Specific cleavage of γ catenin by caspases during apoptosis

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Abstract Caspase-mediated proteolysis of cytoskeletal proteins during apoptosis appears to be commonplace. Enlarging on previous studies we have shown here that γ catenin, like β catenin, was degraded during cisplatin-induced apoptosis, initially giving a major product of 75 kDa. This truncated protein could be co-immunoprecipitated with α catenin. Addition of caspase inhibitors to cells in the presence of cisplatin appreciably reduced the proteolysis of γ catenin as well as the level of apoptosis. Only limited degradation of α catenin was observed even at very late times when over 90% of cells in the culture were apoptotic. Immunohistochemical staining showed that during apoptosis there was a relocation of α, β and γ catenin from the periphery of the cell to the cytoplasm, at the same time as other morphological changes commonly associated with apoptosis occurred. Interestingly, the changes in localisation of the catenins preceded proteolysis by several hours. In the presence of cisplatin and caspase inhibitor no change in distribution of catenins was observed, suggesting that re-localisation requires caspase activity but not necessarily directed against β and γ catenins.

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Key words: Gamma catenin; Plakoglobin; Alpha catenin; Apoptosis; Caspase; Cytoskeleton

1. Introduction

Apoptosis is a process of fundamental importance for the development and well-being of most, perhaps all, multi-cellular organisms. It plays a central role in normal morphogenesis, the functioning of the immune system and homeostasis (reviewed, for example, in [1,2]). Detailed genetic analysis has established the presence of a highly conserved set of regulators (both positive and negative) of apoptosis in organisms as diverse as *Caenorhabditis elegans* and *Homo sapiens* with functional and structural homologues being relatively widespread in viruses (reviewed, for example, in [3–5]).

It appears that the principal effectors of apoptosis are a series of proteases (termed caspases) of the interleukin-1β-converting enzyme (ICE) family [6–8]. The caspases, of which 10 have so far been identified in mammals, form a proteolytic cascade such that certain components serve as substrates and are thereby activated by caspases further up the cascade. Thus, for example, caspase 10 cleaves pro-caspase 3, giving active caspase 3 which can then degrade proteins such as DNA-PK [9], protein kinase C ∂ [10] and the retinoblastoma protein (pRb) [11]. Cleavage sites for caspases share some sequence homology with an absolute requirement for aspartic acid at the P_1 position. The requirements in the P_2 to P_4 positions seem to be dependent on the specific caspase concerned [8].

Over the past year it has become apparent that a very large number of cellular components, including cytoskeletal proteins, are degraded by caspases as apoptosis progresses. Whilst the significance of much of this proteolysis remains unclear at present it seems likely that degradation of cytoskeletal components is associated with the profound morphological changes which occur during apoptosis. It has already been demonstrated that β catenin serves as a substrate for caspase 3 leading to formation of a 65K fragment which has impaired ability to bind α catenin [12]. This disruption of critical adherens junction proteins was considered to be important for loss of cell adhesion during apoptosis. Following on from these studies by Brancolini et al. [12], we have shown the caspase-mediated degradation of γ catenin in apoptotic human cells whilst α catenin remains unaffected despite the presence of potential caspase cleavage sites. In addition, we have shown that α, β and γ catenins are re-located within the cell during the relatively early stages of apoptosis many hours before protein cleavage is apparent. This re-location requires caspase activity but presumably not directed against catenins.

2. Materials and methods

2.1. Cell lines

The Ad2 E1A+N-*ras* HER 313A cell line was produced by transfection of adenovirus 2 early region 1A and mutant N-*ras* DNA into human embryo retinoblasts (HER) as described [13]. Cells were normally maintained in HEPES-buffered DME augmented with 8% FCS and 2 mM glutamine. Additional cell lines used in some experiments were: Ad12 E1A HLBRK3 produced by transfection of adenovirus 12 E1A DNA into hooded Lister baby rat kidney cells; BJAB, an EBV-negative B cell lymphoma cell line; Ramos, an EBV-negative Burkitt lymphoma cell line and Jurkat, a T cell pro-lymphocytic leukaemia cell line. Ad12 E1A HLBRK3 cells were grown on HEPES-buffered DME containing 8% FCS, whilst the remaining cell lines were grown on RPMI containing 10% FCS and glutamine.

2.2. Apoptosis

Cells were usually induced to apoptosis by addition of cisplatin (15–20 μg/ml in the tissue culture medium). In some experiments apoptosis was induced by treatment with UV light (300 J/m^2). Caspases were inhibited by the addition of Z-DEVD-FMK, Z-VAD-FMK, Z-YVAD-FMK and Boc-Asp-FMK (all from Enzyme Systems Products), dissolved in DMSO, to the culture medium at a final concentration of 40 μM.

2.3. Analytical methods

Western blotting: Cells were washed with cold saline, harvested by aspiration and pelleted by centrifugation. Cells were solubilised in 9 M urea, 50 mM Tris-HCl pH 7.4, 0.15 M β-mercaptoethanol and proteins fractionated on 12% polyacrylamide gels run in the presence of 0.1 M Tris, 0.1 M Bicine, 0.1% SDS. Proteins were electrophoretically transferred to nitrocellulose membranes which were incubated with antibodies against α catenin, β catenin and γ catenin (all antibodies obtained from Sigma). Antibodies against α catenin were raised in rabbits and those against β and γ catenins were mouse monoclonals.

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Antibodies were diluted 1/4000, 1/4000 and 1/2000 for detection of α, β and γ catenins respectively. Antigens were visualised by ECL (Amersham).

Immunoprecipitation: Cells were washed with cold saline, harvested by aspiration and pelleted by centrifugation. Cells were solubilised in 0.83 M NaCl, 1% NP40, 20 mM Tris-HCl pH 7.4, sonicated and clarified by centrifugation at 35 K for 30 min. Insoluble material was discarded. Lysates containing equal amounts of protein were pre-cleared by incubation with protein G-agarose (Sigma) for 30 min and then incubated with appropriate antibodies for 1 h. Antibody-antigen complexes were collected on protein G-agarose. After appropriate washing immunoprecipitated proteins were fractionated on 12% polyacrylamide gels and then subjected to Western blotting as described above. Nitrocellulose filters were probed with antibodies against potential binding proteins which were visualised as described above.

Acridine orange staining: Cell suspensions were mixed 1:1 with acridine orange (10 μg/ml) and then viewed by fluorescence microscopy after 5 min. The number of apoptotic cells in a sample of at least 200 cells was counted.

2.4. Immunohistochemistry

Cells were grown on glass multiwell slides and were induced to apoptosis by the addition of cisplatin (16 μg/ml). Apoptotic and viable cells were fixed in paraformaldehyde (4% in PBS) for 30 s and permeabilised with acetone at −20°C (10 min). Fixed cells were stored at −20°C. For staining, cells were thawed to room temperature and non-specific binding sites were blocked in blocking buffer (20% v/v heat inactivated goat serum, 0.1% BSA w/v and 0.1% sodium azide in PBS) for 30 min. Cells were immunostained by diluting the appropriate antibody in the above blocking buffer (α catenin: 1/100, β catenin: 1/200 and γ catenin: 1/20). Cells were incubated for 90 min in a humid box at 37°C. The antibody was removed by washing the slides twice in PBS for 15 min. FITC labelled anti-species antibody was diluted into blocking buffer (1/40) before incubating for 60 min at 37°C. Again the antibody was removed by washing the slides twice in PBS for 15 min. Nuclei were visualised by DAPI (10 μM) staining. The slides were mounted in the presence of DABCO (2% 1,4-diazabicyclo-[2.2.2]octane in 80% glycerol/PBS). Cells were viewed by confocal microscopy using a Zeiss Axiphot fluorescence microscope. Images were recorded with the biovision software package (Impro Vision) and a low-light-level video camera. The two channels were recorded independently and pseudo-colour images generated and superimposed with Adobe Photoshop 3.0 software.

3. Results

It is now well-established that a large number of cellular proteins are substrates for caspases during apoptosis. Of particular interest has been the recent observation that cytoskeletal proteins such as lamin B [14], keratins [15], gelsolin [16] and β catenin [12] are degraded as apoptosis occurs and that this accompanies cytoskeletal reorganisation. With this in mind we have examined the fate of other cytoskeletal components and here describe the degradation of γ but not α catenin.

3.1. β and γ but not α catenins are degraded during apoptosis

Ad2 E1A+N-*ras* HER 313A cells were induced to apoptose by the addition of cisplatin. Cells were harvested at appropriate times and subjected to SDS-PAGE and Western blotting. It can be seen from the data presented in Fig. 1A that γ catenin and β catenin were degraded as cells became more apoptotic whilst α catenin was only slightly affected, even at very late times (Fig. 1B). Thus, at 16 h more than half of the β catenin was cleaved (to give products of 75 kDa and 72 kDa) with 60% of the cells apoptotic by acridine orange staining. In the set of samples Western blotted for γ catenin it can be seen that at 16 h about 60% of the cells were apoptotic with about one third of the protein degraded. In contrast α catenin was cleaved to only a very limited extent 72 h after induction of apoptosis, when over 90% of the cells were apoptotic (about 20% of the protein degraded).

The major cleavage product of β catenin was of 72 kDa which is in reasonably close agreement to the final polypeptide previously seen after caspase 3 action [12]. Degradation of γ catenin only resulted in loss of a short polypeptide from the protein – giving a reduction in molecular weight from 81 to 75 kDa (Fig. 1A). Patterns of γ catenin cleavage, identical to that shown in Fig. 1A, were observed when Ad12 E1A HLBRK3, BJAB, Ramos and Jurkat cells were induced to apoptose by the addition of cisplatin (20 μg/ml) or when Ad2 E1A+N-*ras* HER 313A and Ad12 E1A HLBRK3 cells were treated with high doses of UV light (data not shown).

3.2. Inhibition of caspases

To confirm that proteolysis of γ catenin was directly linked to cisplatin-induced apoptosis the effects of different caspase inhibitors were examined. All of the inhibitors used, with the exception of Z-DEVD FMK (Fig. 2D), reduced appreciably the extent of apoptosis over the 70 h time course of the experiment. Even that inhibitor, although it did not greatly reduce the final level of apoptosis, retarded the onset of cell death such that at 8 and 18 h 3.6% and 47% respectively of cells were identified as apoptotic compared with 35% and 78% apoptotic cells (at 8 and 18 h) with cisplatin in the absence of caspase inhibitors. As well as reducing the overall level of apoptosis the caspase inhibitors appreciably reduced the proteolytic breakdown of γ catenin. This is most marked with the less specific inhibitors Boc-Asp-FMK and Z-VAD-FMK (Fig.

Table 1
Observed and predicted products of caspase-mediated proteolysis of catenins

Catenin Predicted molecular weight (Observed molecular weight [from Fig. 1])	Potential caspase cleavage sites	Predicted products	Observed product (from Fig. 1)
α catenin	aa 143–146 DMAD	15.5 kDa+84.5 kDa	No observed degradation
100 kDa	aa 500–503 DAVD	45 kDa+55 kDa	
(94 kDa)	aa 639–642 DDSD	70.5 kDa+29.5 kDa	
β catenin	aa 78–81 QVAD	8.5 kDa+77 kDa	75 kDa and 72 kDa
85.5 kDa	aa 456–459 DRED	35.5 kDa+50 kDa	
(88 kDa)	aa 761–764 DLMD	2.5 kDa+83 kDa	
γ catenin	aa 693–696 DDMD	6 kDa+75.5 kDa	75 kDa
81.5 kDa	aa 617–620 DAID	14 kDa+67.5 kDa	
(81 kDa)	aa 445–448 DKDD	33 kDa+48.5 kDa	

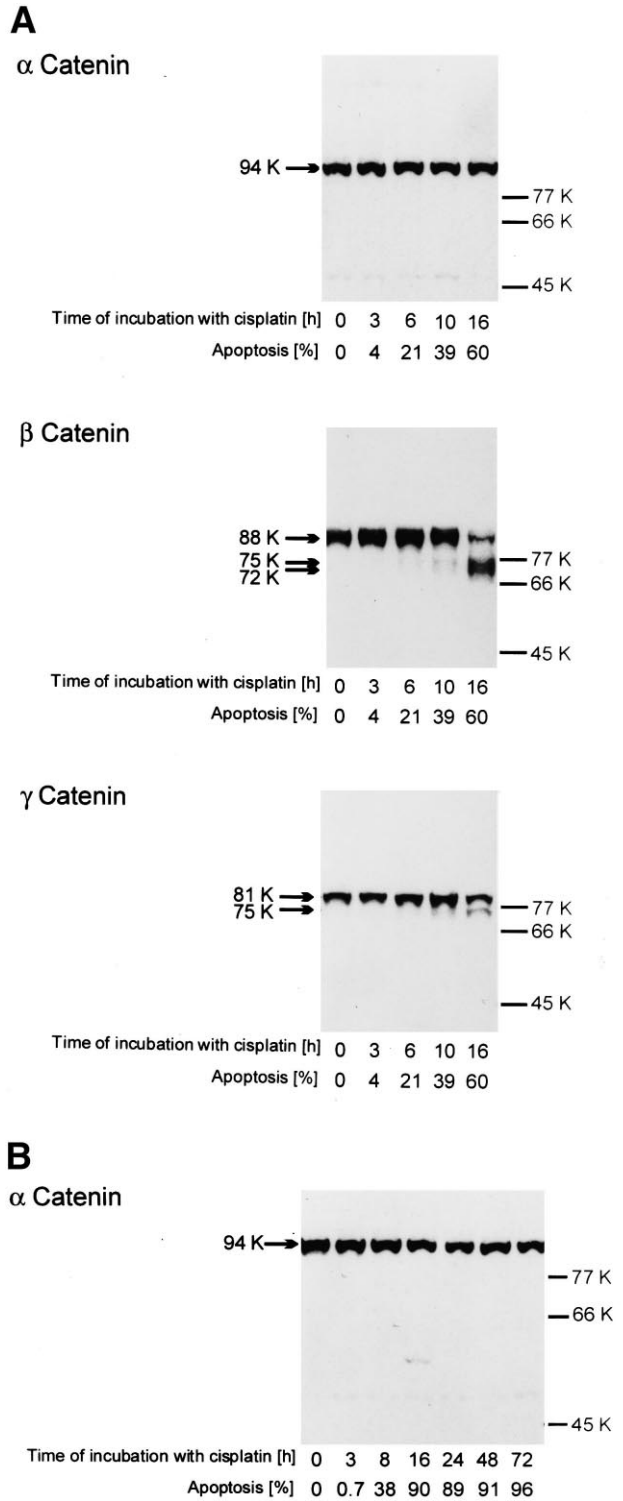

Fig. 1. Expression of catenins in apoptotic cells. Ad2 E1A+N-*ras* HER 313A cells were treated with cisplatin (20 μg/ml in the tissue culture medium) and then harvested at times indicated. Percentage apoptosis was determined by acidine orange staining and is shown under the appropriate time point. Aliquots (containing 50 μg of protein) were fractionated on polyacrylamide gels and subjected to Western blotting using antibodies against α catenin, β catenin and γ catenin. The position of migration of marker proteins is shown on the right-hand side of each panel and the calculated molecular weights of catenins and their cleavage products on the left hand side. A: Samples harvested over a 16 h time course. B: Samples harvested over a longer time course and blotted for α catenin only.

2B,C) which give a high degree of protection from protein degradation. The inhibitor Z-DEVD-FMK did not inhibit γ catenin breakdown as efficiently as Boc-Asp-FMK or Z-VAD-FMK but, as mentioned above, allowed apoptosis to proceed to some extent. This may be explained by the limited uptake of this inhibitor by cells in culture compared to other inhibitors used here (C. Gregory, personal communication). It can be seen from the Western blot in panel E that Z-YVAD-FMK, whilst protecting cells from apoptosis does not stop degradation of γ catenin as effectively as the other compounds. This may suggest that caspases 1 and 4 (for which Z-YVAD-FMK is considered to be a relatively specific inhibitor) are not directly involved in γ catenin breakdown. The observation that Z-DEVD-FMK (Fig. 2D) limits degradation of γ catenin at early times rather more efficiently than Z-YVAD-FMK may suggest the involvement of caspase 3 although this will have to await confirmation using purified proteins.

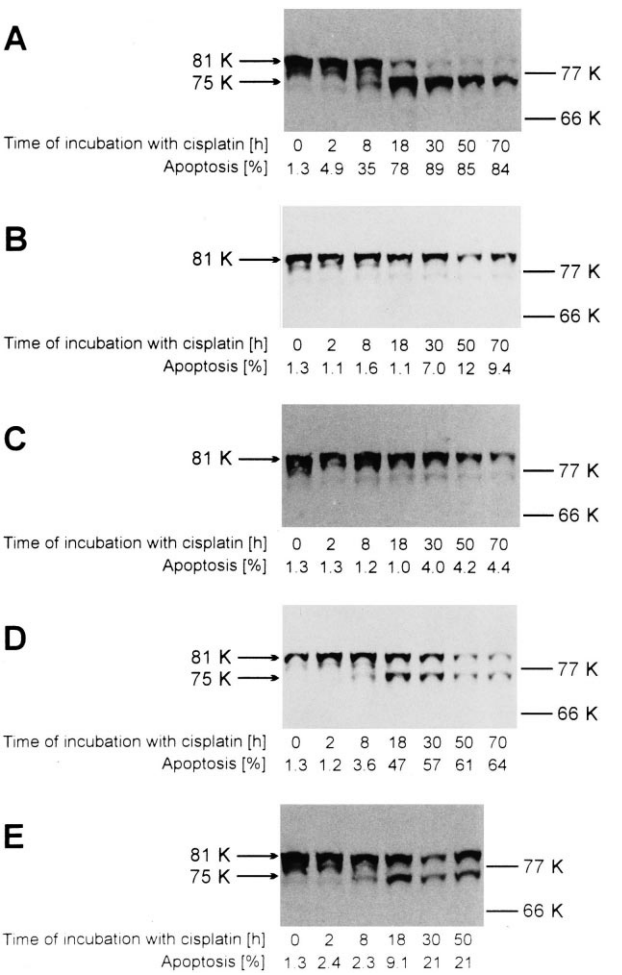

Fig. 2. Inhibition of γ catenin cleavage and apoptosis with caspase inhibitors. Ad2 E1A+N-*ras* HER 313A cells were treated with caspase inhibitors (40 μM) and then with cisplatin (20 μg/ml). Cells were harvested at the indicated times and percentage apoptosis determined by acridine orange staining. Aliquots (containing 50 μg protein) were subjected to Western blotting using antibody against γ catenin. The position of migration of molecular weight markers is shown. The following caspase inhibitors were used: A, no inhibitor; B, Boc-Asp-FMK; C, Z-VAD-FMK; D, Z-DEVD-FMK; E, Z-YVAD-FMK.

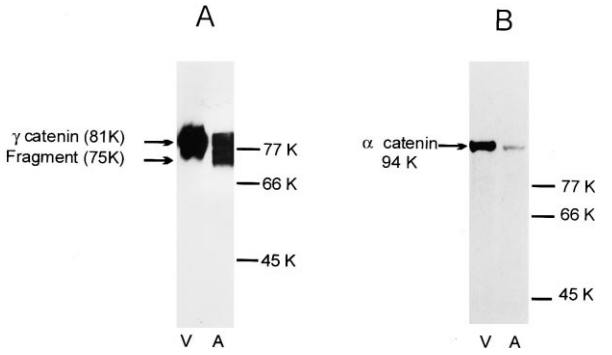

Fig. 3. Co-immunoprecipitation of α and γ catenin. Ad2 E1A+N-*ras* HER 313A cells were treated with cisplatin (20 μg/ml) for 16 h. Treated (apopototic) and untreated (viable) cells were harvested and solubilised. Immunoprecipitation was carried out with antibodies against α (A) and β (B) catenin (Sigma) as described. Immunoprecipitated proteins were fractionated by SDS-PAGE and then electrophoretically transferred to nitrocellulose membranes which were incubated with antibodies raised against γ catenin (A) and α catenin (B). Immunoprecipitated proteins are indicated. The position of molecular weight markers is shown. V, viable cells; A, apoptotic cells.

3.3. Interaction of γ and α catenin in apoptotic cells

The interactions of α catenin with β and γ catenin are now well-established. It has been shown that the 75 kDa β catenin polypeptide formed by caspase activity is unable to bind to α catenin and this has been suggested as a possible cause of disruption of adherens junctions [12]. It would be expected that the 75 kDa γ catenin product formed early in apoptosis would still be able to interact with α catenin as the binding site on γ catenin is between amino acids 109 and 137 [17]. To test the validity of this suggestion α catenin from viable and apoptotic Ad2 E1A+N-*ras* HER 313A cells was immunoprecipitated using the protocol described in Section 2. Bound γ catenin was detected by Western blotting of these immunoprecipitates (Fig. 3A). It can be seen that degradation products of γ catenin were precipitated with α catenin from apoptotic cells, confirming the interaction. Uncleaved γ catenin present in the apoptotic cells was also precipitated. When β catenin was immunoprecipitated from apoptotic cell lysates very little α catenin could be detected (Fig. 3B). Appreciable α catenin could, however, be co-immunoprecipitated with β catenin from viable cells.

3.4. Distribution of γ catenin in apoptotic cells

The morphological changes associated with apoptosis in adherent mammalian cells have previously been carefully analysed. There is a loss of cell-cell and cell-substratum contacts as cells condense and form apoptotic bodies. Collapse of cytoskeletal networks formed by, for example, actin and vimentin is marked such that the filamentous distribution of the proteins is lost with vimentin being concentrated in spherical granules in apoptotic cells ([12] and our unpublished data). In viable cells α and β catenins are normally present in adherens junctions (containing catenin-cadherin complexes) at the cell periphery playing a major role in cell-cell contacts. Staining with antibodies against α and β catenin shows both proteins concentrated primarily around the plasma membrane in viable Ad2 E1A+N-*ras* HER 313A cells (Fig. 4A,E). However, treatment with cisplatin leads to loss of staining of both proteins at the cells periphery and their translocation to the cytoplasm as discrete aggregates as cellular condensation and disruption of the chromatin occurs (Fig. 4B–D,F–H). These changes are readily apparent 3 h after addition of cisplatin (panels B and F). Distribution of γ catenin in viable cells is somewhat different to that seen for α and β catenin (Fig. 4A,E,I; Fig. 5A). Although there is some staining around the periphery of the cell, presumably at the sites of attachment for intermediate filaments (desmosomes) and adherens junctions, a considerable proportion of the γ catenin appears as cytoplasmic granules which are predominantly perinuclear (Fig. 4I), possibly in the endoplasmic reticulum (Fig. 5A). In the apoptotic cells shown in Fig. 4J–L the γ catenin is further concentrated into granules juxtaposed to the nuclei. Again, redistribution of γ catenin is obvious within 3 h of cisplatin treatment (Fig. 4J). The cells shown in Fig. 4B,C,F,G,J,K have changed morphology but few can be classified as apoptotic by acridine orange staining (Fig. 1A). The cells shown in Fig. 4D,H,L are, however, more obviously apoptotic as demonstrated by acridine orange staining (Fig. 1A) and changes in the DAPI-stained nuclei.

When Ad2 E1A+N-*ras* HER 313A cells were treated with cisplatin in the presence of a caspase inhibitor (Z-VAD-FMK) very little change in distribution of catenins was observed. At 10 h much of the γ catenin was still located around the cell periphery (Fig. 5A,C). Z-VAD-FMK alone had no effect on cellular morphology (Fig. 5B). No change in distribution of α and β catenins was observed when cells were treated with cisplatin and Z-VAD-FMK (data not shown).

4. Discussion

It is now clear that morphological changes associated with apoptosis are achieved through widespread disruption of the cytoskeleton and that many cytoskeletal proteins are the substrates for caspases (usually caspase 3). Recently, proteolytic degradation by caspases of β catenin [12], cadherin (our unpublished data), keratins [15], vimentin ([15] and our unpublished data), FAK [18] and actin [19] has been demonstrated. Here we have shown that γ catenin serves as a further substrate for caspases. The major product of caspase activity was a polypeptide of about 75 kDa (Fig. 1). A potential caspase 3 cleavage site occurs in γ catenin between amino acids 693 and 696 (DDMD) which would give rise to a product similar in size to that observed (Table 1). This motif contains an aspartic acid residue at the P_4 position which is considered to be a requirement for caspase 3 cleavage. Indeed, it is worth noting that there is slightly better inhibition of γ catenin proteolysis at earlier (18 h) times by Z-DEVD-FMK (an inhibitor of the caspase 3 family) than by the other caspase inhibitors. However, it is not possible, on the basis of the in vivo inhibitor studies shown in Fig. 2, to reach any firm conclusions as to what caspases may be involved in the cleavage of a particular protein. Lack of specificity of the inhibitors and marked differences in uptake by cells preclude definitive conclusions.

No further cleavage products of γ catenin were observed after 3 days (Fig. 2 and data not shown), even though other potential sites of caspase cleavage are present in the protein (Table 1). However, there is a reduction in the level of the full-length protein in the presence of caspase inhibitors Boc-Asp-FMK and Z-VAD-FMK without a corresponding increase in level of the 75K proteolytic product (seen in apoptotic cells without inhibitor). This may be due to the production of other polypeptides which are not recognised by the monoclo-

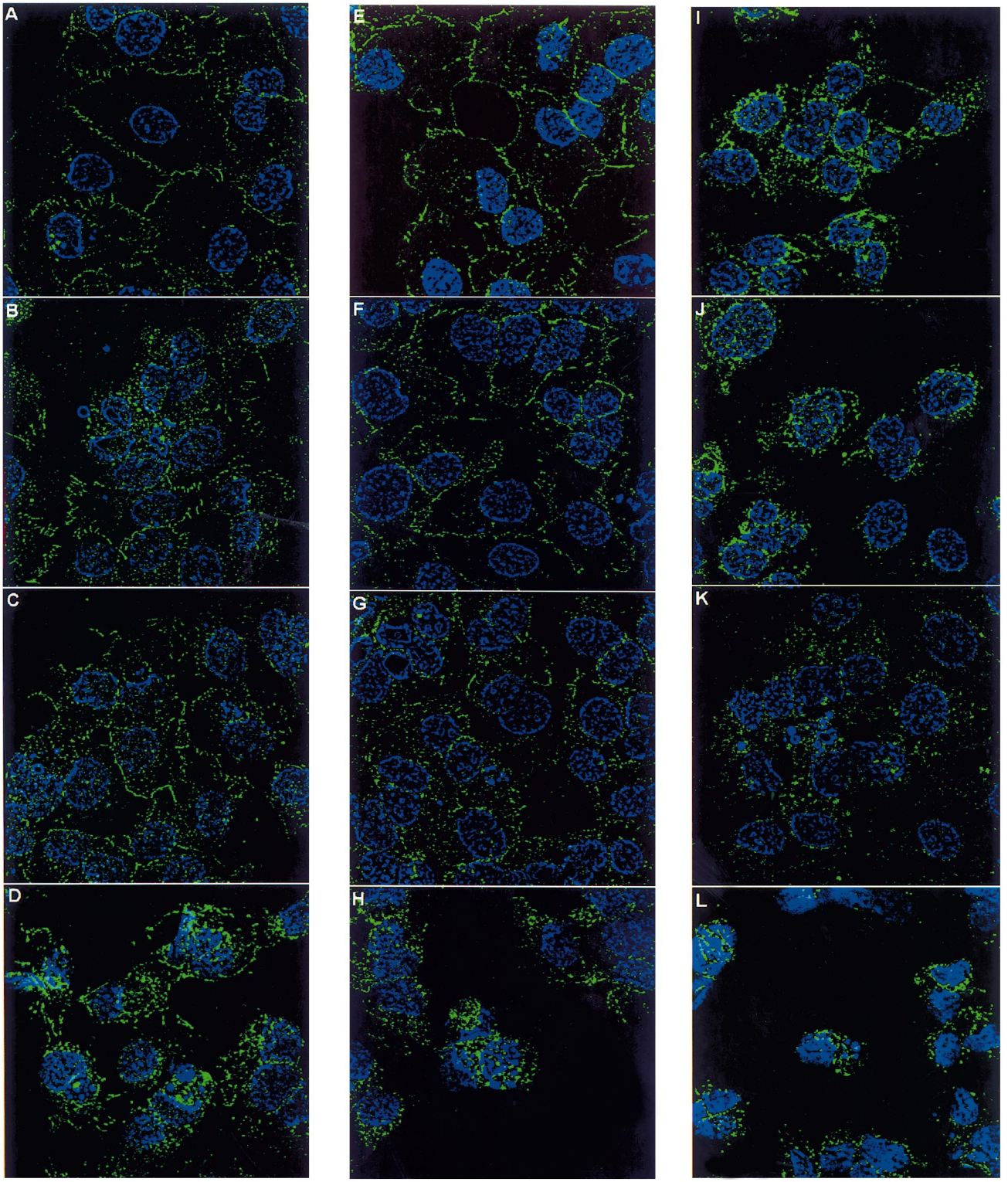

Fig. 4. Subcellular distribution of catenins in viable and apoptotic cells. Ad2 E1A+N-*ras* HER 313A cells were grown on multiwell slides, incubated with cisplatin and then fixed with paraformaldehyde. Cells were stained for α catenin, β catenin or γ catenin (green) and for DNA with DAPI (blue). Cells were viewed by confocal microscopy (see Section 2). A, E, I: Viable cells (not treated with cisplatin); B, F, J: treated with cisplatin for 3 h; C, G, K: treated for 6 h; D, H, L: treated for 10 h. A–D: Stained for α catenin (green); E–H: stained for β catenin (green); I–L: stained for γ catenin (green). (Magnification objective ×20.)

nal antibody (clone 15F11) used in these studies or to a widespread proteolysis of the protein giving rise to very small fragments. Removal of the N- or C-terminal domains from γ catenin has recently been shown to affect interactions with other proteins, with the C-terminus regulating the lengths of desmosomes [20]. It seems likely that the proteolysis of γ catenin described here could play a role in disassembly of adherens junctions, adhesive junctions and desmosomes. However,

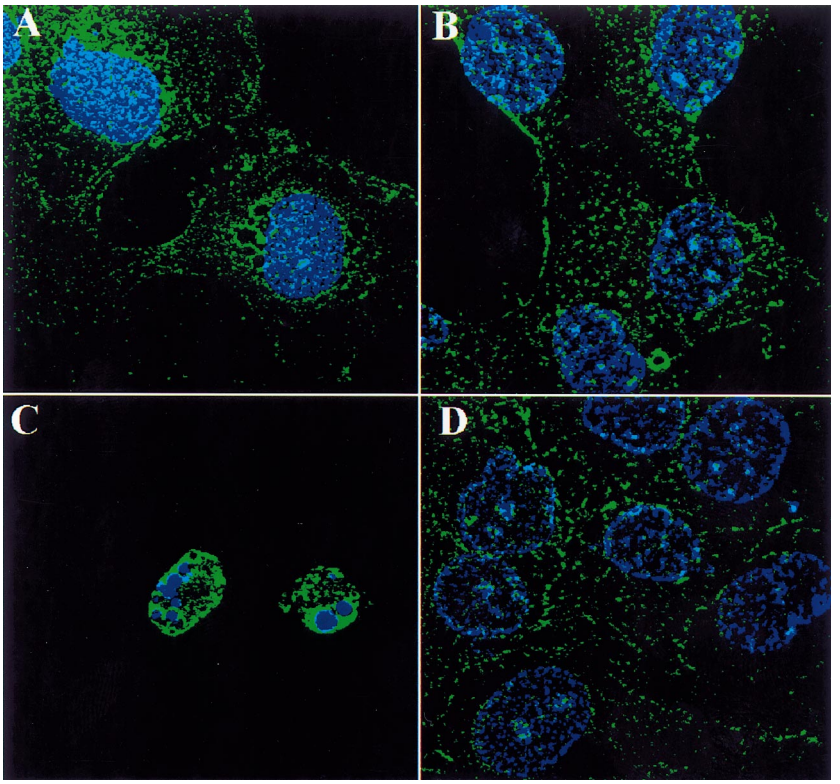

Fig. 5. Subcellular distribution of γ catenin in cells treated with cisplatin and capase inhibitor. Ad2 E1A+N-*ras* HER 313A cells were grown on multiwell slides and incubated with cisplatin in the presence or absence of Z-VAD-FMK. After 10 h cells were fixed with paraformaldehyde and stained for γ catenin (green) and for DNA with DAPI (blue). Cells were viewed by confocal microscopy. A: Viable cells (not treated with cisplatin); B: treated with Z-VAD-FMK only; C: treated with cisplatin; D: treated with Z-VAD-FMK and cisplatin. (Magnification objective ×60.)

loss of 47 amino acids from the C-terminus (or indeed a comparably sized fragment from the N-terminus) would not affect binding to α catenin, the binding site for which has been mapped to a region between amino acids 109 and 137 [17]. This has been confirmed by the co-immunoprecipitation data presented in Fig. 3 where it can be seen that both full-length and truncated γ catenin are precipitated in a complex with α catenin. Notably caspase-mediated cleavage of β catenin during apoptosis removes the α catenin binding site [12]. This is consistent with the data shown in Fig. 3B where there is relatively little β catenin co-immunoprecipitated with the α component. It is possible that caspase-mediated degradation of β catenin in apoptotic cells is of major importance in the disassembly of the adherens junctions, whilst proteolysis of γ catenin plays a more central role in the fate of desmosomes where α and β catenin are absent (reviewed, for example, in [21]).

We have observed similar degradation of β catenin to that reported earlier [12]. It is interesting, however, to note that α catenin is largely unaffected during apoptosis, even at very late times (Fig. 1B). This is somewhat surprising as several potential caspase cleavage sites exist within the protein (Table 1). It seems likely that factors other than the short tetrapeptide sequence must play a part in governing caspase-mediated cleavage of some proteins during apoptosis.

The appreciable changes in localisation of the catenins seen in Fig. 4 occur in the early stages of apoptosis (within 3 h) when caspase-mediated degradation is not detectable (Fig. 1A). For example, the vast majority of β catenin and γ catenin are still full-length at 10 h. In the presence of a caspase inhibitor no change in the location of the catenins was observed (Fig. 5) with the distribution of the proteins being very similar in viable cells and those treated, for 10 h, with cisplatin and Z-VAD-FMK (Fig. 5 and data not shown). It seem likely therefore that the disruption of adherens junctions and desmosomes and re-localisation of the catenins to the cytoplasm does not require the proteolysis of appreciable amounts of β and γ catenin within the cell. However, it is also clear that caspase activity directed against, as yet, unidentified proteins is essential for morphological change (Fig. 5). Determination of the nature of these substrates will have to await further investigation.

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