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**GRP78/BiP is Involved in Ouabain-induced Endocytosis of the Na/K-ATPase
in LLC-PK1 Cells**

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INTRODUCTION

Na/K-ATPase, or sodium pump, is a ubiquitous plasma membrane enzyme that belongs to the family of P-type ATPases and consists of two noncovalently linked alpha and beta subunits (Sweadner 1989; Lingrel et al., 1994). The α -1 subunit, the catalytic subunit, is required for the assembly of the functional enzyme. Recently, we observed that digitalis like substances (DLS) or ouabain induces redistribution of Na/K-ATPase within LLC PK1 cells and ouabain treatment on basolateral side caused a dose and time dependent decrease in α -1 subunit of the Na/K-ATPase (Liu et al., 2002; Liu et al., 2004). We also demonstrated that ouabain induces endocytosis of the α -1 subunit of Na/K-ATPase and mediated by clathrin-dependent pathway.

In this study, our approach is based on two-dimensional (2D) gel electrophoresis and liquid chromatography-tandem mass spectrometry (LC-tandem MS) to identify proteins which are possibly involved in ouabain induced endocytosis in LLC PK1 cells. Using this technique allowed us the separation of complex mixtures of proteins into individual components. We started with analyzing endosome proteins from control and ouabain treated cells. Several proteins were identified by mass spectrometry and one of the proteins that showed high level of up regulation in ouabain treated cells was a 78 kDa Glucose-Regulated Protein (GRP78), also called immunoglobulin heavy chain binding protein (BiP). This Interesting finding confirmed by western blots that determine protein expression by using a specific antibody to bind to the concerned protein.

GRP78/BiP protein is of interest for several reasons: GRP78/BiP is involved in the structural and functional maturation of the Na/K- ATPase (Beggah, et al., 1996; Jorgensen 2003), this protein is essential for the proper folding and assembly of many newly synthesized secretory and membrane proteins (Haas 1994; Mote et al., 1998), its critical role for maintenance of cell homeostasis and prevention of apoptosis (Liu et al., 1997; Yang et al., 2000; Rao et al., 2002) and as a model protein induced following a variety of cell stress conditions (Lee 2001).

LITERATURE

A GRP78 also known as BiP or immunoglobulin heavy chain binding protein (Haas and Wabl 1983), is a stress protein and a member of the 70 kDa heat shock proteins family (HSP70). A GRP78/BIP is a resident protein of the endoplasmic reticulum (ER) and acts as a molecular chaperone (Ellis et al., 1989). This protein associates with a variety of newly synthesized secretory and membrane proteins, binds with misfolded or defective proteins preventing their export from the ER lumen (Gething 1999). As a result assisting proper folding and assembly of oligomeric secretory proteins (Beguin et al., 2000). The GRP78/BIP levels are critical and determine level of many membrane and secreted proteins. In eukaryotic cells, most secretory and membrane proteins enter into the endoplasmic reticulum (ER) lumen on the way to their final destination and GRP78/BIP retains them in a condition fit for proper folding and Oligomerization (Haas 1994).

GRP78/BIP and Na/K-ATPase

Most of the α -1 subunit of Na/K-ATPase set in within the plasma membrane with less of the molecule (~10%) exposed to the ER lumen. The residues ⁸⁶⁸ENGFLIPIHLL⁸⁷⁸ in the L7 8 loop of Na/K-ATPase α -1 subunit exposed to the ER lumen represent definitely GRP78/BIP binding place (Jorgensen et al., 2003). The same loop is in charge for the α and β associations which is necessary for leaving the ER lumen (Beguin et al., 2000). The type II glycoprotein β subunit of Na/K-ATPase has been found to influence the structural and functional maturation of the α -subunit and to smooth the progress of the

passage of Na/K-ATPase to the plasma membrane (Eakle et al., 1995). In *Xenopus* oocytes, different research groups have studied GRP78/BiP and its interactions with other proteins and specifically with Na/K-ATPase subunits. And they showed that the Na/K-ATPase α -1 subunit cannot mature and be translocated to the plasma membrane without the assembly with β -subunit which is necessary for the conformational maturation. The binding of BiP with the catalytic α -subunit of Na/K-ATPase lasts until subunit assembly (Beggah et al., 1996; Beguin et al., 1998, 2000).

GRP78/BiP and the Cell Surface

Recently, many studies in rat pancreatic cells (Takemoto et al., 1992), in Meth A sarcoma cells (Altmeyer et al., 1996), in immature thymocytes (Wiest et al., 1997) and in NG108-15 cell (Xiao et al., 1999) provide evidence that GRP78/BiP is not only located inside the ER, but also exists on the cell surface. And GRP78/BiP with other stress proteins such as GRP60, GRP70, GRP90 can be expressed on cell surface either consecutively or following stress induction. This finding is unexpected since GRP78/BiP is a soluble ER protein, and GRP78/BiP contains a highly conserved sequence (KDEL) which is present at C terminus of GRP78/BiP.

The presence of carboxy terminal KDEL is necessary for preventing the export of misfolded and defected proteins from the ER lumen. This retention mediated by means of KDEL Receptor (Munro and Pelham 1987). Several studies have proposed possible functions of the presence of some ER proteins

on cell surface. The most interesting explanation for presence of KDEL proteins on cell surface of different cells is related to the ability to associate to other proteins, particularly with high molecular weight complexes proteins (Xiao et al., 1999; Delpino and Castelli, 2002)

GRP78/BIP and Calcium

In addition to the “molecular chaperone” function, GRP78/BIP is also known to have Ca binding functions. The depletion of intracellular Ca stores by the calcium ionophore A23187 and thapsigargin (SERCA-2B activity inhibitor) increases grp78/BIP expression (Li et al., 1993). The increased GRP78/BiP expression may be crucial in preventing cells damage and death in the ER (Villa et al., 1991).

GRP78/BIP and ER Stress-induced Cell Death

In general, ER stresses (changes in Ca^{2+} homeostasis, accumulation of misfolded proteins in the ER and underglycosylated proteins) increase the GRP/BIP gene expression. Prolonged ER stress may cause cell death, and GRP78/BIP has protective role in preventing ER stress-induced cell death (Rao et al., 2002).

The cellular response to ER stress is known as the Unfolded Protein Response (UPR). The UPR is characterized by an increase of the ER resident chaperones including GRP78/BIP and is required to ease ER stress and correct the protein folding process, or, if failed (no GRP induction), prompt programmed cell death (apoptosis). Recently, the Caspase 12 (an ER membrane bound protease) is found to be involved in ER stress-induced cell death, and is a

essential mediator in this process. The GRP78 inhibits both caspase activation and caspase-mediated apoptosis through forming a complex with caspase 7 and 12 that blocking release caspase12 from the ER (Nakagawa et al., 2000; Rao et al., 2002).

MATERIALS AND METHODS

Materials

Chemicals of the highest purity available were obtained from Sigma (St. Louis, MO). EZ-Kink sulfo-NHS-ss-Biotin and ImmunoPure immobilized streptavidin-agarose beads were obtained from Pierce Biotechnology (Rockford, IL). PVDF membranes (Hybound-P) were obtained from Amersham Biosciences (Piscataway, NJ).

polyclonal antibodies against GRP78/BIP were obtained from Affinity BioReagents (Golden, CO). Monoclonal antibodies against GRP78/BIP was from BD Biosciences. Polyclonal and monoclonal antibodies against Na/K-ATPase α 1 subunit (clone C464.6). Polyclonal antibodies against Rab5, Rab7, EEA-1 as well as horseradish peroxidase conjugated goat-anti mouse and goat-anti rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against Na/K-ATPase α 1 subunit (clone α 6F) were obtained from Hybridoma Bank (University of Iowa, Iowa City, IA); Alexa Fluor® 488- or Alexa Fluor® 546-conjugated anti-mouse or anti-rabbit secondary antibodies were obtained from Molecular Probes (Eugene, OR). Normal mouse IgG and rabbit IgG were purchased from Sigma.

Cell Culture

The LLC-PK1, the pig renal proximal tubule cell line, was obtained from the American Tissue Type Culture Collection (Manassas, VA), and cultured to confluent condition as described before (Liu et al., 2002). Cell viability was evaluated by Trypan blue exclusion and LDH release measurement. In some

experiments, LLC-PK1 cells were grown as monolayer to confluence (6-7 d). Medium was replaced daily until 12 h before experiments at which time the monolayer was serum starved as reported previously (Liu et al., 2002). Medium was changed daily until the cells reached 80-90% confluence at which time the medium was changed to DMEM with FBS for at least 12 h prior to experiments.

Western Blot

Proteins were separated by 10% SDS-PAGE or NuPAGE 4-12% Bis-Tris gels (Invitrogen), and transferred to PVDF membrane. After transfer, the gel was stained with Coomassie Brilliant Blue to verify uniform loading and transfer. Immunoblotting was performed as described everywhere. Detection was performed using the enhanced chemiluminescence (ECL) super signal kit (Pierce). Multiple exposures were analyzed to assure that the signals were within the linear range of the film. Autoradiograms were scanned with a Bio-Rad GS-670 imaging densitometer (Bio-Rad, Hercules, CA) to quantify the signals (Liu et al., 2002).

Immunoprecipitation of α 1-subunit of Na/K-ATPase, GRP78/BIP

Immunoprecipitation of α 1-subunit and GRP78/BIP was proceeded mainly as described (Sorkin et al., 1995; Haas et al., 2002; Stoddart et al., 2002). Briefly, after washing (2X) with PBS-Ca-Mg and with PBS, cells were solubilized in TGH buffer (1% NP-100, 0.25% sodium deoxycholate, 10% glycerol, 50 mM NaCl, 50 mM Hepes, pH 7.3, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin). After brief centrifugation (13,000 x g for 15 min), supernatants, containing equal amounts of protein, were

immunoprecipitated with a saturating amount of antibodies against $\alpha 1$ subunit or GRP78/BIP at 4°C overnight with end-to-end rotation, and then 2 h with Protein A or G-agarose beads (Upstate). Immunoprecipitates were washed (2X) with TGH and with PBS. Proteins were eluted with (2X) lamml sample loading buffer and resolved on SDS-PAGE followed by immunoblotting. Normal rabbit or mouse IgG was used for as a control.

Preparation of Endosomes

Endosomes were fractionated on a flotation gradient using the technique of Gorvel et al (1991). Briefly, control and treated cells were washed twice with ice-cold PBS-Ca-Mg (PBS containing 100 μ M CaCl_2 and 1 mM MgCl_2) and once with ice-cold PBS. The cells were collected in PBS and centrifuged for 5 min at 4°C and 3,000 x g. The pellet was re-suspended in 3 ml of the homogenization buffer (250 mM sucrose in 3 mM imidazole, pH 7.4) and re-centrifuged for 10 min at 4°C at 3,000 x g. The pellet was re-suspended in 1.0 ml of homogenization buffer (with 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1mM PMSF, and 0.5mM EDTA) and gently homogenized on ice using a Dounce homogenizer (15-20 strokes), followed by a centrifugation (10 min at 4°C at 3,000 x g). The supernatant was adjusted to 46% sucrose using a stock solution of 62% sucrose in 3 mM imidazole (pH 7.4) and loaded at the bottom of a centrifuge tube, to which sequentially added 16% sucrose (4 ml) in 3 mM imidazole and 0.5 mM EDTA in $^2\text{H}_2\text{O}$, 10% sucrose in the same buffer (3 ml), and finally homogenization buffer (1 ml). The gradient was centrifuged for 60 min at 4°C and 130,000 x g in a Beckmann SW 40Ti rotor. Early endosomes were collected at the 16%/10%

sucrose interface, whereas the late endosomes were collected at the homogenization buffer and 10% sucrose interface. The identity of early and late endosomes was determined with polyclonal antibodies raised against Rab5 and Rab7, respectively. Additional confirmation was obtained using EEA-1 which, like Rab 5 was found almost exclusively at the 16%/10% interface.

TCA Protein Precipitation

In preparing endosome samples for one or 2D electrophoresis, trichloroacetic acid (TCA) precipitation is used to concentrate proteins. Samples were precipitated by adding equal volumes of ice cold 20% TCA. The mixture incubated for 30 min on ice and then centrifuged for 15 min at 4 degrees. The supernatant was discarded and 300µl; a cold acetone was added to the pellet with spinning for 5 min at 4 degrees. The supernatant was removed and the pellet dried by placing tubes in room temperature for 5-10 min to drive off acetone.

Biotinylation of Cell Surface Exposed Proteins

Cell surface biotinylation was performed as described above (Gottardi and Caplan 1993; Gottardi et al., 1995; Liu et al., 2002). The LLC-PK1 cells were grown in 35 mm petri dishes. After three washes with ice-cold PBS-Ca-Mg (PBS containing 100 µmol/L CaCl_2 , and 1 mmol/L MgCl_2), the surface proteins were biotinylated with EZ-Kink sulfo-NHS-ss-Biotin (1.5 mg/mL in 10 mmol/L triethanolamine pH 9.0, containing 2 mmol/L CaCl_2 and 150 mmol/L NaCl) for 25 minutes on ice two times. Non-reacted sulfo-NHS-ss-biotin was washed twice with ice-cold PBS-Ca-Mg-glycine (PBS-Ca-Mg containing 100 mmol/L glycine)

and quenched with the same solution for 20 min on ice to make sure the entire biotinylation reagent was quenched. After two washes with PBS-Ca-Mg, cells were lysed with 1.0 mL cell lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 7.5) for 60 min on ice. The cell lysates were clarified by sedimentation at 14,000 $\times g$ for 10 min at 4°C. Biotinylated proteins were separated with a 50% slurry of ImmunoPure immobilized streptavidin-agarose beads (Pierce) overnight at 4°C, with end-to-end rotation. The beads were washed three times with lysis buffer, two times with high-salt washing buffer (0.1% Triton X-100, 500 mmol/L NaCl, 5.0 mmol/L EDTA, 50 mmol/L Tris-Cl, pH 7.5), and two times with no-salt washing buffer (10 mmol/L Tris-Cl, pH 7.5). Proteins bound to the beads were eluted by incubated at 55°C water bath for 30 min in an equal volume of 2 $\times$ Laemmli sample buffer. Proteins then were applied to SDS-PAGE and Western blot as described above.

2D-gel Electrophoresis

For 2D gel electrophoresis (2DE), endosomal fraction protein were lysed in a solution containing 40 mM Tris, 8 M urea, 4% CHAPS, 2 M thiourea, 2 mM TBP, 0.2% Ampholytes, 0.0002% bromophenol blue.

Immobilized pH gradient (IPG) gels (11 cm ReadyStrip IPG strips, PH 3-10 NL, Biorad) were rehydrated with the solubilized sample. Isoelectric focusing was performed on Bio-Rad PROTEIN IEF System. Immediately after focussing, IPG gels were equilibrated in 6 M urea, 2% SDS, 50 mM Tris, pH 6.8 and 30% glycerol, first with 65 mM DTT for 15 min then with 135 mM Iodoacetamide for 15 min before running in the second dimension gel electrophoresis 10% SDS-

PAGE gels. Gels were stained with Colloidal Blue Staining (Invitrogen), scanned, and analyzed using PDQuest software.

Protein Identification by Mass Spectrometry

Differential spots were excised from the coomassie stained 2D gels, which showed the highest expression level for each spot. The selected protein spots were destained with 30% methanol for 3 h at room temperature and treated overnight with modified, sequencing grade trypsin (Promega, Madison, WI) as described previously (Basrur et al., 2003). Briefly, The selected gel spots were covered in 150 μ l of 50% acetonitrile in 0.1 M ammonium bicarbonate buffer, pH 8.0, for 30 min. Gel spots were cut up into 1 mm cubes and dried using a vacufuge (Eppendorf). The diced cubes were reswollen in a small volume of 0.1 M ammonium bicarbonate buffer with trypsin (0.5 μ g). The proteins were digested *italics* for 16 h at 37 C and new trypsin (0.25 μ g) added after 12 h. Peptide fragments were extracted with 150 μ l of 60% acetonitrile containing 0.1% TFA for 30 min, subsequently with 100 μ l of acetonitrile containing 0.1% TFA. Using Vacufuge all extracts were brought to a final volume of 10 μ l. Two μ l of the digest was separated on a reverse phase column (Aquasil C18, 15 μ m tip x 75 μ m id x 5 cm Pico frit column, New Objectives, Woburn, MA) using acetonitrile/1% acetic acid gradient method (5-75% acetonitrile for 35 min after that 95% acetonitrile wash for 5 min) at a flow rate of 250 nl/min. Peptides were analyzed using an ion-trap mass spectrometer (LCQ-Deca XP Plus, ThermoFinnigan) equipped with a nano-spray supply and operated on double play mode to analyze the positive ions. Dynamic exclusion was performed to pull

together two collision induced dissociation (CID) spectra on the most abundant ion and then eliminate it for 2 min.

Protein identification was conducted using TurboSEQUENT (ThermoFinnigan). Results were manually confirmed and any unrevealed CID spectra were searched using the MS-Tag provision of Protein Prospector program(<http://prospector.ucsf.edu>) or SwissProt database.

RESULTS

Proteomic Analysis Identifications

To identify proteins that are involved in ouabain-induced endocytosis, 2D gel electrophoresis of endosomal fraction is applied. After staining with coomassie we compared 2D gels of early endosomal fraction from control and ouabain treated LLC PK1 cells that we purified according to the previously described protocol. Three major protein spots (Fig. 1) we observed were notably higher in the gel of LLC PK1 cells that treated with ouabain (50 nM) for 2 h. these protein spots were identified by mass Spectrometry.

One protein spot with a molecular weight of 55 KDa matching to ATP synthase beta chain (mitochondrial F1 complex), a protein spot of 50 KDa corresponding to Vimentin, and a protein spot of 70 kDa matching to glucose-regulated protein (GRP78) or called Immunoglobulin heavy chain binding protein (BIP).

Westren Blot Analysis Confirms Detected Endosomal Proteins

In order to validate our previously revealed proteins by proteomic analysis, we used Western blot and confirmed presence Vimentin and 78 kDa glucose-regulated protein GRP78/BIP in endosomal fraction. And no band was detected by western blot for ATP synthase beta chain (mitochondrial F1 complex) in endosomal fraction. One possible reason is that reactivity of ATP synthase beta chain antibody with pig species has not been confirmed yet.

(Fig. 1) 2 D Gel Electrophoresis from Early Endosomes Isolated 2 h Following Ouabain Treatment of LLC-PK1 Cells

GRP78 Present on the Surface of LLC PK1 Cells

Using the surface biotinylation method, we determined if the GRP78 exist on the surface of LLC PK1. In this experiment we labeled only the surface exposed proteins with NHS-SS-Biotin. The biotin labeled proteins were subsequently isolated by streptavidin/agrose and analysed by SDS-PAGE. Western blot of the biotinylated fraction shows that the GRP78 can be detected in the surface of LLC PK1 cells.

Ouabain Reduces GRP78 Expression on the Surface of LLC-PK1

We further determined the level of the surface GRP78 in response to ouabain by measuring biotinylated protein densities. In response to 50 nM ouabain applied to monolayers of LLC-PK1 cells following time course study. We found that biotinylated protein content GRP78 decreased in a time-dependent manner (Fig. 2).

(Fig. 2) Ouabain Reduces GRP78 Expression on The Surface of LLC-PK1 In a Time-dependent Manner

Ouabain Appears to Enhance the Interaction of Na/K-ATPase α -1 with GRP78/BiP

In preliminary experiments performed by applying co-immunoprecipitation of the whole cell lysate with antibodies against the Na/K-ATPase α -1 subunit and GRP78/BiP, we found that ouabain treatment increase the protein-protein interaction between the Na/K-ATPase α -1 subunit and GRP78/BiP.

DISCUSSION

Effects of Ouabain on c-fos Expression and GRP 78 /BiP Induction

Recent studies in vascular smooth muscle cells (VMSC) indicates that ouabain able to induce GRP78/BiP expression (Taurin et al., 2002b). However, the underlining mechanism are not fully understood. In this study we have been investigating the involvement of c-Fos in this effect of ouabain on GRP78/BIP. It is significant that ouabain has been shown and other labs were able to inducing c-Fos transcriptions in several cell types including hepatocytes (Cayanis et al., 1992), melanoma cells, mouse fibroblasts, HeLa cells (Nakagawa et al., 1992), cardiomyocytes (Peng et al., 1996), mIMCD-3 cells (Joannidis et al., 1997) and thymocytes (Rodrigues Mascarenhas et al., 2003). The increased mRNAs were proportional to the level of inhibition of Na/K-ATPase and the consequential increase in intracellular calcium concentration. In the muscle cells of rats heart an ouabain-induced rise in intracellular calcium causes activation/expression of the transcription factors c-Fos and c-Jun, which together form the transcription factor AP-1 (Huang et al., 1997). Recently, it also was observed that c-Fos expression is induced quickly by ouabain in calcium-independent pathway through increase of Na/K ratio and elevation of Na (Taurin et al., 2002a). Noteworthy, c-Fos (an element of the AP-1 transcription factor complex) is involved in the singling pathway that mediates GRP78/BIP induction following ER calcium release. and that GRP78/BIP induction is prevented by proteasome-mediated c-Fos degradation (He et al., 2000).

We are proposing that ouabain treatment may induce transcription of genes and one of them encoding c-Fos in so increases AP-1 transcription factor activity and mediates GRP78/BiP induction. More importantly this could explain that inhibition of Na/k-ATPase by ouabain has anti-apoptosis effect which observed in VSMC (Orlov et al., 1999), LLC-PK1 cells, a pig renal proximal tubular cells(Zhou et al., 2001) and recently in VSMC (Taurin et al., 2002a) Ouabain increases expression of GRP78/BiP, which is well documented, and is protective for the cell against toxicity and apoptosis (Yu et al., 1999; Fernandez et al., 2000; Lee 2001; Rao et al., 2002; Reddy et al., 2003).

Given that GRP78/BiP has been primarily characterized as an ER resident chaperone, we sought to confirm the expression of GRP78 /BiP on the surface of LLC PK1 cells via cell surface biotinylation to explore further the finding that GRP78/BiP presence in endosome proteins and possible involvement in endocytosis. In agreement with previously published data, the results reported here demonstrate that the presence of GRP78/BiP on the membrane of LLC PK1 cells was confirmed by using the surface biotinylation method followed by Western blot analysis with an anti-GRP78 /BiP antibody. As we examined whether ouabain caused a reduction in surface GRP78 density, interestingly, we found that ouabain treatment caused decreases in the membrane surface GRP78/BIP in time-dependent manner.

This finding is challenging, because all these proteins contain at their carboxyl terminus the KDEL signal sequence, known to function as signal for retention in

the ER (Munro and Pelham 1987). Our finding that GRP78/BIP can be expressed on the cell surface is in agreement with several previous studies (Altmeyer et al., 1996; Multhoff and Hightower 1996; Berger et al., 1997; Delpino et al., 1998; Xiao et al., 1999; Delpino and Castelli 2002), demonstrating that some ER-resident proteins, including GRP78/BIP, GRP96, calreticulin and protein disulfide isomerase, are not completely limited to the ER lumen, but are also expressed on the cell surface either constitutively or following stress induction.

Molecules are transported from the ER in vesicles to the ER-Golgi intermediate compartment and then to the Golgi apparatus ensuing steps in the secretory pathway involving vesicular travel between different compartments from the Golgi to lysosomes or the plasma membrane. But some proteins must be retained within the ER without proceeding along the secretory pathway, particularly GRP78/BIP must be retained within the ER because of the highly conserved sequence Lys-Asp-Glu-Leu (KDEL) which is present at their carboxy terminus of GRP78/BIP. Noteworthy, the KDEL signal does not prevent soluble ER proteins from being packaged into vesicles and carried through the secretory pathway leading from the ER to the cell surface (Cooper, 2000).

The high lipid affinity of GRP78/BIP, and the ability of this chaperone protein to associate with other newly synthesis proteins during the processes of folding, assembly and maturation may explain the translocation of GRP78/BIP to the cell membrane. In addition, these interactions with other proteins might block the KDEL retention signal sequence, allowing GRP78/BIP to leave the ER and to associate with membrane lipidic proteins, in the form of multiprotein

complexes (Delpino and Castelli 2002). Supporting this hypothesis, it has been found that membrane-exposed GRP78 is indeed tightly associated with many different proteins, forming heterogeneous, high molecular weight, complexes (Xiao et al., 1999).

We previously demonstrated that ouabain induce endocytosis of the Na/K-ATPase and substantial depletion of the sodium pump density and function on the membrane surface in LLC-PK1 cells (Liu et al., 2002). and that the ouabain-stimulated endocytosis of the Na/K-ATPase appears to involve clathrin dependent mechanisms. We are proposing that ouabain could cause a reduction in membrane surface GRP78/BIP by the same mechanism (ouabain-stimulated clathrin dependent endocytosis) which explains why the administration of ouabain also increased the GRP78/BIP content in both early and late endosomes.

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ABSTRACT

We have demonstrated that ouabain causes dose- and time-dependent decreases both in ^{86}Rb uptake and plasmalemmal Na/K-ATPase content of LLC-PK1 cells (Liu et al., 2002). This phenomenon is related to ouabain-induced endocytosis of plasmalemmal Na/K-ATPase in LLC-PK1 Cells by a clathrin-dependent mechanism (Liu et al., 2004). GRP78/BiP is a resident protein of the endoplasmic reticulum (ER) and acts as a molecular chaperone. Recently, several studies have shown GRP78/BiP is also expressed on the cell surface and forms heterogeneous, high molecular weight complexes with other proteins. To identify the proteins that are possibly involved in ouabain-induced endocytosis of the Na/K-ATPase in LLC-PK1 cells, we separated and identified endosomal proteins by 2D gel electrophoresis and MS/MS from both control and ouabain-treated LLC-PK1 cells. GRP78/BiP was identified by MS/MS as one of several up-regulated proteins and confirmed by Western Blot. Interestingly, GRP78/BiP was significantly up-regulated in both early and late endosomes in response to ouabain treatment. By using a cell surface protein biotinylation technique to isolate the cell surface membrane proteins, we found that GRP/BiP is expressed on the cell surface of LLC-PK1 cells and down-regulated in a time-dependent manner in response to ouabain. By comparing the cellular redistribution, our data suggest that both the Na/K-ATPase α -1 subunit and GRP78/BiP follow the same pattern in response to ouabain.