Caspase-resistant Golgin-160 Disrupts Apoptosis Induced by Secretory Pathway Stress and Ligation of Death Receptors

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Golgin-160 is a coiled-coil protein on the cytoplasmic face of the Golgi complex that is cleaved by caspases during apoptosis. We assessed the sensitivity of cell lines stably expressing wild-type or caspase-resistant golgin-160 to several proapoptotic stimuli. Cells expressing a caspase-resistant mutant of golgin-160 were strikingly resistant to apoptosis induced by ligation of death receptors and by drugs that induce endoplasmic reticulum (ER) stress, including brefeldin-A, dithiothreitol, and thapsigargin. However, both cell lines responded similarly to other proapoptotic stimuli, including staurosporine, anisomycin, and etoposide. The caspase-resistant golgin-160 dominantly prevented cleavage of endogenous golgin-160 after ligation of death receptors or induction of ER stress, which could be explained by a failure of initiator caspase activation. The block in apoptosis in cells expressing caspase-resistant golgin-160 could not be bypassed by expression of potential caspase cleavage fragments of golgin-160, or by drug-induced disassembly of the Golgi complex. Our results suggest that some apoptotic signals (including those initiated by death receptors and ER stress) are sensed and integrated at Golgi membranes and that golgin-160 plays an important role in transduction of these signals.

INTRODUCTION

The Golgi complex is central to the regulation of membrane trafficking in eukaryotic cells. It is the primary site for sorting and processing of proteins undergoing both exocytosis and endocytosis (reviewed in Farquhar and Palade, 1998). Under normal conditions, the mammalian Golgi complex is a collection of stacks of cisternal membranes near the microtubule organizing center. Proteins traversing the secretory pathway enter the Golgi at the *cis* face, are processed as they pass through the Golgi stacks, and are sorted at the *trans* face into vesicles bound for their intended destinations. The *trans*-Golgi is also important in sorting proteins being endocytosed or cycling between the plasma membrane and intracellular membrane compartments. This affords the Golgi extensive communication with the rest of the cell and its surroundings, placing it in a prime position to sense and integrate information about the state of the cell and its environment.

The structure of the Golgi is highly dynamic, allowing reversible disassembly during mitosis. Key secretory pathway proteins are phosphorylated in a cell cycle-dependent manner to stop membrane trafficking and disassemble the Golgi (reviewed in Rabouille and Jokitalo, 2003). This rearrangement from a single perinuclear organelle to dispersed vesiculated compartments is thought to ensure partitioning

of the Golgi complex into both daughter cells during cytokinesis. When Golgi disassembly is prevented by addition of antibodies to Golgi reassembly and stacking protein of 65 kDa (GRASP65), cells complete S phase, but they are unable to enter mitosis. This mitotic block can be bypassed by disassembling the Golgi with drugs (Sutterlin *et al.*, 2002). Once mitosis is complete, the Golgi is reassembled to its normal stacked structure and perinuclear position. Thus, the structure of the Golgi complex seems to be an important signal regulating progression of mitosis.

The Golgi complex also disassembles during apoptosis. Whereas elements of mitotic and apoptotic Golgi disassembly seem to have features in common (Sesso *et al.*, 1999), apoptotic Golgi disassembly is irreversible and primarily depends on protein cleavage rather than phosphorylation. During apoptosis, caspases cleave proteins involved in Golgi structure and trafficking, including golgin-160 (Mancini *et al.*, 2000), GM130 (Nozawa *et al.*, 2002; Lowe *et al.*, 2004), giantin (Lowe *et al.*, 2004), GRASP65 (Lane *et al.*, 2002), and p115 (Chiu *et al.*, 2002).

Cleavage of Golgi-resident proteins seems to be important for cessation of membrane trafficking, Golgi disassembly, and possibly initiation of apoptosis. Caspase cleavage of the Golgi t-SNARE syntaxin-5 (Lowe *et al.*, 2004) and Golgi-localized components of the dynein–dynactin motor complex (Lane *et al.*, 2001) have been implicated in termination of membrane trafficking during apoptosis. Expression of caspase-resistant mutants of golgin-160, GRASP65, or p115 delays Golgi disassembly during apoptosis (Mancini *et al.*, 2000; Chiu *et al.*, 2002; Lane *et al.*, 2002). Expression of a construct mimicking the C-terminal caspase-cleavage product of p115 causes Golgi fragmentation and induces apoptosis (Chiu *et al.*, 2002). These results indicate an important role for caspase cleavage of Golgi-resident proteins during apoptosis and suggest involvement of Golgi-resident proteins,

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not just in execution of apoptotic disassembly but also in initiation of apoptosis.

Here, we show that cells expressing a caspase-resistant mutant of golgin-160 demonstrate a remarkable resistance to death induced by a subset of proapoptotic stimuli. Caspase processing is impaired after treatment with this subset of stimuli, suggesting that cleavage of golgin-160 is required for transduction of specific apoptotic signals.

MATERIALS AND METHODS

Materials

Brefeldin-A (BFA), etoposide, staurosporine, anisomycin, cycloheximide (Chx), recombinant human tumor necrosis factor (TNF)- α , dithiothreitol (DTT), thapsigargin, nocodazole, and Hoechst 33258 were from Sigma-Aldrich (St. Louis, MO). Iliquinone (Takizawa *et al.*, 1993) was from Vivek Malhotra (University of California, San Diego, La Jolla, CA). Soluble recombinant TNF-related apoptosis-inducing ligand (TRAIL) was from Calbiochem (San Diego, CA). Concentrated stock solutions were prepared in dimethyl sulfoxide or water and added to culture medium at the final concentrations indicated in the figure legends. Anti-human Fas IgG was from Oncogene Science (Cambridge, MA). Mouse anti-human caspase-2 (ICH1-L) was from BD Biosciences (San Jose, CA), and rabbit anti-green fluorescent protein (GFP) was from Molecular Probes (Eugene, OR). Rabbit anticaspase-8 was from BD Biosciences, and rabbit anticaspase-3 was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-golgin-160 (Hicks and Machamer, 2002) has been described previously. Mouse anti-human poly(ADP-ribose) polymerase (PARP) was from BD Biosciences Pharmingen (San Diego, CA). Fluorescein- and Texas Red secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA), and horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences (Piscataway, NJ).

Cells

HeLa cells were grown in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA). The N-terminally tagged GFP/golgin-160 has been described previously (Mancini *et al.*, 2000). The GFP/golgin-160(3DE) mutant was constructed by site-directed mutagenesis (QuikChange; Stratagene, La Jolla, CA) by sequential mutation of the codons for D59, D139, and D311 to those for glutamic acid. The C-terminally GFP-tagged golgin-160 was constructed by introducing an *EcoRI* site in place of the stop codon and cloning golgin-160 or golgin-160(3DE) into pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA). Stable cell lines expressing GFP-tagged golgin-160 and golgin-160 mutants were selected with 0.4 mg/ml G418 (Geneticin; Invitrogen) and screened as described previously (Mancini *et al.*, 2000). We selected cells that expressed low levels of GFP-tagged golgin-160 to ensure proper targeting. The lines used here expressed GFP-tagged golgin-160 at approximately twofold the level of endogenous golgin-160.

Apoptosis Assays

Cells were plated in six-well dishes 1 to 2 days before treating with apoptotic inducers. Drugs or antibodies were added to cells in 1.5 ml of regular growth medium in a staggered manner so that all wells were harvested at the same time. Cells were scraped into the medium, and spun at 1000 rpm ($150 \times g$) for 20 min. One-tenth of the sample was fixed in 15 μ l of 3% paraformaldehyde containing 2 μ g/ml Hoechst 33258 and analyzed by microscopy. The remainder of the sample was rinsed in 0°C phosphate-buffered saline and respun. Cells were lysed in 0.15 M NaCl, 10 mM Tris-HCl, pH 8.0, 1% Nonidet-P40, 1% deoxycholate, 0.1% SDS, and 10 mM EDTA containing 20 μ g/ml aprotinin, 20 μ g/ml leupeptin, and 2 μ g/ml pepstatin A for 10 min at 0°C, and spun at $16,000 \times g$ for 15 min to remove debris. An aliquot was reserved for protein concentration determination by bicinchoninic acid assay (Pierce Chemical, Rockford, IL). Fourfold concentrated Laemmli sample buffer was added to the remaining lysates for immunoblotting. To determine apoptotic morphology, Hoechst 33258-stained samples were analyzed with an Axioskop (Carl Zeiss, Thornwood, NY) equipped for epifluorescence using the UV filter. At least 300 nuclei were scored for normal or apoptotic morphology. Apoptotic nuclei were defined as those with condensed and marginalized DNA. Each treatment was performed a minimum of three independent times.

Expression of Golgin-160 Cleavage Products

Expression vectors encoding Myc-tagged golgin-160 fragments have been described previously (Hicks and Machamer, 2002). The vector expressing myc-tagged golgin-160 amino acids 312-1498 was made similarly. Site-directed mutagenesis was used where base changes were necessary (QuikChange; Stratagene), and sequences were confirmed using dideoxy sequencing. Cell lines were transfected using FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) according to manufacturer's directions. The percentage of cells expressing each myc-tagged construct was determined

by immunofluorescence by using monoclonal mouse anti-myc (clone 9E10) from Roche Diagnostics. To ensure that any change in apoptosis caused by expression of golgin-160 fragments would be observable, only samples on which at least 12% of the cells were positive for myc expression were analyzed. Expression ranged from 12 to 50%. The fragment consisting of amino acids 140-311 was excluded from this analysis due to poor expression. Cells were treated as described and apoptosis was assessed by cleavage of PARP by immunoblotting. Due to the high sensitivity of this assay, as little as 2% cleavage of PARP could routinely be quantitated.

Immunoblotting

For analysis of PARP, 50 μ g of protein was electrophoresed in a 10% acrylamide gel. Proteins were transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA), and immunoblotting was performed as described previously (Hicks and Machamer, 2002). Mouse anti-PARP was used at 1:2000. Mouse anticaspase-2 was used at 1:400, rabbit anticaspase-8 at 1:3000, and rabbit anticaspase-3 at 1:200. Secondary antibodies were horseradish peroxidase-conjugated goat anti-human IgG, anti-mouse IgG, and anti-rabbit IgG and used accordingly. enhanced chemiluminescence (Amersham Biosciences) was performed according to the manufacturer's directions, and films were scanned and processed using Adobe Photoshop. For quantitation, chemiluminescent signal was measured by a VersaDoc imaging system (Bio-Rad, Hercules, CA) and quantitated using Quantity One software (Bio-Rad). Percentage of cleavage of PARP was determined by measuring full-length and cleaved PARP bands, subtracting background, and then dividing the amount cleaved by the total full-length and cleaved PARP.

For repeated probing of membranes with the same species of primary antibody, membranes were stripped for 30 min at 50°C with occasional agitation in 62.5 mM Tris-HCl, pH 6.8, 100 mM β -mercaptoethanol, and 2% sodium dodecyl sulfate, and then washed in Tris-buffered saline (TBS)-Tween (10 mM Tris-HCl, pH 7.4, .15 M NaCl, and 0.05% Tween 20) and reblocked with 5% milk in TBS-Tween before probing with antibody.

Immunoprecipitation

Cells were labeled for 2 h with 214 μ Ci/ml [35 S]methionine and cysteine (Promix; Amersham Biosciences) in methionine and cysteine-free DMEM. The chase was in normal growth medium for 6 h in the absence or presence of proapoptotic drugs (1 μ M staurosporine, 10 ng/ml TNF- α plus 10 μ g/ml Chx, 0.1 μ g/ml anti-human Fas plus 10 μ g/ml Chx, 10 mM DTT, or 20 ng/ml TRAIL plus 10 μ g/ml Chx). For 4-h drug treatments (staurosporine and TRAIL), cells were chased in normal growth medium for 2 h, and then proapoptotic drugs were added for the remaining 4 h. Lysates were immunoprecipitated with an antibody that recognizes the C terminus of golgin-160 as described previously (Hicks and Machamer, 2002) and analyzed by 10% SDS-PAGE followed by fluorography.

Indirect Immunofluorescence Microscopy

Fixed cells were permeabilized, stained, and imaged as described (Hicks and Machamer, 2002) using an Axioskop fluorescence microscope (Carl Zeiss) and Sensys charge-coupled device camera (Photometrics, Tucson, AZ). Images were collected using IP Lab software (Scanalytics, Fairfax, VA), and imported into Adobe Photoshop.

RESULTS

Apoptosis Is Impaired in Cells Expressing Caspase-resistant Golgin-160

We previously showed that golgin-160, a peripheral Golgi membrane protein, was a substrate for cleavage by caspases during apoptosis (Mancini *et al.*, 2000). Caspase-2 cleaves golgin-160 at D59 and D311. Golgin-160 is also cleaved by caspase-3 at D139 and by caspases-7 and -3 at D311. To evaluate the effects of expressing a noncleavable golgin-160 on apoptosis, we generated HeLa cell lines stably expressing GFP-tagged wild-type golgin-160 or golgin-160 with all three caspase cleavage sites replaced with glutamates [golgin-160(3DE)]. The cell lines chosen for further study expressed a low level of the tagged proteins, less than twofold over that of endogenous golgin-160. Both tagged proteins were targeted normally to the Golgi complex (Figure 1).

We tested the two stable cell lines with a broad spectrum of proapoptotic stimuli to examine the effects of expression of golgin-160(3DE) on apoptosis. We used staurosporine (a broad-spectrum kinase inhibitor), etoposide (a topoisomerase inhibitor), and anisomycin (a protein synthesis inhibitor), which are widely used for induction of apoptosis. We

Figure 1. GFP-tagged golgin-160 proteins are properly targeted in stable cell lines. GFP/golgin-160(wt) and GFP/golgin-160(3DE) localize to the Golgi as shown in fixed cells stained with anti-GM130 as a Golgi marker. Bar, 10 μ m.

also used several drugs that induce the ER stress response, a pathway induced by misfolded or unassembled proteins in the ER (Ma and Hendershot, 2001) that can lead to apoptosis if the stress is not alleviated (Nakagawa and Yuan, 2000). BFA is a fungal metabolite that blocks secretion and causes disassembly of the Golgi complex (Klausner *et al.*, 1992) and with prolonged treatment induces apoptosis (Shao *et al.*, 1996; Guo *et al.*, 1998). Thapsigargin, which blocks the ER calcium pump, and DTT (a reducing agent) also activate the ER stress response. Last, we ligated death receptors by using TNF- α , anti-Fas antibody, and TRAIL in the presence of a sublethal dose of cycloheximide.

We assessed apoptosis induced by this panel of proapoptotic stimuli by morphological criteria and cleavage of PARP, a well-studied nuclear substrate of caspase-3. There were striking differences between the cell lines in response to a subset of these proapoptotic stimuli (Figure 2A). Cells expressing the noncleavable golgin-160 were significantly less sensitive to apoptosis induced by BFA, thapsigargin, and DTT than cells expressing wild-type golgin-160. Cells expressing golgin-160(3DE) also were less sensitive to death induced by TNF- α , anti-Fas, and TRAIL treatments. However, both cell lines were similarly sensitive to staurosporine, anisomycin, and etoposide (Figure 2A). The morphological results largely paralleled cleavage of PARP (Figure 2B). These results show that expression of golgin-160(3DE) impairs apoptosis after proapoptotic stimuli that activate the ER stress response and ligation of death receptors, but not the other stimuli examined. Interestingly, the bulk populations of TNF receptor 1 (Jones *et al.*, 1999), Fas (Bennett *et al.*, 1998), TRAIL receptor 1 and TRAIL receptor 2 (Zhang *et al.*, 2000) are present at the Golgi complex, suggesting a possible link between membrane traffic and Golgi function in transducing apoptotic signals in response to ligation of death receptors.

The cells expressing golgin-160(3DE) were not completely resistant to apoptosis induced by BFA, ligation of death receptors, thapsigargin or DTT. The kinetics of apoptosis induced by BFA and TNF- α (Figure 3) as well as anti-Fas, thapsigargin and DTT (our unpublished data) demonstrated a significantly delayed response compared with etoposide and staurosporine (Figure 3).

The effects of the noncleavable golgin-160 were not due to the N-terminal GFP tag or an artifact of analyzing clonal cell populations. Independently generated cell lines expressing golgin-160 or golgin-160(3DE) tagged at the C terminus responded similarly to proapoptotic stimuli compared with the N-terminally tagged lines. As seen with the N-terminally

Figure 2. Defects in apoptosis in cells expressing golgin-160(3DE). Cells expressing wild-type or noncleavable golgin-160 were treated with 1 μ M staurosporine (Sts), 5 μ g/ml anisomycin (anis.), 100 μ M etoposide (etop.), 1 μ g/ml BFA, 5 μ g/ml thapsigargin (Tg), 10 mM DTT, 10 ng/ml TNF- α plus 10 μ g/ml Chx, 20 ng/ml TRAIL plus 10 μ g/ml Chx, or 0.1 μ g/ml anti-human Fas plus 10 μ g/ml Chx for the times shown. Apoptosis was assessed by morphological criteria (A) or cleavage of PARP by immunoblotting with mouse anti-PARP (B). The morphological data are presented as the average $\pm$ SD for at least three independent experiments. * $p \leq 0.1$ and ** $p \leq 0.01$, as assessed using the Student's *t* test.

Figure 3. Delayed kinetics of apoptosis in cells expressing noncleavable golgin-160. Morphological criteria were used to assess the time course of apoptosis induced by etoposide, staurosporine, BFA, and TNF- α in the two HeLa cell lines. The data are presented as the average $\pm$ SD for three independent experiments.

tagged lines, cells expressing noncleavable golgin-160 were more resistant to apoptosis induced with BFA, TNF- α , and anti-Fas than cells expressing wild-type golgin-160, but not to etoposide or staurosporine (Figure 4). The simplest explanation of our observations is that golgin-160 regulates the transduction of a subset of apoptotic signals that impact the secretory pathway and ligate death receptors.

Disruption of Apoptosis by Caspase-resistant Golgin-160 Cannot Be Bypassed by Predisassembly of the Golgi Complex

We have previously shown that mutation of golgin-160 D59 to alanine delays apoptotic Golgi disassembly (Mancini *et al.*, 2000). As expected, expression of golgin-160(3DE) also delayed apoptotic Golgi disassembly (Figure 5A). Because prevention of Golgi disassembly can arrest cells in mitosis and this block can be bypassed by disassembly of the Golgi with drugs (Sutterlin *et al.*, 2002), we examined whether

Golgi-perturbing drugs could sensitize cells expressing golgin-160(3DE) to apoptosis. For this experiment, we used TNF- α because it induces relatively rapid and synchronous apoptosis. Cell lines expressing wild-type golgin-160 or golgin-160(3DE) were treated for 1 h with BFA, nocodazole, or ilimaquinone. Both cell lines showed normal disassembly of the Golgi in response to the drug pretreatment (Figure 5B). Cells were treated for an additional 6 h with Golgi-perturbing drugs with or without TNF- α and scored for apoptosis by PARP cleavage. Cells expressing golgin-160(3DE) remained resistant to apoptosis, and no significant induction of apoptosis by Golgi-perturbing drugs alone was observed in either cell line (Figure 5C). Assessment of apoptosis in an independent experiment by nuclear morphology (our unpublished data) mirrored the results shown by PARP cleavage. Our results indicate that the delay of Golgi disassembly in cells expressing golgin-160(3DE) was not primarily responsible for their resistance to apoptosis.

Figure 4. Independent stable lines expressing C-terminally tagged golgin-160(3DE) are resistant to death induced by proapoptotic stimuli that cause ER stress and ligation death receptors, but sensitive to other stimuli, similar to the N-terminally tagged lines. Cells were treated 100 μ M etoposide, 1 μ M staurosporine (Sts), 10 ng/ml TNF- α plus 10 μ g/ml Chx, 0.1 μ g/ml anti-human Fas plus 10 μ g/ml Chx, or 1 μ g/ml BFA for the times shown. Apoptosis was assessed by morphological criteria. The data are presented as the average $\pm$ SD for three independent experiments. * $p \leq 0.1$ and ** $p < 0.01$ as assessed using the Student's *t* test.

Expression of Caspase-resistant Golgin-160 Blocks Cleavage of Endogenous Wild-Type Golgin-160 in Response ER Stress and Ligation of Death Receptors

Because all the cell lines used in our experiments also express endogenous wild-type golgin-160, we examined the effects of golgin-160(3DE) on caspase cleavage of endogenous golgin-160. Both cell lines were metabolically labeled, and treated with proapoptotic drugs. Golgin-160 cleavage was assessed after immunoprecipitation with an antibody that recognizes the C terminus of golgin-160. Each sample represents an equal number of cells plated at the beginning of the experiment. The endogenous and stably expressed golgin-160 could be easily distinguished due to the size of the GFP tag. In cells expressing wild-type golgin-160, cleavage of both GFP/golgin-160 and endogenous golgin-160 was observed in response to staurosporine and TNF- α (Figure 6A). In cells expressing the noncleavable golgin-160, GFP/golgin-160(3DE) remained intact after all drug treatments. In cells expressing golgin-160(3DE), most of the endogenous wild-type golgin-160 was cleaved after treatment with staurosporine for 4 h. Interestingly, cleavage of endogenous golgin-160 was not observed after treatment with TNF- α in cells expressing golgin-160(3DE). These results imply that

Figure 5. Drug-induced Golgi disassembly does not sensitize cells expressing golgin-160(3DE) to apoptosis induced by TNF- α . All immunofluorescence images are the same scale (bar, 10 μ m). (A) Golgi disassembly in response to TNF- α is delayed in cells expressing golgin-160(3DE). Cells were treated for 6 h with 10 ng/ml TNF- α plus 10 μ g/ml Chx. Fixed cells were stained with anti-GM130 as a Golgi marker. Cell outlines are marked with dotted lines in immunofluorescence images. Cells also were scored for having a dispersed or intact Golgi complex and graphed as a percentage of the total cells counted. A minimum of 300 cells was counted for each sample. (B) Cells were treated with 1 μ g/ml nocodazole (nocod), 5 μ g/ml BFA, or 25 μ M ilimaquinone (IQ) for 1 h. Fixed cells were stained with antibodies to GM130. Golgi disassembly is similar in cells expressing golgin-160(wt) or golgin-160(3DE). (C) Cells were pre-treated with 1 μ g/ml nocodazole, 5 μ g/ml BFA, or 25 μ M ilimaquinone for 1 h; 10 ng/ml TNF- α plus 10 μ g/ml Chx was added to half the samples and incubation continued for 6 h. Apoptosis was assessed by cleavage of PARP after immunoblotting with mouse anti-PARP antibody.

golgin-160(3DE) dominantly interfered with cleavage of endogenous golgin-160 in response to specific proapoptotic stimuli.

In cells expressing wild-type golgin-160, cleavage of golgin-160 also was observed after treatment with DTT, anti-Fas antibody, and TRAIL (Figure 6B). Production of the C-terminal cleavage fragment of golgin-160 was dramatically reduced in cells expressing golgin-160(3DE). Due to the asynchronous induction of apoptosis in response to the drugs used in Figure 6B, loss of full-length golgin-160 was difficult to assess at the time points examined. However, it is reasonable to presume that the decreased production of the C-terminal fragment of golgin-160 is due to decreased cleavage of endogenous golgin-160 in cells expressing golgin-160(3DE). In additional experiments, treatment with etoposide or thapsigargin induced cleavage of both GFP/golgin-160 and endogenous golgin-160 in cells expressing wild-type golgin-160. In cells expressing golgin-160(3DE), cleavage of endogenous golgin-160 was observed after treatment with etoposide, but not thapsi-

gargin (our unpublished data). Thus, expression of golgin-160(3DE) dominantly interfered with cleavage of endogenous golgin-160 after induction of the ER stress response and ligation of death receptors. These observations suggested that caspase activation after death receptor ligation and activation of ER stress might be inhibited in cells expressing noncleavable golgin-160.

Because cleavage of endogenous golgin-160 was disrupted in golgin-160(3DE) cells treated with proapoptotic stimuli that cause ER stress (DTT and thapsigargin) and ligate death receptors (TNF- α , anti-Fas, and TRAIL), cleavage fragments of golgin-160 were not produced after treatment with these drugs. The disruption of apoptosis in cells expressing golgin-160(3DE) could thus be due to the absence of these golgin-160 cleavage products. To address this possibility, we transiently expressed constructs mimicking each of the potential cleavage fragments of golgin-160 before treatment with TNF- α (see Figure 7A for a schematic diagram of potential golgin-160 caspase cleavage products). We

Figure 6. Cleavage of endogenous wild-type golgin-160 is blocked during apoptosis induced by ER stress and ligation of death receptors in cells expressing golgin-160(3DE). Cells expressing GFP-tagged golgin-160(wt) or golgin-160(3DE) were plated in equal numbers. After 18 h, the cells were metabolically labeled for 2 h with [³⁵S]methionine and cysteine. After a 6-h chase in the absence or presence of proapoptotic drugs, golgin-160 was immunoprecipitated from the lysates using an antibody that recognizes the C terminus. Immunoprecipitates were resolved by SDS-PAGE and detected by fluorography. The 140-kDa fragment representing the C-terminal 312–1498 amino acids of golgin-160 is indicated (C-term. frag.). (A) Cleavage of golgin-160 assessed (by loss of intact golgin-160 and production of the C-terminal cleavage fragment) after treatment with drugs that synchronously induce apoptosis. TNF- α (10 ng/ml) plus 10 μ g/ml Chx or 1 μ M staurosporine were included for the entire 6-h chase or for the last 4 h of the chase, respectively. (B) Cleavage of golgin-160 assessed (by generation of the C-terminal cleavage fragment) in cells treated with drugs that induce asynchronous apoptosis. DTT (10 mM) or 0.1 μ g/ml anti-Fas plus 10 μ g/ml Chx were included for the entire 6-h chase. 20 ng/ml TRAIL was added for the last 4 h of chase.

chose TNF- α for this experiment because cleavage of endogenous and transfected wild-type golgin-160 was complete within 6 h (Figure 6A). Percentage of expression of each piece was determined by indirect immunofluorescence microscopy, and only samples with 12% or greater expression were included in our analysis. This ensured that we could observe any effects on apoptosis due to expression of these golgin-160 fragments by the sensitive PARP cleavage assay. The individual golgin-160 fragments did not induce apoptosis or sensitize cells expressing either golgin-160(wt) (Figure 7B) or golgin-160(3DE) (Figure 7C) to death induced by TNF- α . Expression levels did not correlate with sensitivity to TNF- α , and no consistent changes in sensitivity to TNF- α were observed with any golgin-160 cleavage product. Additional independent experiments in which apoptosis was assessed by nuclear morphology in only transfected cells (identified by indirect immunofluorescence) showed no change in the amount of apoptosis in response to 6 h of TNF treatment in either cell line (our unpublished data). These results suggest that lack of individual cleavage products of golgin-160 is unlikely to be the primary cause of resistance of cells expressing golgin-160(3DE) to death in response to proapoptotic stimuli causing ER stress or ligation of death receptors.

Caspase Processing Is Impaired in Cells Expressing Caspase-resistant Golgin-160

Because caspases are the primary executioners of apoptosis, we examined proteolytic processing of key caspases as an

A. Potential cleavage products of golgin-160

B. golgin-160 (wt)

C. golgin-160 (3DE)

Figure 7. Expression of individual constructs mimicking potential golgin-160 cleavage products does not induce apoptosis or sensitize cells to apoptosis. (A) Schematic of golgin-160 representing potential caspase cleavage products. Cell lines expressing (B) golgin-160(wt) or (C) golgin-160(3DE) were transfected with plasmids expressing the indicated golgin-160 fragment and treated with 10 ng/ml TNF- α plus 10 μ g/ml Chx for the times indicated. Apoptosis was assessed by cleavage of PARP after immunoblotting with mouse anti-PARP antibody. A representative data set of at least three trials is shown. The piece representing amino acids 140–311 was excluded from this analysis due to poor expression.

indirect measure of their activation. Initiator caspases are activated by oligomerization (Boatright *et al.*, 2003), which is typically followed by autocleavage to separate the large and small caspase subunits and remove the prodomain from the procaspase. Caspase-8 is recognized as a primary initiator caspase for death induced by ligation of death receptors (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Caspase-2 is potentially an initiator caspase (Troy and Shelanski, 2003) and shows partial localization at the Golgi complex (Mancini *et al.*, 2000; O'Reilly *et al.*, 2002). Processing of caspases-2 and -8 was directly assessed by cleavage of their proforms on immunoblots. Processing can be observed by loss of the full-length precursor and generation of the first product by cleavage between the large and small subunits. Further cleavage removes the N-terminal prodomain to produce the final product, which we can no longer detect.

caspase-2

caspase-8

Figure 8. Caspase processing is impaired in cells expressing golgin-160(3DE). Cells expressing wild-type or golgin-160(3DE) were treated as indicated (10 μ g/ml Chx was included in samples treated with TNF- α), and lysates were loaded for equal protein amount and immunoblotted for caspase-2 or caspase-8. Caspase processing was visualized by generation of the first cleavage product (created by cleavage between the large and small caspase subunits) and loss of the full-length precursor. The first cleavage product is also lost with further cleavage when the N-terminal prodomain is removed to create the final product, which we can no longer detect. For both caspases, the proform and the product of cleavage between the large and small subunits (33 kDa for caspase-2 and a 43/42-kDa doublet for caspase-8) are indicated.

In cells expressing the wild-type golgin-160, cleavage of procaspase-2 (shown by generation of the first cleavage product, p33; loss of procaspase-2; and subsequent loss of p33 as it is further processed) occurred over time during treatment with anisomycin, staurosporine, TNF- α , etoposide, and BFA (Figure 8). Although caspase-2 was processed normally in anisomycin, staurosporine, or etoposide-treated golgin-160(3DE) cells, significantly less cleavage was observed after treatment with TNF- α or BFA. Cleavage of caspase-2 also was decreased in golgin-160(3DE) cells after treatment with anti-Fas (our unpublished data). Similarly, cleavage of caspase-8 was observed in cells expressing wild-type golgin-160 after treatment with anisomycin, staurosporine, and TNF- α . Caspase-8 processing in cells expressing golgin-160(3DE) was normal in response to anisomycin and staurosporine, but it was substantially decreased in response to TNF- α and BFA. Cleavage of caspase-3 was observed in response to all proapoptotic stimuli in cells expressing wild-type golgin-160. However, in cells expressing golgin-160(3DE), caspase-3 cleavage was dramatically reduced in response to TNF- α and BFA (our unpublished data), suggesting that the downstream caspases fail to be processed as well as initiator caspases. These results suggest that the noncleavable golgin-160 can dominantly interfere with caspase processing in response to certain proapoptotic stimuli. Thus, the block in apoptotic signaling due to expression of golgin-160(3DE) is an early event in apoptosis.

DISCUSSION

In the work reported here, we examined the effects of stable expression of wild-type and a caspase-resistant mutant of

golgin-160 [golgin-160(3DE)]. Cells expressing golgin-160(3DE) displayed a remarkable resistance to a subset of the proapoptotic stimuli examined, including ligation of death receptors and drugs that induce ER stress. However, cells expressing golgin-160(3DE) were sensitive to death after other proapoptotic stimuli. Cells expressing wild-type golgin-160 responded normally to all proapoptotic drugs tested. When cells expressing golgin-160(3DE) were treated with proapoptotic stimuli that induce ER stress or ligate death receptors, endogenous (wild-type) golgin-160 was not cleaved. Our results suggest that this was a result of failure to activate upstream caspases, indicating that an early event in apoptotic signaling was disrupted. The delay in apoptosis in golgin-160(3DE)-expressing cells could not be bypassed by predisassembling the Golgi with drugs or by individually overexpressing constructs mimicking the caspase cleavage products of golgin-160. The simplest interpretation of these experiments is that cleavage of golgin-160 is required for initiation of apoptosis in response to drugs that induce the ER stress response and ligation of death receptors. Alternatively, the mutations introduced to generate the caspase-resistant golgin-160 could modulate golgin-160 function or interactions with other proteins. This work documents for the first time that apoptotic signals can be transduced at the Golgi complex, and supports previous observations that cleavage of Golgi-resident proteins is important during apoptosis.

A number of secretory pathway proteins are substrates for caspase cleavage, including BAP-31 in the ER (Ng *et al.*, 1997), and golgin-160 (Mancini *et al.*, 2000), giantin (Lowe *et al.*, 2004), GRASP65 (Lane *et al.*, 2002), and p115 (Chiu *et al.*, 2002) at the Golgi. Besides contributing to organelle disassembly, cleavage of some of these proteins seems to be important for initiation of apoptosis. For example, in cells expressing the C-terminal caspase cleavage product of p115, the Golgi disassembles and the cells undergo apoptosis. Expression of caspase resistant p115 delays Golgi disassembly during apoptosis, but the sensitivity of these cells to death was not reported (Chiu *et al.*, 2002). The p20 fragment of BAP31 also can induce apoptosis when overexpressed (Breckenridge *et al.*, 2003), and expression of a caspase-resistant BAP31 mutant inhibits apoptosis after ligation of Fas (Nguyen *et al.*, 2000; Wang *et al.*, 2003). Thus, caspase cleavage of secretory pathway resident proteins seems to be important for transduction of apoptotic signals as well as disassembly of the cell.

We found that the failure to generate golgin-160 cleavage products was unlikely to be the primary cause for golgin-160(3DE)-mediated disruption of apoptosis. Overexpression of individual fragments of golgin-160 neither induced apoptosis nor sensitized cells expressing golgin-160(3DE) to TNF- α . A limitation of these experiments is that these cleavage products were not presented to the cell in the same way that they would be when naturally produced during apoptosis. Golgin-160 is phosphorylated in its N-terminal head domain (Cha *et al.*, 2004) and transfected constructs may not be phosphorylated in the same manner as cleavage products made from Golgi-localized golgin-160. Also, the golgin-160 fragments were expressed in cells individually and not in combination, as they might be produced during apoptosis. The golgin-160 cleavage products are potentially very interesting considering that some specifically localize to the nucleus when overexpressed (Hicks and Machamer, 2002). Dissection of the potential functions of nuclear-targeted golgin-160 fragments is currently in progress.

The finding that expression of individual golgin-160 cleavage products could not bypass the block in apoptosis in

cells expressing golgin-160(3DE) suggested another function for golgin-160 cleavage during apoptosis. A likely possibility is that golgin-160 either directly or through interaction with another protein regulates apoptotic signaling.

Cleavage of golgin-160 may sever a link between proteins or release a protein from the Golgi to the cytoplasm, thereby allowing amplification of an apoptotic signal and full activation of caspases. For example, cleavage of golgin-160 may liberate a proapoptotic molecule from a negative regulator, allowing activation of the proapoptotic molecule. Alternatively, the mutations in golgin-160(3DE) could modify its interactions with other proteins, thereby changing its function.

It is also a formal possibility that golgin-160(3DE) can sequester active caspases that bind but cannot cleave at the mutated site. We have been unable to demonstrate an interaction between caspase-2 and caspase-resistant golgin-160 by coimmunoprecipitation from cells or in vitro with recombinant or in vitro-translated proteins (our unpublished data). It is even less likely that caspase-8 would be sequestered by golgin-160(3DE) because caspase-8 does not cleave golgin-160 (Mancini *et al.*, 2000). Therefore, it is unlikely that the disruption of caspase processing observed in the presence of golgin-160(3DE) is a result of sequestration of active caspases by golgin-160(3DE). It is more probable that golgin-160 or its cleavage plays an important role during apoptotic signaling after ER stress and ligation of death receptors. Determining the mechanism of golgin-160(3DE)-mediated inhibition of apoptosis will help us to understand the function of golgin-160 and other Golgi-resident proteins in transduction of proapoptotic signals.

Although a Golgi-localized protein disrupting apoptosis after ligation of death receptors may at first seem surprising, there is increasing evidence that the Golgi complex may have an important role in death receptor-induced apoptosis. The bulk population of TNF receptor I, Fas, TRAIL receptor 1, and TRAIL receptor 2 localize to the Golgi complex at steady state (Cottin *et al.*, 1999; Jones *et al.*, 1999; Zhang *et al.*, 2000; Storey *et al.*, 2002). At the Golgi complex, death receptors may interact with Golgi-resident proteins, including golgin-160. Additionally, overexpression of caspase-resistant BAP31 disrupts Fas-induced apoptosis (Nguyen *et al.*, 2000), implying cross-talk between the secretory pathway and death receptors. Recent reports also suggest that internalization of the TNF receptor I after its ligation is important for initiation of apoptosis (Schutze *et al.*, 1999; Schneider-Brachert *et al.*, 2004), indicating a potential function for membrane trafficking in TNF receptor signaling. Apoptosis also was impaired after treatment with TRAIL and ligation of Fas in cells expressing caspase-resistant golgin-160 (although impairment was less dramatic after Fas ligation than TNF- α or TRAIL treatment). Experiments are currently underway to determine the mechanism by which expression of golgin-160(3DE) disrupts induction of apoptosis after ligation of death receptors.

The fact that expression of a Golgi-localized noncleavable caspase substrate disrupted death induced by proapoptotic agents causing ER stress and ligation of death receptors but not other stimuli suggests that apoptotic signals may be transduced at the Golgi complex in response to specific types of cellular stress. A common link between ligation of death receptors and the ER stress response might be membrane trafficking, in which the Golgi complex plays a central role. Our work implicates the Golgi as a potential stress sensor and scaffold for important signaling events. There are a number of Golgi-localized proteins that are potentially involved in apoptotic signaling. Caspase-2 localizes to the Golgi as well as the nucleus (Mancini *et al.*, 2000; O'Reilly *et*

al., 2002). Apollon, an inhibitor of apoptosis protein, has been reported to be Golgi localized (Hauser *et al.*, 1998; Bartke *et al.*, 2004; Hao *et al.*, 2004). The death receptor adaptor protein TNF-receptor-associated death domain-containing protein also shows localization to the Golgi (Jones *et al.*, 1999). As further studies are performed, the role of the Golgi complex and its resident proteins in apoptotic signaling is becoming increasingly apparent. Understanding how proteins such as golgin-160 regulate apoptotic signal transduction will be important in elucidating the role of the Golgi complex in initiation of apoptosis.

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