Cell. Mol. Life Sci. (2012) 69:1733–1743
DOI 10.1007/s00018-011-0899-8

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Transthyretin binds to glucose-regulated proteins and is subjected to endocytosis by the pancreatic β-cell

Nancy Dekki · Essam Refai · Rebecka Holmberg · Martin Köhler · Hans Jörnvall · Per-Olof Berggren · Lisa Juntti-Berggren

Received: 13 December 2010 / Revised: 8 November 2011 / Accepted: 28 November 2011 / Published online: 20 December 2011

Abstract Transthyretin (TTR) is a functional protein in the pancreatic β-cell. It promotes insulin release and protects against β-cell death. We now demonstrate by ligand blotting, adsorption to specific magnetic beads, and surface plasmon resonance that TTR binds to glucose-regulated proteins (Grps)78, 94, and 170, which are members of the endoplasmic reticulum chaperone family, but Grps78 and 94 have also been found at the plasma membrane. The effect of TTR on changes in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was abolished if the cells were treated with either dynasore, a specific inhibitor of dynamin GTPase that blocks clathrin-mediated endocytosis, or an antibody against Grp78, that prevents TTR from binding to Grp78. The conclusion is that TTR binds to Grp78 at the plasma membrane, is internalized into the β-cell via a clathrin-dependent pathway, and that this internalization is necessary for the effects of TTR on β-cell function.

Keywords Transthyretin · Pancreatic β-cell · Glucose-regulated proteins · Dynasore · Surface plasmon resonance

N. Dekki · E. Refai · R. Holmberg · M. Köhler · P.-O. Berggren · L. Juntti-Berggren (✉)
The Rolf Luft Research Center for Diabetes and Endocrinology, Karolinska Institutet, Karolinska University Hospital L1:03, 171 76 Stockholm, Sweden
e-mail: lisa.juntti-berggren@ki.se

E. Refai · H. Jörnvall
Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden

Introduction

Transthyretin (TTR) consists of four identical 14 kDa subunits [1]. It serves as a transport protein for thyroxine, and in association with retinol-binding protein, for retinol. The major sites for TTR synthesis are in the liver and the choroid plexus of the brain, but it is also synthesized within pancreatic islets [2, 3]. We have demonstrated that TTR has a functional role in the pancreatic β-cell [4]. Thus, β-cells, pre-treated with physiological concentrations of TTR, display a more pronounced increase in $[Ca^{2+}]_i$ than control cells upon stimulation with glucose and depolarizing concentrations of K^+. The changes in $[Ca^{2+}]_i$ were caused by an increase in Ca^{2+}-channel activity at −20 mV. The increase in $[Ca^{2+}]_i$ upon glucose stimulation is followed by an enhancement in insulin release, both in human and mouse β-cells [4]. TTR also protects against apoCIII induced β-cell death [4, 5]. There is evidence for internalization of TTR and for a TTR receptor in the plasma membrane (PM) in different cell types, but the receptor has not been identified [6–10]. We now demonstrate that TTR binds to Grp78 at the cell surface and is internalized by endocytosis.

Materials and methods

Preparation of cells

Pancreatic islets from ob/ob mice were isolated by a collagenase technique [11]. The medium used for both the isolation and the experiments was a HEPES buffer containing (in mM); 125 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 1.28 $CaCl_2$, and 3 glucose, pH 7.4. Bovine serum albumin was added to the medium at a concentration of 1 mg/ml.

Cell suspensions were prepared by washing the islets in phosphate buffered saline (PBS) without $MgCl_2$ and $CaCl_2$, followed by incubation with Versene 1:5,000 (Gibco) for 5 min. The islets were then disrupted into cells in an enzyme free Hanks based solution (Gibco). Cells were seeded onto glass coverslips or kept in suspension in RPMI 1640 culture medium, supplemented with 10% FCS. The islets from ob/ob mice contain about 95% β-cells. We have been using these mice during many years and tested that the cells have a normal function. We have also compared the effects of TTR in human and ob/ob mice islets and have found them to be similar [4].

Preparation of membrane and cytosol fractions from islet homogenates

Islets were homogenized on ice in 100 µl of homogenization buffer (HB) containing in mM: 20 HEPES, 1 $MgCl_2$, and 2 EDTA and a protease inhibitor cocktail (Roche). The homogenate was centrifuged at 1,000× *g* for 1 min and the supernatant was collected and centrifuged at 150,000× *g* in an ultracentrifuge for 40 min at 4°C. The supernatant, which contained the soluble proteins (cytosol) and the pellet (membranes), were collected and re-suspended in 100 µl HB. The protein concentration was measured with the Bradford protein determination assay.

TTR adsorption to Dynabeads RP C18

Magnetic beads (Dynabeads RP C18, Dynal, Invitrogen; 50 mg/ml) were washed with 0.1% trifluoroacetic acid (TFA). TTR, purified from human plasma (Sigma-Aldrich), (40 µg) was added to the magnetic beads and incubated at room temperature (RT) for 2 min. The beads were washed with 0.1% TFA and pre-incubated with cytosol or membrane fractions, for 1 h. Elution was made with 50 µl 60% acetonitrile (ACN) for 2 min. All incubation steps were performed at RT. Controls were made with C18 beads coupled to transferrin (40 µg) [6]. The eluted material was run on two NuPage gels (4–12%). One gel was stained with Coomassie Brilliant Blue and the other was transferred to a polyvinylidene diflouride (PVDF) membrane. The gel bands were manually excised and digested with trypsin using a MassPREP robotic protein handling system (Micromass/Waters), employing a protocol described [12]. Tryptic fragments were analyzed by MALDI-MS (Voyager DE-Pro, Applied Biosystems), using α-cyano-4-hydroxycinnamic acid as matrix (5 mg/ml in methanol:acetonitrile 1:1) and mixed 1:1 (vol/vol) with the sample. Database searches were carried out utilizing the Protein Prospector MS-Fit program. The bands from the PVDF were analyzed with an N-terminal sequencer (cLC, Applied Biosystems) for 15 cycles.

Alexa Fluor 488 labeling of TTR

TTR, at a concentration of 2 mg/ml, was transferred to a vial containing reactive Alexa Fluor 488 dye (Molecular Probes). The reaction mixture was stirred for 1 h at RT. TTR-bound Alexa 488 was purified from unbound Alexa 488 on a resin containing Bio-Rad Biogel P-30 fine. The protein complex was eluted with PBS. Unbound Alexa 488 was used for control experiments.

Digoxigenin labeling of TTR

TTR and Grp78 (Stressgen) were labeled with Digoxigenin-3-0-succinyl-ε-aminocaproic acid-*N*-hydroxy-succinimide ester (DIG-NHS) (Roche) to generate the labeled TTR (TTR-DIG). One mg TTR and 54 µg Grp78 were dissolved in 1 ml PBS each, and were allowed to react with 50 µl DIG-NHS for 2 h at RT during gentle stirring. Non-bound DIG-NHS was separated on a Sephadex G-25 column. Fractions of 0.5 ml were collected and the extinction of each fraction was measured at 280 nm. Fractions containing the highest degree of labeling were pooled and stored at −20°C until used.

Biotinylation of TTR

TTR, dissolved in PBS at a concentration of 1 mg/ml, was incubated at RT for 2.5 h at a ratio of 50:1 with *N*-hydroxysuccinimide-biotin dissolved in DMSO at a concentration of 20 mg/ml. The reaction was terminated by the addition of 3 M acetic acid. Labeled TTR was desalted using SepPak C18 and the eluate was purified by reverse phase HPLC. The fraction with biotinylated TTR was kept at −80°C until used.

Ligand blotting

Aliquots (20–40 µg protein) of equal amounts of protein from cytosol or membrane fractions were run on NuPage (4–12%) electrophoresis. Two wells for each fraction were loaded. The proteins were transferred onto a nitrocellulose membrane which was cut into two strips containing two lanes each. The strips were blocked for 4 h with 5% non-fatty powdered milk in TBS-T, or with blocking solution containing 4 µg/ml TTR, TTR-DIG or TTR-biotin. After washing, the membranes were incubated with polyclonal rabbit anti-human TTR antibody (1:500, DakoCytomation) overnight at 4°C, or with polyclonal sheep anti-DIG Fab fragments conjugated to alkaline phosphatase (1:5,000 in blocking solution) for 30 min, or with streptavidin-HRP (1:500) for 1 h at RT. After washing, the membranes were incubated with secondary antibodies, goat anti-rabbit IgG conjugated to HRP (1:50,000) or with a color substrate solution for alkaline phosphatase. Immunoreactive protein

bands could be visualized as blue precipitates using an ECL Plus™ immunoblotting detection system. The same samples were again electrophoresed and the gel was stained with Coomassie Brilliant Blue. The bands of interest were cut out, in-gel digested with trypsin and constituent peptide fragments mapped by MALDI mass spectrometry.

Grp78 and Grp94 characterization

Grp94 was identified using Western-blot analysis. An equal amount of protein (15 μg) from cytosol and membrane fractions and 1 μg of canine Grp94 (Stressgen) were run on NuPage (4–12%) electrophoresis and transferred to a PVDF membrane. The membrane was probed with a rabbit polyclonal anti-Grp94, also immunoreacting with canine Grp94, and re-probed with rabbit polyclonal anti-Grp78 (Stressgen, 1:2,000 and 1:500). The membrane was washed in TBS-T before incubation with secondary goat anti-rabbit IgG conjugated to HRP (1:50,000). The immunoreactive bands were visualized using ECL Plus™ immunoblotting detection system.

Surface plasmon resonance

The Biacore 3000 was used to evaluate the affinity and kinetics of TTR for Grps. Purified Grp78 and Grp94 proteins were immobilized on research grade sensor chip CM5 using amine coupling chemistry. The chip was activated with 0.05 M *N*-hydroxysuccinimide (NHS) and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Biacore AB). Excess reactive groups were deactivated with 1 M ethanolamine hydrochloride-NaOH, pH 8.5 (Biacore AB). HBS-EP buffer containing 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant p20 (Biacore AB) was used as running buffer and 10 mM glycine–HCl, pH 2, as regeneration buffer. 4000 RU were immobilized on flow cells (Fc) 2 (Grp78) and 4 (Grp94), while 1 and 2 were used as reference surfaces. For binding analysis, TTR was diluted in running buffer to the following concentrations (in nM): 5,000, 2,500, 1,250, 625, 312, and 0, after which it was injected at a flow rate of 20 μl/min over the Fcs for 2 min at 25°C. Between the injections, the surfaces were regenerated by one injection of regeneration buffer for 30 s at a flow rate of 30 μl/min. To correct for refractive index changes, instrument noise and bulk effects, the response data from Fc2 and 4 were submitted to subtractions in two steps. First, the response data from Fc 1 and 3 were subtracted from the response obtained from Fc 2 and 4. Second, the response data for the vehicle of the analyte (HBS-EP) was subtracted from the analytical response data. All experiments were done in duplicates and repeated three times. The evaluation was performed using BIA evaluation 3.2 (Biacore AB). Steady-state affinity interaction models were used to calculate the affinity constant K_D.

TTR-DIG internalization into the β-cell

β-cells were incubated with 8 μg/ml TTR-DIG for different time points: 15, 30, 60, 120, or 180 min. Before addition of TTR-DIG, the cells were kept at 4°C to normalize them at a level of the basal metabolism. The cells were washed with PBS and lysed in a lysis buffer (LB) containing 250 mM NaCl, 50 mM Tris(hydroxymethyl)aminomethane, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, and a protease inhibitor cocktail. Protein concentrations were measured with the Bradford protein determination assay and equal amounts of protein (13–20 μg) were mixed with NuPage sample buffer (Invitrogen), heated for 10 min at 70°C and run on NuPage (4–12%) gel electrophoresis (Invitrogen). The proteins were then transferred to a PVDF membrane. The membrane was first blocked in blocking solution and then incubated with polyclonal sheep anti-DIG Fab fragments conjugated to alkaline phosphatase (1:5,000 in blocking solution) for 30 min. Protein bands were detected with a color substrate solution for alkaline phosphatase containing nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt. Protein bands could be visualized as blue precipitates.

Detection of internalized TTR after pronase digestion

Isolated cells in suspension were incubated for 60 min with TTR-DIG. To verify that TTR had been internalized into the cells, surface bound proteins were removed by proteolytic digestion with 0.1 mg/ml pronase (Roche) in RPMI 1640 medium, containing 20 mM HEPES, pH 7.6. The cells were digested for 50 min at 4°C. The reaction was stopped by addition of an equal volume of 10 mg/ml BSA. The cells were then washed with PBS and lysed in LB. Protein concentration was measured with the Bradford assay and 10 μg of the protein was electrophoresed on NuPage (4–12%) gel, followed by transfer and detection of DIG.

Detection of TTR-DIG and TTR-Alexa 488 in single β-cells

β-cells attached to coverslips were incubated with 8 μg/ml TTR-DIG, 86 μM TTR-Alexa 488 or only Alexa 488 for 15 min, 60 min, and overnight in RPMI 1640 culture medium. Before addition of TTR-DIG, β-cells were kept at 4°C. The cells were washed in PBS and fixed in 4% formaldehyde. Permeabilization was done with 0.3% Tween 20. Detection of DIG was done as described above,

with a concentration of sheep anti-DIG Fab fragments of 1:3,000. Control cells were incubated with Levamisole (1:200) to inhibit endogenous alkaline phosphatase. Alkaline phosphatase precipitates in the β-cells were visualized in a light microscope (Leica). The TTR-Alexa 488 incubated cells were washed in HEPES buffer and the coverslips were mounted in an open chamber with a high rubber ring to keep the cells alive in buffer. The cells were visualized in a TCS SP2 confocal microscope (Leica Microsystems).

Clathrin-coated vesicle preparation

Isolated β-cells (cooled to 4°C) from 5 to 6 ob/ob mice were incubated with 8 μg/ml TTR-DIG and 2 μg/ml Grp78-DIG for 15 min at 37°C. Preparation of a clathrin-coated vesicle (CCV) fraction was performed as previously described [13]. Briefly, after washing the β-cells with PBS, homogenization was performed using a motor pestle homogenizer in a buffer containing 0.1 mM M-2-(*N*-morpholino) ethanesulfonic acid, 1 mM EDTA, 0.5 mM $MgCl_2$, 0.2 mg/ml NaN_3, and protease inhibitors, pH 6.5. The homogenate was centrifuged at 85,000×*g* for 1 h, and the pellet was resuspended in the same buffer and applied to a discontinuous sucrose gradient: 60, 50, 40, 10, and 5%. Samples were centrifuged at 80,000×*g* for 75 min and collected from the 10–40% interface. They were then washed in homogenization buffer and further centrifuged at 85,000×*g* for 1 h. Wheat germ agglutinin was added at a concentration of 1 mg to 10 mg protein and incubated overnight at 4°C. The agglutinated material was sedimented at 20,000×*g* for 15 min. The supernatant, containing the CCV fraction, was run on a NuPage (4–12%) gel electrophoresis and transferred to a PVDF membrane. The upper part of the membrane was incubated with primary mouse anti-human clathrin monoclonal antibodies (1:500, Oxford Biotechnology) overnight at 4°C. After washing with Tris-buffered saline with 0.05% Tween (TBS-T) the membrane was incubated with goat anti-mouse IgG conjugated to horse radish peroxidase (HRP) (1:50,000, Bio Rad) and visualized using the ECL Plus™ immunoblotting detection system (Amersham Pharmacia Biotech). The protein bands between 3 and 98 kDa of the membrane were visualized using the DIG detection system as described above.

Measurements of $[Ca^{2+}]_i$

Pancreatic β-cells from ob/ob mice were attached to coverslips and kept for 5 h in RPMI 1640 culture medium with 10% FCS. Thereafter the cells were washed and incubated for 1 h in medium without FCS with one of the following additions; 150 mg/l TTR, 150 mg/l TTR and 80 μM dynasore or the vehicle for TTR (H_2O) with or without 80 μM dynasore. After incubation, the coverslips were moved to RPMI medium with 10% FCS and left overnight in an incubator. In the experiments with the Grp78- and Grp94 antibodies, the cells were cultured in RPMI medium with 10% FCS and TTR or the vehicle in the presence or absence of 5 μg/ml of the respective antibody. The antibodies were added 20 min before TTR or the vehicle and left in the media during the following 1 h incubation followed by an overnight incubation in RPMI medium with 10% FCS. After the incubations the cells were loaded with 2 μM fura-2/AM (Molecular Probes) for 30 min. Changes in $[Ca^{2+}]_i$ subsequent to depolarization with 25 mM KCl, were measured using a Spex Fluorolog-2 system connected to an inverted epi-fluorescence microscope (Zeiss, Axiovert 35 M). The emissions of the two excitation wavelengths of 340 and 380 nm were used to calculate the fluorescence ratio 340/380, reflecting changes in $[Ca^{2+}]_i$.

Results

TTR interactions with Grps

TTR bound to Dynabeads RP C18 was incubated with the cytosol and membrane fractions from mouse pancreatic islet homogenates. Eluates from the Dynabeads were separated on two NuPage-gels, one for in-gel digestion and fragment mass mapping by MALDI mass spectrometry, the other for transfer to a PVDF membrane and N-terminal sequencer analysis. For the membrane fraction, protein bands of high molecular weight were observed upon the PAGE analysis. Mass analysis of the fragments from protein bands 1, 2 and 3 (Fig. 1a) revealed that those components consisted of the glucose-regulated proteins Grp78, Grp94, and Grp170 (Fig. 1a). The results from sequence analysis for 15 cycles yielded the same protein identifications (Fig. 1b). In both the PAGE gel and the PVDF adsorbed material, TTR was observed, migrating as mono-, di-, and trimers. A preparation of the membrane fraction with transferrin, instead of TTR, bound to the beads did not show analogous binding proteins (Fig. 1c). For the cytosol fraction, the same type of analysis identified a band at 98 kDa as protein Grp94.

Ligand blotting with TTR and anti-TTR, or TTR-DIG for visualization showed TTR-binding proteins with molecular masses of approximately 70, 98, and 150–190 kDa in both the cytosol and membrane fractions (Fig. 2a, b). Similar results were also obtained using biotinylated-TTR (Fig. 2c) and antibodies against Grp78 and Grp94 (Fig. 2d).

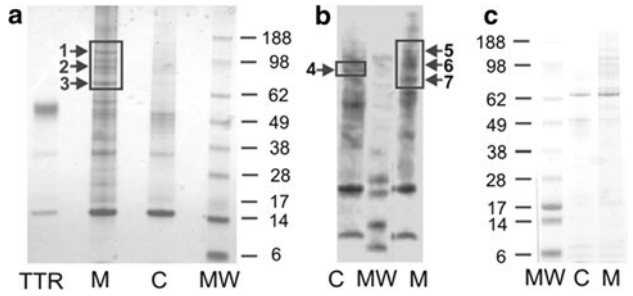


Fig. 1 Interaction of TTR with proteins in the membrane and cytosol fractions. Eluates from TTR or transferrin-coupled magnetic Dynabeads RP C18 incubated with cytosol and membrane fractions were submitted to electrophoresis in two separate gels. The proteins in one NuPage-gel were transferred to a PVDF membrane. The protein bands in the SDS-gels (**a** and **c**) and PVDF membrane (**b**), respectively, were stained with Coomassie Brilliant blue for sequence analysis. *Arrows* show the bands of interest. Protein bands 1, 2, and 3 correspond to Grp170, Grp94, and Grp78, respectively, and 4, 5, 6, and 7 to Grp94, Grp170, Grp94, and Grp78, respectively (**a** and **b**). Transferrin, migrating as a protein of a molecular weight of approximately 70 kDa, did not show similar protein binding pattern as TTR (**c**). M = membrane, C = cytosol and MW = molecular weight. Representative of two experiments

To further evaluate the interaction between TTR and Grps, a surface plasmon resonance assay was developed using immobilized Grp78 or Grp94 as ligand and TTR as analyte. Binding responses were obtained and were reproducibly concentration-dependent ($n = 3$). Binding curves, for five different concentrations of TTR flowing over Grp78 or Grp94 coated cells, show that the binding to both Grp78 and 94 is fast with an association rate (k_a) of 2.02×10^5 and 1.37×10^5 1/Ms, respectively (Fig. 3a, b). The dissociation is also fast as seen from the sensorgrams (Fig. 3a, b). The interactions have weak affinity between the ligand and the analyte, with affinity constants (K_D) within the micromolar range, 5.0 μM for Grp78 and 7.3 μM for Grp94, respectively ($n = 3$).

Upon coincubation of β-cells with TTR and an antibody against Grp78, the TTR effect on $[Ca^{2+}]_i$ was abolished (Fig. 4a), while this was not observed with an antibody against Grp94 (Fig 4b), indicating that TTR binds to Grp78 at the cell membrane.

Time-dependent internalization of TTR into β-cells

Internalization of TTR was monitored by incubation of β-cells with DIG- labeled TTR for different time-points. The DIG detection system was used to enhance the detection efficacy of TTR. Bands, representing the monomer and dimer of TTR, could be visualized after 15 min, indicating a rapid uptake of the protein. The maximal uptake was found after incubation for 60 min (Fig. 5a). No unspecific binding of the anti-DIG antibody could be visualized in the lane containing cell homogenate without the labeled TTR (Fig. 5a lane 1). The internalization was also studied at the single cell level using immunocytochemistry in TTR-DIG incubated fixed β-cells and TTR-Alexa 488 exposure of living β-cells. Quantification of TTR-internalization by counting 1,000 cells twice from two separate isolations, showed that 13% of the β-cells had internalized TTR after 15 min incubation time (Fig. 6b) and 22% after 60 min (Fig. 6c). TTR-Alexa 488 was still present after overnight incubations and displayed punctuate structures throughout the cell. (Fig. 6d).

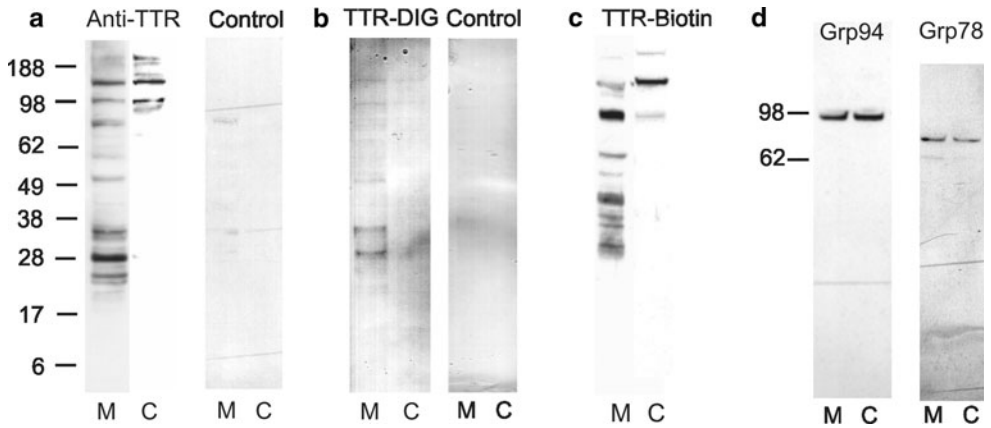


Fig. 2 Ligand blotting of TTR binding of Grps. Ligand blotting shows the presence of TTR binding proteins at molecular weights from approximately 80–190 kDa. TTR binding proteins in cytosol and membrane fractions were detected with **a** TTR- or **b** TTR-DIG incubation, followed by immuno- or DIG detection. No protein bands were detected when omitting TTR or TTR-DIG incubation step. **c** TTR binding proteins in cytosol and membrane fractions were detected with biotinylated TTR. **d** For comparison, Grp78 and Grp94 were identified in cytosol and membrane fractions and commercial Grp94 verified the exact molecular weight. Identification of the protein in the bands of interest was done by MALDI mass-spectrometry. M = membrane and C = cytosol. The experiment was repeated three times

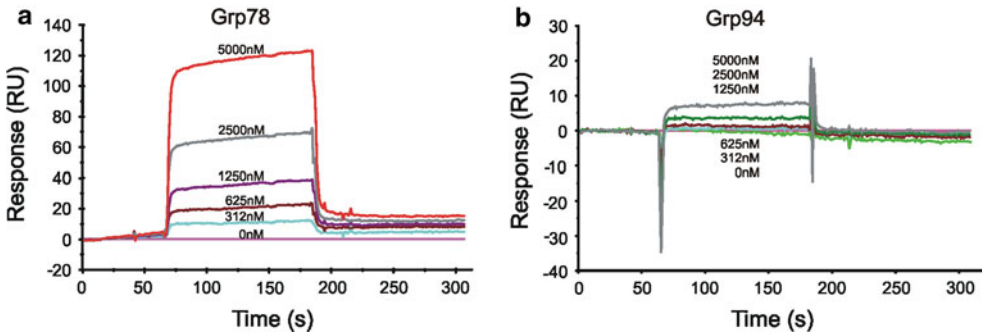


Fig. 3 Interaction between TTR and Grp78 and Grp94 measured with surface plasmon resonance. Sensorgrams from the interaction between TTR and Grp78 and Grp94. Representative sensorgrams obtained from duplicate injections of TTR at a concentration of 5,000, 2,500, 1,250, 625, 312, and 0 nM over a surface of immobilized Grp78 (**a**) and/or Grp94 (**b**) ($n = 3$)

Clathrin-dependent internalization of TTR into the β-cells

To study the TTR and Grp78 internalization process CCV were isolated from β-cells after 15 min pre-incubation with TTR-DIG and Grp78-DIG. Immunodetection of clathrin in the CCV fraction then showed a band at approximately 190 kDa, representing the clathrin heavy chain, together with TTR (Fig. 5c) and Grp78 (Fig. 5d) also present. Since TTR is internalized after 15 min and CCV rapidly shed their clathrin coats after formation, this indicates that TTR is endocytosed. After post-incubation treatment of the β-cells with pronase, TTR was still present, indicating that it was indeed internalized and not just present on the cell surface. A further support for TTR endocytosis is the fact that in β-cells coincubated with TTR and dynasore [14], a compound known to inhibit dynamin GTPase and hence endocytosis, the TTR effect on the changes in $[Ca^{2+}]_i$ was abolished (Fig. 4c).

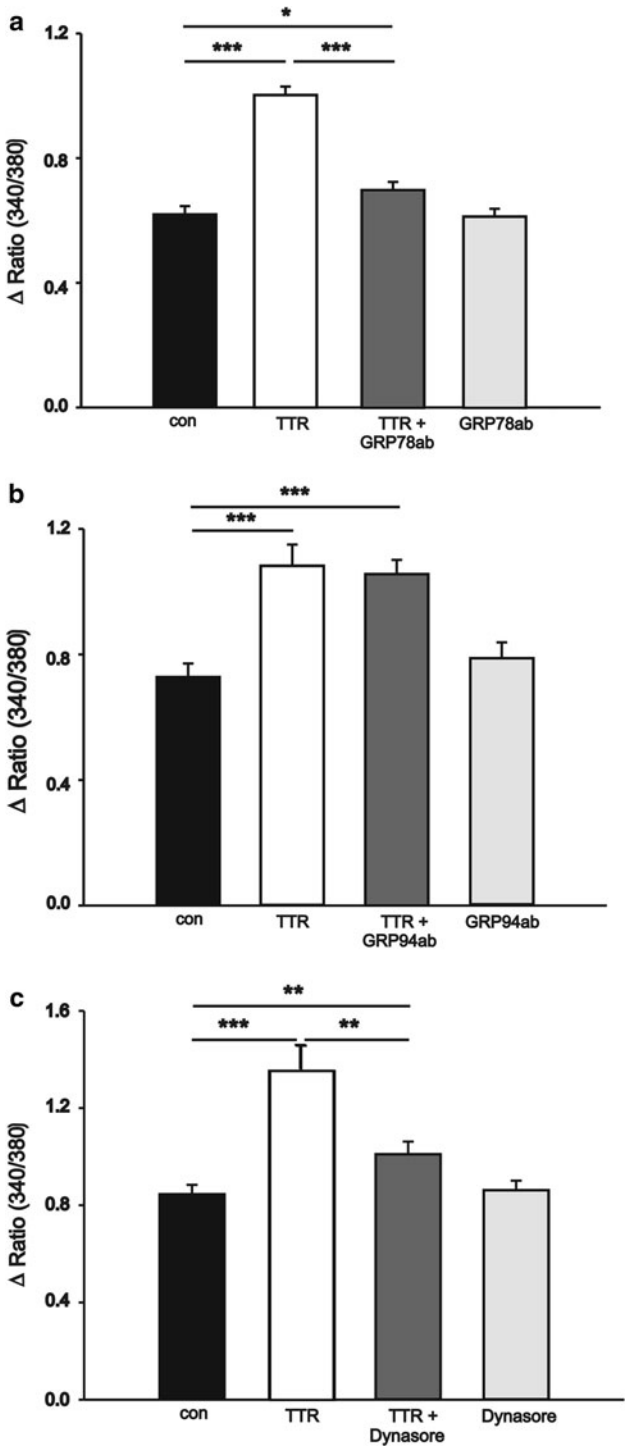
Discussion

In the present study, we investigated mechanisms whereby TTR affects the function of the pancreatic β-cell. Membrane associated proteins of 90, 100, and 115 kDa have earlier been proposed as receptor proteins involved in endocytosis of TTR, although they have not been identified [8–10, 15]. Megalin is a member of the low-density lipoprotein receptor family [16] and it has been shown to be involved in renal and sensory neuronal uptake of TTR [17, 18]. Megalin is an endocytotic receptor expressed in clathrin-coated pits and binds the receptor-associated protein (RAP) which functions as a chaperone. To date, megalin expression has not been found in either endo- or exocrine pancreas. TTR has also been proven to bind to the lysosomal associated membrane protein 1 (LAMP-1) in the circulation and is therefore in a possible interaction with lysosomes [19]. We could demonstrate that TTR binds to partly cytosolic and partly membrane-associated proteins of approximately 70, 100, and 150 kDa. Sequence analysis and mass-determination revealed these proteins to be Grp78, Grp94, and Grp170. They are molecular chaperons of the heat shock protein family and are known as stress proteins, located in the endoplasmic reticulum (ER) [20] and induced by oxidative stress, defective Ca^{2+} homeostasis and glucose deprivation [21]. They are also Ca^{2+}-binding proteins [22–24] and are co-regulators of Ca^{2+}-homeostasis in the cell, affording protection against non-physiological increases in $[Ca^{2+}]_i$ and consequent cell death [25–28].

Grp78 and Grp94 have trans-membrane domains that span the ER and can therefore be seen in both membrane and lumenal cellular fractions [29, 30]. Consequently, they can bind to proteins in the membrane, cytosol, and lumen of the ER.

Grp170 is known to associate with Grp78 and/or Grp94 suggesting that these three Grps may function as a multimeric complex [31–33]. Antibodies against Grp170 were not commercially available, which restricted our ability to evaluate the existence of this protein in the membrane fraction. However, ligand blotting showed clear bands at approximately 150–190 kDa. Mass determinations verified that the protein in these bands was Grp170 present in both the cytosol and membrane fractions. These data suggest that there may be an interaction between TTR and Grp78, Grp94, and Grp170 in the membrane fraction and between Grp94 and Grp170 in the cytosol fraction of the pancreatic β-cell. Surface plasmon resonance shows that TTR binds to both Grp78 and Grp94 with K_Ds in the micromolar range. The fast on- and off rates that we observe indicate that the protein–protein interaction is weak and that the interactions are reversible. Of interest to note is that in previous studies where TTR binding to other proteins has been investigated, although using other methods, the K_D with thyroxine was reported to be 10–14 nM, with metallothionein-2 245 nM, and with retinol-binding protein 350–800 nM [34–36].

Grps are localized to the ER and it has been demonstrated that mutant TTRs interact with Grp78 [37, 38]. When Grp78



◀ **Fig. 4** Dynasore and an antibody against Grp78 block the effect of TTR on $[Ca^{2+}]_i$. Pancreatic β-cells from mice were exposed to TTR and (**a**) in the presence and absence of an antibody against Grp78 ($n = 42$ for control cells, $n = 33$ for TTR treated, $n = 56$ for TTR + Grp78 antibody treated and $n = 49$ for cells incubated with the antibody alone), (**b**) in the presence and absence of an antibody against Grp94 ($n = 41$ for control cells, $n = 22$ for TTR treated, $n = 33$ for TTR + Grp94 antibody treated and $n = 27$ for cells incubated with the antibody alone), and (**c**) in the presence and absence of dynasore ($n = 37$ for control cells, $n = 32$ for TTR treated, $n = 30$ for TTR + dynasore treated and $n = 24$ for cells incubated with dynasore) Changes in $[Ca^{2+}]_i$ were measured when the cells were depolarized with high concentrations of K^+. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

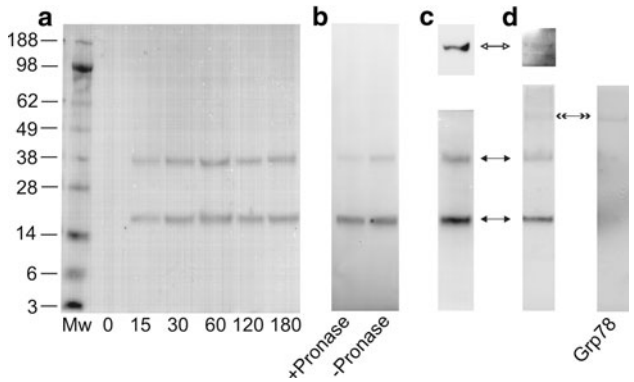


Fig. 5 Time-dependent internalization of TTR and involvement of clathrin-mediated endocytosis. Internalization of TTR-DIG at different time-points. TTR can be detected already after 15 min with a maximum uptake at 60 min. The control lane, with no addition of TTR-DIG, (0 min) shows no presence of TTR. **b** TTR was still detected in cells treated with pronase, showing that TTR is in fact endocytosed and is not only present at the plasma membrane of the β-cell. **c** TTR-DIG was present in the enriched CCV fraction of β-cells after 15 min of incubation. **d** TTR-DIG and Grp78-DIG were both present in the enriched CCV fraction. *Closed arrows* show the TTR dimer and monomer; the *double arrow* shows Grp78 and the *open arrow* shows the position of the clathrin heavy chain

was coexpressed in *Escherichia coli* with the mutant form DIG18G TTR, known to be involved in amyloidosis in the central nervous system, the two proteins formed a complex, while wild-type (wt) TTR was not captured by Grp78 [37]. Also in mammalian HeLa cells D18G TTR and amyloidogenic M-TTRs were retained within ER by binding to Grp78 [38]. These results confirm that Grp78 in the ER functions as a sequestering, protective protein and facilitates degradation of harmful, mutated TTR. However, a number of studies have revealed the presence of Grp78 and Grp94 in the PM of different cell types [39–48]. In liver cells, Grp94 was observed at the PM in coated pits [41], compatible with involvement in receptor-mediated endocytosis, a process described for several receptors [49]. Our results support the suggestion that Grp78, but not Grp94, is expressed in the PM also in pancreatic β-cells, since the effect of wt TTR, mainly existing as a tetramer, on changes in $[Ca^{2+}]_i$ was abolished when the cells were coincubated with TTR and an antibody against Grp78. Grp78 has previously been identified to be the cell surface signaling receptor for α_2-macroglobulin in macrophages [47, 48]. Taken together all data show that Grp78 can have different functions depending on the cellular location.

Time course studies of the uptake of TTR showed that it was rapidly internalized into the β-cell. These data are in

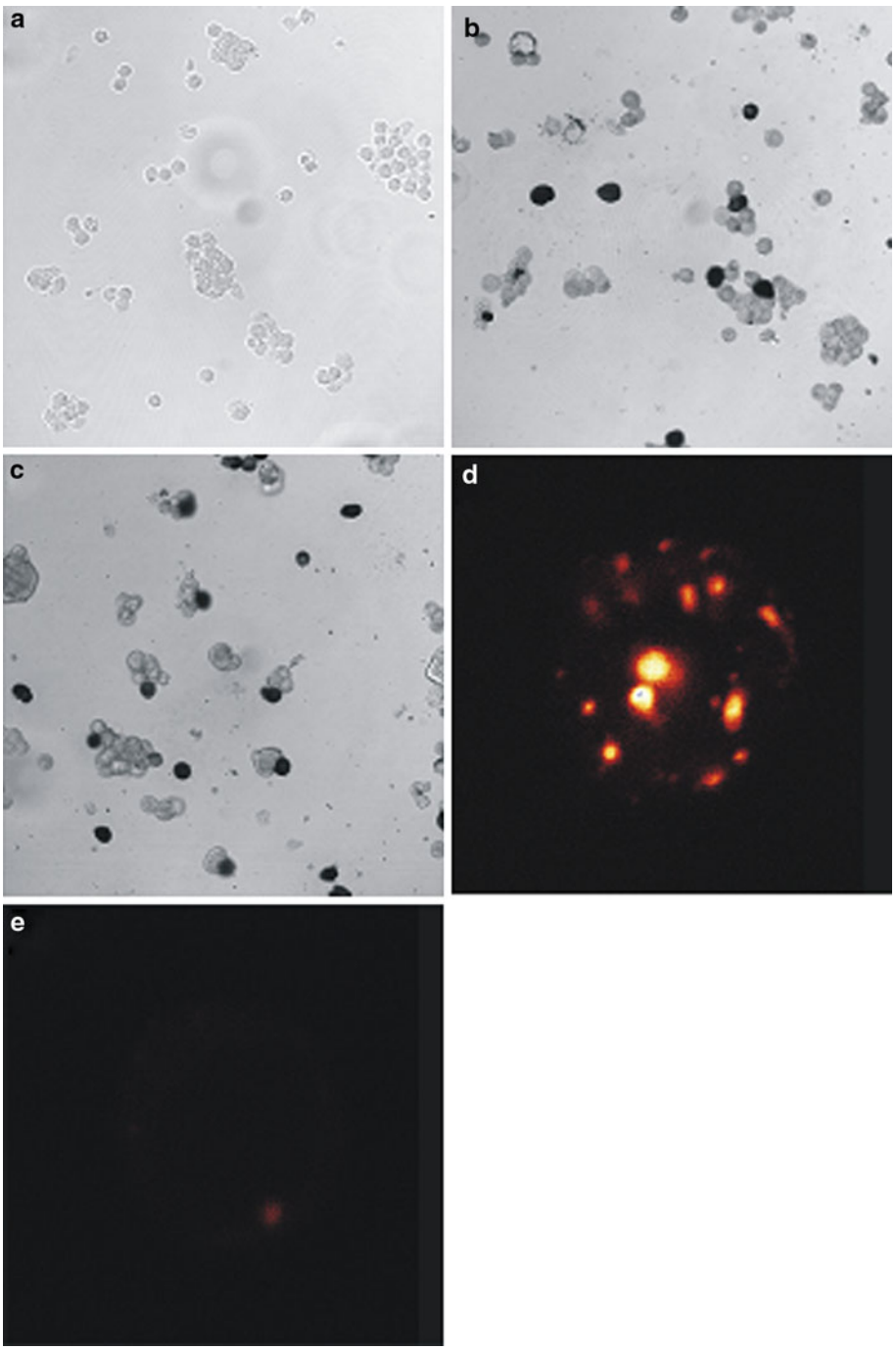


Fig. 6 Cellular distribution of labeled TTR. Distribution of TTR-DIG in fixed cells (**b** and **c**) and of TTR-Alexa 488 in living cells (**d**). Incubation with TTR-DIG show 13% stained cells after 15 min (**b**) and 22% after 60 min (**c**). TTR was still present in specific compartments in the β-cells after overnight incubation with TTR-Alexa 488 (**d**). Control cells, with no addition of TTR, do not show the presence of TTR (**a** and **e**). Representative of two experiments

line with studies of an ependymoma cell line [9]. The internalization was also proven with immunocytochemistry which revealed a higher percentage of stained cells after 60 min than after 15 min. β-cells pre-treated overnight with TTR coupled to Alexa Fluor 488 showed clear punctuate vesicular structures throughout the living cells. In our previous experiments, we saw functional effects of TTR after overnight pre-incubation of β-cells with the protein [4]. The present data verify that TTR is still present in the β-cell after this time. The relatively low number of positive cells with the DIG-detection system can depend on several methodological factors. There is a technical difference between the methods in the sense that cells are attached to coverslips when TTR-DIG is used, but are in suspension and slowly rotating when exposed to the fluorophore. When comparing TTR-DIG treated cells with untreated cells, there was a slightly more intense blue color in all treated cells. However, the only cells that were

counted were those where we could see cell parts clearly stained with the chromophore.

Endocytosis is a cellular process by which eukaryotic cells internalize material from the extracellular medium. A specific and saturable uptake of TTR in hepatomas and astrocytes, consistent with the existence of a TTR-receptor in the PM has been reported [6, 7]. The initial step in receptor-mediated endocytosis occurs through different mechanisms, where the best-characterized one is via clathrin-coated pits. Most receptor-ligand complexes accumulate at clathrin-coated pits in the PM. The cargo material is invaginated in budding vesicles at the PM, yielding CCV, which are thus responsible for most of the receptor-mediated endocytosis from the PM [50–52]. After the vesicles bud off from the PM, the vesicles rapidly shed their clathrin coats before fusing with a cell organelle. This enables the cell to internalize receptor-bound ligands and deliver them to different cell organelles. Internalization of TTR has been described in chicken oocytes to occur through a clathrin-dependent pathway mediated by a specific receptor [8, 15]. Smith et al. [53] showed the presence of TTR in coated-pits in choriocapillaries. However, in another study with an ependymoma cell line, the endocytosis of TTR was suggested to be clathrin-independent and their experiments revealed the presence of a putative TTR receptor on the cell membrane [9]. Since the most common pathway of receptor-mediated endocytosis occurs through CCV, it seems unlikely that TTR should not be detected in the clathrin-coated vesicle fraction during the preparation. The discrepancy between the studies is probably due to a too long pre-incubation time with TTR, of 30 and 60 min, used in the latter study. During this time, most of the endocytosed CCV shed their clathrin coats and cannot easily be detected. To investigate the internalization of TTR and Grp78 in pancreatic β-cells, we enriched CCVs from cells that had been pre-incubated with TTR and Grp78 for a maximum time of 15 min. Distinct protein bands of TTR, migrating as a monomer and dimer, and Grp78 could be observed in the enriched CCV fraction. The Grp78 protein band was confirmed by inclusion of a control lane with only Grp78-DIG. Another proof for a clathrin-dependent internalization of TTR, and also for that TTR exerts its effects in the intracellular compartment, is that dynasore, an inhibitor of dynamin GTPase [14], abolishes the effects of TTR.

In conclusion, we have found that TTR binds to Grp78 at the PM and that both proteins are internalized via a clathrin-dependent pathway. Our data also suggest that the effects of TTR on β-cell function are dependent on this internalization process.

Acknowledgments We thank Prof Tomas Kirchhausen, Harvard Medical School, who kindly provided us with dynasore, synthesized by Dr Henry E Pelish. This work was supported by grants from The Family Erling-Persson Foundation, The Swedish Research Council, the Novo Nordisk Foundation, Swedish Diabetes Association, Barndiabetesfonden, Karolinska Institutet, Magnus Bergwalls Foundation, The Knut and Alice Wallenberg Foundation, the Skandia Insurance Company Ltd., VIBRANT (FP7-228933-2), Strategic Research Program in Diabetes at Karolinska Institutet and Berth von Kantzow's Foundation.

References

1. Blake CC (1981) Prealbumin and the thyroid hormone nuclear receptor. Proc R Soc Lond B Biol Sci 211:413–431
2. Jacobsson B, Carlström A, Platz A, Collins VP (1990) Transthyretin messenger ribonucleic acid expression in the pancreas and in endocrine tumors of the pancreas and gut. J Clin Endocrinol Metab 71:875–880
3. Itoh N, Hanafusa T, Miyagawa J, Tamura S, Inada M, Kawata S, Kono N, Tarui S (1992) Transthyretin (prealbumin) in the pancreas and sera of newly diagnosed type I (insulin-dependent) diabetic patients. J Clin Endocrinol Metab 74:1372–1377
4. Refai E, Dekki N, Yang SN, Imreh G, Cabrera O, Yu L, Yang G, Norgren S, Rossner SM, Inverardi L, Ricordi C, Olivecrona G, Andersson M, Jörnvall H, Berggren PO, Juntti-Berggren L (2005) Transthyretin constitutes a functional component in pancreatic beta-cell stimulus-secretion coupling. Proc Natl Acad Sci USA 102:17020–17025
5. Juntti-Berggren L, Refai E, Appelskog I, Andersson M, Imreh G, Dekki N, Uhles S, Yu L, Griffiths WJ, Zaitsev S, Leibiger I, Yang SN, Olivecrona G, Jörnvall H, Berggren PO (2004) Apolipoprotein CIII promotes Ca^{2+}-dependent beta cell death in type 1 diabetes. Proc Natl Acad Sci USA 101:10090–10094
6. Divino CM, Schussler GC (1990) Receptor-mediated uptake and internalization of transthyretin. J Biol Chem 265:1425–1429
7. Divino CM, Schussler GC (1990) Transthyretin receptors on human astrocytoma cells. J Clin Endocrinol Metab 71:1265–1268
8. Vieira AV, Sanders EJ, Schneider WJ (1995) Transport of serum transthyretin into chicken oocytes. A receptor-mediated mechanism. J Biol Chem 270:2952–2956
9. Kuchler-Bopp S, Dietrich JB, Zaepfel M, Delaunoy JP (2000) Receptor-mediated endocytosis of transthyretin by ependymoma cells. Brain Res 870:185–194
10. Sousa MM, Saraiva MJ (2001) Internalization of transthyretin. Evidence of a novel yet unidentified receptor-associated protein (RAP)-sensitive receptor. J Biol Chem 276:14420–14425
11. Lernmark A (1974) The preparation of, and studies on, free cell suspensions from mouse pancreatic islets. Diabetologia 10:431–438
12. Hirschberg D, Tryggvason S, Gustafsson M, Bergman T, Swedenborg J, Hedin U, Jörnvall H (2004) Identification of endothelial proteins by MALDI-MS using a compact disc microfluidic system. Protein J 23:263–271
13. Hammond TG, Verroust PJ (1994) Trafficking of apical proteins into clathrin-coated vesicles isolated from rat renal cortex. Am J Physiol 266:554–562
14. Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T (2006) Dynasore, a cell-permeable inhibitor of dynamin. Dev Cell 10:839–850
15. Vieira AV, Schneider WJ (1993) Transport and uptake of retinol during chicken oocyte growth. Biochim Biophys Acta 1169:250–256
16. Hussain MM, Strickland DK, Bakillah A (1999) The mammalian low-density lipoprotein receptor family. Annu Rev Nutr 19:141–172

17. Sousa MM, Norden AG, Jacobsen C, Willnow TE, Christensen EI, Thakker RV, Verroust PJ, Moestrup SK, Saraiva MJ (2000) Evidence for the role of megalin in renal uptake of transthyretin. J Biol Chem 275:38176–38181
18. Fleming CE, Mar FM, Franquinho F, Saraiva MJ, Sousa MM (2009) Transthyretin internalization by sensory neurons is megalin mediated and necessary for its neuritogenic activity. J Neurosci 29:3220–3232
19. Chang MH, Hua CT, Isaac EL, Litjens T, Hodge G, Karageorgos LE, Meikle PJ (2004) Transthyretin interacts with the lysosome-associated membrane protein (LAMP-1) in circulation. Biochem J 382:481–489
20. Ni M, Lee AS (2007) ER chaperones in mammalian development and human diseases. FEBS Lett 581:3641–3651
21. Lee AS (1992) Mammalian stress response: induction of the glucose-regulated protein family. Curr Opin Cell Biol 4:267–273
22. Villa A, Podini P, Clegg DO, Pozzan T, Meldolesi J (1991) Intracellular Ca^{2+} stores in chicken Purkinje neurons: differential distribution of the low affinity-high capacity Ca^{2+} binding protein, calsequestrin, of Ca^{2+} ATPase and of the ER lumenal protein, Bip. J Cell Biol 113:779–791
23. Lievremont JP, Rizzuto R, Hendershot L, Meldolesi J (1997) BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of Ca^{2+}. J Biol Chem 272:30873–30879
24. Macer DR, Koch GL (1988) Identification of a set of calcium-binding proteins in reticuloplasm, the luminal content of the endoplasmic reticulum. J Cell Sci 91(Pt 1):61–70
25. Liu H, Miller E, van de Water B, Stevens JL (1998) Endoplasmic reticulum stress proteins block oxidant-induced Ca^{2+} increases and cell death. J Biol Chem 273:12858–12862
26. McCormick TS, McColl KS, Distelhorst CW (1997) Mouse lymphoma cells destined to undergo apoptosis in response to thapsigargin treatment fail to generate a calcium-mediated grp78/grp94 stress response. J Biol Chem 272:6087–6092
27. Miyake H, Hara I, Arakawa S, Kamidono S (2000) Stress protein GRP78 prevents apoptosis induced by calcium ionophore, ionomycin, but not by glycosylation inhibitor, tunicamycin, in human prostate cancer cells. J Cell Biochem 77:396–408
28. Morris JA, Dorner AJ, Edwards CA, Hendershot LM, Kaufman RJ (1997) Immunoglobulin binding protein (BiP) function is required to protect cells from endoplasmic reticulum stress but is not required for the secretion of selective proteins. J Biol Chem 272:4327–4334
29. Reddy RK, Mao C, Baumeister P, Austin RC, Kaufman RJ, Lee AS (2003) Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. J Biol Chem 278:20915–20924
30. Kang HS, Welch WJ (1991) Characterization and purification of the 94-kDa glucose-regulated protein. J Biol Chem 266:5643–5649
31. Lin HY, Masso-Welch P, Di YP, Cai JW, Shen JW, Subjeck JR (1993) The 170-kDa glucose-regulated stress protein is an endoplasmic reticulum protein that binds immunoglobulin. Mol Biol Cell 4:1109–1119
32. Melnick J, Aviel S, Argon Y (1992) The endoplasmic reticulum stress protein GRP94, in addition to BiP, associates with unassembled immunoglobulin chains. J Biol Chem 267:21303–21306
33. Meunier L, Usherwood YK, Chung KT, Hendershot LM (2002) A subset of chaperones and folding enzymes form multiprotein complexes in endoplasmic reticulum to bind nascent proteins. Mol Biol Cell 13:4456–4469
34. Chang L, Munro SL, Richardson SJ, Schreiber G (1999) Evolution of thyroid hormone binding by transthyretins in birds and mammals. Eur J Biochem 259:534–542
35. Goncalves I, Quintela T, Baltazar G, Almeida MR, Saraiva MJ, Santos CR (2008) Transthyretin interacts with metallothionein 2. Biochemistry 47:2244–2251
36. Zanotti G, Folli C, Cendron L, Alfieri B, Nishida SK, Gliubich F, Pasquato N, Negro A, Berni R (2008) Structural and mutational analyses of protein–protein interactions between transthyretin and retinol-binding protein. Febs J 275:5841–5854
37. Sorgjerd K, Ghafouri B, Jonsson BH, Kelly JW, Blond SY, Hammarstrom P (2006) Retention of misfolded mutant transthyretin by the chaperone BiP/GRP78 mitigates amyloidogenesis. J Mol Biol 356:469–482
38. Susuki S, Sato T, Miyata M, Momohara M, Suico MA, Shuto T, Ando Y, Kai H (2009) The Endoplasmic reticulum-associated degradation of transthyretin variants is negatively regulated by BiP in mammalian cells. J Biol Chem 284:8312–8321
39. Arap MA, Lahdenranta J, Mintz PJ, Hajitou A, Sarkis AS, Arap W, Pasqualini R (2004) Cell surface expression of the stress response chaperone GRP78 enables tumor targeting by circulating ligands. Cancer Cell 6:275–284
40. Calvert ME, Digilio LC, Herr JC, Coonrod SA (2003) Oolemmal proteomics–identification of highly abundant heat shock proteins and molecular chaperones in the mature mouse egg and their localization on the plasma membrane. Reprod Biol Endocrinol 1:27
41. de Crom R, van Haperen R, Janssens R, Visser P, Willemsen R, Grosveld F, van der Kamp A (1999) Gp96/GRP94 is a putative high-density lipoprotein-binding protein in liver. Biochim Biophys Acta 1437:378–392
42. Delpino A, Castelli M (2002) The 78 kDa glucose-regulated protein (GRP78/BIP) is expressed on the cell membrane, is released into cell culture medium and is also present in human peripheral circulation. Biosci Rep 22:407–420
43. Reddy RK, Lu J, Lee AS (1999) The endoplasmic reticulum chaperone glycoprotein GRP94 with Ca(2 +)-binding and anti-apoptotic properties is a novel proteolytic target of calpain during etoposide-induced apoptosis. J Biol Chem 274:28476–28483
44. Robert J, Menoret A, Cohen N (1999) Cell surface expression of the endoplasmic reticular heat shock protein gp96 is phylogenetically conserved. J Immunol 163:4133–4139
45. Triantafilou M, Fradelizi D, Triantafilou K (2001) Major histocompatibility class one molecule associates with glucose regulated protein (GRP) 78 on the cell surface. Hum Immunol 62:764–770
46. Wiest DL, Bhandoola A, Punt J, Kreibich G, McKean D, Singer A (1997) Incomplete endoplasmic reticulum (ER) retention in immature thymocytes as revealed by surface expression of "ER-resident" molecular chaperones. Proc Natl Acad Sci USA 94:1884–1889
47. Misra UK, Gonzalez-Gronow M, Gawdi G, Hart JP, Johnson CE, Pizzo SV (2002) The role of Grp 78 in alpha 2-macroglobulin-induced signal transduction. Evidence from RNA interference that the low-density lipoprotein receptor-related protein is associated with, but not necessary for, GRP 78-mediated signal transduction. J Biol Chem 277:42082–42087
48. Misra UK, Gonzalez-Gronow M, Gawdi G, Wang F, Pizzo SV (2004) A novel receptor function for the heat shock protein Grp78: silencing of Grp78 gene expression attenuates alpha2 M*-induced signalling. Cell Signal 16:929–938
49. Brown MS, Goldstein JL (1986) A receptor-mediated pathway for cholesterol homeostasis. Science 232:34–47
50. Brodsky FM, Chen CY, Knuehl C, Towler MC, Wakeham DE (2001) Biological basket weaving: formation and function of clathrin-coated vesicles. Annu Rev Cell Dev Biol 17:517–568
51. Goldstein JL, Anderson RG, Brown MS (1979) Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature 279:679–685

52. Trowbridge IS, Collawn JF, Hopkins CR (1993) Signal-dependent membrane protein trafficking in the endocytic pathway. Annu Rev Cell Biol 9:129–161

53. Smith SS, Pino RM, Thouron CL (1989) Binding and transport of transthyretin-gold by the endothelium of the rat choriocapillaris. J Histochem Cytochem 37:1497–1502