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Apoptosis-specific protein (ASP – 45 kDa) is distinct from human Apg5, the homologue of the yeast autophagic gene *apg5*

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Abstract **We have examined whether the apoptosis-specific protein p45ASP and human Apg5 are identical proteins. Like p45ASP, myc-hApg5 cross-reacted with a c-Jun antibody and ~50% of myc-hApg5 was bound to a Triton X-100-insoluble fraction in HeLa cells. However, soluble myc-hApg5 was degraded during apoptosis induced by staurosporine or TNFα/cycloheximide whilst expression of soluble p45ASP was stabilised. Furthermore, myc-hApg5 degradation was blocked by the caspase inhibitor Boc-Asp(OMe)FMK whilst p45ASP expression was eliminated. Moreover, myc-hApg5 (~32 kDa) never assumed the size of p45ASP (45 kDa). It is therefore likely that p45ASP and human Apg5 are distinct proteins although they do share some common characteristics.**

Key words: Apoptosis; Autophagy; Caspase; Cell death; c-Jun antibody

1. Introduction

During an analysis of apoptosis, a series of proteins were detected by Grand and colleagues on Western blots using an anti-c-Jun antibody raised against the peptide sequence TPTPTQFLCPKNVTD [1]. The most prominent band was a protein of around 45 kDa. To identify this protein – termed apoptosis-specific protein, or ASP – Hammond et al. [2] screened a human foetal liver cDNA expression library and pulled out the 3′ end of ASP cDNA. Sequence analysis of the predicted protein encoded by the full length cDNA demonstrated 26% identity with a yeast gene encoding *apg5*, which is part of a novel conjugating system associated with autophagy in yeast [3]. When Hammond et al. [2] transfected Cos-1 cells with ASP cDNA for 48 h and Western blots were probed using the c-Jun antibody, they found two bands of around M_r 30 and 45 kDa, the latter being similar to the size of p45ASP derived from apoptotic rodent cells. Since *apg5* is predicted to encode a protein of 32.4 kDa, and ASP cDNA was noted to be expressed at this size in bacteria (Hammond et al. [2], data was not shown), it was suggested that the 30 kDa band observed in the transfected cells is 'native' hApg5, while the 45 kDa band is a post-translationally modified form of hApg5. No band of 32.4 kDa, the size expected of native hApg5, was detected before or during apoptosis. Hammond and colleagues further noted that over-expression of hApg5 in Cos-1 cells did not alter cellular morphology and did not induce apoptosis.

The paper proposes that p45ASP (namely the protein of 45 kDa that is induced by apoptotic signals downstream of caspases and which is recognised by the c-Jun antibody) is the mammalian homologue of yeast Apg5. Many reviews in the literature cite this work as providing evidence that apoptosis and autophagy are linked by some common biochemical processes (see for example [4–7]). This idea has been strengthened by our finding that apoptotic inducers can also promote autophagy [8]. However, doubt as to the co-identity of the two proteins has been cast by work from Esquerda and colleagues [9,10], who showed that the reason c-Jun antibody recognises many of the polypeptide 'ASPs' during apoptosis is because they display neo-epitopes generated as a result of caspase-3 cleavage. Nevertheless, no direct evidence has been presented refuting the idea that p45ASP is identical to hApg5 nor is it clear whether hApg5 cross-reactivity with the c-Jun antibody is generated as a result of caspase cleavage. We have attempted to investigate these questions using myc-tagged hApg5. Our data suggest that p45ASP and hApg5 are not the same proteins, although they do share some common characteristics.

2. Materials and methods

Human *apg5* cDNA was subcloned from pSU18-*apg5* (plasmid kindly provided by Roger Grand and Ester Hammond, Birmingham University) into the pRK5-myc vector (kindly provided by Alan Hall, MRC-LCMB, UCL) and 1 μg plasmid was transfected per 35 mm^2 dish of 70% confluent HeLa cells using 3 μl Fugene 6 (Roche) following the manufacturer's instructions. HeLa cells were grown in Dulbecco's modified Eagle's medium and 10% foetal bovine serum.

For immunocytochemistry, cells were fixed with 3% paraformaldehyde for 30 min at room temperature, permeabilised with 0.1% saponin in phosphate-buffered saline containing 1% bovine serum albumin for 20 min, and incubated for 1 h with a mouse monoclonal anti-myc antibody (9E10, BabCo) and a rabbit polyclonal anti-c-Jun(N) antibody (Santa Cruz, SC-45). This c-Jun antibody was raised against the peptide sequence TPTPTQFLCPKNVTD and recognises the same set of proteins as the Oncogene antibody used by Grand et al. [1] and Hammond et al. [2] (H.W. Yung, data not shown). The anti-myc and anti-c-Jun antibodies were visualised with goat anti-mouse FITC and goat anti-rabbit cy3 conjugated antibodies (Jackson ImmunoResearch). Nuclei were visualised using Hoechst 33342 (Sigma). Cells were imaged using a fluorescent microscope, and images were cap-

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Abbreviations: Ab, antibody; CHX, cycloheximide; Sts, staurosporine

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tured using a Photometrics CCD camera. Images shown were collected at the same exposure time.

For studies of protein expression during apoptosis, HeLa cells were transfected for 48 h before treatment with 2.5 μM staurosporine (Sts) (Sigma) or 50 ng/ml TNFα (Peprotech) and 20 μg/ml cycloheximide (CHX) (Sigma). Boc-Asp(O-Me)FMK (BAF) (Enzyme Systems Products) was used to inhibit caspase activity. Cells were lysed at the appropriate time in a gentle lysis buffer containing 1% Triton X-100 [7], spun at 10^3 rpm for 10 min at 4°C and the supernatant (sup) was separated from the pellet, which was lysed in 2% sodium dodecyl sulfate (SDS) lysis buffer [1]. The entire pellet of the relevant sup was loaded per each sample. Protein samples were resolved on a 10% denaturing polyacrylamide gel and analysed by Western blotting using the same antibodies used for immunocytochemistry. Amido black was used to report protein loading.

For studies of hApg5 susceptibility to undergo cleavage by caspases, full length hApg5 cDNA was subcloned into the pIRES vector (a kind gift from Dr Anne Kaminski, Cambridge) and protein was expressed using the TNT-coupled transcription/translation rabbit reticulocyte lysate system (Promega) using the T7 promoter and translation grade [^{35}S]methionine (Amersham Biotech). The cDNA of the Rb(378–938) fragment cloned into the pING vector was obtained from Dr Tony Kouzarides (Cambridge) and was similarly expressed using the SP6 promoter and the TNT system. Translated products were mixed in a caspase-3 assay buffer containing 50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% 3[3-cholaminopropyl diethylammonio]-1-propane sulphonate (CHAPS), 1 mM EDTA, 10% glycerol, 10 mM dithiothreitol (DTT) and 0.3 U/μl recombinant caspase-3 (BIOMOL). Following a 16 h incubation at 30°C, the reaction mixture was resolved using SDS–PAGE and the dried gel was exposed to a phosphorimager screen (Kodak) for 20 h. Image was captured and analysed using ImageQuant software (Molecular Dynamics). Radiolabelled markers (Rainbow) were from Amersham Biotech. For the in vitro assay using cell extracts, an extract (lysate A) was prepared from HeLa cells that had been transfected with myc-hApg5 for 48 h. Control or apoptotic lysates (lysates B) were obtained from HeLa cells left untreated, or treated with either Sts or with TNFα and CHX as described above by extraction into ice-cold buffer containing 10 mM HEPES pH 7.4, 42 mM KCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 1 mM DTT, 1 μg/ml of pepstatin A and leupeptin and 5 μg/ml aprotinin and sonication in a bath sonicator for 5 min in ice water. 16 μg of lysate A in 4 μl and 32 μg of lysate B in 8 μl were mixed into 12 μl of caspase assay buffer containing 20 mM HEPES pH 7.4, 0.1% CHAPS, 1 mM EDTA, 10% sucrose, 5 mM DTT and left to incubate for 16 h at 30°C. Western blots were prepared as described above and probed with 9E10 or with a monoclonal antibody against poly-(ADP-ribose) polymerase (PARP) (a kind gift from Dr Said Aufouchi (Cambridge).

3. Results and discussion

In HeLa cells transfected with myc-hApg5, cells that stained with the anti-myc antibody 9E10 were also co-incidentally stained with the antibody raised against the TPTPTQFLC-PKNVTD epitope in c-Jun (c-Jun(N)Ab from Santa Cruz, SC-45) (Fig. 1A). Little staining was detected with the c-Jun(N)Ab in untreated cells. Myc-hApg5 staining showed a widespread punctate distribution throughout the cytoplasm similar to the pattern for GFP-Apg5 described by Mishizuma et al. [11]. It is likely that the anti-myc antibody recognised hApg5 rather than other cellular proteins that are induced during apoptosis since HeLa cells induced to undergo apoptosis with Sts showed substantially elevated staining with c-Jun(N)Ab, but there was no increase in staining with 9E10. A slight increase in cell death from $1.1 \pm 0.9\%$ to $7 \pm 6\%$ (mean ± range from two independent experiments) was found in the myc-positive transfected cells, but since this increase was not reversed by the general caspase inhibitor BAF, we ascribe this increase to the toxicity of the transfection rather than to apoptosis mediated by the protein.

Apoptotically induced cells that were stained with the anti-c-Jun Ab showed both cytoplasmic and nuclear staining, as discussed previously [7,10,12]. However, there was no difference in the percentages of apoptotic cells scored in the total population of cells ($22.7 \pm 6.9\%$) or in the myc-positive population of cells ($23.2 \pm 4.7\%$) (mean ± range, $n = 2$). Addition of BAF together with Sts inhibited apoptosis in both cell populations, ($0.68 \pm 0.42\%$ and $5.6 \pm 2\%$ in total and myc-positive populations, respectively) as described by Hammond et al. [2]. Thus there is a strong correlation between myc-hApg5 expression and reactivity with c-Jun(N)Ab. However, there was no significant induction of apoptosis in the over-expressing cells, consistent with the results of Hammond et al. [2].

Grand et al. [1] reported that p45ASP was difficult to extract from cells, and that harsh conditions such as 6 M urea or 2% SDS were required to solubilise the protein. Consistent with this result, we also found that myc-hApg5 was difficult to extract from the HeLa cells. However, the relative distribution of the two proteins differed during apoptosis. Fig. 1B shows that when untransfected cells were induced to undergo apoptosis with staurosporine and extracted with gentle lysis buffer containing 1% Triton X-100, about 60% of the total p45ASP was found in the extract, the remainder being found in the 2% SDS-extractable pellet. The same result was found using 6 M urea (data not shown). However, in HeLa cells that were first transfected with myc-hApg5 and then induced to undergo apoptosis with staurosporine, no soluble myc-hApg5 was detected in the Triton X-100 extract, most of the remaining protein being associated with the pellet. There was no increase in hApg5 in the pellet during the period when hApg5 disappeared from the soluble fraction, suggesting that the decrease in the amount of hApg5 in the soluble fraction was not due to a redistribution of the protein into the pellet. Moreover, since hApg5 was detected either with the anti-myc Ab or with anti-c-Jun(N) antibody, which does not recognise the myc epitope (L. Xue, unpublished data) it is unlikely that the reason for the loss of myc-hApg5 expression in the soluble fraction was due to a cleavage of the myc epitope (which terminates in the peptide SEEDL) by caspases.

These data suggested that conditions that stabilise p45ASP during apoptosis promote the degradation of hApg5 and that soluble hApg5 is a labile protein. To examine this question further, we took advantage of the fact that TNFα and CHX stimulate apoptosis in HeLa cells and increase expression of p45ASP whilst preventing protein synthesis. Myc-hApg5 was first expressed for 48 h and then apoptosis was induced with TNFα and CHX for 8 h in the presence or absence of BAF. As predicted from the results with staurosporine, the levels of p45ASP were increased during the stimulus with TNFα and CHX, but hApg5 levels detected with either anti-c-Jun(N) or anti-myc decreased. However, the opposite occurred when cells were treated with TNFα and CHX in the presence of BAF: p45ASP induction was eliminated but hApg5 expression was unaltered.

Thus, hApg5 and p45ASP differ in a number of characteristics: hApg5 is unstable during apoptosis whereas p45ASP levels increase. In contrast, BAF inhibits p45ASP expression but stabilises that of hApg5. These results cannot be explained solely by the fact that one protein is endogenous whilst the other is expressed off a CMV-driven promoter as CMV-EGFP expression is not reduced when HeLa cells are treated with TNFα and CHX. Most crucially, we never observed a p45

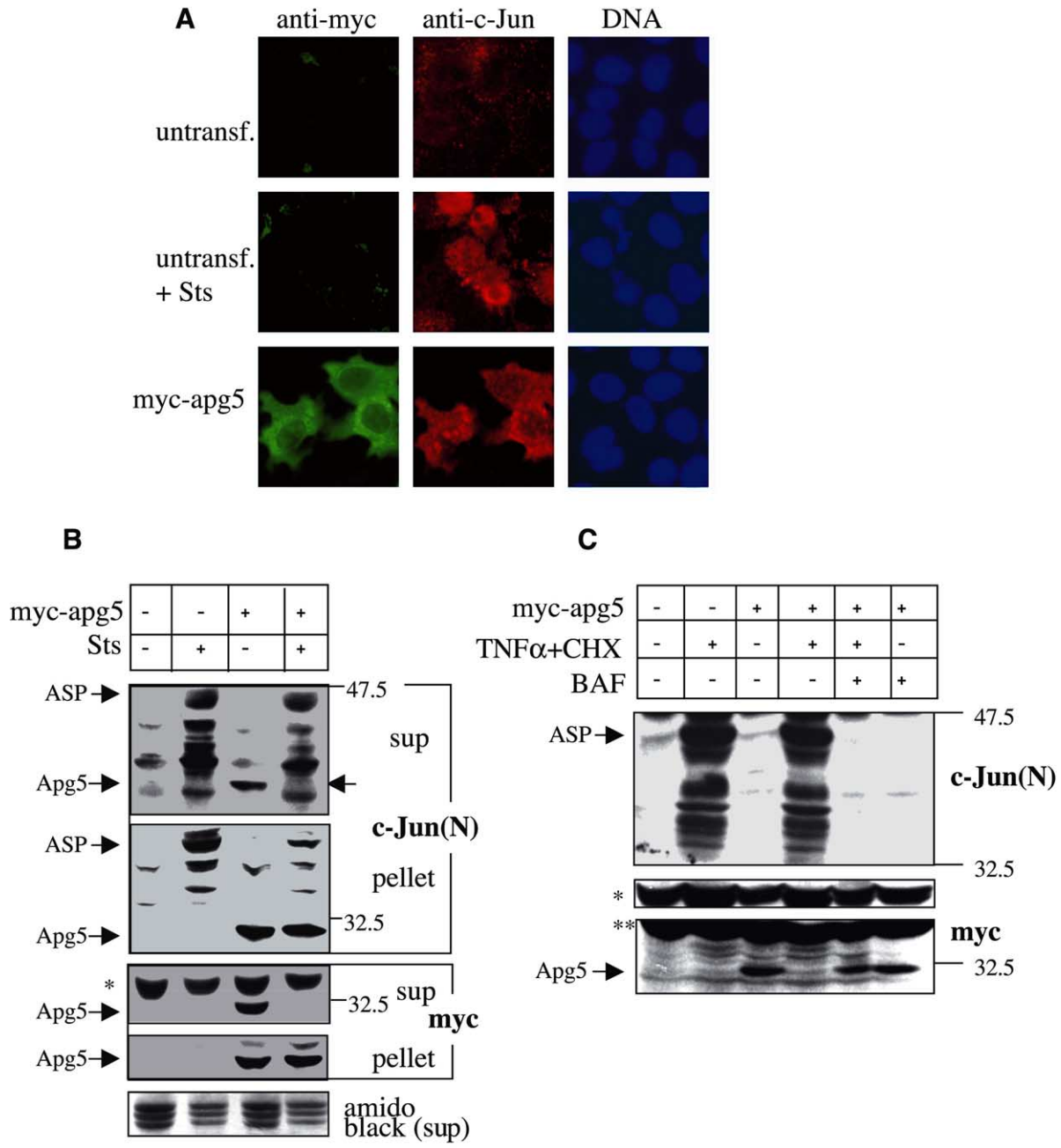

Fig. 1. A: hApg5 cross-reacts with the anti-c-Jun antibody (SC-45). HeLa cells transfected with myc-h*apg5* cDNA were fixed and co-stained with the anti-myc antibody 9E10 and the anti-c-Jun antibody (SC-45) using secondary antibodies conjugated to FITC (myc) or cy3 (c-Jun(N). Nuclei were visualised using Hoechst 33342. Row 1: Untransfected cells. Note low levels of c-myc and 'c-Jun' staining. Row 2: 14 h prior to fixation a set of untransfected cells was treated with 2.5 μM Sts to generate a positive control for ASP. Note lack of myc-positive cells but presence of brightly-stained 'c-Jun'-positive cells, some of which show DNA condensation and fragmentation typical of apoptotic HeLa cells. Row 3: HeLa cells fixed 48 h after transfection. Note lack of apoptotic features and presence of myc- and 'c-Jun'-positive co-stained cells. B: Differential stability and localisation of myc-hApg5 and endogenous p45ASP. HeLa cells transfected for 48 h were treated with 2.5 μM Sts for 14 h. Cells were lysed in a mild lysis buffer containing 1% Triton X-100, spun and the supernatant (sup) was separated from the pellet, which was lysed in 2% SDS lysis buffer. Protein samples (50 μg per lane) were resolved on a 10% denaturing polyacrylamide gel and analysed by Western blotting using the anti-c-Jun(N) antibody SC-45 (panels 1 and 2) or the anti-myc antibody (9E10) (panels 3 and 4). The bottom-most panel shows amido black staining as a loading control. *, non-specific band staining with 9E10. Note that Sts induces the appearance of ASP in the soluble fraction, but causes the concomitant degradation of myc-hApg5, detected with 9E10 or the c-Jun(N) antibody. Myc-hApg5 is apparently more stable than ASP in the pellet fraction. C: HeLa cells were treated with 50 ng/ml TNFα and 20 μg/ml CHX in the absence or presence of BAF for 8 h, after which soluble proteins were extracted and analysed by Western blotting. Note that despite the presence of sufficient CHX to prevent >95% of protein synthesis, ASP is still induced by TNFα and this induction is inhibited by BAF. In contrast, myc-hApg5 expression disappears during TNFα and CHX treatment but BAF prevents this disappearance. Non-specific staining indicated with * for anti-c-Jun(N) or ** for 9E10 (used as loading controls for the upper and lower panels, respectively).

kDa protein of the size of p45ASP to be formed from myc-hApg5 during apoptotic induction.

One remaining question is why – if hApg5 is not an 'ASP' – does it cross-react with the anti-c-Jun(N) antibody? Given that Casas et al. [10] suggested that it is a newly created c-terminal VTD motif that is recognised by the c-Jun antibody in most ASP proteins, and that myc-hApg5 contains a VTD motif at amino acids 47–50, we examined whether hApg5 may be cleaved at this VTD site. Perhaps cleavage at other putative caspase cleavage sites (such as after the sequence EAD at amino acids 131–133) might explain the disappearance of its soluble form from apoptotic cells. To examine whether hApg5 could be cleaved by caspases, we performed two types of assays. In one assay we incubated in vitro translated hApg5 with recombinant caspase-3 or an apoptotic extract. If cleavage at the VTD site were to occur, the size of hApg5 should decrease by about 5 kDa, similar to the decrease in size predicted for an in vitro translated fragment of retinoblastoma (Rb(378–938)) if it were cleaved after D in the DEADG(883-6) sequence. However, no cleavage of in vitro translated hApg5 was observed (Fig. 2A) although Rb(378–938) was cleaved appropriately, showing that caspase-3 was active. Moreover, this cleavage was inhibited by DEVD-CHO. The two proteins were also submitted to cleavage by an apoptotic extract from HeLa cells treated with Sts and again, only Rb(378–938) was cleaved in a DEVD-CHO inhibitable manner. In a second type of assay, a HeLa cell extract containing cellular myc-hApg5 was exposed to a cytosolic extract derived from either Sts or TNFα/CHX-stimulated apoptotic HeLa cells. Once again, no cleavage of myc-hApg5 was observed although PARP was cleaved appropriately in a BAF-inhibitable manner. Thus it is unlikely that the disappearance of soluble hApg5 during apoptosis is due to its cleavage by caspase-3 (or caspase-8, which is activated by TNFα/CHX treatment in HeLa cells).

In summary, we suggest that p45ASP and hApg5 are unrelated proteins that share the property (along with several other proteins) of interacting with c-Jun polyclonal antibodies raised against the TPTPTQFLCPKNVTD motif. If the suggestion that VTD serves as the recognition motif for the c-Jun

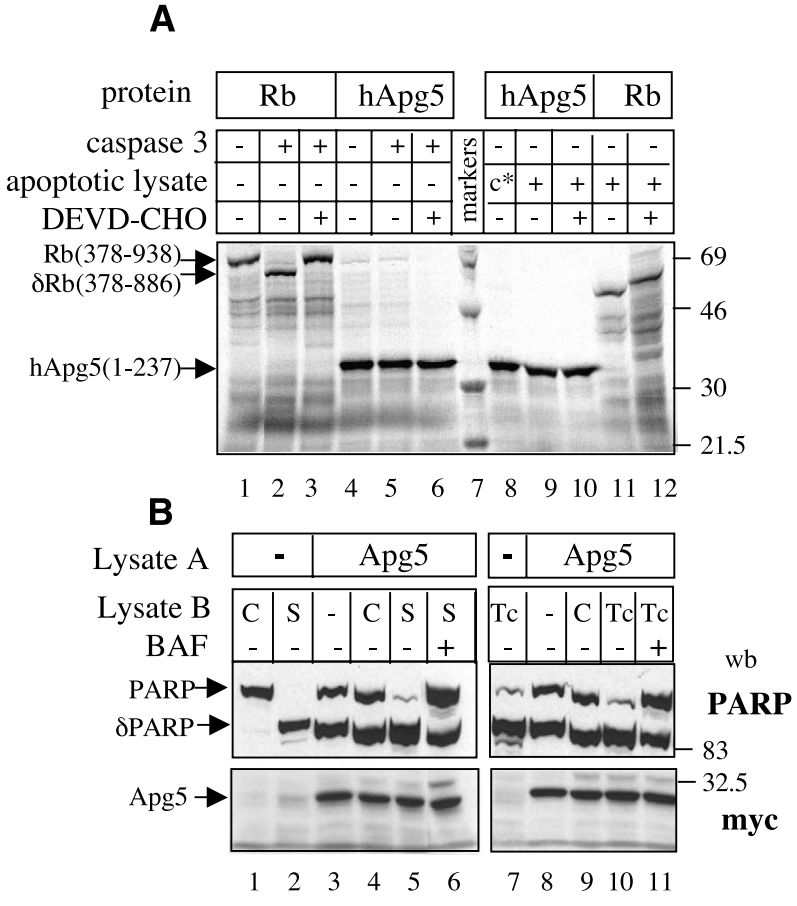

Fig. 2. A: In vitro translated hApg5 (lanes 4–6 and 8–10) or Rb(378–938) (lanes 1–3, 9 and 10) were incubated for 16 h with recombinant caspase-3 (lanes 2, 3, 5 and 6) or with an apoptotic extract derived from HeLa cells treated with Sts for 4 h (lanes 9–12). The extracts were resolved by SDS–PAGE and imaged using a phosphorimager. Lane 8 (C*) signifies addition of a non-apoptotic HeLa cell extract. B: A lysate from myc-hApg5-expressing HeLa cells (lanes 3–6 and 8–11) was either left untouched (duplicate lanes 3 and 8), or mixed with a cell extract derived from untreated (C) (duplicate lanes 4 and 9), or Sts-treated (S) (lanes 5 and 6) or TNFα and CHX (Tc) treated (lanes 10 and 11) HeLa cells and left to incubate for 16 h. BAF (10 μM) was added to the apoptotic lysates (lanes 6 and 11) before mixing with the hApg5-containing extract. Extracts were analysed by Western blotting using the anti-myc antibody 9E10 (lower panels) or an anti-PARP antibody (upper panels) to indicate presence of capsase activity. The controls in lanes 1, 2 and 7 were used to demonstrate the absence (in C) or presence of caspase activity in S and Tc-type B lysates before their addition to the hApg5-containing lysate so they were not incubated for 16 h. Although there was some spontaneous PARP cleavage in the Apg5-containing extract (lanes 3 and 8) incubated for 16 h, cleavage was further enhanced by addition of apoptotic lysates (lanes 5 and 10) and this cleavage was inhibited by BAF (100 μM). The control lysate (lanes 4 and 9) had no effect.

antibody [10] can be extended to native proteins, our data suggest that this epitope is exposed in soluble hApg5 whilst it is buried in the tethered form. Presumably the P1′ lysine in the LVTDK sequence prevents its cleavage by caspases as the amino acids in position P2-4 are not unfavourable to cleavage by a combination of caspases (such as caspase-2, -3 and -8) [13]. Thus the identities of p45ASP and the protease(s) responsible for the disappearance of hApg5 remain to be resolved.

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