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

Jesús Cao, Montserrat Fernández, J. Ignacio Ramos-Martínez, J. Antonio Villamarin*

Departamento de Bioquímica e Biologia Molecular, Facultade de Veterinaria, Universidade de Santiago de Compostela, Campus de Lugo, E-27002 Lugo, Spain

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**Abstract** Several proteins with Mr > 70 kDa from various tissues of the sea mussel *Mytilus galloprovincialis* were specifically recognized in vitro by the regulatory subunit (type RIIc) of cAMP-dependent protein kinase (cAPK) from porcine heart. However, none of these proteins interacted with the regulatory subunit of cAPK from the mussel 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 RII-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 mechanisms 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 NH2 termini of each regulatory subunit proteinor [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.

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 Ria 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 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 PheMeSO2F, 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 PheMeSO2F, 1 mM benzamidine, 1 mg/l pepstatin, 1 mg/l leupeptin 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 RIIc was obtained from porcine heart according to the method of Rannels et al. [19].

2.3. Radiolabeling of R-subunits

Purified porcine heart (RIIc) and *Mytilus* (R*<sub>m</sub>*) regulatory subunits were labeled by incubation with C-subunit of cAPK from mussel and [γ-32P]ATP. The reaction mix contained (final volume of 50 μl) 25 mM potassium phosphate buffer, pH 7.0, 1 mM DTT, 10 mM MgCl2, 10 μM c/γMP, 5 μg R-subunit, 1 μg C-subunit and 0.3 μM [γ-32P]ATP (3,000 Ci/mmol, Amersham). After incubation on ice for 1 h remaining [γ-32P]ATP was removed by gel-filtration chromatography through 1-mL plastic insulin syringes filled with Sephadex G-25 [5]. In the described conditions the incorporation of labeled phosphate was typically ~2×10<sup>6</sup> cpm/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
It should be noted that in all cases labeling was shown to involve in the 8-ametogenic development of the mollusc, various radiolabeled bands were detected in each tissue (lanes 1-10). Moreover, in extracts from gonad and mantle (tissues in mussel tissues (lanes 1-10), in more detail, a 75 kDa protein was present in cytosolic and membrane extracts from five mollusc tissues (c, lanes 1-2), 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).

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 32P-labeled porcine heart RIIα was used as probe (Fig. 1a), several 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 32P-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 32P-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. 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 32P-labeled porcine heart RIIα (a) or 32P-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).

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 32P-R_myt as probe.
cytosolic (e) 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 [35]labeled RIIα or 32P-Rlla 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 32P-Rmyt (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 (Rmyt), 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 Rmyt, 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 Rmyt can form homodimers, but immobilized RIIα was unable to bind radiolabeled Rmyt 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-proteins 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 Haacken et al. [23] and Li and Rubin [24] demonstrated that dimerization and AKAP-binding functions are mediated through distinct amino-acids located at the NH₂-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 Rmyt, 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 [23] for the yeast RII homolog bcy1, 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 Rmyt, 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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