Cleavage of the Actin-capping Protein α-Adducin at Asp-Asp-Ser-Asp<sup>633</sup>-Ala by Caspase-3 Is Preceded by Its Phosphorylation on Serine 726 in Cisplatin-induced Apoptosis of Renal Epithelial Cells*

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Decreased phosphorylation of focal adhesion kinase and paxillin is associated with loss of focal adhesions and stress fibers and precedes the onset of apoptosis (van de Water, B., Nagelkerke, J. F., and Stevens, J. L. (1999) J. Biol. Chem. 274, 13328–13337). The cortical actin cytoskeletal network is also lost during apoptosis, yet little is known about the temporal relationship between altered phosphorylation of proteins that are critical in the regulation of this network and their potential cleavage by caspases during apoptosis. Adducins are central in the cortical actin network organization. Cisplatin caused apoptosis of renal proximal tubular epithelial cells, which was associated with the cleavage of α-adducin into a 74-kDa fragment; this was blocked by a general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk). Hemagglutinin-tagged human α-adducin was cleaved into a similar 74-kDa fragment by caspase-3 in vitro but not by caspase-6 or -7. Asp-Arg-Val-Asp<sup>25</sup>Glu, Asp-Ile-Val-Asp<sup>208</sup>-Arg, and Asp-Asp-Ser-Asp<sup>633</sup>-Ala were identified as the principal kDa fragment by caspase-3

Cleavage of the Actin-capping Protein α-Adducin at Asp-Asp-Ser-Asp<sup>633</sup>-Ala by Caspase-3 Is Preceded by Its Phosphorylation on Serine 726 in Cisplatin-induced Apoptosis of Renal Epithelial Cells*

Apoptosis or programmed cell death is critical for tissue development and homeostasis (1). Uncontrolled apoptosis, which may occur after exposure to toxic chemicals, is a pathophysiological process associated with various human diseases (2, 3). Commitment to apoptosis requires activation of caspases, a family of aspartate-directed cysteinyl-containing proteases (4). The caspase family consists of at least 14 different proteases that can be subdivided based on sequence homology into caspase-1- and caspase-3-like caspases and functionally into upstream caspases (caspases-8, -9, and -10) and terminal executioner caspases (caspases-3, -6, and -7) (4). The caspase-3 subgroup is considered to be critical in the autolytic phase of apoptosis (4). Thus, proteolytic activation of caspase-3 by either of the upstream caspases-8 or -9 results in cleavage of proteins at DXXD sites by caspase-3 (4–6). A variety of proteins, including poly(ADP-ribose) polymerase, DNA-dependent protein kinase, Rh, Mdm2, Bcl-2, and protein kinase C, have been identified as substrates (4). It has been suggested that the net gain or loss of function caused by proteolysis of caspase substrates is required for progression of the apoptotic program. In this respect, it has been proposed that caspase-3-mediated cleavage of cytoskeletal (-associated) components, such as lamin A (7, 8), gelsolin (9, 10), actin (11, 12), focal adhesion kinase (13–15), GAS-2 (16), and fodrin (17–19) may actually initiate morphological alterations observed during apoptosis, i.e. cell shrinkage, loss of cell-matrix and cell-cell interactions and formation of apoptotic bodies.

Cell-matrix and cell-cell interactions provide survival signals (20–23), including activation of phosphoinositide-3 kinase and protein kinase B/Akt signaling cascades (24–26). The actin cytoskeletal network is important in maintaining these interactions, whereas loss of cell-cell and cell-matrix interactions signals caspase activation and apoptosis (20–23, 27, 28). In a recent study (15) we showed that during chemically induced apoptosis of rat proximal tubule epithelial (RPTE) cells, loss of actin cytoskeletal organization and cell-matrix interactions occurs independent of caspase activity. Disruption of the F-actin cytoskeletal network by xenobiotics and loss of both focal adhesion kinase and paxillin from the focal adhesion occurred before caspase activation and was not blocked by caspase inhibitors but was associated with loss of tyrosine phosphorylation of both proteins (15). Caspase cleavage of focal adhesion kinase occurred later (15). The data suggested a model in which loss of focal adhesion integrity results from toxicant-induced changes in signaling. Although caspase-

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1 The abbreviations used are: RPTE cells, rat proximal tubule epithelial cells; Ac-DEVDD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; AMC, 7-amino-4-methylcoumarine; PKA, protein kinase A; PKC, protein kinase C; z-VAD-fmk, benzyloxycarbonyl-Val-Asp-fluoromethyl ketone; HA, hemagglutinin; PBS, phosphate-buffered saline; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfoic acid; PAGE, polyacrylamide gel electrophoresis; MSB, microtubule stabilization buffer; CSK, cytoskeletal.
Phosphorylation and Cleavage of α-Adducin during Apoptosis

**EXPERIMENTAL PROCEDURES**

**Materials**

Dulbecco’s modified Eagle’s medium/Ham’s F-12, α-modified minimal essential medium with ribonucleosides and deoxyribonucleosides, penicillin/streptomycin/amphotericin, and trypsin/EDTA were from Life Technologies, Inc. Fetal calf serum was from Bodinco (Alkmaar, The Netherlands). Bovine serum albumin fraction V, chola toxin, insulin, AMC, doxorubicin, staurosporin, and cisplatin were from Sigma. Epidermal growth factor was from Upstate Biotechnology Inc. (Lake Placid, NY). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk), acetyl-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO), acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (Ac-YVAD-AMC), acetyl-Val-Ile-Asp-7-amino-4-methylcoumarin (Ac-VEID-AMC), calpain inhibitor I and II were from Bachem (Bubendorf, Switzerland). Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVAD-AMC) was from Research Biochemicals International (Natick, MA).

**Plasmids**

An expression vector for hemagglutinin (HA)-tagged human α-adducin (amino acids 1–737; pEF-BOS-HA-α-adducin) was kindly provided by Dr. K. Kaibuchi and described elsewhere (33). For site-directed mutagenesis, the α-adducin-coding region was subcloned into pZErO (Invitrogen) using KpnI. For mutation by polymerase chain reaction of amino acid residue D to A at the following caspase-3 cleavage sites in human α-adducin, the QuickChange mutagenesis kit (Stratagene) was used with the following primers: Asp<sup>29</sup> → Ala, forward primer 5'-GTTACTTCGAGCGAGTAGCTGAGAACAACCC-3' and reverse primer 5'-GGTGTGTTTCTCAGCAGCTACCTCGTCGCAAGTACC-3'; Asp<sup>23</sup> → Ala, forward primer 5'-GGTACTTCGAGCGAGTAGCTGAGAACAACCC-3' and reverse primer 5'-GGTGTGTTTCTCAGCAGCTACCTCGTCGCAAGTACC-3'; Asp<sup>25</sup> → Ala, forward primer 5'-GGGTTGTTCTCAGCAGCTACCTCGTCGCAAGTACC-3'; Asp<sup>33</sup> → Ala, forward primer 5'-GGGTTGTTCTCAGCAGCTACCTCGTCGCAAGTACC-3'. The correct sequences after mutation were confirmed by DNA cycle sequencing. Mutant α-adducin was subcloned back into pEF-BOS. Correct expression of HA-α-adducin and the different Asp → Ala mutants was checked by transient overexpression in COS1 cells followed Western blotting and immunofluorescent staining.

**Cell Culture and Treatment**

RPTE cells were isolated by collagenase perfusion and separated by density centrifugation using Nycodenz as described (54, 55). Cells were cultured on rat tail collagen (Collaborative Research, Bedford, MA), coated dishes in Dulbecco’s modified Eagle’s medium/F-12 containing 1% v/v fetal bovine serum, 10 μg/ml insulin, 10 ng/ml epidermal growth factor, 10 ng/ml chola toxin, and antibiotics as described (complete medium A; Refs. 15 and 56). Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide and fed every other day. RPTE cells were used after they had reached confluence, 6 to 9 days after plating.

For experiments, confluent monolayers of RPTE cells in 24-well dishes containing coated glass coverslips, 6-well or 10-cm dishes (Corning Costar, Acton, MA), were washed with Earle’s balanced salt solution (127 mM NaCl, 5 mM KCl, 0.8 mM MgSO<sub>4</sub>·7 H<sub>2</sub>O, 0.4 mM Na<sub>2</sub>HPO<sub>4</sub>·H<sub>2</sub>O, 1.3 mM CaCl<sub>2</sub>, 4 mM NaHCO<sub>3</sub>, 25 mM HEPES, 5 mM d-glucose, pH 7.4, in a final volume of 1, 2, or 10 ml, respectively, for 8 h. In some experiments the general caspase inhibitor z-VAD-fmk (100 μM; 100 μM stock in Me<sub>2</sub>SO) was added simultaneously with caspase. Following treatment with caspase for 8 h, cells were allowed to recover in complete medium in the presence or absence of z-VAD-fmk.

**Determination of Cell Death**

**Cell Cycle Analysis—Apoptosis** was determined by cell cycle analysis as described (15). Briefly, floating as well as adherent cells that were trypsinized were fixed in 90% ethanol (−20 °C). After washing cells twice with PBS, 1 mM EDTA cells were resuspended in PBS-EDTA containing 7.5 μM propidium iodide and 10 μg/ml RNase A. After a 30-min incubation at room temperature, the cell cycle was analyzed by flow cytometry (FACS-Calibur, Becton Dickinson), and the percentage of cells present in sub-G<sub>0</sub>/G<sub>1</sub> was calculated using the Cellquest software (Becton Dickinson).

**Lactate Dehydrogenase Release**—Cell death was measured by the release of lactate dehydrogenase from cells in the culture medium as described (57). The percentage cell death was calculated from the amount of lactate dehydrogenase release caused by treatment with...
toxins relative to the amount that was released by 0.1% Triton X-100, i.e. 100% release.

Caspase Activity—Briefly, attached and detached cells were harvested and collected by centrifugation as above. The cell pellet was taken up in lysis buffer (10 mM HEPES, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM MgCl₂, and 5 mM EGTA) and subjected to 3 cycles of freezing and thawing. Equal amounts of cell proteins were used in a caspase assay using Ac-DEVD-AMC, Ac-YVAD-AMC, or Ac-VEID-AMC (25 μM; Research Biochemicals) as a substrate. Fluorescence derived from release of the AMC moiety was followed using a fluorescence plate reader (HTS 7000 Bio assay reader; Perkin-Elmer). Caspase activity was calculated as pmol/min/mg of cell protein using AMC as a standard.

In Vitro Caspase-Substrate Cleavage Assay

For transfection, 2 × 10⁵ COS1 cells were plated in 6-well culture clusters. After overnight culturing, COS1 cells were transfected with 0.4 μg of pEF-BOS-α-adducin, pEF-BOS-α-adducin-Asp³⁰⁰→Ala, pEF-BOS-α-adducin-Asp³⁰⁰→Ala, or pEF-BOS-α-adducin-Asp³⁰⁰→Ala using LipofectAMINEPlus (Life Technologies, Inc.) according to the manufacturer’s procedures. After 24 h, cells were harvested in TSE buffer plus inhibitors (10 mM Tris/HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) followed by sonication (3 × 5 s). Protein was determined using the Bradford reagent (Bio-Rad). 15 μg of COS1 cell lysate was dissolved in caspase assay buffer (50 mM HEPES, 2 mM EDTA, 0.1% (w/v) CHAPS, 10% (w/v) sucrose, pH 7.0, containing 10 mM diithiothreitol, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 20 μg/ml pepstatin, and 20 mM phenylmethylsulfonyl fluoride). After the addition of 0.1 μg of recombinant human caspase-3, 6, or 7 (gift from D. Nicholson and N. Thornberry, Merck Frosst, Montreal, Canada), the reaction mixture was incubated for 1 h at 37 °C. Where indicated, 5 μM z-VAD-fmk, 5 μM Ac-DEVD-CHO, or 5 μM calpain inhibitor II was also added. The cleavage reaction was stopped by the addition of Laemmli sample preparation buffer followed by heating for 5 min at 95 °C. Samples were separated for 4 min at room temperature. The homogenate was centrifuged at 85,000 × g for 20 min at 15 °C. The supernatant (soluble fraction) was removed, and the pellet (cytoskeletal fraction) was resuspended in SDS-PAGE buffer. Equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Blots were blocked with 5% w/v nonfat dry milk in TBS-T (0.5M NaCl, 20 mM Tris-HCl, 0.05% v/v Tween 20, pH 7.4) and probed with anti-poly(ADP-ribose) polymerase (monoclonal C2.10, Enzyme System Products, Dublin, CA), anti-α-adducin, and anti-γ-adducin (both polyclonal; Refs. 43 and 58), anti-fodrin (polyclonal, kindly provided by Dr. W. Nelson), anti-HA (clone 3F10, Roche Molecular Biochemicals), or anti-focal adhesion kinase (Transduction Laboratories) followed by incubation with horseradish peroxidase-coupled secondary antibodies (Jackson Laboratories). Visualization was done with the ECL reagent (Amersham Pharmacia Biotech).

Immunofluorescence and Immunoblotting

Cells were scraped in ice-cold PBS and pooled with medium containing floating cells. After centrifugation (5 min, 500 × g, 4 °C), the pellet was resuspended in TSE plus inhibitors. Protein concentration in the supernatant was determined using a fluorescence plate reader (HTS 7000 Bio assay reader; Perkin-Elmer). Caspase activity was calculated as pmol/min/mg of cell protein using AMC as a standard.

Preparation of Cytoskeletal and Soluble Cellular Fractions

To obtain soluble and cytoskeletal fractions, the Triton X-100 extraction method was used (41, 58). Briefly, RPTE cells cultured in 10-cm dishes were treated with cisplatin as above. The medium was removed, and adherent cells were washed twice with PBS and a final wash with microtubule stabilization buffer (MSB; 100 mM PIPES, 2 mM glycerol, 1 mM EGTA, 1 mM magnesium acetate, pH 6.9). Adherent cells were scraped in 500 μl of MSB buffer containing protease and phosphatase inhibitors and 0.2% (w/v) Triton X-100 (MSB Plus). Floating cells were collected from the pooled washes by centrifugation for 5 min at 500 × g. Pelleted cells were mixed with the adherent cells in MSB buffer. Cells were extracted for 4 min at room temperature. The homogenate was centrifuged at 85,000 × g for 20 min at 15 °C. The supernatant (soluble fraction) was removed, and the pellet (cytoskeletal fraction) was resuspended in MSB Plus. Equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Blots were blocked with 5% w/v nonfat dry milk in TBS-T (0.5M NaCl, 20 mM Tris-HCl, 0.05% v/v Tween 20, pH 7.4) and probed with anti-poly(ADP-ribose) polymerase (monoclonal C2.10, Enzyme System Products, Dublin, CA), anti-α-adducin, and anti-γ-adducin (both polyclonal; Refs. 43 and 58), anti-fodrin (polyclonal, kindly provided by Dr. W. Nelson), anti-HA (clone 3F10, Roche Molecular Biochemicals), or anti-focal adhesion kinase (Transduction Laboratories) followed by incubation with horseradish peroxidase-coupled secondary antibodies (Jackson Laboratories). Visualization was done with the ECL reagent (Amersham Pharmacia Biotech).

Immunofluorescence and Imaging Techniques

For immunofluorescence studies, RPTE cells were cultured on collagen-coated glass coverslips in 24-well dishes. After treatment with cisplatin, cells were fixed with 3.7% formaldehyde for 10 min followed
cisplatin-induced apoptosis, (Fig. 2). z-VAD-fmk also prevented the loss of intact caspase activity, RPTE cells were treated with cisplatin (100 \mu M) in the absence or presence of z-VAD-fmk (100 \mu M) for 8 h and allowed to recover for another 16 h in complete culture medium with or without z-VAD-fmk (100 \mu M). Thereafter samples were taken for further analysis by Western blotting for \alpha-adducin, fodrin, poly-(ADP-ribose) polymerase (PARP), and focal adhesion kinase (FAK) cleavage as described under “Experimental Procedures.” Apoptosis was determined by quantitation of the percentage of cells with sub-G0/G1 DNA content by cell cycle analysis. Data shown are representative for four independent experiments (n = 4).

by 3 washes with PBS. After cell lysis and blocking with PBS, 0.2% w/v Triton X-100, 0.5% w/v bovine serum albumin, pH 7.4 (PTB), cells were stained for \alpha-adducin (5 \mu g/ml), \gamma-adducin (5 \mu g/ml), or fodrin (10 \mu g/ml) diluted in PTB. During the staining period of the cells with secondary Alexa488-labeled goat anti-rabbit antibody (Molecular Probes, Eugene, OR), the F-actin cytoskeletal network was labeled with rhodamine-phalloidin at 0.3 units/ml (Molecular Probes). Cells were mounted on glass slides using Aqua-Poly/Mount (Polysciences Inc., Warrington, PA). Cells were viewed using a Bio-Rad 600 MRC confocal laser scanning microscope.

RESULTS

\alpha-Adducin Is Cleaved during Apoptosis—Exposure to the nephrotoxicant cisplatin for 8 h followed by recovery in complete medium resulted in a time- and concentration-dependent cell death in RPTE cells (Fig. 1A). Cell death was associated with markers of apoptosis, including an increase in the percentage of cells with hypodiploid (sub-G0/G1) DNA content and an increase in caspase-3-like activity (Fig. 1, B and C). In addition, poly(ADP-ribose) polymerase, a prototypical caspase substrate, was cleaved to the signature 85-kDa fragment (Fig. 1D). In a similar fashion \alpha-adducin was cleaved into a ~74-kDa fragment in both a time- and concentration-dependent manner (Fig. 1, D and E). Although \alpha-adducin was cleaved during cisplatin-induced apoptosis, \gamma-adducin, the other major adducin in epithelial cells, was not (Fig. 1D).

\alpha-Adducin Cleavage Is Dependent on Caspase Activity—The fact that adducin cleavage coincided with an increase in caspase activity and cleavage of other known caspase substrates (i.e. the cytoskeletal protein fodrin and poly(ADP-ribose) polymerase) suggested that \alpha-adducin is a novel caspase substrate. To test whether \alpha-adducin cleavage was dependent on caspase activity, RPTE cells were treated with cisplatin with or without the general caspase inhibitor z-VAD-fmk. As expected, apoptosis as well as poly(ADP-ribose) polymerase and focal adhesion kinase cleavage was blocked by z-VAD-fmk (Fig. 2). z-VAD-fmk also prevented the loss of intact \alpha-adducin and accumulation of the 74-kDa fragment (Fig. 2). Fodrin interacts with \alpha-adducin and is cleaved by caspases and calpain in other cell types (18, 19, 37, 39). In RPTE cells, cisplatin caused cleavage of fodrin into 145-kDa and 120-kDa fragments (Fig. 2). Formation of the 120-kDa fragment was blocked by z-VAD-fmk, but the 145-kDa fragment was still formed. Formation of the latter is dependent on calpains (19).

\alpha-Adducin Is Cleaved by Caspase-3 but Not -6 and -7—The caspase family consists of “upstream caspases” such as caspase-8 and -9, which activate the “downstream” executioner caspases including caspase-3, -6, and -7 (4). To investigate which of the executioner caspases are capable of cleaving \alpha-adducin, we prepared extracts of COS1 cells expressing a human HA-tagged \alpha-adducin. Recombinant caspase-3, but not caspase-6 and -7, cleaved human \alpha-adducin in cell extracts into a fragment of approximately 74 kDa (Fig. 3), the same molecular mass fragment found in apoptotic cells (see Figs. 1 and 2). In the in vitro cleavage reaction, a fragment of around 78 kDa was also present (Fig. 3A). Caspase-3, but not caspase-6 and -7, cleaved endogenous fodrin into a fragment with a molecular mass of 120 kDa, identical to that observed in RPTE cells (Fig. 3). Despite the inability of recombinant caspase-6 and -7 to cleave \alpha-adducin, these caspases cleaved fluorescent-labeled peptide substrates in in vitro protease assays (data not shown). Altogether, the data indicate that \alpha-adducin is a substrate for caspase-3, and not caspase-6 or -7.

Calpains are calcium-dependent proteases that are also activated during apoptosis and cleave cytoskeletal proteins, including fodrin (Ref. 19; see above). It seemed possible that cleavage of \alpha-adducin by caspase-3 in vitro might involve a pathway whereby caspase-3 would activate calpains and indirectly mediate cleavage of \alpha-adducin. To exclude this possibility we treated cell extracts with caspase-3 either in the absence or presence of calpain inhibitors or caspase-3 inhibitors. Cleavage of \alpha-adducin by caspase-3 was inhibited by both z-VAD-fmk and Ac-DEVD-CHO; however, calpain inhibitor I did not block the caspase-3-mediated processing of \alpha-adducin (Fig. 3). In intact RPTE cells, both calpain inhibitor I as well as calpain inhibitor II were unable to prevent formation of the 74-kDa \alpha-adducin fragment after exposure to cisplatin. In fact, calpain inhibitors themselves caused apoptosis of RPTE cells, which was associated with activation of caspases and cleavage of \alpha-adducin (data not shown). Thus, caspase-3-mediated cleavage is independent of calpain activity and is, most likely, directly mediated by caspase-3.

Since the data suggested that caspase-3 is responsible for cleavage of \alpha-adducin, we focused on potential caspase-3 cleavage sites in \alpha-adducin. Incubation of HA-tagged \alpha-adducin with caspase-3 resulted in the formation of two major cleavage products of around 74 and 78 kDa (see above). However, with prolonged exposure, several minor fragments of approximately of 26, 50, and 60 kDa were also observed on autoradiograms (Fig. 4A). Since the HA-tag contains a caspase-3 cleavage site, staining for HA was not possible, making identification of N-terminal cleavage fragments difficult. To circumvent this, we treated HA-\alpha-adducin with caspase-3 after immunoprecipitation with anti-HA antibody to shield the HA tag. After immunoprecipitation, HA-\alpha-adducin was cleaved by caspase-3 into 2 HA-containing fragments of 28 kDa and 78 kDa (Fig. 4B). Formation of all the above fragments was blocked by incubation with Ac-DEVD-CHO (Fig. 4, A and B). Thus, caspase-3 cleaves \alpha-adducin in two major (74 and 78 kDa) and 4 minor fragments (26, 28, 50, and 60 kDa).

The consensus caspase-3 cleavage site is DEVD. The two aspartic acid residues are absolutely required, but the glutamic acid and valine residues may vary; therefore, any DXDX is a potential site (4). Comparison of rat and human \alpha-adducin revealed three conserved DXDX sequences; two in the N-terminal region at Asp-Arg-Val-Asp\textsuperscript{208}-Glu and Asp-Ile-Val-Asp\textsuperscript{208}-Arg and one in the C terminus at Asp-Xaa-Ser-Asp\textsuperscript{633}-Ala (Fig. 5). These cleavage sites are unique for \alpha-adducin and...
are not present in either β- or γ-adducin (Fig. 5). Cleavage of α-adducin at the N-terminal Asp-Arg-Val-Asp29 or Asp-Ile-Val-Asp208 and the C-terminal Asp-Xaa-Ser-Asp633 sites would result in 9 different fragments with the expected masses shown in Fig. 4C. The 74-kDa major product found in apoptotic cells is consistent with a fragment containing amino acids 30–633. The 4-kDa fragment is not detectable due to its small size but would account for a 78-kDa fragment that contains the 74-kDa product. Of the possible smaller fragments, the predicted 24-kDa fragment cannot be detected because it lacks an epitope recognizable by the α-adducin antibodies used herein. The 12-kDa fragment was not visible and may not be resolved from the 26-kDa fragment due to altered electrophoretic mobility and/or inability to separate these fragments on the gel system used. Regardless, the sequence data indicate that Asp-Arg-Val-Asp29 and Asp-Xaa-Ser-Asp633 are likely cleavage sites for the generation of the major 74-kDa fragment seen in apoptotic cells.

**Asp-Ser-Asp-Ala Is the Key Caspase-3 Cleavage Site in α-Adducin**—To investigate the role of these DXXD sites in α-adducin, COS-1 cells were transfected with 0.4 μg of pEF-BOS-HA-α-adducin. A, 15 μg of total cell lysate was incubated with 100 ng of human recombinant (rh) caspase-3, -6, or -7 as described under "Experimental Procedures," and cleavage of α-adducin, γ-adducin, and fodrin was analyzed by Western blotting. B, 15 μg of total cell homogenate of COS-1 cells expressing HA-α-adducin was incubated with 100 ng of human recombinant caspase-3 in the presence or absence of calpain inhibitor I (5 μM), Ac-DEVD-CHO (5 μM), or z-VAD-fmk (5 μM), and cleavage of α-adducin was determined by Western blotting using the anti-α-adducin antibody.

**Fig. 3. Caspase-3 but not -6 and -7 cleaves HA-tagged α-adducin.** COS-1 cells were transfected with 0.4 μg of pEF-BOS-HA-α-adducin. A, 15 μg of total cell homogenate was incubated with caspase-3 (100 ng), and α-adducin fragments were detected by Western blotting using the clone45 anti-α-adducin polyclonal antibody. B, 25 μg of total cell homogenate was immunoprecipitated (IP) with anti-HA antibody (clone 12CA5) followed by incubation with caspase-3 (100 ng) either in the absence or presence of Ac-DEVD-CHO (5 μM). HA tag-containing fragments were detected by Western blotting using rat anti-HA antibody (clone 3F10).

**Fig. 4. Caspase-3 cleavage products of HA-tagged human α-adducin.** A, 15 μg of total cell homogenate was incubated with caspase-3 (100 ng), and α-adducin fragments were detected by Western blotting using the clone45 anti-α-adducin polyclonal antibody. B, 25 μg of total cell homogenate was immunoprecipitated (IP) with anti-HA antibody (clone 12CA5) followed by incubation with caspase-3 (100 ng) either in the absence or presence of Ac-DEVD-CHO (5 μM). HA tag-containing fragments were detected by Western blotting using rat anti-HA antibody (clone 3F10). IB, immunoblots. C, potential HA-α-adducin cleavage fragments and recognition by either anti-HA or anti-α-adducin antibody.

**Fig. 5. Potential caspase-3 cleavage sites in α-adducin and sequence homology with β- and γ-adducin.** Cleavage sites in human (GenBank® accession number X58141) and rat (GenBank® accession number Z49081) α-adducin. Sequence homology for the two cleavage sites in highly homologous N-terminal head region in human β-adducin (GenBank® accession number X58199) and rat (GenBank® accession number U35775) and human (GenBank® accession number U37122) γ-adducin as well as potential caspase cleavage sites in the variable tail region are indicated.
the formation of the various α-adducin fragments, aspartic acid residue 29, 208, or 633 of human α-adducin were mutated to alanine by site-directed mutagenesis, resulting in HA-α-adducin(Asp29→Ala), HA-α-adducin(Asp208→Ala), and HA-α-adducin(Asp633→Ala). Total cell homogenates of COS-1 cells transiently transfected with either wild-type or any of the mutated α-adducin were incubated with caspase-3. Caspase-3 cleaved wild-type α-adducin into the 74- and 78-kDa fragments; cleavage was blocked by Ac-DEVD-CHO (Fig. 6A). When the concentration of caspase-3 in the incubation was doubled, almost all α-adducin was degraded, including the 74-kDa fragment (Fig. 6A). Although mutation of Asp29→Ala did not block cleavage of α-adducin, the relative amount of α-adducin degraded by caspase-3 was reduced compared with wild-type α-adducin. Furthermore, only one cleavage fragment of approximately 74–78 kDa was observed (Fig. 6A). The Asp208→Ala mutation did not prevent proteolysis of α-adducin by caspase-3 appreciably (Fig. 6A). Mutation of Asp633→Ala blocked the formation of the 74-kDa cleavage product and prevented overall loss of α-adducin even at high concentrations of caspase-3 (Fig. 6A).

Next we investigated which cleavage site is involved in formation of the HA-containing fragments of approximately 28 and 78 kDa. HA-α-adducin was immunoprecipitated followed by incubation with caspase-3 (Fig. 6B). The Asp208→Ala mutation did not block the formation of the 28 and 78 kDa fragments. In contrast, the Asp208→Ala mutation blocked formation of the 28-kDa fragment, whereas the Asp633→Ala mutation blocked formation of the 78-kDa HA-containing fragment as well as the reduction of the overall loss of HA-α-adducin (Fig. 6B).

In conclusion, the caspase-3 cleavage sites Asp-Arg-Val-Asp29-Glu and Asp-Xaa-Ser-Asp633-Ala are both involved in caspase-3-mediated processing of α-adducin to the major 74-kDa cleavage product found in apoptotic cells. The Asp-Arg-Val-Asp208-Arg site may be involved in formation of a 28-kDa N-terminal fragment in vitro cleavage assays, but this species or the corresponding C-terminal fragments of either 50 or 62 kDa are not seen in apoptotic cells.

The 74-kDa α-Adducin Fragment Associates with the Cytoskeletal Fraction in RPTE Cells—The 74-kDa α-adducin fragment lacks the most C-terminal tail region of α-adducin that contains the MARCKS domain, the critical domain for binding of adducin to actin filaments (40). To determine if α-adducin proteolysis altered its association with the cytoskeletal F-actin network, we probed the soluble (Fig. 7, SOL) and cytoskeletal fractions (CSK) from RPTE cells for adducin, the 74 kDa fragment, and fodrin. The 74-kDa fragment, but not intact α-adducin, was found in the CSK fraction after apoptosis (Fig. 7). The loss of intact α-adducin from the CSK fraction was prevented by z-VAD-fmk. Although γ-adducin is not cleaved by caspasas, it also dissociated from the CSK after treatment with cisplatin, which was not prevented by z-VAD-fmk. Loss of fodrin from the CSK after cisplatin treatment was also independent of caspase activity. The 120-kDa caspase-3 fragment of fodrin was present in the soluble fraction.

Loss of α-Adducin from Adherens Junctions Precedes Its Caspase-mediated Fragmentation—Loss of cell-cell contacts is an important phenomenon that occurs in the process of apoptosis. Since adducin family members are important regulators of the cortical F-actin cytoskeletal network, it seemed possible that loss of cell-cell contacts was related to α-adducin cleavage. To test this hypothesis we evaluated the changes in the localization of α-adducin, γ-adducin, as well as fodrin with the adherens junctions in RPTE cells after exposure to cisplatin. Although α-adducin cleavage was observed only after 24 h treatment (Fig. 1), altered localization of both α- and γ-adducin as well as fodrin was already disturbed 8 h after treatment with cisplatin and preceded general loss of the F-actin network (Fig. 8). This indicates that the loss of the adducin isoforms and fodrin from adherens junctions is not caused by α-adducin cleavage. Accordingly, z-VAD-fmk blocked cisplatin-induced α-adducin cleavage at 24 h (see above) but did not affect cisplatin-induced early disturbance of adherens junctions (data not shown).

Loss of α-Adducin from Adherens Junctions Is Associated with Increased Phosphorylation of Ser726—Phosphorylation of α- and γ-adducin at Ser726 and Ser660 precedes loss of both α- and γ-adducin from adherens junctions after treatment of RPTE cells with the phorbol ester phorbol 12,13-dibutyrate (41, 58). To determine whether the cisplatin-induced loss of adducin isoforms from adherens junctions might be due to increased phosphorylation of α- and γ-adducin, we analyzed the phosphorylation of both α- and γ-adducin using a phosho state-specific antibody directed against the PKC/PKA phosphorylation site in the MARCKS domain of adducin (43, 58). Cisplatin

![Image](25810)
treatment for 4 or 8 h caused an increase in the phosphorylation of both α- and γ-adducin (Fig. 9A); α-adducin phosphorylation was most prominent. The increased phosphorylation was independent of caspase activity, since it was not blocked by z-VAD-fmk (Fig. 9B). Finally, we determined whether adducin remained phosphorylated at 24 h. Although most of α-adducin had disappeared after treatment with cisplatin, the remaining α-adducin had a similar extent of phosphorylation compared with control cells (Fig. 10). No phosphorylation was detectable on the 74-kDa caspase-3-mediated fragment of α-adducin as expected since the MARCKS domain is absent (Fig. 10). Although z-VAD-fmk blocked the cleavage of α-adducin at 24 h, it did not affect the phosphorylation of α-adducin (Fig. 10).

DISCUSSION

In the present study we have investigated the temporal relationship between loss of adherens junctions, altered Ser726 phosphorylation and proteolysis of α-adducin, and the induction of chemically induced apoptosis in RPTE cells. Several conclusions can be drawn from these investigations. First, cisplatin-mediated disruption of adherens junctions is associated with increased phosphorylation of α- and γ-adducin. This phosphorylation precedes the complete loss of cell-cell interactions as well as the activation of the apoptotic machinery, i.e. activation of caspases, and is independent on the activity of caspases. Second, we present evidence that α-adducin is a caspase substrate in chemically induced apoptosis of RPTE cells, whereas γ-adducin is not. Cleavage of α-adducin is mediated by the downstream caspase-3, but not -6 or -7. Third, we have identified Asp-Asp-Ser-Asp633-Ala as the key caspase-3 cleavage site that is required for the formation of the major 74-kDa cleavage fragment seen in cells undergoing apoptosis. To our knowledge, chemically induced phosphorylation of adducins as well as caspase-3-mediated proteolysis of α-adducin has not been reported before. Since loss of cellular interactions in (chemically induced) apoptosis is common in renal disorders in humans and animals, our findings may have important consequences for the understanding of the molecular mechanisms of renal cell injury.

The major caspase-mediated cleavage product of α-adducin in cells had a mass of 74 kDa. Not only is the predicted mass of the polypeptide composed of residues 30–633 consistent with a

FIG. 7. Effect of cisplatin on cytoskeletal association of α-adducin. RPTE cells were treated with cisplatin for 8 h followed by recovery in complete medium for 24 h, either in the absence or presence of z-VAD-fmk (100 μM). CSK and soluble (SOL) fractions were prepared as described under “Experimental Procedures.” Equal amounts of protein were separated by SDS-PAGE followed by Western blotting for α-adducin, γ-adducin, phospho-adducin, or fodrin. Results shown are representative for three independent experiments.

FIG. 8. Effect of cisplatin on localization of α-adducin, γ-adducin, and fodrin, and F-actin organization. RPTE cells cultured on collagen-coated glass coverslips were treated with or without cisplatin (100 μM). After 8 h cells were fixed and stained for F-actin using rhodamine-phalloidin and immunostained for either α-adducin (A), γ-adducin (B), or fodrin (C). Pictures are representative of four independent experiments.

74-kDa polypeptide, the experimental evidence also suggested that this fragment arose from caspase-3 cleavage at both Asp-Arg-Val-Asp29-Glu and Asp-Asp-Ser-Asp633-Ala. Mutation of Asp29 did not prevent cleavage, but the resulting fragment coincided with the 78-kDa fragment. Mutation of Asp633 prevented formation of either the 74- or 78-kDa fragments. The Asp208 mutation did not prevent cleavage appreciably. Thus, the data support the notion that the 74-kDa fragment seen in apoptotic cells is derived from cleavage at Asp-Arg-Val-Asp29-Glu and Asp-Asp-Ser-Asp633-Ala to yield amino acids 30–633. Moreover, the 74-kDa fragment lacks the MARCKS domain and was not recognized by the phospho-selective antibody that recognizes the phosphorylated Ser726 in α-adducin. Taken together, the data are consistent with cleavage by caspase-3 to yield a 74-kDa fragment containing amino acid residues 30–633 of α-adducin.

Cleavage of α-adducin into a 74-kDa fragment was not restricted to cisplatin-induced apoptosis of RPTE cells. A fragment with the same size was also observed in RPTE cells that were treated with staurosporin or dichloroacetate as well as in SV40-transformed human renal proximal tubular epithelial cells treated with cisplatin. Moreover, α-adducin proteol-
Although, the exact mechanism of the increased adducin phosphorylation remains unclear, preliminary data indicate that both PKA and PKC inhibitors, e.g. H89 and bisindolylmaleimide, Gö 6983, and Gö 6976, inhibit the cisplatin-induced cell killing of RPTE cells.\(^2\) If this observation is borne out by additional studies, it would suggest that phosphorylation of Ser\(^{726}\) is also important in cell killing by cisplatin. Moreover, the data are consistent with the model proposed previously for dissolution of focal adhesions, i.e. disturbances in phosphorylation precede loss of focal adhesions and cleavage of focal adhesion proteins by caspases.

Although cisplatin caused an increase in the phosphorylation of α-adducin, an increased phosphorylation of cytoskeletal proteins is not a general phenomenon in cisplatin-induced cytotoxicity. Thus, tyrosine phosphorylation of focal adhesion kinase is decreased (15). In addition, focal adhesion kinase dephosphorylation occurs before the onset of apoptosis (15). These observations indicate that changes in the phosphorylation status of cytoskeletal proteins are a common feature in chemically induced cytotoxicity associated with loss of cell-cell and cell-matrix interactions. The data fit with a model in which the net gain and loss of cytoskeletal protein phosphorylation after renal cell damage determines the fate of the cytoskeletal network. As a consequence of cytoskeleton disruption, cell-cell and cell-matrix interactions may be lost, and if these disturbances are severe enough, induction of apoptosis may follow.

In summary, the present findings demonstrate that phosphorylation of α-adducin at Ser\(^{726}\) is associated with its loss from adherens junctions; this precedes caspase-3 activation and cleavage of α-adducin primarily at Asp-Xaa-Ser-Asp\(^{633}\)-Ala. Since cell-cell interactions are important for modulation of epithelial cell survival and proliferation and some adherens junction proteins, e.g. β-catenin, can function as transcription factors to promote expression of potentially pro-apoptotic proteins (49, 59, 60), further investigation on the molecular mechanisms of adherens junction disorganization and the biological consequences in relation to (chemically induced) apoptosis is required. However, a common model for caspase-mediated destruction of focal adhesions and adherens junctions following chemical treatment emerges. Chemical stress causes changes in phosphorylation that result in loss of adherens junctions and focal adhesions contacts with the actin cytoskeleton. Cleavage of key regulatory proteins such as adducin and focal adhesion kinase results in irreversible loss of these structures, ensuring release of the damaged cell from its neighbors and the substratum, a circumstance likely to result in anoikis.

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