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The carboxy-terminal domain of Grp94 binds to protein kinase CK2α but not to CK2 holoenzyme

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Abstract Surface plasmon resonance analysis shows that the carboxy-terminal domain of Grp94 (Grp94-CT, residues 518–803) physically interacts with the catalytic subunit of protein kinase CK2 (CK2α) under non-stressed conditions. A K_D of 4×10^{-7} was determined for this binding. Heparin competed with Grp94-CT for binding to CK2α. CK2β also inhibited the binding of Grp94-CT to CK2α, and CK2 holoenzyme reconstituted in vitro was unable to bind Grp94-CT. The use of CK2α mutants made it possible to map the Grp94-CT binding site to the four lysine stretch (residues 74–77) present in helix C of CK2α. Grp94-CT stimulated the activity of CK2α wild-type but was ineffective on the CK2α K74–77A mutant.

Key words: Protein kinase CK2; Grp94; Surface plasmon resonance; Molecular chaperone

1. Introduction

Glucose-regulated protein of 94 kDa (Grp94) is the endoplasmic resident member of the 90 kDa heat shock protein (hsp90) family sharing 49% identity with hsp90 for human proteins, with major differences in their C-terminal domain [1]. Grp94 is constitutively expressed in all cell types and is upregulated by different stress conditions such as low glucose levels, acidic pH, viral infection and different treatments such as reducing agents, ionophores or tunicamycin [2]. Although Grp94 is mainly an endoplasmic reticulum resident protein it has also been found associated with cytoplasmic proteins and it is a substrate for cytosolic proteases [3–5] suggesting that it may also exist either as a cytoplasmic protein or as a transmembrane protein with the carboxy-terminus on the cytosolic face. Furthermore, Grp94 has been detected on the cell surface of cultured muscle and tumour cells [6,7]. The physiological role of Grp94 is still under discussion but it has been implicated, as a molecular chaperone, in the folding of secretory and membrane proteins. It seems to be preferentially implicated in the folding of advanced folding intermediates or of incompletely assembled proteins [8]. The C-terminal domain of Grp94 contains a peptide binding site [9] and prevents in vitro the aggregation of the catalytic α subunit of protein kinase CK2 [10].

Protein kinase CK2 is a constitutively active enzyme with unknown regulation pathway, which is overexpressed in tumour cell lines and in proliferating tissues (for review see [11]). The catalytic subunit of CK2 (CK2α) is active either alone or when forming the heterotetrameric structure with the regulatory β subunit (CK2β). CK2 has been implicated in the phosphorylation of a broad range of proteins involved in multiple cellular functions (for a review see [12]). The holoenzyme ($\alpha_2\beta_2$) seems to be the most abundant form of the enzyme but it has been shown that the catalytic subunit can also exist either as a free subunit or associated with other cellular proteins, and these forms may play different roles than the holoenzyme [13]. Since Grp94 can interact with CK2 under non-stressed conditions, we hypothesise that Grp94 could play a role in the folding of protein kinase CK2 and in maintaining the pool of free CK2α in a competent state. In this work, we tried to define the regions in CK2α involved in its interaction with the carboxy-terminal domain of Grp94, and the possible consequences of this interaction on CK2α activity.

2. Materials and methods

2.1. Chemicals

[γ-^{32}P]ATP was from Amersham. Ni^{2+}-nitrilotriacetic acid agarose was from Qiagen. Secondary antibodies were from Bio-Rad and all other chemicals were from Sigma. Polyclonal antibody against CK2α (residues 70–89) was from StressGen. Polyclonal antibodies against C-terminal Grp94, CK2β and CK2α were raised by immunisation of rabbits with recombinant proteins.

2.2. Expression and purification

Human C-terminal Grp94 (Grp94-CT), CK2α and CK2β wild-type were expressed in *Escherichia coli* and purified as described previously [10]. CK2α mutants (αK74–77A, αK79R80K83A, αR191K195K198A, αR278K279R280A, αΔ2–12, αΔ2–18, αK77A) were expressed in *E. coli* and purified as described previously [14]. CK2α from *Zea mays* [15] and human CK2α' [16] were expressed and purified according to the procedures described previously.

2.3. Surface plasmon resonance (SPR) analysis

BIAcore X system (BIAcore) was used to detect the interactions. CK2α, CK2β or C-terminal Grp94 were covalently linked to a sensor chip CM5 (BIAcore) using amine coupling chemistry [17]. In the case of C-terminal Grp94 the coupling was done in the presence of 10 mM dithiothreitol to ensure a homogeneous monomeric preparation of

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Abbreviations: CK2, protein kinase CK2 (formerly casein kinase 2 or II); Grp94, glucose-regulated protein of 94 kDa (also known as gp96, endoplasmin or hsp100); hsp90, heat shock protein of 90 kDa; SPR, surface plasmon resonance; wt, wild-type

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C-terminal Grp94. C-terminal Grp94, CK2β, CK2α or different CK2α mutants (see Table 1 for details) were injected over the surface with a flow rate of 10 μl/min in running buffer HBS (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) at 25°C. After injection HBS replaced the protein solutions in a continuous flow rate of 10 μl/min. All samples were run simultaneously over a flow cell containing a blank surface. The kinetic data were calculated using the SPR kinetic evaluation software BIAevaluation 3.0 (BIAcore).

2.4. Protein and phosphorylation assays

Protein concentration in the samples was determined by the Bradford method [18] using bovine serum albumin as standard. Protein kinase CK2 activity was assayed as described previously [14] using 0.1 mM of specific peptide (RRRAADSDDDDD) and 125 μM [γ-^{32}P]ATP as substrates (specific activity 300 cpm/pmol).

3. Results

3.1. SPR analysis of CK2α/Grp94-CT interaction

The interaction between CK2α and Grp94-CT, the latter covalently linked to a sensor chip CM5, was checked using the SPR technique (Fig. 1A). To quantitatively measure the interaction, different concentrations of CK2α (110, 220, 550, 1100, and 2200 nM) were injected on the Grp94-CT chip. An association constant of 4×10^{-7} M was estimated from the data of the binding. Interaction between CK2α covalently linked to a sensor chip CM5 and soluble Grp94-CT was also observed (data not shown). Binding to the Grp94-CT chip was also observed when human CK2α′ or *Z. mays* CK2α were tested (Fig. 1B shows a representative interaction with *Z. mays* CK2α. A similar signal was detected for CK2α′). Human CK2α′ is 70% homologous to CK2α but lacks the 61 amino acids in the carboxy-terminal part of the protein. Comparison of CK2α from *Z. mays* with human CK2α also gave a homology of 70% and again the former lacks 59 amino acids in the carboxy-terminal part of the protein. The fact that both proteins bind to Grp94-CT would indicate that the carboxy-terminal part of CK2α is not involved in their interaction.

Protein kinase CK2 from porcine testis has been reported to bind hsp90 through a region located between residues 62 and 83 [19]. This region contains a polylysine stretch (^{74}KKKKIKREIK^{83}) that has been described to be one of the most peculiar parts of CK2α. This cluster plays a prominent role in heparin binding, downregulation by CK2β, and substrate recognition [14]. To determine if this region of CK2α was also involved in the binding to Grp94-CT, different

Table 1
Summary of proteins checked for interaction on Grp94-CT chip

	Grp94-CT chip
α wt	+++
α′ wt	++
α *Z. mays*	++
αK74–77A	−
αK79R80K83A	+
αR278K279R280A	+
αΔ2–12	++
αΔ2–18	++
αR191R195K198A	+
αK77A	+
$\alpha_2\beta_2$ holoenzyme	−
β wt	−

The symbols indicate interaction (+) or no interaction (−) and the strength of the binding.

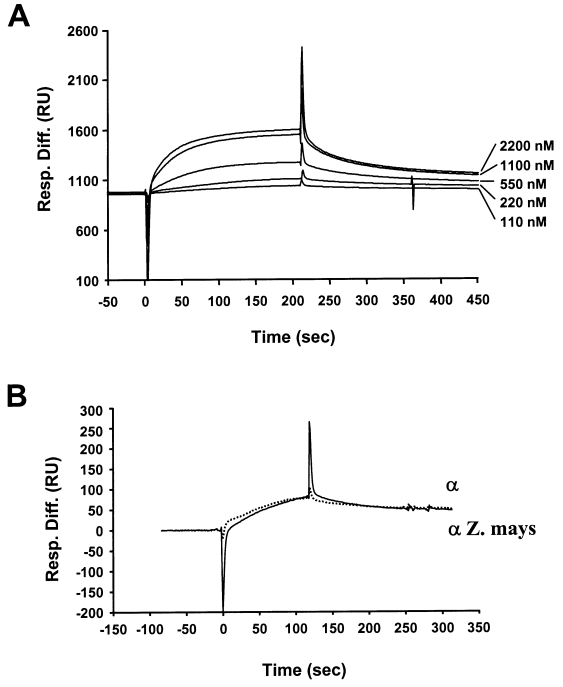

Fig. 1. Interaction of the α subunit of protein kinase CK2 with Grp94-CT by SPR. Representative sensorgrams obtained by injection of 35 μl of sample at a flow rate of 10 μl/min over a sensor surface containing 780 resonance units (RU) of immobilised Grp94-CT. The response obtained with a control sensor surface (without protein immobilised) was subtracted from each sensorgram. The response difference is measured in RU. A: Sensorgrams of injected CK2α at the indicated concentrations. B: Sensorgrams of injected CK2α at 40 nM (continuous line) and CK2α from *Z. mays* (dotted line) at 100 nM.

CK2α mutants were checked for interaction (see Table 1). Fig. 2A shows the sensorgram for the CK2α K74–77A mutant, where a complete inhibition of the interaction can be observed when lysines in this region were mutated to alanines. This is a specific characteristic since, as reported earlier [20,14], this mutant is still able to interact both physically and functionally with the CK2β subunit.

Next, we tested the binding of the mutant CK2α K79R80K83A that contains the mutations on the second part of the basic stretch. As shown in Fig. 2B this mutant was still able to bind Grp94-CT and the interaction was concentration dependent. It is likely that the second half of the basic stretch has also some contribution to the interaction because the shapes of the sensorgrams obtained were slightly different from those of CK2α wt. On the other hand, the CK2α K77A mutant was able to bind Grp94-CT with a kinetic constant similar to that of CK2α wt (data not shown). Residues R191, R195 and K198 in CK2α are also implicated in heparin binding and in the downregulation by CK2β. However, mutation of these residues did not affect the binding of CK2α to Grp94-CT (see Table 1).

To confirm the implication of the polylysine stretch on the interaction between CK2α and Grp94-CT, CK2α plus different concentrations of heparin were injected on the Grp94-CT chip. As shown in Fig. 2C, the binding was completely prevented even at concentrations of heparin as low as 1 nM.

Crystallographic data [21] have shown that the N-terminal region of CK2α makes extensive contacts with the lysine cluster and with the activation loop. In order to explore if this

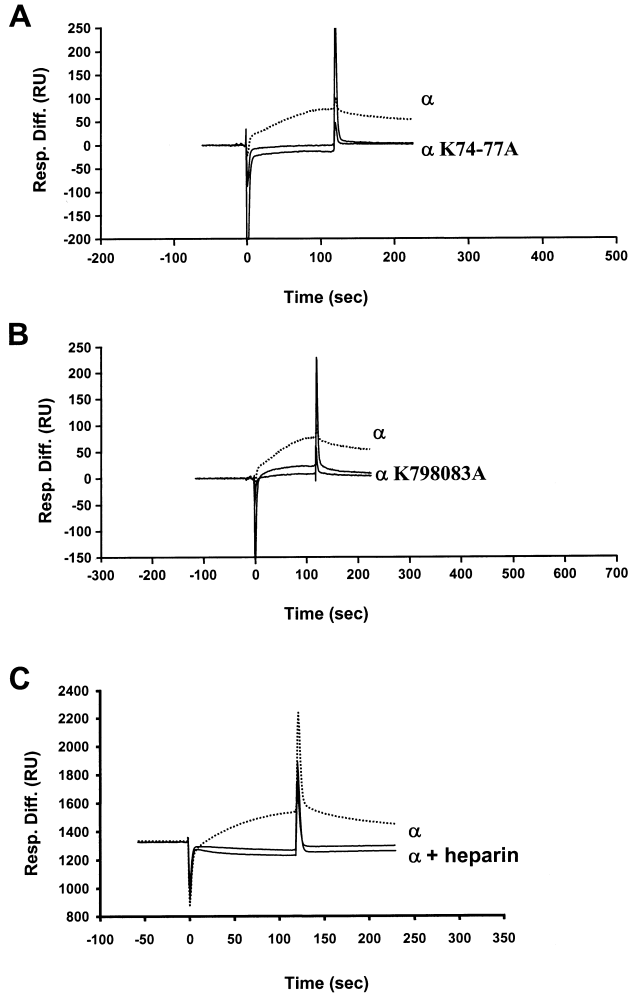

Fig. 2. Analysis of the binding site of CK2α to Grp94-CT. Representative sensorgrams obtained by injection of 35 μl of sample at a flow rate of 10 μl/min over a sensor surface containing 780 RU of immobilised Grp94-CT. The response obtained with a control sensor surface (without immobilised protein) was subtracted from each sensorgram. A: Sensorgrams of injected CK2α 40 nM (dotted line) and 1000 nM and 5000 nM of αK74–77A mutant. B: Sensorgrams of injected CK2α 40 nM (dotted line) and injected CK2α K79R80K83A mutant 40 and 200 nM (continuous lines). C: Sensorgrams of injected CK2α (dotted line) and injected CK2α plus two different concentrations of heparin (1 and 100 nM).

region could influence the interaction with Grp94-CT, deletion mutants of the N-terminal region (CK2α Δ2–12 and Δ2–18) were tested. The binding between the mutants and Grp94-CT had the same association signal as that of CK2α wt (see Table 1).

Binding between CK2β and Grp94-CT was also tested using either Grp94-CT or CK2β immobilised on the chip and injecting the other protein but no interaction was observed (Fig. 3A). Interestingly, no binding was either detected when using reconstituted CK2 holoenzyme ($\alpha_2\beta_2$) as a ligand (Fig. 3B).

3.2. CK2 activity assay in the presence of Grp94-CT

In a previous work [10] it was shown that casein phosphorylation by CK2α seemed to be enhanced by Grp94-CT. However, the fact that Grp94-CT is also a substrate for CK2 [16], with at least five putative phosphorylation sites located in its carboxy-terminal domain, made it difficult to discern if the increase in activity was due to an activation of casein phosphorylation or to a simultaneous phosphorylation of Grp94-CT. The use of the CK2 specific synthetic peptide substrate RRRAADSDDDDD made it possible to work at substrate concentrations 1000-fold higher than those of Grp94-CT, which minimised the contribution of Grp94-CT phosphorylation to less than 1% of total. Under these conditions, Grp94-CT stimulated CK2α wt in a way similar to that caused by CK2β (Fig. 4), although it required Grp94-CT/CK2α molar ratios higher than those of CK2β/CK2α. Interestingly, Grp94-CT was unable to stimulate CK2α K74–77A, whose activity conversely increased in the presence of CK2β. These data reinforce the concept that the 74–77 basic stretch in CK2α is essential for interactions with Grp94-CT.

4. Discussion

The carboxy-terminal domain of Grp94 has been shown to interact in vitro with CK2α and to protect it against heat-induced aggregation [10]. In the present study we show that binding also occurs under non-stressed conditions. Kinetic analysis of the interaction, by SPR, gave an approximate equilibrium dissociation constant (K_D) of 4×10^{-7} M, a value comparable to that reported previously for the interaction between hsp90 and CK2 [19]. Other studies using CK2α subunit as a partner have shown K_D values for the association with either prion protein bPrP [22] or haematopoietic protein HS1 [17] in the same range of that found for Grp94-CT, with the exception of transcription factor ATF1, which showed a

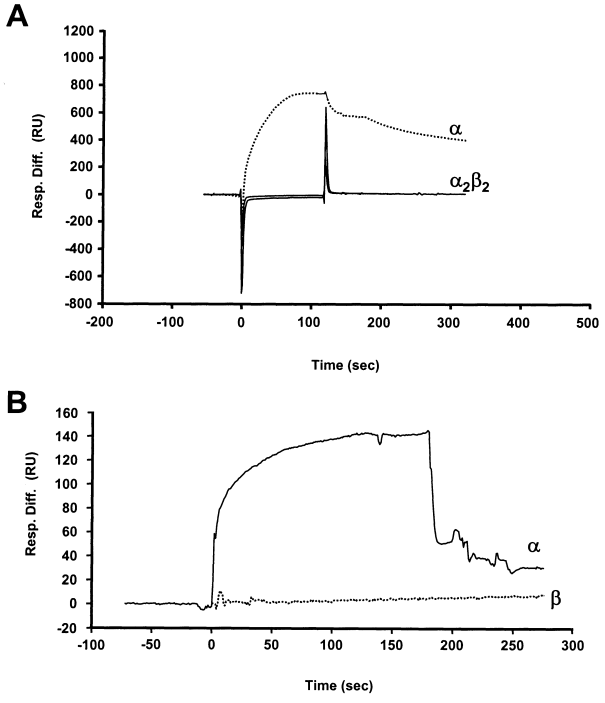

Fig. 3. Analysis of interaction between CK2 holoenzyme and Grp94-CT. Representative sensorgrams obtained by injection of 35 μl of sample at a flow rate of 10 μl/min over a sensor surface containing 780 RU of immobilised Grp94-CT. A: Sensorgrams of injected CK2α (dotted line) and two different concentrations of the holoenzyme ($\alpha_2\beta_2$) reconstituted with CK2α wt or with CK2α K74–77A mutant (continuous lines). B: Sensorgrams of injected CK2α (continuous line) and CK2β (dotted line).

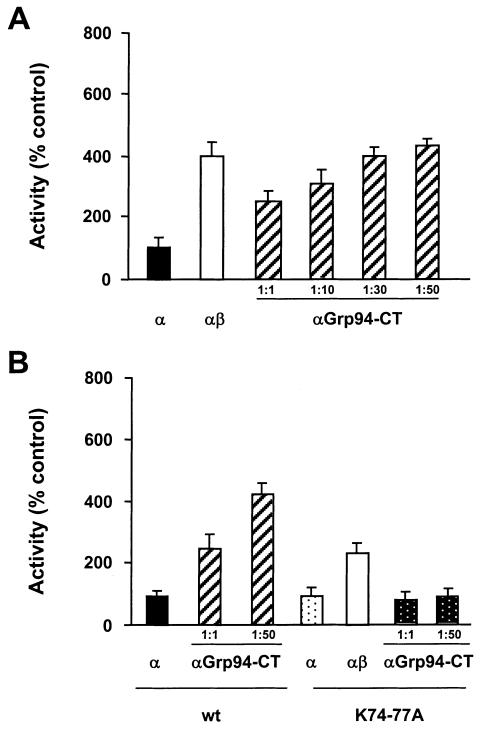

Fig. 4. Effect of Grp94-CT on CK2α and CK2α K74–77A activity. CK2 activity was assayed as described in Section 2. CK2 activity is expressed as a percentage of the activity measured with CK2α wt. A: The activities of CK2α wt alone (solid bar), the holoenzyme reconstituted with equimolar amounts of α and β subunits (open bar) and CK2α plus increasing molar ratios of Grp94-CT (hatched bars) are shown. B: CK2α wt alone (solid bar), CK2α wt plus Grp94-CT at the indicated molar ratios (hatched bars), CK2α K74–77A alone (dotted bar), reconstituted holoenzyme with CK2α K74–77A (open bar) and CK2α K74–77A plus Grp94-CT at the indicated molar ratios (dotted dark bars).

K_D of 1.2×10^{-8} [23], similar to that found for CK2β subunit [17].

The data shown herein demonstrate that the interaction between Grp94-CT and CK2α takes place through the four lysine stretch, located at the start of helix C in CK2α, which is exposed to the solvent in the absence of CK2β [21]. Neither the C-terminal region nor the N-terminal region of CK2α seems to be involved in the interaction. The lysine-rich region was also the proposed site for interaction with hsp90 [19]. However, hsp90 bound to CK2 through an acidic region (residues 227–249) located in its N-terminal domain. The C-terminal domain of Grp94 also contains a negatively charged region (residues 747–803) that could be responsible for the interaction with CK2α. In fact, comparison of Grp94-CT and CK2β showed that this C-terminal region contains a stretch of 15 residues (^{747}**L**NID**PD**A**K**V**EEEPE**E^{761}) with a high homology to the acidic loop of CK2β (^{54}**L**DLE**PD**EE**L**E**D**N**P**NQ^{68}) which could be the interacting domain of Grp94 with CK2α. Interestingly, Grp94-CT bound neither the CK2 holoenzyme nor the free CK2β subunit. It is likely that the interacting regions between CK2α and Grp94-CT are buried in the CK2 holoenzyme and, consequently, not available for interaction. The four lysine stretch of CK2α is inserted in a putative nuclear localisation signal (PVKKKKI) which would be masked by the interaction with the C-terminal domain of Grp94. Thus, the subcellular localisation of CK2α could be influenced by the interaction with Grp94.

It has been reported that the C-terminal domain of CK2β subunit also interacts with CK2α. This binding does not involve the basic cluster of CK2α but the upper lobe of this subunit, and stimulates its catalytic activity [14]. Our present data confirm the existence on CK2α of CK2β binding sites distinct from those interacting with Grp94-CT since CK2β but not Grp94-CT activates the CK2α K74–77A mutant, which is unable to stably interact with Grp94-CT. Nonetheless, interaction with Grp94-CT does not downregulate CK2α catalytic efficiency on the peptide substrate model, as caused by its interaction with the acidic loop of CK2β. In fact, association with Grp94-CT not only maintained CK2α in an active form but also stimulated its activity, suggesting a parallel action of Grp94-CT and CK2β on CK2α.

It has been shown that CK2β exerts a protective role on CK2α against high temperature, acting as a chaperone-like molecule [23], thus Grp94 may keep CK2α in an activated and correctly folded form in solution until it can interact with CK2β to form the holoenzyme, which results in a decrease in its binding to Grp94-CT. This fact implies a possible role of Grp94 in the folding of CK2α and assembling of the holoenzyme. Alternatively, binding to Grp94 may hold CK2α in a competent state to bind other cellular partners. Furthermore the data obtained with CK2α from *Z. mays* suggest that interaction with Grp94-CT may play an important role since it is conserved in animals and plants.

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