ría de Betanzos (Galicia, N.W. Spain). Molluscs were transported to the laboratory within 1 hour of collection and five tissues (mantle, gonad, posterior adductor muscle, foot and gill) were rapidly dissected and immediately stored at −80°C until use. Fresh porcine heart was obtained from a local slaughterhouse and transported to the laboratory on ice. Tissues were homogenized (1:3 (m/v) for mantle, gonad and gill and 1:10 (m/v) for posterior adductor muscle, foot and porcine heart) in ice-cold 25 mM potassium phosphate buffer, pH 7.0, containing 0.25 M sucrose, 2 mM EDTA, 1 mM DTT, 1 mM $PheMeSO_2F$, 1 mM benzamidine, 1 mg/l pepstatin and 1 mg/l leupeptin (buffer A). The homogenate was centrifuged at 100000×*g* for 1 h at 4°C and the resulting supernatant (cytosolic extract) was filtered through glass wool and stored in aliquots at −80°C. The pellet was washed twice with buffer A and then resuspended in 25 mM potassium phosphate buffer, pH 7,0, containing 0.15 M KCl, 2 mM EDTA, 1 mM DTT, 1 mM $PheMeSO_2F$, 1 mM benzamidine, 1 mg/l pepstatin, 1 mg/l leupeptine and 0.25% (v/v) Triton X-100. The suspension was kept at 4°C for 30 min with slight stirring and then centrifuged at 100000×*g* for 60 min. The resulting supernatant (solubilized membrane extract) was stored in aliquots at −80°C.

2.2. Preparation of purified R and C subunits

Regulatory (R) and catalytic (C) subunits of cAPK from *M. galloprovincialis* were purified as previously described [17,18]. Purified mammalian RIIα was obtained from porcine heart according to the method of Rannels et al. [19].

2.3. Radiolabeling of R-subunits

Purified porcine heart (RIIα) and *Mytilus* (R_{Myt}) regulatory subunits were labeled by incubation with C-subunit of cAPK from mussel and [γ-^{32}P]ATP. The reaction mix contained (final volume of 50 μl) 25 mM potassium phosphate buffer, pH 7.0, 1 mM DTT, 10 mM $MgCl_2$, 10 μM cAMP, 5 μg R-subunit, 1 μg C-subunit and 0.3 μM [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham). After incubation on ice for 1 h remaining [γ-^{32}P]ATP was removed by gel-filtration chromatography through 1-ml plastic insulin syringes filled with Sephadex G-25 [3]. In the described conditions the incorporation of labeled phosphate was typically ~2×10^6 cpm/μg R-subunit.

2.4. RII overlay assay

R-binding proteins were detected by a solid-phase overlay technique [9,20]. Samples (50 μg protein) of cytosolic and membrane extracts from *Mytilus* tissues and porcine heart were subjected to SDS-PAGE in a 7.5%-polyacrylamide gel. As molecular mass markers Bio-Rad's kaleidoscope prestained standards were used. The resolved proteins were transferred to 0.45 μm nitrocellulose membranes by applying a 30 V constant current for 24 h at 4°C in a buffer consisting of 25 mM Tris, 192 mM glycine and 20% (v/v) methanol. Membranes were in-

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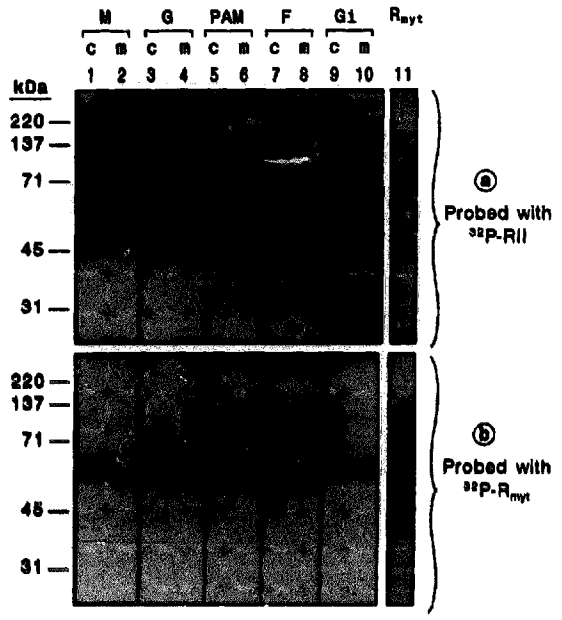

Fig. 1. Autoradiogram of nitrocellulose blots showing R-binding proteins in cytosolic (c) and membrane (m) extracts from various mussel tissues. Samples (50 μg protein) of extracts (lanes 1–10) and a sample (3 μg) of purified regulatory subunit from mussel, R_{myt} (lane 11) were subjected to SDS-PAGE and analyzed by the overlay assay as described in section 2. The blots were incubated with either ^{32}P-labeled porcine heart RIIα (a) or ^{32}P-labeled *Mytilus* regulatory subunit (b). Mussel tissues were: mantle (M, lanes 1 and 2), gonad (G, lanes 3 and 4), posterior adductor muscle (PAM, lanes 5 and 6), foot (F, lanes 7 and 8) and gill (Gi, lanes 9 and 10).

cubated with 10 ml/lane Blotto/BSA (10 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl, 5% (w/v) non-fat dried milk and 0.1% (w/v) BSA) for 15 at 4°C and then with ^{32}P-labeled RIIα or ^{32}P-labeled R_{myt} ($\sim 1 \times 10^5$cpm/ml) in fresh Blotto/BSA (10 ml/lane) for approx. 5 h at room temperature. For monitoring the specificity of the labeling, nitrocellulose membranes were incubated overnight at 4°C with 50-fold excess of cold regulatory subunit prior to the addition of ^{32}P-labeled regulatory subunit In all cases, the membranes were washed three times with 25 ml/lane of Blotto/BSA for 15 min and twice with 25 ml/lane of potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl. Binding of ^{32}P-labeled regulatory subunits to R-binding proteins was detected by autoradiography.

3. Results

To investigate the ability of mussel R_{myt} and mammalian RIIα to specifically recognize R-binding proteins in mussel, cytosolic and membrane extracts from five mollusc tissues were analyzed by the gel overlay technique. When ^{32}P-labeled porcine heart RIIα was used as probe (Fig. 1a), several R-binding proteins with $M_r > 70$ kDa were detected in extracts from mussel tissues (lanes 1–10). In more detail, a ~75 kDa protein was present in cytosolic and membrane extracts from muscular tissues (posterior adductor muscle (lanes 5 and 6) and foot (lanes 7 and 8)) and gills (lanes 9 and 10); additionally, another band with $M_r > 220$ kDa was also detected in cytosolic extract from posterior adductor muscle (lane 5). Moreover, in extracts from gonad and mantle (tissues involved in the gametogenic development of the mollusc), various radiolabeled bands were detected in each tissue (lanes 1–4). It should be noted that in all cases labeling was shown to be specific, since the incubation of nitrocellulose membranes with a 50-fold excess of non-labeled R, completely abolished labeling (not shown).

Parallelly, when in the overlay assay, the nitrocellulose membrane was incubated with ^{32}P-labeled R_{myt}, a single polypeptide with $M_r \sim 54$ kDa was labeled in extracts from all the mussel tissues (Fig. 1b; lanes 1–10). However, none of the bands with $M_r > 70$ kDa revealed by porcine ^{32}P-RIIα were detected in any tissue, which suggests that none of these proteins from mussel were recognized by R_{myt}.

Since the dimer formation on a solid-phase has been noted for R-subunit from other sources [3,20], we suspected that the labeled 54 kDa band could correspond to the mussel regulatory subunit present in extracts that can dimerize on the nitrocellulose membrane with the ^{32}P-R_{myt} used as probe in the overlay assay. In fact, as observed in Fig. 2, when a mussel mantle extract was chromatographed through a cAMP-agarose column and then analyzed by the overlay method for R-binding proteins, the 54 kDa band practically disappears, which indicates that it is the previously isolated and characterized cAMP-binding protein from mussel [17]. Furthermore, when a sample of purified R_{myt} was subjected to SDS-PAGE, transferred to nitrocellulose and probed with ^{32}P-labeled R_{myt}, a radioactive band of 54 kDa was revealed (Fig. 1b, lane 11), which demonstrates the ability of *Mytilus* R-subunit to form homodimers on the nitrocellulose membrane. Contrarily, when ^{32}P-RIIα was used as probe (Fig. 1a, lane 11), a weakly labeled band was observed, suggesting that the formation of heterodimers between regulatory subunits from both molluscan and mammalian sources was very restricted.

To confirm the observed differences between R_{myt} and RIIα in their ability to specifically recognize R-binding proteins, cytosolic and membrane extracts from porcine heart, were subjected to SDS-PAGE, transferred to nitrocellulose mem-

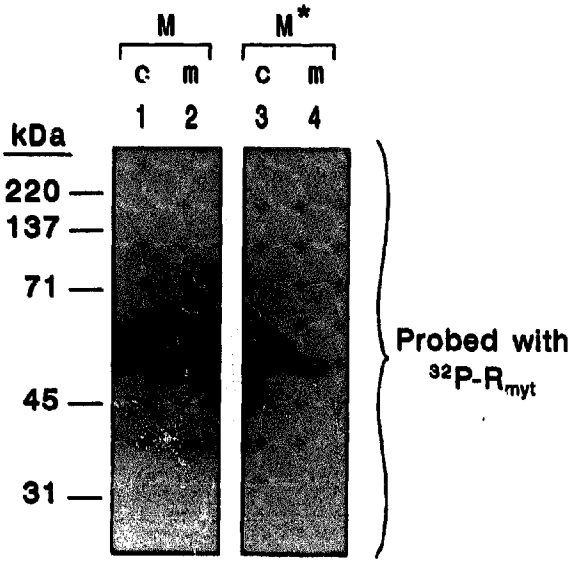

Fig. 2. Mussel mantle 54 kDa protein binds to cAMP agarose. Samples (5 ml) of cytosolic (c) and membrane (m) extracts from mussel mantle tissue were passed through 1-ml cAMP agarose columns. Samples (50 μg protein) of eluates (M*, lanes 3 and 4) and mussel mantle extracts (M, lanes 1 and 2) were subjected to SDS-PAGE and analyzed by the overlay assay as described in section 2 using ^{32}P-R_{myt} as probe.

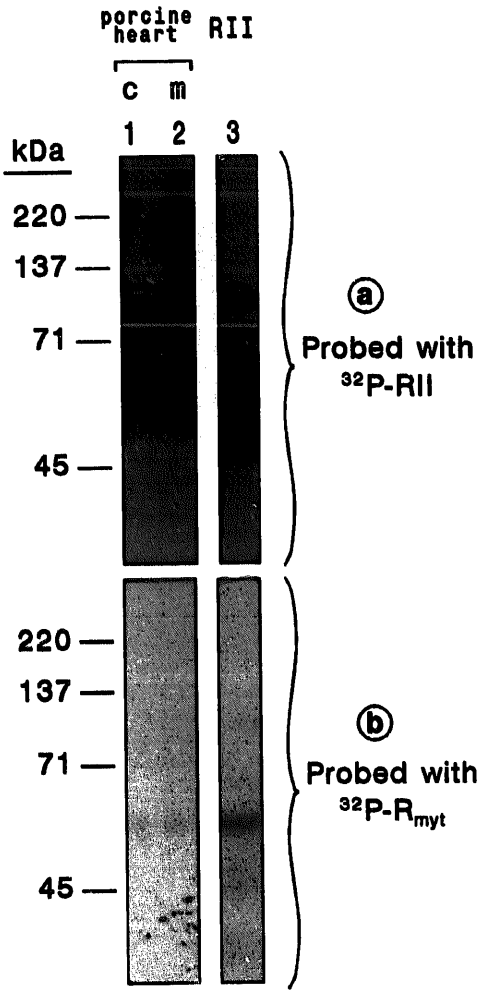

Fig. 3. Porcine heart R-binding proteins. Samples (50 μg protein) of cytosolic (c) and membrane (m) extracts from porcine heart (lanes 1 and 2) and 2 μg of purified RIIα (lane 3) were subjected to SDS-PAGE and R-binding proteins were detected by the overlay assay as described in section 2. Blots were probed with either ^{32}P-labeled porcine heart RIIα (a) or ^{32}P-labeled regulatory subunit from mussel, R_{myt} (b).

branes and analyzed by the overlay procedure using ^{32}P-RIIα and ^{32}P-R_{myt} as probes (Fig. 3). As expected, labeled-RIIα recognized several R-binding proteins, most of which were present in the membrane extract (Fig. 3a; lanes 1 and 2). However, none of these proteins bound ^{32}P-R_{myt} (Fig. 3b; lanes 1 and 2), which confirms the inability of mussel regulatory subunit to recognize R-binding proteins. Again, results obtained with samples of purified porcine heart RIIα (compare lane 3, Fig. 3a,b) confirm that R-subunits from both mammalian and molluscan sources can poorly form heterodimers.

4. Discussion

In a previous paper [17] we reported the isolation of the regulatory subunit of cAMP-dependent protein kinase from the sea mussel *M. galloprovincialis* (R_{myt}), which showed a similar behaviour to mammalian RII-type regulatory subunit regarding its ability to be phosphorylated by C-subunit and the elution pattern from DEAE-cellulose chromatography. However, now that the properties of mammalian RIIα and R_{myt}, related to their ability to specifically recognize R-binding proteins have been analyzed, it can be concluded that both regulatory subunits are quite different at their amino-terminal region, which is responsible for dimerization and AKAP-binding functions [21].In the first place, as assessed by the overlay assay, both porcine RIIα and R_{myt} can form homodimers, but immobilized RIIα was unable to bind radiolabeled R_{myt} and vice versa, which indicates that formation of heterodimers was very restricted. This result suggests that the determinants for dimerization are distinct and/or differently positioned in R-protomers from mammalian and molluscan sources.

Although it is presently accepted that dimerization of mammalian RII is a necessary requisite to interact with AKAPs [22–24], recent site-directed mutagenesis studies carried out by Hausken et al. [23] and Li and Rubin [24] demonstrated that dimerization and AKAP-binding functions are mediated through distinct amino-acids located at the NH_2-terminus of each R-protomer. For instance, isoleucines at positions 3 and 5 of RIIα are crucial residues for interaction with R-binding proteins, but they are not necessary for dimerization. When we investigated the presence of R-binding proteins in various mussel tissues by the overlay technique, several polypeptides were specifically recognized by porcine heart RIIα, but surprisingly none of them was recognized by R_{myt}, although it can form homodimers as previously demonstrated. This result suggests that molluscan regulatory subunit possesses the determinants for dimerization, but, unlike mammalian RIIα, it lacks the specific residues for interaction with R-binding proteins. The inability to bind AKAPs has also been noted by Coghlan and Scott (see reference [23]) for the yeast RII homolog *bcy*1, whose sequence lacks the isoleucines at positions 3 and 5. So, although more investigations are necessary to prove it, it is possible that the ability to cAPK-anchor through interaction with AKAPs is restricted to upper species, in which determinants for AKAP-binding are highly conserved [23].

Unfortunately, very little information about the existence of R-binding proteins in non-mammalian species exists so far. In addition to the results presented here, the presence of RII-binding proteins was only demonstrated in *Aplysia* neurons by the overlay assay [25]. However, since no endogenous regulatory subunit was used as probe, it is unknown if these proteins are also recognized by the RII from *Aplysia* itself. Our results suggest that, at least in mussel tissues, RII-binding proteins are not associated with endogenous R_{myt}, and consequently they should play a different role from cAPK anchoring 'in vivo'. Although most of the investigations have been centered on their R-binding function, it is known that some mammalian AKAPs can also bind proteins different from regulatory subunit of cAPK. For instance, AKAP 75 contains a binding site for calcium-calmodulin [3,26] and AKAP 79 also binds phosphatase 2B (calcineurin) [8,27]. These observations suggest that AKAPs are multifunctional proteins and perhaps they were initially designed to play a different role from cAPK-anchoring.

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