Caspase-mediated cleavage of syntaxin 5 and giantin accompanies inhibition of secretory traffic during apoptosis

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Summary

We report the caspase-dependent cleavage of two Golgi-associated transport factors during apoptosis. The tethering factor giantin is rapidly cleaved both in vitro and in vivo at a conserved site, to generate a stable membrane-anchored domain and a soluble domain that is subject to further caspase-dependent cleavage. The t-SNARE syntaxin 5 is also cleaved rapidly, resulting in the separation of the catalytic membrane-proximal domain from an N-terminal regulatory domain. Cleavage of giantin and syntaxin 5 is accompanied by a cessation of vesicular transport between the ER and the Golgi complex, which first manifests itself as a block in ER exit. The contribution that such an inhibition of trafficking may make towards the generation of an apoptotic phenotype is discussed.

Key words: Golgi apparatus, Apoptosis, SNARE, Vesicle, Caspase

Introduction

Programmed cell death (apoptosis) is a fundamental feature of multicellular organisms (Raff et al., 1994). It is characterised by striking cellular alterations, including fragmentation of the nucleus and activation of endonuclease(s) (Kerr et al., 1972; Wyllie, 1980), cell shrinkage and fragmentation (Wyllie et al., 1980) and plasma membrane blebbing (Mills et al., 1998). Many of these changes, described as the execution phase of apoptosis, are brought about by the activity of conserved cysteine proteases (caspases). Caspase substrates that have been identified in recent years include cytoskeletal proteins, structural proteins of the nuclear envelope, proteins involved in DNA metabolism and repair, and protein kinases (for reviews, see Kidd, 1998; Earnshaw et al., 1999).

Apoptotic cells and their fragments (apoptotic bodies) are rapidly engulfed and degraded by neighbouring cells, an activity thought to be essential for avoiding an inflammatory response (Savill, 1998; Henson et al., 2001). A critical event during apoptosis is therefore the expression of surface markers that permit the selective recognition and phagocytosis of a dying cell. Receptors that might serve for the recognition of apoptotic cells by immune cells have been identified (for reviews, see Savill and Fadok, 2000; Henson et al., 2001). However, the ability of non-specialist cells to phagocytose apoptotic remnants (Parnaik et al., 2000) points to the existence of more generalised recognition motifs. Phosphatidylserine, which appears on the outer leaflet of the plasma membrane during apoptosis (Martin et al., 1995), probably serves as one such motif (Fadok et al., 2000). There is considerable evidence, however, that other surface moieties, including carbohydrates, form part of a general recognition signal (Duvall et al., 1985; Korb and Ahearn, 1997; Ogden et al., 2001).

A distinguishing feature of apoptotic cells is a loss of organised endomembrane structure (Kerr et al., 1972), suggesting that profound changes in the dynamics of membrane trafficking pathways might occur. These in turn may lead to the exposure on the surface of the apoptotic cell of novel carbohydrate-based markers that mediate recognition and engulfment. As a step towards testing this hypothesis we have examined whether membrane transport reactions within the endocytic and secretory pathways are altered during apoptosis. We have demonstrated that inhibition of the endocytic pathway within apoptotic cells correlates with reduced fusion of endosomal membranes, measured using an in vitro assay. This is linked to caspase-3-dependent cleavage of the rab5 effector, rabaptin 5 (Stenmark et al., 1995; Cosulich et al., 1997; Swanton et al., 1999a). More recently, we have shown that apoptosis is accompanied by structural and functional changes in the early secretory pathway. These include the inhibition of microtubule-based movement of the ER, caused by caspase-dependent cleavage of both the intermediate chain of the minus end-directed cytoplasmic dynein motor complex and of the $p150^{Glued}$ subunit of the dynein-associated dynactin complex (Lane et al., 2001). Additionally, the Golgi complex loses its cisternal organisation and is fragmented into dispersed clusters of tubulovesicular elements. This phenotype is partially due to cleavage of the Golgi stacking protein GRASP65 (Lane et al., 2002) and may also involve cleavage of Golgin 160 (Mancini et al., 2000) and the vesicle tethering protein p115 (Chiu et al., (2002).

Although these studies have shown that the structural organisation of the early secretory pathway is affected during apoptosis, they have not addressed the central issue of whether transport to and through the Golgi complex is impaired.

Importantly, fragmentation of the Golgi complex does not necessarily lead to a shut down of the secretory pathway, since dispersal of the Golgi complex by the microtubule depolymerising agent nocodazole does not prevent transport to the cell surface (Rogalski et al., 1984). In addition, the imino sugar N-butyldeoxynojirimycin causes the Golgi to lose its cisternal organisation with no apparent affect on trafficking (Neises et al., 1997). We here report the efficient caspase-dependent cleavage of two proteins that participate in the tethering, docking and fusion of transport intermediates with Golgi cisternae. Cleavage of these proteins is accompanied by a cessation of protein transport between the ER and the Golgi complex as cells enter apoptosis. We discuss how the inhibition of this selective transport step may contribute to the exposure of novel moieties(s) on the surface of the apoptotic cell.

Materials and Methods

Antibodies

The following antibodies were used: MLO7 polyclonal anti-GM130; MLO1 polyclonal anti-p115; 3E1 monoclonal anti-GFP. Monoclonal anti-GS28, anti-GS15 and anti-GM130 were from Transduction Labs (San Diego, CA). M2 monoclonal anti-FLAG was from Sigma, and monoclonal anti-PARP from Calbiochem. Polyclonal anti-calnexin was purchased from Stressgen. FBA24 polyclonal anti-mannosidase I antibodies were from Dr Francis Barr (Max Planck Institute of Biochemistry, Martinsried, Germany). The monoclonal 1D3 antibody (anti-KDEL) was from Dr David Vaux (University of Oxford, Oxford, UK). Polyclonal antibodies to rab1 were from Dr Bill Balch (The Scripps Research Institute, La Jolla, USA). Polyclonal antibodies to membrin, rsec22b, and rbet1 were from Dr Jesse Hay (University of Michigan, Ann Arbor, USA). Monoclonal antibodies to β-COP (mAD) and the lumenal domain of VSV-G were from Dr Rainer Pepperkok (EMBL, Heidelberg, Germany). Polyclonal antibodies raised against full-length and the N terminus of giantin were from Prof. Manfred Renz (Institute of Immunology and Molecular Genetics, Karlsruhe, Germany) and Dr David Shima (Cancer Research UK, London, UK), respectively. Polyclonal antibodies to syntaxin 5 (JSEE1) were raised in rabbits against bacterially-expressed GST-tagged full-length syntaxin 5 and affinity-purified against the recombinant protein. Fluorophore-conjugated secondary antibodies were purchased from Molecular Probes. HRP-conjugated secondary antibodies were purchased from Tago Immunologicals.

Constructs

Amino acid substitutions were introduced into full-length rat syntaxin 5 and giantin cDNA using the QuikChange kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The FLAG tag was introduced into full-length giantin in the pSG5 vector using a modification of the QuikChange method. Instead of one 50 μl PCR reaction containing both primers, two separate reactions of 25 μl were set up, each reaction mix containing 14 pmol of primer FLAG-1 (GCC CAT GGA GCG CTC CTT CAG AGA TGG ATT ACA AAG ATG ACG ATG ACA AGA TGC TGA GCC GAT TAT CGG G) or primer FLAG-2 (CCC GAT AAT CGG CTC AGC ATC TTG TCA TCG TCA TCT TTG TAA TCC ATC TCT GAA GGA GCG CTC CAT GGG C). After 15 cycles, the PCR reactions were pooled and incubated for an additional 10 cycles before Dpn I treatment and transformation into bacteria. All constructs were verified by DNA sequencing.

Cell culture, transfection and drug treatments

HeLa cells were grown in DMEM containing 10% FCS. Plasmid DNA was transfected into cells using Fugene 6 (Roche) according to the manufacturer's instructions. Co-transfection of NAGFP and FLAG-tagged giantin constructs was performed with 0.5 μg NAGFP DNA and 1 μg FLAG-giantin DNA using 3 μl Fugene 6 per 3 cm dish. Cells were used for experiments 16-18 hours post-transfection. To induce apoptosis, cells were treated with 100 J/m^2 UV radiation, 1-2 μM staurosporine (Sigma), or 5 μg/ml anisomycin (Calbiochem). In some cases cells were pre-treated for 15 minutes with 50 μM zVAD.fmk (Calbiochem) prior to apoptotic stimulus.

Preparation of cell extracts

Floating cells were harvested by gentle aspiration of the cell medium and pelleted by centrifugation. After washing with ice-cold PBS, cell pellets were solubilised in boiling SDS sample buffer. Remaining adherent cells were washed with ice-cold PBS and pooled with the extract prepared from floating cells from the same dish to form a total cell population extract. DNA was sheared by vortexing in the presence of glass beads and the extracts cleared by centrifugation at 15,000 ***g*** for 10 minutes. Samples were analysed by SDS-PAGE and immunoblotting with appropriate antibodies.

In vitro cleavage experiments

Preparation of Golgi membranes, control and apoptotic cytosols, and in vitro cleavage experiments were performed as described previously (Lane et al., 2002).

Fluorescence microscopy

Conventional digital epifluorescence images of fixed cells were obtained using an Olympus BX60 upright microscope equipped with a MicroMax cooled, slow-scan CCD camera (Roper Scientific) driven by Metamorph software (Universal Imaging Corporation). Confocal images were obtained using a Leica NT confocal microscope. Unless indicated otherwise, confocal images are shown as projections of optical sections taken in the z-axis at 0.5 μm intervals.

tsO45 VSV-G-GFP trafficking experiments

To assess the transport of VSV-G to the cell surface, HeLa cells were transfected with tsO45 VSV-G-GFP (a generous gift from Patrick Keller and Kai Simons; Max Planck Institute of Molecular Cell Biology and Genetics, Dresden) (Keller et al., 2001), held at 37°C for 2 hours, then incubated at 39.5°C overnight to accumulate tsO45 VSV-GFP in the ER. Cells were then either left untreated (control) or were induced to undergo apoptosis by staurosporine (2 μM) or UV (100 J/m^2) treatment, and were incubated for a further 4 hours at 39.5°C in the presence of 10 μg/ml cycloheximide to block additional protein synthesis. At this point, cells were incubated at 39.5°C or at 31°C for a further 2 hours before fixation in 2% paraformaldehyde. Cells were then processed for immunofluorescence microscopy, without permeabilising, using α-VSV-G antibody and Alexa 594-conjugated secondary antibodies. To quantify the ratio of surface (α-VSV-G) to total (GFP) fluorescence in healthy and apoptotic cells, digital images of transfected cells were obtained using the Micromax camera and Metamorph software using constant image acquisition parameters. Total fluorescence grey levels in the red (α-VSV-G) and green (GFP) channels were recorded, and after subtraction of background fluorescence over untransfected cells, the ratio of surface to total fluorescence was calculated. Apoptotic cells were identified as those having characteristic condensed and marginalised DNA by DAPI fluorescence (see Lane et al., 2002). In some experiments transfected cells were incubated at 39.5°C for 4 hours in the presence of staurosporine and cycloheximide and shifted to 31°C for 25 minutes prior to fixation in 2% PFA, 0.2% gluteraldehyde. Cells were then permeabilised in 0.1% Triton X-100, 0.05% SDS and labelled with antibodies to the Golgi apparatus (anti-GM130) or the ER (anti-calnexin).

Results

Caspase-mediated cleavage of Golgi-associated vesicle tethering and fusion proteins giantin and syntaxin 5

Purified rat liver Golgi membranes were incubated with apoptotic cytosol (generated by addition of cytochrome c to interphase HeLa cytosol preparations), and analysed by western blotting using an array of antibodies against known trafficking components, including coat proteins, tethering factors and SNAREs (Fig. 1A). Giantin, a Golgi transport vesicle-associated tethering factor (Sönnichsen et al., 1998), was efficiently cleaved. In addition, GM130, a tethering factor predominantly associated with the Golgi stack (Lowe et al., 1998b) was cleaved, albeit to a lesser extent. We also observed cleavage of syntaxin 5, a t-SNARE required for the fusion of transport vesicles with Golgi cisternae (Banfield et al., 1994; Dascher et al., 1994), and GS28, a Golgi-associated v-SNARE (Nagahama et al., 1996; Subramaniam et al., 1996). All of these proteins were cleaved in a manner prevented by prior inclusion of the broad-spectrum caspase inhibitor Ac-DEVD-CHO. Cleavage was specific, since there was little change in the general profile of proteins as assessed by Coomassie Blue staining of both membrane and cytosol fractions (see Lane et al., 2002).

Giantin cleavage occurred rapidly, with loss of full-length protein readily detected within the first hour of incubation and largely complete within 2 hours (note that these extracts normally require 15-30 minutes for maximal cytochrome c-dependent activation of caspase-3 (Liu et al., 1996; Swanton et al., 1999b). Cleavage generated a transient 220 kDa product (P1) that was eventually released from the membrane fraction and a more stable 160 kDa product (P2) that remained predominantly membrane-associated. Since giantin is a type II integral membrane protein, the P2 fragment is most likely from

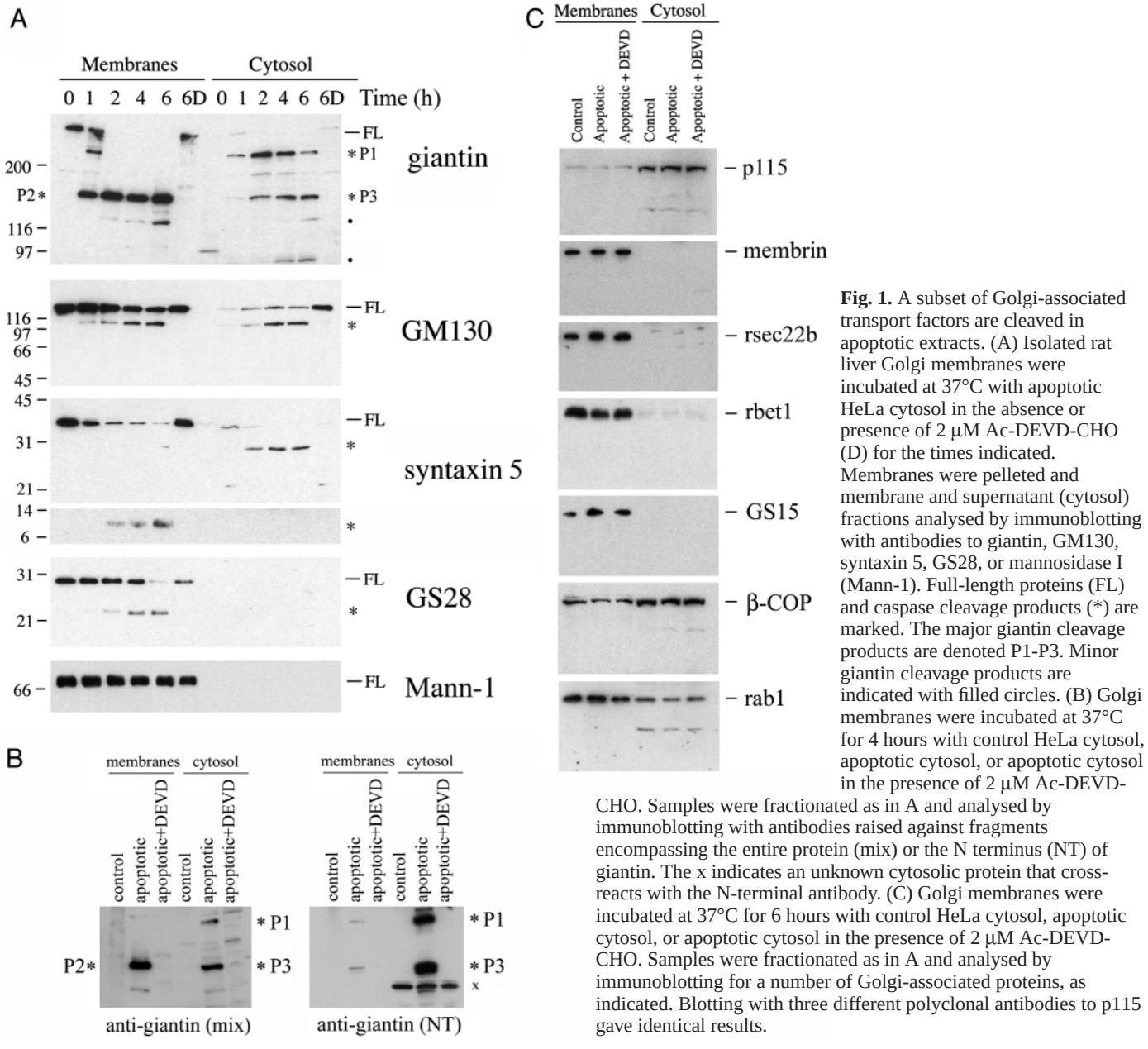

Fig. 1. A subset of Golgi-associated transport factors are cleaved in apoptotic extracts. (A) Isolated rat liver Golgi membranes were incubated at 37°C with apoptotic HeLa cytosol in the absence or presence of 2 μM Ac-DEVD-CHO (D) for the times indicated. Membranes were pelleted and membrane and supernatant (cytosol) fractions analysed by immunoblotting with antibodies to giantin, GM130, syntaxin 5, GS28, or mannosidase I (Mann-1). Full-length proteins (FL) and caspase cleavage products (*) are marked. The major giantin cleavage products are denoted P1-P3. Minor giantin cleavage products are indicated with filled circles. (B) Golgi membranes were incubated at 37°C for 4 hours with control HeLa cytosol, apoptotic cytosol, or apoptotic cytosol in the presence of 2 μM Ac-DEVD-CHO. Samples were fractionated as in A and analysed by immunoblotting with antibodies raised against fragments encompassing the entire protein (mix) or the N terminus (NT) of giantin. The x indicates an unknown cytosolic protein that cross-reacts with the N-terminal antibody. (C) Golgi membranes were incubated at 37°C for 6 hours with control HeLa cytosol, apoptotic cytosol, or apoptotic cytosol in the presence of 2 μM Ac-DEVD-CHO. Samples were fractionated as in A and analysed by immunoblotting for a number of Golgi-associated proteins, as indicated. Blotting with three different polyclonal antibodies to p115 gave identical results.

the C terminus of the protein. Another 160 kDa product (P3) could be distinguished from P2 by its distribution to the cytosolic fraction and by its somewhat slower appearance. The distinct nature of these products was confirmed using antibodies to the N terminus of giantin, which cross-reacted strongly with P3 (and P1) but failed to react with P2 (Fig. 1B). Additional antibody-reactive cleavage products of approximately 130 kDa and 90 kDa were detected at later times (Fig. 1A). Together, these data suggest that the apoptotic cleavage pathway for giantin is complex.

Syntaxin 5 was also rapidly cleaved in apoptotic extracts (Fig. 1A). In this case, the major detected cleavage product of 26 kDa was released from the membrane fraction, though over-exposure of blots revealed an additional 8 kDa membrane-associated product (Fig. 1A). The low reactivity of these products against the antibody versus that of the full-length protein is most likely a consequence of the loss of epitopes. However, we cannot rule out the possibility that further cleavage had occurred. Both GM130 and GS28 were cleaved much more slowly than giantin and syntaxin 5, and the significance of these cleavages remains unclear. Moreover, many other Golgi transport proteins, including the tethering protein p115 (Barroso et al., 1995; Sapperstein et al., 1995), the SNAREs membrin, rsec22b, rbet1 and GS15 (Hay et al., 1997; Xu et al., 1997), the vesicle coat protein β-COP (Allan and Kreis, 1986; Serafini et al., 1991) and the small GTPase rab1 (Tisdale et al., 1992) were unaffected even after prolonged incubations in apoptotic extracts (Fig. 1C). Additionally, the soluble membrane fusion factors α-SNAP and NSF remain intact in apoptotic extracts (Cosulich et al., 1997).

The patterns of cleavage in vitro were largely confirmed by experiments performed in vivo. HeLa cells were incubated for increasing periods with the protein kinase inhibitor staurosporine, a widely used inducer of apoptosis. Cells were then harvested and analysed for protein cleavage (Fig. 2A). As a comparison, the cleavage of PARP, identified as one of the first caspase substrates to be cleaved as cells enter the execution phase of apoptosis (Lazebnik et al., 1994), was also assessed. As expected, PARP was cleaved between 2 and 4 hours after administration of staurosporine. Giantin cleavage was also largely complete over this period. Comparison with the cleavage of giantin from Golgi fractions was complicated slightly by the small difference in length between the human and rat proteins. Nevertheless, the 220 kDa P1 product detected during in vitro incubations was observed but was more transient in vivo, with the 160 kDa P2 product forming the major stable product. A product likely to be P3 migrated just below P2. In addition, the 130 kDa and 90 kDa products were observed at later times. Syntaxin 5 was also cleaved after 2-4 hours staurosporine treatment. Two forms of syntaxin 5 are present in cells, with molecular masses of 35 kDa and 42 kDa (Hui et al., 1997) (generally, only the short form can be detected in purified Golgi fractions since the longer form is highly protease sensitive). Cleavage products of 33 kDa, and 26 kDa and 8 kDa, corresponding to the 26 kDa and 8 kDa products seen in vitro, were detected. As for PARP, proteolysis of giantin and syntaxin 5 was prevented by inclusion of the cell-permeable caspase inhibitor zVAD.fmk. As seen in vitro, cleavage of GM130 in vivo occurred relatively slowly, and GS28 cleavage could not be detected at all. Likewise, those Golgi proteins that were resistant to cleavage in vitro remained intact in apoptotic cells even after prolonged periods (data not shown).

Induction of apoptosis by alternative means generated similar results, indicating that cleavage of giantin and syntaxin 5 is a generalised phenomenon occurring during apoptosis. When cells treated with anisomycin were separated into floating apoptotic remnants and attached unaffected cells (Fig. 2B), the floating cells exclusively contained cleaved PARP, giantin and syntaxin 5, with identically sized final cleavage products to those observed after staurosporine treatment. GM130 was cleaved only moderately, and GS28 not at all.

Cleavage of giantin was also observed using immunocytochemistry. Induction of apoptosis in HeLa cells by UV irradiation led to the dispersal of the Golgi complex into fragments that remained positive for the Golgi matrix protein GM130, as described previously (Lane et al., 2002) (Fig. 2C, top row). This is consistent with the relative resistance of GM130 to apoptotic cleavage. Strikingly, antibodies to the N-terminus of giantin failed to recognise the Golgi fragments in apoptotic cells, in contrast to the distinct perinuclear Golgi staining observed in neighbouring non-apoptotic cells. This is consistent with the lack of reactivity of these antibodies to the membrane-associated P2 fragment (Fig. 1B). As expected, Golgi staining was still observed using the antibody reactive to all cleavage products including P2 (Fig. 2C, bottom row). Loss of giantin N-terminal staining was rapid, occurring soon after apoptotic cells became positive for cleaved caspase (data not shown). Identical results were obtained using NRK cells induced to undergo apoptosis by staurosporine treatment (our unpublished data). Unfortunately, none of the antibodies against syntaxin 5 that we tested worked for immunofluorescence under the conditions used for examining apoptotic cells, so we were unable to confirm the cleavage of syntaxin 5 using this technique.

Giantin and syntaxin 5 are cleaved by caspase-3

GRASP65 is cleaved specifically by caspase-3 (Lane et al., 2002), whilst Golgi-160 can be cleaved in vitro by caspase-2, -3 and -7 (Mancini et al., 2000; Lane et al., 2002). To identify which caspases are responsible for giantin and syntaxin 5 cleavage, we first performed in vitro cleavage experiments in the presence of Casputin^{TM}, a caspase inhibitor that is highly selective for caspases 3 and 7. Apoptosis-specific proteolysis of both giantin and syntaxin 5 was almost completely abolished when Casputin^{TM} was included (Fig. 3A), indicating that caspase-3 or -7 activity is essential for their cleavage in apoptotic lysates.

We confirmed that both giantin and syntaxin 5 are selectively cleaved by caspase-3 by performing cleavage assays using in vitro-translated proteins (Fig. 3B). Control experiments demonstrated that in vitro-translated giantin and syntaxin 5 were cleaved in apoptotic extracts to generate products similar to those derived from Golgi-associated proteins (see Figs 4 and 5). In the case of syntaxin 5, caspase-2 was ineffective at concentrations as high as 240 nM, even though it cleaved a control substrate (in vitro translated procaspase-2) completely. Limited cleavage of syntaxin 5 by caspase-7 was observed, but only at relatively high caspase concentrations. In contrast, caspase-3-mediated cleavage of syntaxin 5 was detected at 0.5 nM protease, slightly higher than the concentration required for

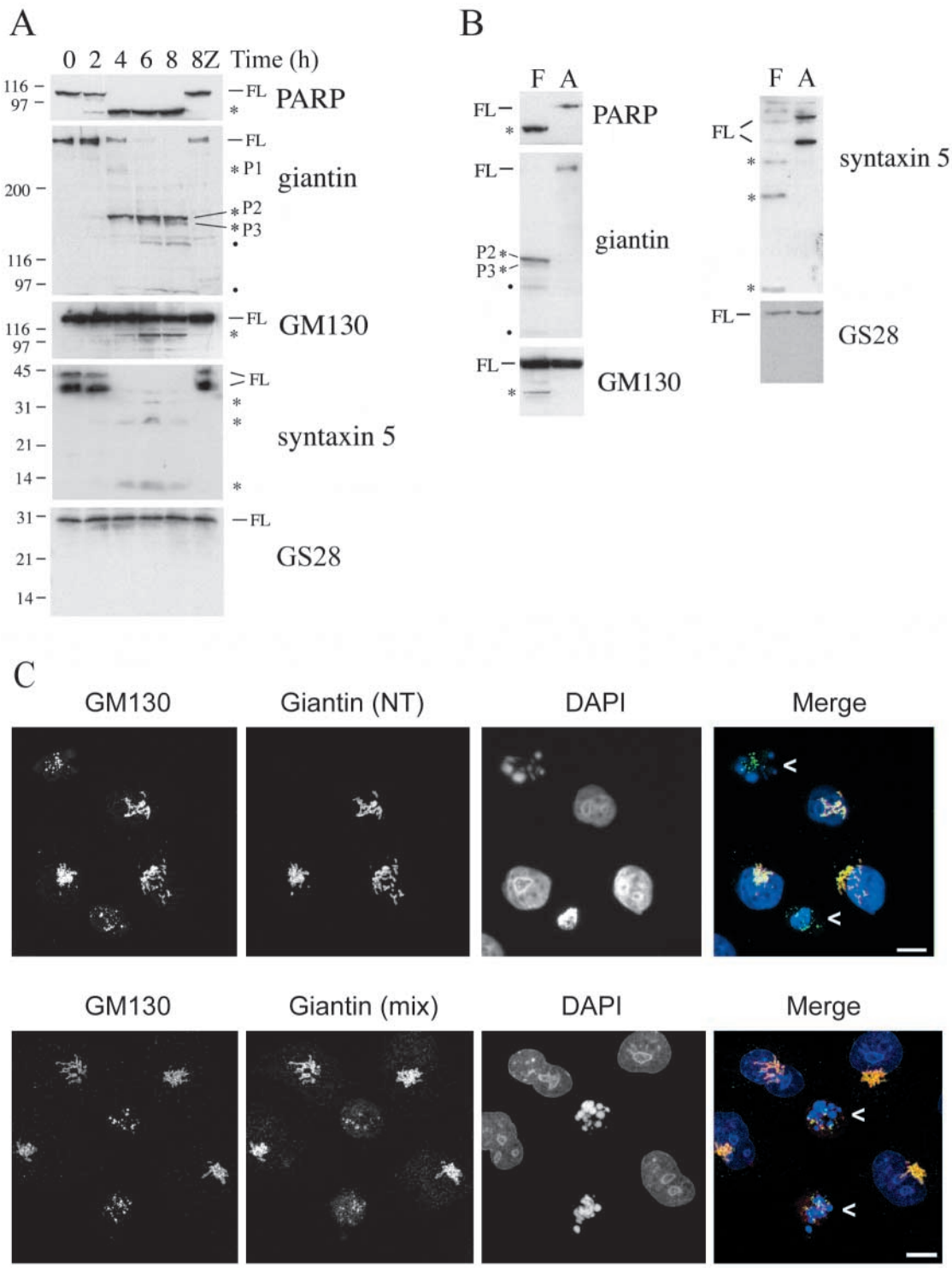

Fig. 2. Giantin and syntaxin 5 are rapidly cleaved during apoptosis in vivo. (A) HeLa cells were treated with 1 μM staurosporine in the absence or presence of 50 μM zVAD.fmk (Z) for the times indicated and analysed by immunoblotting with antibodies to PARP, giantin, GM130, syntaxin 5 and GS28. (B) HeLa cells were treated with anisomycin for 12 hours. Apoptotic cells became detached from the culture dish and these floating cells (F) were harvested by centrifugation. Remaining adherent cells (A) were harvested in parallel, and samples from each population were analysed by immunoblotting with antibodies to PARP, giantin, GM130, syntaxin 5 and GS28. Full-length (FL) and caspase cleavage products (*) are marked. The major giantin cleavage products P1-P3 are shown. Minor cleavage products are indicated with filled circles. (C) Confocal images of HeLa cells treated with UV radiation to induce apoptosis. Cells were incubated for 4 hours post-irradiation and stained with DAPI (blue), antibodies to GM130 (green) and antibodies raised against either the N terminus of giantin (NT) or fragments encompassing the entire protein (mix; red). Regions of overlap between GM130 and giantin are in yellow. Apoptotic cells are marked with arrowheads. Scale bar, 10 μm.

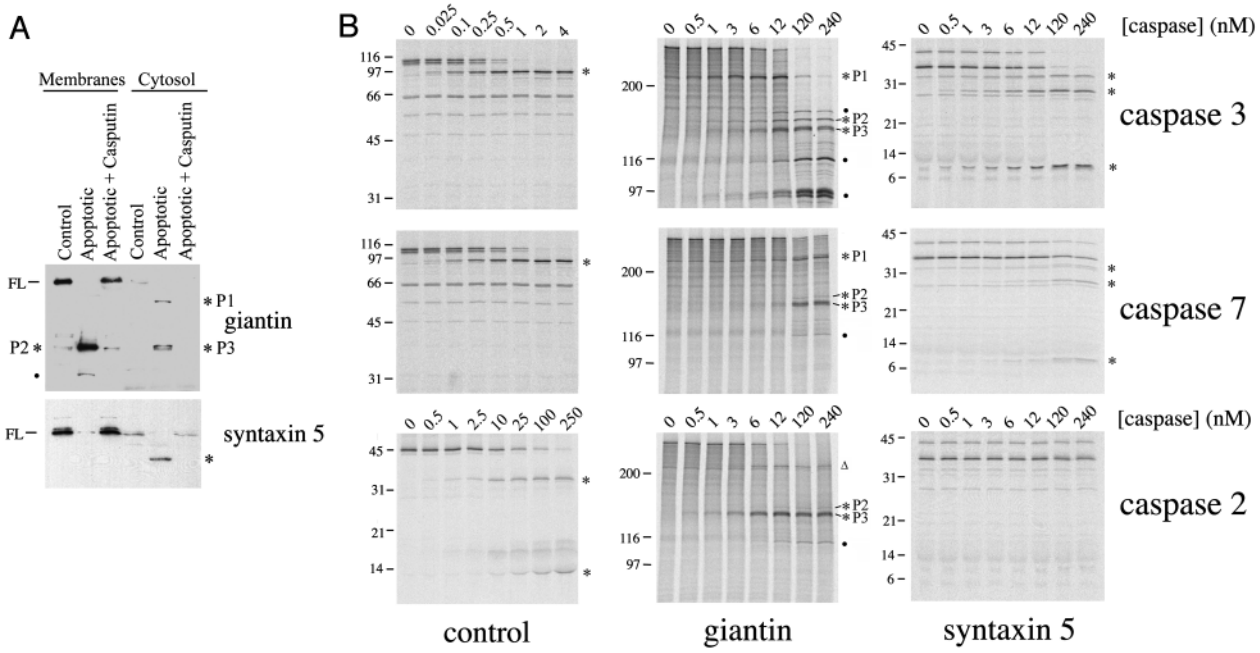

Fig. 3. Giantin and syntaxin 5 are cleaved by caspase-3. (A) Cytosols from HeLa cells were pre-incubated in the absence (control) or presence (apoptotic) of 10 μM cytochrome c for 90 minutes at 37°C to activate endogenous caspases. Golgi membranes were incubated with these control or apoptotic cytosols in the absence or presence of 80 μg/ml Casputin^{TM} for 4 hours at 37°C and analysed by SDS-PAGE and immunoblotting. Full-length (FL) and caspase cleavage products (*) are marked. Filled circles indicate minor giantin cleavage products. (B) In vitro translated ^{35}S-labelled control proteins, giantin or syntaxin 5 were incubated with purified recombinant caspases at the indicated concentrations for 2 hours at 30°C prior to SDS-PAGE and autoradiography. Defined caspase cleavage products (*) are marked. Additional cleavage products that may be non-specific (filled circles) are also marked. The open triangle indicates an additional giantin cleavage product seen only with caspase-2 that may be the cleavage partner for P3 (see also Fig. 5C). Control proteins were PARP (for caspase-3 and -7 at 0, 0.025, 0.1, 0.25, 0.5, 1, 2 and 4 nM) and pro-caspase-2 (for caspase-2 at 0, 0.5, 1, 2.5, 10, 25, 100, 250 nM).

detectable cleavage of the model caspase-3 substrate, PARP. Giantin was also cleaved effectively by caspase-3, with the prominent cleavage product of 220 kDa (P1) being detected using 1 nM caspase-3. In keeping with its transient nature in cells and cell extracts, this product was not seen at higher caspase concentrations, suggesting that it was cleaved at additional, less sensitive site(s). Two bands at approximately 160 kDa, and most likely corresponding to the P2 and P3 cleavage products, were observed (P3 could be immunoprecipitated with antibodies to the N terminus while P2 could not; data not shown). However, additional products were found at higher caspase-3 concentrations (Fig. 3B, indicated by filled circles). These are probably non-physiological cleavage products, since they were not observed using cells or cell lysates (though it is possible that they are products that are poorly detected by immunoblot analysis). It is noteworthy that P3 was generated by caspase-2 and caspase-7 as efficiently as by caspase-3, indicating that cleavage to generate this product is less selective than cleavage to generate P1 and P2.

Identification of the caspase sites within giantin and syntaxin 5

In vitro cleavage of Golgi membrane-associated syntaxin 5 resulted in the release from membranes of a soluble 26 kDa fragment. Since syntaxin 5 is a C-terminally anchored integral membrane protein, this suggested that it is cleaved towards its carboxyl terminus. When this region of the syntaxin 5 sequence was examined, two peptide sequences (DMMD^{249}P and DEQD^{263}S) were found that resembled the canonical DESD caspase-3 cleavage site within PARP and other caspase-3 substrates (Thornberry et al., 1997) (Fig. 4A). Moreover, these peptides were located slightly N-terminal to a coiled coil-forming region of syntaxin 5. Localization of caspase cleavage sites to loops adjacent to helical domains has been documented before, including the major caspase-3 cleavage site within the endosomal vesicle tethering factor rabaptin-5 (Swanton et al., 1999a). To identify which of these peptides is the site of caspase-3 action, wild-type syntaxin 5, syntaxin 5(D249A) and syntaxin 5(D263A) were in vitro translated and assessed for apoptotic cleavage. As shown in Fig. 4B, wild-type syntaxin 5 and syntaxin 5(D249A) were cleaved to identical sized products in apoptotic extracts, but not in control extracts or apoptotic extracts supplemented with Ac-DEVD-CHO. In contrast, no apoptosis-specific proteolysis of syntaxin 5(D263A) was observed, indicating that D^{263} is the site of caspase-3 action.

The apoptosis-specific cleavage of giantin is more complex than that of syntaxin 5. Two conserved peptides (DAGD^{1083}G and DVTD^{1882}A in rat giantin, corresponding to the peptides DASDG and DVTDA in human giantin) that might serve as caspase cleavage sites were identified (Fig. 5A). In vitro cleavage assays using wild-type and mutant giantin in

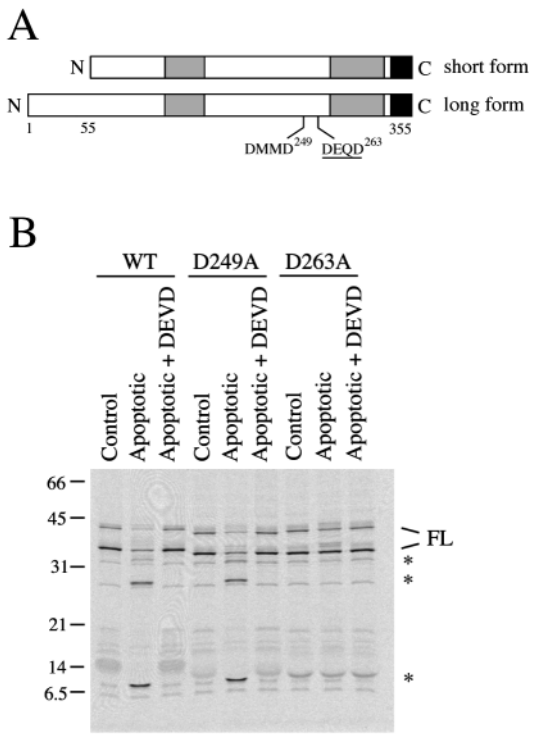

Fig. 4. Identification of the syntaxin 5 cleavage sites. (A) Schematic representation of the structure of syntaxin 5 isoforms showing the putative caspase-3 cleavage sites. Transmembrane domains are indicated in black and coiled coil-forming regions are shaded grey. The actual cleavage site is underlined. (B) In vitro translated ^{35}S-labelled wild-type syntaxin 5 or syntaxin 5 with the indicated aspartic acid to alanine substitutions were incubated with control or apoptotic HeLa cytosol in the absence or presence of 2 μM Ac-DEVD-CHO for 4 hours at 37°C and analysed by SDS-PAGE and autoradiography. Full-length (FL) and caspase cleavage products (*) are marked. Note that the single point mutations give rise to a slight shift in mobility of the full-length protein.

apoptotic cytosol revealed that both of these sites were required to generate the full complement of cleavage patterns (Fig. 5B). However, the mutants differed in the efficiency of their cleavage. The D1083A mutant was cleaved as efficiently as wild-type giantin, generating the 220 kDa P1 product and additional products observed with the wild-type protein, including one at approximately 160 kDa corresponding to P2. The poor cleavage of the D1882A mutant indicated that D^{1882} is the preferred site for caspase cleavage and hence likely to be cleaved first in vivo. Consistent with this, the D1882A mutant failed to produce the P1 and P2 cleavage products, the first that could be detected in vitro from Golgi membranes. However, P3 was seen with the wild-type protein and giantin(D1882A), but not with giantin(D1083A). A faint product (marked with an arrowhead in Fig. 5B) migrating just above P1 and also observed only with giantin D1882A is probably the cleavage partner for P3.

D^{1882} was confirmed as being the major cleavage site in vivo by co-expressing N-terminal FLAG-tagged giantin with the Golgi marker GFP-tagged NAGT1 (NAGFP) (Fig. 5C). FLAG-tagged giantin was localized to the Golgi region when expressed in HeLa cells. The FLAG tag on wild-type giantin was absent from the Golgi region of apoptotic cells that showed a fragmented Golgi complex, confirming that giantin is a caspase substrate in vivo. Weak cytosolic fluorescence was sometimes observed in these cells, though often it could not be detected readily. FLAG labelling was also absent from the Golgi region in cells expressing the D1083A mutant giantin. In contrast, in cells expressing either the D1882A mutant or the double mutant, FLAG staining remained associated with NAGFP-positive Golgi fragments. In all, these data confirm the identity of the major cleavage sites within giantin and suggest the order of cleavage shown in Fig. 5D.

The early secretory pathway is blocked in apoptotic cells

Our previous work demonstrated that the Golgi complex is fragmented during apoptosis (Lane et al., 2002). However, we did not establish whether transport to and through the Golgi complex was seriously affected. Importantly, fragmentation of the Golgi complex does not necessarily lead to a complete shut down of the secretory pathway. Dispersal of the Golgi complex by the microtubule depolymerising agent nocodazole does not prevent transport to the cell surface (Rogalski et al., 1984). In addition, the imino sugar N-butyldeoxynojirimycin causes the Golgi to lose its cisternal organisation with no apparent affect on trafficking (Neises et al., 1997). Given that both giantin and syntaxin 5 are cleaved at an early stage during apoptosis, we anticipated that, at the very least, the delivery of ER-derived transport intermediates to the Golgi would be impaired in apoptotic cells.

To analyse transport through the secretory pathway in apoptotic cells, we transfected cells with the temperature-sensitive tsO45 mutant of VSV-G protein tagged with GFP. At the non-permissive temperature of 39.5°C this mutant accumulates within the ER, because of a reversible folding defect (Rogalski et al., 1984; de Silva et al., 1990). A shift to the permissive temperature (31°C) is followed by the relatively rapid transport of correctly folded VSV-G to the cell surface, which can readily be detected by indirect immunofluorescence of non-permeabilized cells using an antibody directed against the lumenal domain of VSV-G. Hence, we detected negligible fluorescence on the surface of cells that had been held at 39.5°C, whilst cells released to 31°C for 2 hours labelled strongly for surface VSV-G (Fig. 6A, top panel).

Transfected cells held at 39.5°C were treated with UV radiation to induce apoptosis at the restrictive temperature. No difference was observed between 37°C and 39.5°C in the rate of occurrence of apoptosis (data not shown). After a further 4 hours, during which time a substantial portion of cells became apoptotic, the temperature was reduced to 31°C and the incubation continued for a further 2 hours. Apoptotic cells were detected by staining with DAPI. Importantly, UV treatment did not itself substantially affect VSV-G transport, since VSV-G was detected on the surface of non-apoptotic cells that had been shifted to 31°C, but not on the surface of cells that remained at 39.5°C (Fig. 6A, middle panel; Fig. 6B). In addition, control experiments showed that the rate of VSV-G transport was not directly affected by treating cells with UV (data not shown). However, almost no apoptotic cells were found that expressed VSV-G on the surface even after a shift to 31°C, indicating a total block in the secretory pathway (Fig. 6A, middle panel; Fig. 6B). Although a small number of

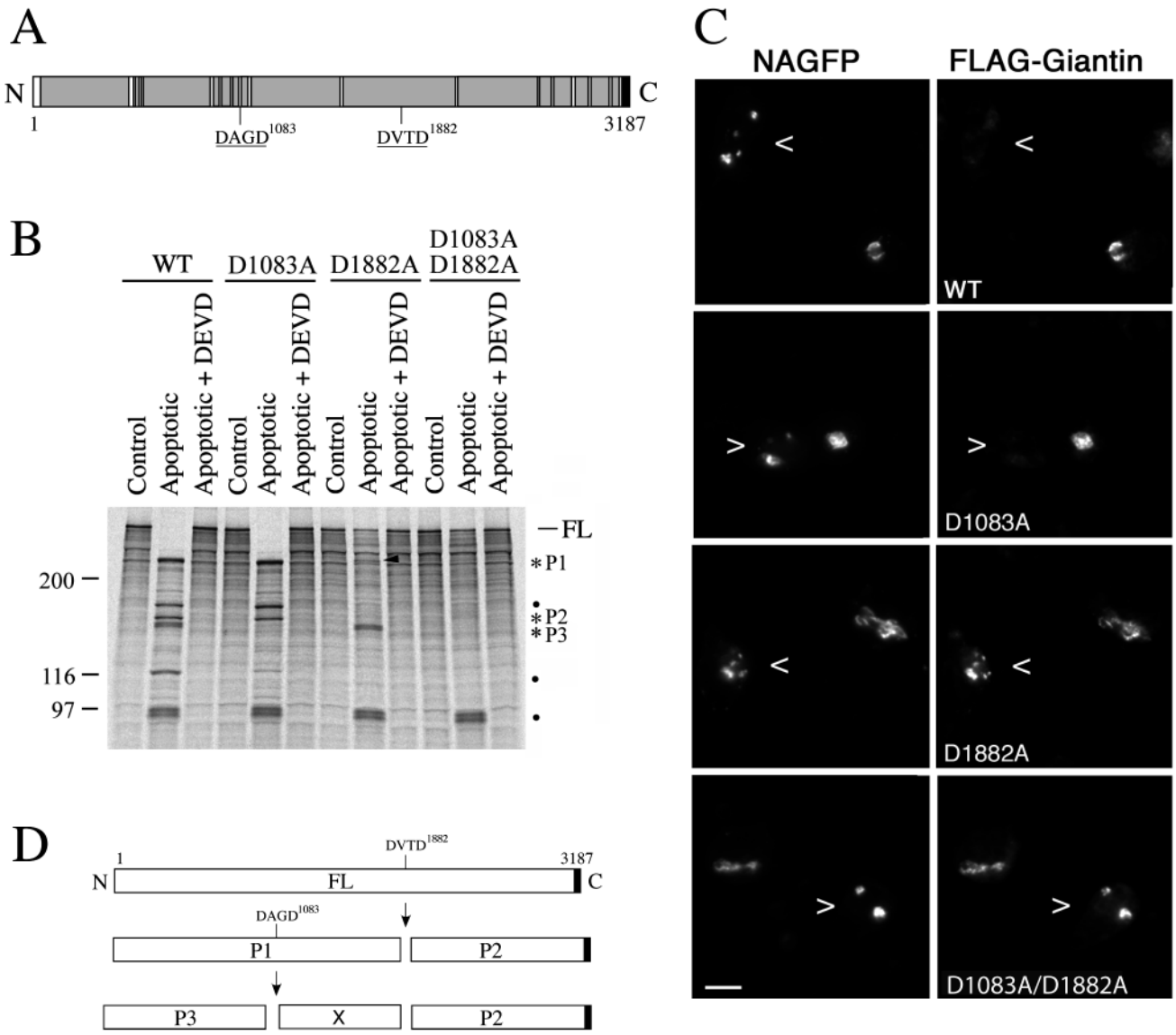

Fig. 5. Identification of the giantin cleavage sites. (A) Schematic representation of the structure of giantin showing the putative caspase-3 cleavage sites that were mutated. The transmembrane domain is shown in black. Potential coiled coil-forming regions are shaded grey. (B) In vitro translated ^{35}S-labelled wild-type giantin or giantin with the indicated aspartic acid to alanine substitutions were incubated with control or apoptotic HeLa cytosol in the absence or presence of 2 μM Ac-DEVD-CHO for 4 hours at 37°C and analysed by SDS-PAGE and autoradiography. Full-length (FL) and defined caspase cleavage products (*) are marked. Additional cleavage products (filled circles) are also shown. The arrowhead marks an additional faint band seen only with the D1882A mutant. (C) HeLa cells expressing GFP-tagged NAGT1 (NAGFP) and FLAG-tagged wild-type giantin, giantin D1083A, giantin D1882A, or the double point mutant were induced with 2 μM staurosporine for 8 hours to undergo apoptosis and visualized for NAGFP and FLAG staining by epifluorescence microscopy. Apoptotic cells are indicated with arrowheads. Scale bar, 10 μm. (D) Scheme to describe the likely order of cleavages occurring within giantin. The P1 and 2 products are produced first by cleavage at D^{1882}. The P3 product and an additional unidentified fragment are generated by secondary cleavage of P1 at DAGD^{1083}.

apoptotic cells expressed detectable levels of VSV-G on their surface, these were most probably cells that had entered apoptosis during the course of the incubation at the permissive temperature and hence may already have transported VSV-G to the cell surface. The block in transport is caspase dependent, since incubation with zVAD.fmk allowed all cells that had been treated with UV to transport VSV-G to the surface (data not shown). Moreover, it is likely to be a generalised property of apoptotic cells, since VSV-G transport was blocked in apoptotic cells generated by treatment with staurosporine (Fig. 6A, bottom panel). The transport block is unlikely to be caused by necrosis, since 97% and 94% of VSV-G-positive UV- and staurosporine-treated cells, respectively, were able to exclude propidium iodide added at the end of the incubations (data not shown).

To identify the site of the apoptosis-specific transport block we closely examined non-apoptotic and apoptotic cells for the localisation of VSV-G (Fig. 7). To analyse whether transport in the early secretory pathway was affected, cells were shifted to the permissive temperature for only 25 minutes rather than

2 hours. In non-apoptotic cells this resulted in the bulk of VSV-G exiting the ER and accumulating in the Golgi apparatus (Fig. 7A). In contrast, in apoptotic cells VSV-G failed to accumulate in the Golgi region and instead remained in the ER (Fig. 7B). This indicates that ER exit is blocked during apoptosis.

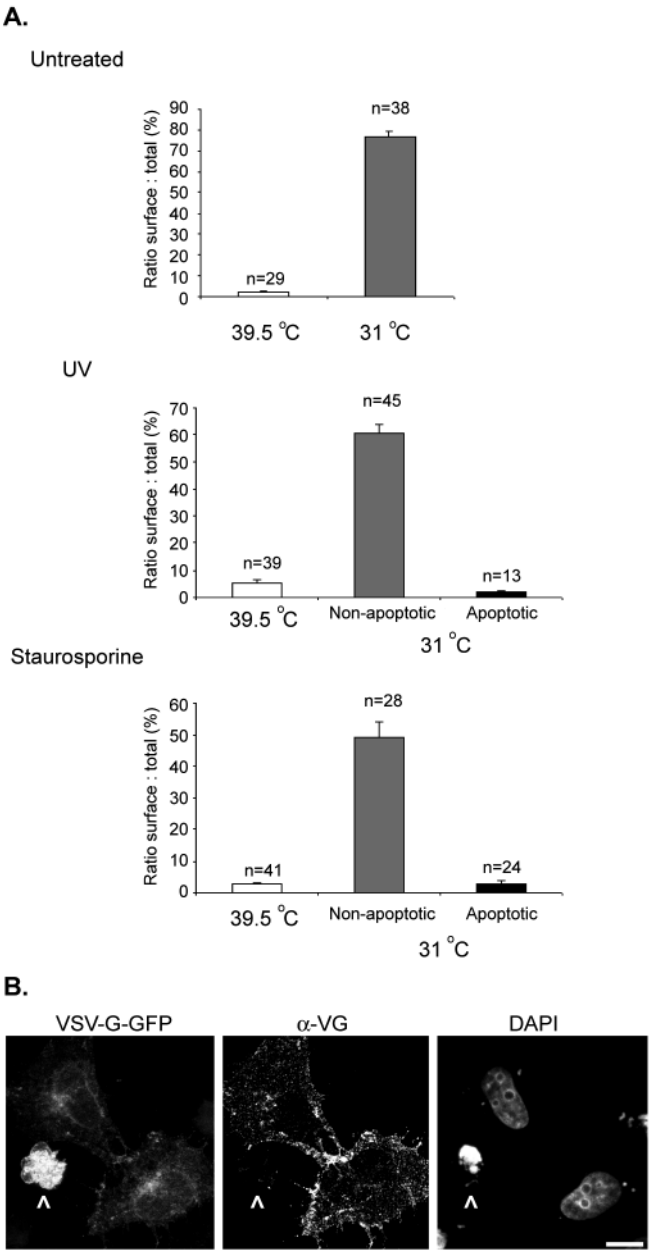

Fig. 6. The early secretory pathway is blocked in apoptotic cells. (A) (Top panel) HeLa cells were transfected with tsO45 VSV-G-GFP and incubated overnight at 39.5°C, then incubated in parallel for 2 hours at 39.5°C or 31°C as shown. They were stained without permeabilization, using anti-VSV-G, to score for transport to the cell surface. (Middle panel) Transfected cells were induced to enter apoptosis by UV irradiation and incubated for a further 4 hours at 39.5°C. Cells were either left at 39.5°C or shifted to 31°C for 2 hours, as indicated, then fixed in PFA. Apoptotic cells were identified using DAPI. (Bottom panel) As in middle panel, but with apoptosis induced by addition of 2 μM staurosporine. Results are presented as the mean ± s.e.m. (*n*=13-45). (B) Fluorescence microscopy of tsO45-VSV-G-GFP trafficking. UV-treated HeLa cells expressing VSV-G-GFP were treated as in A, and examined using confocal microscopy. The non-apoptotic cells show strong surface labelling (α-VG), whilst the apoptotic cell (arrowhead) lacks surface label. Scale bar, 10 μm.

Discussion

In this report we have identified the apoptotic cleavage of two Golgi-associated transport factors, syntaxin 5 and giantin. Syntaxin 5 is a member of the SNARE family of proteins that mediate specific vesicle-target membrane fusion (Söllner et al., 1993; Rothman, 1994). Cognate binding between the membrane-proximal helical domains of specific vesicle-associated (v) and target membrane-associated (t) SNAREs generates a parallel four-helix bundle that bridges the vesicle-target membrane interface (Hanson et al., 1997; Hohl et al., 1998; Sutton et al., 1998). SNAREs are proposed to mediate bilayer fusion, once this bridging, or trans, SNARE complex is formed. Syntaxin 5 partners rbet1, membrin and sec22b during ER-Golgi transport (Hay et al., 1998; Xu et al., 2000) and GS28 (with other unidentified SNAREs) during intra-Golgi transport (Hay et al., 1998). Hence, syntaxin 5 cleavage is likely to affect several membrane fusion events.

There is increasing evidence that efficient membrane fusion requires formation of an array of trans SNARE complexes at the point of membrane contact (Tokumaru et al., 2001). Evidently, this event must be highly co-ordinated. Formation of multiple trans SNARE complexes is controlled, in part, by an N-terminal domain of syntaxin which shields the C-terminal helical region and thus prevents syntaxin from interacting with SNAREs within the same membrane. Release of this domain, when coupled to membrane docking, makes syntaxin available for trans complex formation. This regulatory structure is stabilised by Sec1 proteins (Dulubova et al., 1999; Misura et al., 2000), themselves most probably regulated by Rab proteins (Zerial and McBride, 2001). Indeed, the Sec1 homologue Sly1p modulates the activity of yeast syntaxin 5 via binding to its N-terminal domain (Xu et al., 2000; Yamaguchi et al., 2002). Caspase-3-dependent cleavage of syntaxin 5 would separate its membrane-proximal helical domain from this regulatory domain, thereby impairing efficient SNARE-mediated fusion.

Formation of trans SNARE complexes is also regulated by tethering factors, a diverse family of proteins that includes giantin. Many tethering factors have been identified, each one usually associated with specific membrane transport steps within the cell (Pfeffer, 1999; Waters and Hughson, 2000; Zerial and McBride, 2001). Current models suggest that they are regulated by members of the Rab family of small GTPases, and that they mediate ‘distance’ docking interactions between membranes as a prelude to SNARE binding. An attractive hypothesis to explain giantin function is that it acts as a transport vesicle-associated tethering factor (Sönnichsen et al., 1998), consistent with its localization to Golgi rims and Golgi-associated vesicles (Martinez-Menarguez et al., 2001). Here, it binds to the peripheral Golgi protein p115, which forms an extended dimer. In turn, p115 binds to the Golgi cisternae-associated matrix protein GM130 (Nakamura et al., 1997), and hence acts as a scaffold between the transport vesicle and the Golgi stack. Although other groups have disputed the details of this model (Linstedt et al., 2000), it is clear that cleavage and consequent inactivation of giantin would be predicted to

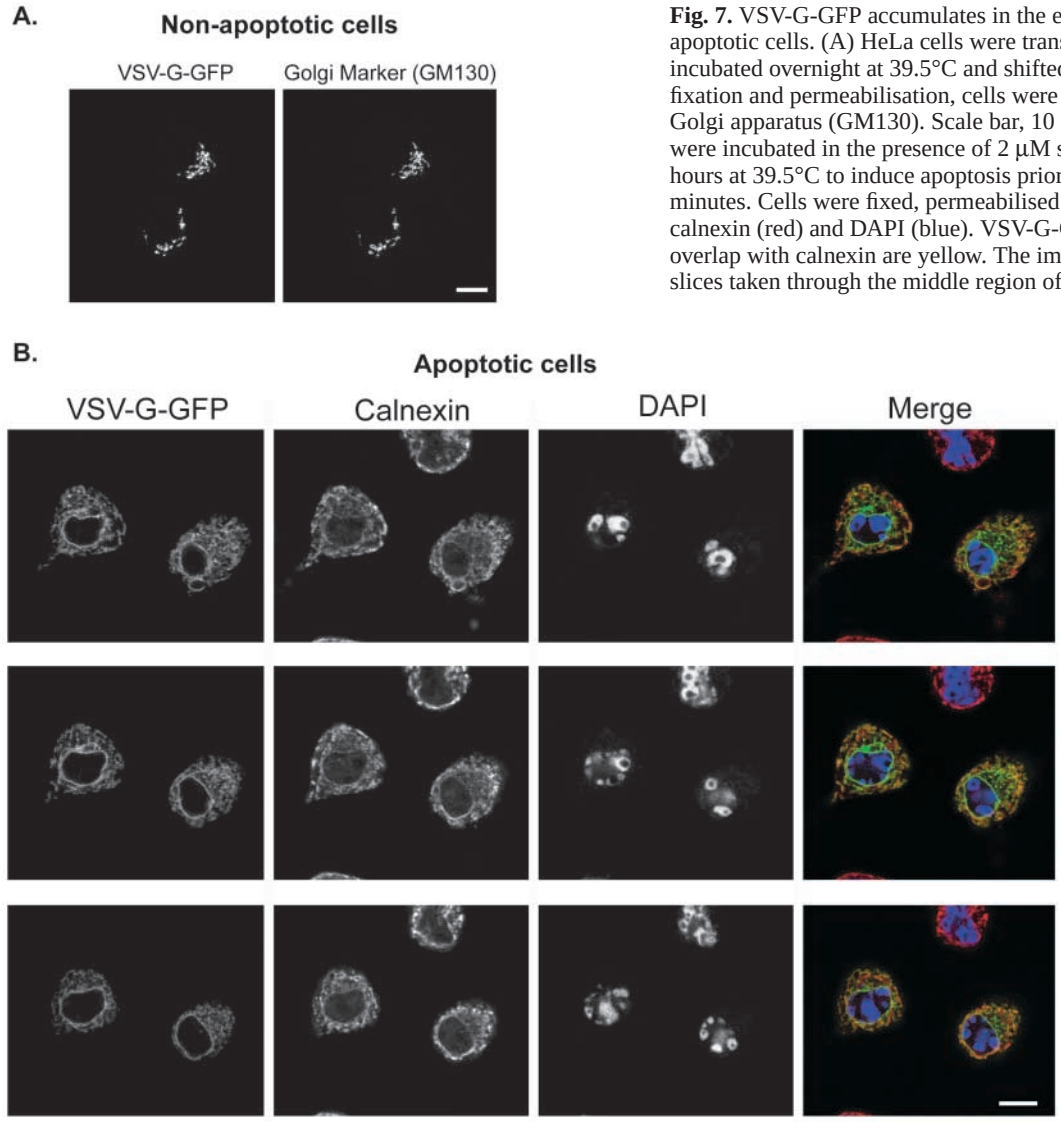

Fig. 7. VSV-G-GFP accumulates in the endoplasmic reticulum of apoptotic cells. (A) HeLa cells were transfected with tsO45 VSV-G-GFP, incubated overnight at 39.5°C and shifted to 31°C for 25 minutes. After fixation and permeabilisation, cells were stained with antibodies to the Golgi apparatus (GM130). Scale bar, 10 μm. (B) Transfected HeLa cells were incubated in the presence of 2 μM staurosporine for an additional 4 hours at 39.5°C to induce apoptosis prior to shifting to 31°C for 25 minutes. Cells were fixed, permeabilised and stained with the ER marker calnexin (red) and DAPI (blue). VSV-G-GFP is green and regions of overlap with calnexin are yellow. The images are three separate confocal slices taken through the middle region of the cell. Scale bar, 10 μm.

prevent efficient SNARE-mediated fusion. In combination with syntaxin 5 cleavage, the inhibition is likely to be profound.

Cleavage of syntaxin 5 and giantin is selective, since many other trafficking components remained uncleaved in our studies. These included membrin, rsec22b, rbet1 GS15, β-COP and rab1. GM130 and GS28 were cleaved to a limited extent and we have not investigated the significance of this any further. In addition and in contrast to the findings of Chiu et al., (Chiu et al., 2002) we failed to detect significant cleavage of p115 either in vitro or in vivo. The reason for this discrepancy is not clear but it is possible that p115 cleavage is cell-type specific, or it may proceed with significantly slower kinetics than cleavage of GRASP65, giantin and syntaxin-5 and was therefore not detectable in the time scale of our experiments.

The primary defect in trafficking in apoptotic cells that we observed was export of VSV-G from the ER. We therefore could not monitor whether transport to or through the Golgi was also blocked, which would be consistent with the established functions of syntaxin 5 and giantin. There are a number of possible reasons for the apoptosis-dependent block in ER exit. It is possible that it is an indirect consequence of impairing docking/fusion reactions at the Golgi complex, since a prolonged delay in docking/fusion would lead to an accumulation, in an inappropriate location, of components required for ER exit that normally recycle between the ER and Golgi complex. Alternatively, SNAREs and tethering factors may be directly involved in promoting ER exit. Indeed, there is accumulating evidence that this might be the case (Allan et al., 2000; Morsomme and Riezman, 2002). Most probably, there are other caspase targets that are involved in the early secretory pathway that we have not yet identified. In this context, we have been unable to test whether co-expression of caspase-resistant syntaxin 5 and giantin is sufficient to restore transport activity to apoptotic cells, since overexpression of wild-type syntaxin 5 itself impairs ER-Golgi trafficking substantially (Dascher et al., 1994). However, consistent with this, we have found that the coatomer subunit β-COP rapidly dissociates from the Golgi complex during apoptosis (our unpublished observations), despite the fact that it remains uncleaved and has no established direct functional links with

either syntaxin 5 or giantin. This implies that β-COP effector(s) may be caspase targets.

Such an inhibition of ER-Golgi trafficking could contribute to the fragmentation of the Golgi complex observed in apoptotic cells, which cannot be explained wholly by cleavage of the stacking factor GRASP65 (Lane et al., 2002). Our reasoning is based on models to explain the reversible disassembly of the Golgi complex that occurs in mitotic cells (Warren et al., 1995). It has been postulated that mitotic vesiculation of elements of the Golgi complex would occur if transport vesicle formation continued under conditions where the consumption of these vesicles (i.e. their docking and fusion with target membranes) were blocked (Misteli and Warren, 1994; Lowe et al., 1998a). In apoptotic cells, incoming traffic is probably blocked, and any remaining outward traffic from the Golgi would contribute to its fragmentation.

The reason for impairing membrane trafficking during apoptosis (as during mitosis) remains a mystery. Certainly, inhibiting trafficking may assist in the breakdown of the Golgi complex and hence its distribution into apoptotic bodies. However, we also favour the idea that altered membrane trafficking pathways contribute to the generation of phagocytic motifs, most probably carbohydrate-based, on the surface of the apoptotic cell. Many studies have implicated carbohydrates in providing at least a portion of a generalised recognition signal that might act in conjunction with or independently of phosphatidylserine (Savill, 1998; Henson et al., 2001). The nature of this signal has yet to be identified. Interestingly, however, several studies have noted that a change in the ratio or pattern of complex and mannose-based oligosaccharides might be a contributory factor (Morris et al., 1984; Duvall et al., 1985; Falasca et al., 1996; Korb and Ahearn, 1997; Ogden et al., 2001). It is conceivable that interdomain cleavage of fusion and tethering factors would not only inhibit normal trafficking pathways but might also permit a limited amount of ‘inappropriate’ fusion reactions to occur by preventing formation of regulatory interactions.

In summary, we have shown that at least two factors required for specific and efficient protein trafficking are cleaved during apoptosis. We propose that these cleavages, in combination with disruption of microtubule-based membrane organisation and Golgi structure (Lane et al., 2001; Lane et al., 2002), could contribute to a breakdown in the selectivity or timing of membrane transport reactions within the early secretory pathway as the cell enters apoptosis. The aim of future work will be to investigate mechanistic links between the breakdown of the normal secretory pathway and the appearance of novel motif(s) on the surface of apoptotic cells.

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