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Interaction of the calcium and calmodulin regulated eEF-2 kinase with heat shock protein 90

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Abstract

Immunoadsorbents containing the 8D3 anti-heat shock protein 90 monoclonal antibodies were prepared. Partly purified preparations of the Ca^{2+} and calmodulin dependent eukaryotic elongation factor eEF-2 specific kinase and crude rabbit reticulocyte lysates were mixed with the immunoadsorbent. After removal of unbound proteins the adsorbed material was released by increasing the salt concentration in the buffer. Analysis of the bound material showed that the eEF-2 kinase was bound to the immunoadsorbent together with hsp 90. The adsorption of the kinase was found to depend on the presence of hsp 90.

Key words: CaM PK III; Elongation factor 2; Heat shock protein 90; Phosphorylation

1. Introduction

Protein synthesis in eukaryotic cells can be regulated at the elongation level via reversible phosphorylation of elongation factor eEF-2. As the phosphorylated eEF-2 has a reduced affinity for the pre-translocation type of ribosome, phosphorylation of the factor causes a shut-off of the translocation step in the elongation cycle.

Phosphorylation of eEF-2 is catalysed by a Ca^{2+} and calmodulin-dependent protein kinase CaM PK III [5,6] while reactivation by dephosphorylation is mainly dependent on phosphoprotein phosphatase 2A [7,8]. The activity of the CaM PK III is dependent on the phosphorylation status of the enzyme and the dephosphorylated kinase shows no catalytic activity [9–11].

Heat shock protein hsp 86, a member of the hsp 90 family of proteins, copurifies with the eEF-2 kinase during isolation [9,10]. Heat shock proteins are known to associate with various other proteins including the steroid receptors and protein kinases [12–16]. One of these protein kinases is the haemin-regulated kinase, HRI, which specifically phosphorylates initiation factor eIF-2 in reticulocyte lysates [15–18]. In this case haemin appears to strengthen the interaction between HRI and hsp 90 [16] and it has been suggested that formation of the HRI-hsp 90 complex is the mechanism by which haemin prevents activation of the kinase [19].

We have recently reported that the CaM PK III activity is inhibited by μM concentrations of haemin in rabbit reticulocyte lysates and in phosphorylation experiments using purified components (manuscript in preparation). The apparent similarities between the eEF-2 and eIF-2 kinases in respect to hsp 90 and haemin prompted us to further investigate the possible association of the eEF-2 kinase with hsp 86 in reticulocyte lysates and at various stages of purification of the kinase.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP was from Amersham Int. (UK). Calmodulin (CaM) was from Boehringer-Mannheim (Germany). Okadaic acid was a gift from Dr. Y. Tsukitani (Fujisawa Pharmaceuticals Co., Tokyo, Japan). Goat anti-mouse IgM was from Sigma (USA). CNBr activated Sepharose was from Pharmacia (Sweden). Monoclonal anti-hsp 90 IgM antibodies 8D3 was provided by Dr. G.H. Perdew (Purdue University). Rabbit reticulocyte lysates and eEF-2 were prepared as previously described [20,21]. The eEF-2 kinase (CaM PK III) was purified from rabbit reticulocyte lysates essentially as previously described [22].

2.2. Immunoadsorption

Goat anti-mouse IgM Sepharose was prepared according to the suppliers recommendation. The immobilized antibodies were allowed to bind the 8D3 monoclonal anti-hsp 90 antibodies as described by Matts et al. [16].

For the adsorption of protein to the anti-hsp 90 Sepharose, reticulocyte lysates or purified preparations of the eEF-2 kinase were mixed with the antibody-Sepharose as described by Matts et al. [16]. Unbound material was removed by repeated washing of the Sepharose with 10 mM Tris-HCl and 10 mM Tris-HCl, pH 7.5, containing 50 mM NaCl [16]. Adsorbed proteins were detached from the Sepharose in 10 mM Tris-HCl, pH 7.5, containing 500 mM NaCl [16].

2.3 Determination of kinase activity

Determination of eEF-2 kinase activity was carried out in the presence of 100 mM KCl, 20 mM Tris-HCl, pH 7.6, 6 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1.0 mM EGTA, 1.5 mM Ca^{2+}, 0.07 mM EDTA, 7% (by vol.) glycerol, 0.25 mM [γ-^{32}P]ATP (specific activity 280 Ci/mol), 120 pmol CaM, 1 μmol eEF-2 and eEF-2 kinase. The reaction mixtures were incubated at 30°C for 5 min.

2.4. Gel electrophoresis

SDS gel electrophoresis using poly acrylamide gradient slab gels were according to Laemmli [23]. The gels were stained with Coomassie brilliant blue and destained. The dried gels were exposed to a X-ray film at −80°C using an intensifying screen.

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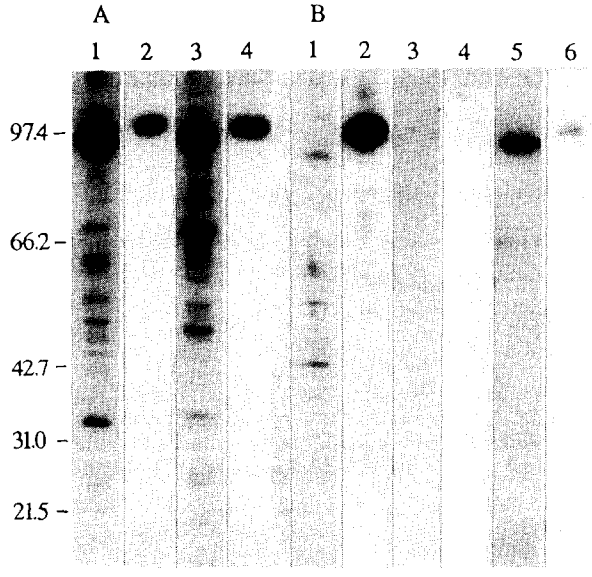

Fig. 1. Analysis of the eEF-2 kinase activity following immunoadsorption to the 8D3 anti-hsp 90 mAb. (A) Preparations containing partially purified eEF-2 kinase (lanes 1 and 2) and reticulocyte lysate (lanes 3 and 4) were allowed to interact with the immunoadsorbent as described in section 2. The adsorbed proteins were released by washing with 0.5 M NaCl and analyzed for eEF-2 kinase activity as described in section 2. Lanes 1 and 3 material detached from the immunoadsorbent at high salt concentration (Stained gels). Lanes 2 and 4 eEF-2 kinase activity in the material detached from the immunoadsorbent (autoradiograms exposed for 1 h). (B) Immunoadsorption of purified eEF-2 kinase lacking hsp 90. Protein composition (lane 1) and kinase activity (lane 2) of the kinase preparation. Protein content (lane 3) and kinase activity of the material detached from the immunoadsorbent at 0.5M NaCl (lane 4). Immunoadsorption of the purified hsp 90 free eEF-2 kinase in the presence of rabbit reticulocyte hsp 90 (lanes 5 and 6). Protein composition (lane 5) and kinase activity of the materal detached at 0.5 M NaCl (lane 6). Lanes 1, 3 and 5 Coomassie brilliant blue stained gel. Lanes 2, 4 and 6 autoradiograms. The autoradigrams were exposed for 1 h (lanes 2) or 24 h (lanes 4 and 6).

3. Results and discussion

Immunoadsorbents based on the 8D3 anti-hsp 90 mAb has been used to demonstrate a direct interaction between hsp 90 and the eIF-2 kinase in rabbit reticulocyte lysates [16,17]. We have used the same immunoadsorbent strategy to investigate the possible interaction of hsp 90 with the Ca^{2+}- and calmodulin-dependent eEF-2 kinase. One advantage with the 8D3 anti-hsp 90 mAb is that the low affinity between the antibody and hsp 90 allows release of the adsorbed hsp 90 and its associated proteins by washing the adsorbent at low salt concentrations [16]. This facilitates the analysis of enzymatic activity among the released proteins. Previous experiments have shown that a fraction of hsp 90 co-purifies with the eEF-2 kinase upon ion exchange chromatography and sucrose gradient centrifugation [9,10,22]. The two proteins also co-purifies upon affinity chromatography on CaM-Sepharose columns (not illustrated). To see if this co-purification could be due to a direct interaction between the kinase and hsp 90 a partially purified kinase preparation was allowed to adsorb to an immunoadsorbent containing the 8D3 anti-hsp 90 mAb. As seen in Fig. 1A, washing of the adsorbents with buffers containing high salt concentrations resulted in a release of considerable amounts of hsp 90 that had been adsorbed from the partially purified kinase preparation. Analysis of the eEF-2 kinase activity of the detached proteins using purified eEF-2 as substrate, showed that the detached proteins were capable of phosphorylating eEF-2. Thus, the eEF-2 kinase was adsorbed to the immunoadsorbent together with hsp 90.

To avoid the possibility that the eEF-2 kinase was adsorbed directly to the matrix of the immunoadsorbent and not via a hsp 90 anti-hsp 90 mAb interaction control adsorption experiments were performed. We have been able to separate the eEF-2 kinase from hsp 90 by repeated chromatography on hydroxylapatite columns using the method of Iwasaki et al. for isolation of hsp 90 [9,24]. These eEF-2 kinase preparations were free from contaminating hsp 90 as judged by electrophoresis (Fig. 1B) and immunoblotting using a combination of hsp 90 specific antibodies (not illustrated). Immunoadsorption experiments using these kinase preparations showed that no kinase activity was found in the salt wash fractions from the adsorbent. This result shows that the eEF-2

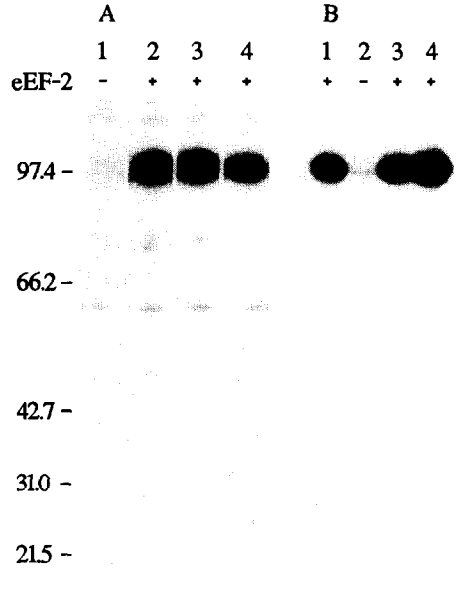

Fig. 2. Autoradiogram showing the eEF-2 kinase activity of the material purified from reticulocyte lysates by immunoadsorption on the 8D3 anti-hsp 90 mAb. (A) Kinase activity in the absence (lane 1) and presence (lanes 2–4) of eEF-2. Kinase activity in the presence of trifluoperazin (TFP) and ocadaic acid (OA), lanes 3 and 4, respectively. (B) Kinase activity in the absence (lane 2) and presence of eEF-2 (lanes 1, 3 and 4). Kinase activity in the presence of 10 mM EGTA (lane 3). Pre-incubation of the kinase for 5 min at 30°C in the presence Ca^{2+} and calmodulin. Thereafter, EGTA (final concentration 10 mM) and eEF-2 were added and the incubation continued as described in section 2 (lane 4). The autoradiograms were exposed for 2 h.

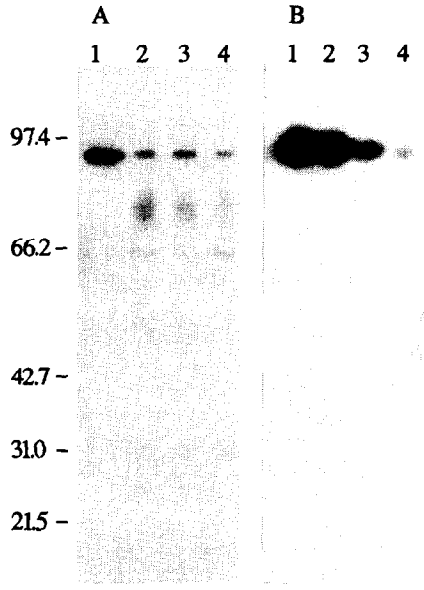

Fig. 3. Co-elution of hsp 90 and the eEF-2 kinase from the 8D3 anti-hsp 90 mAb immunoadsorbent by repeated washing at 0.1 M NaCl (lanes 1–3) and 0.5 M NaCl (lane 4). (A) Coomassie brilliant blue stained gel. (B) Autoradiogram showing the eEF-2 kinase activity of the eluted samples. Exposure was for 2 h.

kinase was not unspecifically adsorbed to the immunoadsorbent. Thus, the observed adsorption of the eEF-2 kinase in preparations containing hsp 90 required an interaction of the 8D3 anti-hsp 90 mAb with hsp 90, suggesting that hsp 90 and the eEF-2 kinase interact in the partially purified kinase preparations.

To see if adsorption of the purified kinase could be restored in the presence of hsp 90, the hydroxylapatite fractions containing the purified kinase and hsp 90, respectively, were recombined. This material was allowed to adsorb to the 8D3 anti hsp-90 mAb. As seen in Fig. 1B, hsp 90 was efficiently adsorbed by the antibodies. However, the adsorbed material contained only trace amounts of the eEF-2 kinase (Fig. 1B, lane 5), indicating that the purified kinase were unable reassociate with hsp 90. Similar problems in reconstituting the interaction between hsp 90 and the steroid receptors have been reported [25,26]. The results also show that the interaction between hsp 90 and the eEF-2 kinase was not induced by the co-presence of both proteins in the solution.

We were interested to see if the eEF-2 kinase interacts with hsp 90 in the lysates or if the interaction seen in the partially purified kinase preparations were an artifact created by the purification procedure. For this purpose reticulocyte lysate samples were allowed to adsorb to the 8D3 anti-hsp 90 mAb immunoadsorbent. As seen in Fig. 1A, washing of the adsorbent with buffers containing increasing salt concentrations liberated considerable amounts of eEF-2 kinase activity together with hsp 90. As seen in Fig. 2A, phosphorylation was dependent on added eEF-2 showing that the phosphorylation seen in the 90 kDa region was not due to phosphorylation of the eluted hsp 90. The eEF-2 kinase adsorbed to the 8D3 anti-hsp 90 mAb showed substantial activity both in the presence of trifluoperazin (TFP) and in the absence of free Ca^{2+} (Fig. 2), suggesting that the immunoadsorbent purified kinase is active even in the absence of Ca^{2+} and calmodulin. However, the activity of the kinase could be increased by preincubation in the presence of Ca^{2+} and calmodulin (Fig. 2B, lane 4). These results are unexpected as no Ca^{2+} and CaM-independent eEF-2 kinase activity has been observed in reticulocyte lysates [7]. Although phosphorylation of eEF-2 using partially purified kinase preparations [9,22] or the hsp 90 free kinase is strictly dependent on Ca^{2+} and CaM (not illustrated) it has recently been reported that purified preparations of CaM PK III can be converted into a Ca^{2+} and CaM-indpendent form presumably by autophosphorylation [10,11]. This may indicate that a similar activation procedure occurs during isolation of the kinase from reticulocyte lysates using the 8D3 anti-hsp 90 mAb immunoadsorbent.

In reticulocytes phospho-eEF-2 is dephosphorylated by the okadaic acid (OA) sensitive phosphoprotein phosphatase PP2A [7,8]. Addition of OA to the phosphorylation reaction containing the immunoadsorbed eEF-2 kinase gave no increase in the eEF-2 phosphorylation (Fig. 2), suggesting that PP2A was not present among the immunoadsorbed proteins.

Attempts to specifically detach the adsorbed kinase without a simultaneous release of hsp 90 using limited salt wash showed that both the kinase and hsp 90 was detached from the adsorbent already at 0.1 M NaCl (Fig. 3). Thus, it was impossible to detach the eEF-2 kinase from the adsorbent without a simultaneous release of the bound hsp 90.

The functional implications of the CaM PK III-hsp 90 interaction is not clear. However, hsp 90 has been reported to regulate the activity of other protein kinases such as casein kinase [27] and the eIF-2 kinase, HRI [19]. It is therefore possible that binding of CaM PK III and HRI to hsp 90 provides the basis for a co-ordinated regulation of the rate of protein synthesis initiation and elongation. Such co-ordination has been observed during heat shock in *Drosophila* cells and in HeLa cells exposed to amino acid analogues [28,29].

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