Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 316 (2004) 6–11
BBRC
www.elsevier.com/locate/ybbrc

Golgi fragmentation during Fas-mediated apoptosis is associated with the rapid loss of GM130☆

Annemieke Walker,* Carol Ward, Tara A. Sheldrake, Ian Dransfield, Adriano G. Rossi, James G. Pryde, and Christopher Haslett

Centre for Inflammation Research, The University of Edinburgh, Medical School, Teviot Place, Edinburgh EH8 9AG, UK

Received 2 February 2004

Abstract

During apoptosis, the Golgi complex becomes fragmented and key proteins (e.g., GRASP65 and p115) are targets for caspase cleavage. GM130, an integral membrane protein, contributes to the maintenance of Golgi structure and facilitates membrane fusion with secretory vesicles. We show that GM130 levels decrease during Fas-induced apoptosis but not during staurosporine-induced apoptosis while in both models p115 levels remain unaffected. We conclude that GM130 is rapidly diminished during Fas-mediated apoptosis associated with Golgi fragmentation in contrast to previous studies which have suggested that loss of GM130 during apoptosis is a late event.

Keywords: Golgi; Apoptosis; p115; GM130; Staurosporine; Fas

The Golgi complex is the 'organising centre' for protein cargo en route from the endoplasmic reticulum to the plasma membrane. During mitosis, this structure becomes fragmented with a concomitant arrest in vesicle trafficking [1]. During apoptosis the Golgi complex also fragments [2–4] and it is possible that the secretory pathway is similarly inhibited, which would in part explain why apoptotic cells fail to respond to various external stimuli [5]. Mitotic Golgi fragmentation has been demonstrated to be dependent on cyclin-dependent-kinase-1 (CDK1) and later GM130 was identified as a CDK1 substrate [6]. GM130, a *cis*-Golgi matrix protein, is associated with the cytoplasmic face of the Golgi via GRASP65 [7], a N-myristoylated peripheral membrane protein [8], and binds the p115 tethering protein present on incoming vesicles [9,10]. Following phosphorylation, GM130 is no longer able to bind to p115 and dissociation of p115–GM130 is thought to contribute to fragmentation of the Golgi complex during mitosis [9]. GM130, GRASP65, and p115 have already been shown to be cleaved during apoptosis in a caspase-dependent manner facilitating Golgi fragmentation during apoptosis [2,3]. These studies concluded that GM130 cleavage during apoptosis is secondary to GRASP65 cleavage and a relatively late event [2,3]. However, these conclusions were based on staurosporine (broad-range kinase inhibitor) -induced apoptosis and work by Cha et al. [11] demonstrates phosphorylation of Golgi-associated targets may modulate cleavage. To address this issue, we followed the fate of GM130 during apoptosis in human J.CaM1.6 cells, a derivative of Jurkat T lymphocyte cells by triggering the physiologically relevant Fas death pathway [12] and for comparison by the broad spectrum kinase inhibitor staurosporine.

Materials and methods

Cell line. J.CaM1.6 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK) and routinely maintained in RPMI-1640 containing 10% FCS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, and 1% non-essential amino acids. Cells were grown in a 5% CO_2 humidified incubator at 37 °C.

☆ *Abbreviations:* z-VAD-fmk, benzylocarbonyl-Val-Ala-Asp-flouromethylketone; PI, propidium iodide; SSP, staurosporine.

* Corresponding author. Fax: +44-131-650-4384.
E-mail address: awalker4@staffmail.ed.ac.uk (A. Walker).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.02.015

Induction of apoptosis. J.CaM1.6 cells were incubated in 96-well plates at 5×10^6 cells/ml. Cells were incubated with either the broad-range kinase inhibitor staurosporine (2 μM) (Calbiochem–Novabiochem, UK) or the anti-Fas monoclonal antibody CH11 (500 ng/ml) (TCS Biologicals, UK) to induce apoptosis. One hundred micromolar of the pan-caspase inhibitor z-VAD-fmk (Bachem, UK) was added to appropriate wells in both staurosporine and CH11 assays and ZB4 (Fas blocking monoclonal antibody, 500 ng/ml, TCS Biologicals, UK) was added to appropriate wells in CH11 assays.

Flow cytometry. Externalisation of phosphatidylserine was monitored by FITC-labelled recombinant human Annexin-V binding. Stock Annexin-V-FITC (Bender MedSystems, Austria) was diluted 1/3000 with Annexin-V-binding buffer (500 ml Hanks' Balanced Salt Solution containing 5 μM $CaCl_2$). Cells were incubated on ice for 10 min with Annexin-V-FITC and 1 min with propidium iodide (PI). Annexin-V-FITC/PI binding was assessed by flow cytometry on a Coulter EPICS (Beckman Coulter, UK) and analysed on associated EXPO software. All experiments were carried out in triplicate ($n = 3$).

Immunofluorescence and image acquisition. The indirect immunofluorescence protocol was as described [13]. Cells were cytocentrifuged (300 rpm for 3 min) onto $1.5 \times 22 \times 22$-mm glass coverslips and fixed in methanol-free 3% (w/v) para-formaldehyde/PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 20 min at 20 °C. Cells were permeabilised with 0.1% (w/v) Triton X-100 and non-specific binding was blocked with 10% (v/v) sheep serum in 0.2% (w/v) fish skin gelatin (Sigma, UK). Primary antibodies used were: anti-active-caspase-3 rabbit polyclonal antibody (BD Pharmingen, UK) and mouse anti-rat GM130 monoclonal antibody (BD Transduction Laboratories, UK). Secondary antibodies used were Alexa 488 (green) goat anti-mouse IgG (highly cross-adsorbed) and Alexa 568 (red) goat anti-rabbit (highly cross-adsorbed) (Molecular Probes, Netherlands). Nuclei were stained with TO-PRO3 (Molecular Probes). Images for each channel were captured separately and assembled into a single file with TCS-MERGE software (Leica Microsystems, Heidelberg, Germany) prior to analysis. All confocal images were processed digitally using Adobe Photoshop 5.02 and Paint Shop Pro 4.

Western blotting. J.CaM1.6 cells (2.5×10^6) were harvested per sample and lysed as described [13]. Each sample was loaded onto a 10% SDS–polyacrylamide gel and proteins were separated and electrophoretically transferred to nitrocellulose. The nitrocellulose membranes were probed with mouse monoclonal antibodies to procaspase-3 (BD Pharmingen, UK), GM130, p115 (BD Transduction Laboratories, UK), and actin (Sigma, UK) followed by HRP-Goat anti mouse IgG (Dako, UK). The membranes were then incubated with ECL reagent (Amersham Biosciences, UK), placed under BioMax MS-1 X-ray sensitive film, and processed through an X-ray developer (X-Ograph Imaging Systems, Wilts, UK).

Statistical analysis. Results are reported either as pooled data from a series of n separate experiments (mean ± SEM) or as individual representative experiments (mean ± SD). Statistical significance was assessed by one-way analysis of variance with comparisons between groups made using the Tukey procedure.

Results

Induction of apoptosis in J.CaM1.6 cells

To examine whether GM130 was affected during Fas-mediated apoptosis, we used J.CaM1.6 cells, a derivative of Jurkat cells, which constitutively expresses Fas. J.CaM1.6 cells were treated with 500 ng/ml CH11 in the presence of FCS, over 6 h and the proportion of apoptotic cells, as assessed by Annexin-V-FITC binding, was monitored at 1, 2, 4, and 6 h (Fig. 1A). At 1 h, the percentage of apoptotic cells was ~12%, significantly ($p < 0.05$) higher than present in control populations (~2%), increasing up to 4 h, where the amount of apoptosis was ~60%, after which the rate of apoptosis began to decline. To ensure that induction of apoptosis occurred via the Fas pathway, control samples were pre-incubated with 500 ng/ml of antagonist Fas antibody ZB4 for 30 min, prior to addition of CH11. ZB4 abolished the apoptotic effect of CH11 upon J.CaM1.6 cells, decreasing the proportion of cells which bound Annexin-V-FITC (~5% not significant ($p < 0.05$) from untreated control levels). In addition, z-VAD-fmk completely inhibited Annexin-V-FITC binding, which was not significantly different from control levels. Fig. 1B shows representative flow cytometry profiles demonstrating that the amount of PI positive cells never increases significantly above control levels.

To follow the kinetics of apoptosis induction in J.CaM1.6 cells by staurosporine, cells were incubated in the presence of 2 μM staurosporine over 4 h (Fig. 2A) and apoptosis was assessed by Annexin-V-FITC binding and exclusion of PI. The results from three separate experiments show that at 1 h, the number of apoptotic cells present, ~14%, was already significantly ($p < 0.05$) increased from control cells ~2%. This trend continued up to 4 h, where ~80% of cells bound Annexin-V-FITC and excluded PI. Apoptosis was inhibited by z-VAD-fmk, demonstrating that the effects of staurosporine were due to caspase activity. Fig. 2B shows representative flow cytometry profiles revealing that staurosporine does not induce significant amounts of necrosis over this time frame.

Immunofluorescence of Golgi fragmentation in apoptotic J.CaM1.6 cells

To examine the fragmentation of the Golgi in apoptotic J.CaM1.6 cells, cells were treated with 500 ng/ml CH11 or 2 μM staurosporine over 4 h to induce apoptosis. Apoptotic cells were identified by their fragmented nuclei and by positive staining with the polyclonal antibody raised against active-caspase-3 (Fig. 3). Many of the cells treated with 500 ng/ml CH11 had caspase-3 activity throughout the cytoplasm and in those cells the nuclei and Golgi were fragmented. In cells that had been pre-treated with the neutralising antibody ZB4 against Fas or the caspase inhibitor z-VAD-fmk, caspase-3 activation, and Golgi fragmentation were prevented. This demonstrated that Golgi fragmentation and nuclear "pebbling" were a direct consequence of signalling through the Fas death pathway in CH11-treated cells and that the morphological changes observed were caspase-dependent. To compare the fragmentation of the Golgi complex during staurosporine-induced apoptosis, J.CaM1.6 cells were treated with 2 μM

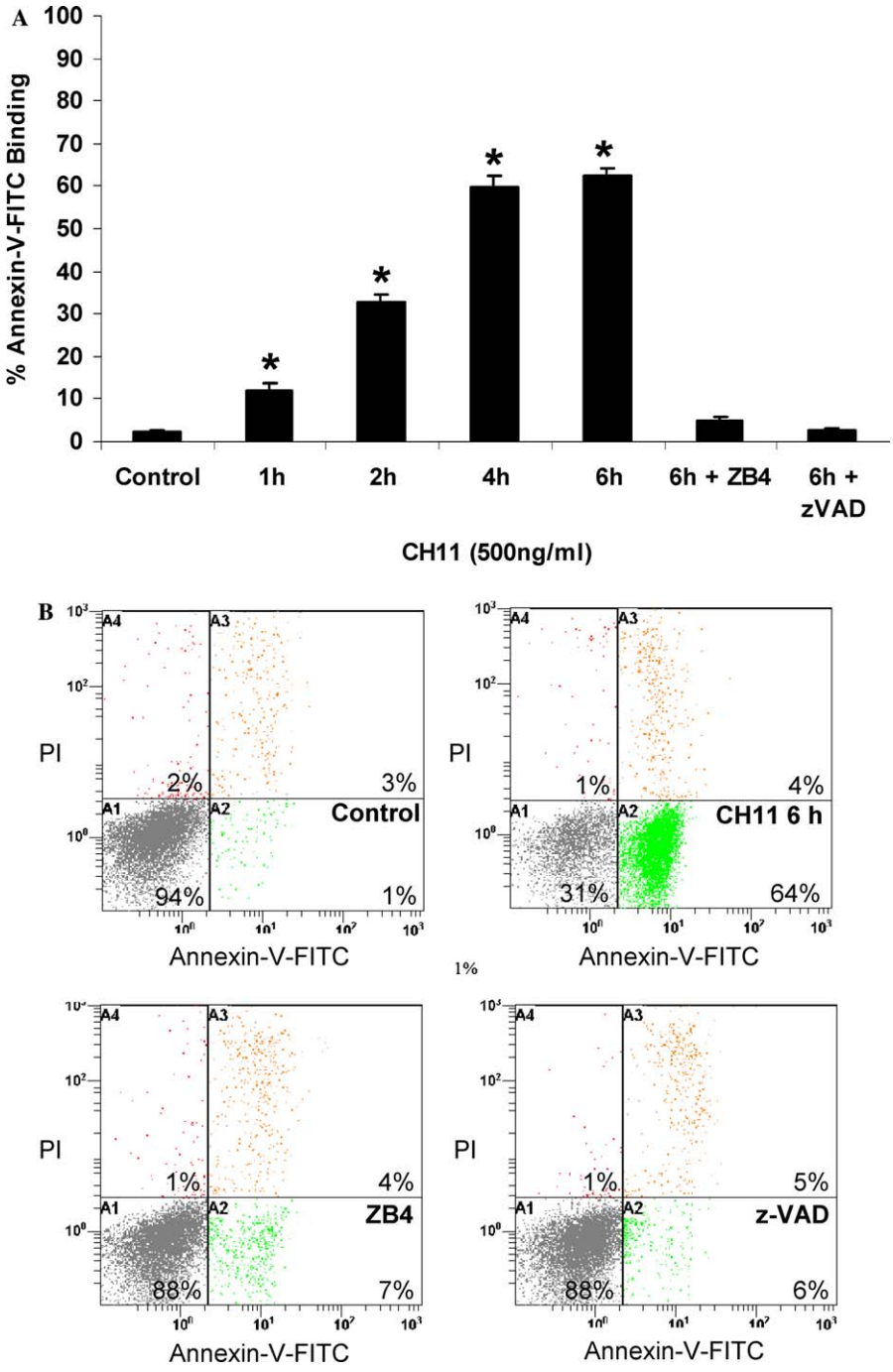

Fig. 1. Fas-induced apoptosis in J.CaM1.6 cells. (A) J.CaM1.6 cells were incubated with the anti-Fas monoclonal antibody CH11 (500 ng/ml) over 6 h. Samples were taken at the time points indicated and the rates of apoptosis and necrosis were assessed by Annexin-V binding and PI staining. The amount of apoptosis increased significantly over 6 h and this was blocked by the Fas neutralising antibody ZB4 (500 ng/ml) and the pan-caspase inhibitor z-VAD-fmk (100 μM). Necrosis as measured by PI staining did not increase significantly during any of the treatments. Data represent means ± SEM of three separate experiments. All experiments were performed in triplicate (*$p < 0.05$ compared with control values). (B) Representative scatter plots demonstrating little PI staining (upper quadrants) in control cell populations and CH11-treated cell populations where Annexin-FITC binding is increased.

staurosporine for 4 h. The proportion of apoptotic cells was higher in staurosporine-treated cells than CH11-treated cells after 4 h with the majority of cells being apoptotic. From the kinetic studies, it can be seen that the majority (~80%) of staurosporine-treated J.CaM1.6 cells are apoptotic at 4 h (Fig. 3). All cells displaying nuclear fragmentation also had caspase-3 activity yet strikingly, a large proportion of these cells still had intact Golgi.

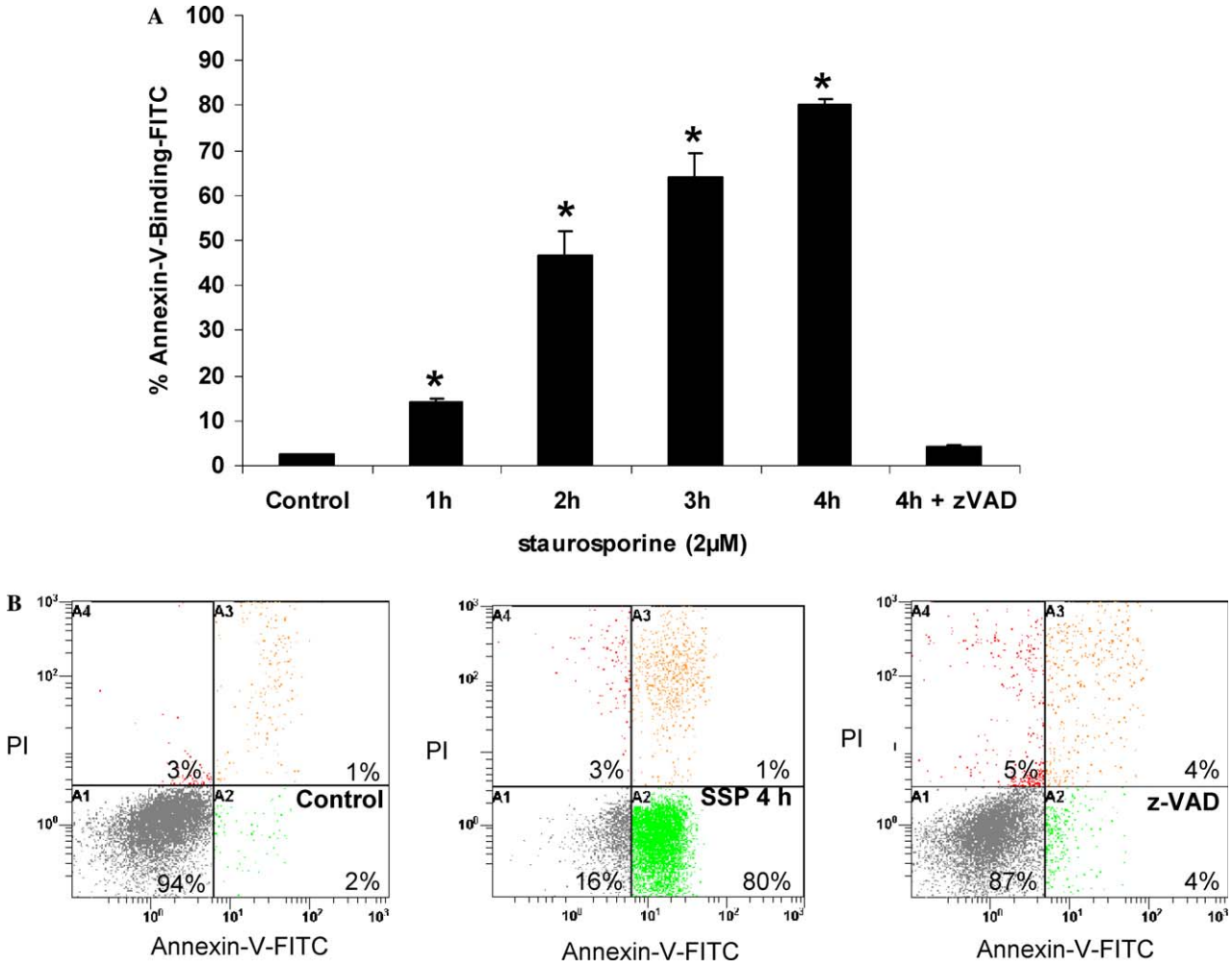

Fig. 2. Staurosporine-induced apoptosis in J.CaM1.6 cells. (A) J.CaM1.6 cells were incubated with 2 μM staurosporine over 4 h. Samples were taken at the time points indicated and the rates of apoptosis and necrosis were assessed by Annexin-V binding and PI staining. The amount of apoptosis increased significantly over 4 h and this was blocked by z-VAD-fmk (100 μM). Necrosis as measured by PI staining did not increase significantly during any of the treatments. Data represent means ± SEM of three separate experiments. All experiments were performed in triplicate (*$p < 0.05$ compared with control values). (B) Representative scatter plot demonstrating little PI staining (upper quadrants) in control cell populations and staurosporine-treated cell populations where Annexin-FITC binding is increased.

GM130 levels decrease during CH11-, but not staurosporine-, induced apoptosis

Immunofluorescence studies suggested that the kinetics of Golgi fragmentation in apoptotic cells treated with staurosporine were different to the kinetics of Golgi fragmentation in cells treated with CH11 (Fig. 3). Of particular interest was the fate of the Golgi matrix protein GM130 and p115 during apoptosis. J.CaM1.6 cells were incubated with 500 ng/ml CH11 over 6 h and the levels of GM130 and p115 were assessed by Western blotting together with procaspase-3 and actin. After 4 h, procaspase-3 was completely cleaved and there was a concomitant decrease in GM130 compared to control levels, although p115 and actin levels were similar to control (Fig. 4). At 6 h, GM130 was barely detectable while p115 and actin levels remained comparable to control levels. Procaspase-3 processing and apoptosis was inhibited by ZB4, demonstrating that apoptosis was specific to the induction of the Fas-mediated death pathway. Procaspase-3 cleavage was also completely inhibited by z-VAD-fmk and the rate of apoptosis was similar to control levels. In both cases, pre-incubation with ZB4 and z-VAD-fmk, GM130 levels were similar to control demonstrating that the reduced levels of GM130 were specific to Fas-mediated apoptosis and were caspase-dependent.

To assess whether GM130 levels also diminished during staurosporine-induced apoptosis we treated J.CaM1.6 cells with 2 μM staurosporine over 4 h and the levels of GM130, p115, procaspase-3, and actin were monitored again by Western blot analysis. By 2 h, procaspase-3 was no longer detected but GM130, p115, and actin were not diminished. After 3 and 4 h, despite

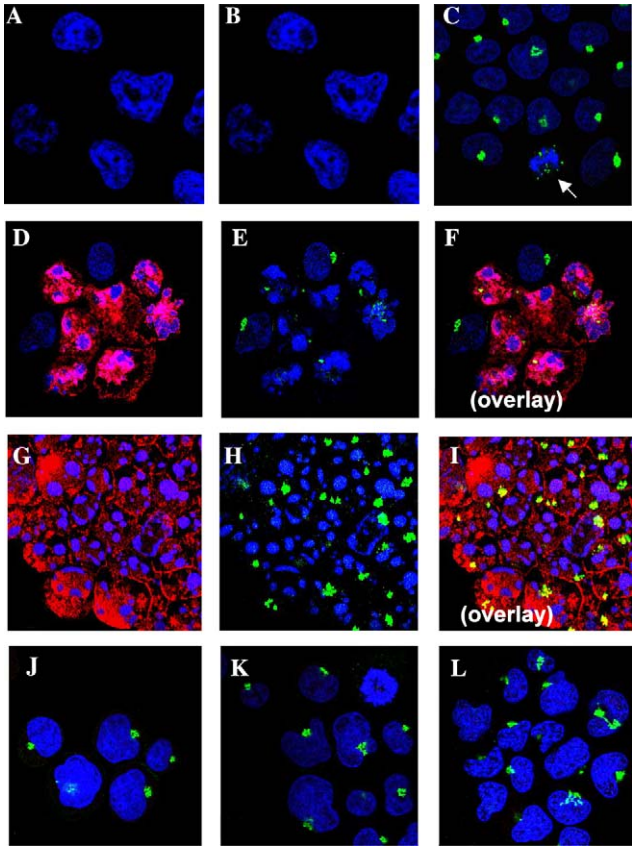

Fig. 3. Golgi morphology in J.CaM1.6 cells treated with 500 ng/ml CH11 or 2 μM staurosporine for 4 h. Cells were stained with anti-active-caspase-3 antibody followed by goat-anti-rabbit-Alexa 568 (red) and anti-GM130 followed by goat-anti-mouse-Alexa 488 (green) and the nuclei with TO-PRO-3 (blue). J.CaM1.6 cells stained with (A) rabbit IgG, goat-anti-rabbit-Alexa 568 and (B) mouse IgG, goat-anti-mouse-Alexa 488. (C) Untreated cells stained with anti-GM130 and anti-active-caspase-3 (arrow indicates mitotic cell). Cells treated with CH11 (500 ng/ml) over 4 h (D–F), where (D) shows anti-active-caspase-3, (E) shows anti-GM130, and (F) is the overlay of (D,E). Cells treated with staurosporine (2 μM) over 4 h (G–I), where (G) shows active-caspase-3 staining, (H) shows GM130 staining, and (I) is the overlay of (G,H). Composite micrograph of cells treated with CH11 in the presence of (J) ZB4 (500 ng/ml), (K) z-VAD-fmk (100 μM). Cells treated with staurosporine in the presence of z-VAD-fmk (L). Data are representative of three experiments.

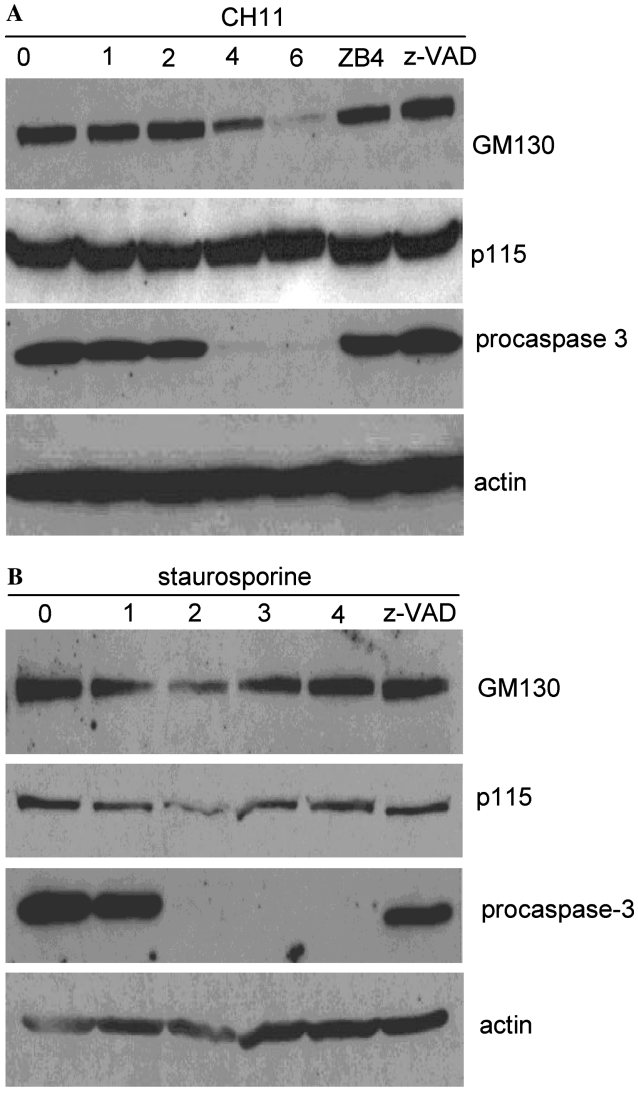

Fig. 4. GM130 levels decrease during CH11-induced apoptosis in J.CaM1.6 cells but not in staurosporine-induced apoptosis. (A) J.CaM1.6 cells were incubated with or without CH11 (500 ng/ml) over 6 h. At the indicated times, cell lysates were prepared for Western blotting using antibodies to GM130, p115, procaspase-3, and actin. After 4 h procaspase-3 was no longer detected and GM130 is diminished as compared to control. At 6 h GM130 is barely detected and this effect is inhibited by ZB4 (500 ng/ml) and by z-VAD-fmk (100 μM). (B) J.CaM1.6 cells were incubated with or without staurosporine (2 μM) over 4 h. At the indicated times, cell lysates were prepared for Western blotting using antibodies to GM130, p115, procaspase-3, and actin. After 2 h procaspase-3 was no longer detected and yet at 4 h GM130 levels were comparable to control. In cells pre-treated with z-VAD-fmk (100 μM), procaspase-3 levels were as in control. Data are representative of three separate experiments.

procaspase-3 processing and ~80% apoptosis (Fig. 2), GM130 was present at levels comparable to untreated cells. Procaspase-3 cleavage was completely inhibited by z-VAD-fmk and similar to control levels. GM130 displays increased resistance to caspase-dependent cleavage in the presence of staurosporine, perhaps reflecting the apparent inhibition of Golgi fragmentation in staurosporine-treated J.CaM1.6 cells (Fig. 3).

Discussion

GM130 is centrally involved in mediating incoming vesicle fusion and is rapidly phosphorylated during mitosis, contributing to the fragmentation of the Golgi complex [6,14]. It therefore seems likely that GM130 would also be an important early target during apoptosis to facilitate Golgi fragmentation. However, studies so far have not borne this out. Most recently, GM130 detection was shown to be diminished by Western blot analysis at the same time as p115 in COS-

7 cells treated with etoposide over 72 h and staurosporine over 48 h [2]. COS-7 cells have an impaired Fas-receptor-mediated signalling pathway [15] and seem to be particularly recalcitrant to the induction of apoptosis via a number of stimuli (personal observation). In contrast, using Fas-sensitive Jurkat cells (J.CaM1.6) we have shown that GM130 levels diminish rapidly following induction of Fas-mediated apoptosis, while p115 levels remained unchanged. One possible explanation for these differing observations would be that the missing components of the apoptotic pathways in COS-7 cells may normally be responsible for the rapid breakdown of GM130 during apoptosis. Although Golgi fragmentation and GRASP65 cleavage during apoptosis have been reported, These studies failed to show early GM130 cleavage [3]. However, this group [3] used the non-selective kinase inhibitor staurosporine extensively, which our data suggest may mask early GM130 cleavage. GM130 remained unaffected at 4 h when treated with staurosporine (Fig. 4B). GM130 levels might decrease at later time point during staurosporine-induced apoptosis, allowing efficient Golgi fragmentation. Interestingly, Nozawa et al. [16] demonstrated that necrosis also diminishes GM130 levels, highlighting the importance of carefully monitoring necrosis levels. Our results clearly show that despite higher levels of apoptosis induced by staurosporine, GM130 remained unaffected, whereas triggering the physiologically relevant Fas death pathway with CH11 resulted in decreasing GM130 levels. In addition, there was also a striking morphological difference in the appearance of the Golgi in staurosporine- and CH11-treated cells. For the staurosporine-treated cells Golgi fragmentation appeared to be incomplete, despite caspase-3 activation (Fig. 3). It is possible that the kinetics of GM130 cleavage and other key Golgi-associated proteins may be cell-type-dependent or dependent on whether the stress pathway or the receptor-mediated pathway of apoptosis is activated. Alternatively, we would speculate that the rapid cleavage of GM130 may involve a phosphorylation step to allow for efficient caspase recognition of the cleavage site as is the case with golgin-160 [11]. The precise kinetics of GM130 depletion during apoptosis in relation to other key Golgi proteins remains to be fully determined. The manner in which apoptosis is induced is important in determining whether key proteins such as GM130 are targeted, for example, we show that apoptosis triggered by the Fas pathway, but not induced by staurosporine, results in rapidly reduced GM130 levels. This is critical since staurosporine-induced apoptosis is a widely used model in the study of Golgi fragmentation during apoptosis.

Acknowledgments

We thank Linda Wilson for help with confocal microscopy. This work was supported by the Norman Salvesen Emphysema Research Trust and the National Asthma Campaign (D1/D42).

References

[1] C. Featherstone, G. Griffiths, G. Warren, Newly synthesized G protein of vesicular stomatitis virus is not transported to the Golgi complex in mitotic cells, J. Cell Biol. 101 (1985) 2036–2046.
[2] R. Chiu, L. Novikov, S. Mukherjee, D. Shields, A caspase cleavage fragment of p115 induces fragmentation of the Golgi apparatus and apoptosis, J. Cell Biol. 159 (2002) 637–648.
[3] J.D. Lane, J. Lucocq, J. Pryde, F.A. Barr, P.G. Woodman, V.J. Allan, M. Lowe, Caspase-mediated cleavage of the stacking protein GRASP65 is required for Golgi fragmentation during apoptosis, J. Cell Biol. 156 (2002) 495–509.
[4] M. Mancini, C.E. Machamer, S. Roy, D.W. Nicholson, N.A. Thornberry, L.A. Casciola-Rosen, A. Rosen, Caspase-2 is localized at the Golgi complex and cleaves golgin-160 during apoptosis, J. Cell Biol. 149 (2000) 603–612.
[5] M.K. Whyte, L.C. Meagher, J. MacDermot, C. Haslett, Impairment of function in aging neutrophils is associated with apoptosis, J. Immunol. 150 (1993) 5124–5134.
[6] M. Lowe, C. Rabouille, N. Nakamura, R. Watson, M. Jackman, E. Jamsa, D. Rahman, D.J. Pappin, G. Warren, Cdc2 kinase directly phosphorylates the *cis*-Golgi matrix protein GM130 and is required for Golgi fragmentation in mitosis, Cell 94 (1998) 783–793.
[7] F.A. Barr, N. Nakamura, G. Warren, Mapping the interaction between GRASP65 and GM130, components of a protein complex involved in the stacking of Golgi cisternae, EMBO J. 17 (1998) 3258–3268.
[8] F.A. Barr, M. Puype, J. Vandekerckhove, G. Warren, GRASP65, a protein involved in the stacking of Golgi cisternae, Cell 91 (1997) 253–262.
[9] N. Nakamura, M. Lowe, T.P. Levine, C. Rabouille, G. Warren, The vesicle docking protein p115 binds GM130, a *cis*-Golgi matrix protein, in a mitotically regulated manner, Cell 89 (1997) 445–455.
[10] B. Sonnichsen, M. Lowe, T. Levine, E. Jamsa, B. Dirac-Svejstrup, G. Warren, A role for giantin in docking COPI vesicles to Golgi membranes, J. Cell Biol. 140 (1998) 1013–1021.
[11] H. Cha, B.L. Smith, K. Gallo, C.E. Machamer, P. Shapiro, Phosphorylation of golgin-160 by mixed lineage kinase 3, J. Cell Sci. 117 (2004) 751–760.
[12] M.E. Peter, P.H. Krammer, The CD95(APO-1/Fas) DISC and beyond, Cell Death Differ. 10 (2003) 26–35.
[13] J.G. Pryde, A. Walker, A.G. Rossi, S. Hannah, C. Haslett, Temperature-dependent arrest of neutrophil apoptosis. Failure of Bax insertion into mitochondria at 15 °C prevents the release of cytochrome *c*, J. Biol. Chem. 275 (2000) 33574–33584.
[14] M. Lowe, N.K. Gonatas, G. Warren, The mitotic phosphorylation cycle of the *cis*-Golgi matrix protein GM130, J. Cell Biol. 149 (2000) 341–356.
[15] S.A. Memon, J. Hou, M.B. Moreno, C.M. Zacharchuk, Apoptosis induced by a chimeric Fas/FLICE receptor: lack of requirement for Fas- or FADD-binding proteins, J. Immunol. 160 (1998) 2046–2049.
[16] K. Nozawa, C.A. Casiano, J.C. Hamel, C. Molinaro, M.J. Fritzler, E.K. Chan, Fragmentation of Golgi complex and Golgi autoantigens during apoptosis and necrosis, Arthritis Res. 4 (2002) R3.