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Phosphorylation by CK2 and MAPK enhances calnexin association with ribosomes

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Calnexin was initially identified as an endoplasmic reticulum (ER) type I integral membrane protein, phosphorylated on its cytosolic domain by ER-associated protein kinases. Although the role of the ER luminal domain of calnexin has been established as a constituent of the molecular chaperone machinery of the ER, less is known about the role of the cytosolic phosphorylation of calnexin. Analysis by two-dimensional phosphopeptide maps revealed that calnexin was *in vitro* phosphorylated in isolated microsomes by casein kinase 2 (CK2) and extracellular-signal regulated kinase-1 (ERK-1) at sites corresponding to those for *in vivo* phosphorylation. In canine pancreatic microsomes, synergistic phosphorylation by CK2 and ERK-1 led to increased association of calnexin with membrane-bound ribosomes. *In vivo*, calnexin-associated ERK-1 activity was identified by co-immunoprecipitation. This activity was abolished in cells expressing a dominant-negative MEK-1. Activation of ERK-1 in cells by addition of serum led to a 4-fold increase in ribosome-associated calnexin over unstimulated cells. Taken together with studies revealing calnexin association with CK2 and ERK-1, a model is proposed whereby phosphorylation of calnexin leads to a potential increase in glycoprotein folding close to the translocon.
Keywords: calnexin/MAPK/phosphorylation/translocation

Introduction

The calnexin family of eukaryotic-specific molecular chaperones couples *N*-linked oligosaccharide modifications on newly synthesized glycoproteins with their productive folding in the endoplasmic reticulum (ER) (Bergeron *et al*., 1994). This family is currently defined as calnexin (CNX), calreticulin and the testis-specific calmegin/calnexin-t (Fliegel *et al*., 1989; Wada *et al*., 1991; Ohsako *et al*., 1994; Watanabe *et al*., 1994). As exemplified by the properties of calnexin, these molecular chaperones are constituents of a glucosylation/deglucosylation cycle. During this cycle, monoglucosylated glycoproteins bind transiently to calnexin (calreticulin, calmegin/calnexin-t), to which the protein folding enzyme ERp57 is recruited, enhancing the rate of productive rearrangement of disulfide bonds for at least one *in vitro* model substrate, RNase B (Zapun *et al*., 1998; Elaine *et al*., 1999). When dissociated from calnexin, glycoproteins are further processed by the removal of the single glucose residue by ER α-glucosidase II (Zapun *et al*., 1997; Trombetta and Helenius, 1998). A glycoprotein folding sensor UDP-glucose:glycoprotein glucosyltransferase (UGGT) recognizes glycoproteins in their non-native conformation and re-adds the glucose residue (Sousa and Parodi, 1995; Wada *et al*., 1997; Zapun *et al*., 1997; Parodi, 1999), enabling these $Glc_1Man_9GlcNAc_2$-containing glycoproteins to rebind to calnexin/calreticulin (Cannon and Helenius, 1999). Properly folded glycoproteins can exit the calnexin cycle as they are ineffective substrates for UGGT (Parodi *et al*., 1983; Trombetta *et al*., 1989; Trombetta and Helenius, 1998; Liu *et al*., 1999).

Calnexin is a type I ER integral membrane protein, with its cytosolic domain *in vivo* phosphorylated in part at the casein kinase 2 (CK2) sites (Ser534, Ser544) and at a protein kinase C/proline-directed kinase (PKC/PDK) site (Ser563; Wong *et al*., 1998). By both cross-linking studies and co-immunoprecipitation analysis, calnexin has been shown to associate with incompletely translocated polypeptide chains (Chen *et al*., 1995; Oliver *et al*., 1996), indicating a close association of calnexin with the translocon. Furthermore, when the HIV-1 envelope protein gp-120 was co-expressed with canine calnexin in Sf-9 cells, transient association of incompletely folded gp-120 with calnexin preceded signal sequence of gp-120 (Li *et al*., 1996). For these studies, calnexin association with oligosaccharide-linked substrates was assessed. However, a direct association of calnexin with membrane-bound ribosomes has not previously been shown. Here, we show that phosphorylation of calnexin results in its association with ribosomes. Phosphorylation of calnexin's cytosolic domain by the concerted action of the protein kinase CK2 at Ser534, Ser544 and extracellular-signal regulated kinase-1 (ERK-1) at Ser563 coincides with calnexin targeting to ribosomes where a direct interaction with ribosomes, independent of that with newly synthesized glycoproteins, is demonstrated.

Results

Phosphorylation of calnexin by CK2 and ERK-1

Calnexin was first characterized as a substrate for kinases associated with the rough ER (Wada *et al*., 1991). One of the kinases was identified as a population of CK2 which was associated with ER membranes (Ou *et al*., 1992; Cala

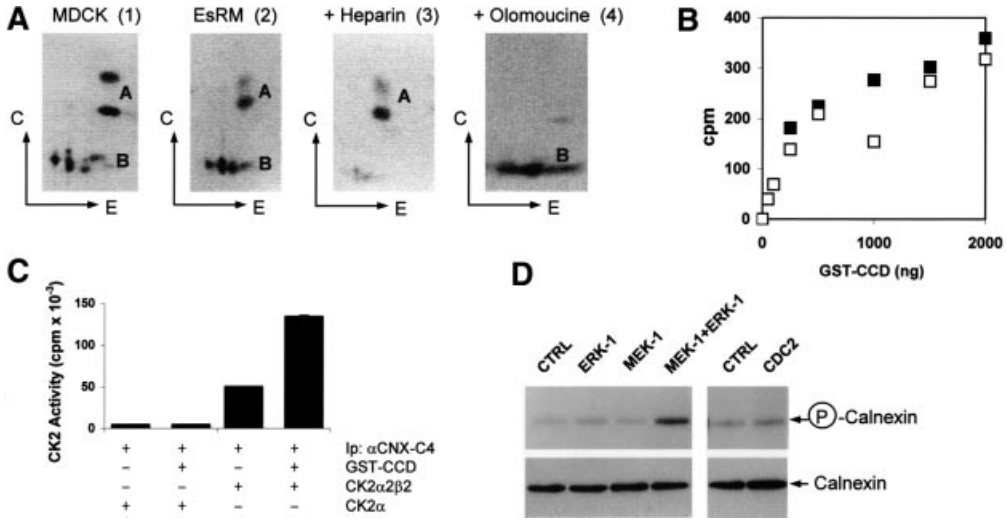

Fig. 1. Phosphorylation of calnexin by CK2 and ERK-1. (**A**) Two-dimensional tryptic phosphopeptide mapping of phosphorylated calnexin from MDCK cells after *in vivo* radiolabeling, calnexin immunoprecipitation and trypsin digestion (map 1). Comparison with two-dimensional analysis of calnexin tryptic phosphopeptides from EDTA-stripped canine pancreatic rough microsomes (EsRM) after *in vitro* phosphorylation (map 2), in the presence of 0.5 mg/ml heparin (map 3) or in the presence of 50 μM olomoucine (map 4). The direction of peptide migration by electrophoresis (E) as well as chromatography (C) are indicated. (**B**) Michaelis–Menten representation of GST–CCD phosphorylation kinetics by CK2α monomer (filled symbols) or α2β2 holoenzyme (open symbols) purified to homogeneity from Sf9 cells. A similar K_m 7.5×10^{-6} M is calculated for each. (**C**) The α subunit (60 ng) or the holoenzyme α2β2 (90 ng) were incubated either in the presence or absence of 0.5 μg of GST–CCD. Complexes were immunoprecipitated with anti-CNX-C4 antibodies and the kinase activity found in the immunocomplex was measured using casein as substrate. Each value is given as counts per minute (c.p.m.) incorporated into casein. (**D**) EsRM were heat-inactivated and incubated in the presence of 0.5 μg of GST–ERK-1, 0.5 μg of GST–MEK-1, both kinases together (1 μg), 0.5 μg of purified $p34^{cdc2}$ or no added kinase, and 2 μCi of [γ-^{32}P]ATP for 30 min at 30°C. Membranes were then solubilized in 1% CHAPS and calnexin was immunoprecipitated with anti-CNX antibodies. Immunoprecipitates were resolved by SDS–PAGE using 10% resolving gel, transferred onto nitrocellulose and directly exposed to X-OMAT AR film for 6 h at –80°C (upper panel) or immunoblotted using anti-CNX-C4 antibodies, and visualized using enhanced chemiluminescence (bottom panel).

et al., 1993). By mass spectrometry, two of the three invariant sites of *in vivo* phosphorylation of calnexin were identified as CK2 phosphorylation motifs Ser534 and Ser544 (human sequence; Wong *et al*., 1998). The third site of *in vivo* phosphorylation of calnexin was identified as a PKC/PDK site (Ser563; Wong *et al*., 1998).

Here, inhibitors have been used to characterize the relevant calnexin-associated kinases. Following either *in vivo* phosphorylation of MDCK cells (Figure 1A, panel 1) or *in vitro* phosphorylation of EDTA-stripped canine pancreatic rough microsomes (EsRM; Figure 1A, panel 2) and analysis by two-dimensional phosphopeptide map, a similar pattern of calnexin phosphopeptides was generated. Hence, *in vitro* phosphorylation of calnexin in canine pancreatic microsomes by resident kinases occurred on sites similar to those identified *in vivo* (Wong *et al*., 1998). Two calnexin phosphopeptide groups were designated as phosphopeptide group A and B (Figure 1A). Heparin, an inhibitor of CK2, attenuated *in vitro* phosphorylation of group B phosphopeptides (Figure 1A, panel 3) while olomoucine, an inhibitor of PDK (Vesely *et al*., 1994) at 50 μM, inhibited the *in vitro* phosphorylation of group A phosphopeptide (Figure 1A, panel 4). When the same experiments were carried out with iso-olomoucine or with the PKC inhibitors calphostin C or chelerythrine chloride, no inhibition was observed (data not shown).

Previous studies have identified CK2 as a relevant calnexin kinase (Ou *et al*., 1992; Cala *et al*., 1993; Wong *et al*., 1998). The protein kinase CK2 holoenzyme consists of two catalytic α or α′ subunits and two regulatory β subunits. Our next experiments were designed to show whether calnexin association with CK2 is dependent on the regulatory β subunit. When *in vitro* phosphorylation was carried out using a glutathione *S*-transferase (GST) fusion protein containing the calnexin cytosolic domain (GST–CCD; 0.5 μg), and either the CK2α subunit alone (60 ng) or the holoenzyme α2β2 (20 ng), a similar level of GST–CCD phosphorylation was observed as quantified in Figure 1B. However, only the holoenzyme stably associated with calnexin as shown by co-immunoprecipitation with antibodies to calnexin. Here, the associated kinase activity (using casein as substrate) was found with the α2β2 holoenzyme (Figure 1C). The experimental design consisted of incubations of CK2α alone or with GST–CCD compared with CK2α2β2 alone or with GST–CCD. Anti-CNX-C4 immunoprecipitates of GST–CCD were then incubated in the presence of casein in an *in vitro* kinase assay. After the reaction, radiolabeled casein was resolved by SDS–PAGE and radioactivity was assessed. No detectable association of CK2α was found with GST–CCD using this technique. Although a high background of casein kinase activity was observed with CK2α2β2 alone, a 2.7-fold increase over this background was found when GST–CCD and CK2α2β2 were incubated together. Thus, we conclude that CK2 requires its β subunit for association with calnexin.

To identify the relevant PDK responsible for the olomoucine sensitive calnexin kinase activity, EsRM were first heat-treated for 10 min at 65°C to inactivate the resident kinases (Ou *et al*., 1992). They were then incubated in the presence of [γ-^{32}P]ATP with either recombinant $p34^{cdc2}$, GST–ERK-1, GST–MEK-1 or GST–MEK-1+ GST–ERK-1 (Figure 1D). An increase in calnexin phosphorylation was observed only with MAPK/ERK kinase-1 (MEK-1)+ERK-1 (Figure 1D, upper panel). This was not due to variation in the amount of calnexin

as shown by an immunoblot of duplicate samples with anti-CNX-C4 antibodies (Figure 1D, bottom panel). The two-dimensional phosphopeptide map of the basal or MEK-1+ERK-1 phosphorylated calnexin mainly revealed the calnexin group A phosphopeptides, corresponding to the olomoucine-sensitive calnexin phosphorylation (data not shown). Hence ERK-1 is a candidate kinase responsible for the *in vitro* phosphorylation of calnexin at its PDK site (Ser563).

ERK-1 binds to calnexin in vivo

To demonstrate a direct association of calnexin and ERK-1, calnexin was immunoprecipitated from proliferating or heat shocked Rat-2 cells followed by an in-gel kinase assay using myelin basic protein (MBP) as substrate. For the immunoprecipitations, two calnexin antibodies were used. The first, termed anti-CNX-C3 (residues 487–505; see Figure 9), was raised against the juxtamembrane and the second, termed anti-CNX-C4 (residues 555–573; see Figure 9), was raised against the extreme C-terminus of calnexin. The anti-CNX-C3 immunoprecipitates contained very weak kinase activity when the in-gel kinase assay was performed using MBP as substrate, resulting in only a very faint phosphorylated band of ~42 kDa. The anti-CNX-C4 immunoprecipitates, however, contained a strong kinase activity as visualized by the phosphorylated band of 44 kDa (Figure 2A, lanes 1 and 2, respectively). The amount of calnexin immunoprecipitated by both antibodies was identical (data not shown). The amount of these kinases which co-immunoprecipitated with calnexin did not change when cells were heat shocked for 15 min at 51°C (Figure 2A, lanes 3 and 4). The difference between the anti-CNX-C3 and anti-CNX-C4 antibodies in co-precipitating the associated kinases suggests an association of this kinase(s) with the juxtamembrane region of calnexin's cytosolic tail, and that the anti-CNX-C3 antibodies select for the calnexin pool, which is not kinase associated. Moreover, the characteristics of the calnexin-associated kinase (molecular mass, capacity to refold after SDS–PAGE and MBP kinase activity) suggest that this protein could belong to the mitogen-activated protein kinase (MAPK) family.

To characterize further the calnexin-associated kinase, we determined the *in vivo* kinase activity associated with calnexin using Rat-2 cells stimulated with 10% fetal bovine serum (FBS) (Troppmair *et al*., 1994). *In vitro* kinase assays with MBP as substrate were carried out respectively on anti-CNX-C4, anti-ERK-1 immunoprecipitates or sequential immunoprecipitates of anti-CNX-C4, followed by release and re-immunoprecipitation with anti-ERK-1 antibodies (Figure 2B, open bars). The MBP kinase activity associated with calnexin was stimulated 9.4-fold by serum treatment, and the activation observed for the total ERK-1 activity was ~3.5-fold. Immunoprecipitation of calnexin followed by sequential anti-ERK-1 immunoprecipitation from the proteins co-precipitated with calnexin demonstrated that only a minor proportion of total ERK-1 activity was associated with calnexin (Figure 2B). *In vitro* kinase assays carried out using GST–CCD as substrate revealed comparable fold-increases upon serum treatment (Figure 2B, filled bars). From these studies, the proportion of ERK-1 activity associated with calnexin using MBP or GST–CCD as substrate was

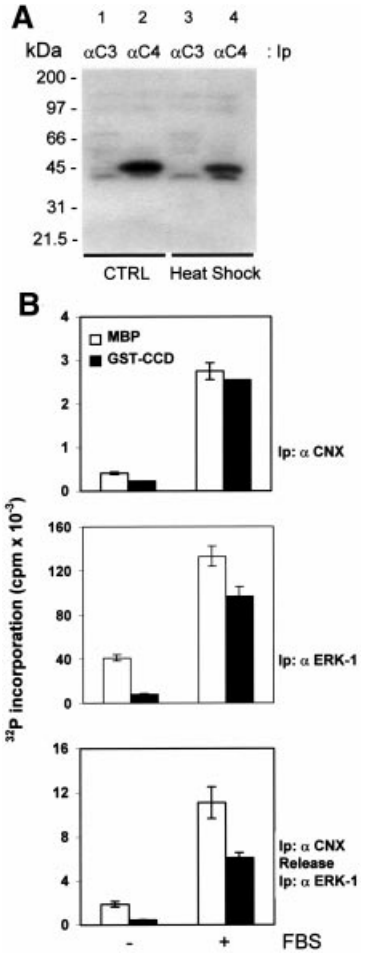

Fig. 2. ERK-1 binds to calnexin *in vivo* and phosphorylates its cytosolic domain. (**A**) Rat-2 control cells (CTRL) or heat shocked cells (15 min at 51°C) were lysed in 1% CHAPS for 30 min at 4°C. Calnexin was then immunoprecipitated using either anti-CNX-C3 or anti-CNX-C4 antibodies. Immunocomplexes were resolved by 10% SDS–PAGE containing 0.5 mg/ml MBP. An in-gel kinase assay was then carried out as described in the Materials and methods. After Coomassie Blue R-250 staining and drying, the gel was radioautographed for 15 h at –80°C. The radioautogram is representative of three separate experiments. (**B**) Rat-2 cells were serum-starved for 20 h and then stimulated for 10 min with 10% FBS in DMEM. After lysis in 1% CHAPS, calnexin or ERK-1 were immunoprecipitated respectively by anti-CNX-C4 or anti-ERK-1 antibodies. Immunocomplexes were released from the beads by heating for 10 min at 95°C in 1% SDS and sequentially immunoprecipitated by anti-ERK-1 antibodies. The kinase activity present in the immunoprecipitates was assayed with [γ-^{32}P]ATP and MBP (open bars) or GST–CCD (filled bars) as substrate. The results represent the mean ± SD for three separate experiments.

estimated to be 5 and 7%, respectively, of the total cellular ERK-1 activity. Taken together, the data of Figure 2 suggest that ERK-1 associates directly or indirectly with the juxtamembrane domain of the calnexin cytosolic tail (in the C3 peptide domain), from where it phosphorylates the C-terminal region at Ser563 (Wong *et al*., 1998).

The association of ERK-1 with calnexin predicts the presence of ERK-1 on ER membranes. Both ERK-1 (p44) and ERK-2 (p42) were found associated with dog pancreatic rough microsomes, but not on high salt washed (750 mM KCl, 1 mM puromycin) membranes or in the purified ribosomal fraction from this wash (Figure 3A). When total membrane fractions [mitochondrial, light

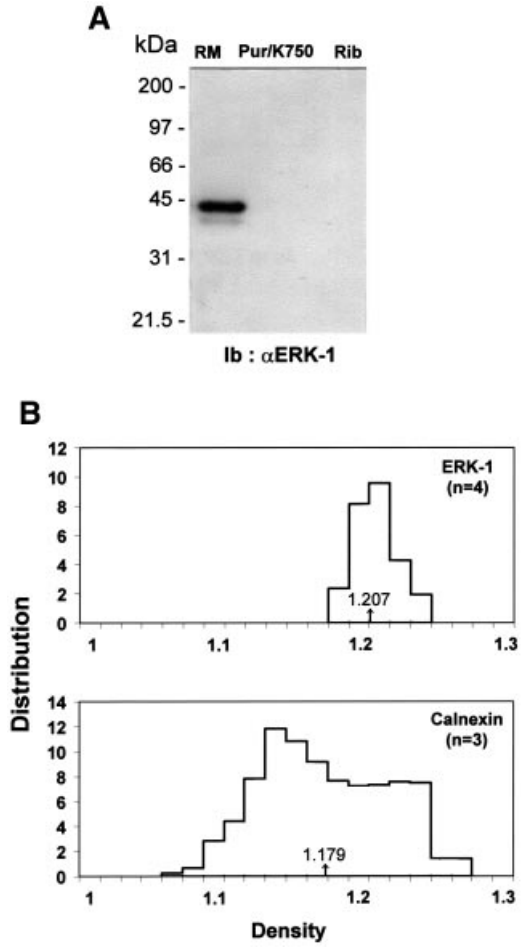

Fig. 3. ERK-1 is associated with the endoplasmic reticulum membrane. (**A**) Canine pancreatic rough microsomes (RM), or 1 mM puromycin, 750 mM KCl (Pur/K750) stripped RM or the purified released ribosomal fraction (Rib) were resolved by 10% SDS–PAGE, and immunoblotted with anti-ERK-1 antibodies, followed by [^{125}I]goat anti-rabbit IgG and visualized by radioautography using Kodak X-OMAT AR film. (**B**) The MLP fraction from rat liver homogenates was centrifuged on linear sucrose gradients as described in the Materials and methods. Equal volumes of each fraction were analyzed for their content of ERK-1 and calnexin by immunoblots, followed by [^{125}I]goat anti-rabbit IgG and visualized by radioautography using Kodak X-OMAT AR film. Quantitation was done by scanning densitometry. The number (*n*) of separate fractionations is indicated. Results were normalized according to the method of Beaufay *et al.* (1964).

mitochondrial and particulate (MLP)] of rat liver were fractionated by isopycnic sucrose gradient centrifugation (Figure 3B), membrane-associated ERK-1 revealed a median density of 1.207, identical to that of the rough ER marker Ribophorin II (not shown). Calnexin revealed a broader distribution than ERK-1 with a median density of 1.179 (Figure 3B).

Ribosome-associated calnexin

In the original strategy used to purify calnexin (as a substrate for ER resident kinases) it co-isolated with the α and β subunits of the ribosome-associated membrane protein TRAP (for translocon-associated protein) as well as gp25L, a member of the p24 family of membrane proteins (Wada *et al*., 1991). To evaluate whether calnexin could be found associated with ribosomes, we used a modification of the methodology employed by Görlich *et al*. (1992) for the identification of ribosome-associated membrane proteins (including TRAP). Canine pancreatic rough microsomes (RM) were solubilized and ribosome-associated proteins were purified through a 1.5 M sucrose cushion. The translocation channel protein Sec61 remained ribosome-associated, as shown previously by Görlich *et al*. (1992). Also using this experimental procedure calnexin, the postulated translocon constituent TRAPα (Görlich *et al*., 1992) and the ER-to-Golgi recycling membrane protein α2p24 (Dominguez *et al*., 1998) were all found to be associated with ribosomes when using either digitonin or CHAPS as detergent (Figure 4A). No ribosome association of the ER luminal proteins BiP (Figure 4C) and GRP94 (not shown) was found. As a control, EsRM were treated identically and the proteins in the 1.5 M sucrose cushion pellets were analyzed by immunoblotting. Neither CNX nor α2p24 (which is in a 35S complex in Golgi fractions; Dominguez *et al*., 1998) were found (Figure 4B). Furthermore, neither TRAPα nor Sec61 were detectable (not shown).

Calnexin–ribosome association was also deduced from protease protection assays. The susceptibility of calnexin to trypsin digestion was assessed by immunoblotting microsomes with antibodies raised against the luminal domain of calnexin (anti-CNX-C1; Figure 4D). In the absence of ribosomes, the mobility of calnexin increased from 90 to 70 kDa (ΔCNX) corresponding to proteolysis of the cytosolic domain (Ou *et al*., 1995; Figure 4D, upper panel). In RM, this proteolysis was attenuated (Figure 4D, lower panel) presumably by the attached ribosomes. Partial protection of calnexin against proteolysis in EsRM was restored by a 30 min pre-incubation with purified ribosomes (Figure 4D, middle panel). TRAPα was also protected from proteolysis by the presence of ribosomes but to a lower extent than CNX (Figure 4E). To distinguish whether the calnexin–ribosome association was dependent on nascent polypeptide chains, RM were treated with 1 mM puromycin to release nascent chains and 750 mM KCl (K750) to release ribosomes or 25 mM KCl (K25) which maintains ribosome association with membranes (Adelman *et al*., 1973). The number of membrane-bound ribosomal particles was quantified by morphometry (Figure 5; Table I). The efficiency of EDTA treatment was comparable to that of K750, while K25 did not lead to ribosome removal. In ribosome sedimentation experiments (Figure 6A), calnexin remained ribosome bound with 1 mM puromycin + 25 mM KCl, while α2p24 did not. Hence, the calnexin–ribosome association was concluded to be direct while that of α2p24 required nascent polypeptide chains. To assess whether calnexin could influence ribosome engagement by the translocation channel, *in vitro* co-translational translocation experiments using alpha-mating factor mRNA were performed on EsRM. No inhibition of translocation was observed when microsomes were pre-incubated with antibodies raised against the calnexin cytosolic domain, nor with antibodies raised against the C-terminus of TRAPα (Figure 6B). Therefore, ribosome-associated calnexin was unlikely to participate in ribosome–membrane interactions via the translocon. This conclusion has been made previously for TRAPα (Migliaccio *et al*., 1992).

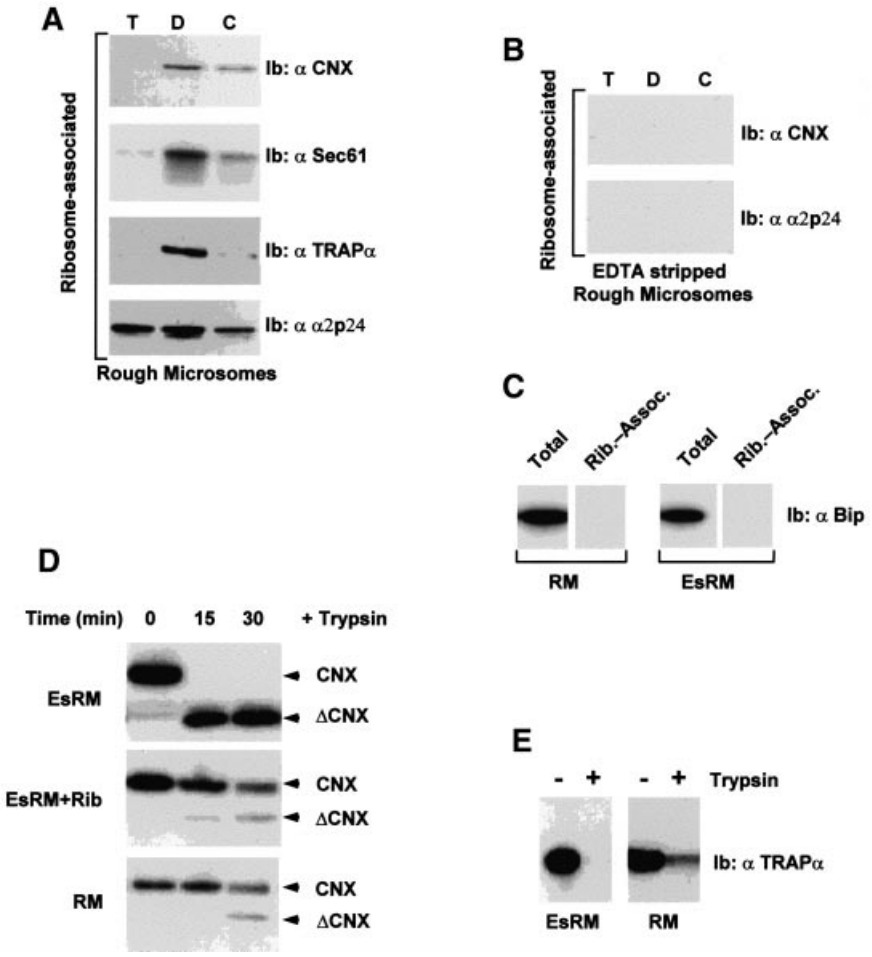

Fig. 4. Calnexin association with ribosomes. (**A**) Canine pancreatic RM were solubilized with 1% Triton X-100 (T), 1.5% digitonin (D) or 1% CHAPS (C). Ribosome-associated protein fractions were purified through a 1.5 M sucrose cushion, the pellet was resuspended in SDS–PAGE Laemmli sample buffer, resolved by SDS–PAGE, transferred onto nitrocellulose and immunoblotted with anti-CNX-C1, anti-Sec61, anti-TRAPα or anti-α2p24 antibodies, followed by [^{125}I]goat anti-rabbit IgG and visualized by radioautography using Kodak X-OMAT AR film. (**B**) The same experiment as in (A) except EsRM were used. Immunoblotting is shown for anti-CNX-C1 and anti-α2p24 antibodies. (**C**) The presence of BiP, an ER luminal protein, was detected by immunoblotting on RM and EsRM and the corresponding ribosome-associated proteins. (**D**) Canine pancreatic rough microsomes (RM), EDTA-stripped rough microsomes (EsRM) or EsRM pre-incubated with competent ribosomes (EsRM+Rib) were treated on ice for 15 or 30 min with trypsin (10 μg/ml). The reaction was stopped by the addition of Laemmli sample buffer. Proteins were resolved by SDS–PAGE, transferred to nitrocellulose and immunoblotted with anti-CNX-C1, followed by [^{125}I]goat anti-rabbit IgG and visualized by radioautography using Kodak X-OMAT AR film. (**E**) RM or EsRM were trypsin digested on ice, then suspended in SDS–PAGE Laemmli sample buffer, resolved by SDS–PAGE, transferred to nitrocellulose and immunoblotted with anti-TRAPα and followed by [^{125}I]goat anti-rabbit IgG and visualized by radioautography using Kodak X-OMAT AR film.

Phosphorylation regulates calnexin association with ribosomes

To determine whether CK2 and ERK-1 phosphorylation of calnexin was relevant to calnexin's ribosome association, heat-inactivated EsRM were either phosphorylated in the absence or in the presence of CK2 or ERK-1 or both enzymes together. Phosphorylated EsRM were then incubated with purified ribosomes, the reconstituted rough membranes were lysed in 1% CHAPS and the ribosome-associated proteins were purified as described in the Materials and methods. As a control, EsRM membranes were used. Figure 7 shows that when CK2 and ERK-1 are exogenously added to heat-inactivated stripped RM to which purified ribosomes are then added, calnexin is found in the ribosomal pellet. Furthermore, the amount of calnexin that is ribosome-associated is comparable to the amount observed in membranes which did not receive heat treatment (Figure 7, compare lanes 1 and 4). In contrast, heat-inactivated EsRM or membranes phosphorylated by CK2 alone revealed a markedly lower association of calnexin with ribosomes (Figure 7, lanes 2 and 3). Moreover, blocking with 10 μg of a peptide containing the PDK site (Ser563), corresponding to the C-terminal 19 amino acids of calnexin abolished the effect of ERK-1 (C4 peptide; Figure 7, lane 6). To test the signaling pathway involved in the phosphorylation of calnexin by ERK-1, we transfected a dominant negative (DN) form of MEK-1 (DN-MEK-1) into Rat-2 cells and carried out an *in vitro* kinase assay after immunoprecipitation by anti-ERK-1 or anti-CNX-C4 antibodies using MBP as substrate (Figure 8A). In Rat-2 cells, the kinase activity associated with calnexin was stimulated 8.7-fold upon FBS treatment while total ERK-1 activity was stimulated 3.7-fold (Figure 8A, open bars). In DN-MEK-1 cells, ERK-1 activity was not enhanced by FBS treatment and little increase in calnexin-associated kinase activity

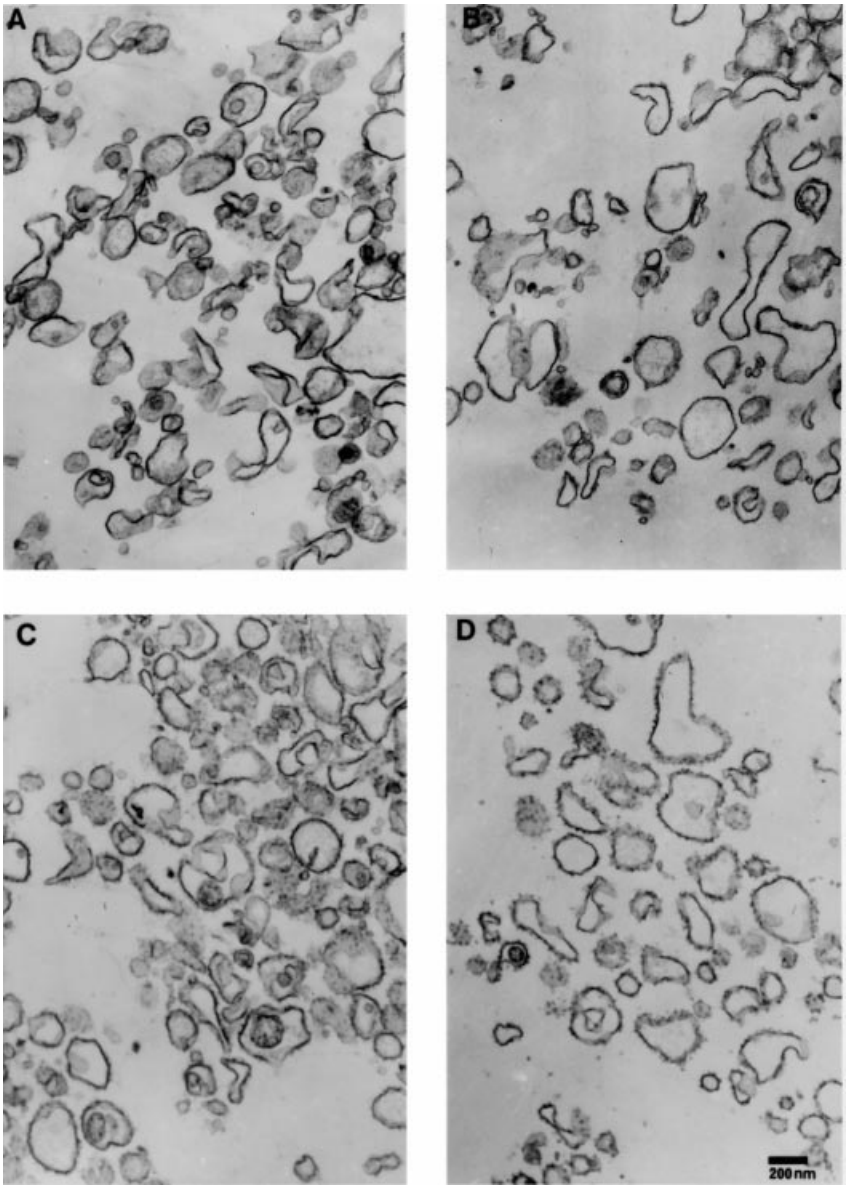

Fig. 5. Ribosome removal from canine pancreatic RM. Canine pancreatic RM were isolated as described in the Materials and methods and treated with (**A**) 750 mM KCl + 1 mM puromycin (K750), (**B**) 2 mM EDTA (EsRM), (**C**) 25 mM KCl + 1 mM puromycin (K25) or (**D**) untreated (control). Membranes were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 0.05% calcium chloride pH 7.4, filtered onto 0.45 μM pore size filters and processed for electron microscopy as described in the Materials and methods.

Table I. Quantitation of membrane-bound ribosomal particles at the surface of ER microsomes

	Ribosomes/ vesicle	No. of vesicles analyzed
750 mM KCl + puromycin	2 ± 2	66
EDTA	3 ± 1	92
25 mM KCl + puromycin	37 ± 5	110
Control	43 ± 3	124

Canine pancreatic RM (control) were stripped as described by Adelman *et al*. (1973). Membranes were then analyzed by electron microscopy as described by Baudhuin *et al*. (1967). The number of ribosomal particles per vesicle was determined by counting on electron micrograph pictures.

was found (Figure 8A, filled bars). These results suggest that calnexin phosphorylation occurs through the MEK-1/ ERK-1 pathway.

The effect of ERK-1 activation on calnexin–ribosome interactions *in vivo* was evaluated. Treatment for 5, 15, 30 or 60 min with 10% FBS of serum-starved Rat-2 and DN-MEK-1 Rat-2 cells was followed by determination of ribosome association of calnexin by their co-sedimentation. In mock-transfected cells, FBS treatment induced a 4-fold relative increase in the association of calnexin with ribosomes (Figure 8B, open squares), while in DN-MEK-1 cells little change was observed (Figure 8B, filled squares). Since ERK-1 enhances protein synthesis (through eIF-4E phosphorylation by Mnk-1 (Hershey *et al*., 1996; Fukunaga and Hunter, 1997; Waskiewicz *et al*., 1997), this may result in an increased number of membrane-bound ribosomes. This was confirmed by measuring the

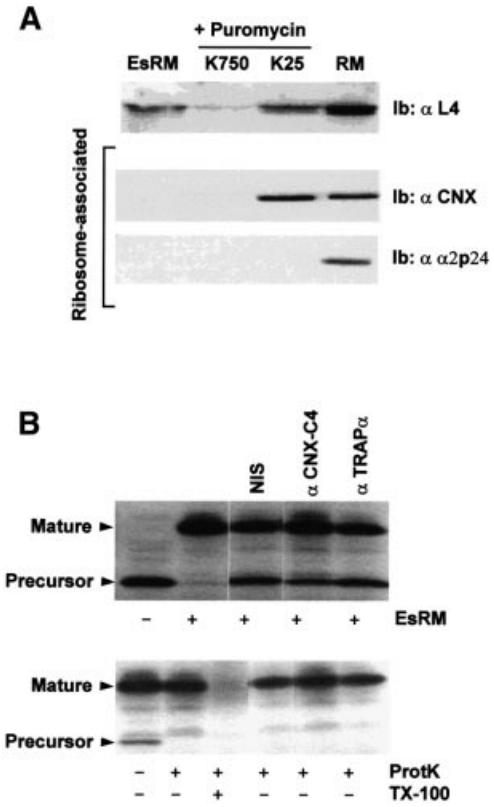

Fig. 6. Characterization of calnexin association with ribosomes. (**A**) Canine pancreatic rough microsomes (RM) were stripped with EDTA (EsRM) or 1 mM puromycin + 750 mM KCl (K750) or 1 mM puromycin + 25 mM KCl (K25) and were immunoblotted with anti-ribosomal L4 antibodies. Ribosome-associated proteins from membranes which had received the above treatments were purified and analyzed by immunoblotting with anti-CNX-C4 or anti-α2p24 antibodies and visualized by enhanced chemiluminescence. (**B**) *In vitro* co-translational translocation of alpha-mating factor was performed using the rabbit reticulocyte lysate system containing canine pancreatic EsRM which had been pre-incubated in the presence of non-immune serum (NIS), anti-CNX-C4 antibody or anti-TRAPα antibodies (upper panel). The translocated products were analyzed for proteinase K (ProtK) resistance (bottom panel) as detailed in the Materials and methods. The ^{35}S-radiolabeled products were processed for fluorography according to the manufacturer's instructions.

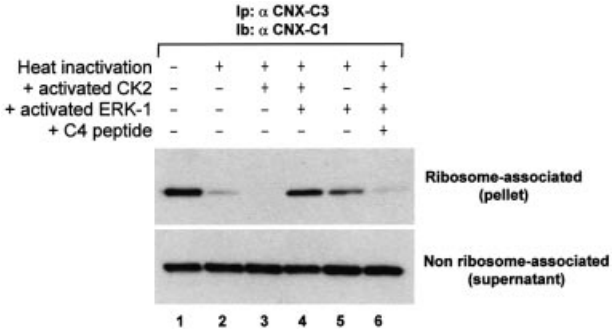

Fig. 7. Phosphorylation regulates calnexin binding to ribosomes *in vitro*. Association of calnexin with ribosomes *in vitro* was carried out as described in the Materials and methods, on EsRM (lane 1) or heat-inactivated EsRM (lane 2) phosphorylated with CK2 (lane 3), or ERK-1 + CK2 (lane 4), or ERK-1 (lane 5) or ERK-1 + CK2 in the presence of 10 μg of C4 peptide (lane 6). After the reaction, samples were separated into ribosomal pellet and supernatant by centrifugation through a 1.5 M sucrose cushion. The pellets and supernatants were denatured and immunoprecipitated with anti-CNX-C3. Immunoprecipitates were resolved by 8% SDS–PAGE, transferred onto PVDF, immunoblotted with anti-CNX-C1 antibodies and visualized by enhanced chemiluminescence.

extent of Sec61–ribosome association (Figure 8B, circles). Nevertheless, at 5–10 min after FBS treatment, significantly more calnexin than Sec61 was ribosome-associated in mock as compared with cells expressing DN-MEK-1. These results indicate that upon FBS treatment the MAPK pathway enhanced calnexin–ribosome association.

Discussion

Regulation by phosphorylation of calnexin association with membrane-bound ribosomes

Using lysed Rat-2 cells and an assay that evaluated the co-sedimentation of ribosomes with ER membrane protein, increased calnexin association with ribosomes was observed after serum stimulation. This increased association was promoted by ERK-1 activation and inhibited by expression of a DN-MEK-1.

The characteristics of the calnexin kinase activity relevant to calnexin–ribosome association were: (i) inhibition by olomoucine; (ii) phosphorylation on a site similar to that of MEK-1 activated ERK-1; (iii) phosphorylation of MBP as well as a GST fusion protein containing the cytosolic domain of calnexin; (iv) apparent molecular mass of 44 kDa; and (v) ability to re-fold in gels after denaturation. Based on these observations, the calnexin kinase was proposed as ERK-1.

This conclusion was supported by the following considerations. First, Ser563 in the calnexin cytosolic domain is in a consensus sequence for a proline-directed kinase such as ERK-1 and a proportion of calnexin is phosphorylated at this site (Wong *et al*., 1998). Secondly, peptide-specific antibodies to the extreme C-terminus of calnexin co-precipitated ERK-1 activity as assessed by an in-gel kinase assay and by sequential immunoprecipitation. The co-immunoprecipitation of calnexin with ERK-1 activity was dependent on serum treatment of starved Rat-2 cells. Approximately 7% of total ERK-1 activity was co-precipitated with anti-CNX-C4 antibody, while this activity was abolished in cells expressing a DN mutant MEK-1. Antibodies to the juxtamembrane region of calnexin (anti-CNX-C3) were unable to co-precipitate ERK-1 activity although calnexin itself was efficiently immunoprecipitated. This suggests that ERK-1 may associate directly or indirectly (via another protein) with the cytosolic domain of calnexin at a region recognized by the anti-CNX-C3 antibody. Consensus docking sites for ERK/MAPK binding to substrates show a conserved FXFP motif and/or a basic region ending in L/I-X-L/I (Jacobs *et al*., 1999). Although the region of calnexin containing the peptide to which the C3 antibody was raised is basic, the exact motifs used as ERK–MAPK docking sites were not found.

MEK-1 activated ERK-1-phosphorylated calnexin when added to heat-inactivated EsRM. This was not observed for p34^{cdc2}. Furthermore, phosphopeptides sensitive to the PDK inhibitor olomoucine were identified as distinct to those sensitive to the CK2 inhibitor heparin. Taken together, these results indicate that ERK-1 is a physiologically relevant kinase which phosphorylates calnexin at Ser563.

By analytical fractionation of total rat liver membranes,

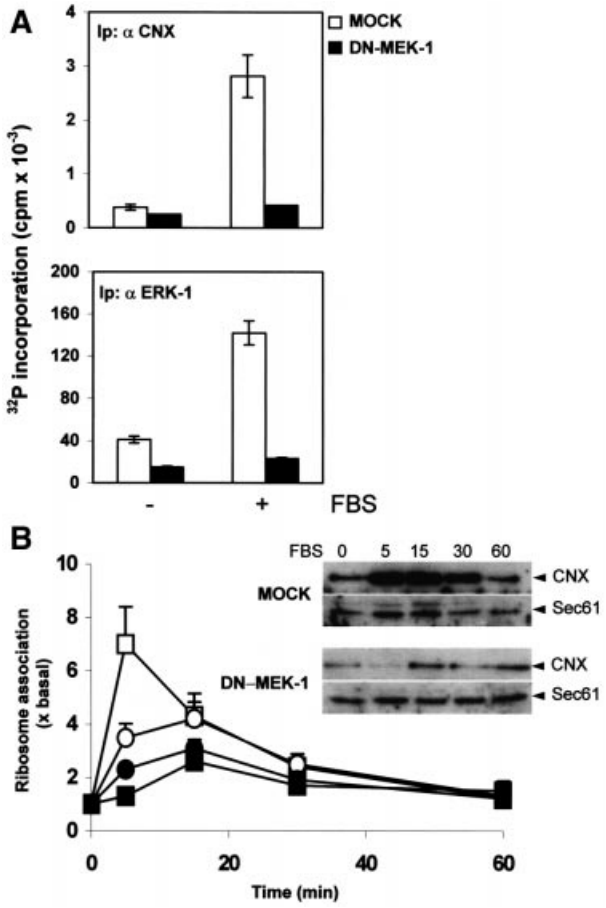

Fig. 8. MEK-1-dependent association of calnexin with ribosomes *in vivo*. (**A**) Rat-2 cells transfected with an empty pCDNA3 (MOCK) or with the same plasmid containing a dominant-negative MEK-1 (DN-MEK-1) were serum-starved for 20 h and stimulated for 10 min with DMEM containing 10% FBS (+FBS). Calnexin and ERK-1 were then immunoprecipitated with anti-CNX-C4 (upper panel) and anti-ERK-1 (lower panel) antibodies. An *in vitro* kinase assay was carried out with MBP as substrate and the immunoprecipitates as the kinase source. These experiments were repeated three times (mean ± SD). (**B**) *In vivo* association of calnexin and Sec61 with ribosomes after addition of 10% FBS for 5, 15, 30 and 60 min. Experiments were carried out four times in Rat-2 cells transfected with pCDNA3 (MOCK, open symbols) or the same plasmid containing DN-MEK-1 (filled symbols). Squares represent the amount of calnexin ribosome-associated and circles the amount of Sec61 which is associated with ribosomes (mean ± SD). Inset is the immunoblot showing the amount of calnexin and Sec61 that is ribosome-associated at the indicated times after FBS stimulation. Visualization was carried out by enhanced chemiluminescence.

membrane-associated ERK-1 was identified in a compartment of median density of 1.207. This corresponded with that of the rough ER marker Ribophorin II. In addition, ERK-1 was detected on highly purified canine RM by immunoblotting. Taken together, these results identify ERK-1 as one of the ER-associated kinases responsible for phosphorylating calnexin both *in vivo* and in purified membranes.

Synergy of CK2 and ERK-1

The protein kinase CK2 holoenzyme has been previously identified on ER membranes (Ou *et al*., 1992). The CK2 holoenzyme (α2β2) or the catalytic subunit alone were capable of phosphorylating the GST fusion protein coupled to the cytosolic domain of calnexin (GST–CCD). However, only the holoenzyme could bind to the GST–CCD.

Phosphorylation of heat-inactivated EsRM by both activated CK2 and activated ERK-1 led to an enhanced association of calnexin to ribosomes subsequently added. The protein kinase CK2α2β2 alone phosphorylated calnexin but diminished basal calnexin–ribosome association. In contrast, activated ERK-1 alone increased this association. Taken together these studies are consistent with a model whereby a coordinated phosphorylation of calnexin by CK2 and ERK-1 leads to ribosome association of calnexin (Figure 9).

Calnexin is associated with ribosomes but not with the translocon

For most studies with cells or purified membranes, CHAPS was used as the detergent for showing calnexin–ribosome association. Screening of various detergents for optimal association of membrane proteins with ribosomes previously led to the suggestion that digitonin but not CHAPS was the most efficient (Görlich *et al*., 1992). Indeed, in probing for the basal level of calnexin–ribosome association in canine RM, we confirmed that a greater proportion of calnexin, Sec61 or TRAPα was associated with ribosomes when using digitonin. However, digitonin associates primarily with cholesterol-enriched membranes, and ER membranes are partially resistant to this detergent (Amar-Costesec *et al*., 1974a). Hence we considered CHAPS as a detergent perhaps more relevant for assessing ribosome–membrane protein interactions. Indeed, Görlich *et al*. (1992) have shown that CHAPS-resistant associations probably represent tighter interactions. Using CHAPS as the detergent, as compared with digitonin, lower but detectable amounts of calnexin and Sec61 were ribosome-associated under basal conditions in canine RM. That this represented a bona fide interaction of calnexin with ribosomes was confirmed by protease protection assays on intact RM. Indeed calnexin was partially protected from trypsin while ribosome removal led to the proteolysis of the entire calnexin cytosolic domain. Ribosome re-binding to EsRM also led to partial resistance of the calnexin cytosolic domain to trypsin digestion. Using CHAPS as detergent instead of digitonin, very little TRAPα was ribosome-associated. As predicted by this result, protection from trypsin hydrolysis by ribosomes was weaker for TRAPα than for calnexin (Figure 4).

The p24 family member α2p24 was concluded to be ribosome-associated with either Triton X-100, digitonin or CHAPS as detergent. However, this interaction was dependent on the presence of newly synthesized proteins since it was abrogated by puromycin treatment. The membrane protein α2p24 was originally identified as a protein which co-purified with calnexin from canine RM (Wada *et al*., 1991). It is known to be partially ER located (Dominguez *et al*., 1998), and in the ER is terminally glycosylated and therefore Golgi-derived (Lavoie *et al*., 1999). The significance of this association of α2p24 with ribosomes remains speculative, but could be distinguished from calnexin binding to ribosomes since the former was dependent on nascent chains. Neither calnexin nor TRAPα (Migliaccio *et al*., 1992) were causally linked to ribosome engagement with the translocon, since saturating concentrations of antibodies to the cytosolic domains of these

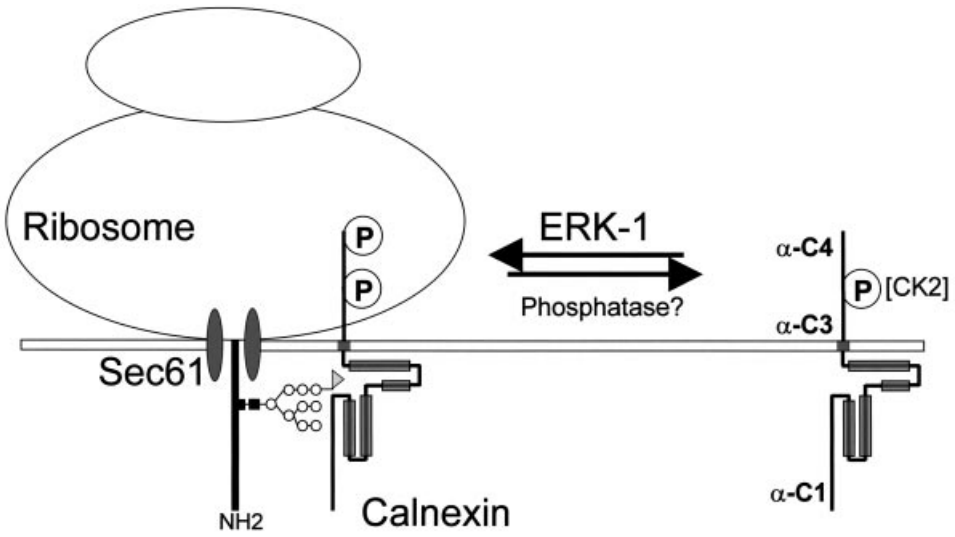

Fig. 9. Summary of calnexin association with ribosomes after phosphorylation. The regions against which antibodies anti-CNX-C1, anti-CNX-C3 and anti-CNX-C4 were raised are indicated (α-C1, α-C3, α-C4). Release from ribosomes is presumably achieved by a phosphatase which remains unidentified. Interestingly, the serine phosphatase inhibitor okadaic acid increased calnexin phosphorylation (Schué *et al*., 1994) and ribosome-associated Ser/Thr phosphatases have also been identified as relevant candidates (Hirano *et al*., 1995; Beullens *et al*., 1996). Grey boxes in the calnexin luminal domain indicate calreticulin-like domains. In the carbohydrate structure, circles indicate mannose residues, and the triangle a glucose residue.

proteins had no effect on the translocation efficiency of alpha-mating factor in canine stripped RM. Hence the association of the cytosolic domain of calnexin with ribosomes is predicted to be external to the Sec61-binding site but partially protected as illustrated in Figures 4 and 9.

Function of regulated calnexin–ribosome association

Conditions that are expected to enhance calnexin abundance near the translocon (i.e. overexpression of calnexin by transient transfection) have been shown to increase the productive folding of co-transfected subunits of the nicotinic acetylcholine receptor (Chang *et al*., 1997) or tyrosinase (Toyofuku *et al*., 1999). The CK2+ERK-1-mediated enhancement of calnexin association with ribosomes provides a regulatory mechanism whereby stimulation of protein synthesis via the MAPK pathway would be rapidly accompanied by an increase in glycoprotein folding capacity near the translocon. Hence, this would be an opposite response to that predicted for the unfolded protein response pathway wherein distinct membrane proteins (IRE1, PEK/PERK) mediate a decrease in protein synthesis near the translocon (Kaufman, 1999).

Materials and methods

Reagents and antibodies

Rabbit anti-sera were raised against three synthetic peptides corresponding to amino acids 30–48 (anti-CNX-C1), 487–505 (anti-CNX-C3) and 555–573 (anti-CNX-C4) of canine calnexin (Ou *et al*., 1995). Anti-α2p24 antibodies were as described previously (Dominguez *et al*., 1998). Anti-Sec61 and L4 antibodies were generously provided by Dr C.Nicchitta (Duke University Medical Center). Anti-TRAPα antiserum was raised against the C-terminal domain of TRAPα and kindly provided by Dr G.Kreibich (NYU Medical Center). Anti-BiP antibodies were a kind gift from Dr L.Hendershot (St Jude Children's Research Hospital). Anti-ERK-1 antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), MBP was from Sigma (St Louis, MO). [γ-^{32}P]ATP (sa: 3000 Ci/mmol) and [^{32}P]orthophosphate (sa: >8000 Ci/mmol) were from Mandel-NEN Life Sciences, Tran^{35}S-label, (sa: 1276 Ci/mmol) and [^{125}I]goat anti-rabbit IgG (sa: 15 μCi/μg) were from ICN Biomedicals (Mississauga, ON). Recombinant GST–ERK-1 and GST–MEK-1 were prepared as described previously (Nantel *et al*., 1998). The canine CCD (89 amino acids) was inserted in pGEX-4T1 (Pharmacia Biotech, Uppsala, Sweden) and the resulting fusion protein was purified to homogeneity using glutathione–Sepharose beads. The protein kinase CK2 was either from Boehringer Mannheim (Mannheim, Germany; results presented in Figure 7), or recombinant CK2α or α2β2 were independently expressed in Sf9 cells and purified to homogeneity (Filhol *et al*., 1992; results presented in Figure 1B and C). Olomoucine was purchased from Calbiochem (San Diego, CA), and heparin was from ICN Biomedicals. Enhanced chemiluminescence reagents were purchased from Mandel-NEN Life Sciences. All other reagents were of the highest grade commercially available.

Subcellular fractionation

Translocation competent canine pancreatic RM were prepared and stripped as described previously (Walter and Blobel, 1983). Stripping was performed with 2 mM EDTA or 1 mM puromycin and 750 mM KCl (Adelman *et al*., 1973). Microsomes (10–12 mg/ml) were stored in aliquots frozen at –80°C in 20 mM HEPES–NaOH, pH 7.4, 50% glycerol, 1 mM dithiothreitol (DTT). The MLP fraction from rat liver homogenate was separated on linear sucrose gradients (0.5–2.3 M) as described previously (Amar-Costesec *et al*., 1974b). ER-associated ribosomes were purified from cells as described previously (Loftus *et al*., 1997).

Cell culture and transfection

Madin–Darby canine kidney (MDCK) cells were cultured and *in vivo* [^{32}P]orthophosphate-labeled as described previously (Wong *et al*., 1998). Rat-2 fibroblasts were passaged every 3 days at 3×10^3 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS at 37°C, 5% CO_2. These cells were transfected with an empty pCDNA3 (mock) vector or with the same vector containing the dominant-negative human MEK-1 (DN-MEK-1) constructed as described previously (Yank and Templeton, 1994). Polyclonal cultures were selected in DMEM containing 10% FBS and 400 μg/ml G-418. Prior to stimulation for 10 min in DMEM containing 10% FBS, Rat-2 MOCK or Rat-2 DN-MEK-1 cells were starved for 20 h in DMEM.

Immunoprecipitations

Cells or microsomal membranes were solubilized for 30 min on ice in 30 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 1 μg/ml leupeptin, 5 KIU/ml aprotinin containing 1% CHAPS (buffer A). The lysate was centrifuged at 100 000 r.p.m. in a Beckman TLA-100.2 rotor for 20 min at 4°C, and the supernatant was incubated overnight at 4°C with the indicated antibody. Protein A–Sepharose beads were added for 1 h at 4°C with rotation then pelleted and washed four times with buffer A. Beads were

either directly resuspended in Laemmli sample buffer or subjected to sequential immunoprecipitations. For this, the beads were resuspended in 50 µl of 30 mM Tris–HCl pH 8.0 containing 1% SDS and heated for 10 min at 95°C, followed by centrifugation for 10 min at top speed in a Brinkmann microfuge. The supernatant was diluted 15× with 30 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM PMSF, 1% Triton X-100 and subjected to immunoprecipitation with the indicated antibody overnight at 4°C. Protein A–Sepharose beads were added for 1 h at 4°C with rotation, following by washing and resuspension in Laemmli sample buffer.

In vitro kinase assays

In vitro phosphorylation of canine pancreatic RM was carried out as previously described (Wada *et al*., 1991). All *in vitro* phosphorylation reactions were performed at 30°C, with the exception of the experiment shown in Figure 1A which was carried out on ice. Phosphorylation of GST–CCD by CK2α or α2β2 was performed using either casein or GST–CCD as substrates as described previously (Filhol *et al*., 1992). The *in vitro* kinase assay with $p34^{cdc2}$ (Boehringer Mannheim) was performed according to the manufacturer's instructions.

In-gel kinase assays

Following immunoprecipitation with either anti-ERK-1 or anti-CNX-C3 or -C4 antibodies, proteins were resolved by SDS–PAGE using 10% resolving gel containing 0.5 mg/ml MBP. Following electrophoresis the gel was washed twice (30 min each) with 30 mM Tris–HCl, 20% isopropanol, pH 7.5, then washed twice (30 min each) in 30 mM Tris–HCl, 2 mM DTT, pH 7.5 and incubated for 45 min in the same buffer containing 6 M urea. The proteins were then subjected to renaturation by three washes of 45 min each in 30 mM Tris–HCl, 2 mM DTT, pH 7.5, containing 0.05% Tween-20 and respectively 3, 1.5 and 0.75 M urea, then washed for 2 h in 30 mM Tris–HCl, 2 mM DTT, pH 7.5, 0.05% Tween-20. The gel was then incubated for 30 min in the kinase reaction buffer containing 30 mM Tris–HCl, 2 mM DTT, 10 mM $MgCl_2$, 10 mM $MnCl_2$, pH 7.5. Phosphorylation was then carried out by incubation of the gel in the same buffer containing 10 µCi/ml [γ-^{32}P]ATP and 0.1 mM ATP for 45 min at room temperature. The gel was then washed extensively and fixed in 5% (v/v) trichloroacetic acid, 1% (w/v) Na-pyrophosphate. Radiolabeled bands were visualized using Kodak X-OMAT AR film.

In vitro co-translational translocation

The experiments were performed essentially as described by Hartmann *et al*. (1989) with the following modifications: 0.1 µg of alpha-mating factor mRNA was translated for 60 min at 30°C in a rabbit reticulocyte lysate system containing 4 equivalents of canine pancreatic stripped RM which had either received no pre-incubation or were pre-incubated with non-immune serum (4 mg/ml total IgG), anti-TRAPα (4 mg/ml total IgG) or anti-CNX-C4 (20 µg/ml affinity-purified on a C4-peptide column) antibodies for 60 min at 4°C. An aliquot of the translocated product was assayed for protease resistance by the addition of proteinase K (200 µg/ml final concentration) in the absence or in the presence of 0.1% Triton X-100 for 60 min on ice. The digestion was stopped by the addition of PMSF (1 mM final concentration), incubated on ice for 5 min followed by the addition of an equal volume of 2× concentrated SDS–PAGE Laemmli sample buffer and analysis by SDS–PAGE (5–15% resolving gel). The gel was processed for fluorography using Enhance (Mandel-NEN). Radiolabeled bands were visualized using Kodak X-OMAT AR film.

Analysis of ribosome-associated membrane proteins

The association of membrane proteins with ribosomes was assayed as described previously (Görlich *et al*., 1992; Kalies *et al*., 1994) except that five equivalents of canine pancreatic microsomes were solubilized in 75 µl of 50 mM Tris–HCl pH 7.6, 400 mM K acetate, 10 mM Mg acetate, 15% glycerol, 5 mM β-mercaptoethanol, protease inhibitors and 1.5% digitonin, or 1% CHAPS or 1% Triton X-100. After incubation for 30 min on ice, the ribosomes were centrifuged through a 100 µl cushion of 1.5 M sucrose in the same buffer, containing only 0.1% of the corresponding detergent. Membrane proteins in the ribosomal pellet were analyzed by immunoblotting after SDS–PAGE. The trypsin protection assay was performed as described previously (Kalies *et al*., 1994).

Ribosome-association assay

Heat-inactivated EsRM were incubated without addition or in the presence of CK2 (0.5 µg, Boehringer Mannheim), or activated ERK-1 (immunoprecipitated from 2 mg cytosol) from epidermal growth factor (EGF)-treated rats as described by Di Guglielmo *et al*. (1994) or a combination of both. After 30 min at 30°C in the presence of 2 µCi [γ-^{32}P]ATP, the reaction was stopped by addition of ATP to a final concentration of 5 mM, then placed on ice and incubated with 50 µg of purified ribosomes for 30 min. Membranes were then solubilized in 1% CHAPS and ribosome-associated proteins purified as described above. Proteins from the supernatant or the pellet were solubilized in 30 mM Tris–HCl pH 7.5, 150 mM NaCl, protease inhibitors, 1% SDS and heated at 65°C for 10 min, then diluted 10-fold in the same buffer without SDS but containing 1% Triton X-100 (final concentration). Calnexin was then immunoprecipitated with anti-CNX-C3, and resolved by SDS–PAGE, followed by immunoblotting with anti-CNX-C1 antibody. Immunoreactive bands were visualized by enhanced chemiluminescence.

Phosphopeptide mapping

Phosphopeptide mapping was carried out (Boyle *et al*., 1991) after either *in vivo* (MDCK cells) or *in vitro* (EsRM) phosphorylation reactions. Phosphorylated calnexin was transferred onto nitrocellulose after separation by SDS–PAGE. Radiolabeled calnexin was detected by radioautography, the corresponding region of the nitrocellulose membrane was excised, and blocked in 0.5% PVP360 in 100 mM acetic acid for 30 min at 37°C. It was then washed extensively with distilled water and twice with 50 mM NH_4HCO_3, pH 7.8. The membrane-bound calnexin was subjected to TPCK-treated trypsin digestion in 50 mM NH_4HCO_3, pH 7.8. The tryptic peptides were lyophilized and subjected to oxidation by freshly prepared performic acid [99% formic acid, 30% H_2O_2; 9:1 (v/v)] for 1 h on ice. The tryptic peptides were lyophilized and washed extensively. The dried tryptic phosphopeptides were dissolved in pH 1.9 buffer [88% formic acid, glacial acetic acid, H_2O; 2.5:7.8:89.7 (v/v/v)]. The peptides were separated first by electrophoresis in pH 1.9 buffer for 30 min at 1 kV employing a Hunter thin-layer electrophoresis system (CBS Scientific, Del Mar, CA), followed by ascending chromatography in isobutyric acid, *n*-butanol, pyridine, glacial acetic acid, H_2O; 625:19:48:29:279 (v/v/v/v/v). Phosphopeptide maps were visualized either by radioautography with enhancing screens or with a Fuji phosphoimager screen. The efficiency of recovery from each step was monitored by Cerenkov counting.

Electron microscopy

Membranes were diluted to reach 0.25 M sucrose final concentration. Samples were fixed with 2.5% gluteraldehyde in 0.1 M cacodylate buffer with 0.05% calcium chloride pH 7.4 for 2.5 h, and filtered onto 0.45 µM pore-size filters (Baudhuin *et al*., 1967), and washed with 0.1 M cacodylate buffer overnight. Samples were treated with a 1:1 solution of 3% potassium ferrocyanide and 2% osmium tetroxide for 1 h on ice (Karnovsky, 1971), and then washed with 0.1 M maleate buffer, pH 5.7, and stained with 5% uranyl acetate for 2 h on ice. After two washes with 0.1 M maleate buffer and one with 0.1 M cacodylate buffer, samples were dehydrated with a graded series of ethanol washes followed by 100% propylene oxide and then placed in a 1:3 mixture of Epon and propylene oxide for 1 h. This was followed by a 1:1 mixture for 1 h and then 100% Epon for 1 h. Samples were then placed in a 60°C oven for polymerization. Thin sections were cut, stained for 2 min in lead citrate and 5 min in uranyl acetate, and micrographs were taken at 37 500 magnification on a JOEL JEM-2000FX electron microscope (Philipps 400) operating at 80.0 kV; prints were magnified a further 1.8×.

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