Cloning and expression of rat caspase-6 and its localization in renal ischemia/reperfusion injury

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Background. Caspase-6 is an important member of the executioner caspases in the caspase family of cell death proteases. The executioner caspases are the major active caspases detected in apoptotic cells and are generally considered to mediate the execution of apoptosis by cleaving and inactivating intracellular proteins. However, the complete characterization of mRNA and protein of caspase-6 in rat and its expression in normal kidney and in disease state has not been previously elucidated.

Methods. A rat kidney cortex cDNA library was screened to isolate the full-length caspase-6 cDNA. The recombinant caspase-6 protein was characterized by expression in bacteria and by transient transfection in mammalian cells. The expression in various tissues was analyzed by Northern blot, and localization in normal and ischemic kidney was performed by immunohistochemistry.

Results. The predicted amino acid sequence of rat caspase-6 contains 277 amino acids, with two potential glycosylation sites, an integrin binding site (KGD), the caspase active site pentapeptide QACRG and the caspase family signature, HX(2,4)(S,C)X(6,7)(L,I,V,M,F)2(S,T)HG (HVDADCFVCVFSLHG). Rat caspase-6 is unique among known caspases by possessing a relatively long 5’ untranslated region. Among various tissues tested, caspase-6 was expressed in varying levels in kidney, liver, spleen, heart, muscle, testis, and lung. Bacterial expression of recombinant rat caspase-6 resulted in production of both the proform and active form of the enzyme suggesting autoactivation. Transient overexpression of rat caspase-6 in COS-1 cells induced DNA fragmentation, a hallmark of apoptosis. We also examined the localization and expression of caspase-6 by immunohistochemistry in kidneys subjected to 40 minutes of ischemia followed by 24 hours of reperfusion injury. Normal kidney showed mostly cytoplasmic and some nuclear staining of the tubules. Kidneys 24 hours after 40 minutes of ischemia showed more intense and diffused cytoplasmic staining with prominent nuclear staining, indicating increased expression and translocation from the cytoplasm to the nuclei. The staining in glomeruli was negative in both normal and ischemic kidney.

Conclusions. These studies demonstrate cloning, expression and characterization of the full-length rat caspase-6 and its localization in normal kidneys and kidneys subjected to ischemia/reperfusion injury. Since caspase-6 is involved in the degradation of nuclear matrix proteins and in activation of caspase-3, it may play an important role during renal ischemic injury.

Caspases are a family of structurally related cysteine proteases that play a central role in the execution of apoptosis [1–6]. On receiving a pro-apoptotic stimulus, the caspases are proteolytically processed to the active forms from their normally synthesized inactive proenzymes. At least fourteen caspases encoded by distinct genes have been cloned and sequenced to date in mammals [1, 7]. Caspase-2, -8, -9, and -10 have large prodomains and initiate the activation of downstream caspases. Caspase-3, -6, and -7 with smaller domains are identified as effector or executioner caspases [1–3, 7]. The executioner caspases are the major active caspases detected in apoptotic cells, and are widely regarded to mediate the execution of apoptosis by cleaving and inactivating intracellular proteins that are essential for cell survival and proliferation [1–3, 8]. The specificity of downstream executioner caspases to cleave cellular proteins is unique because of their different primary sequences and different recognition sites on the target proteins. For example, following activation, caspase-3 primarily recognizes DEVD or DMQD tetrapeptide sequences whereas caspase-6 recognizes the VEID tetrapeptide sequence for cleavage after the aspartate residue on the target proteins [1–3, 6]. Multiple pathways can result in activation of executioner caspases depending on the nature of the death-inducing stimulus. At present, there are two relatively well-characterized cell death pathways that result in the activation of the downstream executioner caspase-3. One is receptor-mediated [9] and the other is mitochondrial-dependent [10]. The receptor-dependent pathway is initiated by activation of cell death receptors (Fas and tumor necrosis factor α) leading to activation of procaspase-8, which in turn cleaves and activates procaspases-3. The mitochondrial-dependent pathway is triggered by cytochrome c release from the mitochondria. The cytosolic cytochrome c recruits procaspase-9, Apaf-1, and dATP and promotes
caspase-9 activation. Activated caspase-9 then cleaves and activates procaspase-3 to its active form [1, 9, 10]. Thus, extensive studies have been done on caspase-3 but very little information is available on the executioner caspase, caspase-6, in the apoptotic pathway.

Many studies have documented the occurrence of apoptosis during renal ischemia/reperfusion injury and other renal diseases [11–15]. Since the executioner caspases are key intracellular mediators of apoptosis, their activation may play an important role in renal injury. To examine the specific role of executioner caspases in renal injury it is essential to know if these caspases are transcribed in the kidney. We have recently shown that rat kidney transcribes the genes for caspase-1, -2, -3, -6, -7, -8, and -9 [16], indicating that all of the executioner caspases are expressed in the kidney. However, the complete characterization of mRNA and proteins of most of these caspases in rat kidney remain to be accomplished. At present, complete identification and characterization of mRNAs and proteins of only three rat caspases, caspase-1 [17], caspase-2 [18] and caspase-3 [19], have been reported. To further examine the specific role of the individual executioner caspases in renal injury, we focused our present study on caspase-6. This study reports the cloning and sequencing of the full-length cDNA of rat caspase-6, and characterization of the recombinant protein expressed in bacteria and by transient transfection in mammalian cells. We also examined the expression of caspase-6 in various tissues and its localization by immunohistochemistry in normal kidney as well as in kidneys subjected to ischemia/reperfusion injury.

METHODS

RT-PCR

Poly A+ RNA was isolated from normal rat kidney and reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously [16]. Briefly, 1 µg of poly A+ was reverse-transcribed using an oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer, Branchburg, NJ, USA). The resulting reverse transcription product was used as a template for PCR using sense and antisense primers based on the human caspase-6 coding sequence (GenBank accession #U20537). The sequences of primers used and their locations in the human caspase-6 coding sequence are: sense, CTAATCTTCAATCACGAAGGTTC (221-243) and antisense, CTCACACAA ATCTTGAA TGTACCA (780-757) (National Bioscience, Plymouth, MA, USA). The criteria to use these primers were described in our previous study [16]. Attempts to amplify rat caspase-6 using degenerate primers designed from the N-terminal initiating domain or from the prodomain of human caspase-6 were unsuccessful. PCR was performed in a Perkin-Elmer Thermocycler 9600 with the following conditions: 94°C for 60 seconds followed by 94°C for 5 seconds, 50°C for 30 seconds; 68°C for two minutes (30 cycles), and a final extension at 68°C for seven minutes. The amplified products were resolved by gel electrophoresis on 1% agarose (FMC BioProducts, Rockland, ME, USA) and visualized by ethidium bromide staining. The appropriate sized band was excised from the gel, purified, and subcloned into a pGEMT vector (Promega, Madison, WI, USA). The cloned cDNA was sequenced using vector primer sites, T7 and SP6.

Cloning of rat caspase-6

The cloned cDNA fragment was excised out from the pGEMT vector, radio-labeled by random priming (Amersham Life Science, Arlington Heights, IL, USA) with [α-32P]dCTP, purified by chromatography on NucTrap Push Columns (Stratagene, La Jolla, CA, USA), and used to screen a rat kidney cortex λgt10 cDNA library (Clontech Inc., Palo Alto, CA, USA). About 5 × 105 plaques were screened using standard protocols [20]. After four cycles of plaque purification six positive clones were purified and the cDNA inserts were excised using EcoRI restriction enzyme. The excised cDNAs were gel purified (Qiagen Inc, Valencia, CA, USA) and subcloned into the vector p ZERO (Invitrogen Inc, Carlsbad, CA, USA).

Sequencing

Sequencing was performed on both strands using T7 and SP6 primers, and thereafter walking along with gene-specific primers using the dideoxy DNA cycle sequencing system (Gibco BRL, Gaithersburg, MD, USA) and confirmed by sequencing on an automated DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA). The sequence for caspase-6 reported in this article was deposited in the GenBank database (accession numbers AF025670, NM031775).

Northern blot analysis

Northern blot analysis was performed as previously described [20]. In brief, 25 µg of total RNA isolated from different adult rat organs was electrophoresed under denaturing condition on a 1.2% agarose gel and blotted to Immobilon N nylon membrane (Millipore, Bedford, MA, USA). The membrane was baked under vacuum for one hour and hybridized with 32P-labeled full-length caspase-6 cDNA as probe under standard condition [20]. After washing, the membrane was subjected to autoradiography at −70°C to detect mRNA signals.

In vitro translation

The full-length rat caspase-6 cDNA was subcloned into the vector pZERO. The recombinant plasmid was
then linearized on the 3’ end of the insert using the NotI restriction enzyme. In vitro translation was performed using the TNT T7-coupled reticulocyte lysate system (Promega Inc.) and [35S]-methionine (Amersham Corp.) as per the manufacturer’s recommendation. In brief, linear plasmid (1 μg), amino acid mix minus methionine (1 mmol/L), ribonuclease inhibitor, [35S]-methionine (1000 Ci/mmol), and T7 polymerase were added to a 50 μL reaction mix and incubated at 30°C for two hours. The reaction products were analyzed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Expression plasmid and transient transfection**

The full-length caspase-6 cDNA was subcloned in the mammalian expression vector pcDNA3.1+ (Invitrogen Inc.) downstream of the cytomegalovirus promoter/enhancer. Transfection was carried out with Lipofectamine (Gibco BRL) according to the manufacturer’s recommendation. In brief, 2.0 × 10⁶ COS-1 cells were transiently transfected in 2 mL of serum free medium with a mixture of 1 μg of pCMV β-galactosidase plus 6 μg of test plasmid. Control cells were transfected with empty vector. After five hours, 4 mL of serum containing growth medium was added. Forty-eight hours post-transfection, cells were washed twice with 1× phosphate-buffered saline (PBS), scraped, and processed for β-galactosidase assay (according to the manufacturer’s recommendation) as well as for DNA damage as previously described in our studies [21].

**Genomic DNA extraction and electrophoresis**

Transfected cells were scraped into medium 48 hours post-transfection and all cells, including the floating cells, were collected. Genomic DNA was isolated by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation as described [21]. The DNA samples were analyzed by electrophoresis on 1.8% agarose gel and DNA was observed by ethidium bromide staining of the gel.

**Expression of recombinant caspase-6 in bacteria**

The coding sequence of rat caspase-6 was PCR amplified using 5’ and 3’ PCR primers (sense: GGTAAGAATTGACGGAGACAGATGGC and antisense: TTGGAGCGGCCGCTTACTTAGGTCCGG) containing Nde and XhoI custom restriction sites, gel purified, and subcloned in-frame into the NdeI and XhoI sites of the bacterial expression vector pET-21a (+) (Novagen). This subcloning placed the rat caspase-6 sequence under the control of the inducible bacteriophage T7-promoter and introduced an NH₂-terminal polyhistidine tag (6 histidines) and S-tag for purification on a nickel-chelating column and for identification by Western blot using s-tag antibody, respectively. An enterokinase cleavage site also was included to allow removal of these tags. The resultant plasmid was transformed into the E. coli strain BL21 (F ompT rB mB mompT). The transformed bacteria were cultured in the presence of 1 mmol/L isopropylthiogalactoside (IPTG; Pharmacia-LKB, Uppsala, Sweden) for three hours. After optimal induction, the bacterial culture was centrifuged and pellets were suspended in ice-cold buffer containing 20 mmol/L HEPES, pH 7.8, 5 mmol/L imidazole, 0.5 mol/L NaCl, and protease inhibitors (10 μg/mL aprotinin, 1 μg/mL antipain, 1 μg/mL pepstatin, 17.5 μg/mL benzamidine), and homogenized. The supernatants were first purified on columns containing nickel bound resin (5 mL) using the His-Bind buffer kit supplied by Novagen. The proteins eluted from the columns were dialyzed extensively in 20 mmol/L HEPES, pH 7.8, containing protease inhibitors and further purified on a Mono Q column by applying a linear gradient of 0 to 0.5 mol/L NaCl in 20 mmol/L Tris-HCl buffer, pH 7.4, using an fast protein liquid chromatography (FPLC) system. Purification of rat caspase-6 in both proenzyme forms and active forms (P10 and P20 subunits) are shown in Figure 6.

**Determination of caspase-6 activity**

Bacterial cell pellets containing recombinant caspase-6 were lysed with 20 mmol/L HEPES, pH 7.5, containing 10% sucrose, 0.1% CHAPS, 2 mmol/L dithiothreitol (DTT), 0.1% NP40, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL leupeptin, and 1 μg/mL pepstatin A at 4°C. The supernatants obtained after centrifugation were used to determine the enzyme activity. The activity of caspase-6 was determined by fluorometric assay using the substrate VEID-AMC that is specifically cleaved by the enzyme at the Asp residue to release the fluorescent leaving group amino-4-methyl coumarin (AMC) [16, 22]. The enzyme extracts containing 50 μg protein were incubated with 100 mmol/L HEPES, pH 7.4, containing 10% sucrose, 0.1% CHAPS, 10 mM DTT, and 50 μmol/L of caspase substrate in a total reaction volume of 0.25 mL. The reaction mixture was incubated for 60 minutes at 30°C. At the end of the incubation, the liberated fluorescent group, AMC, was determined using a fluorescent Spectrofluorometer (Perkin Elmer) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm [22]. AMC was used as a standard. Based on the standard curve made with fluorescence readings with free AMC, the data for caspase activity are expressed as nanomoles of AMC liberated when 50 μg of protein extract was incubated with 50 μmol/L of substrate for 60 minutes at 30°C.

**Ischemia-reperfusion model**

Adult male Sprague-Dawley rats used in the present study were subjected to 40 minutes of ischemia by occluding renal pedicles with smooth vascular clamps as pre-
Previously described [23] and then allowed to recover for 24 hours. Each group consisted of six rats. Blood samples were collected for creatinine and urea nitrogen determination. Animals were anesthetized before tissue harvesting. Rats were perfused through the left ventricle with warm Hanks’ balanced salt solution (HBSS). Kidney tissues were fixed in formalin for histology and immunohistochemistry. The control group consisted of rats undergoing surgical exposure of kidneys without clamping the renal pedicles.

**Immunohistochemistry**

Representative renal sections (4 to 5 μm thick) of formalin-fixed paraffin-embedded tissues of control and ischemic kidney were used. Sections were mounted on frosted micro slides and the paraffin-embedded sections were deparaffinized in xylene and re-hydrated through a graded series of alcohol (100 to 0%). Tissue sections were rinsed in PBS and endogenous peroxidase was inactivated by incubations with 3% H₂O₂ in methanol. The slides were extensively washed with PBS and preincubated with 5% bovine serum albumin (BSA) in PBS for 60 minutes to block nonspecific reaction with the antiseraum. Tissue sections were rinsed in PBS, and then incubated with anti-caspase-6 antibody (1:200; Cell Signaling Technology, Inc., Beverly, MA, USA) at 37°C for two hours, washed in PBS and then incubated with horseradish peroxidase-conjugated-goat anti-rabbit IgG for an hour. After washing with PBS, the sections were developed with DAB and H₂O₂. For negative control, primary antibody was omitted and normal rabbit serum was used at the same dilution.

**Preparation of nuclear and cytosolic fractions**

Nuclei from rat kidney cortex were isolated by homogenization followed by a sucrose gradient as previously described [24]. In brief, nuclei were isolated from minced kidney cortex by homogenization at 0°C in buffer A (20 mmol/L Hepes-NaOH, pH 7.4, 5 mmol/L MgCl₂, 0.2 mmol/L EGTA, 3 mmol/L 2-mercaptoethanol, 0.1 mmol/L PMSF, 1μg/mL leupeptin, 1μg/mL antipain) containing 0.93 mol/L sucrose. The filtered homogenate was centrifuged over a cushion of 1.61 mol/L sucrose in buffer A at 20,000 × g at 4°C for 20 minutes. The pellet was collected as a nuclear fraction and supernatant above the sucrose cushion was further centrifuged at 100,000 × g for 60 minutes, and the resulting supernatant was used as the cytosolic fraction. The nuclear pellets were washed once in 0.25 mol/L sucrose in buffer A and resuspended in 0.6 mol/L NaCl in buffer A containing 10% glycerol.

**RESULTS**

**Isolation and sequencing of rat caspase-6**

To obtain the full-length cDNA for rat caspase-6, we first performed RT-PCR using degenerate primers designed from the corresponding human sequences. Our attempts to obtain full-length cDNA by RT-PCR were unsuccessful. However, we were able to amplify a 559-bp cDNA fragment by RT-PCR using primers based on the conserved internal sequences of human caspase-6. The amplified cDNA fragment obtained by RT-PCR was gel purified, and subcloned into the vector pGEMT and sequenced using vector primer sites. Two out of five clones sequenced were found to be identical and showed significant homology to the human caspase-6 alpha gene (we tentatively called this fragment the rat caspase-6 cDNA fragment). The remaining cDNA fragments showed no homology to the caspases and were not pursued further.

We screened a normal rat kidney λgt10 cDNA library using the subcloned rat caspase-6 cDNA fragment as a probe to obtain the full-length cDNA. A total of 5 × 10⁵ plaques were screened and several clones were purified after four rounds of screening. Four clones (clone 1: 1.2 kb; clone 2: 1.5 kb; clone 3: 1.8 kb and clone 4: 2.2 kb) were sequenced using vector primer sites and thereafter walking along with primers. Sequencing revealed that clone 2 contained a total of 1446 nucleotides and represents the full-length rat caspase-6 cDNA. It contained the entire 559 bp sequence of the probe, initiating methionine, stop codon, and poly adenylation signal (Fig. 1). Clone 1 was identical to clone 2 but was incomplete. Although clones 3 and 4 did contain the entire sequence of clone 2, the sequence was interrupted with small stretches of nucleotide sequences of unknown homology suggesting they represented the possibility of incompletely processed pre mRNAs, thus these clones were not pursued further.

The open reading frame (ORF) of clone 2 encodes 277 amino acids (Fig. 1), which is relatively smaller than that reported for human caspase-6. In vitro translation of clone 2 using TNT T7 coupled reticulocyte lysate system resulted in the both proform and the active subunits (Fig. 2). Formation of smaller subunits from the larger precursor represents the processing to form the active enzyme [2–5]. In comparison, rat caspase-3 translation to the proform and the active subunits is also shown. The published full-length cDNA for mouse caspase-6 and its initiating codon [26] corresponds to the initiating codon of the rat caspase-6 cDNA suggesting that this ATG site is conserved in rodents. To further ensure that the cloned cDNA is in fact full-length, we performed RACE using extreme 5’ end of the rat caspase-6 as primer and obtained the same cDNA sequence as in the cloned cDNA obtained from library screening. The initiating codon ATG in the cloned cDNA follows the KOZAK rule and is flanked by purine (A) at −3 and +4 position [25]. In addition, an in-frame stop codon TAA (bp 233–235) is preceded by the initiating codon ATG in the cloned cDNA, indicating that the initiation
Fig. 1. Full-length sequence of rat caspase-6. Features in bold include the start and stop codon, putative aspartic acid cleavage sites, potential glycosylation sites (NVTQ, NGSW), and a potential integrin binding site (KGD). The linker region is underlined. The active site is in italic and underlined.

Fig. 2. In vitro translation of the full-length cDNA of rat caspase-6. In vitro translation was performed using the TNT T7-coupled reticulocyte lysate system (Promega Inc.) and [35S]-methionine (Amersham Corp.) of caspase-6 expression should begin at this ATG site as per the manufacturer’s recommendation. The reaction products were analyzed on 12% SDS-PAGE followed by autoradiography. Translation products of caspase-6 and caspase-3 are shown further confirms the site of initiation at the ATG site at bp 239–243.

The 5’-UTR in rat caspase-6 is unexpectedly longer than that reported for other species including human and mouse. On the other hand, the 3’-UTR is shorter than in human caspase-6. Like mouse caspase-6, the location of the initiating methionine in rat caspase-6 corresponded with the second methionine (19th amino acid) of human caspase-6 (Fig. 3). Blast analysis of total nucleotide sequences revealed 69% identity with human caspase-6, however, GAP analysis of the ORF sequence revealed highest 84% homology at the nucleotide level and 87.7% homology at the amino acid level with the human caspase-6, and 40 to 50% identity to the other chicken and trout.

The predicted amino acid sequence of rat caspase-6 showed two potential glycosylation sites, an integrin binding site (KGD), the conserved pentapeptide QACRG sequence of the active site, and the caspase family signature HX2-4(S,C)X4(L,I,V,M,F)2(S,T)HG (that is, HVDADCFVCVFLSHG) where X can be any amino acid. Amino acids involved in catalysis (Cys-146 and His-104), and those known to form the P1 carboxylate binding pocket (Arg-47, Glu-144, Arg-200 and Ser-206) were also conserved (Fig. 3). The location of aspartic acids at positions 16, 162 and 173 suggest that this molecule is produced as a proenzyme since subunits of p18 and p11 are produced after aspartic acid cleavage. The phylogenetic tree analysis showed a tight grouping with other caspase-6 genes. Like the mouse gene, the prodomain in rat is relatively short for a caspase-6 gene, being just five amino acids in length, compared to human, chicken and trout.

Northern analysis and tissue distribution

Northern analysis of caspase-6 showed a single mRNA transcript corresponding to a size of approximately 1.6 kb. To determine the tissue distribution of rat caspase-6, we performed Northern blot analysis using poly A+ RNA from various rat tissues. The results indicated that
Fig. 4. Tissue distribution of rat caspase-6 mRNA. Total RNA from each tissue was isolated as described in the text. A total of 25 μg total RNAs from various tissues of an adult rat were analyzed by Northern blot analysis. The positions of 18S and 28S RNAs are shown as indicated.
degradation of nuclear matrix proteins and in caspase-3 activation. Therefore, we examined the expression and localization of caspase-6 by immunohistochemistry in normal kidney as well as kidneys subjected to 40 minutes of ischemia followed by 24 hours of reperfusion injury. Normal kidney showed mostly cytoplasmic and some nuclear staining of the proximal tubules. Kidneys 24 hours after 40 minutes of ischemia showed more intense and diffused cytoplasmic staining, with nuclear staining primarily in the tubular segments of the cortex, indicating increased expression and nuclear translocation (Fig. 7). Only occasional staining was observed in the distal tubules within the cortex. The staining in the glomeruli was negative in both normal and ischemic kidney. The cytosolic and nuclear fractions from the control and ischemic kidneys also were examined for the expression of caspase-6 by Western blot analysis. As shown in Figure 8, the increased expression of the cytosolic and nuclear caspase-6 in ischemic kidney corresponds very well to the immunoperoxidase staining data. Nuclear fractions isolated from the control and ischemic kidneys also were examined for the cleavage of nuclear matrix protein, lamin A. Our data demonstrate that lamin A is cleaved to a 46 kD lamin A fragment during ischemic injury (Fig. 9). This cleavage is attributed to the action of caspase-6 since this caspase not other caspases has been shown to cleave lamin A [27, 28].

**DISCUSSION**

Our studies demonstrate the cloning, sequencing, and characterization of the rat caspase-6 cDNA and its expression in the kidney. The cloned cDNA shows highest homology with the known caspase-6 genes and close homology with other executioner caspases. Like other caspases, the rat caspase-6 contains the caspase family signature and the conserved active site pentapeptide QACRG required for proteolytic activity. In common with executioner caspases and caspase-14, caspase-6 has a relatively short prodomain [29], and a linker region where conserved Asp residues allow cleavage by other caspases to release the active heterodimer. Within the prodomain, rat caspase-6 shows close homology at the amino acid level to the mouse caspase-6 but relatively poor homology to the human, chicken and trout caspase-6. Among the three predicted cleavage sites for the human caspase-6 prodomain [30] that occur at Asp23-Ala24, Asp32-Pro-33, and Asp40-His 40, only the last site is conserved within the rat sequence. The rat caspase-6 is unique among known caspases in that it possesses a relatively long 5'-UTR. On long 5'-UTRs Cap-dependent ribosomal scanning has been shown to be severely hampered [30, 31]. Long 5'-UTRs are often found in mRNAs encoding regulatory proteins like proto-oncogenes, growth factors and their receptors, and...
homeodomain proteins and are suspected to provide a regulatory mechanism [31, 32]. However, the mechanisms involved in 5′-UTR-mediated control are not well understood. It is possible that the long 5′-UTR of rat caspase-6 is involved in its translational regulation. As with all caspases, the rat gene shows conservation of residues known to form the P1 carboxylate-binding pocket reflecting the absolute requirement for an Asp at position P1 for substrate cleavage [33]. Residues that form the P2-P4 binding pocket are less conserved between caspases, accounting for differences in substrate specificity.

Although caspase-6 is an important member of the executioner caspases, the steps involved in its activation and its relative contribution in the cell death pathway are not as well understood as for caspase-3. The identity of some target cellular proteins for caspase-6 in response to various apoptotic stimuli has recently been reported. The nuclear matrix protein, lamin A, is the only protein exclusively targeted and cleaved by the activated caspase-6 [28, 33]. Caspase-6, but not caspase-3, is activated during neuronal apoptosis and is responsible for the processing of amyloid precursor protein (APP) to generate increased production of Alzheimer’s amyloid beta peptide (A β) [34–36]. However, in sharp contrast, much information is available on the protein targets for caspase-3 and its role has been extensively studied. Caspase-3 has been reported to degrade many key structural and regulatory proteins including DNA repair enzymes [1, 37–39], ICAD (inhibitor of caspase-activated DNase) or DNA fragmentation factor [40, 41], nuclear structural proteins [1, 42, 43], cytoskeleton proteins [1, 33, 44], transcription factors [1, 45–48], and regulatory proteins including the
proteins involved in the signal transduction pathway [1, 49–52]. Recent studies have demonstrated the cleavage of some intracellular proteins both by caspase-6 and caspase-3. For example, focal adhesion kinase (FAK) is cleaved by both caspase-3 and caspase-6 in apoptotic Jurkat T cells [53, 54]. The vimentin filament network is cleaved in human macrophages apoptosis induced by oxidized low-density lipoprotein (LDL) [55–57]. AP2α [48] is cleaved by both caspase-3 and caspase-6 in a sequence-specific manner at different sites of the protein during tumor necrosis factor-α (TNF-α)-induced apoptosis. At present, it is not precisely known whether caspase-6 activation occurs before, simultaneously, or after caspase-3 activation. Some relationship between caspase-3 and caspase-6 activation has become apparent from recent studies in cell free systems. Active caspase-3 has been shown to activate caspase-6 [57, 58]; however, the relevance of these observations has not been demonstrated in an in vivo set up, since ultraviolet or gamma irradiation-treated caspase-3 (−/−) hepatocytes showed caspase-6 activation [59], indicating that caspase-3 is not involved in caspase-6 activation. On the other hand, some recent studies have demonstrated that caspase-6 can activate caspase-3 in response to apoptotic cell stimuli [60, 61]. Future studies using caspase-6 null mice will reveal the precise relationship between caspase-3 and caspase-6 and the various steps involved in caspase-6 activation in response to an apoptotic stimulus.

At present there is virtually no information on the expression and localization of executioner caspases in kidney in normal or in disease state. We have previously shown distinctive pattern of expression of these caspases in kidney following induction of ischemia [19], indicating that caspase expression is under strict transcriptional control during ischemia/reperfusion injury. Caspase-6 mRNA was moderately increased during ischemia/reperfusion injury. Our present study provides the first demonstration, to our knowledge, of expression of rat caspase-6 in the kidney, and shows by immunohistochemistry its cytoplasmic localization and some nuclear staining of the tubules in cortex and outer medulla. Following ischemia/reperfusion injury we detected increased localization of caspase-6 in the nuclei, indicating some translocation from the cytoplasm to the nuclei during the renal injury. In non-renal tissue, a transient increase in caspase-6 immunoreactivity in astrocytes has been reported in permanent focal ischemia [62]. Thus, further studies are required to identify the potential role of caspase-6, the molecular ordering of the caspase-cascade as well as the cellular proteins as targets of the caspase-6 in renal injury.

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