Homology between a human apoptosis specific protein and the product of *APG5*, a gene involved in autophagy in yeast

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Abstract Apoptosis specific proteins (ASP) are expressed in the cytoplasm of cultured mammalian cells of various lineages following induction of apoptosis. The cDNA encoding *ASP* has been cloned from a human expression library and has significant homology to the *Saccharomyces cerevisiae APG5* gene which is essential for yeast autophagy. The *ASP* gene, known as *hAPG5*, can be transcribed to give mRNAs of 3.3 kbp, 2.5 kbp and 1.8 kbp which are present at comparable levels in viable and apoptotic cells, demonstrating that protein expression must be regulated at the translational level. These data indicate a possible relationship between apoptosis and autophagy and suggest evolutionary conservation in mammalian apoptosis of a degradative process present in yeast.

Key words: Apoptosis specific protein; *Saccharomyces cerevisiae*; Apoptosis

1. Introduction

Apoptosis is an active form of cell death and is of fundamental importance in tissue development and homeostasis [1–3]. The ability to initiate apoptosis appears to be a property of virtually all metazoan cells, with the cell death mechanism being highly conserved throughout evolution [4]. A closely related series of well-characterised activators, inhibitors and effectors are responsible for the regulation of the process in most cell types [5–7]. For example functionally and structurally homologous cell survival factors occur in mammals (encoded by *bcl*-2), *Caenorhabditis elegans* (encoded by the *ced*-9 gene) [8] and viruses (for example adenovirus E1B and EBV BHRF1 gene [9,10]). Similarly the *C. elegans ced*-3 gene encodes a protein related to the caspase family of vertebrate cysteine proteases [11]. These proteins appear to play important roles in the apoptotic process under certain, but not necessarily all, circumstances (see for example [6,7]).

Much less well-understood, however, are the changes in protein expression or structure which may be associated with morphological changes which are common to apoptotic cells. It has been known for some time that the degradation of nuclear lamins precedes DNA fragmentation in the nucleus of apoptotic cells [12]. In addition, a number of cellular enzymes and regulators such as FAK [13], PARP [14] and Rb [15] are known to be degraded during apoptosis. These events, however, are probably more closely linked to the action of the caspases than to morphological change. Perhaps more surprisingly we have recently demonstrated a dramatic increase in level of expression, in apoptotic cells, of a 45 kDa protein which appears to be associated with the cytoskeleton [16]. This protein was first observed in Burkitt's lymphoma cells and transformed retinoblasts [16] but has since been detected in all mammalian cells examined following induction of apoptosis. Here we report the isolation and characterisation of the cDNA encoding this apoptosis specific protein (which we have termed ASP). DNA sequencing demonstrates the human cDNA to be novel but to be homologous to the yeast *APG5* gene which is necessary for autophagy. We conclude that apoptosis and autophagy may be related processes employing some of the same proteins and/or biochemical mechanisms.

2. Materials and methods

2.1. Cloning a cDNA encoding an apoptosis specific protein

Previously apoptosis specific proteins have been identified by cross-reactivity of a c-Jun antibody (Ab2, Oncogene Science). Using this antibody a human foetal liver ZAP Express *Eco*RI/*Xho*I library was screened (Stratagene). The ExAssist Helper Phage (Stratagene) was then used to generate phagemids from positive bacteriophage. This generated the 3′ end of the cDNA. The 5′ sequence was generated using a human foetal brain RACE library (Clontech). The gene specific primers used were: 1, 5′-CTGTGATGTTCCAAGGAAGAG-CTG-3′ and 2, 5′-GATAATGCCATTTCAGTGGTGTGCCTTC-3′. A 373A ABI Gene Sequencer and Prism FS Dye terminator kit were used for sequencing.

2.2. Southern blotting

The commercially available filter used consisted of 4 μg of genomic DNA per lane extracted from nine eukaryotic species (Clontech). The probe used for hybridisation was a ^{32}P-labelled 1 kbp PCR product from the *hAPG5* sequence. Hybridisation was at 65°C for approximately 16 h using the buffer conditions suggested by the manufacturer. Signals were then visualised using Kodak XAR-2 film at −70°C over a period of 2–3 days.

2.3. Northern blotting

The Northern blot contained 2 μg of poly(A)$^+$ mRNA extracted from the human tissues specified separated on a formaldehyde agarose gel (Clontech). The filter was hybridised with a ^{32}P-labelled *hAPG5* specific probe for approximately 16 h at 42°C. The prehybridisation and hybridisation buffer used was 5×SSPE, 10×Denhardt's, 100 μg/ml salmon sperm and 50% de-ionised formamide. Signals were visualised as before over a period of 4–5 days.

2.4. Western blotting

Cells were rinsed with cold saline, harvested by centrifugation and solubilised in 9 M urea, 50 mM Tris-HCl pH 7.3, 0.15 M β-mercaptoethanol. Aliquots, containing 50 μg of protein, were fractionated by SDS-PAGE and subjected to Western blotting using an antibody against human c-Jun (Oncogene Science Ab2) at a dilution of 1 in 200. Antigens were visualised using ECL (Amersham).

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2.5. DNA transfections

Transient transfections were carried out using the LipoTAXI (Stratagene) and the protocol supplied by the manufacturer. 20 μg of DNA was used per transfection.

3. Results

Apoptosis specific proteins were originally demonstrated [16] in apoptosing human and rodent cell lines by cross-reactivity of an antibody (Oncogene Science) raised against a peptide equivalent to part of the human c-Jun sequence (amino acids 73–87, TPTPTQFLCPKNVTD). In addition, it has recently been shown to be expressed in human tissues, such as tonsil, liver and pancreas undergoing apoptosis (our unpublished data).

These initial studies indicating the widespread distribution of ASP in apoptotic cells of diverse origin suggested that the protein might play an important role in the apoptotic process, possibly within the modified cytoskeleton [16]. If this were the case, it might be expected that ASP expression would be a relatively 'late' event in cells undergoing apoptosis. To investigate this further, BOC-ASP-CH_2F OMe (100 μM), a caspase inhibitor, was added to Cos-1 cells at the same time as they were induced to apoptose with cisplatin (50 μg/ml). It can be seen from the Western blot shown in Fig. 1 that no ASP was expressed after the addition of the caspase inhibitor together with cisplatin, whereas the DNA damaging agent alone caused ASP expression after 10 h and caused the morphological changes normally associated with apoptosis (data not shown).

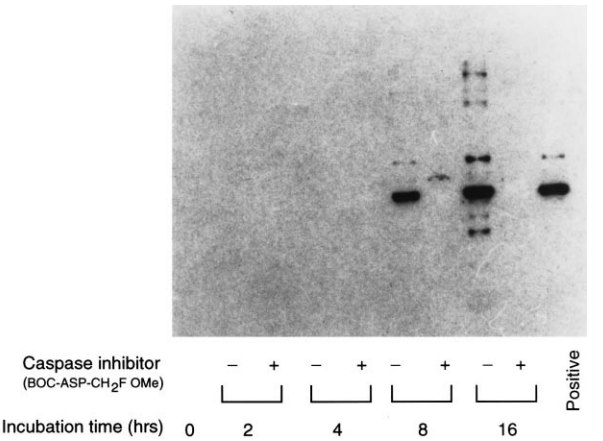

Fig. 1. The effect of a caspase inhibitor on ASP expression. Cos-1 cells were incubated with cisplatin (50 μg/ml) either in the presence or in the absence of BOC-ASP-CH_2F OMe (100 μM). At the times indicated, cells were harvested and aliquots (containing 50 μg protein) subjected to PAGE and Western blotting using an antibody to c-Jun. The positive control used was apoptotic rodent cells.

Thus, it can be seen that ASP is expressed only after events attributable to caspase activity. In an attempt to obtain further clues to its function the gene encoding ASP was cloned by screening human cDNA libraries with the antibody against c-Jun. Initially a human foetal liver ZAP Express *Eco*RI/*Xho*I library was screened. ExAssist Helper phage were used to generate phagemids from positive bacteriophage. By this approach the 3′ end of the ASP cDNA was isolated. The re-

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ASP     1 MTDDKDVLRDVWFGRIPTCFTLYQDEITEREAEPYYLLLPR....VSYLT 46
APG5    1 MNDIKQLLWNGELNVLVSIDPSFLMKGSPREIAVLRIRVPRETYLVNYMP 50
         47 LVTDKVKKHFQKVMRQEDISEIWFEYEGTPLKWHYPIGLLFDLLASSSA. 95
         51 LIWNKIKSFLSFDPLTDSEKYFWFEHNKTPIPWNYPVGVLFDCLAGKSAT 100
         96 ..............LPWNITVHFKSFPEKDLLHCPSKDAIEAHFMSCMKE 131
        101 FTTSFENQVKDVLTFLRIHLVMGDSLPPTIIPIASSKTQAEKFWFHQWKQ 150
        132 AD.ALKHKSQVINEMQKKDHKQLWMGLQNDRFDQFWAINRKLMEYPAEEN 180
        151 VCFILNGSSKAIMSLSVNEARKFWGSVITRNFQDFIEISNKISSSRP... 197
        181 GFRYIPFRIYQTTTERPFIQKLFRPVAADGQLHTLGDLLKEVCPSAIDPE 230
        198 ..RHIPLIIQTSRTSGT.....FRISQPTISMTGVNPTLKDIEGDILDVK 240
        231 DGEKKNQVMI..HGIEPMLETPLQWLSEHLSYPDNFLHISIIPQPTD*.. 276
        241 EGINGNDVMVICQGIEIPWHMLLYDLYSKLRSFDGFLYITLVPIKGGDKA 290

        291 SSEL* 294
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Fig. 2. Primary structure of ASP and sequence homology to APG5 from *S. cerevisiae*. The predicted amino acid sequence of human ASP is shown. The start site 3′ end of the original cDNA is at amino acid 5. *APG5* was translated from ORF YPL149W on *S. cerevisiae* chromosome XVI. A potential glycosylation site was identified at N99, casein kinase II phosporylation sites at T29, S106, T192, T214, S225 and S259, protein kinase C phosphorylation sites at T28, T49 and T194 and a tyrosine kinase site at Y36. The amino acid sequence of ASP was aligned with APG5 using the Genetics Computer Group GAP program. | indicates an identical match; : amino acids of strong similarity; · amino acids of weak similarity. … indicates a gap introduced into the sequence to give maximum homology. A possible match to the c-Jun peptide used to raise the antibody used in this work is underlined. Homology analyses and motif searches were performed with the Genetics Computer Group package (Version 8, Madison Wisconsin) or BLAST [19] with the National Centre for Biotechnology Information BLAST network service using the non-redundant protein database. Information on *S. cerevisiae* ORFs was obtained from the *S. cerevisiae* Genome Database at Stanford University via the worldwide web. The *hAPG5* sequence is available from EMBL/GenBank/DDBJ under accession number Y711588.

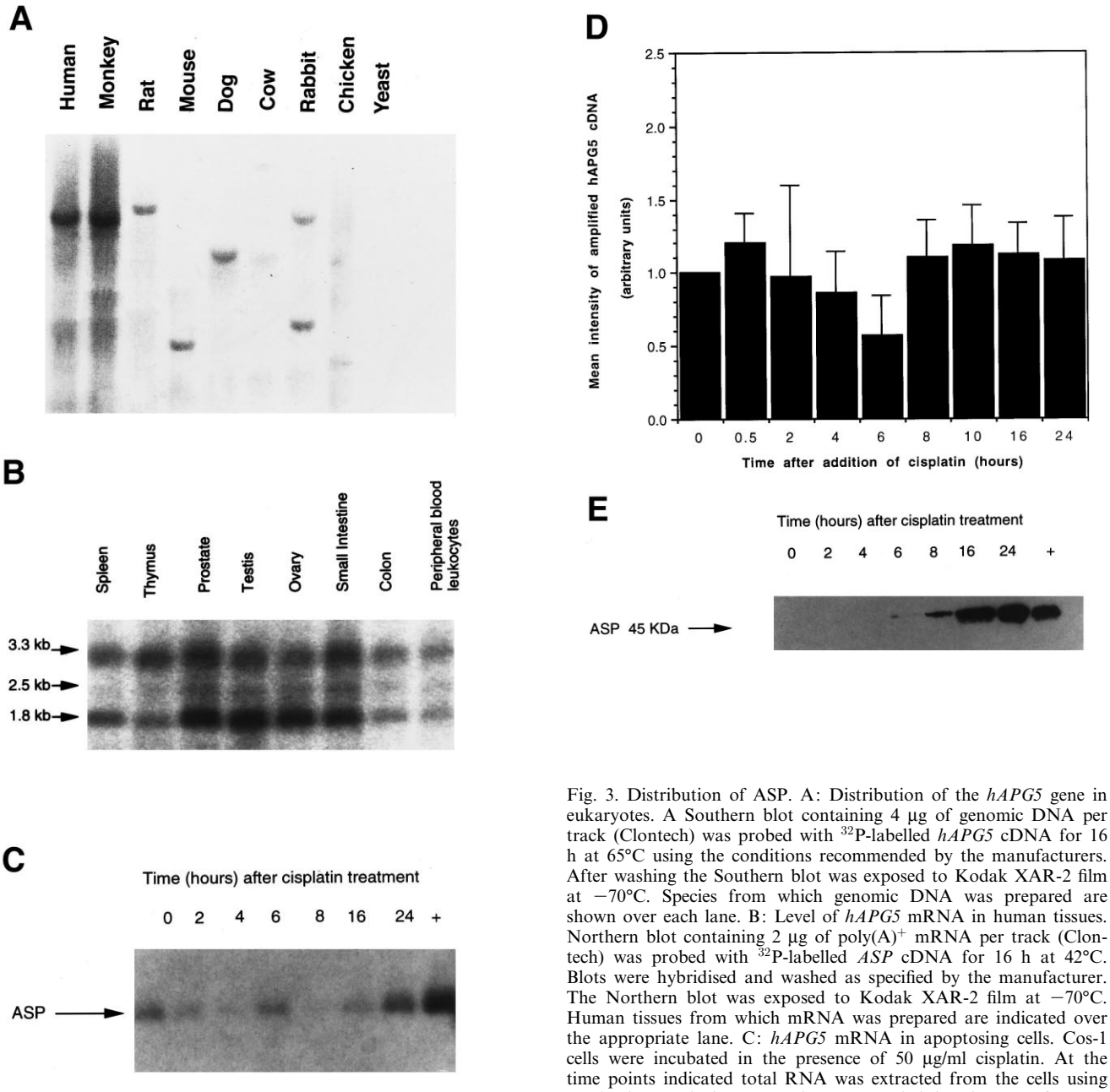

Fig. 3. Distribution of ASP. A: Distribution of the *hAPG5* gene in eukaryotes. A Southern blot containing 4 μg of genomic DNA per track (Clontech) was probed with ^{32}P-labelled *hAPG5* cDNA for 16 h at 65°C using the conditions recommended by the manufacturers. After washing the Southern blot was exposed to Kodak XAR-2 film at −70°C. Species from which genomic DNA was prepared are shown over each lane. B: Level of *hAPG5* mRNA in human tissues. Northern blot containing 2 μg of poly(A)$^+$ mRNA per track (Clontech) was probed with ^{32}P-labelled *ASP* cDNA for 16 h at 42°C. Blots were hybridised and washed as specified by the manufacturer. The Northern blot was exposed to Kodak XAR-2 film at −70°C. Human tissues from which mRNA was prepared are indicated over the appropriate lane. C: *hAPG5* mRNA in apoptosing cells. Cos-1 cells were incubated in the presence of 50 μg/ml cisplatin. At the time points indicated total RNA was extracted from the cells using RNAzol B and the protocol recommended by the manufacturer. 5 μg of the RNA obtained was used to produce cDNA using the Superscript kit from Gibco. A PCR was then performed on 2 μl of the resultant cDNA using the following primers: 5′-3′ AGC TGG ATC CAT GAC AGA TGA CAA AGA TGT GCT TCG AGA TGT GTG G and 3′-5′ GGG TGA CAT GCT CTG GGA TCC CCC ATT TAA GGA TG. In each case 24 cycles were conducted to ensure the reactions remained in the exponential phase. Relative levels of gapdh were quantified using a densitometer and were found to be constant, whilst the relative quantities of *hAPG5* cDNA were determined by hybridisation with a *hAPG5* cDNA specific probe. The positive control shown (+) was obtained using *hAPG5* plasmid DNA in the PCR. D: Data from RT-PCRs. The experiment described in C was performed four times. Blots similar to that shown in C were subjected to densitometric scanning. Data obtained for each time point were normalised to the level of *hAPG5* cDNA seen for the untreated, viable cells. These data are displayed in the histogram shown, $n=4$. E: ASP expression in apoptotic Cos-1 cells. Cos-1 cells were treated with cisplatin (50 μg/ml) as in C. Cells were harvested at the times indicated and samples subjected to Western blotting using the c-Jun (Ab2) antibody. All lanes contain a total of 50 μg of protein.

maining 5′ sequence was generated using a human foetal brain RACE library. The DNA isolated was 3.3 kbp in length with an open reading frame of 828 bp, the remaining nucleotide sequence containing 3′ and 5′ untranslated regions. The encoded protein comprises 276 amino acids with a preponderance of acidic residues. A potential glycosylation site is present at amino acid N99. In addition potential casein kinase II, protein kinase C and tyrosine kinase sites are present as indicated in the legend to Fig. 2.

A data base search revealed a significant homology between the amino acid sequence of ASP and the predicted product of the *Saccharomyces cerevisiae APG5* gene – a member of a large family of genes involved in yeast autophagy [17]. The *APG5* product is believed to be necessary for the correct processing of autophagic bodies under conditions of nitrogen starvation [17,18] (Fig. 2). 26% of the amino acids were identical in ASP and the *APG5* product with 46% considered to be

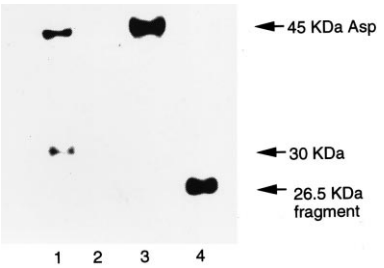

Fig. 4. Transient transfection of Cos-1 cells with pSG5/*hAPG5*. Cos-1 cells were transfected using the LipoTAXI reagent [20]. 48 h after transfection the cells were harvested and the proteins extracted. 50 μg of each sample was electrophoresed on a SDS gel. A Western blot was carried out using the c-Jun (Ab2) antibody. Cells were transfected with either pSG5/*hAPG5* (lane 1) or the vector alone pSG5 (lane 2). Apoptotic rodent cells were used in lane 3 as an ASP positive control. Lane 4 shows a fragment of *hAPG5*.

similar. The homology was reasonably well distributed throughout the sequence which suggests that the similarity is not attributable to the conservation of a common domain or motif.

Southern blotting has established the presence of the *hAPG5* gene in a number of different mammalian species as well as in birds (Fig. 3A), consistent with our previous Western blotting data using the c-Jun antibody ([16]; our unpublished data) which suggested that the protein was widely distributed. A number of human tissues were examined for the presence of mRNA transcribed from the *hAPG5* gene It can be seen (Fig. 3B) that the message is present in all tissues examined in approximately equal abundance. Two major transcripts of 3.3 kbp and 1.8 kbp were seen and a minor one of 2.5 kbp. It is presumed that these products arise through differential splicing although homology with a related gene cannot be ruled out.

It has previously been shown that expression of ASP increases dramatically as cells undergo apoptosis [16]. To determine whether this increase was regulated transcriptionally or translationally the level of mRNA in Cos-1 cells induced to apoptose by the addition of cisplatin (50 μg/ml for a period of 16 h) was examined using RT-PCR. A representative blot is shown in Fig. 3C. It can be seen that, whilst the overall level of *hAPG5* mRNA remains reasonably constant, some variations are apparent. To determine whether the detected fluctuations in level of mRNA are significant, four separate experiments were carried out. After densitometric scanning of the blots the intensity of the band for each time point was normalised to that obtained for the viable, untreated cells. The data from the four blots are presented in graphical form in Fig. 3D. We conclude that there is no change in *hAPG5* mRNA following the addition of cisplatin and consequent apoptosis (confirmed by acridine orange staining, data not shown). However, Western blotting of similarly treated samples showed an appreciable increase in ASP expression (Fig. 3E).

The *hAPG5* open reading frame encodes a protein of 276 amino acids with a theoretical molecular weight of 32.4 kDa which is appreciably smaller than the major 45 kDa ASP band observed during Western blotting of apoptotic cells. When the ASP cDNA was expressed in *Escherichia coli* the major product detected was of the predicted molecular weight 32 kDa. However, when *hAPG5* cDNA was transiently transfected into Cos-1 cells two bands were detected by Western blotting – a protein of 32 kDa similar to that seen after expression in *E. coli* as well as a 45 kDa protein similar to that seen in apoptotic cells (Fig. 4). We have concluded that a post-translational modification is responsible for the large discrepancy in molecular weights between ASP expressed in bacteria and mammalian cells.

4. Discussion

It has previously been shown that the expression of a protein of 45 kDa molecular weight is dramatically increased in a variety of cell lines in response to stimuli which induce apoptosis [16]. Here we present evidence to show that this protein is encoded by a novel gene with an open reading frame of 828 bp which is homologous to a yeast gene required for autophagy [18]. Autophagy has been shown to be a general response of mammalian cells to serum deprivation and usually involves the sequestration of intracellular organelles and cytoplasm in autophagosomes which subsequently fuse with lysosomes (see for example [21,22]). A comparable process occurs in yeast [23]. Thus it appears that whilst autophagy and apoptosis have distinct features, similar proteins are involved in each process. The functions of ASP (*hAPG5*) and APG5 are not well understood, but on the basis of our previous studies we consider that the proteins may be involved in the maintenance of the integrity of apoptotic and autophagic bodies. Whilst the similarities in the amino acid sequences of ASP and APG5 suggest a functional homology, we have no evidence to support this at present. The complementation of yeast with a 'knocked out' *APG5* phenotype with the *hAPG5* cDNA will be required to resolve this point unequivocally. It is worth noting, however, that a number of proteins with proven functional conservation in yeast and humans have a comparable amino acid identity to that seen for APG5 and ASP. For example, the identity between *Schizosaccharomyces pombe* and human cofillin is 35.1%, elongation factor 1γ 36.9% and rad 54 and ERC6 33.5%.

The observation that *hAPG5* mRNA is present at similar levels in viable and apoptotic cells strongly suggests that increases in ASP expression are largely, perhaps entirely, attributable to increases in translation of pre-existing mRNA. This conclusion is consistent with our inability to detect *hAPG5* mRNA in a number of different cell types by in situ hybridisation (data not shown). Interestingly no increase in APG5 mRNA was seen in yeast undergoing autophagy [18] indicating that regulation of expression of ASP and APG5 may be by a similar mechanism. It seems likely that the ASP observed after transient transfection of the cDNA (Fig. 4) is attributable to the temporary very high levels of message such that in this case, unusually, ASP expression can occur in the absence of the remainder of the apoptotic process.

The introduction of ASP into viable Cos-1 cells (Fig. 4) appeared to have no effect on cellular morphology and did not induce apoptosis. This is consistent with data presented in Fig. 1 which demonstrated that ASP expression was a relatively late event in the apoptotic process occurring downstream of caspase activity.

The discrepancy between the predicted and observed molecular weights for ASP is not simply explained. However, it has been shown that enzymes present in eukaryotic, but not prokaryotic, cells are necessary for the conversion. Further-

more, it appears that the increase in molecular weight does not require apoptosis. We suggest that the addition of moieties such as carbohydrates may be responsible, although we have no evidence to support this view at present. Most of the potential modification sites shown in the legend to Fig. 2 are not conserved in APG5 but it is not clear whether this is significant as no protein product has yet been identified in yeast.

In conclusion, the data presented has shown that the apoptosis specific protein ASP is encoded by a gene present in all mammalian species as well as in birds. The low levels of mRNA seen in viable cells as well as in apoptotic cells indicates that the high levels of protein seen are largely attributable to increased translation of pre-existing mRNA. In addition the significant homology between ASP cDNA and APG5 from yeast has two important implications. Firstly it suggests that apoptosis and autophagy are related processes or at least employ some common mechanisms. Secondly it confirms the conservation of a common biochemical pathway involved in autophagy and apoptosis in all eukaryotes.

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