Catalytic unit-independent phosphorylation and dephosphorylation of type II regulatory subunit of cyclic AMP-dependent protein kinase in rat liver plasma membranes

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Rat liver plasma membranes contain a 55 kDa protein which proved to be identical with type II regulatory subunit (R^{II}) of the cyclic AMP-dependent protein kinase (kinase A) by several criteria (gel electrophoretic behaviour, peptide map, position of the autophosphorylated site). Analysis of phosphopeptide maps revealed that the membrane-bound R^{II} was phosphorylated by a kinase which is unrelated to the catalytic unit (C) of kinase A. Dephosphorylation of the membrane-bound R^{II} by an endogenous phosphatase was stimulated by both cyclic AMP and fluoride. Addition of C did not stimulate dephosphorylation even in the presence of ADP; moreover, protein inhibitor of C did not modify the effects of cyclic AMP or fluoride. The effects of both cyclic AMP and fluoride were, however, inhibited by C. Results indicate that rat liver plasma membranes contain a phosphorylation–dephosphorylation system for which R^{II} is a relatively specific substrate.

INTRODUCTION

The only proven mechanism by which cyclic AMP alters the function and metabolism of mammalian cells is phosphorylation of proteins by the cyclic AMP-dependent protein kinase (kinase A). In most tissues two forms of kinase A have been observed, namely type I and II (Krebs & Beavo, 1979; Flockhart & Corbin, 1982; Beavo & Mumby, 1982) which are distinguished by their different regulatory subunits (R^I and R^{II} respectively). R^{II} of bovine heart, unlike R^I , is autophosphorylated by its catalytic subunit (C) in a serine residue at position 95 (Takio *et al.*, 1982; Carmichael *et al.*, 1982). In addition, serines at positions 74 and 76 are also phosphorylated by casein kinase II (Carmichael *et al.*, 1982; Hemmings *et al.*, 1982) and serines at 44 and 47 by glycogen synthase kinase 3 (Hemmings *et al.*, 1982). The function of R phosphorylation is not known in all respects. From a recent study it appears that simultaneous phosphorylation of two sites, mediated by C and other kinase respectively, increases the affinity of R^{II} for cyclic AMP, i.e. would decrease the concentration of cyclic AMP required for the activation of holoenzyme (Rymond & Hofmann, 1982).

A major function of both R^I and R^{II} is the inhibition of activity of C in the holoenzyme (Krebs & Beavo, 1979). There are an increasing number of indications, however, that R^{II} may have additional roles as well. This assumption is based mainly on the demonstrated interaction of R^{II} with other proteins including the microtubule-associated protein 2 (MAP 2) (Vallee *et al.*, 1981; Miller *et al.*, 1982), a protein which copurifies with it (Lohmann *et al.*, 1984), the calcium + calmodulin-activated phosphoprotein phosphatase (calcineurin) (Hathaway *et al.*, 1981) and a calmodulin-binding protein, also called P75 (Sarkar *et al.*, 1984). This latter

protein is most abundant in liver plasma membranes. Such interactions between R^{II} and other proteins may ensure close localization of holoenzyme to its substrates: this assumption is supported by the fact that most of these proteins are substrates for kinase A. Alternatively, the existence of these R^{II} -binding proteins may indicate a multifunctional regulatory role for R^{II} . The recently reported inhibition of a high- M_r protein phosphatase by R^{II} (Khatra *et al.*, 1985) strongly supports such a possibility. In this latter case, phosphorylation of R^{II} increased its inhibitory potency. Thus, as with R^{II} –C interactions, the phosphorylation state of R^{II} is probably also important in the interactions between R^{II} and other proteins.

In various tissues, with the exception of brain, most R molecules are present in soluble form (Rubin *et al.*, 1979) and studies, including those on their phosphorylation, were usually performed on this form. The role of membrane-bound R^{II} as well as the nature of enzymes which phosphorylate and dephosphorylate it is less known.

In this present paper we describe various properties of phosphorylation and dephosphorylation of R^{II} in purified rat liver plasma membranes which lack functional C by several criteria.

MATERIALS AND METHODS

Materials

Acrylamide, SDS, ammonium persulphate and NNN' -tetramethylethylenediamine (Temed) were bought from Serva. Catalytic subunit of cyclic AMP-dependent protein kinase, protein inhibitor of catalytic subunit and *Staphylococcus aureus* V8 proteinase were

Abbreviations used: C, catalytic subunit of cyclic AMP-dependent protein kinase (kinase A); R^I and R^{II} , type I and II regulatory subunits of the cyclic AMP-dependent protein kinase respectively; PI, protein inhibitor of catalytic unit.

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from Sigma. Ampholines (pH range 3.5–10 and 5–7) were the product of LKB. 8-(6-Aminohexylamino)-cyclic AMP–Sephacrose 4B was obtained by coupling 8-(6-aminohexylamino)-cyclic AMP (prepared by the method of Guilford *et al.*, 1972) to CNBr-activated Sepharose 4B (Pharmacia) according to Weber & Hilz (1978). [γ - 32 P]ATP (> 1000 Ci/mmol) was prepared by the Isotope Institute of the Biological Research Center (Szeged). X-ray film (Medifort RP) and photographic chemicals were from Forte Photochemical Industry (Vác, Hungary) and intensifying screens ('Lightning plus') were from Du Pont.

Methods

Membrane and enzyme preparation. Female Wistar rats (150–180 g body wt.) that had been starved overnight were used. Liver plasma membranes were prepared according to the method of Neville (1968). R^{II} from bovine heart was prepared and purified on a 8-(6-aminohexylamino)-cyclic AMP–Sephacrose 4B affinity column by the method of Rannels *et al.* (1983) and was phosphorylated with purified catalytic subunit according to Erlichman *et al.* (1983). When the purified R^{II} was separated by two-dimensional gel electrophoresis (10% acrylamide in the second dimension) the 32 P activity was present only in R^{II} (80%) and in a 39 kDa protein (20%) which, according to its peptide map, is most probably a proteolytic product of R^{II} .

Assay of R^{II} phosphorylation. The standard components of the phosphorylation mixture (70 μ l) were 25 μ M [γ - 32 P]ATP (20000 c.p.m./pmol), 10 mM-MgCl₂, 30 mM-Tris/HCl (pH 7.6) and 100 μ g of plasma membrane proteins. The mixture was preincubated at 37 °C for 2 min and then for either 30 s or 5 min after addition of membranes as will be indicated. In the latter case, after 2.5 min of incubation ATP (in 5 μ l) was added again to restore the original ATP concentration (both labelled and unlabelled) which had dropped below 5 μ M by that time. There was no further increase in protein phosphorylation after 5 min even if labelled ATP was repeatedly added. Incubations were stopped by adding 1 ml of ice-cold acetone. Samples were left on ice for 15 min followed by centrifugation at 10000 *g* for 2 min. Pellets were dissolved in 50 μ l of lysis buffer (O'Farrell, 1975). Identical results were obtained when proteins were precipitated with 10% trichloroacetic acid; however, removal of traces of trichloroacetic acid, which was necessary to obtain reproducible gel patterns, was more time consuming.

Assay of R^{II} dephosphorylation. Membrane proteins (2 mg) were first incubated (in 0.6 ml volume) at 37 °C for 5 min in the presence of 30 mM-Tris/HCl (pH 7.6), 10 mM-MgCl₂ and [γ - 32 P]ATP. The labelled ATP was added at the beginning of incubation in a 5 μ l volume to achieve a final 25 μ M concentration (10⁵ c.p.m./pmol). Since ATP is rapidly degraded by membranes, 5 μ l of labelled ATP solution was added after each 1 min of incubation. The last addition of ATP occurred just before centrifugation of the membranes at 10000 *g* for 2 min. The ATP-free pellet (resuspended in 0.4 ml of 20 mM-Tris/HCl, pH 7.6) was used immediately (0.1 mg of protein) for the assay of dephosphorylation at 37 °C in the presence of 30 mM-Tris/HCl (pH 7.6) and 10 mM-MgCl₂. The reaction was stopped as before.

Two-dimensional polyacrylamide-gel electrophoresis. The O'Farrell (1975) two-dimensional procedure was used with the modifications described by Steinberg & Coffino (1979). For all samples of a single experiment, first-dimension isoelectric-focusing gels were loaded with equal amounts of proteins (the acid-precipitable radioactivity of samples differed by less than 10%) and run for 7000 V · h · second-dimension SDS-containing gels were 10% (w/v) in polyacrylamide. Labelled proteins were detected either by direct autoradiography of dried gels at room temperature or at –70 °C by using Du Pont 'Lightning plus' intensifying screens.

Peptide mapping of R^{II} . Peptide mapping by *Staphylococcus aureus* V8 proteinase was performed by the method of Cleveland *et al.* (1977), overlaying excised gel pieces (containing R^{II} either from liver plasma membrane or bovine heart cytosol) with 1 ml of 0.9% (w/v) agarose containing 5 μ g of enzyme. The time left for digestion in the stacking gel (4% acrylamide) was 30 min. Peptides were stained with silver by the method of Merrill *et al.* (1981). Protein was determined according to the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Addition of C to rat liver plasma membranes resulted in increased phosphorylation of several proteins, indicating that these membranes contain many substrates for this kinase (Fig. 1). All these effects of C were blocked by the protein inhibitor (PI) of C (added at a 4-fold higher concentration). Phosphorylation of other proteins was not affected by PI (results not shown), suggesting that plasma membranes did not contain dissociated, i.e. already activated, C.

Beside stimulating protein phosphorylation, C also appeared to decrease the 32 P label in a 55 kDa protein (Fig. 1). This phenomenon was observed if the concentration of C was at least 50 μ g/ml and the incubation time was short (30 s). As will be shown later (Fig. 4), the 55 kDa protein, whose phosphorylation in the membrane was independent of C, was phosphorylated mostly by the added C under the above conditions. The amount of C needed to provoke the above events was fairly high. For example, the 55 kDa protein comprises only about 0.2–0.3% of the total membrane protein pool: in this special case about a 20-fold excess of C is required for maximum effects. Since the activities of different batches of C were similar, this phenomenon probably cannot be explained by partial inactivation of C. It is more likely that membrane-bound proteins, including the 55 kDa protein, are not readily accessible to C.

In contrast with C, addition of cyclic AMP or dibutyryl cyclic AMP up to 0.1 mM concentration did not increase phosphorylation of any of these or other proteins (Fig. 2). When the range of protein separation was extended by using 7.5 and 15% gels as well, we observed increased phosphorylation of at least 30 proteins by C, but none of these changes were elicited by cyclic AMP. This indicated that purified membranes also lacked a cyclic AMP-sensitive holoenzyme. In view of the fact that, unlike R, C is readily released from membranes (Corbin *et al.*, 1977), it is not surprising that the lengthy procedure used for membrane isolation resulted in a C-depleted preparation. Cyclic AMP had only one effect on protein phosphorylation: it inhibited 32 P-labelling of a 55 kDa

Fig. 1. Phosphorylation of liver plasma membrane proteins by the catalytic subunit (C) of cyclic AMP-dependent protein kinase

Plasma membrane proteins (100 µg) were phosphorylated in the absence (a) or presence (b) of 75 µg of C/ml for 30 s. Phosphoproteins (1.3×10^5 acid-precipitable c.p.m. were applied) were subjected to two-dimensional gel electrophoresis and dried gels were exposed for autoradiography for 6 days. Numbered arrowheads indicate the position of those phosphoproteins whose phosphorylation was increased by C. In this and the next experiments gel patterns are shown with acidic ends of isoelectric focusing gel dimensions to the right.

Fig. 2. Effect of cyclic AMP on phosphorylation of plasma membrane proteins

Membrane proteins were phosphorylated by incubation for 5 min in the absence (a) or presence (b) of 100 µM-cyclic AMP. Phosphoproteins (9×10^4 acid-precipitable c.p.m. were applied) were separated as before and dried gels were exposed for 6 days. Arrowheads indicate the position of a 55 kDa protein whose phosphorylation is decreased by cyclic AMP.

protein (Fig. 2b). Phosphorylation of this protein, on the other hand, was not affected either by Ca^{2+} (0.5 mM), or by a partially purified and activated lipid-dependent kinase (kinase C) or by PI (tested up to 0.3 mg/ml). When the 55 kDa protein was excised from gels, trypsin-treated as described by Steinberg & Kiss (1985) and separated by SDS/polyacrylamide-gel electrophoresis, most ^{32}P label was present in a 16–17 kDa fragment (results not shown). A similar peptide map was generated from R^{II} after phosphorylation with casein kinase II (Carmichael *et al.*, 1982), suggesting possible identity of the 55 kDa protein with R^{II} and the protein kinase involved in its phosphorylation with casein kinase II.

It is well known that binding of cyclic AMP in cells occurs only to R^{I} and R^{II} (Ekanger *et al.*, 1985) except perhaps to *S*-adenosylhomocysteinase (Ueland & Doskeland, 1978; Doskeland & Ogreid, 1981; Hohman & Vernon, 1984). This latter enzyme is, however, unrelated to protein phosphorylation. Therefore, it was most possible that the 55 kDa protein is an R^{II} whose

occupation by cyclic AMP decreases its phosphorylation. Identity of this protein with R^{II} was confirmed by the following data. Purified nonphosphorylated R^{II} from bovine heart and the phosphorylated 55 kDa protein ran into very similar positions on the two-dimensional gels. When both proteins were excised from dried gels and subjected to proteolysis by *S. aureus* V8 proteinase in a second slab gel (Cleveland *et al.*, 1977) the resultant peptide maps were nearly identical (Fig. 3). In the next experiment we compared phosphopeptide maps of the 55 kDa protein and purified R^{II} . In Fig. 4(a) the 55 kDa protein was labelled in the presence of 50 µg of C/ml for only 30 s. Its phosphopeptide pattern, showing one major phosphorylated band of about 18 kDa, is virtually identical with that of autophosphorylated bovine heart R^{II} (Fig. 4b). This further confirms that the 55 kDa protein is an R^{II} . If the labelling was performed in the absence of C we observed a quite different phosphopeptide pattern of the 55 kDa protein (Fig. 4d), the most prominent band being of 10 kDa. The larger fragments, which were less intense, probably indicate a lower degree of digestion and not different phosphorylated sites. The results indicated that phosphorylation of membrane

Fig. 3. Peptide mapping of the 55 kDa protein and the purified R^{II} from bovine heart

Coomassie-Blue-stained 55 kDa protein was identified by the aid of radioactive tracings, cut out from the gel and placed on the top of a second gel (4% acrylamide for stacking gel and 15% acrylamide for the separating gel). Gel pieces were overlaid with 1 ml of 0.9% (w/v) agarose containing 5 μ g of *S. aureus* V8 proteinase and electrophoresis proceeded until the dye front (Bromophenol Blue) was only 1 cm above the separating gel. The electrophoresis was stopped at this point for 30 min. R^{II} from bovine heart was also separated first by the two-dimensional technique. Peptides were stained by silver (Merrill *et al.*, 1981).

bound R^{II} was almost exclusively catalysed by a protein kinase different from C. As mentioned earlier, this enzyme may possibly be casein kinase II. It should be noted that after longer incubations of membranes with C, or at lower concentrations of C, phosphopeptide maps showed mixed patterns of Figs 4(a) and 4(d). Also, after longer periods of incubation with C, double-phosphorylated forms of R^{II} appeared with a horizontal shift (from pH 5.3 to about pH 5.2) to the more acidic end and with an upward shift (by about 2 kDa) (results not shown). Phosphorylation of R^{II} at new sites is often accompanied, for unknown reasons, by decreased electrophoretic mobility (Robinson-Steiner *et al.*, 1984). Fig. 4(c) shows that cyclic AMP decreases ³²P label in the various phosphopeptides without major discrimination.

Binding of cyclic AMP could, in principle, render R^{II} a less efficient substrate for a protein kinase or a better substrate for a phosphatase. To distinguish between these possibilities membrane proteins were first prelabelled

Fig. 4. Phosphopeptide analysis of 55 kDa protein and R^{II}

In all cases phosphopeptides were generated by V8 proteinase and autoradiograms were exposed for 5 days at -70°C using Du Pont 'Lightning plus' intensifying screens. (a) Distribution of ³²P label in peptides from the 55 kDa protein when plasma membranes were first phosphorylated in the presence of 75 μ g of C/ml for 30 s and phosphoproteins were separated by the two-dimensional technique (3×10^5 acid-precipitable c.p.m. were applied). (b) Phosphopeptide pattern of autophosphorylated R^{II} (500 c.p.m. was applied) purified from bovine heart. (c) Decrease by 100 μ M-cyclic AMP of ³²P label in all phosphopeptide fragments of 55 kDa protein in an experiment analogous to that described in the legend to Fig. 2. (d) Distribution of ³²P label in peptides from the 55 kDa protein when membrane proteins were first phosphorylated without C for 5 min (in c and d 3×10^5 acid-precipitable c.p.m. were applied for three-dimensional separation).

with ³²P (Fig. 5a) in the absence of C and after removal of labelled ATP membranes were further incubated for 5 min in the absence (Fig. 5b) or presence (Fig. 5c) of cyclic AMP. There was no significant dephosphorylation of R^{II} in the control membranes during that period of time (compare Figs. 5a and 5b), while nearly complete dephosphorylation took place in the presence of 100 μ M-cyclic AMP.

When the autophosphorylated form of bovine heart

Fig. 5. Stimulation of dephosphorylation of membrane-bound R^{II} (55 kDa protein) by cyclic AMP and NaF

Membrane proteins were first prelabelled with ^{32}P and then separated from labelled substrate by centrifugation at 10000 g for 2 min. Aliquots of the resuspended pellet (100 μg of protein) were either precipitated by acetone immediately (*a*) serving as a 0 min control or were further incubated for 5 min (37 $^{\circ}\text{C}$) without further additions (*b*) or in the presence of 100 μM -cyclic AMP (*c*) or 20 mM-NaF (*d*). Phosphoproteins (7×10^4 acid-precipitable c.p.m. were applied) were separated as before and dried gels were exposed for 7 days. Arrowheads indicate the position of the 55 kDa protein (R^{II}).

R^{II} was added to liver membranes, cyclic AMP did not significantly promote its dephosphorylation unless ADP was present (results not shown). Similarly, cyclic AMP had only a marginal effect on dephosphorylation when the membrane-bound R^{II} was first phosphorylated by added C. Thus, the membrane-bound phosphatase does not seem to dephosphorylate the autophosphorylated site on R^{II} .

Curiously, fluoride, which is an inhibitor of most phosphatase activities and also inhibits the phosphatase which acts on cytosolic R^{II} (Uno, 1980), promoted almost complete dephosphorylation of R^{II} at 20 mM concentration (Fig. 5*d*). Half-maximal effects were observed with 5 mM-fluoride. Similarly to cyclic AMP, fluoride did not promote dephosphorylation of autophosphorylated bovine heart R^{II} , suggesting the involvement of the same phosphatase. The stimulatory effect of fluoride on dephosphorylation was highly specific for R^{II} because, beside R^{II} , we observed similar effects on only three other phosphoproteins each having a molecular mass higher than 70 kDa (results not shown). Neither the effect of cyclic AMP nor of fluoride on dephosphorylation was reversed by PI (tested up to 0.3 mg/ml). This indicated that, similarly to phosphorylation, dephosphorylation is also not catalysed by C. This question was, however, further investigated by adding C to membranes (prelabelled with ^{32}P) in the absence of labelled ATP. As is

shown on Fig. 6, unlike cyclic AMP (Fig. 6*b*), C does not induce dephosphorylation of R^{II} (Fig. 6*c*). It is known that reversal of autophosphorylation by C requires ADP as a phosphate acceptor (Rosen & Erlichman, 1975). In separate experiments ADP, tested up to 2 mM (37 $^{\circ}\text{C}$, 5 min, pH 7.6) did not promote dephosphorylation by C. This indicates that C can catalyse dephosphorylation of the autophosphorylated site only. On the other hand, addition of C partially prevented stimulation of dephosphorylation of R^{II} by cyclic AMP (Fig. 6*d*). This suggests that a fluoride-activated phosphatase acts only on free R^{II} provided that C does not directly inhibit this phosphatase.

Brautigan *et al.* (1981) described a membrane-associated phosphotyrosine-specific phosphatase activity which was stimulated by fluoride and inhibited by low concentrations of Zn^{2+} . Under the experimental conditions described in Fig. 5, 50 μM - Zn^{2+} completely prevented the stimulatory effect of fluoride on the dephosphorylation of R^{II} (results not shown). However, when gels were immersed into 1 M-KOH solution at 55 $^{\circ}\text{C}$ for 1 h (a treatment to which only phosphotyrosine is resistant; Cooper & Hunter, 1981), the ^{32}P activity was released from R^{II} , indicating that the phosphate group was bound to either serine or threonine.

In summary, our results indicate that rat liver plasma membranes contain a phosphorylation-dephosphoryla-

Fig. 6. Inhibition of cyclic AMP effect on R^{II} dephosphorylation by C

Membrane proteins were first prelabelled with ^{32}P and then separated from labelled substrate as described in the legend to Fig. 5. Aliquots of resuspended pellet (100 μ g of protein) were incubated further for 5 min (37 °C) without further additions (a) or in the presence of 100 μ M-cyclic AMP (b), 75 μ g of C/ml (c) and 100 μ M-cyclic AMP plus 75 μ g of C/ml (d). Phosphoproteins (3×10^5 acid-precipitable c.p.m. were applied) were separated as before and dried gels were exposed for 5 days. Only small parts of the gels are shown: arrowheads indicate the position of the 55 kDa protein (R^{II}).

tion system which is independent of C and for which R^{II} is a relatively specific substrate. Dephosphorylation of R^{II} is 'unconventional' in the sense that it is activated by fluoride. Most phosphatase activities characterized so far are inhibited by fluoride. The exception is a membrane-bound phosphatase whose activity is inhibited by Zn^{2+} and stimulated by fluoride (Brautigan *et al.*, 1981). It is possible that this enzyme, which does not seem to be absolutely specific for phosphotyrosine, is involved in dephosphorylation of R^{II} . Further work is required to prove this possibility.

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