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The tight association of protein kinase CK2 with plasma membranes is mediated by a specific domain of its regulatory β-subunit

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Abstract

Previous immunocytochemical studies have shown that protein kinase CK2 is mostly detected both in the cytoplasm and the nucleus of most cells. In the present study, CK2 was detected in highly purified plasma membrane preparations from rat liver. The protein kinase could be released from the membranes by high salt extraction (>1 M NaCl). Plasma membranes prepared from SF9 insect cells expressing the α- and β-subunits of CK2 also contained a significant amount of oligomeric CK2. Furthermore, it was demonstrated in this cell system as well as in rat liver plasma membranes, that the β-subunit of the kinase is the targeting subunit which mediates the tight association of the enzyme to plasma membrane components. Binding studies using membranes and recombinant proteins corresponding to different regions of the β-subunit suggest that a functional domain previously shown to be involved in the binding of polyamines may also participate to the binding of CK2 to membranes. Modification of membranes by trypsin and phospholipases indicated that the binding process may require both membrane protein(s) and phospholipids. Interestingly, it was observed that the amount of membrane-bound CK2 in liver of embryos and new born rats increases dramatically after birth and persists during the postnatal stages of development. © 1998 Elsevier Science B.V.

Keywords: Protein kinase CK2; Regulatory subunit; Membrane association

1. Introduction

The protein kinase CK2 (CK2) from most sources is a tetrameric enzyme composed of two α- (and/or α′-) subunits and two β-subunits [1,2]. The α- and α′-subunits contain all of the conserved consensus motifs of protein kinase family members [3], and bear the catalytic site [4–7]. The precise functions of β-subunit are not known, but it does appear to have a role in regulating the kinase activity of the a subunit [8–10]. Complementary DNAs encoding the α-, α′- and β-subunits of CK2 have been isolated from human and chicken libraries [11–15]. These studies indicated that the α- and α′-subunits are closely related proteins that are encoded by distinct genes. Several studies have demonstrated that cellu-

Abbreviations: CK2, protein kinase CK2; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MBP, maltose binding protein

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lar mammalian CK2 exists as homotetramers ($\alpha_2\beta_2$ or $\alpha'_2\beta_2$) as well as heterotetramers ($\alpha\alpha'\beta_2$) [16,17]. At present, distinct functions for CK2α and CK2α′ have not been clearly defined. Immunocytochemical studies of the intracellular localisation of the two isomeric forms of CK2 have been mostly devoted to the characterisation of the nuclear and cytoplasmic distribution of the kinase, although with somewhat controversial conclusions [18,19]. However, previous studies have shown that several membrane receptors, such as the insulin and IGF-II receptors and the cation independent mannose 6-phosphate receptor are phosphorylated by CK2 in intact cells [20–23]. These observations suggest that CK2 may play a role in the regulation of membrane components such as intrinsic receptors. Thus, it may be suspected that varied subcellular locations of CK2 isoforms may connote differences in biochemical functions.

Here we report results of our effort aiming at exploring the functions of CK2 isoforms by localising these proteins within the cell. We find that an heterotetrameric form of CK2 is tightly associated with highly purified rat liver plasma membranes. The associated protein kinase could be released from the membrane upon addition of recombinant isolated β-subunit. Similarly a plasma membrane bound form of CK2 was isolated from SF9 cells overexpressing the CK2 subunits. Using this cell system we have shown that a specific domain of the β-subunit previously shown to be involved in the binding of polyamines may target the kinase to the membranes. In addition, membrane treatment by trypsin or phospholipases, suggests that the reversible binding of CK2 may require both protein(s) and phospholipids.

A possible involvement of membrane-bound CK2 in postnatal development is suggested by the increase in CK2 found associated with rat liver plasma membranes after birth in new born rats.

2. Materials and methods

[γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham. Casein, phosvitin were obtained from Sigma. Phosphocellulose was purchased from Whatman. Recombinant human basic FGF was provided to us by J.-J. Feige (INSERM Unité 244, BRCE, Grenoble). Antiserum Rα is an antiserum generated against the recombinant α-subunit of CK2 purified from baculovirus-infected SF9 cells [4]. Antiserum Ab-Pα′21 is an anti-peptide antiserum generated against the 21 C-terminal amino acids of the α′-subunit [16]. The αβ-antiserum which was provided by C.V.C. Glover (University of Georgia, Athens, GA, USA) is a rabbit antiserum generated against oligomeric *Drosophila* CK2 [24]. Bovine CK2 was purified to homogeneity using a previously described method [24].

CK2 activity assay was performed using a synthetic peptide (RRREEETEEE) as substrate, as previously described [8].

2.1. Immunoblotting

Samples were run on 12% SDS-polyacrylamide gels and proteins were electrotransferred (1 h, 100 V) onto immobilon P membranes (Millipore). CK2 subunits (α, α′ and β) were detected using rabbit antisera at a 1/500 dilution.

The membranes were then incubated 1 h with horseradish peroxidase-labelled anti-rabbit IgG antibody. The immunocomplexes were visualised using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham).

2.2. Preparation and purity of liver plasma membranes

Plasma membranes were prepared essentially by the method of Ray [25], from liver of male albino Wistar rats (150 g). This preparation was further purified by a 5–50% (w/w) continuous sucrose gradient (100 000 × *g* for 30 min).

Enrichment and purity of final plasma membrane preparations have been estimated using specific marker enzymes, as described by Sarrouilhe et al. [26]. The purity of the membrane preparation has been also controlled by electron microscopy which has not revealed any contaminant.

2.3. Preparation of plasma membrane from infected SF9 cells

SF9 cells were infected with the recombinant viruses EV55 Dmα and EV55 Dmβ to express respec-

tively the α- and β-subunits of CK2, as previously described [8].

At 3 days postinfection (15×10^6), cells were harvested and homogenised in 20 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethyl sulphonyl fluoride and 50 mg/ml leupeptin. The cell lysate was sonicated for 3 min and centrifuged at $15\,000\times g$ for 20 min. The supernatant was then centrifuged at $23\,000\times g$ for 30 min and the pellet was resuspended in 10 mM Tris HCl, pH 7.4, 1 mM DTT, and centrifuged again over a cushion of 20% sucrose in the same buffer at $30\,000\times g$ for 1 h. The pellet was finally resuspended in 10 mM Tris HCl pH 7.4, 1 mM DTT, 2% glycerol (TDG buffer) containing 0.5 M NaCl and the suspension was centrifuged at $23\,000\times g$ for 30 min. The pellet was resuspended in TDG buffer and frozen by aliquots at −80°C.

2.4. Partial purification of CK2 extracted from rat liver plasma membranes

Highly purified rat liver plasma membranes (6 mg protein) were extracted in 0.3 ml TDG buffer containing 1.5 M NaCl for 15 min at 4°C. After centrifugation for 15 min at $15\,000\times g$, the supernatant was diluted in 3 ml of TDG buffer and applied to a phosphocellulose column (0.2 ml). The column was eluted stepwise by 0.5 ml of TDG buffer containing increasing NaCl concentrations.

2.5. Protein kinase CK2 radioactive labelling

Protein kinase CK2 radioactive labelling was carried out as previously described [27].

2.6. Protein expression

Chicken CK2 β-subunit was produced in SF9 cells and purified as described [8].

Chicken CK2 β-subunit was also expressed in *E. coli* as a fusion protein with the maltose binding protein (MBP) The sequences coding for the chicken β-subunit (representing amino acids 1–215) or β-subunit fragment corresponding to amino acids 51–110 were amplified by PCR from the corresponding cDNA (kindly provided by Dr. E. Nigg). The PCR amplification led to DNA fragments carrying a *Bam*HI site and a *Hin*dIII site located respectively at the

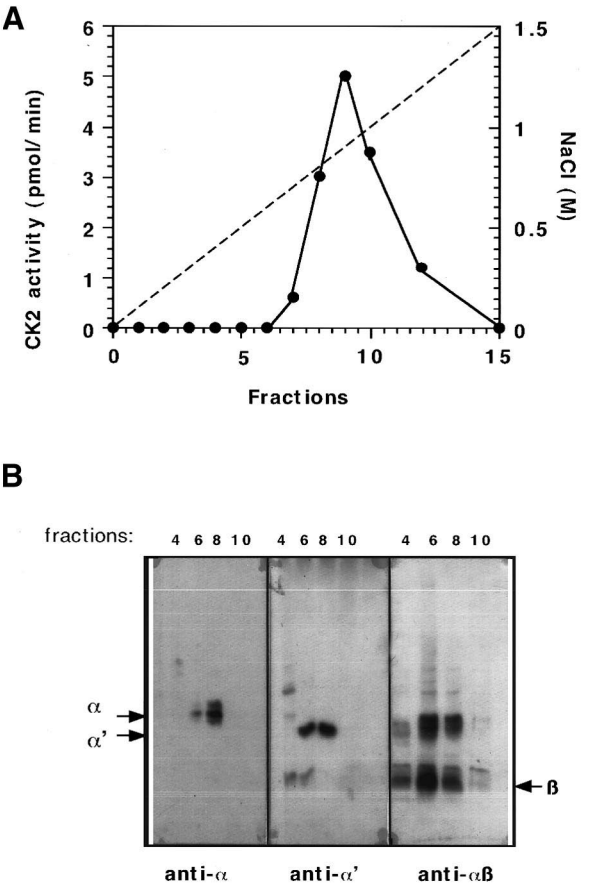

Fig. 1. Isolation of a protein kinase CK2 activity from rat liver plasma membranes. (A) Highly purified rat liver plasma membranes (6 mg protein) were extracted by shaking in 0.3 ml of TDG buffer containing 1.5 M NaCl for 15 min at 4°C. After centrifugation for 15 min at $15\,000\times g$, the supernatant was diluted in 3 ml of TDG buffer and applied to a phosphocellulose column (2 ml). The column was eluted by a linear NaCl gradient in TDG buffer. Protein kinase activity in each fraction was determined using the specific peptide substrate for CK2. The assays were carried out as described in Section 2. (B) Fractions from the phosphocellulose column which contained CK2 activity were analysed on a 12% SDS-PAGE and the proteins were transferred on an immobilon membrane. The membrane was probed with the Rα antibody (anti-α), the Ab-Pα′21 antibody (anti-α′), and the αβ antibody (anti-αβ).

5′- and 3′- extremities of the coding sequence. PCR products were cloned in the pMAL-C2 vector (protein fusion and purification system, New England Biolabs) at the corresponding restriction sites.

The resulting recombinant vectors were used to transform *Escherichia coli* strain BL21. The culture was induced with 0.3 mM isopropyl thiogalactopyranoside (IPTG). The cell pellet was resuspended in

cold lysis buffer (10 mM phosphate, 30 mM NaCl, 0.25% Tween 20, 10 mM EDTA, 10 mM EGTA, pH 7.0). After a thermal shock (−70 to +20°C) and a 3×2-min sonication, the lysate was adjusted to 0.5 M NaCl and subjected to centrifugation at 9000×*g* for 20 min. The supernatant was then mixed with amylose resin (New England Biolabs) at 4°C during 1 h and the fusion protein was eluted with 10 mM maltose added to column buffer (10 mM phosphate, 0.5 M NaCl, 1 mM azide, 1 mM EGTA, pH 7.0). The recombinant protein MBP-β chicken was finally concentrated on a centricon cell up to 4 mg/ml.

Recombinant mouse p53 was prepared as previously described [27].

2.7. Embryo collection

Sprague–Dawley rat embryos were surgically removed from anaesthetised mothers (plug day = day 0) placed under an optical microscope. Intact embryos were immediately processed for liver plasma membrane preparation as described above.

3. Results

3.1. Characterisation of a protein kinase CK2 activity in rat liver plasma membrane

Highly purified rat liver plasma membrane were extracted with a buffer containing 1.5 M NaCl and the extracted proteins were chromatographed on a phosphocellulose column and eluted with increasing salt concentrations. Assay of protein kinase activity in the different collected fractions using as a substrate a synthetic peptide which has been shown to be specific for CK2, disclosed the presence of a single peak of protein kinase activity eluting between 0.6 and 1.5 M NaCl (Fig. 1A). The protein kinase activity present in these fractions was further characterised using different anti-CK2 antibodies.

A Western blot analysis of the corresponding fractions was performed with anti-CK2 antibodies recognising specifically the α-, α′- and β-subunits. The results illustrated in Fig. 1B show that the antibodies recognised polypeptides with molecular weight corresponding to those of the α-, α′- and β-subunits of the

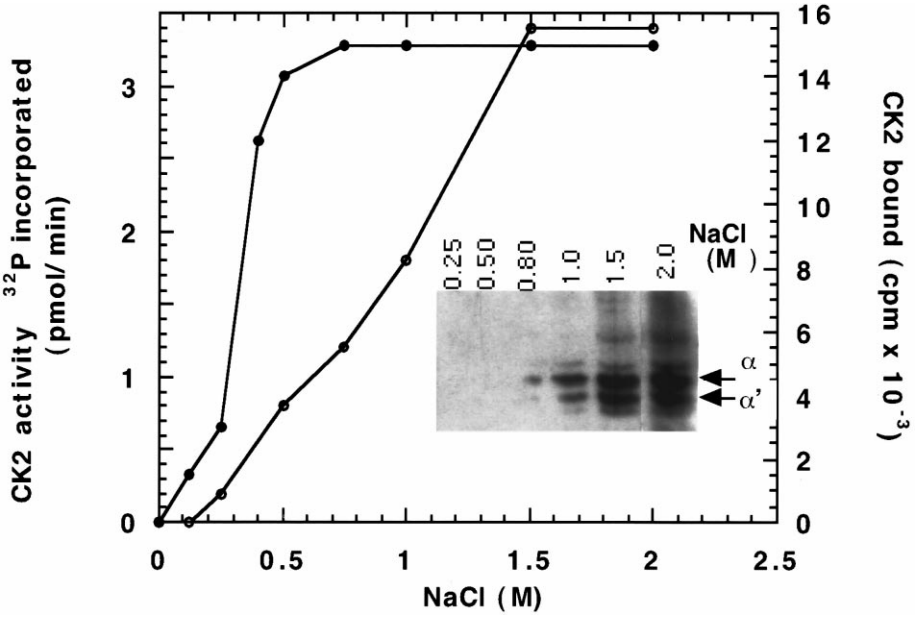

Fig. 2. Salt extraction of protein kinase CK2 from rat liver plasma membranes. Aliquots of plasma membrane (250 μg) were extracted for 20 min at 4°C in 30 μl TDG buffer containing increasing NaCl concentrations. After centrifugation of the extracts, the supernatants were used for determination of CK2 activity (○) and the extracted proteins were separated by SDS-PAGE and Western blotted on an immobilon membrane. The presence of catalytic CK2 subunits was revealed on the membrane using an anti-CK2α antibody. Membranes were also incubated with ^{32}P-autophosphorylated recombinant CK2 for 30 min at room temperature. Membranes were washed and extracted in TDG buffer containing increasing NaCl concentrations. After centrifugation, the radioactivity present in the supernatants was counted (●).

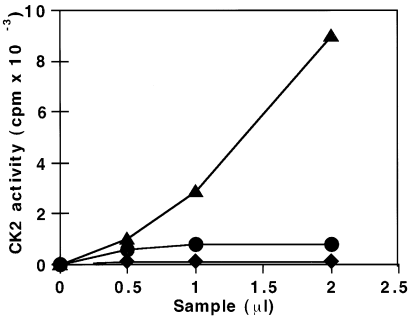

Fig. 3. CK2 activity in intact membranes and in membrane extracts. CK2 activity was determined on aliquots of either intact membranes (♦) or after extraction with 2 M NaCl (▲) or solubilisation with 1% Triton X-100 (●).

mammalian CK2 [5]. A gel filtration analysis disclosed that the protein kinase isolated on phosphocellulose was eluting from the column with an apparent molecular weight of 130–150 kDa (not shown). We therefore conclude that the kinase activity which is associated with the plasma membrane is an oligomeric protein kinase CK2 composed by the association of α-, α′- and β-subunits as described for CK2 purified from either nuclear or cytoplasmic fractions [28].

3.2. Salt extraction of protein kinase CK2 associated with plasma membranes

Aliquots of plasma membranes were extracted by increasing concentrations of NaCl. After centrifugation the amount of CK2 activity and of immunoreactive α- and α′-subunits were determined in the supernatants. The results illustrated in Fig. 2 show that the membrane bound kinase could be extracted by high salt concentrations. A maximum recovery was achieved for concentrations above 1 M NaCl. At a concentration of 1.5 M NaCl, almost all the CK2 subunits present in the membranes were recovered in the supernatant. Plasma membranes were also incubated with ^{32}P-autophosphorylated re-

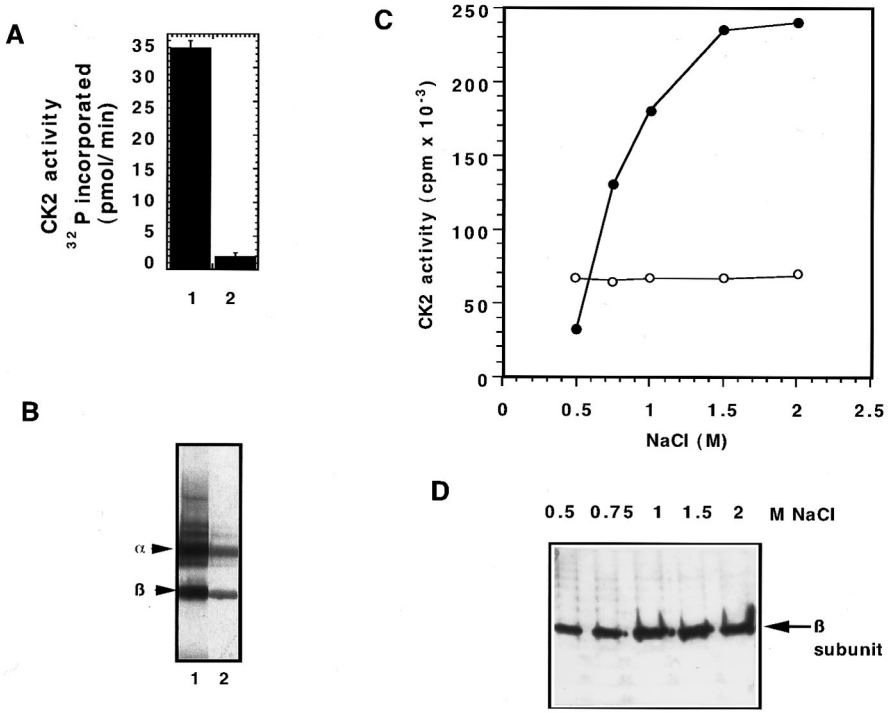

Fig. 4. Detection of protein kinase CK2 in plasma membranes from SF9 cells. At 3 days postinfection, SF9 cells (15×10^6) either uninfected (control cells) or infected with EV55 Dmα+EV55 Dmβ were used to prepare whole cell extract or plasma membranes. Aliquots of whole cell extract (1) or membrane extract (2) were analysed for CK2 activity (A) and for the presence of CK2 α- and β-subunits by Western blotting detection (B). (C) Aliquots of plasma membranes expressing either the oligomeric CK2 or its isolated subunits were extracted by increasing NaCl concentrations. CK2 activity was determined in the different salt extracts of membranes from cells expressing the oligomeric CK2 (●) or the isolated α-subunit (○). (D) A Western blotting detection was performed on the salt extracts of membranes from cells expressing the isolated β-subunit.

combinant CK2. After incubation, the membranes were extracted by increasing NaCl concentrations. It was observed that the exogenously added CK2 bound to membranes but the kinase was easily extracted by salt concentrations lower than 0.5 M NaCl.

3.3. CK2 activity in intact membranes

CK2 activity associated with rat liver plasma membranes was determined on intact membranes, and on NaCl or Triton X-100 extracts of the same membrane samples (Fig. 3). Surprisingly, no kinase activity was detectable either in intact membranes nor in Triton X-100 extracts. In contrast, a high salt extract of membrane exhibited a high CK2 activity, suggesting the presence of inhibitory compounds which were separated from the kinase by salt extraction of membranes.

3.4. Plasma membrane-bound CK2 in SF9 insect cells overexpressing the CK2 subunits

SF9 cells were co-infected with the baculoviruses encoding for the α- and β-subunits of CK2. Plasma membranes and whole cell extract were prepared and extracted by 1.5 M NaCl. The amount of CK2 subunits was quantified in each extract by CK2 activity determination and by Western blotting (Fig. 4A,B). A significant amount (~8%) of the expressed recombinant oligomeric CK2 was recovered in the plasma membrane fraction. SF9 cells were also separately infected with bacoloviruses encoding for the α- and β-CK2 subunits. It was observed that the extraction of the oligomeric recombinant CK2 present in the plasma membranes required high salt concentrations (Fig. 4C), a situation already observed for the extraction of CK2 associated with rat liver plasma membranes (Fig. 2). In contrast, the isolated α-subunit present in the insect cell membrane was easily recovered in 0.5 M NaCl. Membranes from SF9 cells expressing the β-subunit alone were extracted in the same conditions. Interestingly, the salt concentrations which were necessary to extract the bound β-subunit were similar to those required to extract the oligomeric CK2 (Fig. 4D).

Taken together, these experiments suggest that whereas the α-subunit has a low affinity for plasma membrane, the tight association of the isolated β-subunit is reminiscent of the binding properties of the oligomeric form of the kinase.

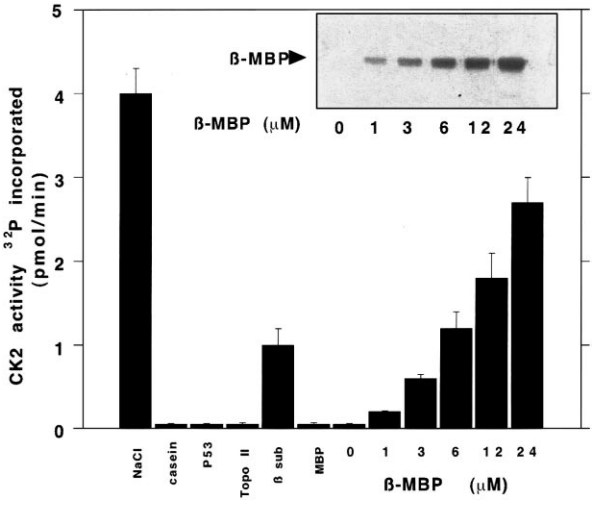

Fig. 5. Release of protein kinase CK2 from rat liver plasma membranes upon incubation with recombinant CK2 β-subunit. Aliquots of plasma membranes were incubated for 30 min at 4°C with the following additions: 1.5 M NaCl, 30 μM casein, 1 μM p53, 1 μM topoisomerase II, 1 μM recombinant β-subunit expressed in SF9 cells, 10 μM MBP, or increasing concentration of β-MBP fusion protein expressed in *E. coli*. After centrifugation of the extracts, aliquots of the different supernatants were used for CK2 activity determination. Membrane pellets were analysed for the presence of the β-MBP fusion protein by Western blotting. The membrane was probed with anti-MBP antibody (inset). This experiment is representative of three separate experiments.

3.5. Release of plasma membrane-bound CK2 by recombinant CK2 β-subunit

Aliquots of rat liver plasma membranes were incubated with different CK2 substrates such as casein, p53 protein and topoisomerase II. After centrifugation, the protein kinase activity present in the supernatants was determined. As shown in Fig. 5, no CK2 activity could be detected in the resulting supernatants. However, incubation of membranes with the isolated CK2 recombinant β-subunit led to the recovery of some CK2 activity in the supernatant. Similarly, incubation of membranes with increasing concentrations of β-MBP fusion protein expressed in *E. coli* could release CK2 activity. More than 60% of the amount of CK2 extracted by 1.5 M NaCl was released upon incubation of membranes with 24 μM

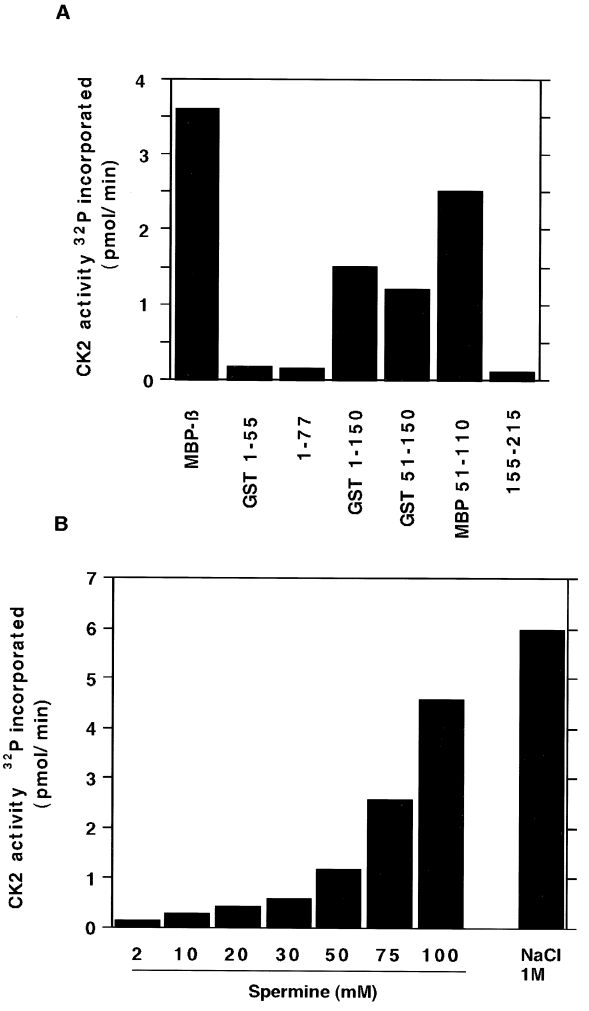

Fig. 6. Release of protein kinase CK2 from rat liver plasma membranes upon incubation with a specific CK2 β-subunit domain. (A) Aliquots of plasma membranes were incubated for 30 min at 4°C in the presence of 20 μM of fusion proteins containing different β-subunit regions or synthetic peptide corresponding to the β-subunit sequences 1–77 and 155–215. After centrifugation of the extracts, aliquots of the different supernatants were used for CK2 activity determination. (B) Aliquots of plasma membranes were incubated for 30 min at 4°C with increasing concentrations of spermine or with 1 M NaCl. After centrifugation, aliquots of the different supernatants were assayed for CK2 activity. This experiment is representative of two separate experiments.

of the β-MBP fusion protein. Under these experimental conditions, the β-MBP fusion protein exhibited a saturable binding to plasma membranes (Fig. 5, inset) At the same concentrations MBP alone had no effect. Thus these experiments provide evidence that the regulatory subunit of the membrane-bound form of CK2 is the specific interacting subunit mediating the attachment of CK2 to plasma membrane components.

To delineate further the region of the β-subunit which is involved in this interaction, aliquots of rat liver plasma membranes were incubated with synthetic peptides or fusion proteins corresponding to different regions of the CK2 β-subunit (Fig. 6A).

The amino terminal domain (GST^{1-55} or $peptide^{1-77}$) was without effect on the membrane-bound CK2. However fragments of the β-subunit which contain the region between residues 51 and 110 (GST^{1-150}, GST^{51-150}, MBP^{51-110}) could release CK2 activity. More than 70% of the amount of CK2 extracted by the β-MBP fusion protein was released upon incubation of membranes with 20 μM of MBP^{51-110}. Under the same conditions, $peptide^{155-215}$ which corresponds to the C-terminal domain of the β-subunit was without effect.

Altogether, this experiment suggests that the association of CK2 with plasma membrane components is mediated by a specific β-subunit domain localised between residues 51 and 110. It has been shown previously that this region represents a regulatory domain which binds polybasic compounds like polyamines. Indeed it was observed that when plasma membranes were incubated with increasing concentrations of spermine, this polyamine was competing against CK2 binding, causing the release of the kinase from the membrane (Fig. 6B).

3.6. Nature of the CK2 binding sites in plasma membranes

To better elucidate the nature of the CK2 receptor site(s), plasma membranes from SF9 cells expressing the CK2 subunits were either extracted by 1 M NaCl or incubated in the presence of Trypsin or phospholipase D. At the end of the incubation, membranes were centrifuged and the presence of CK2 in the supernatants was quantified (Fig. 7A). It was observed that both NaCl extraction or trypsin treatment resulted in a similar release of CK2 activity which correlated with the presence of α- and β-subunits in the supernatants (Fig. 7A, inset). Treatment of membranes with phospholipase D also led to a substantial release of CK2 activity. Quantitation of the data showed that treatment of membranes with

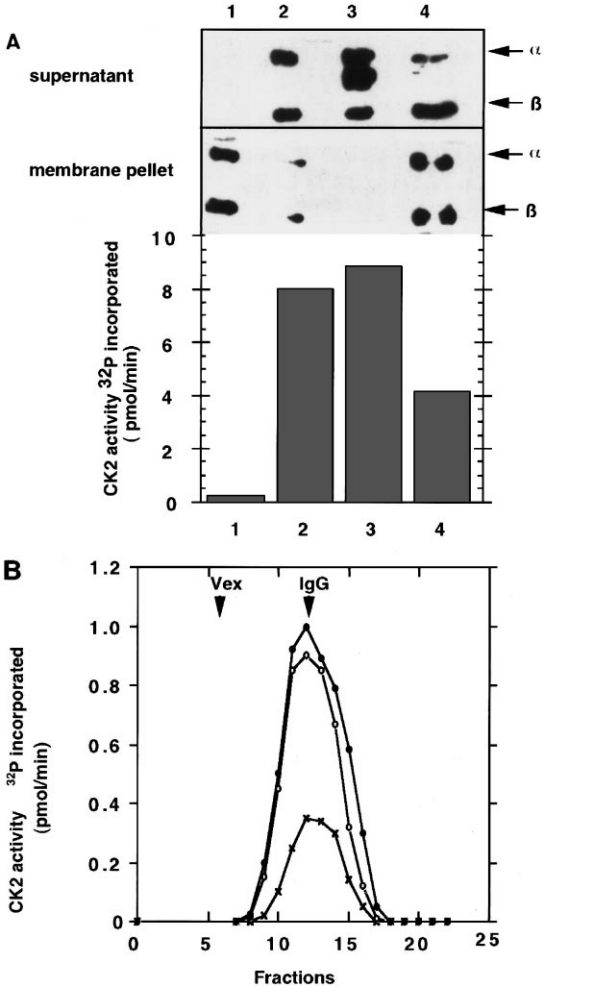

Fig. 7. Nature of the CK2 binding sites in plasma membranes. (A) Aliquots of membranes from SF9 cells overexpressing CK2 subunits were incubated for 20 min at 30°C with the following additions: (1) TDG buffer; (2) 1 M NaCl; (3) 50 μg/ml trypsin; and (4) 9 units of Phospholipase D. After centrifugation of the extracts, aliquots of the different supernatants were used for CK2 activity determination. Inset: aliquots of the membrane pellets and of the supernatants were analysed by Western blotting and the membrane was probed with anti-CK2 subunit antibodies. (B) Aliquots of the supernatants were also loaded on a ACA 34 Ultrogel column (0.7×0.9 cm) equilibrated in TDG buffer containing 1 M NaCl. Aliquots of the collected fractions (0.15 ml) were assayed for CK2 activity. NaCl (○), trypsin (●), phospholipase D (×). The exclusion volume and the position of the elution of goat IgG (MW 150 kDa) are indicated.

either NaCl or trypsin promoted the release of almost 100% of the kinase subunits present in the membranes. Under the same conditions, phospholipase D released 30% of the membrane bound CK2. Phospholipase A2, C and phosphatidylinositol specific phospholipase C were without effect (not shown). A gel filtration analysis was carried out on CK2 fractions released by either NaCl, trypsin or phospholipase D treatments. The results depicted in Fig. 7B showed that for each condition, the released CK2 activity, eluted from the column at a position corresponding to the whole heterotetrameric holoenzyme.

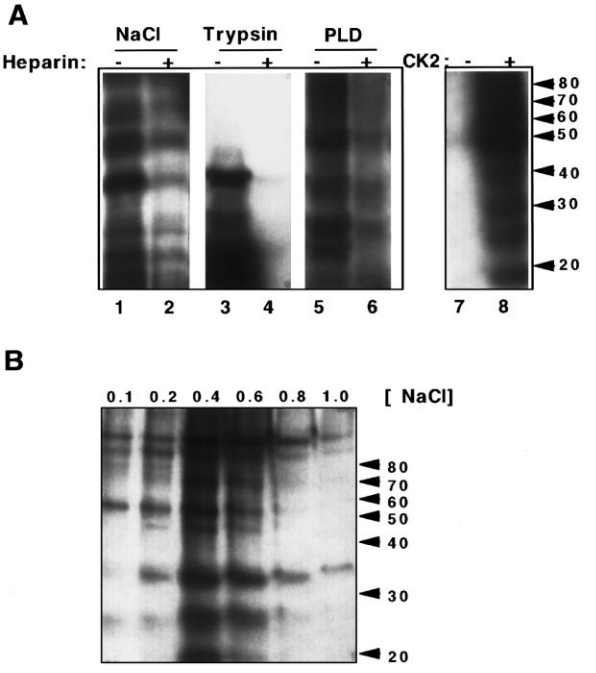

Fig. 8. Release of CK2 substrates from plasma membranes. (A) Aliquots of plasma membranes from SF9 cells overexpressing CK2 subunits were incubated for 20 min at 20°C in the presence of 1 M NaCl (lanes 1 and 2), 50 μg/ml Trypsin (lanes 3 and 4), or 9 units of PLD (lanes 5 and 6). Membranes from non-infected SF9 cells were extracted by 1 M NaCl (lanes 7 and 8). After centrifugation of the extracts, aliquots of the supernatants were incubated for 10 min at 30°C with 50 μM [γ-^{32}P]ATP+10 mM $MgCl_2$ in the absence or presence of heparin (lanes 1 and 6) or in the absence or presence of 0.1 μg recombinant CK2 (lanes 7 and 8). Proteins were separated by SDS-PAGE and the dried gel was exposed for autoradiography. (B) Plasma membranes from SF9 cells overexpressing the CK2 subunits were extracted with 1 M NaCl. After centrifugation of the extract, the supernatant was incubated for 10 min at 30°C with 50 μM [γ-^{32}P]ATP+10 mM $MgCl_2$. The sample was then loaded on an amylose column containing the MBP-β fusion protein. The column was eluted stepwise by increasing NaCl concentrations. The phosphorylated proteins present in the different fractions were separated by SDS-PAGE and visualised by autoradiography.

3.7. Presence of CK2 substrates in plasma membranes

We checked whether the kinase was released together with membrane proteins which might be CK2 substrates. It was observed that several proteins with molecular weight ranging from 20 to 80 kDa were extracted from the membrane by NaCl or by incubation with trypsin or phospholipase D. These proteins were phosphorylated by the released CK2 because their phosphorylation was strongly inhibited by heparin (Fig. 8A). The same proteins were also extracted by phospholipase D treatment of membranes of non infected SF9 cells and were also phosphorylated by exogenously added CK2 (Fig. 8A, lanes 7 and 8).

A high salt extract of membranes from SF9 cells overexpressing the CK2 subunits was incubated in the presence of [γ-^{32}P]ATP/$MgCl_2$ and then loaded on a column of β-MBP fusion protein immobilised on amylose resin. As shown in Fig. 8B, most of the CK2 substrates were bound to the β-MBP fusion protein and were eluted in the presence of 0.4–0.6 M NaCl. As a control, these CK2 substrates were not retained on a column of immobilised MBP (data not shown).

3.8. Increased CK2 association with plasma membranes in postnatal rat liver

The membrane-bound CK2 was examined during the liver development of embryonic and postnatal rats. The presence of CK2 was probed in purified plasma membrane by CK2 activity determination and by Western blotting (Fig. 9) Membrane-bound CK2 level was low in prenatal liver (embryonic day 16.5 to birth). Then, a striking increase in CK2 was observed during the first week after birth and high level of membrane-bound CK2 persisted during several weeks. A Western blot analysis disclosed that the kinase contained a stoichiometric amount of α- and α′-subunits.

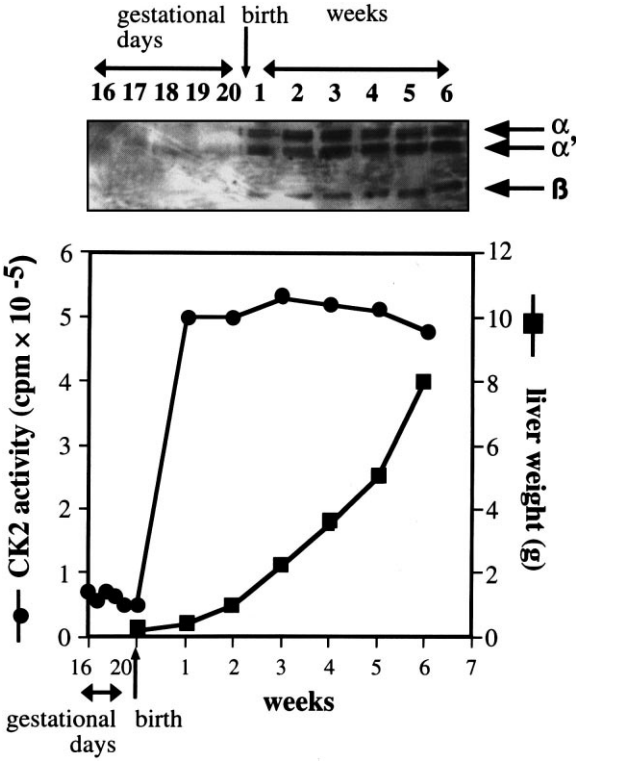

Fig. 9. Membrane-bound CK2 during embryonic and postnatal rat liver development. Plasma membranes were prepared from liver of embryonic (E17–E20) and postnatal rats. The different samples were normalised for their protein content and CK2 activity was determined in 2 M NaCl extract of membranes (●). The liver weight at each stages was also determined (■). Inset: sample of the extracted proteins were separated by SDS-PAGE and Western blotted on an immobilon membrane. The presence of CK2 subunits was revealed on the membrane using anti-CK2 antibodies.

4. Discussion

Protein kinase CK2 itself, as well as its potential specific intracellular substrates are found in the different compartments of the cell [2], indicating a localisation-immanent specification of the enzyme. Immunocytochemical studies have shown that in most cell types, CK2 is detected both in the cytoplasm and the nucleus [18,19,29]. Detection of CK2 activity in plasma membrane preparations has been previously reported [32–34].

The presence of an ecto-protein kinase associated with plasma membrane from HeLa cells [30,31] and human neutrophils [33] has been described by several groups. A casein kinase 2-like enzyme was also identified in caveolin-rich membranes [34]. Similarly, the presence of casein kinase I associated with rat liver plasma membrane has already been observed [35]. Here we report the presence of an oligomeric form of CK2 tightly associated with highly purified rat liver plasma membrane preparations.

The protein kinase activity which eluted at 0.8–0.9 M NaCl on phosphocellulose, phosphorylated intensively a peptide substrate established as being specific for CK2. Using specific antibodies recognising the different subunits of CK2, we found that this protein kinase contained the two catalytic α- and α′-subunits and the regulatory β-subunit characteristic of CK2 purified from different sources. The apparent molecular weight (≅140 kDa) suggests that the protein kinase is oligomeric, thus the enzyme has all the hallmarks of authentic CK2.

Several CK2 isoforms containing two different catalytic subunits have been described in different tissues [16] and rat liver homogenates contained several forms of CK2 activity that could be separated by ion exchange chromatography [36]. Interestingly, CK2 activity extracted from rat liver plasma membrane was found to eluate as a single peak on different chromatographic steps. A Western blot analysis showed that this purified CK2 contained both α- and α′-subunits. The amount of CK2 associated with purified plasma membrane was calculated to represent 2.4 and 8% of the CK2 activity present in rat liver and in CK2-expressing SF9 cells, respectively.

The presence of CK2 associated with plasma membranes is not restricted to rat liver since we have also detected this protein kinase in plasma membranes derived from A431 cells.

Until now, the nature of the association of CK2 with membranes has not been examined in detail. We do not know the topographical orientation of the protein kinase within the plasma membrane. Therefore, we cannot assess if this membrane bound form of CK2 is an ectoprotein kinase [30,31] or a peripheral membrane protein exposing its catalytic domain toward the cytoplasm. However the casein kinase 2-like enzyme associated with caveolin-rich membrane domains was shown to phosphorylate the cytoplasmic domain of caveolin [34], suggesting that the kinase is oriented toward the cytoplasm. Interestingly, the enzyme associated with rat liver plasma membranes was tightly bound to membranes and its release occurred upon extraction with high ionic strength (>1 M NaCl). In contrast, exogenous CK2 was able to bind to membrane, but was easily extracted by low salt concentrations. Similarly it has been observed that most of the nuclear bound CK2 is readily extracted by 0.5 M NaCl (unpublished results). It is not known if the attachment of CK2 to membranes requires a specific covalent modification, but our observation that the kinase is extracted by high salt concentrations suggests that electrostatic interactions between protein domains and membrane lipids or protein–protein interactions may contribute to binding. Using the SF9 cell system, we found that the extractibility of the isolated β-subunit recovered from the plasma membrane fraction was identical to the one observed for the native liver CK2 and the recombinant oligomeric CK2 in SF9 cells. From these experiments, it is tempting to speculate that the β-subunit is the targeting subunit which is responsible for the tight anchoring of the oligomeric enzyme in the plasma membrane. This notion is strengthened by the competition experiments described in Fig. 5, showing that whereas several CK2 substrates could not release the membrane-bound kinase, the recombinant β-subunit bound to membranes and could efficiently displace the kinase from its binding sites. These observations imply that the catalytic CK2 subunits are not involved in this interaction, but rather provide evidence for a major role for the regulatory subunit in mediating this membrane attachment.

As far as the exact mechanism of the interaction of CK2 with plasma membrane is concerned, the molecular mechanism of this association is not known. The fact that both baculovirus and *E. coli*-expressed β-subunits disclosed the same potency in displacing the membrane-bound CK2, supports the notion that no covalent modification of this targeting subunit is required for the interaction.

To delineate the region of the β-subunit which is involved in this interaction, different fragments of this subunit were tested in competition experiments. A fusion protein containing the region between residues 51 and 110 was found to release specifically the membrane-bound CK2. This observation could be interpreted as an indication that this region is indeed a CK2-membrane targeting domain.

It has been recently observed that this region represents a regulatory domain which binds polybasic compounds such as polyamines and mediates the polyamine-stimulation of CK2 [37]. Interestingly, the membrane-bound CK2 could be released by high spermine concentrations. The fact that this do-

main can easily displace the membrane-bound CK2 indicates that the kinase may interact with polycationic membrane components. We have observed that CK2 activity could not be detected in intact membranes unless a high salt extraction was performed. Similarly, Triton X-100 extracts from membrane did not exhibit a detectable CK2 activity although a Western blot analysis indicated that the kinase was indeed solubilised in the presence of detergent. Thus, plasma membranes may contain CK2-inhibitory components which, like CK2, are extractable in the presence of Triton X-100. The same inhibitory components are apparently not released after salt extraction of plasma membranes. Altogether, these observations lend support to the notion that plasma membranes harbor a cryptic form of CK2 which is under a tight inhibitory control.

To elucidate the nature of the CK2 receptor site(s), membranes were incubated in the presence of trypsin or phospholipases. It was observed that both trypsin and phospholipase D treatments are able to release the whole heterotetrameric holoenzyme from the membranes. This observation may indicate that membrane proteins and/or membrane phospholipids are involved in the binding of CK2 to membranes. However, it should be pointed out that the enzymatic extractions of the kinase was carried out only to gain evidence for different binding sites for the association of the enzyme and not to define the biochemical composition of the CK2 binding sites. The results illustrated in Fig. 7A only suggest that both membrane proteins and phospholipids are required at, or near, specific binding sites to mediate this type of membrane association.

The fact that both enzymatic treatments or salt extraction of membranes released a limited number of membrane proteins which are CK2 substrates, raises the possibility that some of these proteins may represent potential CK2 anchoring proteins present in the plasma membranes. This notion is supported by the observation illustrated in Fig. 8B showing that these CK2 substrates are interacting with recombinant CK2-β-subunit.

The membrane bound form of CK2 observed in the liver of adult rat is undetectable in the liver of rat embryos. Interestingly, a striking increase in the level of the kinase associated with plasma membranes was observed after birth and the presence of the enzyme in this compartment persists during the postnatal stages of development. Based on these observations, we conclude that the membrane localisation of CK2 is developmentally regulated. In this context, it has been previously observed that CK2 activity is elevated in 12-day mouse embryos as compared to other embryonic stages and 6-day-old mice [38]. However, this study was performed in low salt extract of whole mouse embryos and the presence of membrane-bound CK2 was not addressed. More recently, it was observed that foetal hepatic CK2 was largely localised to the nucleus until birth. In contrast, CK2 was absent from adult nuclear extract [39]. This study, together with our own, demonstrate that a complex intracellular redistribution of CK2 takes place in late foetal and postnatal rat liver development.

It is not known at this time what functions this membrane bound form of CK2 might serve. Compartmentalisation of protein kinases resulting from their binding to specific anchoring proteins is a well documented observation [40]. In this context, the association of CK2 to plasma membranes would place the kinase close to certain substrates but also cluster it with enzymes that may regulate its activity.

The challenge now is to pinpoint which important cellular phosphorylation events are regulated by this membrane-bound form of the enzyme.

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