RESEARCH COMMUNICATION

Cyclic AMP-dependent protein kinase phosphorylates rabbit reticulocyte elongation factor-2 kinase and induces calcium-independent activity

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The catalytic subunit of cyclic AMP-dependent protein kinase (PKA) phosphorylated purified calcium/calmodulin-dependent eukaryotic elongation factor-2 (eEF-2) kinase, isolated from rabbit reticulocyte lysates. It maximally incorporated about 1 mol of phosphate/mol of eEF-2 kinase. The K_m of eEF-2 kinase for PKA was calculated to be 7 μM. Phosphorylation of eEF-2 kinase by PKA induced calcium-independent activity which amounted to 40–50 % of the total activity measured in the presence of calcium. Furthermore, the level of calcium-independent activity induced by phosphorylation by PKA was similar to that induced by the calcium-stimulated autophosphorylation of eEF-2 kinase. Phosphopeptide mapping of eEF-2 kinase labelled by autophosphorylation and by PKA revealed a number of common phosphopeptides. This suggests that PKA may phosphorylate the same site(s) which are phosphorylated autocatalytically and which are responsible for the induction of calcium-independent activity. The possible implications these findings have for the control of translation are discussed.

INTRODUCTION

Eukaryotic elongation factor-2 (eEF-2) mediates the translocation step in the elongation phase of protein synthesis in eukaryotic cells [1–3]. The activity of this protein can be regulated by phosphorylation mediated by eEF-2 kinase, a highly specific calcium/calmodulin-dependent protein kinase (CaM PK) [4,5]. The kinase is very specific for eEF-2 and phosphorylates it on two threonine residues (Thr^{56} and Thr^{58}) [6]. The phosphorylation is an ordered process: phosphorylation of Thr^{56} appears to be a prerequisite for the phosphorylation of Thr^{58}. However, phosphorylation of Thr^{56} alone seems to inhibit completely the activity of the factor [6–8]. Phosphorylation of eEF-2 has been observed *in vivo* under conditions where the cytoplasmic calcium concentration has been raised by hormones and growth factors, or by calcium ionophores [9–11].

We have recently purified eEF-2 kinase from rabbit reticulocytes and have shown that it is a monomeric protein of approx. 103 kDa [12]. The kinase undergoes autophosphorylation in a calcium-dependent manner and, in common with CaM PK II [13] and CaM PK IV [14], autophosphorylation of eEF-2 kinase induces calcium-independent kinase activity. This may have important implications for the control of these kinases *in vivo* by hormones which raise the cytoplasmic calcium concentration, since the autophosphorylated kinases could continue to be active even after the calcium concentration had returned to basal levels.

Here we show that the catalytic subunit of cyclic AMP (cAMP)-dependent protein kinase (PKA) can phosphorylate eEF-2 kinase and that this also induces calcium-independent kinase activity. The possible implications of this for the control of eEF-2 kinase are discussed.

EXPERIMENTAL

Chemicals and biochemicals were obtained as before [12,15]. eEF-2 and eEF-2 kinase were purified from rabbit reticulocytes as described [12], using CaM–Sepharose in the purification of eEF-2 kinase. The catalytic subunit of PKA from bovine heart was purified as described [16], except that an additional Mono S column was used after the hydroxyapatite step. One unit of PKA activity was that which incorporated 1 μmol of phosphate·min^{-1} into histone 2A under standard assay conditions [16]. The peptide inhibitor of PKA and histone 2A were obtained from Sigma Chemical Co.

eEF-2 kinase activity was measured as described [12]. The assay buffer used here contained no added calcium since there appeared to be enough calcium in the solutions used to maximally activate eEF-2 kinase. In the experiments described below, those incubations which are described as containing calcium contained no added calcium and no EGTA, while those which are described as containing no calcium contained 1 mM EGTA. Two-dimensional phosphopeptide mapping and SDS/PAGE were performed as described [12]. Protein concentrations were determined as described [17].

RESULTS

Phosphorylation of eEF-2 kinase by PKA

We have previously reported that eEF-2 kinase undergoes autophosphorylation in the presence of Ca^{2+}/CaM, as shown in Figure 1(a) (lane 1). The preparation of eEF-2 kinase used here contained the 103 kDa and 95 kDa forms of the kinase [12]. Autophosphorylation of the kinase decreases the mobility of both forms of the kinase protein on SDS/polyacrylamide gels, leading to the appearance of several radiolabelled bands as can also be seen in Figure 1(a) (lane 1). The lower-molecular-mass peptides which become phosphorylated are degradation products of the kinase. Incubation of eEF-2 kinase in the absence of calcium and in the presence of the catalytic subunit of PKA resulted in the phosphorylation of both forms of eEF-2 kinase (Figure 1a, lane 3).

The incorporation of phosphate into eEF-2 kinase by PKA in the absence of calcium was about 7-fold higher than that

Abbreviations used: CaM PK, calcium/calmodulin-dependent protein kinase; eEF-2, eukaryotic elongation factor-2; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase.

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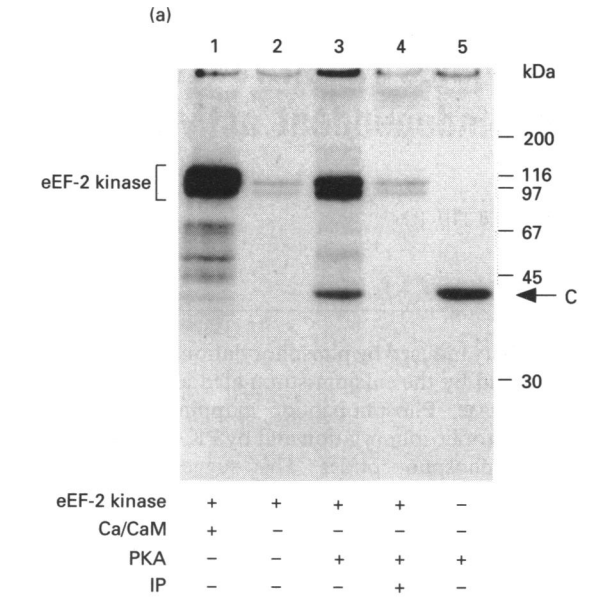

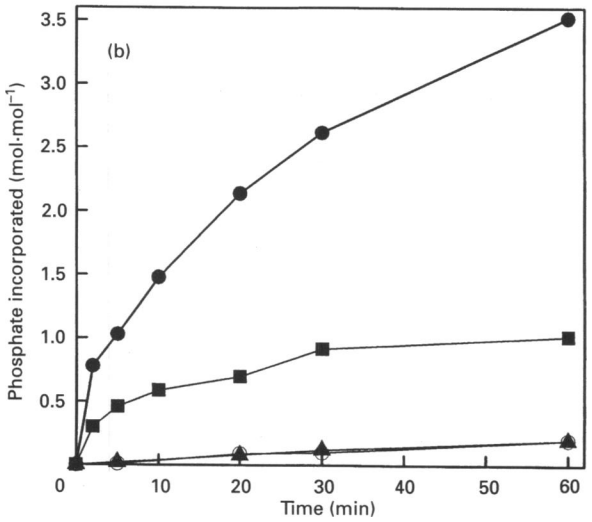

Figure 1 Phosphorylation of eEF-2 kinase by the catalytic subunit of PKA

(**a**) eEF-2 kinase (2 pmol) was incubated in the presence of [γ-^{32}P]ATP (3000 Ci·mmol^{-1}; 500 μCi·ml^{-1}) with or without Ca^{2+}/CaM, the catalytic subunit of PKA (0.65 m units) or PKA inhibitor peptide (IP) (10 μM) as indicated in a final volume of 20 μl for 20 min at 30 °C. After incubation the samples were denatured by boiling in sample buffer and were run on a polyacrylamide gel which was then dried and subjected to autoradiography. The molecular mass markers were as follows: myosin (200 kDa), β-galactosidase (116 kDa), glycogen phosphorylase (97 kDa), BSA (67 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa). The positions of eEF-2 kinase and the catalytic subunit of PKA (C) are indicated. (**b**) A similar experiment to that in (**a**) was performed. eEF-2 kinase was incubated with Ca^{2+}/CaM (●), without Ca^{2+}/CaM (○), with PKA (32.5 munits·ml^{-1}) (■) or with PKA and the peptide inhibitor (10 μM) (▲) At the indicated times, samples were removed and run on a polyacrylamide gel. Gel chips containing eEF-2 kinase were excised and the associated radioactivity determined by Čerenkov counting.

incorporated into eEF-2 kinase when incubated alone without calcium (cf. lanes 2 and 3, Figure 1a). When the PKA inhibitor peptide was included in the incubation in the absence of free calcium, it completely blocked the phosphorylation of eEF-2 kinase by PKA (cf. lanes 2 and 4), showing that the phosphorylation in the absence of calcium was mediated by PKA.

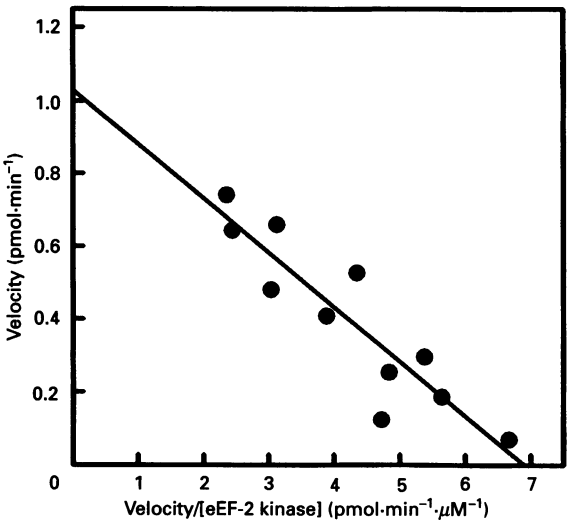

Figure 2 Kinetics of the phosphorylation of eEF-2 kinase by PKA

Various concentrations of eEF-2 kinase were incubated with PKA (1 munit) for 1 min. The incorporation of phosphate into eEF-2 kinase was measured as described in the legend to Figure 1. The data are from two separate experiments and are presented as an Eadie–Hofstee plot.

The maximum level of phosphorylation of eEF-2 kinase by PKA was 1 mol of phosphate/mol of eEF-2 kinase (S.E.M. = 0.1, $n = 4$) (Figure 1b). Incubation with higher concentrations of PKA did not increase the maximum stoichiometry. As reported before [12], and as can be seen in Figure 1(b), eEF-2 kinase autophosphorylates to a much higher stoichiometry.

The activity of PKA against eEF-2 kinase between a concentration of 0.3 and 0.01 μM was measured; the K_m of eEF-2 kinase for PKA was calculated as 7 μM and the $V_{max.}$ was 1 μmol·min^{-1}·mg^{-1} (Figure 2). These values are not untypical of other PKA protein substrates [18,19].

Effect of phosphorylation by PKA on eEF-2 kinase activity

We have recently found that, in common with CaM PK II and CaM PK IV, autophosphorylation of eEF-2 kinase rapidly (within 0.5 min) induces a calcium-independent state [12]. That is, if calcium is removed after allowing the kinase to autophosphorylate, the enzyme remains active although at a reduced level. It was therefore of importance to examine the effects of phosphorylation by PKA on eEF-2 kinase. As shown in Figure 3, eEF-2 kinase was allowed to autophosphorylate in the presence of calcium and then its activity was measured in the presence and absence of calcium. The activity of autophosphorylated eEF-2 kinase measured in the absence of calcium was about 40% of the maximal activity in the experiment illustrated. Maximal calcium independence was generally observed to be between 40 and 50% of the activity measured in the presence of calcium. If autophosphorylation of eEF-2 kinase was prevented by preincubation in the absence of calcium, then essentially no calcium-independence was observed as previously reported. Preincubation in the presence of PKA and in the absence of calcium was also shown to induce calcium-independent eEF-2 kinase activity. Preincubation of eEF-2 kinase in the presence of both free calcium and PKA resulted in a small increase in total activity when subsequently measured in the presence of calcium (15–20% increase relative to eEF-2 kinase preincubated and assayed in the presence of calcium alone) (results not shown).

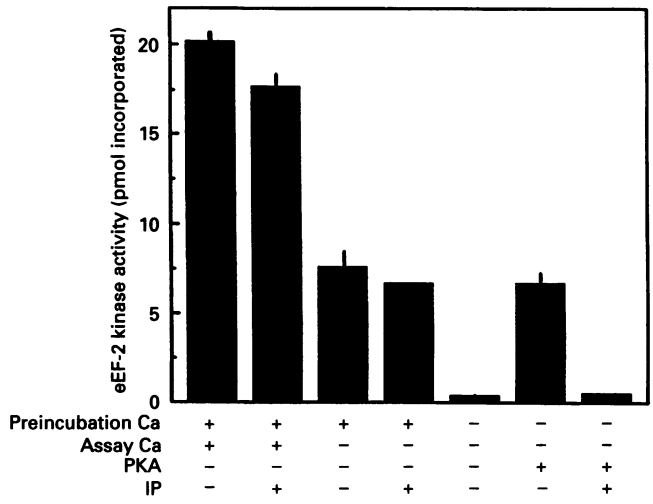

Figure 3 Induction of eEF-2 kinase calcium-independent activity by phosphorylation by PKA

eEF-2 kinase (0.05 pmol) was preincubated for 20 min in the presence or absence of calcium, PKA catalytic subunit (2.6 munits) and PKA inhibitor peptide (IP; 13.8 μM) in a final volume of 16 μl. EGTA (1 mM final concentration) was then added to some incubations to remove free calcium. eEF-2 kinase activity was then assayed by the addition of eEF-2 (60 pmol) and [γ-^{32}P]ATP to the incubations for 0.5 min. The samples were then denatured and run on an SDS/polyacrylamide gel. Gel chips containing the eEF-2 were excised and the associated radioactivity was determined by Čerenkov counting.

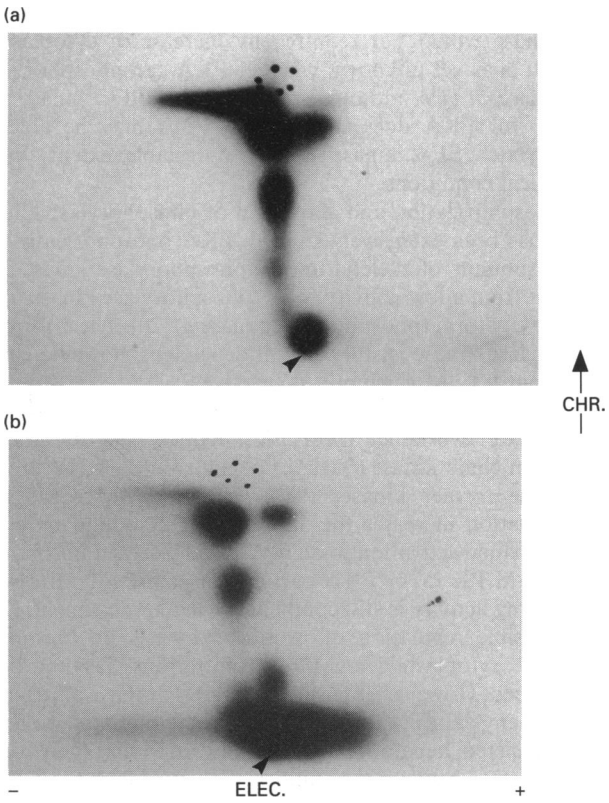

Figure 4 Two-dimensional phosphopeptide mapping of eEF-2 kinase

eEF-2 kinase was phosphorylated to a stoichiometry of 0.9 mol·mol^{-1} by PKA in the presence of 1 mM EGTA (**a**) or was allowed to autophosphorylate in the absence of EGTA to a stoichiometry of 3.5 mol·mol^{-1} (**b**). After incubation, the samples were run on an SDS/polyacrylamide gel and the gel chip containing the eEF-2 kinase was subjected to trypsinolysis overnight. The peptides eluted from the gel chips were redigested overnight and two-dimensional phosphopeptide mapping was performed as described. The broken circle indicates the position of a dinitrophenyl-lysine marker and the arrowhead indicates the position of the origin. CHR. and ELEC. refer to the directions of ascending chromatography and electrophoresis respectively.

The induction of eEF-2 kinase calcium-independent activity by PKA phosphorylation was completely abolished by the presence of the PKA inhibitor peptide, showing that the effect on eEF)2 kinase was mediated by PKA phosphorylation, although the inhibitor peptide did have a modest (10–15%) direct inhibitory effect on eEF-2 kinase activity at the concentration used here. It is not clear why this was so, since the sequence around the phosphorylation sites in eEF-2 bears little resemblance to the inhibitor peptide sequence and the latter is therefore unlikely to be acting as a competitive inhibitor of eEF-2 kinase. Under the experimental conditions described, PKA did not directly phosphorylate eEF-2 (results not shown).

Phosphopeptide mapping of eEF-2 kinase phosphorylated by PKA

The tryptic phosphopeptide map derived from eEF-2 kinase phosphorylated by PKA to a stoichiometry of about 0.9 mol·mol^{-1} revealed the presence of a number of phosphopeptides (Figure 4). Several of the phosphopeptides were also evident in the phosphopeptide map of autophosphorylated eEF-2 kinase (stoichiometry of 3.5 mol·mol^{-1}).

Since autophosphorylation and phosphorylation by PKA induce calcium-independent eEF-2 kinase activity, it is possible that the site(s) phosphorylated in peptides common to eEF-2 kinase phosphorylated by both means may be responsible for the observed effect. Since no sequence data are yet available for eEF-2 kinase, it is impossible to speculate on the sequences around the phosphorylation sites or whether any of the peptides obtained contain recognition motifs for PKA. It is not known why some five or six tryptic phosphopeptides should be obtained from eEF-2 kinase phosphorylated to a stoichiometry of only 0.9 mol·mol^{-1}. The labelled eEF-2 kinase was digested twice overnight with trypsin to ensure complete cleavage. Nonetheless, it is possible that the large number of peptides results from partial cleavage around the phosphorylation sites, since phosphorylation close to a cleavage site can partially inhibit the activity of trypsin. Trypsin is likely to cleave close to phosphorylation sites in eEF-2 kinase phosphorylated by PKA, since multiple basic residues form part of the recognition sequence for PKA. Therefore multiple peptides could result containing differing numbers of basic residues at the N-terminus, and since trypsin is not an exopeptidase, an N-terminal basic residue will not be cleaved by trypsin even after prolonged incubation.

Phosphoamino acid analysis showed that PKA phosphorylated eEF-2 kinase exclusively on serine residues (results not shown).

DISCUSSION

We have shown here that eEF-2 kinase can be phosphorylated *in vitro* by the catalytic subunit of PKA and have thus identified eEF-2 kinase as a novel substrate for PKA. Phosphorylation by PKA activates eEF-2 kinase in the absence of calcium. The potential importance of this observation is that the phosphorylation of eEF-2 kinase via the increase in cAMP and activation of PKA *in vivo* could constitute a pathway for the activation of eEF-2 kinase which is distinct from that involving an increase in

cytoplasmic calcium ion concentrations and therefore activation of the kinase would not require any increase in cytoplasmic calcium. It is as yet unknown whether PKA can phosphorylate eEF-2 kinase *in vivo*, although the fact that eEF-2 kinase has a low K_m for PKA does suggest that PKA may be able to phosphorylate eEF-2 kinase to an appreciable extent under physiological conditions.

The phosphorylation and activation of phosphorylase kinase by PKA has been extensively studied. PKA phosphorylates the α- and β-subunits of skeletal muscle phosphorylase kinase and converts it from a low activity form (phosphorylase kinase *b*) to a high activity form (phosphorylase kinase *a*) [20]. The *a*-form of phosphorylase kinase is still calcium-dependent, however, since below about 0.1 μM calcium it is inactive.

The catalytic subunit of PKA has also been shown to phosphorylate two other CaM PKs, namely smooth muscle myosin light chain kinase (SMMLCK) and CaM PK IV. In the case of the former kinase, PKA inhibits SMMLCK since phosphorylation increases the amount of calmodulin required for half-maximal activation some 10–20-fold [21,22]. Phosphorylation of CaM PK IV by PKA partially inactivates it. However, the remaining activity is still completely calcium-dependent [23].

The possible regulation of protein synthesis by hormones which raise cytoplasmic cAMP concentrations has not been widely studied. However, it has been found that the α-adrenergic agonist, phenylephrine, inhibited protein synthesis in isolated, calcium-depleted, hepatocytes [24]. It was found that there was a close correlation between the abilities of phenylephrine to inhibit protein synthesis and to increase cAMP concentrations. These authors did not determine whether this inhibition was exerted mainly at the initiation or the elongation stage of peptide synthesis.

Glucagon also increases the cellular cAMP content and inhibits protein synthesis in rat liver [25–27]. Intriguingly, it was shown that glucagon treatment increased the polypeptide chain completion time and increased the average polysomal size, indicating an inhibition of the elongation rate. It was also shown that the initial decrease in protein synthesis correlated well with the increase in cAMP, in that 5 min after glucagon administration, the protein synthesis rate had reached its lowest value and at this time cAMP was maximally elevated. However, after 20 min, protein synthesis had returned to normal while the concentration of cAMP was still elevated, although it was only 36% of the maximum concentration observed [25]. It was suggested that this indicated that changes in cAMP were not directly involved in the control of protein synthesis in the liver by glucagon. Rather, it was suggested that glucagon decreased the [NAD^+]/[NADH] and [ATP]/[ADP] ratios, which in turn led to the reduced protein synthetic rate [26]. This does not explain, however, why the control exerted by glucagon should occur primarily at the elongation phase of protein synthesis.

Nonetheless, in view of the results presented here, it does seem possible that the activity of eEF-2 kinase and hence protein synthesis could be regulated *in vivo* by hormones which increase cytoplasmic cAMP. A possible role for the inhibition of protein synthesis by hormones which raise cAMP could be to divert amino acids away from incorporation into proteins for their use as precursors for gluconeogenesis.

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REFERENCES

1 Proud, C. G. (1992) Curr. Top. Cell. Regul. **32**, 243–369
2 Moldave, K. (1985) Annu. Rev. Biochem. **54**, 1109–1149
3 Moazed, D. and Noller, H. F. (1989) Nature (London) **342**, 142–148
4 Palfrey, H. C. (1983) FEBS Lett. **157**, 183–190
5 Ryazanov, A. G., Natapov, P. G., Shestakova, E. A., Severin, F. F. and Spirin, A. S. (1988) Biochimie **70**, 619–626
6 Redpath, N. T., Price, N. T., Severinov, K. V. and Pround, C. G. (1993). Eur. J. Biochem. **213**, 689–699
7 Ryazanov, A. G., Shestakova, E. A. and Natapov, P. G. (1988) Nature (London) **334**, 170–173
8 Nairn, A. C. and Palfrey, H. C. (1987) J. Biol. Chem. **262**, 17299–17303
9 Palfrey, H. C., Nairn, A. C., Muldoon, L. L. and Villereal, M. L. (1987) J. Biol. Chem. **262**, 9785–9792
10 Hinke, M. T. and Nairn, A. C. (1992) Biochem. J. **282**, 877–882
11 Mackie, K. P., Nairn, A. C., Hampel, G., Lam, G. and Jaffe, E. A. (1989) J. Biol. Chem. **264**, 1748–1753
12 Redpath, N. T. and Proud, C. G. (1993) Eur. J. Biochem. **212**, 511–520
13 Miller, S. G. and Kennedy, M. B. (1986) Cell **44**, 861–870
14 Frangakis, M. V., Ohmstede, C.-A. and Sahyoun, N. (1991) J. Biol. Chem. **266**, 11309–11316
15 Redpath, N. T. and Proud, C. G. (1989) Biochem. J. **262**, 69–75
16 Reimann, E. M. and Beham, R. A. (1983) Methods Enzymol. **99**, 51–55
17 Bradford, M. M. (1976) Anal. Biochem. **77**, 248–254
18 Bylund, D. B. and Krebs, E. G. (1975) J. Biol. Chem. **250**, 6355–6361
19 Munday, M. R. and Hardie, D. G. (1984) Eur. J. Biochem. **141**, 617–627
20 Cohen, P. (1980) Eur. J. Biochem. **111**, 563–574
21 Conti, M. A. and Adelstein, R. S. (1981) J. Biol. Chem. **256**, 3178–3181
22 Nishikawa, M., de Lanerolle, P., Lincoln, T. M. and Adelstein, R. S. (1985) J. Biol. Chem. **259**, 8429–8436
23 Kameshita, I. and Fujisawa, H. (1991) Biochem. Biophys. Res. Commun. **180**, 191–196
24 Menaya, J., Parrilla, R. and Ayuso, M. S. (1987) Biochem. J. **248**, 903–909
25 Ayuso-Parrilla, M. S., Martin-Requero, A., Perez-Diaz, J. and Parrilla, R. (1976) J. Biol. Chem. **251**, 7785–7790
26 Martin-Requero, A., Perez-Diaz, J., Ayuso-Parrilla, M. S. and Parrilla, R. (1979) Arch. Biochem. Biophys. **195**, 223–234
27 Martin-Requero, A., Martinez-Izquierdo, J. A., Parrilla, R. and Ayuso-Parrilla, M. S. (1981) Horm. Metab. Res. **13**, 22–24

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