

SDS/PAGE characteristics of protein kinases tightly associated with chick embryo brain ribosomes

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Protein kinases tightly associated with chick embryo brain ribosomes washed with Triton X-100 and KCl were characterized by their ability to phosphorylate ribosomes and two exogenous substrates, histone IIA and casein. c-AMP-dependent kinase (PKA) and casein kinases (CK1, CK2) were examined in the presence of specific modulators by SDS/PAGE followed by renaturation in gel assay according to Kameshita & Fujisawa (*Anal. Biochem.* 1989, 183, 139-143). Basing on these data it can be presumed that PKA activity increases, but the levels of CK2 and CK1 decrease during chick embryo development.

Protein phosphorylation systems provide an important regulatory mechanism in protein synthesis. Activity of several initiation and elongation factors as well as aminoacyl-tRNA synthetases depends on their phosphorylation states [1, 2]. Phosphorylation of ribosomal protein S₆ is one of the mechanisms which control the speed and/or fidelity of mRNA translation [3]. Phosphorylation of the acidic ribosomal proteins (P-proteins) stimulates the rate of peptide chain elongation [4, 5].

We have shown previously that the intensity of phosphorylation of brain ribosomal pro-

teins *in vivo* decreased during development of chick embryo and in the 19-day old embryos was seventy times lower than in 5 day old embryos [6]. These changes were not correlated with the overall cytosolic protein kinases activity [7].

Our earlier observations [8] indicate that protein kinases responsible for ribosomal protein phosphorylation are tightly bound to ribosomes.

In the present paper we have applied SDS/PAGE analysis of protein kinases tightly bound to brain ribosomes.

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; PKA, cAMP-dependent protein kinase; CK2, protein kinase CK2 („casein kinase-2”); CK1, protein kinase CK1 („casein kinase-1”); PKI, Walsh inhibitor of PKA.

MATERIAL AND METHODS

Preparation of chick brain cell free extracts and ribosomes. Brains from 8-, 14- and 18-day old chick embryos were removed, minced and washed free of blood with ice cold buffer A (50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 10 mM KCl, 10 mM 2-mercaptoethanol, 1 mM PMSF) containing 0.25 M sucrose, and homogenized in 5 vol. (w/v) of the same buffer. To remove connective tissue and other cellular debris, the obtained homogenate was centrifuged at 20 000 × *g* for 40 min.

Ribosomes were separated from cell free extracts by centrifugation at 100 000 × *g* for 2.5 h. The microsomal fraction was resuspended in buffer A containing additionally 1% Triton X-100, and membrane free ribosomes (crude ribosomes) were sedimented by centrifugation as above. Crude ribosomes resuspended in buffer A supplemented with KCl to 1 M concentration were sedimented in the conditions described before. Washed ribosomal preparations were stored in 40% glycerol in buffer A [8].

Endogenous phosphorylation of ribosomes in vitro. Ribosomes were used as both an enzyme and a substrate source. The standard reaction mixture in a volume of 100 μl, contained: 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 60–100 μg of ribosomes, 0.09 mM [γ -³²P]ATP (1000 c.p.m./pmol). After 10 min incubation at 30°C the reaction was stopped by the addition of Laemmli sample buffer [9]. Proteins were then separated by SDS/PAGE and their phosphorylation level was determined after autoradiography.

Electrophoretic methods. SDS/PAGE was performed in 10% gel or in 5–15% gradient according to Laemmli [9]. Gels were stained with Coomassie Brilliant Blue R-250. The molecular mass of proteins was calculated on the basis of the following marker proteins (Pharmacia): phosphorylase b (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean

trypsin inhibitor (20 kDa), and α -lactalbumin (14.4 kDa).

Renaturation and assay of protein kinases after SDS/PAGE (gel assay). Protein kinases were renatured and detected according to Kameshita & Fujisawa [10].

Preparation of acidic ribosomal proteins (P-proteins). The acidic proteins were extracted from brain ribosomes (20 mg/ml) with 0.25 M NH₄Cl/50% ethanol at 0°C for 20 min as described by Sanchez-Madrid *et al.* [11].

Reagents. All the reagents used including Walsh inhibitor of PKA were from Sigma.

RESULTS AND DISCUSSION

Purified ribosomes from yeast [12, 13] and liver [13] are apparently free of protein kinase activities, however, brain ribosomes isolated from chick embryos are different, since being at same stage of purification still show protein kinase activities [8].

To study endogenous phosphorylation the washed ribosomes were incubated with [γ -³²P]ATP, and ³²P-labelled proteins were analysed by SDS/PAGE and autoradiography. Over ten radioactive bands were detected (Fig. 1a, lane C) proving the association of protein kinase activity with brain ribosomes. Out of the visible radioactive bands, the proteins of 50–58 kDa were phosphorylated with the highest intensity.

When the effect of specific modulators of different protein kinases was tested under the same conditions, small but qualitative and quantitative differences were distinct (marked by asterisks).

Cyclic-AMP (lane A) increased phosphorylation of the 32 kDa and 16 kDa proteins and stimulated phosphorylation of two additional proteins of 22 and 23 kDa. Phosphorylation of these proteins was inhibited by PKI, the Walsh inhibitor of PKA (lane I), while the proteins of 58, 45, 20, 13 kDa were phosphorylated more intensively. It is of in-

terest that these proteins serve probably as phosphorylation substrates for CK2, since their phosphorylation is stimulated by spermine [14] (lane S), and inhibited by heparin [15] (lane H). It is worth noting that heparin completely inhibited phosphorylation of the 13 kDa band, corresponding to acidic ribosomal proteins (P-proteins) (Fig. 1b), which are known to be phosphorylated by CK2 [8,

G). These data point to the presence of CK2. However, the presence of other kinases which preferentially phosphorylate casein and which are not inhibited by heparin can not be excluded.

More information on the ribosome-associated protein kinases was obtained by renaturation in the gels containing histone IIA or casein following SDS/PAGE ("in gel as-

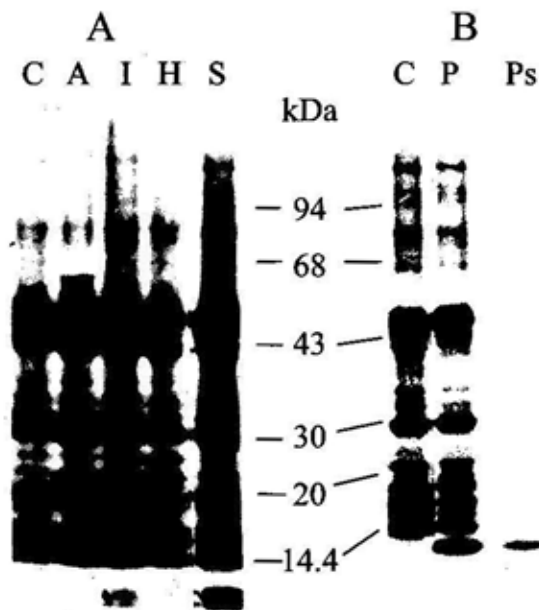


Figure 1. Endogenous phosphorylation of chick embryo brain ribosomes in the presence of PKA and CK2 effectors (a), and phosphorylation of acidic ribosomal proteins (P-proteins) by brain ribosomes (b).

Washed brain ribosomes (60 μ g) were incubated with [γ - 32 P]ATP and analysed by SDS/PAGE (5–15% gradient gel) and autoradiography. Lanes: C, control (ribosomes alone); A, cAMP (5 μ M); I, Walsh inhibitor of PKA (10 μ g); H, heparin (50 μ g/ml); S, spermine (0.2 mM); P, acidic ribosomal proteins (3 μ g); Ps, P-proteins alone (3 μ g) stained with Coomassie Brilliant Blue. Positions of the main differences are indicated by asterisks. The acidic proteins (P-proteins) were extracted from brain ribosomes with 0.25 M NH_4Cl /50% ethanol [11].

12, 13]. Furthermore, heparin stimulated phosphorylation of additional proteins of 43, 27 and 24 kDa. Similar stimulation of phosphorylation by heparin in a substrate-dependent manner was observed for the CK1 γ subfamily [16] and neuronal Cdc-2-like kinase [17].

To characterize ribosomal protein kinases, we used a small amount of ribosomes (2 μ g) as a source of protein kinases and histone IIA or casein as exogenous substrates. Phosphorylation of histone by the ribosome bound kinase was significantly increased by cAMP and completely abolished by the PKA inhibitor (Fig. 2a), confirming the presence of PKA bound to ribosomes. Phosphorylation of casein (Fig. 2b) was significantly stimulated by spermine (lane S) and distinctly lowered by heparin (lane H). Addition of GTP also decreased phosphorylation of casein [18] (lane

say"). The activity of denatured and renatured kinases was examined by autoradiography. When histone IIA was used as a substrate in the renaturation gel assay [10] the radioactive band of 40 kDa, corresponding in its mobility to the catalytic subunit of PKA was detected (Fig. 3a). When casein was phosphorylated in the same gel assay (Fig. 3b) two radioactive bands of 35–42 and 50–58 kDa corresponding to the catalytic subunit of CK2 and probably of CK1 respectively, were detected. In Fig. 3ab lanes 1, 2 and 3 represent the activity of ribosomal kinases on the 8, 14 and 18 day of chick embryo development, illustrating the age-related changes. As can be seen (Fig. 3a) PKA activity increased, while the activity of CK2 (Fig. 3b) decreased during chick embryo development. Quantitative evaluation of these results points to a significant concentration of CK2, particularly, that, in the gel assay,

only activity of the catalytic subunit is determined, and reconstitution of the holoenzyme is known to enhance this activity 2–5-fold [19,

tory subunits (R IIB) of PKA. It is known that CK2 mainly localized to nuclei and cytosol [18] is also tightly associated with 80S ribo-

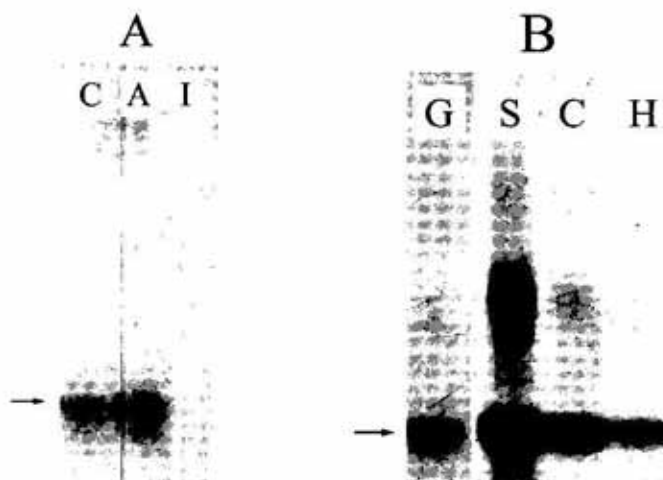


Figure 2. Effect of some modulators of PKA and CK2 on phosphorylation of histone IIA (a) and casein (b).

Histone IIA or casein and washed brain ribosomes (2 μ g) used as a source of protein kinases, were incubated with [γ - 32 P]ATP and analysed by SDS/PAGE. Lanes: C, no additions; A, cAMP; I, Walsh inhibitor of PKA; G, GTP (0.3 mM); S, spermine; H, heparin. Positions of phosphorylated substrates were indicated by arrows.

20]. The activity of the enzyme phosphorylating casein of 50–58 kDa, also decreased during embryogenesis. Its molecular mass corresponds to protein kinase CK1 identified in the cytosol fraction of chick embryo brain (Sanecka-Obacz, Szyszka, unpublished).

In the central nervous system, c-AMP dependent protein kinase IIB (PKA IIB) is the

somes of many eukaryotic organisms. Two ribosomal proteins, L5 [23] and L41 [24] interact with beta subunit of CK2. Moreover, it was found that CK2 forms a complex with liver pp45 [25], a protein, which is composed of the beta and gamma subunits of translation initiation factor eIF-2 [26]. In all cases the regulatory subunits determine substrate specificity

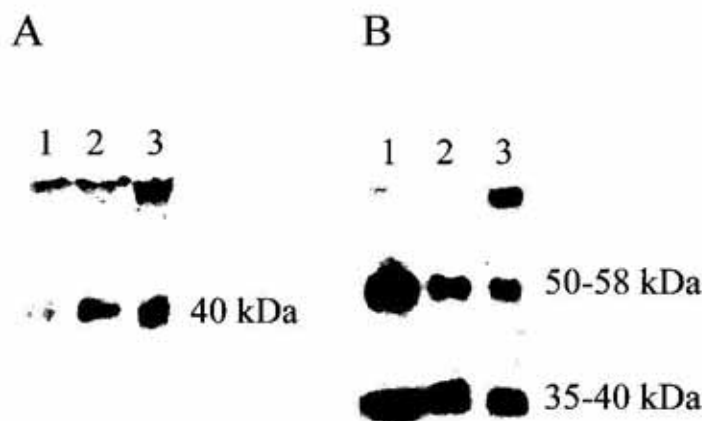


Figure 3. Detection of ribosomal protein kinases by renaturation after SDS/PAGE according to Kameshita & Fujisawa (gel assay) [10] with histone (a) or casein (b) as substrates.

Washed ribosomes (50 μ g) from chick embryo brain, were electrophoresed on SDS/polyacrylamide slab gels (10%), containing 1 mg/ml histone IIA (A), or 1 mg/ml casein (B), and renatured *in situ* after treatment with 6 M guanidine HCl. The gels were incubated for 2 h in phosphorylation buffer containing [γ - 32 P]ATP. Then, the gels were washed, dried and submitted to autoradiography. Lanes 1, 2, 3 present day 8, 14 and 18 of chick embryo development, respectively.

predominant PKA isoform [21, 22]. More than 70% of PKA IIB is bound *via* a high affinity interaction of regulatory subunit (R IIB) with anchor proteins of cytoskeleton, membranes, and other organelles of neurons [22]. It seems probable that proteins of 50–58 kDa represent such a complex containing regula-

and physiological function, but anchor proteins may regulate both the activity and subcellular localization of PKA and CK2.

To our knowledge, CK1 was not detected in ribosomes, but was found in microsomes [18], membranes [27], cytoskeleton [28], and neurofilaments [29]. In animals CK1 exists in

multiple isoforms [16, 30], having molecular masses of 40–60 kDa [16, 18]. Hybridization to mRNA points to a tissue-specific distribution of CK1 isoforms [16, 31], and to the presence of CK1 α and CK1 γ 3 subfamilies in brain [16, 32], where the latter isoform has the highest molecular mass. As substrates for CK1 serve some components of the translation apparatus including initiation factors 4B, 4E and 5 [33], dopamine- and cAMP regulated phosphoprotein (32 kDa) [34], all three neurofilaments [29], and the microtubule associated protein-tau [35]. Phosphorylation of these elements is a key event in axonal growth and regeneration, and in neurofibrillary pathology. Tau is hyperphosphorylated in brain of Alzheimer's disease patients [29].

Basing on these informations and our data, it can be presumed that in chick embryos PKA, CK2 and probably CK1 are tightly bound to brain ribosomes. Their specific isoforms may reflect adaptation of brain to specific functions.

Protein phosphorylation systems are much more active in nervous tissue than in non-nervous ones [36], which explains the fact that so many protein kinases copurify with ribosomes.

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