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Perturbations in maturation of secretory proteins and their association with endoplasmic reticulum chaperones in a cell culture model for epithelial ischemia

(thyroglobulin/glucose-regulated proteins/heat shock proteins/ATP depletion/antimycin A)

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ABSTRACT **The effects of ischemia on the maturation of secretory proteins are not well understood. Among several events that occur during ischemia-reperfusion are a rapid and extensive decrease in ATP levels and an alteration of cellular oxidative state. Since the normal folding and assembly of secretory proteins are mediated by endoplasmic reticulum (ER) molecular chaperones, the function of which depends on ATP and maintenance of an appropriate redox environment, ischemia might be expected to perturb folding of secretory proteins. In this study, whole animal and cultured cell models for the epithelial ischemic state were used to examine this possibility. After acute kidney ischemia, marked increases in the mRNA levels of the ER chaperones glucose-regulated protein (grp)78/immunoglobulin-binding protein (BiP), grp94, and ER protein (ERp)72 were noted. Likewise, when cellular ATP was depleted to less than 10% of control with antimycin A, mRNA levels of BiP, ERp72, and grp94 were increased in kidney and thyroid epithelial cell culture models. Since the signal for the up-regulation of these stress proteins is believed to be the accumulation of misfolded/misassembled secretory proteins in the ER, their induction after ischemia *in vivo* and antimycin treatment of cultured cells suggests that maturation of secretory proteins in the ER lumen might indeed be perturbed. To analyze the effects of antimycin A on the maturation of secretory proteins, we studied the fate of thyroglobulin (Tg), a large oligomeric secretory glycoprotein, the folding and assembly of which seems to require a variety of ER chaperones. Treatment of cultured thyroid epithelial cells with antimycin A greatly inhibited (>90%) the secretion of Tg. Sucrose density gradient analysis revealed that in antimycin A-treated cells Tg associates into large macromolecular complexes which, by immunofluorescence, appeared to localize to the ER. Furthermore, coimmunoprecipitation studies after antimycin A treatment demonstrated that Tg stably associates with BiP, grp94, and ERp72. Together, our results suggest that a key cellular lesion in ischemia is the misfolding of secretory proteins as they transit the ER, and this leads not only to increased expression of ER chaperones but also to their stable association with and the subsequent retention of at least some misfolded secretory proteins.**

Ischemia is a major cause of epithelial and other tissue injury and leads to a variety of cellular lesions. These include disruption of tight junctions, the subcortical actin cytoskeleton, cell-substratum interactions mediated by integrins, and possibly of other cellular structures and processes involved in the generation and maintenance of apical-basolateral protein polarity (1–3). On a biochemical level, ischemia and subsequent reperfusion are manifested by a variety of changes that include a rapid and severe decline in cellular ATP levels (4–7), alterations of cellular redox state, and perturbations of Na^+/K^+ ratio, intracellular pH, and intracellular Ca^{2+} homeostasis (7–9). Ischemia also leads to a cytosolic stress response manifested by impressive induction of the heat shock proteins (Hsps), especially the members of the Hsp70 family (6, 10, 11). Preinduction of Hsp70 appears to protect from future ischemic insult (12–15).

Hsp70, like a number of other proteins believed to function as molecular chaperones, participates in the ATP-dependent folding and assembly of a variety of newly synthesized cytosolic proteins (16). Proteins belonging to the Hsp70 family are also involved in the detection of misfolded or misassembled proteins and may play a role in targeting them for degradation (17). In ischemia, it is believed that the resulting ATP depletion leads to misfolding and aggregation of proteins (e.g., the actin cytoskeleton) and that Hsp70 participates in the refolding of these proteins and/or their degradation (18).

Secretory (membrane and secreted) proteins are translocated into the lumen of the endoplasmic reticulum (ER) shortly after initiation of their synthesis. The ER lumen serves as a site for folding, assembly and degradation of secretory proteins. As in the case of Hsp70-catalyzed folding of cytosolic proteins, the maturation of secretory proteins in the ER is dependent on ATP and sensitive to the local redox environment; folding reactions are mediated by a set of specific molecular chaperones, most of which are resident ER lumenal proteins (19, 20). The best studied of these are immunoglobulin-binding protein [BiP; also known as glucose-regulated protein (grp)78], which is an ER homolog of Hsp70, and grp94, an ER homolog of Hsp90 (19). BiP and grp94 have been shown to bind ATP (20–22). At least in the case of BiP, the role of ATP in its chaperone function appears to be similar to the role of ATP in the function of Hsp70 in that both chaperones possess an ATPase activity and utilize ATP for dissociation from their polypeptide substrates (23). Other likely ER chaperones include members of the thioredoxin superfamily that are thought to participate in disulfide isomerization (protein disulfide isomerase, ERp72, and ERp50) (20, 24, 25, 33); proteins related to peptidyl-prolyl isomerases (FK506-binding protein 13 and ER cyclophilins) (26–28); and calcium-binding proteins calreticulin and calnexin (20, 29, 30).

In recent years, many studies on the molecular mechanisms of cellular injury due to ischemia have focused largely on the functions of cytosolic stress proteins, leaving the involvement of molecular chaperones localized to other cellular compartments, including the ER, much less explored. However, many of the epithelial proteins whose function is believed to be disrupted by ischemia are secretory proteins initially folded and assembled in the ER. Perturbation of ATP-dependent and

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Abbreviations: ER, endoplasmic reticulum; BiP, immunoglobulin-binding protein; grp, glucose-regulated protein; Hsp, heat-shock protein; ERp, endoplasmic reticulum protein; Tg, thyroglobulin; Tn, tunicamycin; MDCK, Madin-Darby canine kidney.

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redox environment-sensitive folding and assembly of polypeptides in the ER in the face of ischemia might be expected to play an important part in creation of the ischemic epithelial cell phenotype, and understanding the role of ER molecular chaperones is essential. Furthermore, it might be anticipated that the ability to recover from ischemic insult would to some extent depend on the epithelial cell's capacity to (re)fold and (re)assemble newly synthesized integrins, transmembrane junctional components, polarized transporters and other secretory proteins, the maturation of which requires assistance of ER chaperones.

Thus, this study was undertaken to better understand how ischemic conditions might affect ER chaperone function in maturation of newly synthesized secretory proteins. Expression of ER chaperones in whole kidney ischemic-reperfused preparations as well as in cultured kidney and thyroid epithelial cell models for ischemia were examined. In the cell culture models, antimycin A, a specific inhibitor of mitochondrial oxidative phosphorylation, which causes rapid ATP depletion and possibly altered cellular redox potential (31, 32) was employed. We have also studied the effects of antimycin A treatment on the secretion and folding of a model epithelial cell secretory protein, thyroglobulin (Tg), the maturation of which is known to involve a variety of ER chaperones (33, 34). Our results suggest that metabolic changes associated with ischemia cause marked induction of mRNA coding for ER molecular chaperones as well as perturbation of folding and/or assembly of secretory proteins in the ER. Our findings also suggest that the misfolded secretory proteins which accumulate after ischemia are retained in the ER in stable complexes with ER molecular chaperones.

EXPERIMENTAL PROCEDURES

Materials. cDNA probes were kindly provided by the following investigators: BiP by M.-J. Gething (University of Texas Southwestern Medical Center); ERp72 and grp94 by M. Green (St. Louis University). Hsp70 cDNA was from American Type Culture Collection (ATCC). PCCl3 rat thyroid cell line was a gift from M. T. Berlingieri (Universita degli studi di Napoli, Italy). Madin-Darby canine kidney (MDCK) cells and FRTL-5 rat thyroid cells were purchased from ATCC. The mouse monoclonal antibody against BiP and rat monoclonal antibody against grp94 were obtained from StressGen Biotechnologies (Sidney, Canada). Rabbit polyclonal antiserum against Tg was from Dako. Rabbit polyclonal antiserum against ERp72 was kindly provided by M. Green. Secondary antisera were obtained from Jackson ImmunoResearch Laboratories.

Induction of Renal Ischemia. Adult male Munich–Wistar rats at an average body weight of 300 g were anesthetized with phenobarbital (50 mg/kg) and the body temperature of rats was maintained between 36–37°C. Renal ischemia was induced by clamping the left renal artery for 15 (mild) or 45 (severe) min, followed by reperfusion for 5 h. The rats were reanesthetized and both the left ischemic kidney and the right nonischemic kidney (control) were removed, frozen in liquid nitrogen and total RNA was isolated as described below. In addition, left nonischemic (control) kidneys from separate age-matched rats were also removed and frozen in liquid nitrogen.

Cell Culture. MDCK cells were grown in Dulbecco's minimal essential medium supplemented with 5% fetal bovine serum. FRTL-5 and PCCl3 rat thyroid cells were grown in Coon's modified Ham's F-12 medium (Sigma) supplemented with 5% bovine calf serum and a six-hormone mixture (35). Intracellular ATP concentrations were measured by a modification of the luciferase method using a kit purchased from Sigma.

RNA Isolation and Northern Blot Analysis. Whole rat kidneys were homogenized, and RNA was prepared in 4 M guanidium isothiocyanate. mRNA was recovered after centrifugation through 5.7 M CsCl. In cultured kidney and thyroid cells, total RNA was isolated by phenol:chloroform extraction (28). Isolated RNA was electrophoresed on 1% formamide/formaldehyde agarose gels, transferred to nitrocellulose, and hybridized with random primed ^{32}P-labeled cDNA probes.

Immunoprecipitation. Rat thyroid cells were washed twice with PBS and suspended in PBS, pH 7.5, containing 5 mM EGTA and 5 mM EDTA (33). The cells were lysed on ice by addition of 1% Triton X-100 in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 5 μg/ml each leupeptin, pepstatin A, and antipain). Tg was immunoprecipitated from samples of cell lysates as described (33). Immunoprecipitates were analyzed by SDS/PAGE (36) followed by Western blot analysis (20). Immunoblots were developed using the SuperSignal Substrate system (Pierce).

Sucrose Density Gradient Analysis. Rat thyroid cell lysates were layered on top of linear 5–20% sucrose gradients prepared in PBS, pH 7.5, containing 5 mM EGTA and 5 mM EDTA (33). The gradients were centrifuged at 32,000 rpm for 20 h at 4°C using a Beckman SW40TI ultracentrifuge rotor. Seventeen fractions were collected using a Buchler Auto Densi-Flow IIC gradient fractionator. Trichloroacetic acid at a final concentration of 10% was added to fractions to precipitate proteins. Trichloroacetic acid precipitates were washed once with ice-cold acetone, dissolved in sample buffer, and analyzed by SDS/PAGE followed by Western blot analysis with anti-Tg antiserum.

Immunofluorescence. PCCl3 cells grown on coverslips were fixed in methanol at −20°C for 5 min, washed twice in PBS, pH 7.5, and incubated with rabbit polyclonal anti-Tg antiserum for 1 h at 37°C. Coverslips were then washed in PBS and incubated with fluorescein-conjugated anti-rabbit immunoglobulin antiserum. Staining was examined using a Nikon Labphot compound microscope equipped for epifluorescence.

RESULTS AND DISCUSSION

Ischemia causes an impressive induction of cytosolic chaperones in a variety of tissues, including the kidney (6, 10, 11, 16). However, the effect of ischemia on the expression of ER chaperones and on maturation of secretory proteins remains poorly understood. Nevertheless, previous studies have found increased expression of glucose-regulated proteins, which may have been ER chaperones, after exposure of cultured cells to anaerobic conditions followed by reoxygenation (37). To determine whether glucose-regulated proteins of the ER as well as other ER molecular chaperones are similarly induced under ischemic conditions, rat kidneys were subjected to mild (15 min) or severe (45 min) ischemia by renal arterial ligation and then reperfused. By Northern blot analysis, ischemia produced significant and consistent increases in mRNA levels of BiP, grp94, and ERp72 (Fig. 1, lanes 1). The increases in mRNA levels for ER molecular chaperones were more pronounced after mild ischemia (Fig. 1*B*, lane 1), possibly due to increased cell death following severe ischemia (Fig. 1*A*). These findings indicate that *in vivo* ischemic injury in rat kidneys induces the ER molecular chaperones in a manner comparable to that seen in tissue culture cells treated with either tunicamycin (Tn), a nucleoside antibiotic that blocks N-linked glycosylation (38), or with calcium (Ca^{2+}) ionophores, which deplete ER Ca^{2+} stores (39). These treatments are believed to cause an accumulation of unfolded proteins in the ER, thereby signaling the transcription of ER molecular chaperones (19, 40, 41). Thus, it appears that along with its other deleterious effects on epithelial cell surface proteins, ischemic injury affects the internal environment of the ER.

Ischemia followed by reperfusion represents multiple kinds of injury, including ATP depletion associated with the ischemic phase followed by oxidative damage during the reperfu-

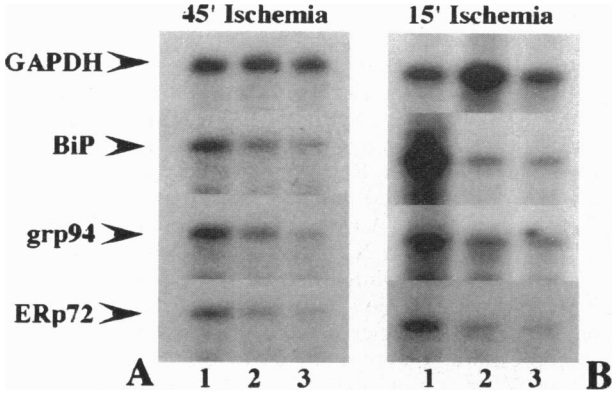

FIG. 1. Whole kidney ischemia causes an increase in level of the mRNAs encoding several ER molecular chaperones. Northern blot analysis of total RNA from either ischemic left kidney (lane 1) or nonischemic kidney (right kidney from ischemic animal, lane 2; and left kidney from nonischemic animal, lane 3). Blots were probed with ^{32}P-labeled cDNA probes for molecular chaperones; glyceraldehyde-3-phosphate-dehydegenase (GAPDH) was used to determine the equivalence of gel loading. (*A*) Ischemia (45 min) followed by reperfusion (5 h). (*B*) Ischemia (15 min) followed by of reperfusion (5 h).

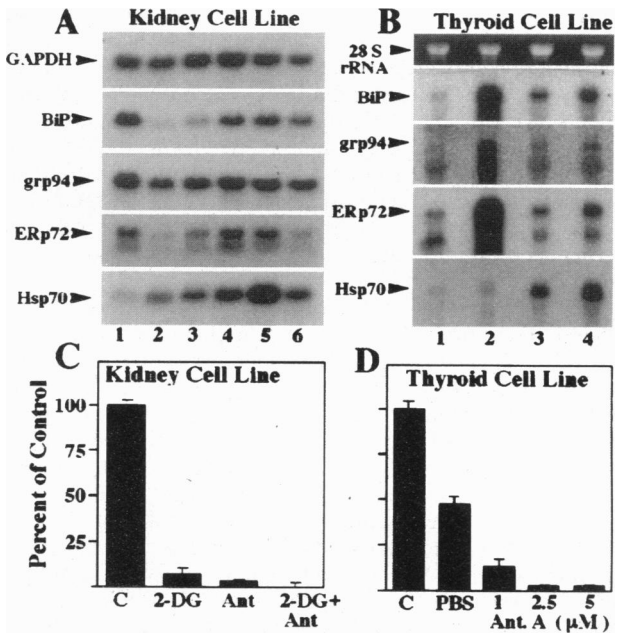

FIG. 2. ATP depletion in cultured cells causes an induction of ER molecular chaperones. (*A*) MDCK cells were incubated for 90 min in normal growth medium (lane 2), HBSS (lane 3) or in HBSS with ATP-depleting agents: 10 mM 2-deoxyglucose (lane 4); 10 μM antimycin A (lane 6); and a combination of these two agents (lane 5). (Lane 1) Tn (10 μg/ml) was used as a positive control for maximal induction of ER chaperones. Cells were then transferred to normal growth medium and incubated for additional 4 h. Total RNA was isolated and subjected to Northern blot analysis with ^{32}P-labeled cDNA probes for molecular chaperones; GAPDH cDNA was used to determine the equivalence of gel loading. (*B*) PCCl3 cells were incubated for 90 min in normal growth medium (lane 1) or in PBS containing 1.5 mM $CaCl_2$ and 2 mM $MgCl_2$ with 1 μM (lane 3) or 5 μM (lane 4) antimycin A. (Lane 2) Tn (10 μg/ml) was used as a positive control for maximal induction of ER chaperones. Cells were then transferred to normal growth medium and incubated for additional 4 h. Total RNA was isolated and subjected to Northern blot analysis using ^{32}P-labeled cDNA probes for molecular chaperones. Ethidium bromide staining of the 28s rRNA was used to indicate the equivalence of gel loading. (*C* and *D*) Intracellular ATP levels in MDCK cells (*C*) and PCCl3 cells (*D*). MDCK cells were incubated for 90 min in either normal growth medium (control) or HBSS with 10 mM 2-deoxyglucose, 10 μM antimycin A, or combination of the two agents. HBSS reduced cellular ATP levels to ≈ 10% of control (data not shown). PCCl3 cells were incubated for 60 min in either normal growth medium (control) or PBS with antimycin A (1, 2.5, and 5 μM). Intracellular ATP levels are expressed as percent of control.

sion phase (11), as well as alterations of cellular pH and cation balance (7–9). To further investigate the effects of ischemia on the ER and the role that ER molecular chaperones might play in the cellular response to an ischemic insult, we employed a cell culture model for ischemia. In this model, cultured canine kidney (MDCK) and rat thyroid (PCCl3) cells were subjected to inhibition of oxidative and/or glycolytic metabolism by treatment with antimycin A, a specific inhibitor of mitochondrial oxidative phosphorylation (31) and/or 2-deoxyglucose, a nonhydrolyzable analog of glucose. These agents have been used extensively to create an *in vitro* cell culture model for ischemia (42, 43) and were shown to produce rapid and severe ATP depletion, although both agents are likely to induce other biochemical changes. Antimycin A, for example, has been shown to cause an increase in hydrogen peroxide concentration in tissues in a manner similar to that which happens after reperfusion of ischemic tissues (7). Intracellular ATP levels were reduced to virtually zero after 90 min of treatment of canine kidney cells with either 10 mM 2-deoxyglucose, 10 μM antimycin A, or a combination of these two agents (Fig. 2*C*). Rat thyroid cells showed even greater sensitivity to the treatment: 60 min of treatment with 2.5 μM antimycin A alone reduced intracellular ATP to 2% of control (Fig. 2*D*). The degree and rapidity of ATP depletion resembles that described by other investigators and is comparable to that seen during the onset of renal ischemia *in vivo* (5). In both cell types, the mRNA level for Hsp70, the cytosolic chaperone, was increased after antimycin A treatment (Fig. 2 *A* and *B*), consistent with other studies that demonstrate that energy depletion causes up-regulation of Hsp70 (16, 18). Likewise, consistent increases in the mRNA levels for BiP, the Hsp70 homolog in the ER, were seen in both cell types with either 2-deoxyglucose or antimycin A (Fig. 2 *A* and *B*). In cultured kidney cells, the increases in BiP message levels after antimycin A treatment were roughly comparable to those achieved with Tn (Fig. 2*A*), one of the most potent and specific inducers of ER chaperones described. Thyroid epithelial cells proved to be extremely sensitive to treatment with Tn, and increases in the mRNA levels for ER chaperones induced by Tn were much greater than those induced by antimycin A. This is likely to be due to the fact that Tg, the major secretory product of thyroid cells [≈13% of total thyroid protein synthesis (44)] is a highly glycosylated protein. Thus, inhibition of glycosylation and subsequent retention in the ER (unpublished observation) of such a large fraction (even if all other secretory activity in thyroid epithelial cells is disregarded) of the cellular secretory products would therefore represent a major stress and could account for the observed extreme sensitivity of thyroid cells to Tn. As expected, the Hsp70 mRNA levels were not induced by Tn treatment in either cell type.

Treatment of MDCK cells with 2-deoxyglucose alone or in combination with antimycin A also caused increases in the mRNA levels for the ER chaperones ERp72 and grp94, in addition to BiP (Fig. 2*A*, lanes 4 and 5). Antimycin A alone produced a somewhat lesser increase in the mRNA levels of these chaperones (Fig. 2*A*, lane 6). This is consistent with the notion that 2-deoxyglucose, as a nonmetabolizable analog of glucose, could have additional effects on cell functions. For example, N-linked glycosylation in the ER is likely to be affected by this agent in a manner similar to Tn. For this reason (and due to our finding that thyroid cells are exquisitely sensitive to inhibition of N-linked glycosylation, as discussed above), we chose not to employ 2-deoxyglucose as a tool in investigating the effects of ATP depletion on the functions of ER molecular chaperones. Furthermore, in rat thyroid epi-

thelial cells, antimycin A alone induced BiP, grp94, and ERp72 (Fig. 2*B*, lanes 3 and 4). Thus, although there are some differences in the magnitude of induction, antimycin A treatment of kidney epithelial cells and thyroid epithelial cells (Fig. 2 *A* and *B*) induces the ER molecular chaperones in a manner similar to that seen in ischemic rat kidneys *in vivo* (Fig. 1). These findings support the validity of the cell culture model and suggest that ER molecular chaperones play a role in the cellular response to ischemic injury.

Considerable evidence suggests that the signal for up-regulation of ER chaperones is the accumulation of misfolded/misassembled proteins in the ER (16). Since both ATP and the redox environment are known to be essential for proper folding and assembly of secretory proteins, it seems likely that a key cellular lesion caused by ischemia could be the retention of incompletely folded or misassembled proteins in the ER, possibly through interactions with molecular chaperones. This might place demands on the cell to increase the synthesis of ER molecular chaperones to compensate for the presence of increased amounts of malprocessed polypeptides in the ER. Also, in the event the misfolding and misassembly are irreversible for some secretory proteins essential to the polarized epithelial phenotype (e.g., cell adhesion and junctional molecules, and integrins), increased ER chaperone synthesis might provide the cell with enhanced capacity to recover by rapidly replacing the nonfunctional molecules with newly synthesized counterparts. The increase in levels of mRNA coding for the ER molecular chaperones we have observed in ischemic tissue and cells (Figs. 1 and 2) is consistent with this hypothesis. We therefore investigated the effect of antimycin A on the secretion of a model secretory protein, Tg by cultured thyroid epithelial cells, as well as on the interaction of Tg with ER molecular chaperones.

As discussed above, treatment of rat thyroid epithelial cells with antimycin A resulted in a rapid and severe decrease in intracellular ATP levels (Fig. 2*D*). Treatment with antimycin A also resulted in nearly complete inhibition of secretion of Tg into the culture medium (Fig. 3*A*, lanes 3–5). Incubation in PBS in the absence of antimycin A affected secretion of Tg only minimally (Fig. 3*A*, lane 2).

The folding of secretory proteins and their dissociation from ER molecular chaperones are not the only energy-dependent steps in the intracellular transport pathway; ER to Golgi transport and release of proteins from the secretory vesicles are also steps that require ATP. Therefore it was important to determine the site of intracellular accumulation of Tg in antimycin A-treated cells. By immunofluorescence, nearly all intracellular Tg in untreated cells localized to a perinuclear site consistent with the ER (Fig. 3*C*). The predominance of the ER staining in untreated cells suggests that the exit of Tg from the ER represents a rate-limiting step in its intracellular transport. Because Tg is a large dimeric glycoprotein with multiple disulfide bonds, the chaperone-assisted folding and assembly of Tg is likely to be more complex than for many other secretory proteins, and this may explain why Tg requires a long ER transit time. After antimycin A treatment, Tg was likewise found almost exclusively in the ER (Fig. 3 *B* and *D*), suggesting that the primary block in intracellular maturation of Tg imposed by ATP depletion (and/or alterations in the redox environment induced by antimycin A) is prior to the exit of the protein from the ER.

Incompletely folded and/or processed Tg has a tendency to transiently form large molecular weight aggregates in the ER in the course of the protein's normal maturation (45). To examine the possibility that Tg is retained in the ER of antimycin A-treated cells as large molecular weight aggregates, we analyzed lysates from untreated and treated cells by sucrose density gradient centrifugation (Fig. 4). In untreated cells (Fig. 4*A*) and in cells incubated in PBS without antimycin A (Fig. 4*B*), Tg sedimented mainly in moderate density fractions of the gradient (fractions 8–13). A small amount of the protein (6%

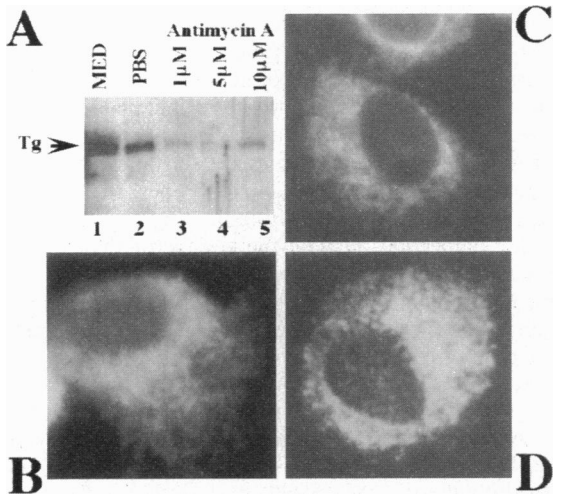

FIG. 3. ATP depletion blocks secretion of Tg in thyroid epithelial cells. (*A*) PCCl3 cells were incubated for 60 min in either culture medium (lane 1) or PBS containing 1.5 mM $CaCl_2$ and 2 mM $MgCl_2$ and without (lane 2) or with (lanes 3–5) antimycin A (1, 5, and 10 μM). Aliquots of media were collected, and any floating cells were removed by centrifugation. Media samples were subjected to 5% SDS/PAGE followed by Western blot analysis with anti-Tg antiserum. The arrow denotes the polypeptide band corresponding to Tg (300 kDa). (*B–D*) Rat thyroid epithelial cells grown on coverslips were incubated for 60 min in PBS containing 1.5 mM $CaCl_2$ and 2 mM $MgCl_2$ without (*C*) or with antimycin A [1 μM (*B*) and 10 μM (*D*)]. Following the treatment, the cells were fixed in methanol and analyzed for Tg immunofluorescence.

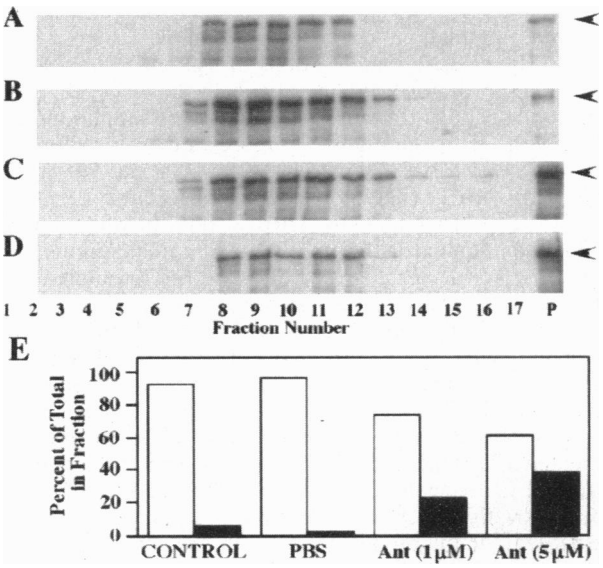

FIG. 4. ATP depletion causes Tg to associate into high molecular weight complexes. PCCl3 cells were incubated for 60 min in culture medium (*A*) or PBS containing 1.5 mM $CaCl_2$ and 2 mM $MgCl_2$ either without (*B*) or with antimycin A (*C*, 1 μM; *D*, 5 μM). Aliquots of cell lysates were layered on top of linear 5–20% sucrose density gradients and centrifuged to equilibrium. Seventeen fractions, as well as material pelleted at the bottom of each gradient (fraction "P"), were collected from each gradient. Protein precipitates from each fraction as well as pellets (P) were analyzed by SDS/PAGE followed by Western blot analysis with anti-Tg antiserum (*A–D*). (*E*) Immunoblots were quantified using the HP Scanjet II scanner and National Institutes of Health IMAGE software. For the gradients presented in *A–D*, the amounts of Tg in fractions 7–13 (open bars) and in fraction P (filled bars) were expressed as percent of total Tg recovered from the gradient.

and 3%, respectively, for untreated cells and cells incubated in PBS, [Fig. 4*E*, solid bars]) was also present in material pelleted at the bottom of the gradient. This portion of Tg presumably represents the aggregates formed by incompletely folded and/or assembled protein in the course of its normal maturation. In antimycin A-treated cells, there was a marked increase in the fraction of Tg that sedimented at the bottom of the gradient (Fig. 4 *C* and *D*, fraction P). Fig. 4*E* (solid bars) shows that 23% and 38%, respectively, of total intracellular Tg has shifted to the pellet fractions of the gradients in cells incubated with 1 and 5 μM antimycin A. As shown in Fig. 2*D*, 1 and 5 μM antimycin A deplete intracellular ATP levels to 12% and 2%, respectively, of control. Thus, Tg association into extremely large macromolecular complexes correlated with the degree of ATP depletion. These complexes could conceivably represent aggregated and/or incompletely assembled Tg stably complexed with one or more ER molecular chaperones.

We have previously shown that at least three ER lumenal molecular chaperones interact with Tg during its maturation. These include BiP, ERp72, and grp94 (33). With the exception of BiP, interactions of Tg and ER molecular chaperones are normally weak and transient, and chemical crosslinking is generally necessary to detect these associations (33). Since ATP has been shown to be required for proper dissociation of secretory proteins from ER molecular chaperones (20, 33, 46, 47), ATP depletion might be expected to inhibit dissociation of chaperones like BiP and grp94 from Tg; this could at least in part explain the presence of Tg-containing high molecular weight protein complexes in the ER. Similarly, the folding of Tg, which contains many disulfide bonds, is also likely to be very sensitive to changes in the local redox environment. Although it is difficult to assess the effects of ischemia-reperfusion and antimycin A on the redox environment of the ER, the fact that whole cell hydrogen peroxide levels increase (7) raises the possibility that the redox environment of the ER is altered. This could also play a role in the generation of Tg-containing high molecular weight complexes

To analyze possible associations of Tg with ER molecular chaperones, we subjected metabolically labeled cell lysates from control and antimycin A-treated cells to immunoprecipitation with the specific anti-Tg antiserum. Autoradiographs revealed at least two bands of 90–100 kDa and 70–80 kDa associating with Tg (data not shown), consistent with possible association of grp94 and BiP/ERp72 (both of which migrate similarly on standard SDS/PAGE). Therefore, immunoprecipitates were further analyzed by Western blot analysis with antisera against various ER proteins (Fig. 5). In untreated thyroid cells and cells treated with PBS alone, only BiP associated with Tg (Fig. 5, lanes 1 and 2). In antimycin A-treated cells, there was an increase in the amount of BiP associating with Tg (Fig. 5, lanes 3–5). A considerable amount of grp94 also coimmunoprecipitated with Tg in antimycin A-treated cells (lanes 3–5). There was also a detectable amount of ERp72 in association with Tg in antimycin A-treated cells, especially with higher concentrations of antimycin A (lanes 4 and 5). Thus, in this model for ischemia, the normally transient associations of BiP, grp94, and ERp72 with Tg in the ER are stabilized.

Taken together, our results suggest that, among other deleterious effects on cellular function, ischemia leads to misfolding/aggregation of at least some secretory proteins, resulting in formation of stable complexes of misfolded proteins with ER molecular chaperones. It is known that a number of characteristics of the internal environment of the ER (lumenal Ca^{2+} content, redox potential, and availability of ATP) are essential for the optimal maturation of secretory proteins as well as for their export from the organelle. Newly synthesized secretory proteins undergo cycles of continuous folding, unfolding, and refolding (48), and ATP has been proposed to be required for at least the folding/refolding steps of the cycle

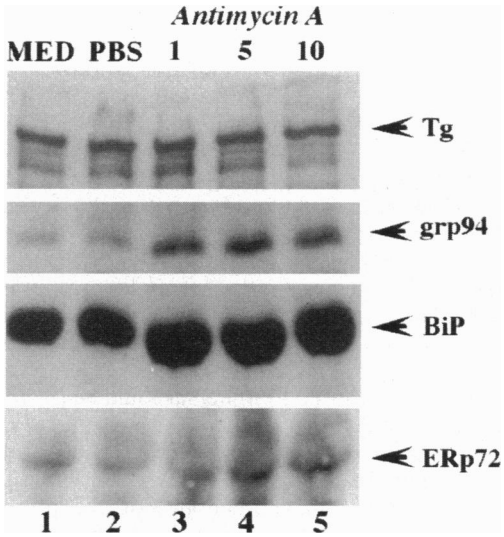

FIG. 5. ATP depletion results in stable association of Tg with ER molecular chaperones. Rat thyroid epithelial cells were incubated for 60 min in normal growth medium (lane 1) or in PBS containing 1.5 mM $CaCl_2$ and 2 mM $MgCl_2$ either with (lane 3, 1 μM; lane 4, 5 μM; and lane 5, 10 μM) or without (lane 2) antimycin A. Tg was immunoprecipitated from cell lysates. Immunoprecipitates were analyzed by SDS/PAGE followed by Western blot analysis with anti-Tg, anti-grp94, anti-BiP, and anti-ERp72 antisera.

(48). Presumably, these are the steps assisted by the ER molecular chaperones and most affected by ATP depletion. But it is also likely that other aspects of ischemia and reperfusion (changes in intracellular redox potential and alterations in intracellular Ca^{2+} homeostasis) contribute to perturbations of secretory protein maturation.

The ER is the site of initial post-translational maturation of both membrane and secreted proteins, including those the function of which appears to be very sensitive to ischemia (e.g., transmembrane junctional proteins, polarized transporters, and integrins). Thus, the effects of ischemia on folding/assembly processes in the ER lumen could play a central role in generation of the ischemic epithelial cell phenotype. Moreover, to the extent that recovery from ischemic injury requires *de novo* synthesis, folding and assembly of these proteins, ER chaperones could conceivably play a major role in the recovery process. In addition to cytosolic chaperones, the cell's ability to elicit a stress response by up-regulating expression of ER molecular chaperones may be the key factor that determines cellular survival following ischemic insult. In support of this notion, a preliminary report suggests that prior treatment with tunicamycin (which upregulates ER chaperones) appears to exert a protective effect against ATP depletion due to antimycin A (49).

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