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The major histocompatibility complex class I antigen-binding protein p88 is the product of the calnexin gene

(human calnexin/chaperone/immunoglobulins/endoplasmic reticulum)

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ABSTRACT **A 90-kDa phosphoprotein (p90) of the endoplasmic reticulum was identified by a monoclonal antibody generated against human hepatoma cells. Pulse–chase experiments with [^{32}P]phosphate and [^{35}S]methionine demonstrated that p90 formed both stable and transient complexes with other cellular proteins, suggesting its role as a molecular chaperone. This protein associates with heavy chains of major histocompatibility complex class I proteins, suggesting that it is the human homolog of the recently described 88-kDa protein that transiently associates with murine class I molecules in the endoplasmic reticulum. The p90 protein also associates in B lymphocytes with membrane immunoglobulin μ heavy chains and may serve as a chaperone for many membrane-bound polypeptides. A partial human p90 cDNA was cloned from a λgt11 expression library and identified as the human homolog of calnexin, a major canine calcium-binding protein found to be associated with the signal-sequence receptor in endoplasmic reticulum membranes.**

In studies on the mechanism of translocation of signal-peptide-containing proteins into the endoplasmic reticulum (ER), a 90-kDa phosphoprotein was found to be associated with the 35-kDa signal-sequence receptor α chain in canine pancreatic microsomes (1). Purification of this protein revealed that it was identical to a ubiquitous 90-kDa ER-specific protein originally described by Louvard *et al.* (2) using polyclonal antibodies specific for the ER. Molecular cloning of the canine gene that encodes this 90-kDa protein indicated that it is an integral membrane, resident ER, calcium-binding protein, which was consequently named calnexin (1).

In the course of investigating the phenotype of human hepatoma cells with monoclonal antibodies, we identified an antibody (AF18) that was specific for the ER. In immunoblotting experiments with a variety of human tissues, the AF18 antibody identified a ubiquitous 90-kDa protein (p90). In our studies, immunoprecipitation of [^{32}P]phosphate-labeled cells with the AF18 antibody revealed that p90 is a phosphoprotein that forms a stable complex with another, 35-kDa phosphoprotein, suggesting that p90 is the human homolog of canine calnexin. In keeping with these observations, p90 was subsequently identified by molecular cloning as human calnexin.† However, pulse–chase experiments with [^{35}S]methionine-labeled cells demonstrated that human calnexin was complexed not only to the 35-kDa phosphoprotein but also to many other cellular proteins. The localization of human calnexin to the ER and its transient association with different polypeptides suggested that it might serve as a molecular chaperone in eukaryotic cells.

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A murine ER protein of similar molecular mass (88 kDa) that transiently associates with class I histocompatibility molecules has been described (3). Recent studies (4) have demonstrated that the association of major histocompatibility complex (MHC)-encoded class I molecules with this 88-kDa protein is a prelude to the oligomerization of MHC molecules into tetramers made up of four heavy chains and four β_2-microglobulin moieties (4). We show here that human calnexin associates with MHC class I heavy chains as well as with immunoglobulin μ heavy chains. Thus, calnexin may be involved in the transmembrane assembly of numerous oligomeric proteins including the signal-sequence receptor, the MHC class I antigen, and the B-cell antigen receptor.

MATERIAL AND METHODS

Production of Monoclonal Antibody AF18, Immunoperoxidase Staining, and Western Immunoblotting. Mouse monoclonal antibody AF18 was produced by immunization of a mouse with a human hepatoma cell line named Focus (5), as described (6). Antibody-secreting hybridomas were injected into BALB/c mice to obtain ascites, which were used directly or after purification of AF18 antibody on protein A-Sepharose (7). Immunoperoxidase and Western immunoblotting techniques have been described (6, 7).

Metabolic Labeling of Cells and Pulse–Chase Experiments. Focus and Huh 7 hepatoma cells were used for metabolic labeling with [^{35}S]methionine (7). The Hep G2 hepatoma cell line was grown to 70% confluency as described (7), incubated with phosphate-free Dulbecco's modified Eagle's medium for 3 hr and labeled in the same medium containing 100 μCi of [^{32}P]orthophosphate (New England Nuclear; specific activity, 9000 Ci/mmol; 1 Ci = 37 GBq) per 2 ml per 10-cm plate for 3 hr. Pulse–chase experiments with [^{35}S]methionine were performed as described (7). Metabolic labeling of murine lymphoid cell lines and immunoprecipitation with anti-μ and anti-κ antibodies were performed as described (8).

Cell Fractionation and *in Vitro* Phosphorylation of Microsomes. Huh-7 human hepatoma cells were grown to confluency, washed three times in phosphate-buffered saline, suspended in 5 volumes of homogenization buffer (0.25 M sucrose/10 mM Tris acetate, pH 7.4/1 mM EDTA) and homogenized with a Dounce homogenizer. The cell homogenate was centrifuged at 9500 rpm (Sorvall SS34 rotor) for 10 min at 4°C to pellet cell debris, nuclei, and mitochondria. The supernatant was centrifuged at 40,000 rpm (Sorvall AH650 rotor) for 90 min at 8°C to separate microsomes and Golgi

Abbreviations: ER, endoplasmic reticulum; MHC, major histocompatibility complex.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M98452).

membranes (pellet) from cytosolic proteins (supernatant). Microsomes (130 μg of protein) were resuspended in 100 μl of GTP buffer (40 mM Tris·HCl, pH 7.5/2.5 mM $MnCl_2$/7.5 mM $MgCl_2$/30 mM KCl) and labeled with 25 μl (50 μCi) of [γ-^{32}P]GTP (New England Nuclear; specific activity, 30 Ci/mmol) for 30 min at 4°C as described by Wada *et al.* (1). ^{32}P-labeled microsomes were solubilized with 1 ml of lysis buffer (50 mM Tris·HCl, pH 7.4/250 mM NaCl/5 mM EDTA/50 mM NaF/0.1% Triton X-100) for 2 hr and used for immunoprecipitations as described (7). For studies on the subcellular localization of p90, microsome-enriched, nuclear-enriched, and cytosolic fractions were solubilized in SDS sample buffer (9) containing 5% 2-mercaptoethanol and analyzed by Western immunoblotting using AF18 antibody (1:500 dilution of ascites) and peroxidase-conjugated rabbit anti-mouse IgG (1:3000 dilution; DAKO, Carpinteria, CA) as first and second antibodies, respectively. Antigen–antibody complexes were detected using the ECL Western blotting kit (Amersham) according to the manufacturer's recommendations.

Construction of a cDNA Expression Library, Cloning of p90 cDNA, and Northern Blot Analysis. The construction of a λgt11 expression library from Focus human hepatoma cells and techniques used for antibody screening have been described (10). After screening of 10^6 plaque-forming units, 1 clone with consistent positive immunoreactivity was identified. The cDNA insert was amplified from purified phages by PCR (11), cut with *Eco*RI, and ligated into plasmid pGEM-7Z (Promega). A recombinant plasmid (p181) was identified by color selection and by hybridization to the original phage clone, amplified in *Escherichia coli* DH5α cells, and purified on a cesium chloride gradient. Nucleic acid sequence was determined with a Sequenase kit (Pharmacia). For Northern blot analysis, total RNA extracted from Focus cells with guanidinium salts (10) was used directly or after selection of poly(A)$^+$ RNA with an oligo(dT)-cellulose column (12). RNAs were analyzed by Northern blotting using formaldehyde-based denaturing gel conditions (12). Blots were hybridized to a 1.6-kilobase cDNA fragment prepared from *Eco*RI-digested p181 by isolating a 1.6-kilobase insert in an agarose gel, eluting it with the Geneclean kit (Bio 101, La Jolla, CA), and ^{32}P labeling by the random priming method (12).

Crosslinking and Analysis of MHC Class I and IgM Immunoprecipitates for the AF18 Antigen. Murine 3320 B cells were metabolically labeled for 18 hr with [^{35}S]methionine, lysed with 1% digitonin, and immunoprecipitated with either an anti-μ antibody or an anti-κ antibody (7). The immunoprecipitated complexes were analyzed in a 12.5% polyacrylamide/SDS gel.

Human Jijoye B cells were lysed with 1% digitonin and crosslinked with dithiobis(succinimidyl propionate) (Pierce), a cleavable bifunctional crosslinking reagent, as described (4). The crosslinked lysate was divided into portions that were separately immunoprecipitated with (*i*) the AF18 antibody, (*ii*) a mouse monoclonal anti-human MHC class I antibody (HC10; ref. 13), (*iii*) a monoclonal anti-μ antibody (Zymed Laboratories) in conjunction with a rat anti-mouse IgG antibody (Zymed Laboratories), (*iv*) the rat anti-mouse IgG antibody alone, and (*v*) an irrelevant mouse IgG (an anti-mouse MHC class II antibody, Y-3P; ref. 14). The last two immunoprecipitations were performed as negative controls. Following immunoprecipitation, samples were electrophoresed in a 9% polyacrylamide/SDS gel and transferred to an Immobilon-P membrane (Millipore). The presence of the AF18 protein in these immunoprecipitates was assayed by an immunoblot assay using anti-AF18, a peroxidase-conjugated rabbit anti-mouse IgG antibody, and the ECL detection system (Amersham).

RESULTS

Initial Characterization of p90. A monoclonal antibody (AF18) produced by immunizing a mouse with human hepatoma cell line Focus was initially selected by its high levels of binding to the cell line used as immunogen. When tested by the immunoperoxidase staining method, the AF18 antibody showed a distinct pattern of ER-specific staining in many cell lines, including the human hepatoma cell line Huh-7 (Fig. 1*A*). Triton X-100-soluble extracts from various human autopsy tissue specimens were tested for AF18 immunoreactivity by Western immunoblotting. The AF18 antibody identified a major 90-kDa polypeptide in liver, skin, adrenal gland, kidney, heart, colon, testes, and ovary (Fig. 1*B*).

Identification of p90 as a Phosphoprotein Transiently Associating with Other Cellular Proteins. Human hepatoma cells (Hep G2) were labeled with [^{32}P]phosphate, lysed with Triton X-100, and analyzed by immunoprecipitation. The AF18 antibody identified a 90-kDa phosphoprotein in addition to

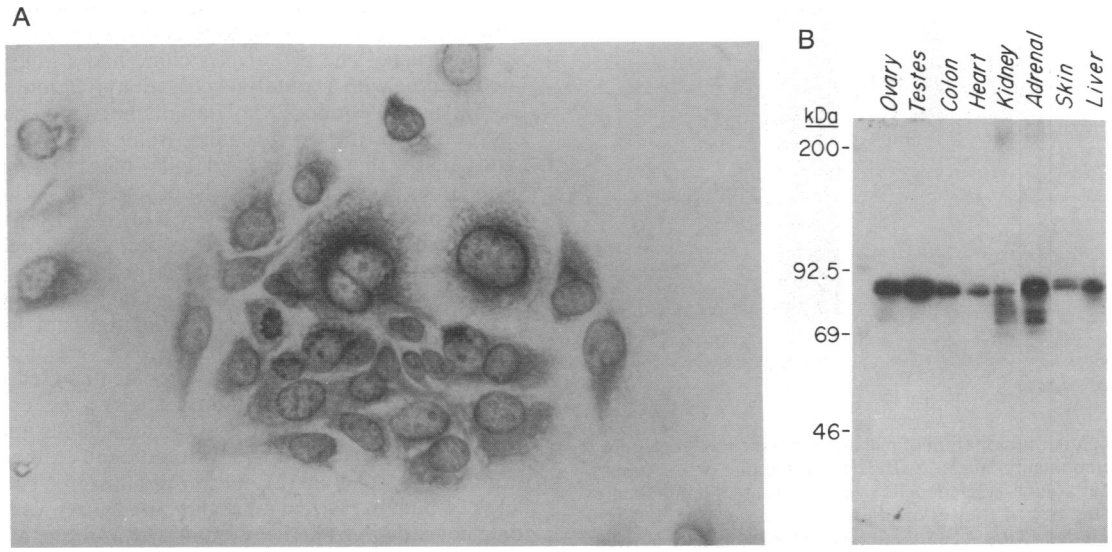

FIG. 1. (*A*) Immunoperoxidase staining of Huh-7 human hepatoma cells with AF18 monoclonal antibody. (*B*) Western immunoblot analysis of normal tissue extracts with AF18 antibody.

another phosphoprotein with an apparent molecular mass of 35 kDa (Fig. 2, lane 6). Since the 35-kDa phosphoprotein (p35) was not identified by AF18 on immunoblots, this result suggested that p90 is complexed to p35 in Hep G2 cells. A 35-kDa phosphoprotein associated with p90 was also detected in six other cell lines tested. Studies on the turnover of p90 performed after ^{32}P labeling indicated that p90 and p35 were stable and remained associated for at least 24 hr (data not shown). When similar experiments were performed with [^{35}S]methionine-labeled cells, p90 was found to be associated to a large number of proteins as shown in Huh-7 cells (Fig. 2, lane 2) and Focus cells (lane 4). However, after 3 hr of chase, the majority of these p90 associated-proteins were no longer detected (Fig. 2, lanes 3 and 5). These results suggested that p90 forms complexes with a large number of cellular proteins, but only one complex (with phosphoprotein p35) is stable, all others being transient. Since p90 is localized to the ER, we hypothesized that p90 could form complexes with certain cellular proteins during ER transit.

p90 Complexes with MHC Class I Molecules and Immunoglobulin μ Chains. Degen and Williams (3) had demonstrated the transient association of murine MHC class I heavy chains with an 88-kDa protein in the ER by using a crosslinking approach. In crosslinking experiments involving short periods of metabolic labeling, a complex of this 88-kDa protein with MHC class I heavy chains was observed, although p88 itself was not metabolically labeled under those conditions. Metabolic labeling for extended periods led to the incorporation of [^{35}S]methionine into this protein. Krishna *et al.* (4) have extended these observations and have shown that association of MHC class I heavy chains with the putative p88 chaperone is a prelude to their assembly into a higher-order oligomer. When murine B cells were metabolically labeled for 16–18 hr, lysed with digitonin, and immunoprecipitated with anti-μ or anti-κ antibodies, we observed that an ≈90-kDa protein was specifically associated with incompletely assembled IgM complexes (Fig. 3*A*, lane 2, anti-μ), but not with mature complexes (lane 3, anti-κ). Since a murine 88/90-kDa protein associates transiently with MHC

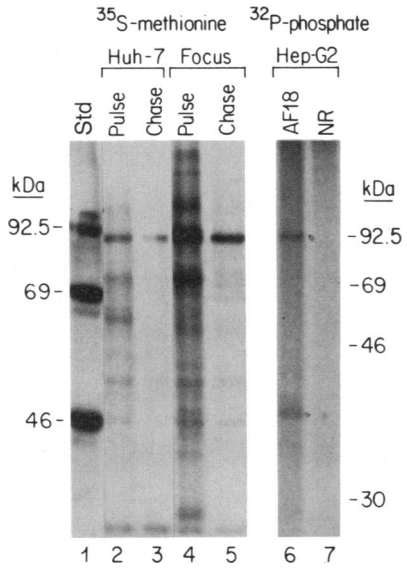

FIG. 2. Immunoprecipitation of p90 and its associated molecules from cells metabolically labeled with [^{35}S]methionine or [^{32}P]phosphate. [^{35}S]Methionine-labeled cells were harvested immediately after the labeling period (pulse) or after a 3-hr chase; lysates were immunoprecipitated with AF18 antibody. [^{32}P]Phosphate-labeled cells were harvested immediately after labeling; lysates were immunoprecipitated with AF18 antibody or a nonrelevant (NR) antibody. Std, molecular size standards.

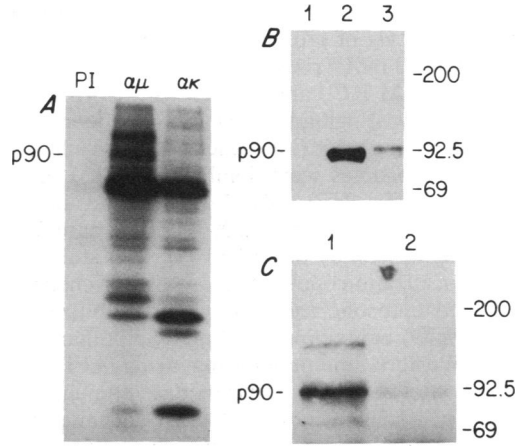

FIG. 3. Identification of p90 in association with MHC class I molecules and immunoglobulin μ chains. (*A*) In murine B cells a 90-kDa protein is associated with incomplete (anti-μ, $\alpha\mu$) but not complete (anti-κ, $\alpha\kappa$) membrane IgM complexes. PI, preimmune rabbit IgG used as a control. (*B* and *C*) AF18 antibody recognizes the 90-kDa protein associated with MHC class I and IgM molecules. Jijoye B-cell lysates were immunoprecipitated with anti-MHC (*B* lane 3 and *C* lane 1), anti-IgM (*C* lane 2), anti-AF-18 (*B* lane 2), or irrelevant antibody (*B* lane 1). Immunoprecipitated proteins were separated by SDS/PAGE and immunoblotted samples were probed with AF18 antibody.

class I molecules as well as with incomplete B-cell receptor complexes, we wished to ascertain whether the human p90 could be crosslinked with MHC class I molecules and with IgM. In the combined immunoprecipitation/immunoblot assay, p90/AF18 was associated both with human MHC (HLA) class I heavy chains (Fig. 3*B*, lane 3, and Fig. 3*C*, lane 1) and with IgM heavy chains (Fig. 3*C*, lane 2). No AF18 antigen was seen when samples were originally immunoprecipitated with rat anti-mouse IgG antibodies (Fig. 3*B*, lane 1) or with an irrelevant mouse IgG antibody (data not shown).

Molecular Cloning of p90 and Its Identification as Human Homolog of Calnexin. The nucleic acid and deduced amino acid sequences of p90 cDNA are shown in Fig. 4*A*. The 1560-base-pair clone obtained by λgt11 screening had a single major open reading frame, nucleotides 1–1068, capable of encoding 356 amino acids. The reading frame did not have a methionine start codon but was in phase with the phage-derived *lacZ* gene reading frame, indicating that the AF18 antibody recognized an epitope coded by the deduced open reading frame. The p90 cDNA clone had a long 3′ untranslated region with a putative polyadenylylation signal (AAGAAA) at nucleotide 1283. The calculated molecular mass of the product of the open reading frame was 35 kDa, significantly below the apparent molecular mass of 92 kDa of p90, suggesting that we had isolated a partial cDNA clone. To test this hypothesis, we performed a Northern Blot analysis on RNA from Focus cells as well as from other cell lines and from human liver and kidney. A major transcript of 4.6 kilobases with two minor transcripts of 3.8 and 3.0 kilobases were detected in all specimens tested (data not shown). This analysis indicated that the p90 cDNA was authentic but shorter than the smallest RNA species detected in cells.

p90 cDNA and deduced amino acid sequences were compared with the available data in the data banks and literature and p90 was found to be highly homologous to two major ER proteins; dog calnexin (2) and calreticulin (15). The homology between human p90 and dog calnexin cDNAs was the highest (data not shown). A higher homology was found at the protein level (Fig. 4*B*). Out of 355 amino acids compared, 333 were identical between dog calnexin and human p90 (94% identity). Ten of the 22 amino acid residues that were different

A

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   1 CTTTACACACTAATCTTGAATCCAGATAATAGTTTTGAAATACTGGTTGACCAATCTGTG 60
     L  Y  T  L  I  L  N  P  D  N  S  F  E  I  L  V  D  Q  S  V
  61 GTGAATAGTGGAAATCTGCTCAATGACATGACTCCTCCTGTAAATCCTTCACGTGAAATT 120
     V  N  S  G  N  L  L  N  D  M  T  P  P  V  N  P  S  R  E  I
 121 GAGGACCCAGAAGACCGGAAGCCCGAGGATTGGGATGAAAGACCAAAAATCCCAGATCCA 180
     E  D  P  E  D  R  K  P  E  D  W  D  E  R  P  K  I  P  D  P
 181 GAAGCTGTCAAGCCAGATGACTGGGATGAAGATGCCCCTGCTAAGATTCCAGATGAAGAG 240
     E  A  V  K  P  D  D  W  D  E  D  A  P  A  K  I  P  D  E  E
 241 GCCACAAAACCCGAAGGCTGGTTAGATGATGAGCCTGAGTACGTACCTGATCCAGACGCA 300
     A  T  K  P  E  G  W  L  D  D  E  P  E  Y  V  P  D  P  D  A
 301 GAGAAACCTGAGGATTGGGATGAAGACATGGATGGAGAATGGGAGGCTCCTCAGATTGCC 360
     E  K  P  E  D  W  D  E  D  M  D  G  E  W  E  A  P  Q  I  A
 361 AACCCTAGATGTGAGTCAGCTCCTGGATGTGGTGTCTGGCAGCGACCTGTGATTGACAAC 420
     N  P  R  C  E  S  A  P  G  C  G  V  W  Q  R  P  V  I  D  N
 421 CCCAATTATAAAGGCAAATGGAAGCCTCCTATGATTGACAATCCCAGTTACGAGGGAATC 480
     P  N  Y  K  G  K  W  K  P  P  M  I  D  N  P  S  Y  Q  G  I
 481 TGGAAACCCAGGAAAATACCAAATCCAGATTTCTTTGAAGATCTGGAACCTTTCAGAATG 540
     W  K  P  R  K  I  P  N  P  D  F  F  E  D  L  E  P  F  R  M
 541 ACTCCTTTTAGTGCTATTGGTTTGGAGCTGTGGTCCATGACTCTGACATTTTTTTTTGAC 600
     T  P  F  S  A  I  G  L  E  L  W  S  M  T  L  T  F  F  F  D
 601 AACTTTATCATTTGTGCTGATCGAAGAATAGTTGATGATTGGGCCAATGATGGATGGGGC 660
     N  F  I  I  C  A  D  R  R  I  V  D  D  W  A  N  D  G  W  G
 661 CTGAAGAAAGCTGCTGATGGGGCTGCTGAGCCAGGCGTTGTGGGGCAGATGATCGAGGCA 720
     L  K  K  A  A  D  G  A  A  E  P  G  V  V  G  Q  M  I  E  A
 721 GCTGAAGAGCTCCCTTGGCTGTGGGTAGTCTATATTCTAACTGTAGCCCTTCCTGTGTTC 780
     A  E  E  L  P  W  L  W  V  V  Y  I  L  T  V  A  L  P  V  F
 781 CTGGTTATCCTCTTCTGCTGTTCTGGAAAGAAACAGACCAGTGGTATGGAGTATAAGAAA 840
     L  V  I  L  F  C  C  S  G  K  K  Q  T  S  G  M  E  Y  K  K
 841 ACTGATGCACCTCAACCGGATGTGAAGGAAGAGGAAGAAGAGAAGGAAGAGGAAAAGGAC 900
     T  D  A  P  Q  P  D  V  K  E  E  E  E  E  K  E  E  E  K  D
 901 AAGGGAGATGAGGAGGAGGAAGGAGAAGAGAAACTTGAAGAGAAACAGAAAAGTGATGCT 960
     K  G  D  E  E  E  E  G  E  E  K  L  E  E  K  Q  K  S  D  A
 961 GAAGAAGATGGTGGCACTGTCAGTCAAGAGGAGGAAGACAGAAAACCTAAAGCAGAGGAG 1020
     E  E  D  G  G  T  V  S  Q  E  E  E  D  R  K  P  K  A  E  E
1021 GATGAAATTTTGAACAGATCACCAAGAAACAGAAAGCCACGAAGAGAGTGAAACAATCTT 1080
     D  E  I  L  N  R  S  P  R  N  R  K  P  R  R  E  *
1081 AAGAGCTTGATCTGTGATTTCTTCTCCCTCGTCCCCTGCAAGAGTGGTCCTAGGAGAGGA 1140
1141 CCTGGCACACCTTAGGTTGACATTCAGAAAAGTTCAAGACATCACCATCAGCAGGCTCCA 1200
1201 GTTGAACACTAGTCTGTGTAACTTTAAACATCTAGCAGTAAATACTTGCAGTTGTGATAT 1260
1261 AAAGGACCCTGTTTCTGTAGAAaagaaaACATTTAACATAATGGTTGTGAATGTAACATG 1320
1321 AAGCAAACTAACTTTTTTTTTTTTTAACATCTTTGTTTTTAAAATAGAATGATAGAACTTT 1380
1381 CCCAGTCTTTAAGATCTTGGCTTAATTTAATGTATTAATCTGTTTGTGCAAACATAATAC 1440
1441 CACCATTTAAAAATGTTAGGGAGATGAGTTGCAGTTTTTATAATAGATTTTTTTTAAAGT 1500
1501 TTGGTATTGTAAAACATTCACACCTCTGTCCCTCAAAATTGATAATTACGTTTAAAGTGC 1560
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B

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Human LYTLILNPDNSFEILVDQSVVNSGNLLNDMTPPVNPSREIEDPEDRKPED
      ||||||||||||||||||:||||||||||||||||||||||.||||
  Dog LYTLILNPDNSFEILVDQSIVNSGNLLNDMTPPVNPSREIEDPEDQKPED 267

Human WDERPKIPDPEAVKPDDWDEDAPAKIPDEEATKPEGWLDDEPEYVPDPDA
      ||||||||||:|||||||:||||||||||||||:|||||||||||||||
  Dog WDERPKIPDPDAVKPDDWNEDAPAKIPDEEATKPDGWLDDEPEYVPDPDA 317

Human EKPEDWDEDMDGEWEAPQIANPRCESAPGCGVWQRPVIDNPNYKGKWKPP
      ||||||||||||||||||||||:|||||||||||||:||||||||||||
  Dog EKPEDWDEDMDGEWEAPQIANPKCESAPGCGVWQRPMIDNPNYKGKWKPP 367

Human MIDNPSYQGIWKPRKIPNPDFFEDLEPFRMTPFSAIGLELWSMTLTFFFD
      |||||.|||||||||||||||||||:|||||||||||||| .:|||
  Dog MIDNPNYQGIWKPRKIPNPDFFEDLEPFKMTPFSAIGLELWSMTSDIFFD 417

Human NFIICADRRIVDDWANDGWGLKKAADGAAEPGVVGQMIEAAEELPWLWVV
      |||:|:|||:|||||||||||||||||||||||||||||| ||||||
  Dog NFIVCGDRRVVDDWANDGWGLKKAADGAAEPGVVGQMIEAAEERPWLWVV 467

Human YILTVALPVFLVILFCCSGKKQTSGMEYKKTDAPQPDVKEEEEEKEEEKD
      |:|||||||||||||||||.|.:|||||||||||||||||||||||||
  Dog YVLTVALPVFLVILFCCSGKKQSSPVEYKKTDAPQPDVKEEEEEKEEEKD 517

Human KGDEEEEGEEKLEEKQKSDAEEDGGTVSQEEEDRKPKAEEDEILNRSPRN
      ||||||||||||||||||||||||.||||:||||||||||||||||||
  Dog KGDEEEEGEEKLEEKQKSDAEEDGGTASQEEDDRKPKAEEDEILNRSPRN 567

Human RKPRRE*
      ||||||
  Dog RKPRRE*
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FIG. 4. (*A*) Partial cDNA sequence and deduced amino acid sequence of p90. (*B*) Homology between dog calnexin and human p90.

between the two species were homologous. Thus, the homology between the known sequence of human p90 and dog calnexin was 97%, strongly suggesting that p90 is the human homolog of calnexin (Fig. 4*B*). Dog calnexin was initially identified as a major ATP- and GTP-phosphorylated protein of microsomes and forms a complex with a 35-kDa phosphoprotein of the ER that was identified as the signal-sequence receptor α chain by Wada *et al.* (1). To further confirm the

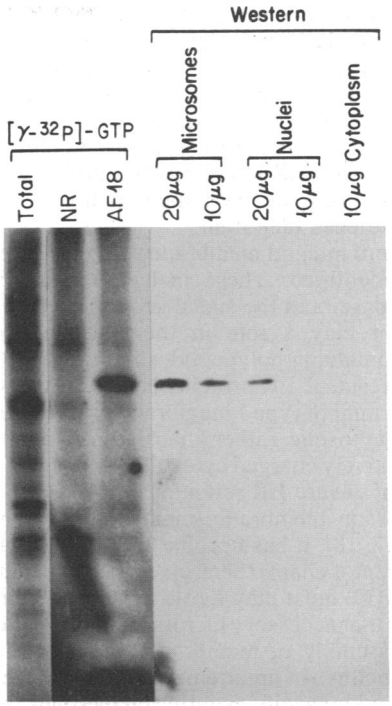

FIG. 5. *In vitro* phosphorylation of human hepatoma microsomes, immunoprecipitation, and Western immunoblotting with AF18 antibody. (*Left*) Microsomes were phosphorylated *in vitro* with [^{32}P]GTP and immunoprecipitated with AF18 antibody or a nonrelevant mouse IgG (NR). (*Right*) Microsomes as well as nuclear and cytoplasmic fractions from Huh-7 cells were coelectrophoresed with ^{32}P-labeled extracts (*Left*), transferred onto Immobilon-P membranes, and probed with AF18 antibody.

identity of p90 as human calnexin, we analyzed p90 in various subcellular fractions of human hepatoma cell line Huh-7.

Huh-7 cells were homogenized in a sucrose-containing buffer and subcellular fractions enriched in nuclear, microsomal, or cytoplasmic proteins were prepared by differential centrifugation. Equal amounts of protein from each fraction were analyzed by Western immunoblotting for p90. The microsomal fraction had the highest levels of p90, although the nuclear fraction also had detectable p90 (Fig. 5). In contrast, p90 was not detectable in the cytosolic fraction (Fig. 5). To test whether p90 can be phosphorylated *in vitro* by GTP, microsomal proteins were incubated with [γ-^{32}P]GTP, lysed with Triton X-100, and immunoprecipitated with the AF18 antibody. Incubation of human microsomes with GTP resulted in phosphorylation of several proteins (Fig. 5, lane 1). By immunoprecipitation with AF18 we identified p90 as a [^{32}P]GTP-labeled microsomal protein (Fig. 5).

DISCUSSION

Proteins that are resident in the ER are assumed to serve functions related to the translocation, proper folding, assembly, posttranslational modification, and transport of proteins along the secretory pathway. This pathway includes proteins that are primarily destined for export either to the cell surface or to lysosomes, as well as resident Golgi proteins that in turn may perform similar or related functions later in the secretory pathway.

Luminal ER proteins include Bip, an ER-specific member of the heat shock family, and protein disulfide isomerase, both of which possess carboxyl-terminal Lys-Asp-Glu-Leu (KDEL) sequences that play a role in their retention in the ER (for a recent review see ref. 16). Bip binds to secretory immunoglobulin heavy chains and may play a role in the

identification of misfolded proteins in the ER. Protein disulfide isomerase plays an important role in the proper folding and assembly of proteins in the ER. Other luminal ER proteins that play a role in glycosylation and in the trimming of N-linked carbohydrates have also been identified. Bip shares with other luminal ER proteins, such as calreticulin, the ability to bind calcium, and structural features of these proteins that correlate with their ability to complex with calcium have been identified.

A number of integral membrane resident ER proteins have also been identified. These include the KDEL receptor, signal peptidase, and the signal-sequence receptor proteins, which might play a role in the translocation of signal-sequence-containing polypeptides into the ER (16). Integral membrane resident ER proteins lack KDEL sequences (the carboxyl termini of type I membrane proteins resident in the ER face a cytosolic rather than luminal milieu) but often contain positively charged cytosolic residues (at positions −2 and −4) that ensure ER retention.

In studies on membrane immunoglobulin assembly and transport (17, 18), it has become apparent that factors other than the receptor chains themselves (heavy chain, light chain, mb-1, and B29) must play a role in the transport process. It is also clear that accessory factors must exist that specifically influence assembly of membrane as opposed to secretory immunoglobulins. In an attempt to identify such membrane immunoglobulin-specific putative chaperones, we have identified a 90-kDa protein, p90, that is associated with incomplete B-cell receptors but not with the complete receptor. Since this 90-kDa protein (p88/p90/calnexin) appears to be involved in the assembly of MHC class I and membrane immunoglobulin molecules, we presume that it may serve as a molecular chaperone for a number of integral membrane multisubunit proteins. There are a number of potential roles that calnexin may play in the biogenesis of class I molecules and of the B-cell antigen receptor. Calnexin may conceivably directly facilitate the folding and assembly of these transmembrane multisubunit proteins. Alternatively, as has been suggested for Bip, calnexin may serve as a sensor for misfolded variants of class I molecules and of the antigen receptor; its transient association with these proteins may be a reflection of the scrutiny of all ER forms of these proteins during assembly. A third and extremely likely role of calnexin (see below) is its potential role in the higher-order oligomerization of its target proteins.

The observed association of calnexin with polypeptides of the signal-sequence receptor suggest that this putative chaperone may not actually be a component of the receptor as has been suggested (1), but that it might actually play a role in the assembly of this receptor. The observation that the transient association of p88 with MHC class I heavy chains precedes the higher-order oligomerization of 60-kDa MHC heterotrimers into 240-kDa tetrameric complexes (4) suggests that this putative chaperone may play a role in the higher-order oligomerization of a subset of integral membrane multisubunit proteins.

In recent studies, Brenner and coworkers (19) have found a 90-kDa intracellular protein (IP90) associated with a number of immune receptors. Peptide sequence obtained from this protein and cDNA sequencing have confirmed (M. Brenner, personal communication) that IP90 is indeed identical to calnexin.

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