TREBALL II

Resum del treball II. *The C-terminal domain of human grp94 protects the catalytic subunit of protein kinase CK2 (CK2α) against thermal aggregation. Role of disulphide bonds.*

El domini C-terminal de la grp94 humana es va clonar i expressar en *E. Coli* com a proteïna de fusió amb una cua de sis histidines localitzada a l'extrem N-terminal de la proteïna. Les dades prèvies amb grp94 de fetge de rata indicaven que la proteïna nativa era un dímer unit per ponts disulfur i pensàvem que els dímers i oligòmers es formaven per oxidació de dues cisteïnes localitzades a l'extrem C-terminal. La grp94 humana recombinant pot formar dímers i oligòmers que poden ser dissociats en monòmers en presència de DTT i aquesta dissociació no és deguda al procediment experimental utilitzat. La dissociació del domini C-terminal no implica pèrdua d'estructura ja que els espectres d'estructura secundaria obtinguts per dicroisme circular són iguals per la grp94 tractada i no tractada amb DTT.

D'altra banda hem pogut establir un mètode d'assaig xaperona, similar a l'utilitzat per hsp90, que demostra que el domini C-terminal de grp94 confereix protecció a la subunitat α de CK2 enfront d'agregació induïda per xoc tèrmic però no la protegeix contra inactivació. La inhibició de l'agregació és dosi depenent i s'observa un màxim de protecció a relacions molars de 4:1. La presència de DTT i por tant la dissociació dels oligòmers redueix la capacitat d'inhibir l'agregació sense alterar les característiques de solubilitat de la proteïna. Així doncs, el domini C-terminal de grp94 té activitat xaperona i requereix el manteniment de la seva estructura quaternària per assolir màxima activitat xaperona. L'estructura quaternària sembla que està estabilitzada per ponts disulfur.

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The C-terminal domain of human grp94 protects the catalytic subunit of protein kinase CK2 (CK2α) against thermal aggregation

Role of disulfide bonds

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The C-terminal domain (residues 518–803) of the 94 kDa glucose regulated protein (grp94) was expressed in *Escherichia coli* as a fusion protein with a His_6-N-terminal tag (grp94-CT). This truncated form of grp94 formed dimers and oligomers that could be dissociated into monomers by treatment with dithiothreitol. Grp94-CT conferred protection against aggregation on the catalytic subunit of protein kinase CK2 (CK2α), although it did not protect against thermal inactivation. This anti-aggregation effect of grp94-CT was concentration dependent, with full protection achieved at grp94-CT/CK2α molar ratios of 4 : 1. The presence of dithiothreitol markedly reduced the anti-aggregation effects of grp94-CT on CK2α without altering the solubility of the chaperone. It is concluded that the chaperone activity of the C-terminal domain of human grp94 requires the maintenance of its quaternary structure (dimers and oligomers), which seems to be stabilised by disulphide bonds.

Keywords: aggregation; chaperone assay; grp94; protein kinase CK2.

The 94 kDa glucose-regulated protein (grp94) is a glycoprotein with multiple Ca^{2+} binding sites that participates in the cellular response to different types of stressors [1]. Grp94 is abundant in the lumen of the endoplasmic reticulum (ER), but several lines of evidence have challenged the assumption of an exclusive luminal localization. Grp94 has been found to associate with cytoplasmic proteins and to serve as substrate for cytosolic proteases [1–3], suggesting that it may also exist either as a cytoplasmic protein or as a transmembrane protein with a cytoplasmic C-terminus. Furthermore, grp94 has been detected on the cell surface of cultured muscle and tumour cells [4,5]. Grp94 is known to participate in protein folding and assembly, in protein secretion, and to protect cells against apoptosis. Furthermore, in tumour and virus-infected cells, grp94 mediates immunogenicity that is conferred not by itself but due to its ability to bind immunogenic peptides [1,6], a function with potential applications in cancer immunotherapy.

Hsp90 is the cytosolic counterpart of grp94, with which it shares approximately 50% homology [1]. The Hsp90 structure is composed of two clearly distinguishable domains linked through a relatively flexible, highly charged loop. A similar structure has been suggested for grp94 [1]. Both hsp90 and grp94 form homodimers through tail to tail interactions [7,8], and oligomers through a less defined mechanism that, in the case of hsp90, also involves the C-terminal domain [9]. Recent studies on grp94 have mapped its peptide binding site to the C-terminal domain of the protein in close juxtaposition to the dimerization domain, and a possible role for dimerization in stabilizing peptide binding has been proposed [10]. Dimerization of grp94 seems to be largely due to hydrophobic interactions [8,11]. However, dissociation of native rat liver grp94 into monomers requires the presence of reducing agents [12]. Furthermore, disulphide bonds have been detected in recombinant murine grp94 [13].

Hsp90 and grp94 bind to a wide range of cellular proteins, including different protein kinases [1]. It is well known that hsp90 binds to protein kinase CK2 and forms soluble complexes that protect CK2 against thermal aggregation and inactivation [14]. Interaction occurs between the heparin-binding and DNA-binding sites of the catalytic subunit of the kinase (CK2α) and, probably, two acidic regions in the N-terminal domain of hsp90 [14]. These N-terminal regions contain the CK2 phosphorylation sites, which are known to be phosphorylated in hsp90 *in vivo* [14,15]. Evaluation of the ATP requirement for peptide binding to grp94 revealed that it was dispensable and that the ATP binding and hydrolytic activities of grp94 preparations were not due to this protein but to trace contamination with protein kinase CK2 [16]. Grp94 is also a substrate for CK2, and the phosphorylation sites are present both at the N-terminal domain and, more importantly, in the C-terminal tail of the protein [17]. Reassociation of CK2 and grp94 to form immunoprecipitable complexes has been observed [18]. However, the potential role of grp94 in protecting CK2 remains unexplored.

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Abbreviations: CK2, protein kinase CK2 (formerly casein kinase 2 or II); ER, endoplasmic reticulum; grp94, glucose regulated protein of 94-kDa (also known as gp96, endoplasmin or hsp100); hsp90, heat shock protein of 90-kDa.

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The data reported here show that the C-terminal domain of human grp94 protects the catalytic subunit of CK2 (CK2α) against thermal-induced aggregation and this effect is markedly influenced by the presence of reductants, which also disturb the quaternary structure of this grp94 domain.

EXPERIMENTAL PROCEDURES

Chemicals

[γ-^{32}P]GTP was from Amersham. Ni^{2+}/nitrilotriacetic acid agarose and pQE-30 were purchased from Qiagen. Bacto-tryptone Bacto yeast extract and agar were from Difco. Ampicillin and kanamycin were from Gibco. *Bam*HI, dNTPs, Pwo polymerase and synthetic oligonucleotides were from Roche. Secondary antibodies and SDS/PAGE standards were from Bio-Rad, protein A-agarose was from Pierce and all other chemicals were from Sigma. Polyclonal antibody against CK2α (residues 70–89) and polyclonal antibody against C-terminal Grp94 (residues 787–802 of mouse Grp94) were from StressGen. Antibodies against grp94-CT were raised by immunization of rabbits with recombinant proteins and the immunoglobulin fraction was obtained from sera by protein A-agarose chromatography.

Plasmids

Expression plasmid pQE-30, which codes for the C-terminal domain (residues 518–803) of human grp94 was constructed as follows: PCR was carried out using a HeLa cDNA library (Matchmaker cDNA library, Clontech) as a template and the synthetic oligonucleotides 5′-CGG-GATCCCCAACTGACATTACTAGCC-3′ and 5′-CGGG-ATCCTTACAATTCATCTTTTTCAG-3′ as primers (the underlined sequence represents the GRP94 ORF and the other sequence is the site for restriction enzyme *Bam*HI). pQE-30 and the PCR product were digested with *Bam*HI and ligated with T4DNA ligase (Roche). The ligated product was used to transform the strain XL-1 Blue. The sequence of the insert was verified using the type III sequencing primers and reverse as per the manufacturer's instructions.

Expression and purification

Grp94-CT, CK2α and CK2β were overexpressed from pQE-30 plasmids in the M15[pREP4] *E. coli* strain, as fusion proteins with an N-terminal His_6-tag, and the soluble fraction purified by Ni/nitriloacetic acid agarose chromatography according to the manufacturer's instructions. The purified samples were pooled and dialysed against NaCl/P_i pH 7.5. The purified fusion proteins were used in assays described below and to raise rabbit polyclonal antibodies. The amino acid sequence grp94-CT was verified by N-terminal sequencing using a Beckman LF3000 gas-phase sequencer at the Protein Chemistry Facility of the Universitat Autònoma de Barcelona.

Circular dichroism measurements

Far-UV CD spectrometry was used to analyse grp94-CT secondary structure in a JASCO-715 spectrometer. grp94-CT (100 µg) were untreated or treated with 20 mM dithiothreitol, dialysed against 10 mM phosphate buffer pH 7.8, lyophilized and resuspended in 10 mM phosphate buffer pH 7.8 (0.3 mL). The mean residue ellipticity was calculated using a mean residue molecular mass of 115 Da.

SDS/PAGE and native-PAGE

Proteins were subjected to electrophoresis under denaturing conditions (SDS/PAGE) according to the Laemmli method [19] or nondenaturing conditions (native-PAGE) according to the Andrews method [20]. Low range prestained SDS/PAGE standard molecular markers were used for nondenaturing and denaturing electrophoresis. Bovine serum albumin (monomer 66 kDa, dimer 132 kDa, trimer 198 kDa) was also used under nondenaturing electrophoresis.

Two-dimensional double SDS/PAGE

Grp94-CT was resolved under nonreducing conditions using 10% SDS/PAGE. The lane was excised and layered on the second dimension under the same conditions. After the second dimension electrophoresis the proteins were stained with Coomassie Brilliant Blue.

Protein and phosphorylation assays

Protein concentration in the samples was determined by the Bradford method [21] using bovine serum albumin as standard. Protein kinase CK2 activity was assayed as described previously [18] using 4 mg·mL^{-1} β-casein and 125 µM [γ-^{32}P]GTP as substrates (specific activity 300 cpm·pmol^{-1}). One unit of protein kinase activity is defined as the amount that catalyses the transfer of 1 nmol of phosphate from [γ-^{32}P]GTP to β-casein per min at 30 °C.

Protein aggregation assays

Protein aggregation was induced by incubating the samples at 40 °C or 45 °C, as indicated. Aggregation was monitored over a period of 60 min by measuring light scattering at 360 nm on a Perkin-Elmer 650-40 spectrofluorimeter interfaced to a Perkin-Elmer data station 3600 with the samples incubated at 40 °C or in a Cary-400 spectrophotometer with the samples incubated at the same wavelength and time. To perform protein kinase CK2 assays, aliquots of the samples were taken at different times during the aggregation assay, stored on ice and assayed as described previously. Alternatively the samples of CK2α alone or with grp94-CT either untreated or treated with 5 mM dithiothreitol were centrifuged at 16 000 *g* for 60 min, at 4 °C, and the supernatants were dialysed against 25 mM Tris pH 7.5 and lyophilized. Both the sediments and the lyophilized supernatants were resuspended in SDS/PAGE sample buffer and subjected to electrophoresis and western blotting. Prior to incubation with antibodies against CK2α or grp94-CT, the membranes were stained with Ponceau-S.

RESULTS

Characterization of recombinant grp94-CT

The recombinant protein corresponding to the His-tagged C-terminal fragment (residues 518–803) of human grp94

(grp94-CT) was expressed in *E. coli* and recovered in the soluble fraction, from where it could be purified by a single chromatography step on Ni/nitriloacetic acid-agarose. About 50 mg of purified protein were obtained from 400 mL of culture.The recombinant protein migrated as a 36 kDa band under reducing conditions, which agreed with the 34 475 M_r value estimated from the sequence (ProtParam tool, ExPASy). Direct N-terminal analysis of grp94-CT revealed the sequence MRGS*HHHHHH*GSPT, which corresponds to the His-tag (in italics) followed by the first two residues expected for the 518–803 grp94 fragment. Immunoblotting with the commercial antibody raised against the synthetic peptide EEEEETEKESTEKDEL, which corresponds to residues 787–802 in the C-terminus of mouse grp94, confirmed the presence of a homologous region in the recombinant protein (data not shown).

Dimerization and oligomerization of grp94-CT are reverted by dithiothreitol

SDS/PAGE analysis of grp94-CT was routinely carried out under reducing conditions, and only the 36 kDa band was observed. However, a similar analysis in the absence of reductants revealed that grp94-CT existed mainly as dimers and multiple oligomeric forms (Fig. 1). The pattern of bands observed in the SDS/PAGE gels corresponded to monomers, dimers, trimers, tetramers and oligomeric structures of higher M_r, which cannot be resolved on this gel. Different oligomeric bands were also observed when grp94-CT was analysed by electrophoresis in nondenaturing gels (data not shown).

Dithiothreitol concentration dependence analysis using SDS/PAGE gels showed that the high M_r oligomeric forms were dissociated at 1 mM dithiothreitol (Fig. 1A). However higher concentrations were required to completely dissociate the dimers into monomeric grp94-CT. Treatment of the samples with 8 M urea did not dissociate the oligomers, but seemed to potentiate their conversion first into dimers and

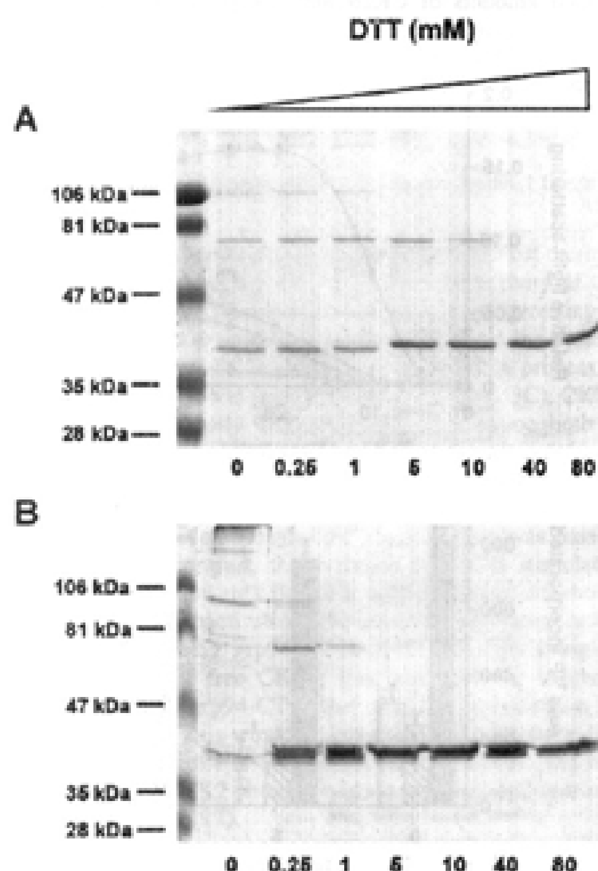

Fig. 1. The presence of disulphide bonds in grp94-CT. (A) Aliquots of 4 µg of protein were incubated with different dithiothreitol concentrations for 15 min at room temperature and then subjected to SDS/PAGE. (B) Aliquots of 4 µg of protein were incubated in the presence of 8 M urea with different concentrations of dithiothreitol for 15 min at room temperature and then subjected to urea/SDS/PAGE. In both cases, the proteins were visualized by Coomassie Brilliant Blue staining.

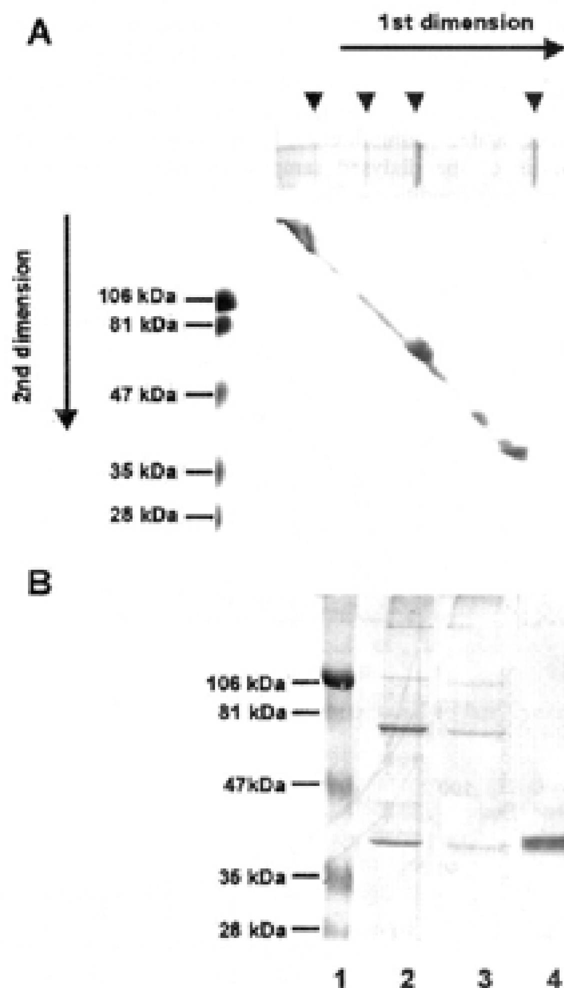

Fig. 2. Analysis of the quaternary structure of grp94-CT by two-dimensional double SDS/PAGE. (A) grp94-CT (40 µg) was subjected to SDS/PAGE under nonreducing conditions. After the first dimension the lane was excised and layered on a second SDS/PAGE dimension under nonreducing conditions. The proteins were stained with Coomassie Brilliant Blue. (B) grp94-CT (15 µg) untreated (lane 2), dialyzed against NaCl/P_i (lane 3) or treated with dithiothreitol for 30 min at room temperature and dialyzed against NaCl/P_i (lane 4) was subjected to SDS/PAGE and stained with Coomassie Brilliant Blue. Prestained SDS/PAGE standards (Bio-Rad) (lane 1) were electrophoresed as molecular mass markers.

then into monomers with further dithiothreitol treatment (Fig. 1B).

The presence of different forms of grp94-CT confirmed that it contained the oligomerization domain, as expected from previous reports in pig [8] and barley grp94 [22]. Detection of these forms in SDS/PAGE gels was in contrast to the lack of differences in the apparent M_r of pig grp94 observed when analysed either in the presence or absence of dithiothreitol [11], but it agreed with previous observations by other groups using cultured cells and rat liver [1,18]. The possibility that the dimers were artefacts originating during electrophoresis was tested by two-dimensional double analysis in SDS/PAGE under nonreducing conditions. The obtained results showed that the different spots were located in a diagonal (Fig. 2A), which effectively negates the possibility of interconversion during the analysis. The possible generation of dimers from monomers as a result of artefactual oxidation due to the absence of reductants in the buffers was also explored. For this purpose, a sample of grp94-CT was treated with 5 mM dithiothreitol and then subjected to extensive dialysis. A control sample, without added dithiothreitol, was treated in parallel. Analysis of the dialysed samples by SDS/PAGE under nonreducing conditions (Fig. 2B) revealed the presence of dimers and monomers in the control sample whereas only monomers were detected in the sample treated with dithiothreitol. This suggested that dimerization through disulphide bonds was not simply the result of spontaneous oxidation.

Circular dichroism spectra at 25 °C of untreated and dithiothreitol treated grp94-CT were very similar, which indicated the lack of major changes in protein secondary structure as a result of dissociation into monomers (data not shown).

Grp94-CT protects CK2α against thermal aggregation

Previous studies have shown that hsp90 protects protein kinase CK2 against aggregation and thermal denaturation through a direct interaction of hsp90 with the protein kinase CK2 catalytic subunit (CK2α) [14]. When incubated alone at 45 °C, recombinant human CK2α forms aggregates that are detectable by changes in the absorbance at 360 nm. Aggregation begins during the first 5 min and reaches a plateau at 20 min (Fig. 3B), which highlights the thermal instability of the CK2 subunit. In contrast to CK2α, the CK2 holoenzyme, reconstituted *in vitro* by mixing equimolar amounts of CK2α and CK2β, did not aggregate.

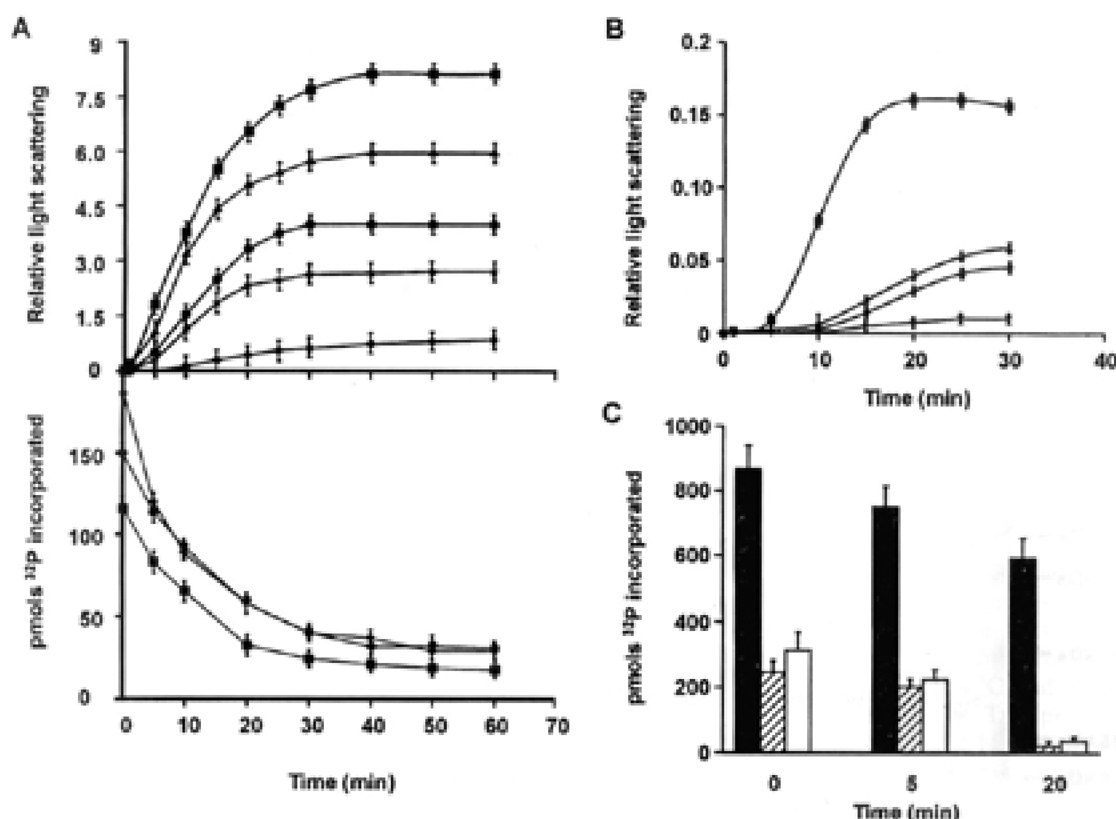

Fig. 3. **Effect of grp94-CT on thermal aggregation and thermal inactivation of CK2α.** (A) Upper panel: an aggregation assay performed at 40 °C for 60 min with CK2α alone (■), CK2α/grp94CT at 1 : 1 (▲), 1 : 2 (●) and 1 : 4 (◆) ratios and CK2α/CK2β at a 1 : 1 ratio (★). Lower panel: 10 µL aliquots of the samples were taken during the aggregation assay at 40 °C at the indicated times to test protein kinase CK2 activity. CK2α alone (■), CK2α with grp94-CT at 1 : 2 (●) and 1 : 4 (◆) ratios were assayed in parallel. (B) An aggregation assay was perfomed at 45 °C for 30 min with CK2α alone (■), reconstituted CK2 holoenzyme (▼) (CK2α/CK2β 1 : 1) and CK2α/grp94CT at 1 : 1 (▲) and 1 : 2 (●) ratios. Protein aggregation at 40 °C or at 45 °C was monitored by measuring light scattering at 360 nm. (C) Protein kinase CK2 activity assay of 10 µL aliquots of samples previously assayed for aggregation at 45 °C. Reconstituted CK2 holoenzyme ($\alpha_2\beta_2$), solid bars; CK2α, shaded bars; and CK2α grp94-CT (1 : 1), open bars. Results are shown as the average and standard deviation of three different experiments.

A small increase in absorbance was detected when an equimolar amount of CK2β was added to CK2α at the beginning of the incubation at 45 °C (Fig. 3B), as expected from previously published results [23,24]. Addition of a fourfold molar excess of albumin did not modify the kinetics of CK2α aggregation (data not shown) but progressive protection was achieved by the presence of increasing amounts of grp94-CT (Fig. 3B). In contrast, grp94-CT did not affect aggregation of malate dehydrogenase under these conditions (data not shown). Neither CK2β alone nor grp94-CT formed aggregates under these conditions (note that CK2β is always a dimer) [25].

Aggregation of CK2α at 40 °C was also monitored by light scattering in a Perkin-Elmer 650-40 spectrofluorimeter (Fig. 3A, upper panel). At this temperature, aggregation kinetics were slower and reached a plateau after 60 min. As indicated above, the presence of grp94-CT also protected against aggregation in a concentration-dependent manner. An increase in light scattering was also detected when an equimolar amount of CK2β was added to CK2α at the beginning of the incubation at 40 °C. Comparison of the data obtained with CK2β and grp94-CT would suggest that, under these conditions, grp94-CT at 4 : 1 ratio with CK2α would exert a stronger protective effect than CK2β at 1 : 1 ratio with CK2α. However, the increase in light scattering may result from the formation of oligomeric CK2 structures of high M_r when the enzyme was being reconstituted during the incubation at 40 °C [25].

Grp94-CT does not protect CK2α activity against thermal inactivation

It is known that hsp90 protects CK2 activity against thermal inactivation, although it did not restore the activity of the thermal-inactivated enzyme [14]. Thus, it was interesting to check the effect of grp94-CT on the changes in the protein kinase activity of CK2α during the aggregation process at 40 °C (Fig. 3A, lower panel) and at 45 °C (Fig. 3C). CK2α activity progressively decreased with aggregation both at 40 °C (compare Fig. 3A upper and lower panel) and 45 °C (compare Fig. 3B,C). However, inactivation of CK2α was also observed at both temperatures in the samples containing grp94-CT, even though CK2α aggregation was mostly prevented. In contrast, the presence of CK2β stimulated CK2α activity towards β-casein and conferred protection against thermal inactivation. Phosphate incorporation in the samples containing grp94-CT was always slightly higher than in those of free CK2α; this could be due to phosphorylation of grp94-CT rather than to stabilization of CK2α activity. This would be consistent with previous data showing that grp94 is a substrate for free CK2α [26] and the presence of CK2 phosphorylation sites in the C-terminal region of grp94 [17].

Protection of CK2α aggregation by grp94-CT is strongly decreased by the presence of dithiothreitol

The possible influence of grp94-CT oligomeric structure on the protective effect on CK2α stability was explored by testing the effect of dithiothreitol on the aggregation reaction (Fig. 4A). In the presence of 5 mM dithiothreitol, the protective effect of grp94-CT on CK2α was strongly reduced. On the other hand, 5 mM dithiothreitol did not alter the aggregation observed with CK2α alone, which argues against dithiothreitol having a substrate-directed effect.

In order to ascertain if grp94-CT was maintaining CK2α in solution, samples containing a fixed amount (1 μM) of this catalytic subunit were incubated at 40 °C for 60 min either alone or in the presence of grp94-CT, dithiothreitol, or both, and then centrifuged at 16 000 g for 60 min at 4 °C. The supernatants were dialysed against 20 mM Tris, pH 7.5, and concentrated by lyophilization. Both supernatants and pellets were resuspended in the same volume (50 μL) and 20 μL aliquots were subjected to SDS/PAGE and western blotting to analyse the protein content. This allowed a direct comparison of the band intensity detected in the pellets and supernatants obtained from each sample. Staining of the gels with Ponceau-S revealed the presence of a discrete set of bands in the pellets of the samples with CK2α alone, which corresponded to the intact recombinant subunit and its partially proteolysed forms (Fig. 4B). These

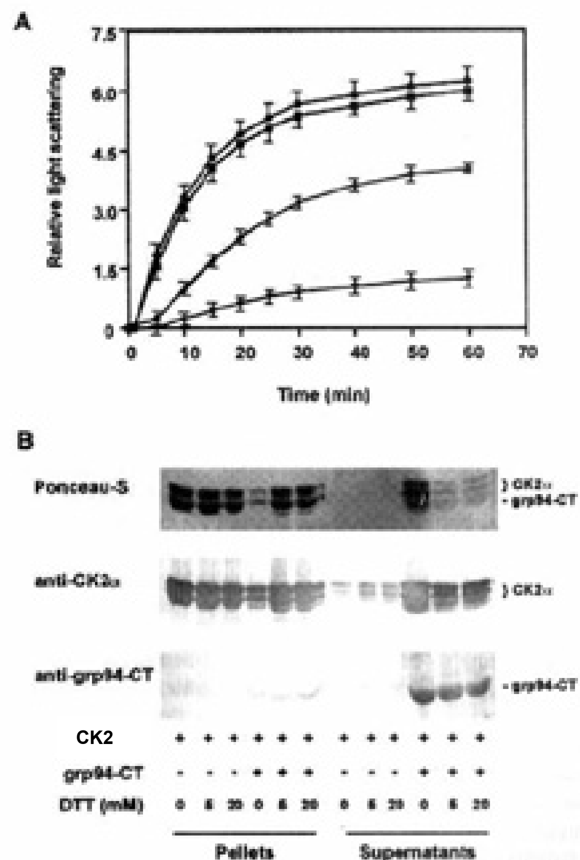

Fig. 4. Effect of dithiothreitol on the protective function of grp94-CT on CK2α. (A) CK2α was incubated either alone (●, ■) or with grp94-CT (ratio 1 : 4) in the absence (▼) or presence (♦) of 5 mM dithiothreitol for 60 min at 40 °C and the aggregation was monitored in a Perkin-Elmer spectrofluorimeter. Results are shown as the average and standard deviation of three different experiments. (B) After 60 min of aggregation assay the samples were centrifuged at 16 000 g for 60 min at 4 °C and the proteins in the supernatants and pellets were subjected to SDS/PAGE and transferred onto an Immobilon-P membrane. The membranes were stained with Ponceau-S and incubated with antibodies against CK2α and grp94-CT.

bands were scarcely visible in the supernatants derived from the same samples. In contrast, the set of bands corresponding to CK2α were clearly evident in the supernatants from the samples incubated in the presence of grp94-CT. This was reflected in a decrease in the amount of CK2α detected in the corresponding pellets. Inclusion of dithiothreitol in the incubation mixture did not alter the pattern observed with CK2α alone, but decreased markedly the amount of this subunit detected in the supernatants from the samples supplemented with grp94-CT, with a concomitant increase in the pellets. A similar pattern was observed when the presence of CK2α was detected immunologically (Fig. 4B, anti-CK2α). In this case, the conditions for immunodetection were maximally optimized to detect the CK2α present in the supernatants from the samples containing only this subunit. The level of CK2α was very low in the supernatant from the control sample but was clearly detectable in the samples supplemented with grp94-CT in the absence of dithiothreitol. A decrease in the CK2α content in the supernatant, with a concomitant increase in the pellets, was observed when dithiothreitol was added. On the other hand, detection with anti-grp94-CT antibodies showed that most of this protein remained in the supernatant, with only a small part sedimenting in the samples treated with dithiothreitol (Fig. 4B, anti-grp94-CT). These data suggest that a small portion of grp94-CT interacted with aggregated CK2α. They also indicate that the red-ox state of the two cysteinyl residues present in this region of grp94 are of great importance for maintaining grp94-CT structure, and contribute to its protective effect on CK2α aggregation, although the influence of other features cannot be disregarded.

DISCUSSION

Studies on the molecular chaperone activity of hsp90 are made easier by the ability to evaluate it *in vitro* by either its ability to bind antigenic peptides or its anti-aggregation effects on different denatured proteins. Recent studies have shown that both the N-terminal and C-terminal domains of hsp90 prevent the aggregation of denatured proteins, but they show different specificity towards peptide binding, ATP-dependence, and inhibition by geldanamycin and cisplatin [27,28]. In contrast, the chaperone activity of grp94 seems to have been monitored *in vitro* exclusively by its peptide binding capability, which resides in its C-terminal domain [10]. Our data show that this domain of grp94 also possesses anti-aggregation activity that can be easily assessed with CK2α. It is interesting to point out that no such activity was detected with malate dehydrogenase under conditions similar to those used to detect the chaperone activity of calreticulin [29]. Whether this simply reflects the high tendency of CK2α to form aggregates or obeys to specific determinants absent in malate dehydrogenase is unknown. On the other hand, grp94-CT has little effect on the inactivation of CK2α present in the soluble complexes. This is similar to some other molecular chaperones of citrate synthase [30] that form stable chaperone–substrate complexes with 'late' unfolding intermediates that can not be easily reactivated.

The protective effect of grp94-CT on CK2α aggregation was strongly diminished by the presence of dithiothreitol. Reductants are known to affect CK2 by two means. They either alter the structure of the protein substrate and accessibility of the phosphorylation sites to CK2α [31], thus promoting its dissociation from the nuclear matrix, a process that involves disulphide bonding through the regulatory CK2β subunit [32], or they directly activate the phosphotransferase activity of CK2 holoenzyme [32]. However, although CK2α contains two cysteine residues, dithiothreitol has no effect on the catalytic activity of this free subunit [32]. Furthermore, our data indicate that the kinetics of CK2α aggregation were not affected by dithiothreitol. Therefore, this identified grp94-CT as the component altered by dithiothreitol.

Exposure of culture cells to reductants affects folding and assembly of certain membrane and secretory proteins [33,34]. *In vitro* association of the molecular chaperone BiP with protein substrates is favoured in the presence of reductants [33,35], whereas grp94 seems to interact with substrates preferentially (or exclusively) after removal of the reductant [33]. Native grp94 from different sources forms dimers and oligomers [1]. Hydrophobic interactions between grp94 C-terminal domains participate in forming these structures, but the role of disulphide bonds in stabilizing them has been a matter of discussion. However, the dimeric form of native rat liver grp94 requires dithiothreitol to dissociate into monomers [12] and Cys117 in murine recombinant grp94 has been reported to participate in the formation of a disulphide bonded homodimer [13]. Our data on the lack of spontaneous reversibility *in vitro* of the disulphide bonds between grp94-CT (Fig. 2), together with the fact that reductants (either 2-mercaptoethanol or dithiothreitol) have been included occasionally in the buffers used to isolate grp94 [11,36], may help to explain these different observations. It is possible that dimerization of grp94-CT may still occur in the presence of dithiothreitol, probably through hydrophobic interactions, but that in the expressed protein intersubunit disulphide bonds serve to stabilize the dimer. Intramolecular disulphide bonds may also exist in the monomers, as suggested by the small changes in its mobility in SDS/PAGE after dithiothreitol treatment. Disulphide bonds are essential for oligomerization, as the oligomers were not disrupted by urea (Fig. 1B). dithiothreitol treatment induces the progressive dissociation of oligomers to the monomeric state. Grp94-CT secondary structure does not seem to be affected by dissociation, which agrees with the previous observation that the activation of porcine grp94 peptide binding activity after different treatments was not accompanied by changes in its secondary structure [37]. The fact that the anti-aggregation effect of grp94-CT was lost after exposure to 5 mM dithiothreitol suggests that either the oligomeric structures are required or that reduced Cys residues are strongly detrimental. The first possibility is supported by recent data showing that grp94–peptide complexes exists as dimers or higher order structures [38].

The primary structure of human grp94 (Swiss-Prot accession number P14625) contains only three Cys residues, two of which are located in the fragment corresponding to grp94-CT. These Cys residues are present in the sequences 571PVDEYCIQALP581 and 643SPCALVASQ651, which are conserved in mammalian grp94. No other Cys residues are present in the region homologous to grp94-CT in either animals or plants. Interestingly, Cys645 in human grp94 precedes the sequence 652YGWXXNMERIMKAQA666,

which is fully conserved in almost all animal grp94s, and also in almost all animal hsp90αs (residues 603–617 in human hsp90α, Swiss-Prot accession number P07900) and hsp90βs (residues 595–609 in human hsp90β, Swis-Prot accession number P08238), with XX being SG in grp94 and TA in both hsp90α and hsp90β. Interestingly, the peptide binding site in grp94 has been mapped as part of the sequence DKALKDK [10], which is only 15 residues ahead of Cys645. This suggests that the region encompassing Cys645 is of great importance in grp94 function, in addition to the region 698–740 (676–719 in the mature protein), which is involved in dimerization through hydrophobic interactions [8]. Taken together it is clear that both Cys576 and Cys645 in grp94-CT must participate in maintaining the oligomeric structures in which the monomers are held together by disulphide bonds.

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REFERENCES

1. Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z. & Nardai, G. (1998) The 90-kDa molecular chaperone family: structure, function and clinical applications. A comprehensive review. *Pharmacol. Ther.* 79, 129–168.
2. Hoshino, T., Wang, J., Devetten, M.P., Iwata, N., Kajigaya, S., Wise, R.J., Liu, J.M. & Youssoufian, H. (1998) Molecular chaperone GRP94 binds to the Fanconi anemia group C protein and regulates its intracellular expression. *Blood* 91, 4379–4386.
3. Reddy, R.K., Lu, J. & Lee, A. (1999) The endoplasmic reticulum chaperone glycoprotein GRP94 with Ca^{2+}-binding and antiapoptotic properties is a novel proteolytic target of calpain during etoposide-induced apoptosis. *J. Biol. Chem.* 274, 28476–28483.
4. Srivastava, P.K., DeLeo, A.B. & Old, L.J. (1986) Tumor rejection antigens of chemically induced sarcomas of inbred mice. *Proc. Natl Acad. Sci. USA* 83, 3407–3411.
5. Gorza, L. & Vitadello, M. (2000) Reduced amount of the glucose-regulated protein GRP94 in skeletal myoblasts results in loss of fusion competence. *FASEB J.* 14, 461–475.
6. Tamura, Y., Peng, P., Liu, K., Daou, M. & Srivastava, P.K. (1997) Immunotherapy of tumors with autologous tumor-derived heat shock protein. *Science* 278, 117–120.
7. Nemoto, T., Ohara-Nemoto, Y., Ota, M., Takagi, T. & Yokoyama, K. (1995) Mechanism of dimer formation of the 90-kDa heat-shock protein. *Eur. J. Biochem.* 233, 1–8.
8. Wearsch, P.A. & Nicchitta, C.V. (1996) Endoplasmic reticulum chaperone GRP94 subunit assembly is regulated through a defined oligomerization domain. *Biochemistry* 35, 16760–16769.
9. Nemoto, T. & Sato, N. (1998) Oligomeric forms of the 90-kDa heat shock protein. *Biochem. J.* 330, 989–995.
10. Linderoth, N.A., Popowicz, A. & Sastry, S. (2000) Identification of the peptide-binding site in the heat shock chaperone/tumor rejection antigen gp96 (Grp94). *J. Biol. Chem.* 275, 5472–5477.
11. Wearsch, P.A. & Nicchitta, C.V. (1996) Purification and partial molecular characterization of GRP94, an ER resident chaperone. *Prot. Exp. Purif.* 7, 114–121.
12. Trujillo, R., Miró, F., Plana, M., José, M., Bollen, M., Stalmans, W. & Itarte, E. (1997) Substrates for protein kinase CK2 in insulin receptor preparations from rat liver membranes: identification of a 210-kDa protein substrate as the dimeric form of endoplasmin. *Arch. Biochem. Biophys.* 344, 18–28.
13. Qu, D., Mazzarella, R.A. & Green, M. (1994) Analysis of the structure and synthesis of GRP94, an abundant stress protein of the endoplasmic reticulum. *DNA Cell Biol.* 13, 117–124.
14. Miyata, Y. & Yahara, I. (1995) Interaction between casein kinase II and the 90-kDa stress protein, HSP90. *Biochemistry* 34, 8123–8129.
15. Lees-Miller, S.P. & Anderson, C.W. (1989) Two human 90-kDa heat shock proteins are phosphorylated *in vivo* at conserved serines that are phosphorylated *in vitro* by casein kinase II. *J. Biol. Chem.* 264, 2431–2437.
16. Wearsch, P.A. & Nicchitta, C.V. (1997) Interaction of the endoplasmic reticulum chaperone GRP94 with peptide substrate is adenine nucleotide-independent. *J. Biol. Chem.* 272, 5152–5156.
17. Cala, S.E. & Jones, L.R. (1994) GRP94 resides within cardiac sarcoplasmic reticulum vesicles and is phosphorylated by casein kinase II. *J. Biol. Chem.* 269, 5926–5931.
18. Riera, M., Roher, N., Miró, F., Gil, C., Trujillo, R., Aguilera, J., Plana, M. & Itarte, E. (1999) Association of protein kinase CK2 with eukaryotic translation factor eIF-2 and with grp94/endoplasmin. *Mol. Cell. Biochem.* 191, 97–104.
19. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* 227, 680–685.
20. Andrews, A.T. (1986) *Electrophoresis: Theory, Techniques, Biochemical and Clinical Applications.* Oxford University Press, Oxford, UK.
21. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
22. Nemoto, T., Matsusaka, T., Ota, M., Takagi, T., Collinge, D. & Walther-Larsen, H. (1996) Dimerization characteristics of the 94-kDa glucose-regulated-protein. *J. Biochem.* 120, 249–256.
23. Boldyreff, B., Meggio, F., Pinna, L.A. & Issinger, O.G. (1994) Protein kinase CK2 structure function relationship: effects of the beta subunit on reconstitution and activity. *Cell. Mol. Biol. Res.* 40, 391–399.
24. Valero, E., DeBonis, S., Filhol, O., Wade, R.H., Langowoski, J., Chambaz, E.M. & Cochet, C. (1995) Quaternary structure of casein kinase 2. Characterization of multiple oligomeric states and relation with its catalytic activity. *J. Biol. Chem.* 270, 8345–8352.
25. Issinger, O.-G. (1993) Casein kinases: pleiotropic mediators of cellular regulation. *Pharmacol. Ther.* 59, 1–30.
26. Shi, Y., Brown, E.D. & Walsh, C.T. (1994) Expression of recombinant human casein kinase II and recombinant heat shock protein 90 in *Escherichia coli* and characterization of their interaction. *Proc. Natl Acad. Sci. USA* 91, 2767–2771.
27. Young, J.C., Schneider, C. & Hartl, F.U. (1997) *In vitro* evidence that hsp90 contains two independent chaperone sites. *FEBS Lett.* 418, 139–143.
28. Itoh, H., Ogura, M., Komatsuda, A., Wakui, H., Miura, A.B. & Tashima, Y. (1999) A novel chaperone-activity-reducing mechanism of the 90-kDa molecular chaperone HSP90. *Biochem. J.* 343, 697–703.
29. Saito, Y., Ihara, Y., Leach, M.R., Cohen-Doyle, M.F. & Williams, D.B. (1999) Calreticulin functions *in vitro* as a molecular chaperone for both glycosylated and non-glycosylated proteins. *EMBO J.* 18, 6718–6729.
30. Buchner, J., Grallert, H. & Jakob, U. (1998) Analysis of chaperone function using citrate synthase as non-native substrate protein. *Methods Enzymol.* 290, 323–338.
31. Scotto, C., Mély, Y., Ohshima, H., Garin, J., Cochet, C., Chambaz,

E. & Baudier, J. (1998) Cysteine oxidation in the mitogenic S100B protein leads to changes in phosphorylation by catalytic CKIIα subunit. *J. Biol. Chem.* **273**, 3901–3908.
32. Zhang, P., Davis, A.T. & Ahmed, K. (1998) Mechanism of protein kinase CK2 association with nuclear matrix: role of disulphide bond formation. *J. Cell. Biochem.* **69**, 211–220.
33. Melnick, J., Dul, J.L. & Argon, Y. (1994) Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. *Nature* **370**, 373–375.
34. Gelman, M.S. & Prives, J.M. (1996) Arrest of subunit folding and assembly of nicotinic acetylcholine receptors in cultured muscle cells by dithiothreitol. *J. Biol. Chem.* **271**, 10709–10714.
35. Jamsa, E., Simonen, M. & Makarow, M. (1994) Selective retention of secretory proteins in the yeast endoplasmic reticulum by treatment of cells with a reducing agent. *Yeast* **10**, 355–370.
36. Welch, W.J. & Feramisco, J.R. (1982) Purification of the major mammalian heat shock proteins. *J. Biol. Chem.* **257**, 14949–14959.
37. Wearsch, P.A., Voglino, L. & Nicchitta, C.V. (1998) Structural transitions accompanying the activation of peptide binding to the endoplasmic reticulum hsp90 chaperone GRP94. *Biochemistry* **37**, 5709–5719.
38. Sastry, S. & Linderoth, N. (1999) Molecular mechanism of peptide loading by the tumor rejection antigen/heat shock chaperone gp96 (GRP94). *J. Biol. Chem.* **274**, 12023–12035.