Conserved in vivo Phosphorylation of Calnexin at Casein Kinase II Sites as Well as a Protein Kinase C/Proline-directed Kinase Site

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Calnexin is a lectin-like chaperone of the endoplasmic reticulum (ER) that couples temporally and spatially N-linked oligosaccharide modifications with the productive folding of newly synthesized glycoproteins. Calnexin was originally identified as a major type I integral membrane protein substrate of kinase(s) associated with the ER. Casein kinase II (CK2) was subsequently identified as an ER-associated kinase responsible for the in vitro phosphorylation of calnexin in microsomes (Ou, W-J., Thomas, D. Y., Bell, A. W., and Bergeron, J. J. M. (1992) J. Biol. Chem. 267, 23789¿23796). We now report on the in vivo sites of calnexin phosphorylation. After 32PO4 labeling of HepG2 and Madin-Darby canine kidney cells, immunoprecipitated calnexin was phosphorylated exclusively on serine residues. Using nonradio-labeled cells, we subjected calnexin immunoprecipitates to in gel tryptic digestion followed by nanoelectrospray mass spectrometry employing selective scans specific for detection of phosphorylated fragments. Mass analyses identified three phosphorylated sites in calnexin from either HepG2 or Madin-Darby canine kidney cells. The three sites were localized to the more carboxyl-terminal half of the cytosolic domain: S524DAE (CK2 motif), S544QEE (CK2 motif), and S563PR. We conclude that CK2 is a kinase that phosphorylates calnexin in vivo as well as in microsomes in vitro. Another yet to be identified kinase (protein kinase C and/or proline-directed kinase) is directed toward the most COOH-terminal serine residue. Elucidation of the signaling cascade responsible for calnexin phosphorylation at these sites in vivo may define a novel regulatory function for calnexin in cargo folding and transport to the ER exit sites.

Calnexin was originally identified and purified as a constituent of a complex of four co-isolated integral membrane proteins, two of which were phosphorylated in microsomes by ER-associated kinase(s) (1). This phosphorylation was exclusively on serine residues and by controlled protease digestion found to be cytosolically oriented (1, 2). As a consequence of the cDNA cloning of this phosphoprotein, it was predicted and subsequently confirmed to be a type I integral membrane protein with extensive sequence similarity in its luminal domain to the ER luminal resident protein, calreticulin (1, 2). Calnexin and then calreticulin were found to be novel molecular chaperones of the ER. These chaperones act as lectins to couple oligosaccharide modifications to newly synthesized N-linked glycoproteins with productive glycoprotein folding. The lectin specificity of these chaperones has been identified as the recognition of high mannosyl oligosaccharides terminating in monoglucosyl residues linked α1–3 (3–13).

Purification of the ER-associated kinase that phosphorylated calnexin in microsomes led to the identification of CK2 (14). The properties of this kinase were consistent with the conditions that originally revealed calnexin phosphorylation (1, 14). Furthermore, purified CK2 has been found to phosphorylate calnexin on putative CK2 sites found within the cytosolic domain of calnexin (14, 15).

Calnexin is phosphorylated in vivo (16–18). Phosphorylated calnexin has been shown to associate with the null Hong Kong mutant of α1-antitrypsin, coinciding with retention of this misfolded glycoprotein within the lumen of the ER (16). Phosphorylated calnexin was also found in association with newly synthesized major histocompatibility complex class I allotypes, which egressed from the ER at slow rates. Those allotypes transported to the Golgi apparatus at more rapid rates were associated preferentially with nonphosphorylated calnexin (17). Prolonged association of newly synthesized major histocompatibility complex class I heavy chains with calnexin was found in a B lymphoblastoid cell line transfected with HLA-B701 after incubation with the phosphatase inhibitor cantharidin or okadaic acid (19). Furthermore, when human synovial epithelial (McCoy) cells were treated with okadaic acid, the major cellular protein whose phosphorylation was shown to increase (based on two-dimensional gels followed by protein microsequencing) was calnexin (18). Remarkably, calnexin phosphorylation also increased 3-fold when McCoy cells were treated with Clostridium difficile cytotoxin B (18), a protein that glucosylates Rho proteins of the Ras superfamily (20).

Although some progress has been made on the kinases and sites of phosphorylation of calnexin in vitro phosphorylation of intact microsomes (14, 15) little is known of the sites of calnexin phosphorylation in vivo. Here we report on their identification in two mammalian cell lines, HepG2 cells (human) and Madin-Darby canine kidney MDCK cells. Both cell lines lose; MS/MS, tandem mass spectrometry; CK2, casein kinase II; MDCK, Madin-Darby canine kidney; PKC, protein kinase C; PDK, proline-directed kinase; PVDF, polyvinylidene difluoride.
revealed phosphorylation of the cytosolic domain of calnexin exclusively on serine residues within CK2 motifs as well as a protein kinase C (PKC) and/or proline-directed kinase (PDK) motif.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit antibodies raised against a synthetic peptide, corresponding to amino acid residues 487–505 of canine calnexin, described previously were used (3). [32P]Orthophosphoric acid (specific activity > 8000 Ci/mmol) was purchased from NEN Life Science Products. Protein A-Sepharose beads were from Amersham Pharmacia Biotech. Pyridine, phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and HEPES were from Sigma. Sequencing grade bovine trypsin was from Boehringer Mannheim. TLC microcrystalline plates (0.1-mm thickness) were from EM Science (Gibbstown, NJ). Kodak XAR-5 OMAT film was purchased from Picker International Canada Inc. (Montreal, Quebec). Reagents for SDS-PAGE and protein determination were from Bio-Rad. All other reagents were from Sigma, Anachemia Canada Inc. (Lachine, Quebec), or Boehringer Mannheim.

**Media and Cell Lines**—Dulbecco’s modified Eagle’s medium, phosphate-deficient Dulbecco’s modified Eagle’s medium, dialyzed FBS, penicillin, and streptomycin were purchased from Life Technologies, Inc. FBS was obtained from HyClone Laboratories, Inc. (Logan, UT). Both human hepatoma (HepG2) cells and MDCK cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) FBS, 500 units/ml penicillin, and 500 μg/ml of streptomycin. Cells were maintained in a 37 °C incubator with 5% atmospheric CO2 and were used when they were 80% confluent.

In vivo [32P]P4 Labeling of Cultured Cells and Immunoprecipitation—Cells were radiolabeled as described previously (16, 17) with the following modifications. Briefly, cells were washed in phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 1% dialyzed FBS followed by incubation in the same medium for 1 h at 37 °C. Cells were then labeled by the addition of 2 μCi/ml [32P]orthophosphate for 3 h. At the end of labeling, the cells were lysed as described previously (3). Briefly, cells were washed twice with ice-cold phosphate-buffered saline (20 mM NaPO4, pH 7.5, 150 mM NaCl) and once with ice-cold HEPES-buffered saline (50 mM HEPES, pH 7.6, 200 mM NaCl) before harvesting. Cells were then lysed in 2% CHAPS/HEPES-buffered saline lysis buffer (2% (w/v) CHAPS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each leupeptin and aprotinin, 10 mM NaF, 10 mM NaPPi, 0.4 mM NaVO4, and 5 mM NaMoO4) for 30 min on ice. The same procedures were used to immunoprecipitate calnexin from both [32P]labeled and cold-stable, [32P]-labeled cellular extracts as described previously (3). Immuneplexed calnexin was isolated by SDS-PAGE. The gel was dried for 2 h at 80 °C under vacuum, or the proteins were transferred onto PVDF membrane (1). Radioactive bands were visualized by radioautography at room temperature.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described (21). Briefly, in vitro phosphorylated calnexin was resolved by SDS-PAGE and electroblotted onto a PVDF membrane. [32P]-Labeled calnexin was detected by radioautography, and the corresponding PVDF bands were excised. The membrane containing phosphorylated calnexin was washed extensively with distilled water and subjected to acid hydrolysis, immersed in 6 N HCl at 110 °C for 60 min. The hydrolysate was transferred to a microcentrifuge tube, lyophilized, and dissolved in pH 1.9 buffer (88% formic acid, glacial acetic acid, H2O; 2.5:7.8:89.7 (v/v/v)). Phosphoamino acid analysis was by two-dimensional electrophoresis on TLC plates in the presence of phosphoamino acid standards; phosphoserine, phosphothreonine, and phosphotyrosine. First dimension electrophoresis was carried out in pH 1.9 buffer for 20 min at 1.3 kV employing a Hunter thin layer electrophoresis system (C.B.S. Scientific, Del Mar, CA). Second dimension electrophoresis was carried out in pH 3.5 buffer (pyridine, glacial acetic acid, H2O; 0.5:5.9:94.5 (v/v/v)) at 1.5 kV for 20 min. The standards were visualized by spraying a 0.25% (w/v) ninhydrin acetone solution followed by incubation at 65 °C for 10 min. The radiolabeled amino acids were detected by radioautography with an enhancing screen at 70 °C. Recovery from each step was monitored by Cerenkov counting.

**In Gel Detection and Mass Spectrometry** (DE-MALDI MS and nanoESI MS)—Calnexin immunoprecipitated from nonradiolabeled HepG2 and MDCK cell lysates were resolved by SDS-PAGE, visualized by Coomassie Blue staining (stain was 0.2% (w/v) Coomassie Brilliant Blue R250 in 50% (v/v) methanol in water containing 2% (v/v) acetic acid; destain was 50% (v/v) methanol in water containing 2% (v/v) acetic acid); and the corresponding gel slice was excised. Coomassie stain was removed by extraction (twice) with 50% (v/v) acetonitrile/H2O followed by two cycles each of extracting with acetonitrile and swelling with 100 mM NH4HCO3. Calnexin was in gel reduced and alkylated with 15 mM dithiothreitol and 1.3 mM iodoacetamide and then in gel digested with 15 μg/ml bovine trypsin in the presence of 5 mM CaCl2 as described (22). Tryptic peptides were first extracted with acetonitrile, followed by two cycles each of swelling with 100 mM NH4HCO3 and extracting with 50% (v/v) acetonitrile/H2O and then two cycles each of swelling with 5% formic acid and extracting with acetonitrile. The efficiency of the extraction of calnexin tryptic phosphorylated fragments from gel pieces was evaluated with radiolabeled calnexin and Cerenkov counting. Greater than 70% of the radioactivity was recovered and extracted (data not shown). For DE-MALDI-ToF MS, dried peptide extracts were redissolved in 5% formic acid containing 5% methanol (10 μl) (22). An aliquot (0.4 μl) was spotted onto a stainless steel target precoated with α-cyano-4-hydroxycinnamic acid. The target was allowed to air dry before being washed with an aqueous solution containing 1% trifluoroacetic acid. Excess wash solution was blown off the target using compressed air. The target was loaded into the mass spectrometer for analysis of DE-MALDI-ToF
HepG2 and MDCK calnexins, respectively.

Peptides containing phosphate produce characteristic P0^+ ion fragment at m/z 79 (26, 27). Molecular ions for these peptides were recorded by scanning the first quadrupole between m/z 300 and 1400 with the third quadrupole set to transmit m/z 79 only (precursors m/z 79 scans). Argon gas was used in the collision cell (quadrupole 2) at a collision gas thickness of 250 units. Scanning the first and third quadrupoles with an offset of m/z 48 profiled phosphopeptides by loss of H_3PO_4 (molecular mass of 98) from doubly charged molecular ions (Constant Neutral Loss scans) (28). For MS/MS detection, Q1 was set to transmit a mass window of 2 Da for product ion scans. Product ion spectra were accumulated with a 0.2-Da mass step size. Dwell time was 1.0 ms, and collision energy was optimized to obtain the MS/MS spectra. Spectra interpretation was performed using BioMultiView (Sciex) software.

Computer Analysis—Candidate kinases for the cytosolic serine residues of calnexin were predicted based on consensus sequence motifs employing the PROSITE data base of EXPASY^2 (29). Predicted peptide m/z values were evaluated employing tools provided by ProteinProspector. Calnexin and calmegin sequence alignments were initially generated by BLASTp (30), FASTA (31), and MSA (32) and optimized manually.

**RESULTS**

**Calnexin In Vivo Phosphorylation on Serine Residues in HepG2 and MDCK Cells**—Calnexin was originally identified as a major substrate of ER-associated kinase(s) by *in vitro* phosphorylation of intact microsomes with [γ-^32^P]GTP as phosphate donor (1). In order to determine the *in vivo* sites of phosphorylation for calnexin, both HepG2 and MDCK cells were *in vivo* labeled with [32^P]orthophosphate followed by immunoprecipitation with anti-calnexin antibodies. SDS-PAGE-resolved immunoprecipitates were electrosprayed onto PVDVF membranes and similar levels of phosphorylated calnexin from both cell types were revealed by radioautography (Fig. 1A, lanes 1 and 2). The bands corresponding to phosphorylated calnexin were excised from the PVDVF membranes and subjected to phosphoamino acid analyses. Radioautograms of the two-dimensional TLC plates for both human and canine calnexins revealed only ^32^P-labeled serine that comigrated with the nonradiolabeled phosphoserine standard as detected by ninhydrin.
Calnexin in Vivo Phosphorylation Sites

Identity and in Vivo Sites of Phosphorylation—Analyses of Tryptic Digests of Calnexin by DE-MALDI-ToF mass analyses of tryptic fragments of calnexin. Positive ion mode DE-MALDI-ToF mass spectra are shown for tryptic digests of calnexin from MDCK (A) and HepG2 (B). The identities of the numbered peaks are shown in Table II. The x axis indicates the mass-to-charge (m/z) values, and the y axis indicates the relative ion intensity.

drin staining (left and right parts of Fig. 1B, respectively). The phosphorylated residues were not altered with longer (up to 24 h) in vivo radiolabeling (data not shown). Hence, calnexin was exclusively in vivo phosphorylated on serine residues in both cell types.

In isolated ER microsomes, in vitro phosphorylation by ER-associated kinase(s) is exclusively on the cytosolic domain of calnexin (1). The cystolic COOH-terminal domains of canine (MDCK) and human (HepG2) calnexins (1, 33) share 94% identity (Fig. 2). The cytosolic domain of canine calnexin contains six serines, and the cystolic domain of human calnexin contains five equivalent serines and a threonine. The five conserved cystolic serine residues are in primary sequence motifs that are predicted to be recognized by CK2 (34), PKC (35, 36), PDK (37, 38), or protein kinase A (39, 40) (Table I). To identify those that are predicted to be recognized by CK2 (34), PKC (35, 36), and PDK (37, 38), or protein kinase A (39, 40) (Table I). To identify those

Fig. 3. DE-MALDI-ToF mass analyses of tryptic fragments of calnexin. Positive ion mode DE-MALDI-ToF mass spectra are shown for tryptic digests of calnexin from MDCK (A) and HepG2 (B). The identities of the numbered peaks are shown in Table II. The x axis indicates the mass-to-charge (m/z) values, and the y axis indicates the relative ion intensity.

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- PO4, phosphate group; Met-ox, methionine sulfoxide; pyro-Q, pyro-glutamic acid.

staining, calnexin was in gel digested with trypsin as described under “Experimental Procedures.” DE-MALDI-ToF mass spectra for the tryptic peptides of calnexin from MDCK and HepG2 were collected (Fig. 3, A and B). Peptide masses not observed in the mock in gel digest (data not shown), were employed to confirm the identity of MDCK and HepG2 calnexins. Total coverage for calnexin from MDCK cells was 165 of 573 (29.8%) amino acid residues, and coverage from HepG2 cells was 171 of 572 (29.9%) amino acid residues. With respect to the cytosolic domain of calnexin, however, the degree of coverage was 58.4 and 33.7% from MDCK and HepG2 cells, respectively. This coverage by DE-MALDI-ToF included phosphopeptides observed as peak 1 and peak 7 in Fig. 3A for MDCK calnexin and as summarized in Table II. Coverage of the cytosolic domain was increased by nano-ESI MS and was greater than 90% by combination of both mass spectrometric techniques (see below). Poor coverage of the luminal domain was consistent with the generation of a protease-resistant core in the presence of Ca2+ (2), conditions employed during in gel trypsin digestion.

Analyses of Tryptic Digests of Calnexin by Nano-ESI MS: Identity and in Vivo Sites of Phosphorylation—The Q1 positive ion spectrum between m/z 800 and 1150 of HepG2 calnexin tryptic digests displays four ions (P1–P4) tentatively assigned as tryptic fragments of calnexin by comparison with mock in gel trypsin digest (Figs. 4, A and B). By comparison of the observed masses and the cDNA predicted calculated average masses (33), P1 (m/z 817) and P2 (m/z 868.3) correspond to the doubly positively charged states of the tryptic fragments 91ESKLPG-DKGLVLMSR–205 (calculated average mass, 1631.0 atomic mass units) and 91APVTGEVYFAFDSFDR–57 (calculated average mass, 1771.9 atomic mass units), respectively, of calnexin. The P3 (m/z 903) and P4 (m/z 910) ions correspond to the triply positively charged states of the tryptic fragments 151PTELNL-DQFHKDTPYTIMFGDPK–733 (calculated average mass,
2709.1 atomic mass units) and $^{191}$GIYEEKHAKRPDADLKTYFTDK$^{213}$ (calculated average mass, 2728.0 atomic mass units), respectively, of calnexin. The tentative assignment of the major non-trypsin tryptic peptide ion P2 ($m/z$ 886.3), the $+2$ charged tryptic fragments of $^{42}$APVPTGEVFADSFDR$^{57}$ (calculated average mass, 1771.9 atomic mass units); P3 ($m/z$ 903), the $+3$ charged tryptic fragment of $^{151}$TELNLQFDHKTPTYNMGFPDK$^{173}$ (calculated average mass, 2709.1 atomic mass units); and P4 ($m/z$ 910), the $+3$ charged tryptic fragment of $^{151}$GITYEEKHAKRPDADLKTYFTDK$^{173}$ (calculated average mass, 2728.0 atomic mass units) of HepG2 calnexin. Inset, positive ion MS/MS profile of $m/z$ 886.3 (P2), identifying the $y$ ion series corresponding to $(\ldots)(V)PT(GE)(V)\ldots$ (weak assignments identified by parentheses; see “Results”). The $x$ axis indicates the mass-to-charge ($m/z$) values, and the $y$ axis indicates the relative ion intensity.

Calnexin in Vivo Phosphorylation Sites

In this figure, we present a comprehensive analysis of calnexin phosphorylation sites in vivo. The identities of the labeled peaks are as follows: P1 ($m/z$ 817), the $+2$ charged tryptic fragment of $^{91}$ESKLPGD-KGLVLMSR$^{105}$ (calculated average mass, 1631.0 amu); P2 ($m/z$ 886.3), the $+2$ charged tryptic fragments of $^{42}$APVPTGEVFADSFDR$^{57}$ (calculated average mass, 1771.9 atomic mass units); P3 ($m/z$ 903), the $+3$ charged tryptic fragment of $^{151}$TELNLQFDHKTPTYNMGFPDK$^{173}$ (calculated average mass, 2709.1 atomic mass units); and P4 ($m/z$ 910), the $+3$ charged tryptic fragment of $^{151}$GITYEEKHAKRPDADLKTYFTDK$^{173}$ (calculated average mass, 2728.0 atomic mass units) of HepG2 calnexin. Inset, positive ion MS/MS profile of $m/z$ 886.3 (P2), identifying the $y$ ion series corresponding to $(\ldots)(V)PT(GE)(V)\ldots$ (weak assignments identified by parentheses; see “Results”). The $x$ axis indicates the mass-to-charge ($m/z$) values, and the $y$ axis indicates the relative ion intensity.

2709.1 atomic mass units) and $^{191}$GIYEEKHAKRPDADLKTYFTDK$^{213}$ (calculated average mass, 2728.0 atomic mass units), respectively, of calnexin. The tentative assignment of the major non-trypsin tryptic peptide ion P2 ($m/z$ 886.3) was confirmed by generation of sequence-specific sequence tags by MSMS. Prominent peptide fragment ions of 1120.6, 1306.4, 1407.6, and 1504.8 $m/z$ values correspond to $y$-ion series of singly positively charged ions, $y_9$ (calculated $m/z$ 1120.2), $y_{11}$ (calculated $m/z$ 1306.4), $y_{13}$ (calculated $m/z$ 1407.5), and $y_{14}$ (calculated $m/z$ 1504.6) for collision-induced fragmentation of the calnexin tryptic peptide $^{42}$APVPTGEVFADSFDR$^{57}$ (using ProteinProspector search tools). This identification is unambiguous (search parameters: mass tolerance of 0.5 Da for both parent and fragmented ions using ProteinProspector tools), and the partial sequence $(\ldots)(\text{Val}^{44})$-Pro-Thr-(Gly-Glu)-(Val$^{49})\ldots$ (weak assignments shown in parentheses) can be assigned from the MS/MS spectrum, taking into account potential weak signals for $y_8$ (calculated $m/z$ 1021.1), $y_{10}$ (calculated $m/z$ 1249.3) and $y_{14}$ (calculated $m/z$ 1603.7) (Fig. 4, inset).

Only two phosphopeptides were detected by DE-MALDI-ToF MS (Table II). Hence, we proceeded to characterize in greater detail the phosphorylated peptides in the desalted total tryptic digests of nonradiolabeled phosphocalnexin from both MDCK and HepG2 cells employing two selective techniques for identification of phosphorylated fragments: scans for precursors $m/z$ 79 in negative ion mode (26, 27) or scans for constant neutral loss (CNL) of $\text{H}_3\text{PO}_4$ ($m/z$ 49 for doubly charged peptides) in positive ion mode (28). Precursors $m/z$ 79 scans reveal

**Fig. 4.** Nano-ESI MS and MS/MS of tryptic fragments of calnexin. A and B, positive ion Q1 scans (800–1150 $m/z$) of calnexin and mock in gel tryptic digest, respectively. The identities of the labeled peaks are as follows: P1 ($m/z$ 817), the $+2$ charged tryptic fragment of $^{91}$ESKLPGD-KGLVLMSR$^{105}$ (calculated average mass, 1631.0 amu); P2 ($m/z$ 886.3), the $+2$ charged tryptic fragments of $^{42}$APVPTGEVFADSFDR$^{57}$ (calculated average mass, 1771.9 atomic mass units); P3 ($m/z$ 903), the $+3$ charged tryptic fragment of $^{151}$TELNLQFDHKTPTYNMGFPDK$^{173}$ (calculated average mass, 2709.1 atomic mass units); and P4 ($m/z$ 910), the $+3$ charged tryptic fragment of $^{151}$GIYEEKHAKRPDADLKTYFTDK$^{173}$ (calculated average mass, 2728.0 atomic mass units) of HepG2 calnexin. Inset, positive ion MS/MS profile of $m/z$ 886.3 (P2), identifying the $y$ ion series corresponding to $(\ldots)(\text{V})PT(GE)(V)\ldots$ (weak assignments identified by parentheses; see “Results”). The $x$ axis indicates the mass-to-charge ($m/z$) values, and the $y$ axis indicates the relative ion intensity.
precursor ions that fragment to produce a characteristic product ion of \( m/z 79 \) that corresponds to the phosphate anion, \( \text{PO}_3^{2-} \).

CNL scans reveal the masses of parent ions that lose the phosphate group, \( \text{H}_3\text{PO}_4 \) (molecular mass of 98), as a neutral fragment. A loss of \( m/z 49 \) would be observed from doubly charged precursor ions, \([\text{M}+2\text{H}]^{2+}\). Precursors \( m/z 79 \) scans of the tryptic peptides from MDCK calnexin were dominated by three major phosphorylated peptide ions (Fig. 5A). Two of the three \( m/z \) values (753.4 and 502.2) correspond to the doubly and triply negatively charged states of the phosphorylated fragment \( 555\text{AEEDEILRPSPR}^{566} \) (calculated average mass, 1509.5 atomic mass units) (A) and the multiply charged states of the clustered ion series (B) are indicated. The x axis indicates the mass-to-charge (\( m/z \)) values, and the y axis indicates the relative ion intensity.

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Precursor ions that fragment to produce a characteristic product ion of \( m/z 79 \) that corresponds to the phosphate anion, \( \text{PO}_3^{2-} \). CNL scans reveal the masses of parent ions that lose the phosphate group, \( \text{H}_3\text{PO}_4 \) (molecular mass of 98), as a neutral fragment. A loss of \( m/z 49 \) would be observed from doubly charged precursor ions, \([\text{M}+2\text{H}]^{2+}\).

Precursors \( m/z 79 \) scans of the tryptic peptides from MDCK calnexin were dominated by three major phosphorylated peptide ions (Fig. 5A). Two of the three \( m/z \) values (753.4 and 502.2) correspond to the doubly and triply negatively charged states of the phosphorylated tryptic fragment \( 555\text{AEEDEILRPSPR}^{566} \) (where pS represents phosphoserine; calculated average mass, 1509.5 atomic mass units). The ion at \( m/z 405.0 \) is a commonly observed nonspecific background peak.\(^4\) CNL \( m/z \) 49 scans of the tryptic fragments of MDCK calnexin (Fig. 6A) confirmed the former assignment, since the doubly charged phosphopeptide \( 555\text{AEEDEILRPSPR}^{566} \) (\( m/z \) 754.6) was observed.

Mass spectral analyses of the tryptic peptides from HepG2 calnexin by precursors \( m/z 79 \) scans revealed a clustered series of multiply charged ions (Fig. 5B) that upon deconvolution identified several large phosphorylated partial tryptic fragments derived from the cytosolic domain of HepG2 calnexin (see below). Analyses of this tryptic digest of HepG2 calnexin by CNL \( m/z 49 \) scans detected a \([\text{M}+2\text{H}]^{2+}\) peptide ion with an \( m/z \) value of 755.2 (Fig. 6B), equivalent to the fragment similarly observed for MDCK calnexin (Fig. 5A). This fragment was not observed as strongly by precursors \( m/z 79 \) scans of the trypsinized HepG2 calnexin as it was observed for trypsinized MDCK calnexin (Fig. 5, compare A and B). The selectivity of the CNL \( m/z 49 \) scans, however, confirmed the presence of this same fragment \( 554\text{AEEDEILRPSPR}^{566} \) in the HepG2 calnexin tryptic digest.

The clustered series of multiply charged ions recorded by precursors \( m/z 79 \) scans of trypsinized HepG2 calnexin was subjected to deconvolution employing the Hypermass deconvolution software (41). The reconstruct algorithm was initially carried out over the range of 2,000–10,000 Da and then focused in on the range of interest. No discernible species could be identified at other masses. The reconstruct profile (Fig. 7)

\(^4\) M. A. Ward, unpublished observations.
indicated that the clustered series of multiply charged ions was
derived from partial digests of the same phosphorylated region
of the cytosolic domain of HepG2 calnexin. The partial tryptic
fragments (Fig. 7) corresponded to the region K^{496}T^{497}DAP-
QPDVKEEEKKKDKDEEKEEKLKEDRQKSDAE-
DGTVSQEEDKPKAEDEILNRSP^{565}NR^{567} of calnexin
(boldface type represents potential sites of phosphorylation).
The observed masses and their calculated monoisotopic and
average masses are tabulated (Fig. 7). These phosphorylated
partial tryptic fragments contained three of the five serine
residues, Ser^{534}, Ser^{544}, and Ser^{563}, of the cytosolic domain
of human calnexin, and these fragments contained either two or
three phosphate groups. Thus, these three serine residues rep-
resent the in vivo phosphorylation sites for calnexin. Close
inspection of the precursors m/z 79 scans for phosphorylated
tryptsinized MDCK calnexin revealed a similar clustered series
of multiply charged ions (Fig. 5, A and B), albeit these signals
were weaker relative to the dominant ions (m/z 405.0, 502.2,
and 753.4) but consistent with those observed in Fig. 5B. Par-
tial digestion of phosphorylated MDCK calnexin was probably
preferential but to a lesser extent in this sample. Attempts to
deconvolute these clusters of multiply charged ions (Fig. 5A)
were unsuccessful due to the strong signals from the three
abundant ions. Differences in sensitivity to trypsinization is
unclear. As a precaution to autodigestion, trypsinization was
performed in the presence of 5 mM Ca^{2+} (22, 23). This precau-
tion may have contributed to an increased proteolytic resist-
ance of the cytosolic domain, since Ca^{2+} binds to this domain
(42). This caveat is similar to that indicated above for poor
coverage of the luminal domain (Fig. 3, A and B), resulting from
an increased proteolytic resistance induced by millimolar con-
centrations of Ca^{2+} (2). Nevertheless, these data are consistent
with MDCK calnexin being phosphorylated on the equivalent
three serine residues, i.e. Ser^{535}, Ser^{545}, and Ser^{564} (summar-
ized in Fig. 8).

**DISCUSSION**

A recently uncovered family of resident ER proteins has
revealed properties of novel lectin-like molecular chaperones.
These recognize N-linked glycoproteins and couple N-linked
oligosaccharide modification with productive glycoprotein folding
(3–13, 43). The family is composed of calnexin, a type I
transmembrane protein of the ER (1, 2); calreticulin, a KDEL-
terminated soluble ER-resident protein (44); and calmegin, a
testis-specific ER transmembrane protein with sequence con-
servation at the predicted luminal domain (45).

Major differences among the three proteins are found at
their COOH termini. Mammalian calnexins reveal 89 cytosoli-
cally oriented residues (1, 2, 33, 42), which were here shown to
be phosphorylated at three of the four invariant serine resi-
dues. The calmegin deduced protein sequences predict 119
amino acids cytosolically oriented (45, 46) with six conserved
(human and mouse) potential serine phosphorylation sites. The
calmegin conserved potential serine phosphorylation sites are,
as with the observed sites of serine phosphorylation in cal-
nexin, also in the COOH-terminal half of the respective cyto-
solic domain. Five of the six potential serine phosphorylation
sites of calmegin are within motifs similar to those observed for
calnexin; three are in CK2 motifs, one is in a PKC motif, and
another (mouse sequence only) is in a PDK motif (Fig. 9). Thus,
calmegin is predicted to be phosphorylated on equivalent
serines to those in calnexin (Fig. 9). The alignment of the
cytosolic domains of calnexins and calmegins identifies three
major (and three minor) loops (boxed in Fig. 9) that are unique
to the calmegins. Furthermore, this alignment reveals that the
cytosolic domains of both calnexin and calmegin can be divided
into four subdomains: a juxtamembrane basic, lysine-rich sub-
domain; a central acidic, glutamic acid-rich subdomain; a phos-
phorylation signaling subdomain; and a putative COOH-termi-

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**Fig. 7. Reconstruct profile of precursors m/z 79 scans of HepG2 calnexin.** Deconvolution of the clustered series of multiply charged ions of HepG2 calnexin (Fig. 5B) employing the Hypermass reconstruction software (41). The identities of the numbered fragments are tabulated. Na" and K" adducts are indicated. mi, monoisotopic; av, average. The x axis indicates the mass values (atomic mass units (amu)), and the y axis indicates the relative ion intensity.

**Fig. 8. Schematic representation of in vivo phosphorylation sites of cal-
exin.** The cytosolic domain of HepG2 (human) calnexin is presented. Phos-
phorylated fragments detected either by pre-
cursors m/z 79 or CNL scans of either HepG2 or MDCK calnexin are indicated. The numbering indicates amino acid resi-
dues of mature human calnexin. Two diphasporlated peptides (496–565 and 497–567, see Fig. 7) that contain three potential serine phosphorylation sites are indicated by 2 of 3".
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FIG. 9. Sequence alignment of the cytosolic domains of known mammalian calnexins and calmeins. Predicted cytosolic domains of canine (Ca; Refs. 1 and 14), rat (Ra; Ref. 42), mouse (Mo; Ref. 42), and human (Hu; Ref. 33) calnexin (cnx) and human and mouse calmeins (cmg; Refs. 45 and 46) are shown. CC and CW (italic type), amino acid residues predicted to be the last two residues of the transmembrane domains of calnexin and calmein, respectively. Calnexin identified (closed symbols) and calmein putative (open symbols) phosphorylation sites are shown. Potential kinases are as follows: CK2 (arrows), PKC (arrowheads), and PDK (asterisks). Residue numbers refer to the mature calnexins or calmeins. Serine residues are in boldface type; amino acid differences among the calnexins and among the calmeins are indicated; and identities are indicated by dashes. Gaps introduced to optimize alignments are shown as dots. Calnexin alignment identifies two deletions of one amino acid at position 507 and 524 of rodent calnexins; similarly, calmein alignment identifies two deletions of one (Mo, 510) or two (Hu, 528) amino acids. Calnexin/calmein alignment identifies six deletions/insertions of 2, 6, 8, 3, and 11 residues and 1 residue (enclosed boxes, calmein). Consensus sequence is as follows: invariant (boldface uppercase type) and five of six identities (bold lowercase type). Boldface dots indicate differences. The four subdomains (i.e. the juxtamembrane basic, acidic, phosphorylation, and the predicted ER retrieval (47) domains) are indicated.

Ca2+ luminal core of calnexin (2). The next COOH-terminal serine residue was conserved in Hep2G2 (Ser563, a potential CK2 phosphorylation site) and MDCK (Ser561, a potential CK2/PDK phosphorylation site) calnexins but not rodent calnexins (Fig. 9). This site was detected only as a nonphosphorylated fragment by MS analysis of the Hep2G2 tryptic digests (Fig. 3B).

As summarized in Fig. 8, only Ser534, Ser535, and Ser563 (human numbering, invariant in mammalian calnexins; see Fig. 9) were in vivo phosphorylated as detected by two selective nano-ESI MS techniques for detection of phosphorylated peptides. Two of these three invariant serine phosphorylation sites, Ser534 and Ser535, are within well recognized CK2 motifs (34). This coincides with earlier observations that calnexin in microsomes was in vitro phosphorylated by CK2 (14, 15) and that CK2 was purified as an ER membrane-associated kinase (14). The identification of a third site of calnexin phosphorylation (Ser563 in Hep2G2, Ser564 in MDCK) was not predicted from previous in vitro studies (1, 14, 15). This site, invariant in mammalian calnexins, is within a motif potentially recognized by either PKC (35, 36) or PDK (37, 38).

We have presented evidence for diphosphorylated and tripophosphorylated (Figs. 7 and 8) calnexins but no conclusive data for a monophosphorylated form, i.e. with only one of the two CK2 sites or only the S563PR site being phosphorylated, since only singly phosphorylated fragments encompassing the three sites were identified. Nonphosphorylated peptides encompassing the observed two CK2 sites of serine phosphorylation were identified by DE-MALDI-ToF MS analyses:532QKS-DAEDGTVSVEEDDRKPK554 of Hep2G2 calnexin and 532QKS-DAEDGTVSVEEDDRKPK554 of MDCK calnexin (Figs. 2 and 3, and Table II). The strongest evidence for only one of the two CK2 sites being phosphorylated was observed from MDCK trypsinized calnexin (Fig. 3A and Table II). The peptide ion, m/z 2130.9 (Fig. 3A and Table II) corresponds to the partial tryptic fragment 532QKS-DAEDGTVSVEEDDRKPK554, containing one phosphate group and cyclization of the NH2-terminal glutamine (calculated average mass, 2130.9) (52). The cyclization of the NH2-terminal glutamine of this tryptic fragment was suggested also by Cala and co-workers (15). For both

calnexins, the two phosphorylated CK2 sites (Ser$^{354}$ and Ser$^{544}$, HepG2 calnexin) are contained within the same tryptic fragment, and thus by our strategies, these two phosphorylation sites could not be characterized individually. Evidence for a nonphosphorylated state of the most COOH-terminal serine residue was not obtained. This may be a consequence of complete proteolytic digestion of this nonphosphorylated form and subsequent loss of the corresponding tryptic tripeptide, (RS$^{563}$PR, during desalting/washing steps prior to MS analysis. However, the S$^{563}$PR site in the diphosphorylated large partial tryptic fragment (Fig. 7) that contains three potential sites of phosphorylation may correspond to a nonphosphorylated Ser$^{363}$ site. On this basis, there are six potentially different (three mono- and three diphosphorylated) partially phosphorylated states of calnexin that probably represent a regulatory mechanism for calnexin action.

The phosphorylation results presented in this paper extend the previous finding by Capps and Zuniga (17) and Le et al. (16) that a significant proportion of calnexin was phosphorylated in vivo. The identification of phosphorylated calnexin in association with a subset of incompletely folded major histocompatibility complex class I alleles (17) or of the misfolded null Hong Kong mutant of α-1-antitrypsin (16) is suggestive of a coincident and perhaps regulatory role with the action of the luminal domain of calnexin in glycoprotein folding and quality control. Conservation of the three serine targets of protein kinases as elucidated here predicts that this conservation and their phosphorylation are under strict control. Elucidation of the signaling cascades that trigger calnexin phosphorylation at the PKC/PDK site as well as the CK2 site may lead to new insights in the regulation of cargo folding and transport from the ER. These studies may also lead ultimately to a rationale for the evolution of three distinct genes in mammals that encode this family of molecular chaperones.

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Conserved in Vivo Phosphorylation of Calnexin at Casein Kinase II Sites as Well as a Protein Kinase C/Proline-directed Kinase Site

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