FEBS Letters 484 (2000) 202–206 FEBS 24263

# Limited caspase cleavage of human BAP31

Juha Määttä[a], Outi Hallikas[b,1], Saara Welti[a,1], Pekka Hildén[a,1], Jim Schröder[b], Esa Kuismanen[a,*]

[a] *Department of Biosciences, Division of Biochemistry, Viikki Biocenter, Viikinkaari 5, 00014 University of Helsinki, Helsinki, Finland*
[b] *Department of Biosciences, Division of Genetics, Viikki Biocenter, Viikinkaari 5, 00014 University of Helsinki, Helsinki, Finland*

Received 26 September 2000; accepted 10 October 2000

First published online 24 October 2000

Edited by Felix Wieland

**Abstract Human BAP31 was cleaved at both of its two identical caspase cleavage sites in two previously reported models of apoptosis. We show here that only the most carboxy-terminal site is cleaved during apoptosis induced in HeLa cells by tunicamycin, tumor necrosis factor and cycloheximide, or staurosporine. Similar results were obtained in HL-60 cells using Fas/APO-1 antibodies, or cycloheximide. This limited cleavage, which is inhibited by several caspase inhibitors, removes eight amino acids from human BAP31 including the KKXX coat protein I binding motif. Ectopic expression of the resulting cleavage product induces redistribution of mannosidase II from the Golgi and prevents endoplasmic reticulum to Golgi transport of virus glycoproteins.** 

*Key words:* Apoptosis; BAP31; Caspase cleavage; Endoplasmic reticulum; Golgi apparatus

## 1. Introduction

Human BAP31 is an integral endoplasmic reticulum (ER) membrane protein, which is also recycled between the ER and the Golgi [1,2]. Its cytosolic tail ends with the KKXX coat protein I binding motif [3]. BAP31 has been proposed to control the export of cellubrevin from the ER [1].

Previous findings also suggest that BAP31 may take part in the control of programmed cell death. Human BAP31 can bind Bcl-2/Bcl-$X_L$, caspase-8 pro-enzyme, and Ced-4 when this nematode protein is expressed in human cells [2,4]. During apoptosis, induced by adenovirus E1A oncoproteins in KB cells [2] or by photodynamic therapy in HeLa cells [5], human BAP31 was cleaved at the two identical caspase cleavage sites of the cytosolic tail (Fig. 2A).

We report here that in several model systems of apoptosis, human BAP31 is cleaved only at its most C-terminal caspase cleavage site. We show, using caspase inhibitors, that this cleavage is caspase dependent. When expressed ectopically, the cleavage product (BAP31−8aa) leads to the redistribution of mannosidase II (Man II) from the Golgi into the ER and prevented virus glycoprotein transport from the ER to the Golgi.

## 2. Materials and methods

### 2.1. Materials

Biochemicals, enzymes, and cell culture supplies were from Sigma (St. Louis, MO, USA) unless otherwise stated. The polyclonal Man II antibody was a gift from K. Moremen and M. Farquhar (University of Georgia, Athens, GA, USA). The polyclonal Semliki Forest virus (SFV) glycoprotein antibody was a gift from S. Keränen (Technical Research Centre of Finland, Espoo, Finland). The polyclonal poly-ADP-ribose-polymerase (PARP) antibody (H-250) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

FuGENE-6 transfection reagent was purchased from Boehringer Mannheim GmbH (Mannheim, Germany) and transfections were performed according to the manufacturer's instructions.

### 2.2. Plasmids

The wild type BAP31 sequence (the coding region and 117 bp upstream of the start codon and 424 bp downstream of the stop codon) from a human placental lgt11 cDNA library (Clontech, Palo Alto, CA, USA) was subcloned into the *Eco*RI site of pcDNA I/Amp (Invitrogen Corp., Carlsbad, CA, USA). This construct encoded a protein designated BAP31wt.

The following oligonucleotides were used to generate a construct that encoded a protein designated BAP31−8aa (BAP31 minus last eight amino acids): 5′-TAATACGACTCACTATAGGG-3′ and 5′-AAAGAATTCTTAATCTACTGCAGCCTGCAGC-3′. BAP31wt construct was used as a template. The PCR product was subcloned into the *Bam*HI–*Eco*RI site of pcDNA I/Amp.

To generate glutathione-*S*-transferase (GST) or $His_6$ fusion proteins of BAP31, a PCR fragment encoding the C-terminal cytosolic part of BAP31 (amino acids 151–246) was produced using the oligonucleotides 5′-AAAGGATCCATGGAGGAGAATGACCAGC-3′ and 5′-AAAGAATTCTTACTCTTCCTTCTTGTCCAT-3′. The PCR product was subcloned into the *Bam*HI–*Eco*RI cut pGEX-2T (Amersham Pharmacia Biotech AB, Uppsala, Sweden) or pRSET-A (Invitrogen BV, NV Leek, The Netherlands) expression vectors. The resulting constructs encoded BAP31 fusion proteins GST-BAP31 and $His_6$-BAP31, respectively.

### 2.3. Production of fusion proteins and antibodies

Expression of GST-BAP31 fusion proteins was performed in *Escherichia coli* strain BL21 according to the supplier's instructions (Stratagene, La Jolla, CA, USA). Bacteria were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (Promega Corp., Madison, WI, USA). Homogenization of bacteria and purification of GST fusion proteins were performed according to the manufacturer's instructions (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

*Corresponding author. Fax: (358)-9-191 59068.
E-mail: esa.kuismanen@helsinki.fi

[1] These authors contributed equally to this article.

*Abbreviations:* BAP31−8aa, BAP31 minus last eight amino acids; BAP31mab, monoclonal BAP31 antibody; BAP31pab, polyclonal BAP31 antibody; BAP31wt, wild type BAP31; CHX, cycloheximide; HL-60, human promyelocytic leukemia; Man II, mannosidase II; NRK, normal rat kidney; PARP, poly-ADP-ribose-polymerase; SFV-ts1, Semliki Forest virus temperature sensitive mutant; STS, staurosporine; TNF, tumor necrosis factor

0014-5793/00/$20.00 
PII: S0014-5793(00)02159-1

To produce monoclonal BAP31 antibodies (BAP31mab), four BALB/c female mice were immunized three times at 2 week intervals intraperitoneal with 100 μg of GST-BAP31 fusion proteins. The first immunization was given in Freund's complete adjuvant and the subsequent immunizations in Freund's incomplete adjuvant. A booster immunization with 100 μg of antigen in saline was given intravenous 4 days before the fusion. The spleen cells were harvested and fused to Ag-8 myeloma cells at 10:1 ratio in the presence of 50% polyethylene glycol 1500 (Boehringer Mannheim GmbH, Mannheim, Germany). After the fusion, hybrid selection was performed in HAT culture medium: $1\times10^5$ hybridoma cells per well were cultured in 0.2 ml of RPMI 1640 supplemented with 2 mM L-glutamine, 100 U of penicillin, 100 mg of streptomycin sulfate per ml, 20% (v/v) donor horse serum (Biological Industries, Beit Haemek, Israel), 111 ng of Na pyruvate per ml (Bio Whittaker, Walkersville, MD, USA), MEM-non-essential amino acids solution (Biological Industries, Beit Haemek, Israel), and 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (Boehringer Mannheim GmbH, Mannheim, Germany). Antibody production was screened using enzyme-linked immunosorbent assay (ELISA) as described previously [6]. $His_6$-BAP31 fusion proteins were produced for ELISA screening. Proteins were produced and purified according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). The product was further purified using FPLC-anion exchange chromatography (Amersham Pharmacia Biotech AB, Uppsala, Sweden). BAP31mab antibodies were affinity-purified using a chromatography column where GST-BAP31 fusion proteins were bound to CNBr-activated Sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

To generate polyclonal antibodies against the cytosolic tail of BAP31, a New Zealand white rabbit was immunized subcutaneously with 400 μg of GST-BAP31 fusion proteins that were treated with 0.5% sodium dodecyl sulfate (SDS) in 85°C for 10 min and emulsified with 600 μl of Freund's complete adjuvant. The rabbit was bled every 2 weeks, and the antisera were tested by immunoblotting [7]. Five booster immunizations were performed in the same manner as the primary immunization except that the amount of the protein was reduced to 200 μg and Freund's incomplete adjuvant was used as adjuvant.

In the indirect immunofluorescence staining of HeLa cells, both the BAP31mab and the polyclonal BAP31 antibody (BAP31pab) gave identical ER staining (not shown). BAP31mab did not stain endogenous BAP31 of BHK-21 and normal rat kidney (NRK) cells at the detection level used in this study. Both antibodies detected an ~30 kDa protein from HeLa cell lysate on a Western blot corresponding to the size of previously described human BAP31 (Fig. 1). In addition, BAP31pab and BAP31mab were capable to recognize both of the caspase cleavage products of BAP31 in immunoblotting (Fig. 1).

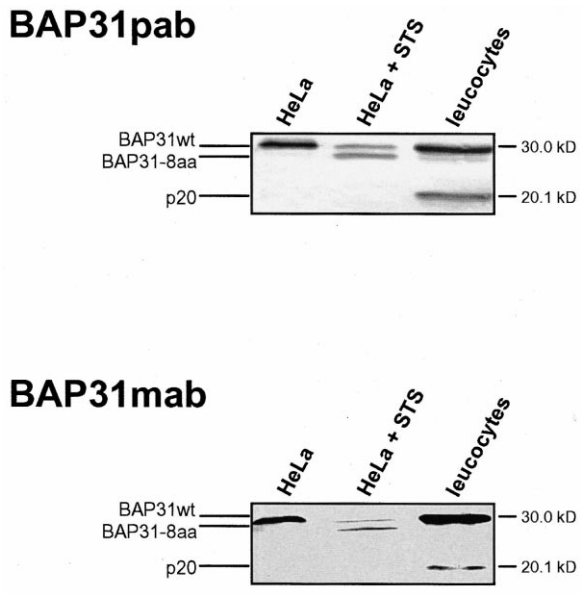


Fig. 1. Characterization of the antibodies. Both rabbit polyclonal antiserum BAP31pab and anti-BAP31 monoclonal antibodies BAP31mab recognize full length and cleaved forms of BAP31. In uninduced HeLa cells (HeLa), only full length BAP31 is seen. Both antibodies recognize BAP31 cleaved at the most carboxy-terminal caspase site (BAP31−8aa) in HeLa cells treated with STS for 24 h (HeLa+STS). Both BAP31pab and BAP31mab recognize also the p20 cleavage product, as shown in the third lanes (leucocytes). To demonstrate this, the gel sample was prepared from peripheral blood buffy coat cells obtained from the Finnish Blood Transfusion Service (Helsinki, Finland). These cells had been treated with Sendai virus for interferon production, and therefore the majority of the cells is likely to be in necrotic state.

### 2.4. Cell culture

BHK-21, HeLa, and NRK cells were grown at 37°C in 5% $CO_2$ in DME supplemented with 2 mM L-glutamine, 100 U of penicillin, 100 mg of streptomycin sulfate per ml, and 10% (v/v) fetal calf serum (Biological Industries, Beit Haemek, Israel). HL-60 (human promyelocytic leukemia) cells were grown in RPMI 1640 (Biological Industries, Beit Haemek, Israel) supplemented as DME. Hybridoma cells were grown in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U of penicillin, 100 mg of streptomycin sulfate per ml, 20% (v/v) donor horse serum (Biological Industries, Beit Haemek, Israel), 111 ng of Na pyruvate per ml (Bio Whittaker, Walkersville, MD, USA), and MEM-non-essential amino acids solution (Biological Industries, Beit Haemek, Israel).

### 2.5. Induction of apoptosis and protein cleavage analyses

Medium of overnight cultures was first replaced by serum-free culture medium. Apoptosis was induced by adding to the cell medium tunicamycin (20 μg/ml), tumor necrosis factor (TNF) (Boehringer Mannheim GmbH, Mannheim, Germany) (50 ng/ml), together with cycloheximide (CHX) (10 μg/ml), monoclonal Fas/APO-1 antibodies (Boehringer Mannheim GmbH, Mannheim, Germany) (0.25 μg/ml), CHX (50 μM), or staurosporine (STS) (1 μM) [8]. All incubations were performed at 37°C in 5% $CO_2$. After incubation, induced cells and non-induced control cells were collected to Laemmli sample buffer. SDS–PAGE [9] and immunoblotting [7] were done according to established procedures. BAP31pab and polyclonal PARP antibodies were used. Secondary antibodies were alkaline phosphatase-conjugated anti-rabbit IgG antibodies.

### 2.6. Caspase inhibitor assays

Caspase inhibitors (Calbiochem, La Jolla, CA, USA) were added directly from stocks to HeLa cells in serum-free culture medium 1 h before adding TNF (20 μg/ml), together with CHX (10 μg/ml). After the addition of TNF and CHX, cells were incubated for 6 h in the presence of inhibitors and collected as described above. BAP31 and PARP cleavage was detected as above.

### 2.7. Other methods

Indirect immunostaining was performed essentially as described previously [10]. However, 0.1 M phosphate buffer (pH 7.4) was used instead of phosphate-buffered saline.

BHK-21 cells were infected with SFV temperature sensitive ts-1 mutant (SFV-ts1) as described previously [11]. Incubations at reduced temperature were carried out in water baths as previously described [10] using $NaHCO_3$-free DME supplemented with 20 mM HEPES, pH 7.4.

Fluorescence images were captured using an Olympus AX70 fluorescence microscope (×60, 1.4 NA) with a SenSys CCD camera (Photometrics, Ltd., München, Germany). Images were converted using the Image-Pro Plus version 3.0 software (Media Cybernetics, Silver Spring, MD, USA).

## 3. Results

### 3.1. BAP31 was cleaved only at the C-terminal caspase cleavage site in the tested apoptotic cells

We examined BAP31 cleavage during apoptosis in vivo in HeLa cells in which apoptosis was induced either by tunicamycin [12] or by TNF, together with CHX [13]. In HL-60

cells, apoptosis was induced either by Fas/APO-1 antibody [14] or by CHX [13]. BAP31 cleavage was analyzed by immunoblotting using BAP31pab.

In the tested cells, only one cleavage product of BAP31 was formed corresponding to the cleavage at the C-terminal caspase cleavage site (to be referred to as limited cleavage of BAP31 in this article) (Fig. 2B,C). No p20 cleavage product was formed. PARP cleavage was used as a control for activation of caspase cascade. In every case, PARP cleavage was observed with similar kinetics to BAP31 processing (Fig. 2B,C). To confirm that the observed cleavage product is a result from the most carboxy-terminal caspase cleavage, we transfected NRK cells with the BAP31−8aa construct which produces BAP31 protein deleted after the amino acid 238. The protein product produced in transfected NRK cells showed the same apparent size as the cleavage product in apoptotic HeLa cells (Fig. 3).

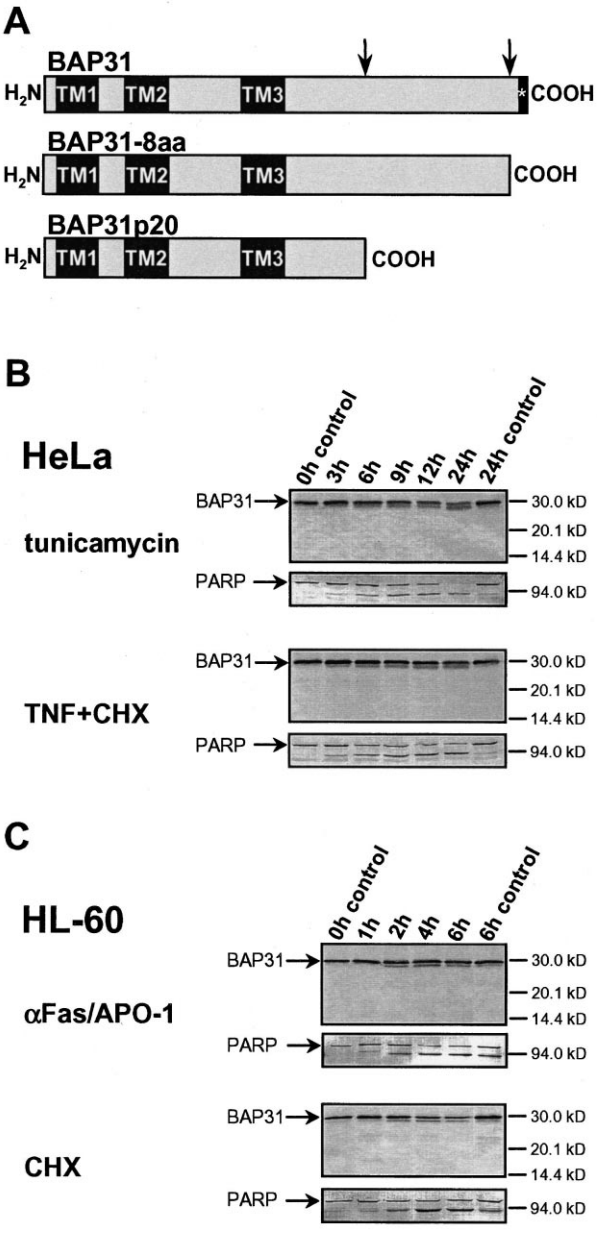


Fig. 2. BAP31 and PARP cleavage in apoptotic HeLa and HL-60 cells. (A) A schematic representation of human BAP31 indicating three transmembrane regions (TM1, TM2, and TM3), two caspase cleavage sites (arrows), and the location of the KKXX sequence motif (asterisk). Two possible caspase cleavage products, BAP31−8aa and BAP31p20, are shown below. In HeLa cells (B), apoptosis was induced by tunicamycin or by TNF, together with CHX, and in HL-60 cells (C) by Fas/APO-1 antibody or by CHX. Sample collection timepoints after induction of apoptosis are indicated. Control cells were not induced. Both in B and C, appearance of BAP31−8aa cleavage product follows the kinetics of the PARP cleavage.

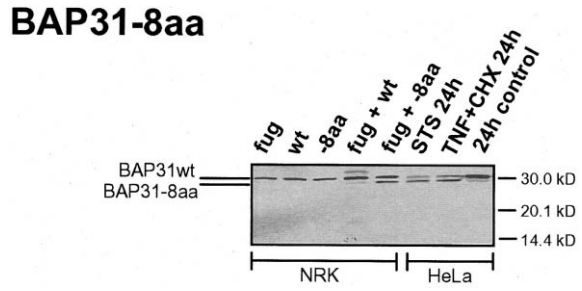


Fig. 3. The protein expressed from BAP31−8aa is identical with the cleavage product of BAP31 in apoptotic cells. NRK control cells treated with FuGENE (fug), BAP31wt (wt), or BAP31−8aa (−8aa) only showed endogenous rodent BAP31. The intensity of the BAP31 band increased when human BAP31wt was transfected with FuGENE (fug+wt). BAP31−8aa transfected cells (fug+−8aa) showed, additionally to endogenous rodent BAP31wt, a band corresponding to human BAP31 without the last eight amino acids. This band is equal in size as the cleavage product seen in HeLa cells treated for 24 h with STS (lane STS 24 h), or TNF and CHX (lane TNF+CHX 24 h). The 24 h control HeLa cells were not induced and show only uncleaved BAP31.

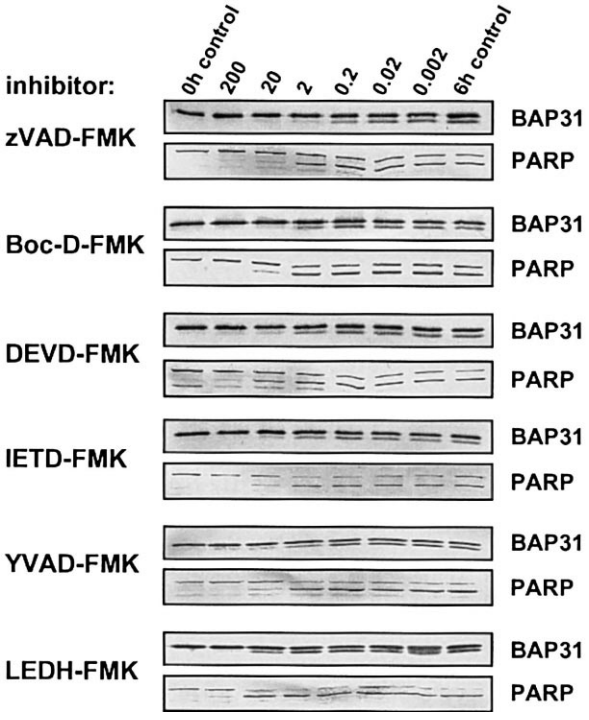


Fig. 4. Effect of caspase inhibitors (0.002–200 μM) on BAP31 and PARP cleavage. Apoptosis was induced by TNF/CHX treatment of HeLa cells (no induction in 0 h control). No inhibitors were present in the control cells. Each of the tested caspase inhibitors inhibited both BAP31 and PARP cleavage at similar inhibitor concentrations.

### 3.2. Limited cleavage of BAP31 is caspase dependent

Caspase inhibitors were used to study the involvement of caspases in the observed BAP31 cleavage. All the tested caspase inhibitors inhibited BAP31 cleavage after TNF/CHX treatment (Fig. 4). PARP cleavage was always prevented at the same inhibitor concentrations as BAP31 cleavage, indicating that BAP31 was always cleaved when caspase cascades were active. Depending on the inhibitor used, a different inhibitor concentration was needed to efficiently prevent BAP31 and PARP cleavage after TNF/CHX treatment.

### 3.3. In ectopic expression BAP31−8aa induced redistribution of Man II and prevented virus glycoprotein transport from the ER to the Golgi

Ectopic expression of the caspase cleavage product BAP31−8aa in BHK-21 or NRK cells induced formation of BAP31−8aa positive membrane blebs (Fig. 5A,B, arrows). We observed that the formation of these membrane structures was accompanied by redistribution of Golgi marker Man II. In these cells, Man II had disappeared from the Golgi and had an ER-like staining pattern (Fig. 5C,D, note the stained nuclear membrane, arrow in Fig. 5C).

Similar membrane blebs have previously been observed as a result of ectopic expression of the transmembrane fragment of human BAP31 [1]. In the same study, cellubrevin transport from the ER was prevented in cells having these membrane structures. Therefore, we wanted to study whether inhibition of ER to Golgi transport could be the reason for the redistribution of Man II in BAP31−8aa expressing cells.

BHK-21 cells were first transfected with BAP31wt or BAP31−8aa using FuGENE-6 transfection reagent. After 24 h incubation at 37°C, the transfected cells were infected with SFV-ts1. Then, the cells were incubated at 38°C for 2.5 h to arrest SFV-ts1 glycoproteins into the ER [15]. After this, the membrane blebs of the BAP31−8aa expressing cells were positive for virus glycoproteins, showing that these structures were composed of ER membranes (Fig. 6E,F, arrows). When the cells were further incubated at 20°C for 2 h to allow the ER to Golgi transport of SFV-ts1 glycoproteins [11], virus proteins were transported to the Golgi in the BAP31wt expressing cells (Fig. 6D, arrow). In contrast, virus glycoproteins were retained in the ER and in the membrane blebs in BAP31−8aa expressing cells at 20°C (Fig. 6H, arrow). No Golgi-like virus glycoprotein staining was observed in these cells.

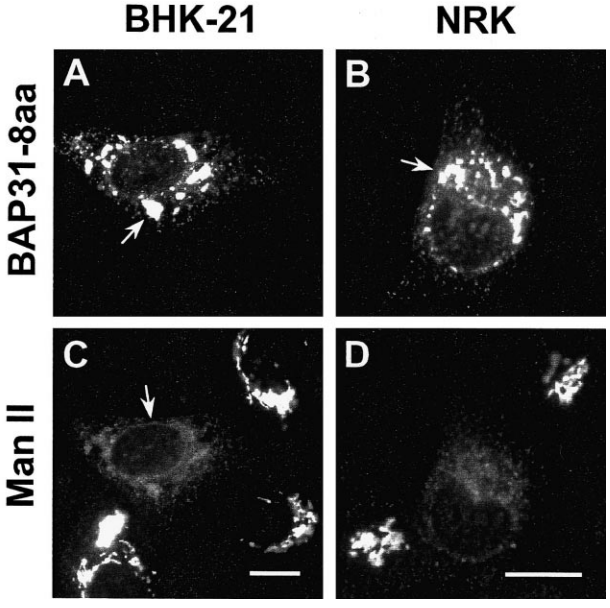


Fig. 5. BAP31−8aa positive membrane blebs and redistribution of Man II. BHK-21 and NRK cells were transfected with BAP31−8aa using FuGENE-6 transfection reagent and were double stained with BAP31mab and anti-Man II antibodies after 24 h expression. The arrows in A and B indicate BAP31−8aa positive membranes. Note the disappearance of the Man II positive Golgi structure in cells expressing BAP31−8aa. Bars, 10 μm.

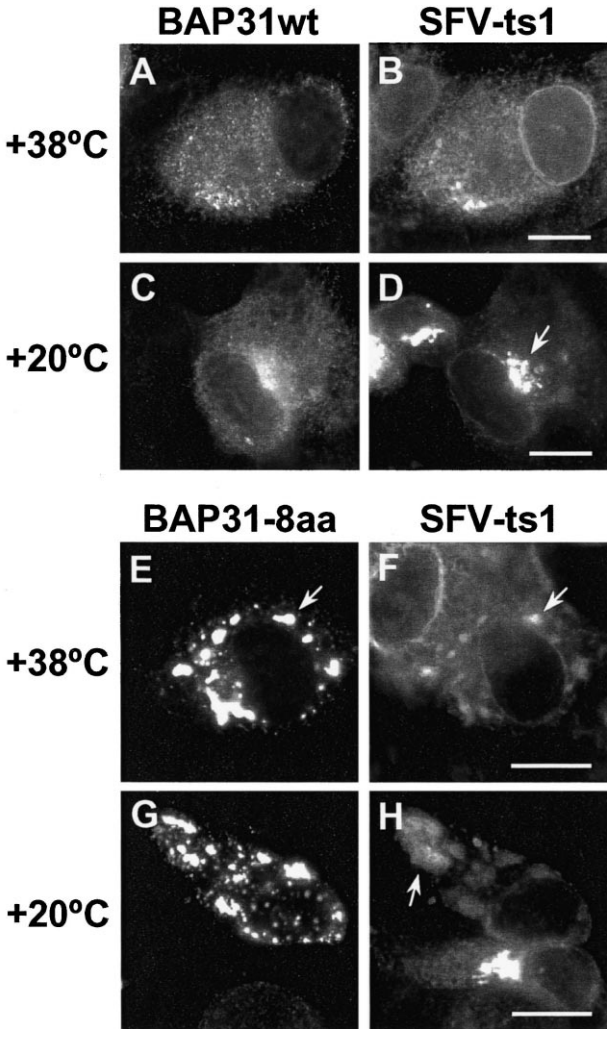


Fig. 6. Ectopic expression of BAP31−8aa prevents ER to Golgi transport of SFV-ts1 glycoproteins. After fixation, the cells were double stained with BAP31mab and anti-SFV antibodies. The upper panel (A–D) demonstrates the transport of SFV-ts1 glycoproteins to the Golgi in BAP31wt expressing cells (arrow in D). The lower panel (E–H) demonstrates that the transport of SFV-ts1 glycoproteins to the Golgi is prevented in the cells having BAP31−8aa positive membrane blebs. Note the normal Golgi transport of SFV glycoproteins in the absence of BAP31−8aa expression (shown in H). Bars, 10 μm.

## 4. Discussion

Previous studies have shown that in some apoptotic cells both the caspase cleavage sites of BAP31 are processed [2,5]. The findings presented in this study show, however, that in

many other models of apoptosis, human BAP31 is processed only at its most C-terminal caspase cleavage site. This variation in the cleavage of BAP31 raises the possibility that the function of BAP31 during apoptosis could be controlled by alternative caspase cleavage.

Ectopic expression of BAP31−8aa caspase cleavage product induced formation of BAP31−8aa positive membrane blebs and caused Man II to redistribute into the ER. In a previous study, Cole et al. [16] presented evidence that Golgi proteins are constitutively recycled into the ER. This kind of recycling would result in the redistribution of Golgi proteins into the ER if the transport from the ER is prevented. This could be the explanation for Man II redistribution after BAP31−8aa expression, since the anterograde transport was prevented in the cells expressing BAP31−8aa: SFV-ts1 glycoproteins were not transported from the ER to the Golgi. An interesting subject for further studies is to solve whether this transport defect is responsible for some alterations that take place in dying cells during programmed cell death. Further studies are also required to find out if there are any differences in the function of the two caspase cleavage products of BAP31.

*Acknowledgements:* We thank M. Farquhar, S. Keränen, and K. Moremen for antibodies and V. Mäkiranta for skillful and diligent technical assistance. This study was supported by research grants from the Academy of Finland.

## References

[1] Annaert, W.G., Becker, B., Kistner, U., Reth, M. and Jahn, R. (1997) J. Cell Biol. 139, 1397–1410.
[2] Ng, F.W.H., Nguyen, M., Kwan, T., Branton, P.E., Nicholson, D.W., Cromlish, J.A. and Shore, G.C. (1997) J. Cell Biol. 139, 327–338.
[3] Adachi, T., Schamel, W.W.A., Kim, K.-M., Watanabe, T., Becker, B., Nielsen, P.J. and Reth, M. (1996) EMBO J. 15, 1534–1541.
[4] Ng, F.W.H. and Shore, G.C. (1998) J. Biol. Chem. 273, 3140–3143.
[5] Granville, D.J., Carthy, C.M., Jiang, H., Shore, G.C., McManus, B.M. and Hunt, D.W.C. (1998) FEBS Lett. 437, 5–10.
[6] Engvall, E. and Perlmann, P. (1972) J. Immunol. 109, 129–135.
[7] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[8] Jacobson, M.D., Weil, M. and Raff, M.C. (1996) J. Cell Biol. 133, 1041–1051.
[9] Laemmli, U.K. (1970) Nature 227, 680–685.
[10] Kuismanen, E. and Saraste, J. (1989) Methods Cell Biol. 32, 257–274.
[11] Saraste, J. and Kuismanen, E. (1984) Cell 38, 535–549.
[12] Perez-Sala, D. and Mollinedo, F. (1995) J. Cell. Physiol. 163, 523–531.
[13] Boldin, M.P., Goncharov, T.M., Goltsev, Y.V. and Wallach, D. (1996) Cell 85, 803–815.
[14] Itoh, N. and Nagata, S. (1993) J. Biol. Chem. 268, 10932–10937.
[15] Saraste, J., von Bonsdorff, C.-H., Hashimoto, K., Kääriäinen, L. and Keränen, S. (1980) Virology 100, 229–245.
[16] Cole, N.B., Ellenberg, J., Song, J., DiEuliis, D. and Lippincott-Schwartz, J. (1998) J. Cell Biol. 140, 1–15.