

# Cleavage of the Actin-capping Protein $\alpha$ -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\*

Received for publication, February 25, 2000, and in revised form, May 16, 2000  
Published, JBC Papers in Press, May 22, 2000, DOI 10.1074/jbc.M001680200

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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  $\alpha$ -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  $\alpha$ -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>29</sup>-Glu, Asp-Ile-Val-Asp<sup>208</sup>-Arg, and Asp-Asp-Ser-Asp<sup>633</sup>-Ala were identified as the principal caspase-3 cleavage sites; Asp-Asp-Ser-Asp<sup>633</sup>-Ala was key in the formation of the 74-kDa fragment. Cisplatin also caused an increased phosphorylation of  $\alpha$ -adducin and  $\gamma$ -adducin in the MARCKS domain that preceded  $\alpha$ -adducin cleavage and was associated with loss of adducins from adherens junctions; this was not affected by z-VAD-fmk. In conclusion, the data support a model in which increased phosphorylation of  $\alpha$ -adducin due to cisplatin leads to dissociation from the cytoskeleton, a situation rendered irreversible by caspase-3-mediated cleavage of  $\alpha$ -adducin at Asp-Asp-Ser-Asp<sup>633</sup>-Ala.

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 pro-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, Rb, Mdm2, Bcl-2, and protein kinase C  $\delta$ , 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)<sup>1</sup> 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

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 pathological process associated with various human diseases (2, 3). Commitment to apoptosis requires activation of caspases,

\* These studies were supported by Dutch Organization for Scientific Research Grants 902-21-208 and 902-21-217 and a fellowship from the Royal Netherlands Academy of Arts and Sciences (to B. v. d. W.) as well as United States Public Health Service Grants DK47267 and ES07847 (to J. L. S.) and CA71607 (to S. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: RPTE cells, rat proximal tubule epithelial cells; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; AMC, 7-amino-4-methylcoumarin; PKA, protein kinase A; PKC, protein kinase C; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; HA, hemagglutinin; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; MSB, microtubule stabilization buffer; CSK, cytoskeletal.

cleavage did not initiate the disruption by inducing a gain or loss of function, it did render the process irreversible, ensuring that the cell is committed to apoptosis.

Preliminary observations also suggested that cell-cell contact was also disrupted during xenobiotic-induced apoptosis. Cell-cell interactions are mediated through homophilic interactions between E-cadherins at the adherens junctions (29). E-cadherin is linked through  $\beta$ - and  $\alpha$ -catenin to the cortical F-actin network (29); the latter interacts with the cortical spectrin cytoskeletal network (30). This cortical F-actin network is regulated by various regulatory proteins, such as Tiam-1, c-Src, and Rho-family members as well as cytoskeletal-associated proteins, including adducins (29, 31–33). Cell-cell interactions are disrupted during apoptosis, and some proteins of the adherens junction are caspase-3 substrates (17–19). However, little is known about the involvement of caspases in disruption of the cortical actin cytoskeleton during drug-induced apoptosis. Therefore, we investigated the fate of the cortical actin-capping protein adducin in relation to cisplatin-induced apoptosis in renal epithelial cells.

Adducins are cytoskeletal proteins composed of highly related  $\alpha$  and  $\beta$  or  $\alpha$  and  $\gamma$  subunits that bind to the fast-growing ends and the sides of actin filaments (34–38). Adducins consist of three distinct domains: an N-terminal head, involved in dimerization, a neck, and C-terminal tail domain containing the MARCKS-related domain that recruits spectrin/fodrin to the fast-growing ends of actin filaments (35–37, 39, 40). Adducin function is modulated by several kinases: Rho kinase, protein kinase A (PKA), and protein kinase C (PKC) (33, 41–44). Rho kinase phosphorylates adducin in the neck domain, resulting in increased association of  $\alpha$ -adducin with F-actin (33, 45). PKA phosphorylates  $\alpha$ -adducin at Ser<sup>408</sup>, Ser<sup>436</sup>, and Ser<sup>481</sup> in the neck domain, and PKC and PKA phosphorylate at Ser<sup>726</sup> in the MARCKS domain (42). PKA-mediated phosphorylation of adducin results in reduced formation of actin-spectrin complexes (42), whereas PKC-mediated phosphorylation results in an accumulation of phosphorylated adducin in the cytosol (41, 43). Although adducin is a cytoskeletal component important for controlling the cortical cytoskeletal network, to our knowledge there is little or no data available on its involvement in cytoskeleton rearrangements before apoptosis or the fate of adducin during apoptosis. To investigate this we have used nephrotoxicant-induced apoptosis of renal proximal tubular epithelial cells as a model.

RPTE cells are a target for many chemicals as well as ischemia/reperfusion injury (46, 47), which may cause apoptosis *in vitro* and *in vivo* (15, 48, 49), a process associated with loss of cell-cell and cell-matrix interactions (15, 50–53). Since we used the RPTE cell model to investigate the role of caspases in loss of focal adhesion integrity during apoptosis, we used RPTE cells to address the relationship between adherens junction organization, adducin phosphorylation, and proteolysis in chemically induced apoptosis. Apoptosis of RPTE cells was preceded by an early increased phosphorylation of primarily  $\alpha$ -adducin at Ser<sup>726</sup>, most likely by a PKC isoform that correlated with a loss of adducin in adherens junctions. This was followed by activation of caspases and induction of apoptosis.  $\alpha$ -Adducin (but not  $\gamma$ -adducin) was cleaved by a caspase-3-dependent manner at Asp-Arg-Val-Asp<sup>29</sup>-Glu and Asp-Asp-Ser-Asp<sup>633</sup>-Ala to yield a 74-kDa fragment in RPTE cells. The data indicate that phosphorylation of  $\alpha$ -adducin and disruption of cellular adherens junctions precede the caspase-3-mediated degradation of  $\alpha$ -adducin.

## EXPERIMENTAL PROCEDURES

### Materials

Dulbecco's modified Eagle's medium/Ham's F-12,  $\alpha$ -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, cholera 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), acyl-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO), acyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (Ac-YVAD-AMC), acyl-Val-Glu-Ile-Asp-7-amino-4-methylcoumarin (Ac-VEID-AMC), calpain inhibitor I and II were from Bachem (Bubendorf, Switzerland). Acyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was from Research Biochemicals International (Natick, MA).

### Plasmids

An expression vector for hemagglutinin (HA)-tagged human  $\alpha$ -adducin (amino acids 1–737; pEF-BOS-HA- $\alpha$ -adducin) was kindly provided by Dr. K. Kaibuchi and described elsewhere (33). For site-directed mutagenesis, the  $\alpha$ -adducin-coding region was subcloned into pZErO (Invitrogen) using *Kpn*I. For mutation by polymerase chain reaction of amino acid residue D to A at the various caspase-3 cleavage sites in human  $\alpha$ -adducin, the QuickChange mutagenesis kit (Stratagene) was used with the following primers: Asp<sup>29</sup>  $\rightarrow$  Ala, forward primer 5'-GGTACTTCGACCGAGTAGCTGAGAACAACCC-3' and reverse primer 5'-GGGTTGTTCTCCAGCTACTCGGTGCGAAGTACC-3'; Asp<sup>208</sup>  $\rightarrow$  Ala, forward primer 5'-GGTACTTCGACCGAGTAGCTGAGAACAACCC-3' and reverse primer 5'-GGGTTGTTCTCAGCTACTCGGTGCGAAGTACC-3'; Asp<sup>633</sup>  $\rightarrow$  Ala, forward primer 5'-GGAGATGACAGTGCTGCTGCACCTTTAAGC-3' and reverse primer 5'-GCTTAAAGGTGGCAGCAGCATCTCATCTCC-3'. The correct sequences after mutation were confirmed by DNA cycle sequencing. Mutated  $\alpha$ -adducin was subcloned back into pEF-BOS. Correct expression of HA- $\alpha$ -adducin and the different Asp  $\rightarrow$  Ala mutants was checked by transient overexpression in COS1 cells followed Western blotting and immunofluorescent staining.

### Cell Culture and Treatment

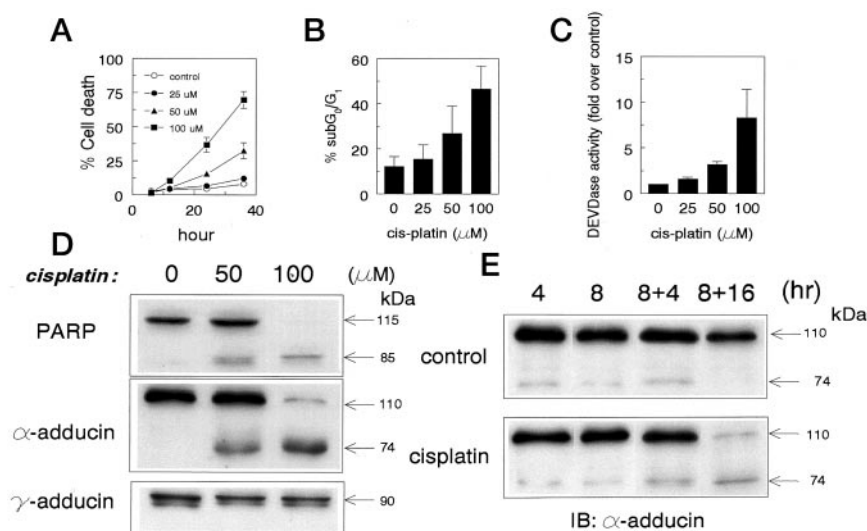
RPTE cells were isolated by collagenase perfusion and separated by density centrifugation using Nycodenz as described (54, 55). Cells were cultured on rat tail collagen (Collaborative Research, Bedford, MA)-coated dishes in Dulbecco's modified Eagle's medium /F-12 containing 1% v/v fetal bovine serum, 0.5 mg/ml bovine serum albumin, 10  $\mu$ g/ml insulin, 10 ng/ml epidermal growth factor, 10 ng/ml cholera 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 once. Thereafter, cells were treated with cisplatin in Hanks' balanced salt solution (137 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>·2 H<sub>2</sub>O, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 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  $\mu$ M; 100 mM stock in Me<sub>2</sub>SO) was added simultaneously with cisplatin. Following treatment with cisplatin 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  $\mu$ M propidium iodide and 10  $\mu$ g/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



**FIG. 1.  $\alpha$ -Adducin is cleaved during cisplatin-induced apoptosis of RPTE cells.** RPTE cells were treated with varying concentrations of cisplatin for 8 h and allowed to recover for another 16 h in complete culture medium, after which samples were taken for further analysis. Cell death was analyzed by measuring lactate dehydrogenase release (A) or by quantitation of percentage of cells with sub- $G_0/G_1$  DNA content by cell cycle analysis (B) as described under "Experimental Procedures." Ac-Asp-Glu-Val-Asp-AMC cleavage activity (DEVDase activity) (C) was determined in cell extracts using Ac-DEVD-AMC as a substrate and expressed as fold over control; DEVDase activity in untreated RPTE cells was  $165 \pm 71$  pmol/min/mg ( $n = 4$ ). Cleavage of poly(ADP-ribose) polymerase (PARP),  $\alpha$ -adducin, and  $\gamma$ -adducin at 24 h (D) as well as the time course of  $\alpha$ -adducin cleavage (E) in total cell homogenates was determined by Western blotting as described under "Experimental Procedures." Data shown are the mean  $\pm$  S.E. of (A–C) or representative for (D and E) four independent experiments ( $n = 4$ ). IB, immunoblots.

toxicants relative to the amount to that released by 0.1 w/v 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  $\beta$ -glycerophosphate, 50 mM NaCl, 2 mM  $MgCl_2$ , 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  $\mu$ M; 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/mg of cell protein-min using AMC as a standard.

#### *In Vitro Caspase-Substrate Cleavage Assay*

For transfection,  $2 \times 10^5$  COS1 cells were plated in 6-well culture clusters. After overnight culturing, COS1 cells were transfected with 0.4  $\mu$ g of pEF-BOS- $\alpha$ -adducin, pEF-BOS- $\alpha$ -adducin-Asp<sup>29</sup>  $\rightarrow$  Ala, pEF-BOS- $\alpha$ -adducin-Asp<sup>208</sup>  $\rightarrow$  Ala, or pEF-BOS- $\alpha$ -adducin-Asp<sup>633</sup>  $\rightarrow$  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 dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin) followed by sonication ( $3 \times 5$  s). Protein was determined using the Bradford reagent (Bio-Rad). 15  $\mu$ g 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 dithiothreitol, 20  $\mu$ g/ml leupeptin, 20  $\mu$ g/ml aprotinin, 20  $\mu$ g/ml pepstatin, and 20 mM phenylmethylsulfonyl fluoride). After the addition of 0.1  $\mu$ g 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  $^{\circ}$ C. Where indicated, 5  $\mu$ M z-VAD-fmk, 5  $\mu$ M Ac-DEVD-CHO, or 5  $\mu$ M calpain inhibitor I or 5  $\mu$ M 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  $^{\circ}$ C. Samples were separated by SDS-PAGE followed by Western blotting.

For immunoprecipitation of HA-tagged human  $\alpha$ -adducin, 25  $\mu$ g protein of total COS1 cell lysate was dissolved in 500  $\mu$ l of radioimmune precipitation buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1.0% (w/v) deoxycholate, 1.0% (w/v) Triton X-100, 0.2% (w/v) sodium dodecyl sulfate, pH 7.4, containing the above protease inhibitors) followed by pre-clearing by centrifugation (30 min, 13,000 rpm). The supernatant was incubated with anti-HA antibody (clone 12CA5) for 1 h at 4  $^{\circ}$ C followed by incubation with protein G-Sepharose beads (Sigma) for 1 h at 4  $^{\circ}$ C. After 3 washes with low salt buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.1% (v/v) Nonidet P-40, pH 7.4, containing protease

inhibitors), beads were resuspended in caspase assay buffer. Next, 0.1  $\mu$ g of human recombinant caspase-3 was added, and the mixture was incubated for 1 h at 37  $^{\circ}$ C with or without 5  $\mu$ M Ac-DEVD-CHO. The cleavage reaction was stopped by the addition of Laemmli sample preparation buffer followed by heating for 5 min at 95  $^{\circ}$ C. Samples were separated by SDS-PAGE followed by Western blotting.

#### *Preparation of Cytoskeletal and Soluble Cellular Fractions*

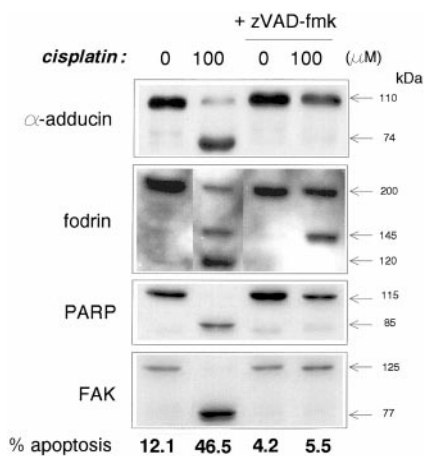
To obtain soluble and cytoskeletal fractions, the Triton X-100 extraction method was used (41, 58). Briefly, RPTE cells cultured in 10-cm dishes were treated with cisplatin as above. The medium was removed, and adherent cells were washed twice with PBS and a final wash with microtubule stabilization buffer (MSB; 100 mM PIPES, 2 M glycerol, 1 mM EGTA, 1 mM magnesium acetate, pH 6.9). Adherent cells were scraped in 500  $\mu$ l 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  $500 \times g$ . 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  $85,000 \times g$  for 20 min at 15  $^{\circ}$ C. The supernatant (soluble fraction) was removed, and the pellet (cytoskeleton fraction) was resuspended in MSB Plus. Equal amounts of protein were separated by SDS-PAGE followed by Western blotting.

#### *Gel Electrophoresis and Immunoblotting*

Cells were scraped in ice-cold PBS and pooled with medium containing floating cells. After centrifugation (5 min,  $500 \times g$ , 4  $^{\circ}$ C), the pellet was resuspended in TSE plus inhibitors. Protein concentration in the supernatant was determined using the Bradford protein assay using IgG as a standard. Equal amounts of total cellular protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). Blots were blocked with 5% w/v nonfat dry milk in TBS-T (0.5 M 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- $\alpha$ -adducin, and anti- $\gamma$ -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



**FIG. 2. The caspase inhibitor z-VAD-fmk blocks  $\alpha$ -adducin cleavage during apoptosis.** RPTE cells were treated with or without 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- $G_0/G_1$  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- $G_1/G_0$ ) 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  $\sim$ 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).

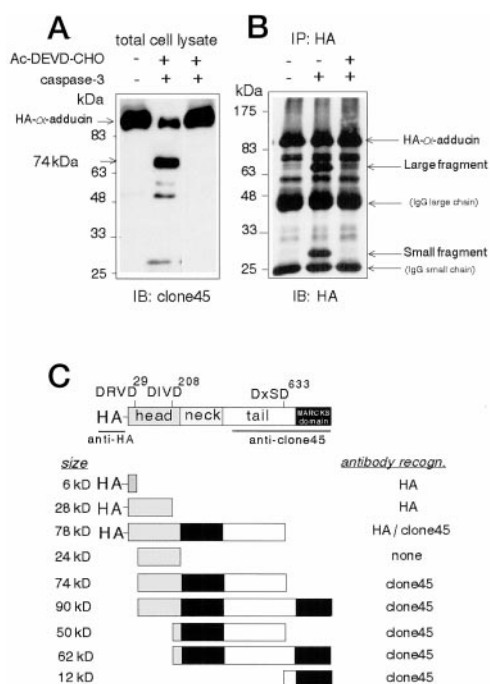
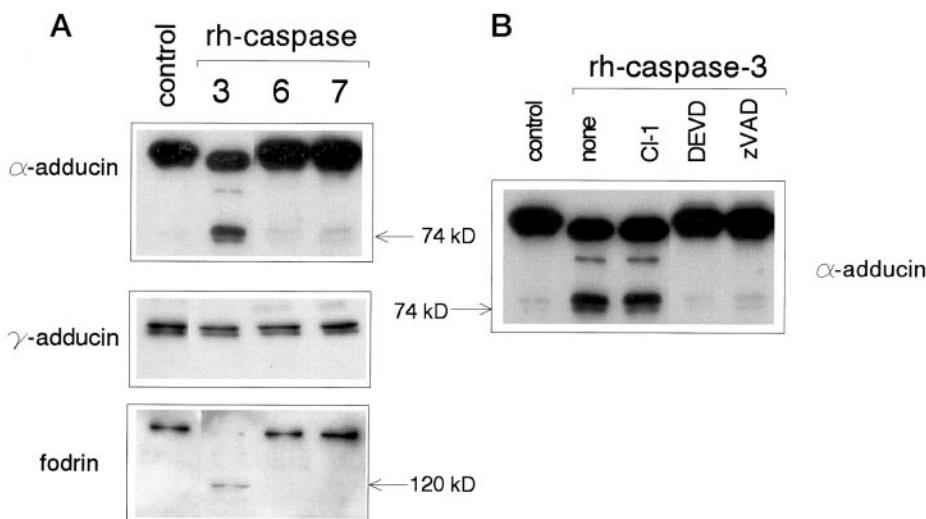
**$\alpha$ -Adducin Is Cleaved by Caspase-3 but Not -6 and -7**—The caspase family consists of "upstream caspases" such as caspase-8 and -9, which activate the "downstream" executioner caspases including caspase-3, -6, and -7 (4). To investigate which of the executioner caspases are capable of cleaving  $\alpha$ -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 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 to 2 major (74 and 78 kDa) and 4 minor fragments (26, 28, 50, and 60 kDa).

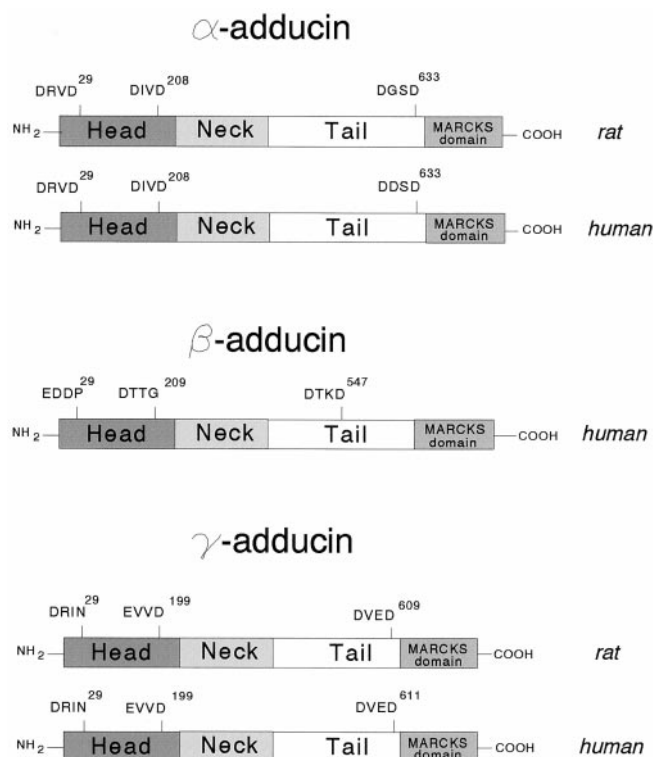
The consensus caspase-3 cleavage site is DEVD. The two aspartic acid residues are absolutely required, but the glutamic acid and valine residues may vary; therefore, any DXXD is a potential site (4). Comparison of rat and human  $\alpha$ -adducin revealed three conserved DXXD sequences; two in the N-terminal region at Asp-Arg-Val-Asp<sup>29</sup>-Glu and Asp-Ile-Val-Asp<sup>208</sup>-Arg and one in the C terminus at Asp-Xaa-Ser-Asp<sup>633</sup>-Ala (Fig. 5). These cleavage sites are unique for  $\alpha$ -adducin and

**FIG. 3. Caspase-3 but not -6 and -7 cleaves HA-tagged  $\alpha$ -adducin.** COS-1 cells were transfected with 0.4  $\mu$ g of pEF-BOS-HA- $\alpha$ -adducin. **A**, 15  $\mu$ g 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  $\alpha$ -adducin,  $\gamma$ -adducin, and fodrin was analyzed by Western blotting. **B**, 15  $\mu$ g of total cell homogenate of COS-1 cells expressing HA- $\alpha$ -adducin was incubated with 100 ng of human recombinant caspase-3 in the presence or absence of calpain inhibitor I (5  $\mu$ M), Ac-DEVD-CHO (5  $\mu$ M), or z-VAD-fmk (5  $\mu$ M), and cleavage of  $\alpha$ -adducin was determined by Western blotting using the anti- $\alpha$ -adducin antibody.



**FIG. 4. Caspase-3 cleavage products of HA-tagged human  $\alpha$ -adducin.** COS-1 cells were transfected with 0.4  $\mu$ g of pEF-BOS-HA- $\alpha$ -adducin. **A**, 15  $\mu$ g of total cell homogenate was incubated with caspase-3 (100 ng), and  $\alpha$ -adducin fragments were detected by Western blotting using the clone45 anti- $\alpha$ -adducin polyclonal antibody. **B**, 25  $\mu$ g 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  $\mu$ M). HA tag-containing fragments were detected by Western blotting using rat anti-HA antibody (clone 3F10). **IB**, immunoblots. **C**, potential HA- $\alpha$ -adducin cleavage fragments and recognition by either anti-HA or anti- $\alpha$ -adducin antibody.

are not present in either  $\beta$ - or  $\gamma$ -adducin (Fig. 5). Cleavage of  $\alpha$ -adducin at the N-terminal Asp-Arg-Val-Asp<sup>29</sup> or Asp-Ile-Val-Asp<sup>208</sup> and the C-terminal Asp-Xaa-Ser-Asp<sup>633</sup> 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

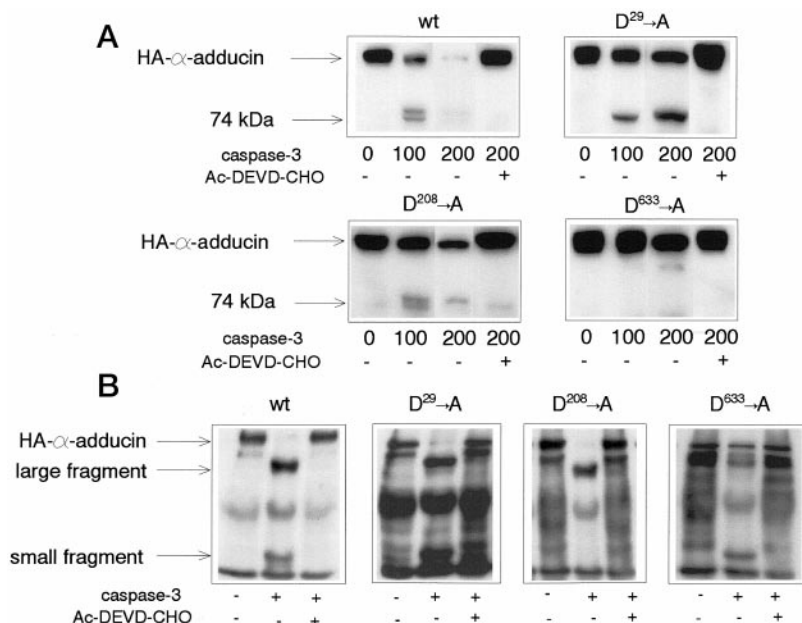


**FIG. 5. Potential caspase-3 cleavage sites in  $\alpha$ -adducin and sequence homology with  $\beta$ - and  $\gamma$ -adducin.** Cleavage sites in human (GenBank<sup>®</sup> accession number X58141) and rat (GenBank<sup>®</sup> accession number Z49081)  $\alpha$ -adducin. Sequence homology for the two cleavage sites in highly homologous N-terminal head region in human  $\beta$ -adducin (GenBank<sup>®</sup> accession number X58199) and rat (GenBank<sup>®</sup> accession number U35775) and human (GenBank<sup>®</sup> accession number U37122)  $\gamma$ -adducin as well as potential caspase cleavage sites in the variable tail region are indicated.

recognizable by the  $\alpha$ -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 an inability to separate these fragments on the gel system used. Regardless, the sequence data indicate that Asp-Arg-Val-Asp<sup>29</sup> and Asp-Xaa-Ser-Asp<sup>633</sup> are likely cleavage sites for the generation of the major 74-kDa fragment seen in apoptotic cells.

*Asp-Asp-Ser-Asp<sup>633</sup>-Ala Is the Key Caspase-3 Cleavage Site in  $\alpha$ -Adducin*—To investigate the role of these DXXD sites in

**FIG. 6. Effect of mutation Asp-Arg-Val-Asp<sup>29</sup> → Ala, Asp-Ile-Val-Asp<sup>208</sup> → Ala and DDS<sup>633</sup> → Ala on caspase-3-mediated cleavage of human HA- $\alpha$ -adducin.** COS-1 cells were transfected with either wild-type (*wt*), Asp-Arg-Val-Asp<sup>29</sup> → Ala, Asp-Ile-Val-Asp<sup>208</sup> → Ala, or Asp-Asp-Ser-Asp<sup>633</sup> → Ala HA- $\alpha$ -adducin. **A**, cell homogenates were incubated with 0, 100, or 200 ng of recombinant human caspase-3 as described in Fig. 5 in the absence or presence of Ac-DEVD-CHO (5  $\mu$ M). **B**, HA- $\alpha$ -adducin was immunoprecipitated followed by incubation with 100 ng of human recombinant caspase-3 as described in Fig. 5. After separation by SDS-PAGE and transfer to polyvinylidene difluoride membranes, blots were either probed with anti- $\alpha$ -adducin (**A**) or anti-HA antibodies (**B**) followed by ECL detection. Data are representative for three independent experiments.



the formation of the various  $\alpha$ -adducin fragments, aspartic acid residue 29, 208, or 633 of human  $\alpha$ -adducin were mutated to alanine by site-directed mutagenesis, resulting in HA- $\alpha$ -adducin(Asp<sup>29</sup> → Ala), HA- $\alpha$ -adducin(Asp<sup>208</sup> → Ala), and HA- $\alpha$ -adducin(Asp<sup>633</sup> → Ala). Total cell homogenates of COS-1 cells transiently transfected with either wild-type or any of the mutated  $\alpha$ -adducin were incubated with caspase-3. Caspase-3 cleaved wild-type  $\alpha$ -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  $\alpha$ -adducin was degraded, including the 74-kDa fragment (Fig. 6A). Although mutation of Asp<sup>29</sup> → Ala did not block cleavage of  $\alpha$ -adducin, the relative amount of  $\alpha$ -adducin degraded by caspase-3 was reduced compared with wild-type  $\alpha$ -adducin. Furthermore, only one cleavage fragment of approximately 74–78 kDa was observed (Fig. 6A). The Asp<sup>208</sup> → Ala mutation did not prevent proteolysis of  $\alpha$ -adducin by caspase-3 appreciably (Fig. 6A). Mutation of Asp<sup>633</sup> → Ala blocked the formation of the 74-kDa cleavage product and prevented overall loss of  $\alpha$ -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- $\alpha$ -adducin was immunoprecipitated followed by incubation with caspase-3 (Fig. 6B). The Asp<sup>29</sup> → Ala mutation did not block the formation of the 28 and 78 kDa fragments. In contrast, the Asp<sup>208</sup> → Ala mutation blocked formation of the 28-kDa fragment, whereas the Asp<sup>633</sup> → Ala mutation blocked formation of the 78-kDa HA-containing fragment as well as the reduction of the overall loss of HA- $\alpha$ -adducin (Fig. 6B).

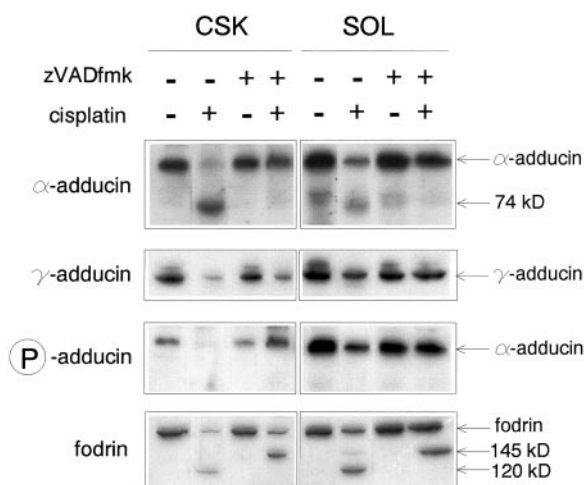
In conclusion, the caspase-3 cleavage sites Asp-Arg-Val-Asp<sup>29</sup>-Glu and Asp-Xaa-Ser-Asp<sup>633</sup>-Ala are both involved in caspase-3-mediated processing of  $\alpha$ -adducin to the major 74-kDa cleavage product found in apoptotic cells. The Asp-Ile-Val-Asp<sup>208</sup>-Arg site may be 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  $\alpha$ -Adducin Fragment Associates with the Cytoskeletal Fraction in RPTE Cells**—The 74-kDa  $\alpha$ -adducin fragment lacks the most C-terminal tail region of  $\alpha$ -adducin that contains the MARCKS domain, the critical domain for binding

of adducin to actin filaments (40). To determine if  $\alpha$ -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  $\alpha$ -adducin, was found in the CSK fraction after apoptosis (Fig. 7). The loss of intact  $\alpha$ -adducin from the CSK fraction was prevented by z-VAD-fmk. Although  $\gamma$ -adducin is not cleaved by caspases, 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  $\alpha$ -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  $\alpha$ -adducin cleavage. To test this hypothesis we evaluated the changes in the localization of  $\alpha$ -adducin,  $\gamma$ -adducin, as well as fodrin with the adherens junctions in RPTE cells after exposure to cisplatin. Although  $\alpha$ -adducin cleavage was observed only after 24 h treatment (Fig. 1), altered localization of both  $\alpha$ - and  $\gamma$ -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  $\alpha$ -adducin cleavage. Accordingly, z-VAD-fmk blocked cisplatin-induced  $\alpha$ -adducin cleavage at 24 h (see above) but did not affect cisplatin-induced early disturbance of adherens junctions (data not shown).

**Loss of  $\alpha$ -Adducin from Adherens Junctions Is Associated with Increased Phosphorylation of Ser<sup>726</sup>**—Phosphorylation of  $\alpha$ - and  $\gamma$ -adducin at Ser<sup>726</sup> and Ser<sup>660</sup> precedes loss of both  $\alpha$ - and  $\gamma$ -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  $\alpha$ - and/or  $\gamma$ -adducin, we analyzed the phosphorylation of both  $\alpha$ - and  $\gamma$ -adducin using a phospho state-specific antibody directed against the PKC/PKA phosphorylation site in the MARCKS domain of adducin (43, 58). Cisplatin



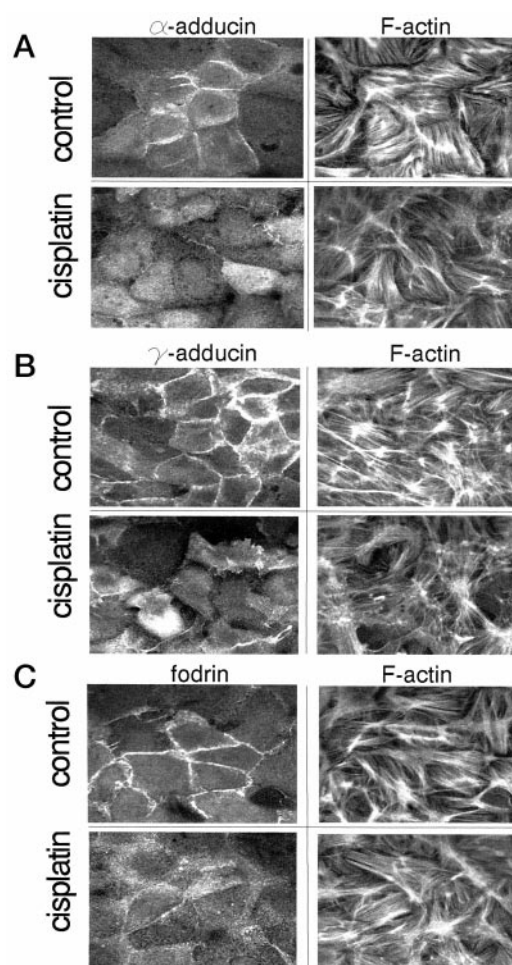
**FIG. 7. Effect of cisplatin on cytoskeletal association of  $\alpha$ -adducin.** RPTE cells were treated with cisplatin for 8 h followed by recovery in complete medium for 24 h, either in the absence or presence of z-VAD-fmk (100  $\mu$ M). CSK and soluble (SOL) fractions were prepared as described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

treatment for 4 or 8 h caused an increase in the phosphorylation of both  $\alpha$ - and  $\gamma$ -adducin (Fig. 9A);  $\alpha$ -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  $\alpha$ -adducin had disappeared after treatment with cisplatin, the remaining  $\alpha$ -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  $\alpha$ -adducin as expected since the MARKS domain is absent (Fig. 10). Although z-VAD-fmk blocked the cleavage of  $\alpha$ -adducin at 24 h, it did not affect the phosphorylation of  $\alpha$ -adducin (Fig. 10).

#### DISCUSSION

In the present study we have investigated the temporal relationship between loss of adherens junctions, altered Ser<sup>726</sup> phosphorylation and proteolysis of  $\alpha$ -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  $\alpha$ - and  $\gamma$ -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  $\alpha$ -adducin is a caspase substrate in chemically induced apoptosis of RPTE cells, whereas  $\gamma$ -adducin is not. Cleavage of  $\alpha$ -adducin is mediated by the downstream caspase-3, but not -6 or -7. Third, we have identified Asp-Asp-Ser-Asp<sup>633</sup>-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  $\alpha$ -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  $\alpha$ -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

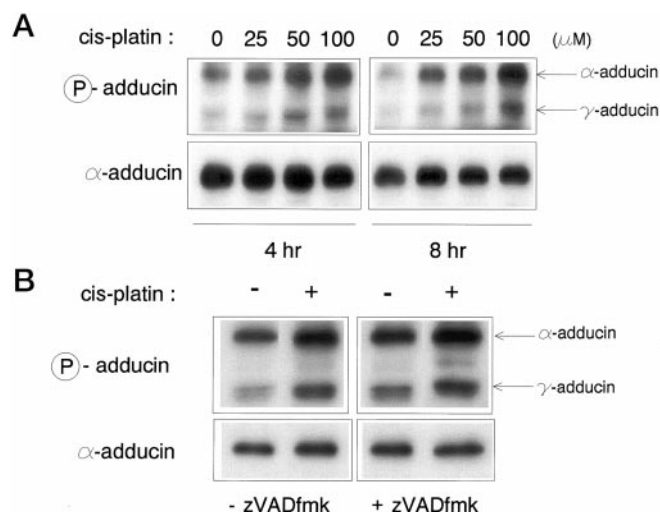


**FIG. 8. Effect of cisplatin on localization of  $\alpha$ -adducin,  $\gamma$ -adducin, and fodrin, and F-actin organization.** RPTE cells cultured on collagen-coated glass coverslips were treated with or without cisplatin (100  $\mu$ M). After 8 h cells were fixed and stained for F-actin using rhodamine-phalloidin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

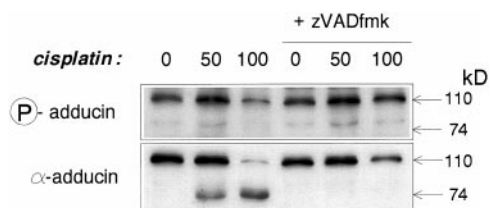
74-kDa polypeptide, the experimental evidence also suggested that this fragment arose from caspase-3 cleavage at both Asp-Arg-Val-Asp<sup>29</sup>-Glu and Asp-Asp-Ser-Asp<sup>633</sup>-Ala. Mutation of Asp<sup>29</sup> did not prevent cleavage, but the resulting fragment coincided with the 78-kDa fragment. Mutation of Asp<sup>633</sup> prevented formation of either the 74- or 78-kDa fragments. The Asp<sup>208</sup> 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-Asp<sup>29</sup>-Glu and Asp-Asp-Ser-Asp<sup>633</sup>-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 Ser<sup>726</sup> in  $\alpha$ -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  $\alpha$ -adducin.

Cleavage of  $\alpha$ -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 dichlorovinylcysteine as well as in SV40-transformed human renal proximal tubular epithelial cells treated with cisplatin.<sup>2</sup> Moreover,  $\alpha$ -adducin proteol-

<sup>2</sup> B. van de Water, A. Verbrugge, and G. J. Mulder, unpublished observations.



**FIG. 9. Effect of cisplatin on phosphorylation of  $\alpha$ -adducin on serine 726.** RPTE cells were treated with or without cisplatin (25, 50, or 100  $\mu$ M) for either 4 or 8 h. Total cell homogenate was separated by SDS-PAGE followed by Western blot analysis for phosphorylation of serine 726 phosphorylation using phospho-site-specific antibody directed against serine 726 and serine 660 in  $\alpha$ -adducin and  $\gamma$ -adducin, respectively (43, 58). After stripping, blots were reprobed for  $\alpha$ -adducin using the clone45 antibody. Data are representative of four independent experiments.



**FIG. 10. Effect of z-VAD-fmk on cisplatin-induced phosphorylation of  $\alpha$ -adducin.** RPTE cells were treated with cisplatin (0, 50, or 100  $\mu$ M) for 8 h followed by recovery for 24 h in complete medium in the absence or presence of z-VAD-fmk (100  $\mu$ M). Equal amounts of total cell homogenates were separated by SDS-PAGE followed by Western blot analysis for phospho-adducin (top panel) and  $\alpha$ -adducin (lower panel). Data are representative for three independent experiments.

ysis into a 74-kDa fragment was also observed in the apoptosis of rat mammary adenocarcinoma cell line MTLn3 caused by anticancer drugs,<sup>2</sup> indicating that proteolysis of  $\alpha$ -adducin is not restricted to renal epithelial cells and may well be a general phenomena in apoptosis of various cell types.

The caspase-3-mediated cleavage of  $\alpha$ -adducin was not involved in the loss of  $\alpha$ -adducin from adherens junctions. Localization of  $\alpha$ -adducin was already disturbed before the 74-kDa  $\alpha$ -adducin fragment was detected (Fig. 8); moreover, the general caspase inhibitor z-VAD-fmk did not affect the translocation of  $\alpha$ -adducin. The  $\alpha$ -adducin redistribution correlated better with increased phosphorylation at the PKA/PKC phosphorylation site in the MARCKS domain of both  $\alpha$ - and  $\gamma$ -adducin; this was already evident 4 h after treatment with cisplatin. Indeed, treatment of RPTE cells with the PKC activator phorbol 12,13-dibutyrate (300 nM), but not with the cell-permeable PKA activator dibutyryl-cAMP (up to 1 mM), results in a phosphorylation of  $\alpha$ - and  $\gamma$ -adducin and a translocation of adducin to the cytosolic fraction, whereas there is no association with the cytoskeleton (58).<sup>2</sup> The broad spectrum PKC/PKA inhibitor staurosporin (50 nM) was able to inhibit the cisplatin-induced phosphorylation of both  $\alpha$ - and  $\gamma$ -adducin,<sup>2</sup> but staurosporin was toxic to RPTE cells, making it difficult to evaluate the potential role of Ser<sup>726</sup> phosphorylation in cytotoxicity.

Although, the exact mechanism of the increased adducin phosphorylation remains unclear, preliminary data indicate that both PKA and PKC inhibitors, *e.g.* H89, bisindolylmaleimide, Gö 6983, and Gö 6976, inhibit the cisplatin-induced cell killing of RPTE cells.<sup>2</sup> If this observation is borne out by additional studies, it would suggest that phosphorylation of Ser<sup>726</sup> 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  $\alpha$ -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  $\alpha$ -adducin at Ser<sup>726</sup> is associated with its loss from adherens junctions; this precedes caspase-3 activation and cleavage of  $\alpha$ -adducin primarily at Asp-Xaa-Ser-Asp<sup>633</sup>-Ala. Since cell-cell interactions are important for modulation of epithelial cell survival and proliferation and some adherens junction proteins, *e.g.*  $\beta$ -catenin, can function as transcription factors to promote expression of potentially proapoptotic 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.

**Acknowledgment**—We are indebted to Dr. Fred Nagelkerke as well as other members of the lab for discussion and helpful suggestions. We thank Drs. Don Nicholson, Nancy Thornberry, James Nelson, and Kozo Kaibuchi for kindly providing antibodies and cDNA constructs.

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*J. Biol. Chem.* 2000, 275:25805-25813.

doi: 10.1074/jbc.M001680200 originally published online May 22, 2000

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Access the most updated version of this article at doi: [10.1074/jbc.M001680200](https://doi.org/10.1074/jbc.M001680200)

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