Misfolded growth hormone causes fragmentation of the Golgi apparatus and disrupts endoplasmic reticulum-to-Golgi traffic

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SUMMARY

In some individuals with autosomal dominant isolated growth hormone deficiency, one copy of growth hormone lacks amino acids 32-71 and is severely misfolded. We transfected COS7 cells with either wild-type human growth hormone or Δ32-71 growth hormone and investigated subcellular localization of growth hormone and other proteins. Δ32-71 growth hormone was retained in the endoplasmic reticulum, whereas wild-type hormone accumulated in the Golgi apparatus. When cells transfected with wild-type or Δ32-71 growth hormone were dually stained for growth hormone and the Golgi markers β-COP, membrin or 58K, wild-type growth hormone was colocalized with the Golgi markers, but β-COP, membrin and 58K immunoreactivity was highly dispersed or undetectable in cells expressing Δ32-71 growth hormone. Examination of α-tubulin immunostaining showed that the cytoplasmic microtubular arrangement was normal in cells expressing wild-type growth hormone, but microtubule-organizing centers were absent in nearly all cells expressing Δ32-71 growth hormone. To determine whether Δ32-71 growth hormone would alter trafficking of a plasma membrane protein, we cotransfected the cells with the thyrotropin-releasing hormone (TRH) receptor and either wild-type or Δ32-71 growth hormone. Cells expressing Δ32-71 growth hormone, unlike those expressing wild-type growth hormone, failed to show normal TRH receptor localization or binding. Expression of Δ32-71 growth hormone also disrupted the trafficking of two secretory proteins, prolactin and secreted alkaline phosphatase. Δ32-71 growth hormone only weakly elicited the unfolded protein response as indicated by induction of BiP mRNA. Pharmacological induction of the unfolded protein response partially prevented deletion mutant-induced Golgi fragmentation and partially restored normal TRH receptor trafficking. The ability of some misfolded proteins to block endoplasmic reticulum-to-Golgi traffic may explain their toxic effects on host cells and suggests possible strategies for therapeutic interventions.

Key words: Golgi apparatus, Growth hormone, Unfolded protein response

INTRODUCTION

Growth hormone (GH) is a soluble, monomeric protein, which is secreted by the somatotrophs of the anterior pituitary gland and is necessary to confer normal postnatal growth and development. After synthesis of GH, cleavage of the signal peptide results in a 191 amino acid, 22 kDa mature peptide that undergoes no further post-translational modification (de Vos et al., 1992). In some individuals with isolated GH deficiency type II, a single base transition in the intron 3 donor splice site causes a skipping of the entire exon 3 of GH mRNA, resulting in a GH peptide lacking amino acids 32-71 (Δ32-71-GH) (Cogan et al., 1994; Phillips and Cogan, 1994; Cogan et al., 1995). Because residues 32-71 normally comprise a large interconnecting loop between the first and second α-helices of a four-helix bundle, deletion of this region results in a protein that cannot fold normally. Usually, heterozygous GH deletions are phenotypically recessive, but individuals heterozygous for the Δ32-71-GH mutation exhibit severe clinical GH deficiency. The basis of the dominant negative phenotype associated with Δ32-71-GH expression is not fully understood. As a relatively small, soluble protein, Δ32-71-GH would not be expected to interfere with folding of wild-type GH (wt-GH). Lee et al. showed that transient expression of Δ32-71-GH in neuroendocrine cells causes a 50% decrease in the secretion of wt-GH that results from a decrease in the stability of wild-type hormone (Lee et al., 2000). Accelerated degradation of wt-GH may contribute to the GH deficiency in heterozygotes carrying the Δ32-71-GH mutation, but it is not clear from available data if increased intracellular degradation of wt-GH is sufficient to account for the observed severity of GH deficiency.

In addition to autosomal dominant GH deficiency, there are many other disorders in which misfolding of a protein leads to a deficiency or malfunction of the protein in question (Kuznetsov and Nigam, 1998; Perlmutter, 1999). When proteins misfold, they are often retained in the endoplasmic reticulum (ER) and targeted for degradation (Ellgaard et al., 1999). Cells can exhibit a variety of responses to the accumulation of misfolded protein in the ER. One such reaction, the unfolded protein response, involves the

transcriptional activation of genes encoding a wide range of proteins necessary for protein folding and secretion (Chapman et al., 1998; Sidrauski et al., 1998; Mori, 2000; Travers et al., 2000). Other cellular strategies for handling accumulated misfolded protein have been demonstrated in association with specific misfolded proteins. For example, an accumulation of misfolded protein called an aggresome is formed when mutant cystic fibrosis transmembrane conductance regulator accumulates (Johnston et al., 1998). In some disorders, accumulation of misfolded protein in the ER may cause damage to the host cell (Teckman et al., 1996; Ito and Jameson, 1997).

These studies were designed to learn how cells respond to the expression of the misfolded Δ32-71-GH and whether intracellular accumulation of the Δ32-71-GH protein has consequences that might damage a somatotroph over time and contribute to the dominant negative phenotype. Here we show that the misfolded GH, when expressed in COS cells, is retained in the ER. Furthermore, we provide direct experimental evidence that the expression of misfolded GH causes fragmentation of the Golgi apparatus and interferes with the trafficking of other, nonmutant proteins, and that these effects may be due to failure of Δ32-71-GH to induce an adequate unfolded protein response. These findings provide a possible mechanism for cellular toxicity in some diseases of protein misfolding, demonstrate the heterogeneity of cellular responses to misfolded proteins and suggest possible therapeutic strategies for prevention of cellular damage in clinical disorders associated with ER protein accumulation.

MATERIALS AND METHODS

Monolayer cultures of COS cells were grown in DMEM supplemented with 5% fetal bovine serum and transfected using Superfect Transfection Reagent (Qiagen, Valencia, CA). Plasmids were used at: 1 μg/ml ER-targeted yellow cameleon YC3er (from Roger Tsien, University of California, San Diego, CA); 2.5 μg/ml human GH, Δ32-71-GH or P89L-GH, which were cloned into pcDNA3, or empty pcDNA3; 5 μg/ml HA-tagged rat thyrotropin-releasing hormone (TRH) receptor (from Graeme Milligan, University of Glasgow, UK); 5 μg/ml rat prolactin in pcDNA3, obtained by transferring prolactin sequences from pCR2-1 (from James Shull, University of Nebraska, Omaha, NE); and 5 μg/ml FLAG-bax in pcDNA3 (from Robert Freeman, University of Rochester, Rochester, NY). Plasmid encoding rat preproinsulin with two mutations in the A chain, V3L and C7S, was prepared from pBC12BI from the American Type Culture Collection encoding the genomic sequence for rat preproinsulin II by the overlapping primer technique described by Ho et al. (Ho et al., 1989). Following a 3 hour transfection, cells were plated on 25 mm glass coverslips and 24 hours later they were fixed and permeabilized for 10 seconds in ice-cold 50% methanol/50% acetone. Coverslips were then washed three times with PBS, incubated for 60 minutes at room temperature with primary antibody in PBS containing 0.2% Nonidet P-40 and 5% goat serum, washed three times with PBS, and finally incubated for 20 minutes with secondary antibody and washed three times for 5 minutes in PBS and mounted in mowiol supplemented with 2.5% 1,4-diazabicyclo-[2,2,2]-octane. Primary antibodies were used at: 1 μg/ml rabbit anti-natural human GH (from A. F. Parlow, National Hormone and Pituitary Program, Harbor-ULCA Medical Center, Torrance, CA); 0.4 μg/ml anti-GFP (a mixture of two mouse monoclonal antibodies from Boehringer Mannheim (Indianapolis, IN); 5 μg/ml anti-β-COP (clone maD, Sigma, St Louis, MO); 2 μg/ml mouse anti-membrin (StressGen Biotechnologies, Victoria, BC); 4 μg/ml anti-Golgi 58K (Sigma); 1.5 μg/ml mouse anti-α-tubulin (Amersham Life Science, Arlington Heights, IL); 1.5 μg/ml mouse anti-HA epitope (Babco, Richmond, CA); 1:500 of ascites fluid of mouse anti-prolactin (from Jonathan Scammell, University of South Alabama, Mobile, AL); 1:1000 of rabbit antibody to insulin (Affinity Bioreagents, Golden, CO); and 0.1 μg/ml mouse anti-FLAG M2 (Sigma). Rhodamine-labeled anti-rabbit IgG and fluorescein-labeled anti-mouse IgG (Molecular Probes, Eugene, OR) were used at 1:200. Cells were examined on a Nikon inverted fluorescence microscope or Leica TCS SP spectral confocal microscope. Where numbers are given, 40 cells from at least ten fields and at least two slides were counted, except in Hoechst staining experiments, in which 200 cells from ten or more fields were counted.

For northern blots, total RNA was isolated by RNeasy (Qiagen) and 5 μg RNA were loaded per lane. The probe for BiP was a 1.5 kb EcoR1-PstI fragment from p3C5, a plasmid containing the cDNA for hamster BiP (from Amy S. Lee, University of Southern California, Los Angeles, CA), and labeled by random priming. For western blots, transfected cells were lysed in Laemmli sample buffer and proteins were resolved by SDS-PAGE on 12% gels. Proteins were transferred to nitrocellulose and blots incubated with antibody to GH or β-COP (1:1000). Bands were identified using ECL (NEN Life Science Products, Boston, MA), for β-COP, or [^{125}I]protein A (NEN) for GH. Specific binding of 5 nM [^{3}H]MeTRH was measured at 37°C as described (Yu and Hinkle, 1998).

For Hoechst staining, methanol:acetone-fixed cells were incubated for 5 minutes in a 1.5 μg/ml aqueous solution of Hoechst 33432 (Molecular Probes). In some experiments, Hoechst staining was done after immunocytochemical staining for GH. Staining was observed using a UV-1A filter set from Chroma Technology (Brattleboro, VT).

A plasmid encoding secreted alkaline phosphatase (SEAP) cloned in pcDNA3 was obtained from Sven-Ulrik Gorr, University of Louisville School of Dentistry, Louisville, KY. Cells were transfected with 2.5 μg SEAP and 2.5 μg either wt-GH or Δ32-71-GH. Medium was collected 24 or 48 hours after transfection and secreted enzyme activity was measured using the Phospha-Light chemiluminescent assay from Tropix (Foster City, CA). The amount of SEAP activity obtained when cells were cotransfected with wt-GH was set at 100%.

The measurements of prolactin synthesis in cells cotransfected with wt-GH or Δ32-71-GH were performed by [^{35}S]amino acid incorporation for 10 minutes followed by immunoprecipitation from the cell lysate and gel electrophoresis as described (Lee et al., 2000).

RESULTS

Subcellular localization of Δ32-71-GH

To compare the subcellular localization of mutant and wt-GH, we transfected COS cells with plasmid vectors encoding wt-GH, Δ32-71-GH, or GH containing a naturally occurring point mutation (P89L-GH), which is reported to cause a mild autosomal dominant GH deficiency (P. Duquesnoy et al. (1998) Endocrine Society Annual Meeting Abstracts). Mutation of Pro89, an invariant residue in the second α-helix of the GH protein, likely causes a mild degree of misfolding. An immunoblot of whole cell extracts prepared 24 hours after transfection revealed bands at the expected positions and indicated that the three GHs were expressed at similar levels (Fig. 1). Cells were cotransfected with each of the GH constructs and an ER marker, the YC3er green fluorescent protein (GFP)-based ‘cameleon’ calcium indicator targeted to the ER with a calreticulin signal sequence and C-terminal KDEL (Miyawaki et al., 1997), and then immunostained 24 hours later for GH and GFP (Fig. 2). Both wt-GH and P89L-GH were concentrated in a discrete perinuclear location shown

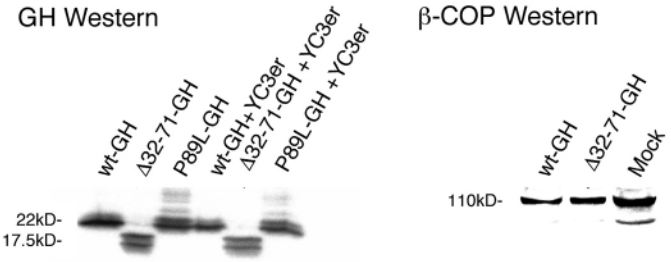

Fig. 1. Western blots for GH and β-COP. Cells were transfected with wt-GH, Δ32-71-GH or P89L-GH as shown at the top of gels. As shown on the left, wt-, P89L- and Δ32-71-GH were expressed at similar levels. The predicted size for wt- and P89L-GH is 22 kDa and for Δ32-71-GH is 17.5 kDa. As shown on the right, transfection of cells with wt-GH or Δ32-71-GH does not affect expression of endogenous β-COP, so the disappearance of β-COP staining in cells expressing Δ32-71-GH was not due to decreased β-COP expression.

below to coincide with Golgi markers (Fig. 3), whereas Δ32-71-GH immunoreactivity was found in a reticular pattern throughout the cell, largely coincident with the ER marker (Fig. 2). In each case, over 90% of cells expressing detectable YC3er also expressed GH.

Effects of Δ32-71-GH on the Golgi apparatus

Because Δ32-71-GH immunocytochemical staining was not found in a Golgi-like pattern, we asked whether the Golgi apparatus was morphologically normal in cells expressing the misfolded GH mutant. To answer this question we stained cells expressing wt-GH or Δ32-71-GH for three Golgi markers, β-COP, Golgi 58K protein and membrin. β-COP is the major component of the COP-I protein complex, which is critical for vesicular traffic between the ER and Golgi and useful as a marker for the vesicular-tubular clusters found at the cis face of the Golgi stack, as well as the cis-Golgi itself (Oprins et al., 1993). Golgi 58K, a formiminotransferase cyclodeaminase, is used as a selective marker for the cytoplasmic face of the Golgi apparatus (Gao et al., 1998). Although β-COP and Golgi 58K are cytoplasmic proteins that associate with Golgi membranes, membrin is a 27 kDa integral Golgi membrane protein that serves as a t-SNARE in ER-to-Golgi-transport (Lowe et al., 1997). Wt-GH immunoreactivity colocalized extensively with all three Golgi markers, indicating that the native hormone is transported to the Golgi apparatus (Fig. 3). β-COP, Golgi 58K and membrin immunostaining patterns were identical in mock-transfected and wt-GH-transfected cells (not shown). Table 1 shows that Δ32-71-GH was retained in the ER of nearly all cells, but wt-GH and P89L-GH were found predominantly in the Golgi apparatus.

The immunocytochemical staining patterns of the three Golgi markers were dramatically altered in cells expressing Δ32-71-GH. In some cells expressing the deletion mutant (Fig. 3, arrowheads), β-COP immunostaining seemed almost to disappear, but immunoblots indicated similar levels of β-COP in cells expressing wt-GH and Δ32-71-GH (Fig. 1). In other cells expressing Δ32-71-GH (Fig. 3, arrows), β-COP staining was visible but found in a much more dispersed pattern than that seen in adjacent cells not expressing the mutant GH. These results indicate that misfolded Δ32-71-GH did not cause decreased expression of β-COP but altered its localization. We examined β-COP immunostaining at 4, 6, 12 and 24 hours after transfection of COS cells with Δ32-71-GH. At 4 hours and 6 hours post-transfection, GH was not yet detectable by immunocytochemistry. At 12 hours after transfection, 40% of cells with detectable Δ32-71-GH showed dispersal of β-COP staining, and by 24 hours after transfection 80% of the Δ32-71-GH-positive cells exhibited Golgi fragmentation. The presence of Δ32-71-GH was also associated with a locally dispersed Golgi 58K staining pattern rather than the tightly clustered pattern in wt-GH-positive cells, again documenting Golgi fragmentation (Fig. 3). Similarly, membrin immunostaining colocalized with wt-GH, but was dispersed in cells expressing Δ32-71-GH (Fig. 3). Because membrin is an integral membrane protein, these results suggest that the changes in β-COP and Golgi 58K staining patterns induced by Δ32-71-GH were not simply due to redistribution of those Golgi-associated proteins to the cytoplasm, but that fragmentation of the Golgi apparatus occurred. Taken together, these morphological data indicate that Δ32-71-GH causes fragmentation of the Golgi apparatus and suggest that the mutant hormone may cause a global disruption of ER-to-Golgi transport and Golgi function.

Table 1. Pattern of GH localization in COS cells expressing wt-GH, Δ32-71-GH or P89L-GH

GH expressed	Golgi alone (%)	Golgi predominant (%)	ER alone (%)	ER predominant (%)
wt-GH	37.5	52.5	0	10
Δ32-71-GH	0	0	87.5	12.5
P89L-GH	65	35	0	0

Localization of growth hormones was quantified by visual counting of at least 40 cells in ten fields (see legends to Figs 2 and 3).

Effect of Δ32-71-GH on cytoplasmic microtubules

Golgi stacks in COS cells are normally juxtaposed to the centrosome, the major organizing center for microtubules in the cytoplasm (Thyberg and Moskalewski, 1999), and microtubule depolymerization leads to redistribution of the Golgi from the centrosomal location to peripheral sites of protein exit from the ER (Cole et al., 1996). Therefore, we asked if the Golgi fragmentation induced by expression of Δ32-71-GH was associated with changes in cytoplasmic microtubular arrangement. Staining for α-tubulin revealed no microtubule depolymerization in cells expressing either wt-GH or Δ32-71-GH. Most cells expressing wt-GH had well-defined microtubule-organizing centers typical of normal interphase cells. However, most cells expressing Δ32-71-GH did not contain distinct microtubule-organizing centers (Fig. 4). Visual counting of wt-GH-positive cells and Δ32-71-GH-positive cells (80 cells from two separate experiments for each GH construct) revealed that 76% of cells expressing wt-GH had obvious microtubule-organizing centers, whereas this microtubular pattern was seen in only 8% of cells expressing Δ32-71-GH.

Effect of Δ32-71-GH on TRH receptor trafficking

To test the effect of the Δ32-71-GH on trafficking of an intrinsic membrane protein, we cotransfected COS cells with plasmids encoding wt-GH or Δ32-71-GH and a plasmid encoding an HA epitope-tagged TRH receptor and studied the

cellular localization of the TRH receptor in GH-positive cells using antibody against the HA epitope. The TRH receptor is a G protein-coupled receptor with seven transmembrane domains that is normally localized on the plasma membrane following synthesis in the ER and *N*-glycosylation in the ER and Golgi (Ashworth et al., 1995). TRH receptor immunoreactivity was appropriately localized on the surface of cells co-expressing wt-GH (Fig. 5), as it was in cells expressing no detectable wt-GH (not shown). By contrast, cells expressing Δ32-71-GH showed no appreciable plasma membrane TRH receptor staining. Instead, both Δ32-71-GH and TRH receptor staining were found in a typical ER pattern. [^{3}H]MeTRH binding data corroborated the immunocytochemical results. [^{3}H]MeTRH, a membrane-impermeant peptide, binds only surface receptors. Cells expressing the Δ32-71-GH bound significantly less [^{3}H]MeTRH than cells expressing wt-GH (11.9±0.5 vs 17.3±1.0 cpm/μg, respectively, P<0.01). Only 40% of cells staining for TRH receptor costained for GH, so the binding data underestimated the deficiency in TRH receptor trafficking in cells expressing Δ32-71-GH. Following immunoprecipitation, TRH receptors from cells transfected with Δ32-71-GH ran close to the predicted protein MW of 47 kDa on western blots, indicating that receptor was not appreciably glycosylated, whereas receptors from cells transfected with wt-GH ran as a broad band at 60-80 kDa, indicative of glycosylation (data not shown). These differences are not sufficient to explain the differences in TRH receptor trafficking to the plasma membrane. The TRH receptor immunocytochemistry results, summarized in Table 2, and binding data combine to indicate that expression of misfolded GH results in a functional severe reduction in trafficking of an unrelated membrane protein.

Effect of Δ32-71-GH on prolactin trafficking

We also tested whether expression of Δ32-71-GH altered trafficking of a normal soluble secretory protein. We studied the effect of Δ32-71-GH on trafficking of prolactin, a hormone that is structurally related to GH and, like GH, undergoes no post-translational modification beyond cleavage of the signal peptide. Confocal images of cells cotransfected with a plasmid encoding rat prolactin and either wt-GH or Δ32-71-GH (Fig. 6) show that wt-GH and prolactin colocalize in the Golgi apparatus. By contrast, prolactin immunoreactivity was localized in a diffuse pattern in cells expressing Δ32-71-GH. Prolactin and Δ32-71-GH immunoreactivity did not overlap completely, but the patterns were similar, indicating that prolactin, like Δ32-71-GH, was probably in the ER. Rates of prolactin synthesis, measured by [^{35}S]amino acid incorporation followed by immunoprecipitation from cell

Table 2. Pattern of prolactin and TRH receptor immunocytochemical staining in COS cells expressing either wt-GH or Δ32-71-GH

GH expressed	Golgi predominant (%)	ER predominant (%)	Plasma membrane predominant (%)	No staining (%)
	Prolactin			
wt-GH	85	7.5	0	7.5
Δ32-71-GH	5	92.5	0	2.5
	TRH receptor			
wt-GH	0	7.5	80	12.5
Δ32-71-GH	0	77.5	20	2.5
Δ32-71-GH 10 μg/ml tunicamycin	0	55	45	0

Localization of prolactin and TRH receptor was quantified by visually counting at least 40 cells from ten fields (see legends to Figs 5 and 6).

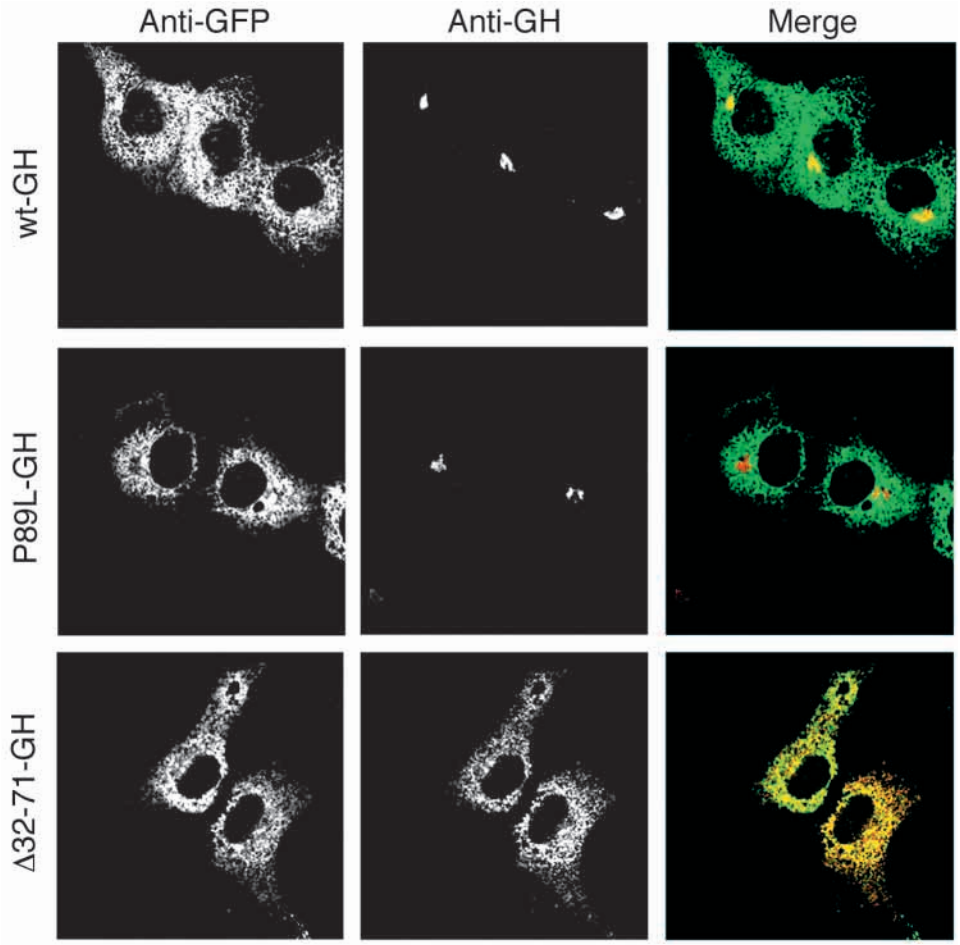

Fig. 2. ER marker and GH staining. Cells cotransfected with wt-GH, Δ32-71-GH or P89L-GH and YC3er were stained with antibody to GFP, which labels the ER marker YC3er (left panels), and with antibody to GH (middle panels). Right panels show merged images with YC3er in green and GH in red; colocalized staining appears yellow. Only Δ32-71-GH colocalized with the ER marker.

lysates, were not significantly different between cells expressing wt-GH or Δ32-71-GH, averaging 388±70 vs 323±13 arbitrary units by densitometry, respectively (data not shown). Quantification of the immunocytochemical staining patterns indicates that expression of Δ32-71-GH interferes with the normal trafficking of prolactin as well as the TRH receptor (Table 2).

Δ32-71-GH decreases secretion of alkaline phosphatase

To test further the effects of the GH deletion mutant on protein secretion we transfected COS cells with secreted alkaline phosphatase, a constitutively secreted protein (Berger et al., 1988), along with wt-GH or Δ32-71-GH. Secreted alkaline phosphatase activity was measured in the media 24 and 48 hours after transfection. No activity was detected in the media from nontransfected cells. The cells expressing Δ32-71-GH secreted 44±7% of the alkaline phosphatase activity that cells expressing wt-GH secreted after 24 hours, and 21±6% after 48 hours. These results further indicate that Δ32-71-GH interferes with global protein secretion.

Δ32-71-GH weakly induces the unfolded protein response

To determine whether Δ32-71-GH induces the unfolded protein response, we used northern analysis to quantify the amount of mRNA for BiP, also called GRP78, in cells transfected with empty vector or plasmids encoding wt-GH or Δ32-71-GH. BiP was induced only weakly and to the same extent (twofold) by wild-type or misfolded GH. BiP was induced strongly (11-fold) by treatment of COS cells with dithiothreitol (DTT), a strong reducing agent known to induce the unfolded protein response (Molinari and Helenius, 1999) (Fig. 7). Tunicamycin, which interferes with glycoprotein synthesis and is also used to induce the unfolded protein response, caused fourfold and sevenfold increases in BiP mRNA when used at 1 μg/ml and 10 μg/ml, respectively. Expression of a mutant insulin that does not fold properly increased BiP mRNA fivefold (Fig. 7). These experiments show that Δ32-71-GH does not markedly induce the unfolded protein response, which would be

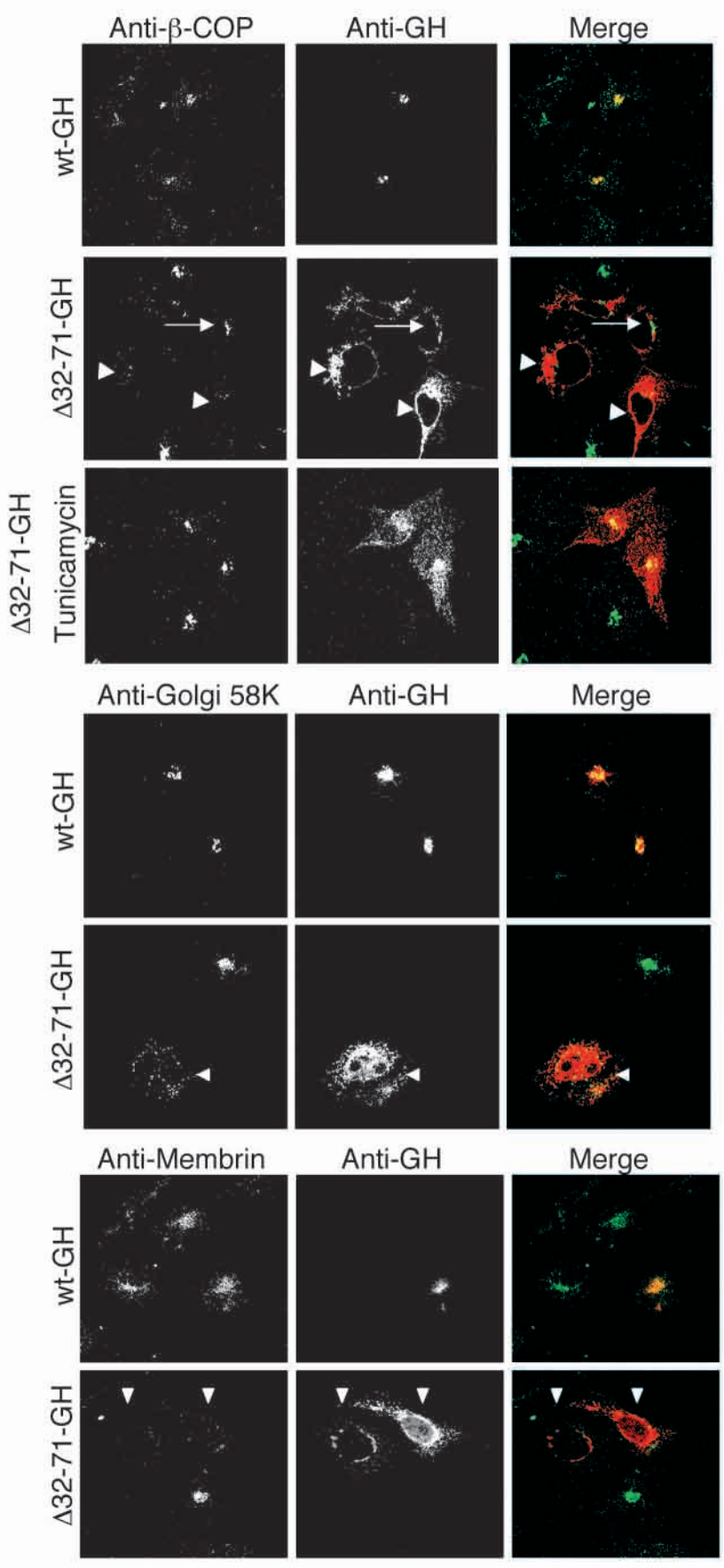

Fig. 3. Golgi marker and GH staining. Cells cotransfected with wt-GH or Δ32-71-GH were stained with antibody to the Golgi markers β-COP (top left panels), Golgi 58K (middle left panels), or membrin (bottom left panels), and antibody to GH (center column of panels). Merged images show GH in red and Golgi markers in green; colocalized staining appears yellow. Where noted, 10 μg/ml tunicamycin was added for 3 hours during transfection and then removed. Arrowheads denote cells in which the Golgi almost disappeared and arrows show cells in which the Golgi was visible but dispersed.

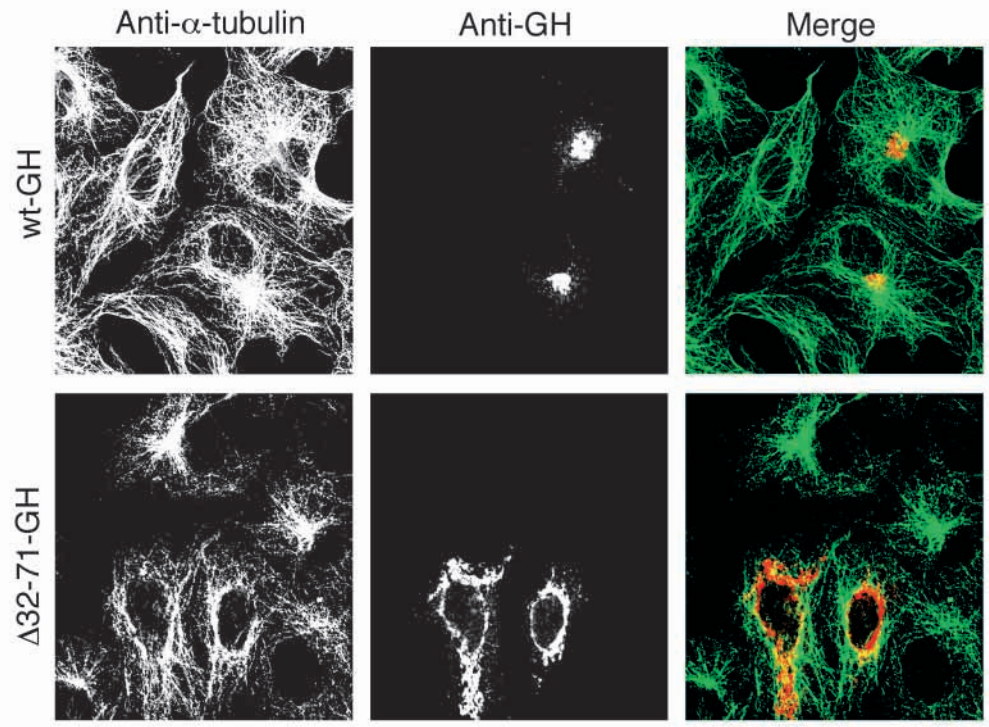

Fig. 4. Disruption of microtubule-organizing centers in cells expressing mutant GH. Cells cotransfected with wt-GH or Δ32-71-GH were stained with antibody to α-tubulin (left panels) and antibody to GH (middle panels). Right panels show GH in red and α-tubulin in green; colocalized staining appears yellow. Microtubule polymers can be seen emanating from distinct microtubule-organizing centers in cells expressing wt-GH, which is concentrated in the Golgi apparatus, and in cells not expressing any GH. The lower panels show that Δ32-71-GH is retained in the ER, and cells expressing Δ32-71-GH show no distinct microtubule organizing centers.

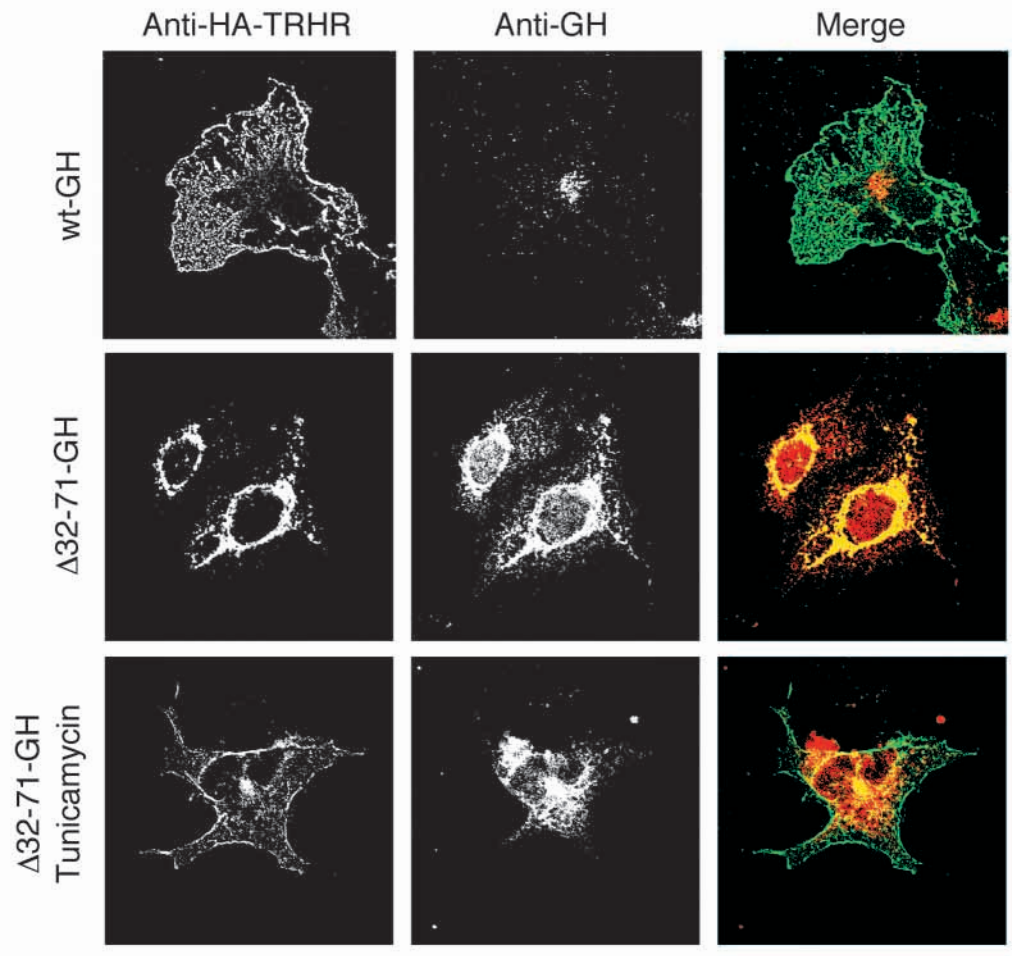

Fig. 5. Mislocalization of membrane TRH receptor in cells expressing mutant GH and reversal with tunicamycin treatment. Cells cotransfected with wt-GH or Δ32-71-GH and HA-epitope-tagged TRH receptor were stained with antibody to the HA epitope (left panels) and antibody to GH (middle panels). Right panels show GH in red and HA in green; colocalized staining appears yellow. Approximately 80% of cells were positive for TRH receptor and 32% for GH. TRH receptor is localized on the plasma membrane in cells expressing wt-GH but retained in the ER in cells expressing the GH mutant. In cells expressing the GH mutant and treated with 10 μg/ml tunicamycin, plasma membrane localization of TRH receptor was restored in 45% of cells (bottom panels). TRHR, TRH receptor.

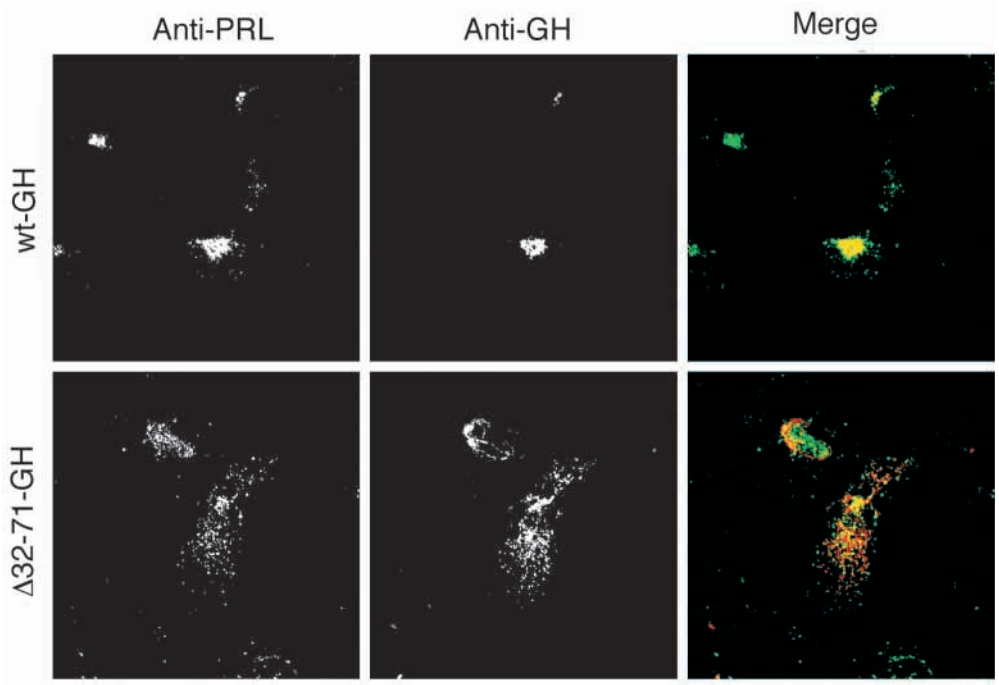

Fig. 6. Mislocalization of prolactin in cells expressing mutant GH. Cells cotransfected with wt-GH or Δ32-71-GH and human prolactin were stained with antibody to prolactin (left panels) and antibody to GH (middle panels). Right panels show GH in red and prolactin in green; colocalized staining appears yellow. Prolactin is found in the Golgi apparatus of cells expressing wt-GH but retained in the ER in cells expressing Δ32-71-GH. PRL, prolactin.

expected to help protect the cells against toxic effects resulting from unfolded proteins.

Reversal of Δ32-71-GH effects by unfolded protein response induction

Because Δ32-71-GH failed to induce the unfolded protein response strongly, we asked whether artificial induction of the response would prevent the Golgi fragmentation and TRH receptor trafficking blockade caused by the GH mutant. The proportion of cells with Golgi fragmentation was in fact reduced from 80% to 57.5% when Δ32-71-GH-transfected cells were cotransfected with the insulin mutant. Similarly, Golgi fragmentation was seen in 57.5% of cells treated with 1 μg/ml tunicamycin (Fig. 3), and in only 32.5% of cells treated with 10 μg/ml tunicamycin. Tunicamycin also increased the localization of TRH receptor on the plasma membrane in cells expressing Δ32-71-GH, suggesting that inducing the unfolded protein response partially restores normal protein trafficking (Table 2) (Fig. 5). Tunicamycin increased from 12.5% to 35% the fraction of cells in which some or all of Δ32-71-GH was localized in the Golgi apparatus.

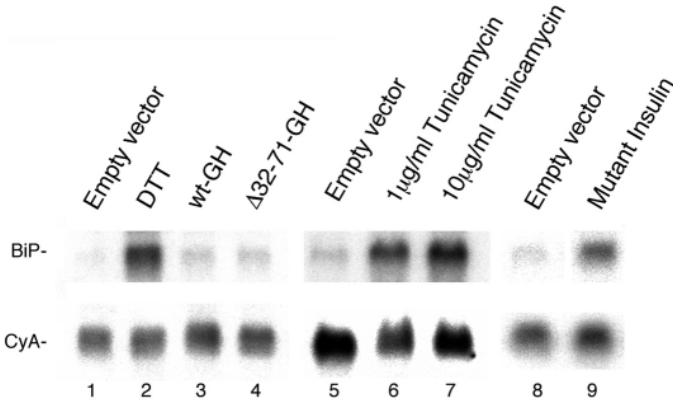

Fig. 7. Northern blots for BiP. Lanes 1-4: cells were transfected with GH or empty vector and incubated without or with 10 mM DTT for 6 hours. Lanes 5-7: cells were transfected with empty vector and treated with 1 or 10 μg/ml tunicamycin for 3 hours, then switched to drug-free medium. Lanes 8 and 9: cells were transfected with empty vector or mutant preproinsulin encoding insulin with V3A and C7S in the A chain. RNA was isolated after 24 hours. Blots were probed for BiP mRNA, then stripped and reprobed for cyclophilin A (CyA).

Effect of Δ32-71-GH on nuclear chromatin pattern

Golgi fragmentation occurs during apoptosis (Sesso et al., 1999). We tested whether Δ32-71-GH induces condensation and fragmentation of nuclear chromatin to investigate the possibility that the Δ32-71-GH-induced Golgi fragmentation was secondary to an overall increase in cell death by apoptosis. In one experiment, cells were transfected with either wt-GH, Δ32-71-GH, empty pcDNA3 vector or, as a positive control, with epitope-tagged bax, a strong inducer of apoptosis. After 24 hours, cells were costained for either GH (wt-GH and Δ32-71-GH transfections) or the FLAG epitope (bax-FLAG transfections) and with Hoechst dye, which identifies nuclear chromatin, and scored for condensed or fragmented nuclear chromatin, a consistent characteristic of apoptotic cells. Apoptotic nuclei were observed in 177/200 (88.5%) of cells expressing bax, but in only 35/200 (17.5%) and 39/200 (19.5%) of cells expressing wt- or Δ32-71-GH, respectively. No GH or FLAG immunoreactivity was seen in cells transfected with the empty plasmid. These results suggest that expression of Δ32-71-GH does not increase apoptosis as compared to cells expressing wt-GH and that the Golgi fragmentation we observed is probably not due to the general disassembly of cellular organelles reported in cells undergoing apoptosis (Kohler et al., 1990).

DISCUSSION

Some disorders, such as cystic fibrosis, are due to altered trafficking of incorrectly folded protein (Kim and Arvan, 1998; Kuznetsov and Nigam, 1998). In others, accumulation of misfolded protein in the ER may cause damage. The mutant rhodopsin molecules responsible for some forms of autosomal dominant retinitis pigmentosa accumulate in the ER when expressed in COS or HEK 293 cells (Sung et al., 1993; Kaushal and Khorana, 1994). Mutant vasopressin precursors that cause autosomal dominant neurohypophyseal diabetes insipidus accumulate in the ER when expressed in differentiated Neuro2A cells, and ultimately cause those cells to die (Ito and Jameson, 1997). The PiZ variant of human α_1-antitrypsin (α_1-ATZ) accumulates as insoluble aggregates in the ER of hepatocytes and the accumulation may result in liver damage. Fibroblasts from individuals with α_1-ATZ-associated liver damage have a reduced ability to degrade α_1-ATZ compared to fibroblasts from individuals with the mutation but without liver damage, so fibroblasts from susceptible individuals accumulate more of this misfolded protein (Wu et al., 1994). Accumulation of misfolded protein in the ER can be associated with cellular toxicity, but how misfolded proteins in the ER are detrimental to cells is not known.

We examined the expression of Δ32-71-GH in COS cells, and found that this misfolded protein, like many others, accumulates in the ER. We further found that expression of Δ32-71-GH leads to fragmentation of the Golgi apparatus and aberrant trafficking of other proteins destined for the plasma membrane or for secretion. Such changes seem likely to lead to cellular toxicity and may provide an explanation for why some proteins that cannot fold correctly exert dominant negative effects.

In previous studies, we found that Δ32-71-GH did not accumulate in several neuroendocrine cell lines over a 24 hour period (Lee et al., 2000). Our finding that cotransfected Δ32-71-GH accumulated in COS cells and caused altered trafficking of prolactin may reflect differences in protein expression and secretory pathways between cell types. Δ32-71-GH may resemble α_1-ATZ in that it becomes more toxic in situations where it accumulates. The neuroendocrine cells lines, however, may not be good models for the normal somatotroph through all its stages of development and maturation. If expression of Δ32-71-GH leads to degradation of Golgi structure and function, similar to what we have observed, at any stage of maturation, this disruption would be expected over time to damage or destroy pituitary somatotrophs. This presents a plausible explanation for the dominant phenotype seen in isolated GH deficiency type II. This hypothesis is supported by recent studies in transgenic mice in which expression of Δ32-71-GH caused selective disappearance of somatotrophs by 3 weeks of age (I. C. Robinson, personal communication). Histological studies of pituitary glands from people heterozygous for the Δ32-71-GH mutation have not been reported.

Transport of proteins from the ER occurs in COPII vesicles that bud from selected sites, and some proteins that cannot achieve their normal configuration accumulate at these sites (Raposo et al., 1995). Aridor et al. used transmission electron microscopy and morphometric analysis to show that expression of a temperature-sensitive mutant of vesicular stomatitis glycoprotein (VSV-G) or α_1-ATZ reduced the number of vesicles budding from the ER (Aridor et al., 1999). These experiments suggest that correctly folded and assembled cargo proteins are involved in the formation of their own COPII-coated ER-to-Golgi transport vesicles. Studies examining formation of COPII vesicles in vitro supported this conclusion. The disruption of protein transport and Golgi morphology caused by Δ32-71-GH in our studies could result from a similar failure of COPII vesicles to bud. Additional studies are needed to pinpoint the mechanism underlying abnormal protein trafficking in cells expressing Δ32-71-GH. In light of the results of Aridor et al. (Aridor et al., 1999), incorrectly folded proteins could interfere with transport of other, normal proteins out of the ER. Our findings that Δ32-71-GH interferes with the flow of prolactin, TRH receptors and secretory alkaline phosphatase confirm this prediction. It is important to note that not all incorrectly folded proteins disrupt protein transport and Golgi morphology. In these studies, we found that an insulin mutant accumulates in the ER but does not alter Golgi staining. In another example, Hobman et al. examined accumulation of Rubella virus E1 glycoprotein in the ER (Hobman et al., 1998); E1 is only transported further along the secretory pathway if it is complexed with E2. They found that E1 accumulates at specific sites of exit from ER but does not affect the distribution of COPII staining.

Golgi fragmentation has been observed previously as a late step in cells undergoing apoptosis (Watanabe et al., 2000). We considered the possibility that the Golgi fragmentation we observed was secondary to apoptosis induced by Δ32-71-GH, but we did not detect apoptotic features in cells expressing the misfolded hormone.

Another well-known condition associated with Golgi fragmentation is microtubule depolymerization (Cole et al., 1996). When microtubule polymers are disrupted, Golgi membrane components redistribute to peripheral sites of protein exit from the ER; Golgi function is preserved under these conditions despite the structural changes. The patterns of α-tubulin staining we observed in cells expressing Δ32-71-GH, however, are not consistent with microtubular depolymerization. Rather, microtubule-organizing centers were disrupted. Similar disordering of cytoplasmic microtubules has been seen in cells infected with vaccinia virus (Ploubidou et al., 2000) and in cells treated with a DNA polymerase inhibitor (Tanaka et al., 1998). The effects of Δ32-71-GH and other misfolded proteins on microtubular dynamics bears further study. Rowe et al. reported disassembly of the Golgi apparatus in neurosecretion-defective PC12 clones and NRK fibroblasts transfected with cDNA encoding syntaxin 1A, a t-SNARE involved in synaptic exocytosis (Rowe et al., 1999). This Golgi disassembly was prevented by cotransfection of rbSec1, a protein that interacts with syntaxin 1A in neurosecretory exocytosis. It is unlikely that the Golgi fragmentation in cells expressing Δ32-71-GH was due to absence of a protein like rbSec1, however, as Golgi disassembly did not occur in cells expressing either wt-GH or misfolded insulin.

Golgi fragmentation has also been reported in cells treated with 1-butanol, an inhibitor of phosphatidylinositol(4,5)bis-phosphate formation (Siddhanta et al., 2000). It will be of interest to determine whether phosphatidylinositol(4,5)bis-phosphate concentrations are reduced in cells expressing Δ32-71-GH.

Not all proteins that aggregate in the ER are toxic. For example, the Mody mouse has an autosomal dominant mutation that results in a Tyr for Cys substitution in insulin 2. This mutation prevents proper folding and processing of wild-type insulins 1 and 2, so that they aggregate in the ER, but the pancreatic β cells are not destroyed, and insulin mRNA is transcribed normally (Wang et al., 1999). Mice with this mutant overexpress BiP, a molecular chaperone in the ER (Wang et al., 1999). By contrast, Δ32-71-GH causes little or no induction of BiP, and in this respect resembles the α_1-ATZ variant, which also accumulates in the ER without inducing BiP mRNA (Cresteil et al., 1990; Graham et al., 1990). Although BiP binds to many unfolded proteins, Graham et al. were unable to detect BiP binding to α_1-ATZ (Graham et al., 1990), and we have been unable to detect BiP binding to Δ32-71-GH (T.K.G. and P.M.H., unpublished). The unfolded protein response, including increased transcription of BiP mRNA, is induced by activation of the transmembrane kinase Ire1p, and binding of BiP to the lumenal portion of Ire1p is thought to keep the kinase from dimerizing and activating itself (Bertolotti et al., 2000). If this activation mechanism is correct, proteins that fail to bind BiP will not induce the unfolded protein response because they fail to compete with BiP binding to Ire1p. Over 300 genes are induced in the unfolded protein response in yeast, and many of these gene products have functions in the secretory pathway, including translocation into the ER, protein folding and modification, protein degradation through the proteasome pathway, and transport from the ER to the Golgi complex and from the Golgi complex to lysosomes or the cell surface (Travers et al., 2000). Thus, the unfolded protein response is a useful protective mechanism against damage resulting from unfolded proteins. Proteins such as Δ32-71-GH and α_1-ATZ that induce the unfolded protein response poorly are more likely to cause cellular toxicity. This is supported by our finding that strategies to induce the unfolded protein response decreased Golgi apparatus fragmentation, and partially rescued normal TRH receptor trafficking in cells expressing mutant GH. It is notable that induction of the unfolded protein response with tunicamycin, as well as by expression of a misfolded insulin mutant, gave similar results. It will be of interest to examine whether the failure to induce the unfolded protein response contributes to the mechanism of cytotoxicity seen in other protein misfolding diseases. It will also be interesting to test the notion that therapeutic induction of the unfolded protein response might alleviate the cellular damage caused by the accumulation of the misfolded protein.

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