The Death Domain of Kidney Ankyrin Interacts with Fas and Promotes Fas-Mediated Cell Death in Renal Epithelia

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Abstract. Ankyrins are a ubiquitously expressed family of conserved proteins that mediate the linkage of integral membrane proteins such as transporters and channels with the underlying cytoskeleton. Ankyrins possess a conserved death domain, the functional significance of which has remained puzzling. In this study, the death domain of AnkG190, the isoform of ankyrin expressed in kidney tubules, was used as bait in a yeast two-hybrid screen to identify interacting partners. One of these interactions was with the proapoptotic molecule Fas. This was confirmed by coimmunoprecipitation, colocalization, and glutathione S-transferase pull-down assays in cultured renal epithelial (MDCK) cells. Site-directed mutagenesis of a conserved arginine (R1496 in AnkG190), previously shown to be critical for the binding of Fas (R234 in Fas) to FADD, abolished the interaction of ankyrin’s death domain with Fas. Overexpression of constructs containing ankyrin’s death domain promoted Fas-mediated apoptosis in MDCK cells. The linkage between ankyrin and Fas was confirmed in vivo in mouse kidney tubule cells by coimmunoprecipitation and colocalization. In an established mouse model of renal ischemia-reperfusion injury characterized by apoptotic tubule cell death, the expression of both ankyrin and Fas was markedly induced, and the interaction between these molecules remained intact. The results identify a novel tethering interaction between ankyrin and Fas in kidney epithelia and suggest that AnkG190 may play a role as an adapter molecule in renal tubule cell death.

Tethering interactions between membrane proteins and the underlying spectrin-based cytoskeleton play key roles in several cellular activities, including organization of plasma membrane domains (1). Ankyrins are a ubiquitously expressed family of conserved proteins that have emerged as critical adapter molecules mediating such linkages because they possess binding sites for spectrin as well as increasing numbers of integral membrane proteins (1–5). Three distinct ankyrin genes encode for a variety of alternatively spliced and tissue-specific isoforms. Although the ANK1 and ANK2 gene products are largely restricted to red cells and brain, respectively, the ANK3 gene transcribes isoforms that display a general tissue distribution and are hence termed AnkG. These include a 480-kD isoform localized at the axonal initial segment and node of Ranvier (6), a 190-kD kidney ankyrin isoform expressed at the plasma membranes of kidney tubule cells (7,8), and truncated isoforms associated with the Golgi apparatus (9,10), lysosomes (11), and sarcoplasmic reticulum (12). The majority of ankyrins described to date are modular proteins comprising three conserved domains, including an aminoterminal domain containing a varying number of ankyrin repeats, a spectrin-binding domain, and a death domain domain located near the carboxyl terminal (1–8). Several structurally and functionally diverse proteins interact with the repeats domain of ankyrin, including α-Na, K-ATPase, anion exchangers, the voltage-dependent sodium channel, sodium/calcium exchanger, calcium channels, IP3 receptor, ryanodine receptor, clathrin, tubulin, and cell adhesion molecules such as CD44 and the L1 family (1–5).

The “death domain” was initially reported as a region of sequence homology within the intracellular portions of the proapoptotic receptors Fas and TNFR1 (13,14). These domains were involved in protein-protein interactions, enabling the subsequent identification of several additional key death domain-containing proapoptotic proteins such as FADD, TRADD, and RIP (15–18). Database searches have since identified over a dozen proteins that possess the death domain, including red cell ankyrin (14,19). The death domain within kidney ankyrin (8) displays an even greater similarity to those within proapoptotic molecules than that of red cell ankyrin. However, the functional significance of ankyrin’s death domain has hitherto remained puzzling (3). In this study, we show that kidney ankyrin’s death domain interacts with the death domain of Fas and promotes Fas-mediated apoptosis in renal epithelial cells both in vitro and in vivo.
Yeast Two-Hybrid Screen

The yeast two-hybrid screen has proven to be a valuable technique for the study of protein-protein interactions involving death domains (20). We used the Matchmaker Two-Hybrid System (Clontech, La Jolla, CA) as recommended by the manufacturer. The death domain of AnkG190, the isoform of ankyrin that is expressed at the plasma membranes of kidney tubule cells (7,8), was used as bait. On the basis of published sequence homologies (13–18), a cDNA clone spanning bp 4449 to 4691, or residues 1479 to 1559 of AnkG190 (GenBank accession number AF069525) (8) was inserted into the DNA binding domain vector pGBT7 (DNA/BD). A premade human kidney cDNA library cloned into the activating domain vector pGADT7 (AD/library) was obtained from Clontech. To verify the absence of autonomous activation, the DNA/BD and AD/library constructs were independently transformed into the yeast AH109 strain, with parallel positive and negative controls as recommended by the manufacturer. To assess for protein-protein interactions, the DNA/BD and AD/library were cotransformed in high stringency medium (SD/-Ade/-Leu/Tre/-His) with the DNA/BD and AD/library constructs. Positive clones were isolated and the plasmids purified, transformed into *Escherichia coli*, and sequenced to determine their identity.

Comunmunoprecipitations In Vitro

MDCK cells (American Type Culture Collection, Manassas, VA) were cultured in complete Dulbecco modified Eagle medium with 10% FBS, and communoprecipitations performed as described previously (21,22). These cells were chosen because they represent kidney distal/collecting tubule epithelial cells that normally express all of the proteins pertinent to this study, including AnkG190, Fas, and FADD (8–10,21–24). Briefly, cells were lysed for 20 min at 4°C in immunoprecipitation buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.5% deoxycholate, 1% Nonidet P-40, and 1× complete protease inhibitor): the lysates were pre cleared with preimmune serum and a 50% protein A-Sepharose solution and incubated overnight at 4°C with either preimmune serum or a polyclonal antibody to AnkG190 (22). After an additional incubation with a 50% protein A-Sepharose solution for 2 h, the lysates were centrifuged and washed, and pellets were subjected to SDS-PAGE and Western blot analysis with antibodies to AnkG190 at 1:500 (22), Fas at 1:2000, and FADD at 1:200 (both from Transduction Laboratories, Lexington, KY). Aliquots of cell lysates were probed with antibody to tubulin (1:10,000; Sigma, St. Louis, MO) before precipitation to verify equal loading of samples.

Glutathione S-Transferase Pull-Down Assay

Ankyrin binding assays were performed as described previously (21–24). Briefly, the death domain of ankyrin (DD) was expressed in bacteria as a fusion protein with glutathione S-transferase (GST) using the pGEX prokaryotic expression system (Pharmacia, Nutsley, NJ) and purified with glutathione-agarose. GST alone was expressed as a control peptide. Proteins were analyzed by SDS-PAGE followed by Coomassie blue staining. Each fusion protein (GST or GST-DD, 50 μg) was conjugated to 50 μl of a 50% glutathione-agarose slurry for 1 h at 4°C, and incubated overnight with 1 ml of cell lysate obtained by extracting MDCK cells with a buffer containing 10 mM PIPES, 500 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 0.5% Triton X-100, and 1× complete protease inhibitors. The beads were pelleted, washed, and analyzed by Western blotting with antibodies as above.

Colocalization In Vitro

Colocalization studies were performed as described previously (9,10,22). Briefly, MDCK cells cultured to confluence on glass slides were fixed with acetone, blocked in goat serum for 30 min, simultaneously incubated in primary antibodies to AnkG190 (polyclonal, 1:200) and Fas (monoclonal, 1:2500) for 1 h, washed, incubated in secondary antibodies conjugated to Cy2 and Cy3 (Amersham, Piscataway, NJ), and visualized with a fluorescence microscope (Zeiss Axiophot).

Site-Directed Mutagenesis

Because a conserved arginine residue within the death domain of Fas (R234) has been shown to be critical for Fas-FADD interactions (25), the corresponding arginine within the death domain of AnkG190 (R1496) was substituted to alanine (AGG to GCC) by using recombinant PCR (26). Briefly, a primary PCR product was obtained with a sense primer targeting the 5′ end of ankyrin’s death domain and an antisense primer containing the AGG to GCC substitution. A second PCR product was obtained using a primer containing the substitution as the sense primer and an antisense primer targeting the 3′ end of the death domain. The PCR products were then used as templates to amplify a “zipper” product with the outside primers. The final product was sequenced to verify the presence of the R to A substitution and cloned into the pGEX system for GST pull-down assays.

Eukaryotic Overexpression as Green Fluorescence Protein Fusion

The original death domain of AnkG190 (DD) and the mutated death domain (ΔR) were cloned into the eukaryotic expression vector pEGFP-N1 (Clontech) for expression of fusion proteins with green fluorescence protein (GFP). MDCK cells were stably transfected with either of these constructs under G418 selection (400 μg/ml; Invitrogen, Carlsbad, CA) as described previously (8–10). Stable expression was confirmed by immunofluorescence, and transfecants were subjected to immunoprecipitation with antibody to GFP (Clontech), followed by Western blot analysis with antibodies to GFP, Fas, and FADD.

AnkG190 Constructs, Eukaryotic Overexpression, and Apoptosis Assays

The engineering, stable transfection, and expression of a series of overlapping AnkG190 constructs, each containing the eight-residue FLAG tag (Kodak, New Haven, CT), using the pcDNA3 eukaryotic expression system (Invitrogen), have been previously reported (8). Two additional clones were constructed for this study using PCR. First, a construct encoding for only the death domain of AnkG190 devoid of the death domain. Constructs were stably transfected into MDCK cells, and their expression was verified by Western blot analysis as described previously (8). Stable transfecants were exposed to stimulatory Fas monoclonal antibody (clone DX2, Clontech) at 200 ng/ml for 4 h and subjected to the DNA laddering assay for detection of apoptosis as described previously (21). Briefly, cells were resuspended in lysis buffer (1% SDS, 25 mM EDTA, 1 mg/ml proteinase K, pH 8) at 50°C overnight, digested with ribonuclease A (10 mg/ml), and the chromosomal DNA extracted and analyzed by agarose gel electrophoresis. To confirm and quantitate apoptosis, we performed the terminal deoxynucleotidyl transferase (TUNEL) assay (ApoAlert Assay Kit; Clontech) as described previously (21). Briefly, cells grown on coverslips were fixed with 4% formaldehyde for 30 min at 4°C, permeabiliized.
with 0.2% Triton X-100 for 15 min at 4°C, incubated with a mixture
of nucleotides and TdT enzyme for 60 min at 37°C in a dark,
humidified chamber, the reaction terminated with 2× SSC, and the
coverslips mounted on glass slides. Apoptotic nuclei were detected by
visualization with a fluorescence microscope and quantitated as a
percentage of all nuclei visualized by phase contrast microscopy. Only
cells that displayed the characteristic morphology of apoptosis, in-
including nuclear fragmentation, nuclear condensation, and intensely
fluorescence nuclei by TUNEL assay, were counted as apoptotic.
Merely TUNEL-positive cells, in the absence of morphologic criteria,
were not considered apoptotic. As another confirmatory assay, we
used the annexin V-FITC cell membrane labeling assay (ApoAlert
Annexin V Kit, Clontech) to detect translocation of phosphatidyliner-
ine from the inner face of the plasma membrane to the outer cell
surface, where it binds an annexin V-FITC conjugate and serves as an
early and specific marker of apoptosis (21). Briefly, cells grown on
coverslips were washed, incubated with annexin V-FITC for 15 min
at room temperature in the dark, and visualized by fluorescence
microscopy. Apoptosis was quantitated as the number of annexin
positive cells per 100 cells examined.

Mouse Model of Renal Ischemia-Reperfusion Injury

We used well established murine models in which the structural
and functional consequences of brief periods of renal ischemia have
been previously documented (27–30). Briefly, male Swiss-Webster
mice (Taconic Farms, Germantown, NY) weighing 25 to 35 g were
housed with a 12:12 h light:dark cycle and were allowed free access
to food and water. The animals were anesthetized with sodium pen-
toobarbital (50 mg/kg intraperitoneally) and placed on a warming table
to maintain a rectal temperature of 37°C. The renal pedicles were
occluded with a nontraumatic vascular clamp for 30 min, the clamps
released, the kidney observed for return of blood flow, the incision
sutured, and the mice allowed to recover in a warmed cage. After 0,
3, 12, or 24 h of reperfusion, the animals were reanesthetized, and
blood obtained by puncture of the inferior vena cava for serum
creatinine determination by quantitative colorimetric assay kit (Sigma,
St. Louis, MO). The animals were killed, the kidneys perfusion-fixed
in situ with 4% paraformaldehyde in PBS, and both kidneys har-
vested. At least five separate animals were examined at each of the
reflow periods. Half of one kidney was snap-frozen in liquid nitrogen
and stored at −70°C until further processing; a sample was fixed in
formalin, embedded in paraffin, and sectioned (4 μm). Paraffin sec-
tions were stained with hematoxylin and eosin and examined histo-
logically as well as by TUNEL staining for apoptosis as described
previously (28–30).

For the TUNEL assay, we used the ApoAlert DNA Fragmentation
Assay Kit (Clontech). Paraffin was removed from the sections by
xylene and descending grades of ethanol. The sections were fixed with
4% formaldehyde/PBS for 30 min at 4°C, permeabilized with pro-
teinase K at room temperature for 15 min and 0.2% Triton X-100/PBS
for 15 min at 4°C, and incubated with a mixture of nucleotides and
TdT enzyme for 60 min at 37°C. The reaction was terminated with 2×
SSC and the sections washed with PBS, and they were mounted with
Crystal/mount (Biomeda, Foster City, CA). TUNEL-positive apopto-
tic nuclei were detected by visualization with a fluorescence micro-
scope. Only cells that displayed the characteristic morphology of
apoptosis, including nuclear fragmentation, nuclear condensation, and
intensely fluorescence nuclei by TUNEL assay, were counted as
apoptotic. Merely TUNEL-positive cells, in the absence of morpho-
logic criteria, were not considered apoptotic.

The other half of one kidney was embedded in optimal cutting
temperature compound (Tissue-Tek; Miles, Naperville, IL), and fro-
zen sections (4 μm) were obtained for immunohistochecmistry. The
second kidney was processed for Western blotting and immunopre-
cipitations as follows. Whole kidneys were homogenized in ice-cold
lysis buffer (20 mM Tris, pH 7.4, 250 mM sucrose, 150 mM NaCl, 1% NP-40, and 1× complete protease inhibitors) with a Polytron homog-
izer. The homogenates were incubated on ice for 30 min, centri-
fuged at 1000 × g for 5 min at 4°C to remove nuclei and cellular
debris, and analyzed for protein content by the Bradford assay (Bio-
Rad, Hercules, CA). By means of this mouse model, others (27) and
we (28–30) have previously documented the presence of tubule cell
apoptosis and necrosis, and the induction of Fas in apoptotic tubule
cells.

Coimmunoprecipitations In Vivo

Whole kidney lysates were precleared with a 50% protein A-
Sepharose solution and incubated overnight at 4°C with a monoclonal
antibody to AnkG190 (Zymed, San Francisco, CA). After an addi-
tional incubation with a 50% protein A-Sepharose solution for 2 h, the
lysates were centrifuged and washed, and pellets were subjected to
SDS-PAGE and separate Western blot analysis with polyclonal anti-
bodies to AnkG190 (22), Fas (Santa Cruz Biotechnology, Santa Cruz,
CA), or FADD (Santa Cruz Biotechnology). Aliquots of cell lysates
were probed with antibody to tubulin (1:10,000, Sigma) before pre-
cipitation to verify equal loading of samples.

Colocalization In Vivo

Colocalization studies for ankyrin and Fas were performed in
mouse kidneys as described previously (28–30). Briefly, frozen sec-
ions were permeabilized with 0.2% Triton X-100 in PBS for 10 min,
bloated with goat serum for 1 h, and incubated with primary anti-
bodies to AnkG190 (polyclonal) and Fas (monoclonal) for 1 h at room
temperature. Slides were then incubated with secondary antibodies
conjugated with either Cy5 (for Fas) or Cy3 (for ankyrin) and visu-
alyzed with rhodamine or fluorescein filters, respectively. For colo-
calization of Fas with apoptotic cells, serial sections were subjected to
either the TUNEL assay or to immunohistochemistry as described
previously (28,29). Visualization with rhodamine filters revealed cells
that stained positive for Fas, and examination with fluorescein filters
identified TUNEL-positive nuclei in serial sections.

Results

Identification of Proteins that Interact with Kidney
Ankyrin’s Death Domain

The yeast two-hybrid system was used to screen for proteins
that interact with the death domain of kidney ankyrin. The identi-
fied proteins and their putative function are shown in Table 1.

Table 1. Proteins interacting with the death domain of AnkG
in a yeast two-hybrid assay

<table>
<thead>
<tr>
<th>Number of Positive Clones</th>
<th>Sequence Identity</th>
<th>Putative Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Fas death domain</td>
<td>Pro-apoptotic</td>
</tr>
<tr>
<td>4</td>
<td>AnkG death domain</td>
<td>Pro-apoptotic</td>
</tr>
<tr>
<td>4</td>
<td>MDM2</td>
<td>p53 inhibition</td>
</tr>
<tr>
<td>2</td>
<td>Siva death domain</td>
<td>Pro-apoptotic</td>
</tr>
<tr>
<td>2</td>
<td>Toll-like/IL-1 receptor</td>
<td>NF-κB signaling</td>
</tr>
<tr>
<td>2</td>
<td>Tollip</td>
<td>NF-κB inhibition</td>
</tr>
</tbody>
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The most abundant interacting clones encoded the death domain of Fas, a proapoptotic protein belonging to the TNF receptor family (13,14). Several clones representing the death domain of AnkG190 were also isolated, suggesting an ability of this peptide to dimerize with itself. Interestingly, all identified proteins play putative roles in regulation of apoptosis. For this study, we chose to further characterize the interaction between the death domains of AnkG190 and Fas.

Ankyrin’s Death Domain Forms Complexes with Fas and FADD In Vitro

The ability of kidney ankyrin’s death domain to interact with Fas was tested in MDCK cells. These cells were chosen because they represent kidney distal/collecting tubule epithelial cells that normally express all of the proteins pertinent to this study, including AnkG190, Fas, and FADD (8–10,21–24). Furthermore, these cells have been shown to respond to partial ATP depletion by inducing Fas and FADD and undergoing programmed cell death (21). By using recently described antibodies (22), AnkG190 was found to form a specific complex with Fas and FADD in MDCK cell lysates, as shown in Figure 1A. This complex was absent from lysates immunoprecipitated with preimmune serum (Figure 1A, Pre). This interaction was confirmed using GST pull-down assays, in which a fusion peptide of GST and ankyrin’s death domain specifically interacted with Fas and FADD, as shown in Figure 1B. The GST peptide alone did not form a complex with Fas or FADD.

Ankyrin Colocalizes with Fas In Vitro

It was next of interest to assay for the intracellular localization of ankyrin and Fas. MDCK cells were double stained with polyclonal antibodies to AnkG190 and monoclonal antibodies to Fas. AnkG190 in these polarized epithelial cells was largely restricted to the plasma membranes (Figure 2, red), whereas Fas was present both at the cell surface and in an intracellular distribution (Figure 2, green). The merged images revealed that ankyrin colocalizes with Fas predominantly at the plasma membrane (Figure 2, yellow).

A Conserved Arginine Residue Is Critical for Ankyrin-Fas Interaction

We next defined the residues within AnkG190 that were critical for interaction with Fas. An alignment of protein sequences within several known death domains is shown in Figure 3. We noted that of the four residues on Fas (arrows) required for FADD binding (25), only one (R234 in Fas and R1496 in AnkG190) is fully conserved in all death domains examined. By means of site-directed mutagenesis, we accomplished a R1496→A substitution in AnkG190. A GST fusion containing this substitution failed to interact with Fas and FADD in GST pull-down assays of MDCK cell lysates, as shown in Figure 4A, labeled ΔR. To confirm this observation, we performed stable transfections of MDCK cells with either wild-type or mutated (R1496→A substitution) AnkG190 death domain.
domain cloned in the eukaryotic expression vector pEGFP-N1. Immunoprecipitation of lysates with GFP antibody revealed a functional complex between the AnkG190 death domain, Fas, and FADD in cells transfected with the wild-type construct (Figure 4B, labeled DD). This interaction was abolished by the R1496→A substitution (Figure 4B, labeled ΔR).

**The Death Domain of Kidney Ankyrin Promotes Fas-Mediated Apoptosis In Vitro**

To explore the role of ankyrin-Fas interactions, we used MDCK cells stably transfected with a series of constructs spanning the coding region of AnkG190, as illustrated in Figure 5. The engineering, transfection, and expression of these constructs have been previously reported (8). In preliminary experiments, the cell lines demonstrated comparable viability, and the overexpression of AnkG190 constructs containing the death domain did not in itself induce apoptosis (data not shown). We have also previously shown that although nontransfected MDCK cells retain Fas-dependent apoptotic pathways, stimulatory monoclonal Fas antibodies resulted in DNA laddering only after prolonged exposure (21). We tested the ability of AnkG190 constructs to promote Fas-mediated apoptosis after short incubation periods. As shown in Figure 5, cells transfected with constructs containing the death domain underwent apoptosis after only 4 h of stimulatory Fas antibody incubation. This included constructs I (full-length AnkG190), V (regulatory domain of AnkG190 with the death domain included), and VI (death domain of AnkG190 alone). However, the constructs varied in their ability to induce Fas-mediated apoptosis, as illustrated in Figure 6.

By means of two rigorous and complementary methods for apoptosis detection and quantitation, control untransfected cells responded only minimally to short-term Fas antibody incubation (4 ± 2% apoptosis by TUNEL assay and morphology, and 5 ± 3% by annexin staining). Cells transfected with full-length AnkG190 (construct I) exhibited the most dramatic response (64 ± 10% apoptosis by TUNEL and morphology, and 75 ± 5% by annexin). Cells expressing truncated AnkG190 constructs that contained the death domain showed an intermediate degree of apoptosis (18 ± 5% by TUNEL/morphology and 22 ± 6% by annexin for construct V, and 15 ± 5% by TUNEL/morphology and 25 ± 6% by annexin for construct VI).

**Kidney Ankyrin Interacts with Fas In Vivo**

The ability of kidney ankyrin to interact with Fas was also tested in vivo. By using recently described antibodies (22), AnkG190 was found to form a specific immunoprecipitatable complex with Fas and FADD in whole kidney lysates, as shown in Figure 7 (lane marked Con). This was confirmed by immunofluorescence studies, for which kidney sections were stained with antibodies recognizing AnkG190 and Fas. The results indicated that ankyrin and Fas are co-localized in kidney tissue, consistent with the in vitro observations.

*Figure 3.* Sequence comparison reveals that of the four residues on Fas (arrows) required for FADD binding (25), only one (R234 in Fas and R1496 in AnkG190) is fully conserved in all death domains examined.

*Figure 4.* A conserved arginine residue is critical for ankyrin-Fas interactions. (A) Results of glutathione S-transferase (GST) pull-down assay. CB, Coomassie blue stain of purified GST-ankyrin death domain fusion (DD) or the mutant protein containing the R1496→A substitution (ΔR). Blots were probed with antibodies as shown on the right. Antibodies used are shown on the right. (B) Results of immunoprecipitation with GFP antibody of cells stably transfected with either ankyrin death domain (DD) or the R1496→A substitution (ΔR) cloned into the expression vector pEGFP-N1. Lysates were probed with anti-tubulin (Tub) before immunoprecipitation and with anti-GFP antibody after immunoprecipitation to verify equal loading of samples. The blots represent three separate experiments.

*Figure 5.* Ankyrin’s death domain promotes Fas-mediated apoptosis. The panel on the left shows the various AnkG190 constructs (labeled I to VII) used in this study. Each construct was tagged with the FLAG epitope at the 3’ end. MDCK cells were stably transfected with each construct, exposed to stimulatory Fas monoclonal antibody for 4 h, and subjected to the DNA laddering assay as shown on the right panel. Results were reproducible in three experiments.
double stained with polyclonal antibodies to AnkG190 and monoclonal antibodies to Fas (Figure 8). The distribution of ankyrin immunoreactivity was noted to be strongest in the outer medullary region, in distal tubular cells and ascending limb of Henle’s loop, as described previously by others (7) and by us (8). Importantly, Fas was also noted to be expressed predominantly in the same nephron segments, as described previously by others (27). In control kidneys, ankyrin staining (green) was largely restricted to the plasma membranes of tubule cells (Figure 8, top panel, arrows), whereas Fas (red) was present both at the cell surface and in an intracellular distribution. The merged images (yellow) revealed that ankyrin colocalizes with Fas predominantly at the plasma membrane.

Renal Ischemia-Reperfusion Injury Results in Induction of Tubule Cell Apoptosis, Fas, and Ankyrin

Because overexpression of ankyrin promoted Fas-mediated apoptosis in cultured renal epithelial cells, it was next of
importance to examine an analogous in vivo situation. We used a well established mouse model of early renal ischemia-reperfusion injury characterized by the presence of tubule cell apoptosis and necrosis (28–30). The characteristic functional derangements and histopathologic features of ischemic injury were readily evident in the 24-h reperfusion samples. These included an elevation in serum creatinine (2.5 ± 0.6 mg/dl in the 24 h reflow animals versus 0.6 ± 0.3 mg/dl in the control group, P < 0.05), loss of brush border membranes, tubular dilation, flattened tubular epithelium, luminal debris, and an interstitial infiltrate (Figure 9, top panel, IRI). Also evident were tubule epithelial cells that displayed the characteristic morphology of apoptotic nuclei as described previously (28–30), consisting of condensed, fragmented, and intensely staining nuclei (Figure 9, top panel, arrows). The presence of apoptotic tubule epithelial cells was confirmed by TUNEL assay, which revealed the characteristic intensely fluorescence, condensed, and fragmented nuclei (Figure 9, middle panel, arrows). By means of both morphologic and TUNEL-positive criteria, apoptosis was detected in 10 ± 3 cells per 100 counted in the 24-h reflow animals versus 2 ± 1 cells per 100 counted in the control group (P < 0.05). The number of apoptotic cells counted were similar when counted using morphology (hematoxylin and eosin) or TUNEL staining.

Apoptosis was most evident in the outer stripe of the outer medulla, with occasional occurrence in the cortex. Apoptosis was predominantly localized to distal tubular cells and ascending limb of Henle’s loop, both in detached cells within the lumen as well as attached cells, as described previously by others (27) and by us (28–30). Occasional proximal tubular cells were also noted to be apoptotic, but the glomeruli were essentially devoid of apoptosis. Importantly, apoptosis was noted to occur largely in the same nephron segments in which ankyrin and Fas were maximally expressed, namely the distal segments.

After ischemia-reperfusion injury, the expression of ankyrin, Fas, and FADD proteins were markedly induced by Western blot analysis of kidney lysates (Figure 10) and by ankyrin immunoprecipitations (Figure 7). By densitometry, ankyrin expression was upregulated by approximately threefold at 3 and 12 h of reperfusion, but fell to baseline by the 24 h reflow period (Figure 11). In contrast, Fas and FADD expression remained upregulated by approximately threefold at all time periods examined. Immunohistochemistry (Figure 8, bottom panel) confirmed the upregulation of ankyrin (green) and Fas (red) in comparison with control kidneys, both of which were now distributed at intracellular sites as well as the plasma membranes of ischemic kidney tubule cells. Importantly, the upregulation of ankyrin and Fas was noted to occur largely in the same nephron segments in which apoptosis was maximally detected, namely the outer stripe of the outer medulla. Indeed, in colocalization studies done on serial sections, tubule cells displaying Fas induction after ischemia-reperfusion were also punctuated by TUNEL-positive nuclei that revealed the characteristic morphology of apoptosis (Figure 9, bottom panel, arrows).

The Interaction between Ankyrin and Fas Remains Intact after Ischemic Injury

To implicate the ankyrin-Fas interaction in renal tubule cell apoptosis, it was essential to demonstrate a continued interac-
tion between these molecules after an ischemic insult. The ability of ankyrin to form a specific immunoprecipitatable complex with Fas and FADD in whole kidney lysates remained intact after ischemia, as shown in Figure 7. Indeed, the immunoprecipitates examined early after ischemia (at the 3- and 12-h reperfusion periods) consistently displayed a quantitatively increased amount of complexed Fas and FADD when compared with control, perhaps reflective of induction of these proteins. This was confirmed by immunofluorescence studies, for which kidney sections after 24 h of reperfusion were double stained with polyclonal antibodies to AnkG190 and monoclonal antibodies to Fas. The merged images revealed that ankyrin

Figure 9. Renal ischemia-reperfusion injury results in induction of tubule cell apoptosis. Kidney sections from control (Con) or after 24 h of reperfusion after ischemia (IRI), stained with hematoxylin and eosin (top) or terminal deoxynucleotidyl transferase (TUNEL) (middle). By rigorous morphologic and TUNEL criteria (see Materials and Methods), apoptosis (arrows) was detected predominantly in the outer stripe of the outer medulla in distal tubule cells. Evidence for necrosis was also present (asterisks), consisting of cellular disintegration and granular cell debris in the lumen. The panel on the extreme right shows high-power (HP) images of cells undergoing apoptosis (arrows). The bottom panel shows serial sections of kidneys after 24 h of reperfusion after ischemia stained with Fas antibody (red) or TUNEL (green), showing that cells overexpressing Fas also reveal TUNEL-positive nuclei (arrows). Figure represents four experiments.
colocalizes with Fas both at the plasma membranes and within intracellular sites (Figure 8, bottom panel, yellow).

Discussion

The yeast two-hybrid system identified Fas as a protein that interacts with kidney ankyrin’s death domain. This interaction was confirmed both in vitro and in vivo by means of a variety of complementary studies. Overexpression of constructs containing the death domain of kidney ankyrin promoted Fas-mediated apoptosis in vitro. After renal ischemia-reperfusion injury, the expression of both ankyrin and Fas was upregulated in the same nephron segments as those displaying apoptosis, namely the outer medullary regions, and the interaction between these molecules remained intact. Our results identify a novel tethering interaction between ankyrin and Fas in kidney epithelia, and suggest that ankyrin may play a role as an adapter molecule in renal tubule cell death.

An important prerequisite for Fas-mediated apoptosis appears to be trafficking of intracellular Fas to the cell surface (31,32), where cross-linking with Fas ligand leads to its oligomerization (33–35). Although the pathways responsible for Fas trafficking remain unknown, our results provide a possible mechanism for tethering Fas at the plasma membrane of kidney tubule cells after its delivery. Ankyrins are adapter molecules that interact with and maintain the plasma membrane distribution of an increasing number of proteins (1–5), and the study presented here adds Fas to that list. Although the majority of integral membrane proteins bind to ankyrin’s repeats domain, Fas appears to use the well documented ability of death domains to mediate a protein-protein interaction with ankyrin.

Most death domains described to date induce apoptosis when overexpressed in mammalian cells, but the study presented here indicates that ankyrin’s death domain is a notable exception. Generation of cell lines stably transfected with either the AnkG190 death domain alone or the death domain with other parts of the molecule did not result in any loss of cell viability. However, overexpression of AnkG190 death domain–containing constructs rendered the cells sensitive to Fas-mediated cell death, lending support to the notion that ankyrin plays a facilitatory role in this process. It is interesting to note that the various constructs differed in their ability to promote Fas-mediated apoptosis. The full-length AnkG190 construct exhibited the most dramatic apoptotic response to stimulatory Fas antibodies, whereas constructs containing either the death domain alone or the entire regulatory domain displayed an intermediate response.

These results suggest that in addition to the death domain itself, other sequences within AnkG190 also play a role in promoting Fas-mediated apoptosis. An analogous situation has been demonstrated in the case of FADD, which contains a C-terminal death domain as well as an N-terminal “death effector domain” that interacts with procaspase 8 and initiates the caspase cascade of apoptosis (33,34). The proapoptotic limb downstream of both Fas and TNF-R1 activation appears to be dependent on the death effector domain of FADD. However, although overexpression of the death effector domain of FADD alone can induce apoptosis, AnkG190 constructs devoid of the death domain did not promote Fas-mediated apoptosis in the study presented here. Our results suggest that the presence of a death effector domain within AnkG190 is unlikely, and they provide a possible explanation for the inability of ankyrin overexpression per se to induce apoptosis. The mechanisms whereby other sequences within AnkG190 modulate the apoptosis-promoting ability of the death domain remain unknown.

It is intriguing to speculate that the identified interaction may contribute to apoptotic cell death and organ dysfunction in pathophysiologic states characterized by upregulation of ankyrin and/or Fas. For example, it has been shown that ischemic injury to the kidney is associated with apoptosis (21,28–30), upregulation of Fas (21,27,29), and enhanced ankyrin expression (22) in tubular epithelial cells, and Fas-deficient mice are protected from ischemic renal injury (27). In the study presented here, we have shown that in a model of early renal ischemia-reperfusion injury characterized by apoptotic tubule cell death, the expression of both ankyrin and Fas was markedly induced, and the interaction between these molecules remained intact. Apoptosis after Fas induction has been implicated in a myriad of clinical situations, including response to chemotherapy and irradiation, infections such as HIV, and autoimmune disorders such as lupus (33). It will be important...
in future studies to explore the role of ankyrin-Fas interactions in promoting cell death in these conditions.

Interestingly, all proteins interacting with the death domain of AnkG190 identified by our yeast two-hybrid screen appear to play putative roles in apoptosis regulation. For example, we identified the death domain of Siva, which is a known ligand for CD27, another member of the TNF receptor family (36). Siva is upregulated after various apoptosis-inducing stimuli, including kidney ischemia (37), viral infection (38), oxidative stress (39), and chemotherapy (40), and overexpression of Siva induces apoptosis in several cell lines (36). In several of these instances, Siva-mediated apoptosis may occur independent of its previously documented ligands such as CD27. On the basis of our current findings, it is attractive to speculate that a ubiquitously expressed adapter molecule such as AnkG190 may provide an important permissive role in Siva-dependent apoptotic pathways.

In summary, the study presented here has identified a novel interaction between the death domain of kidney ankyrin and Fas. We speculate that this binding serves to tether Fas at the cell surface, and it promotes Fas-mediated apoptosis by facilitating interactions with downstream adapter molecules. Interruption of this interaction may represent a therapeutic tool in clinical conditions characterized by activation of Fas-dependent cell death pathways.

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References