

Characterization of a Novel Human Serine Protease That Has Extensive Homology to Bacterial Heat Shock Endoprotease HtrA and Is Regulated by Kidney Ischemia*

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We report the isolation and characterization of a cDNA encoding the novel mammalian serine protease Omi. Omi protein consists of 458 amino acids and has homology to bacterial HtrA endoprotease, which acts as a chaperone at low temperatures and as a proteolytic enzyme that removes denatured or damaged substrates at elevated temperatures. The carboxyl terminus of Omi has extensive homology to a mammalian protein called L56 (human HtrA), but unlike L56, which is secreted, Omi is localized in the endoplasmic reticulum. Omi has several novel putative protein-protein interaction motifs, as well as a PDZ domain and a Src homology 3-binding domain. Omi mRNA is expressed ubiquitously, and the gene is localized on human chromosome 2p12. Omi interacts with Mxi2, an alternatively spliced form of the p38 stress-activated kinase. Omi protein, when made in a heterologous system, shows proteolytic activity against a nonspecific substrate β -casein. The proteolytic activity of Omi is markedly up-regulated in the mouse kidney following ischemia/reperfusion.

Mammalian cells are often exposed to adverse environmental conditions brought about by changes in temperature, pH, infections by bacteria and viruses, reactive oxygen species, toxins, or ischemia/reperfusion. Some of these stress conditions can result in the accumulation of damaged and/or denatured proteins within cells. Spontaneous mutations can also cause misfolding of a particular polypeptide. To monitor and assist the proper folding of proteins, cells have numerous specialized proteins, including chaperones and proteases. Accumulation of misfolded proteins in the endoplasmic reticulum (ER)¹ leads to the activation of the unfolded protein response (1), an intracellular pathway present in all eukaryotic cells that originates in the ER. When activated, the unfolded protein response controls

the transcription of genes involved in protein folding (2). This results in the proper folding or removal of damaged proteins in the ER, which is necessary for the recovery and ultimate survival of cells following environmental stress. In bacteria exposed to elevated temperature or oxidative stress, damaged and denatured proteins are removed by the action of the HtrA (high temperature requirement A) endoprotease (reviewed in Ref. 3). HtrA is localized in the periplasmic space of bacteria, and its presence is necessary for bacterial thermotolerance. HtrA is an active serine protease and has two PDZ domains known to be involved in protein-protein interactions (4–6). It has recently been found that bacterial HtrA has a dual role acting as chaperone at normal temperatures and an active protease at elevated temperatures (7). Here we report the isolation and characterization of a mammalian homologue of HtrA, which we call Omi. Omi is a novel serine protease that has extensive homology to the bacterial HtrA heat shock protease. Omi also has homology with L56, a cDNA isolated in a differential screen for mRNAs repressed by SV40 transformation of human fibroblasts (8). The L56 cDNA (human HtrA) was also independently isolated as a differentially expressed protein in human osteoarthritic cartilage (9). The homology among Omi, L56, and HtrA is restricted to the carboxyl terminus of the polypeptides; the amino terminus for each protein is unique. Furthermore, Omi is immunolocalized exclusively in the ER, whereas L56 is secreted. Omi has numerous protein-protein interaction motifs, including a SH3-binding domain, a novel motif that is repeated three times in Omi, a PDZ domain, and a putative transmembrane region. Via its PDZ domain, Omi can specifically bind the carboxyl terminus of Mxi2, an alternatively spliced form of p38 stress-activated kinase (10). Baculovirus-expressed Omi shows proteolytic activity in an assay that uses β -casein as a generic substrate. We used a mouse model system of kidney ischemia/reperfusion to investigate whether the proteolytic activity of Omi is regulated by stress. The same model system has been extensively used to study regulation of stress-activated kinases (11, 12) or heat shock proteins, such as heat shock protein 70 (13). In this model, the proteolytic activity of Omi was substantially increased following kidney ischemia/reperfusion. This suggests activation of Omi may play a role in the biochemical changes that follow kidney ischemia and may serve as a cellular protective response.

EXPERIMENTAL PROCEDURES

Isolation of Omi—A HeLa cDNA library was screened using a modified yeast two-hybrid system (14, 15). We used the full-length Mxi2 fused to LexA_{1–220} as a bait. EGY48 (*MATatrp1ura3his3leu2::p3LexAop-Leu2*) yeast strain was used, which has an integrated

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF020760.

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¹ The abbreviations used are: ER, endoplasmic reticulum; aa, amino acid(s); GST, glutathione S-transferase; HA, hemagglutinin; kb, kilobase pair(s); PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SH3, Src homology 3; X-gal, 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside; BiP, immunoglobulin heavy chain-binding protein.

LEU reporter gene and upstream LexA operators as well as a pSH18–34 (LexAop-lacZ) 2 μ plasmid that directs the synthesis of β -galactosidase (14). Of the 1 million primary yeast colonies screened, 88 colonies were initially isolated by their ability to grow on Gal-Ura-His-Trp-Leu-selective plates. When these were tested on Ura-His-Trp-X-gal glucose and Ura-His-Trp-X-gal galactose plates, several colonies had a galactose-dependent phenotype. The cDNA library plasmids were isolated from these colonies and introduced into KC8 *Escherichia coli* by electroporation. One of the Mxi2-specific interactors was further characterized by PCR analysis and partial restriction digest. The complete sequence of the cDNA was determined by sequencing both strands with a commercially available kit (United States Biochemicals). Sure-RACE PCR (Origene) was used to obtain the complete 5' sequence of Omi cDNA. Sequence searches were performed using the BLAST program on the NCBI network server. The sequence data of the human Omi cDNA is accessible under GenBank™ accession number AF020760.

Immunoprecipitation—HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10% (v/v) fetal calf serum (Sigma). All transient transfections were done on 100-mm dishes using the calcium phosphate method (16). Briefly, the transfection mixture was prepared by resuspending 10 μ g of DNA in 500 μ l of 250 mM CaCl₂ and incubating for 30 min at room temperature; the CaCl₂ mixture was then added to 500 μ l of Hepes buffer, pH 7.2, while bubbling air through it. The mixture was left at room temperature for 20 min and then added to the cells. Cells were washed with PBS and lysed using 400 μ l of 20 mM Hepes, pH 7.5, 25% glycerol, 250 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1% Nonidet P-40. Lysates were incubated on ice for 30 min and then centrifuged for 15 min at 14,000 rpm. Pre-clearing was done by adding 350 μ l of TS buffer (20 mM Tris, pH 7.5, 140 mM NaCl) and 100 μ l of protein G-agarose to a 200- μ l aliquot of the cell lysate and incubating with rocking for 4 h at 4 °C. The protein G-agarose was then removed, and the pre-cleared lysates were incubated with 5 μ l of Omi polyclonal antibody for 1 h on ice. After this time, 20 μ l of protein G-agarose beads was added, and the lysates were incubated overnight at 4 °C. The beads were washed four times with TS buffer and resuspended in 30 μ l of 2 \times SDS sample buffer. The samples were boiled for 5 min and resolved by SDS-polyacrylamide gel electrophoresis and electro-transferred onto PVDF membranes (NEN Life Science Products) using a Trans-Blot (Bio-Rad). Nonspecific binding was blocked by saturating the membrane in 5% nonfat dry milk in PBS. The membrane was exposed to 1:1000 diluted monoclonal antibody against the HA tag (Roche Molecular Biochemicals) in 5% nonfat dry milk.

Mapping the Domains and Amino Acid Sequences Involved in Mxi2-Omi Interaction—The following constructs expressing LexA-Mxi2 or LexA-p38 fusion proteins in yeast were used: LexA-Mxi2 aa 1–297 (full-length), LexA- Δ 2Mxi2 aa 1–295, LexA- Δ 4Mxi2 aa 1–293, and LexA- Δ 6Mxi2 aa 1–291. Furthermore, a LexA-Mxi2+PSD was made that had the 80-amino acid-long unique carboxyl-terminal domain (PSD) of p38, corresponding to amino acids 281–360, fused to its carboxyl terminus. LexA-p38 aa 1–360 (full-length p38) fusion and a LexA-p38+MSD that had the unique 17 carboxyl-terminal amino acids of Mxi2 (MSD) fused to its end. All these constructs were made using PCR and specific primers that introduced an early terminator codon. The LexA-p38+MSD was made using the primers shown below in a PCR. The 3' end primer was used to introduce the specific carboxyl terminus Mxi2 sequence (MSD) encoding 17 amino acids onto p38. Primers were as follows: sense primer p38, 5'-CCG CAA TTG CCA TGT CTC AGG AGA GGC CC-3'; antisense primer p38+MSD, 5'-CGC GGA TCC TCA TAT CAT AAC CAA TTC AAT ATC CAT GAG GTG AGG ATA TAT GGT CAA CTT ACC GGA CTC CAT CTC TTC TTG-3'. To make the Mxi2+PSD construct, two PCRs were used. The resulting products were then used as templates in a final PCR. The first two primers used to amplify the 5' portion of the chimeric protein were as follows: sense primer Mxi2, 5'-CCG CAA TTG CCA TGT CTC AGG AGA GGC CC-3'; antisense primer Mxi2+PSD, 5'-AAG GGC TTG GGC CGC TGT TAT CAT AAC CAA TTC AAT-3'. The 3' end portion of the construct was made using the following primers: sense primer Mxi2+PSD, 5'-ATT GAA TTG GTT ATG ATA ACA GCG GCC CAA GCC CTT-3'; antisense primer p38, 5'-CGC GGA TCC TCA GGA CTC CAT CTC TTC TTG GTC-3'. The final PCR was performed using the sense primer Mxi2 5' and the antisense primer p38. The final PCR product encoding Mxi2+PSD was digested with MfeI/BamHI and cloned into EcoRI/BamHI sites of pL202 vector. DNA sequence was verified by sequencing both DNA strands using a commercially available kit (Amersham Pharmacia Biotech).

The following pair of primers was used to PCR amplify a DNA fragment encoding the PDZ domain of Omi corresponding to amino acids 381–458: OMI-PDZ 5' end, 5'-GAT GTG CCA GAT TAT GCC TCT

CCC GAA TTC GAA CCA AGC TTT CCC GAT GTT-3'; 3' end, 5'-CTC TGG CGA AGA AGT CCA AAG CTT CTC GAG ATT CTG TGA CCT CAG GGG TCA-3'.

The following primers were used to subject to PCR a DNA fragment encoding the PDZ domain of L56/Hum.HtrA, corresponding to amino acids 402–480: L56 PDZ 5' end, 5'-GAT GTG CCA GAT TAT GCC TCT CCC GAA TTC CAC CGG GAC TTC CCA GAC GTG-3'; L56 PDZ 3' end, 5'-CTC TGG CGA AGA AGT CCA AAG CTT CTC GAG CTA TGG GTC AAT TTC TTC GGG-3'.

The PCR products were cloned into the yeast vector pJG4–5 vector (14) by homologous recombination as described in Ref. 17. Expression of the fusion proteins was confirmed by Western blot analysis of yeast extracts using anti-HA antibody. Interaction of these fusion proteins with Mxi2 in yeast was detected using standard procedures (15).

Northern Blot Analysis—Human mRNA tissue and cancer blot (CLONTECH) were hybridized at 42 °C with a radiolabeled probe corresponding to full-length Omi cDNA, as described previously (18). Blots were washed at 65 °C with 2% SSC, 0.1% SDS for 10 min followed by 0.1% SSC, 0.1% SDS for 30 min. The blots were then subjected to autoradiography.

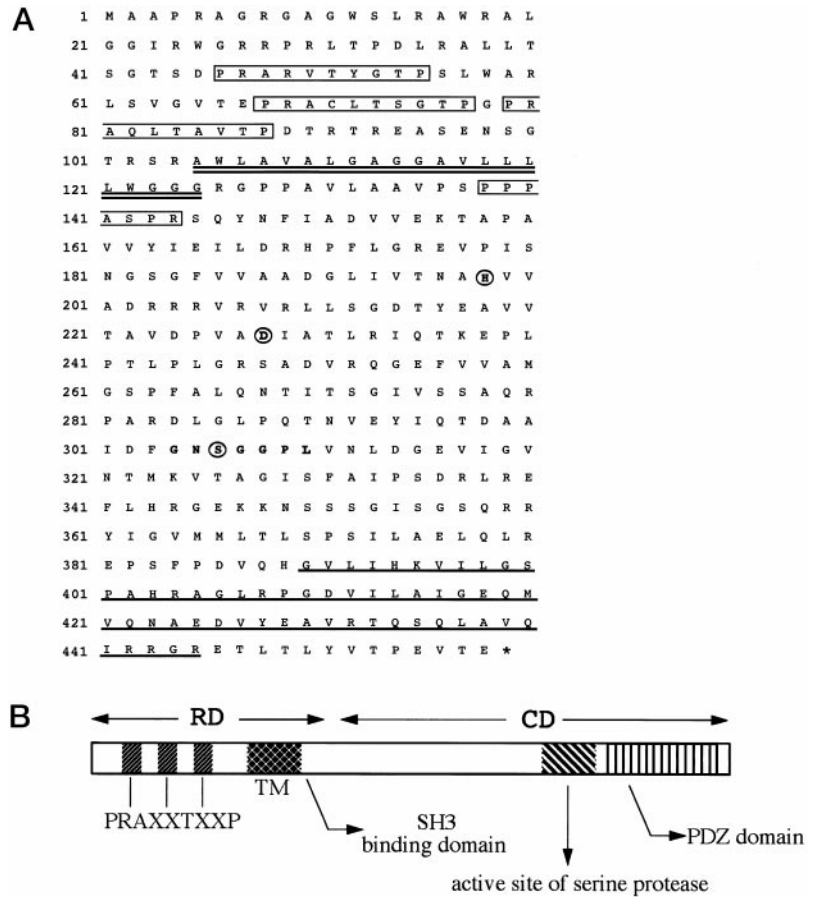
Preparation of Omi Polyclonal Antibody—Anti-Omi antibodies were prepared by immunizing rabbits with the carboxyl-terminal region of Omi protein (residues 217–458) fused to the maltose-binding protein (New England Biolabs). The fusion protein was expressed in *E. coli* and purified using maltose-amylose resin (New England Biolabs). Affinity purified maltose-binding protein-Omi was resolved on polyacrylamide gels. Acrylamide gel pieces containing the maltose-binding protein-Omi fusion protein were excised and used to inject rabbits (Cocalico Biologicals, Inc). Anti-Omi antibodies were affinity purified on a column of maltose-binding protein-Omi covalently linked to Sepharose. Immunoblotting was performed using the anti-Omi antiserum at 1:1000 dilution. The secondary antibody used was a goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad). Visualization of antibody binding was carried out using the ECL detection system (Amersham Pharmacia Biotech).

Production of Recombinant Baculovirus—The cDNA coding for the full-length Omi was subcloned into the pAcGHTL (Pharmingen) vector, downstream of the coding sequence of glutathione *S*-transferase (GST). In order to make the OmiS306A mutant, a four primer, two-reaction protocol was used. Briefly, two overlapping PCR fragments were generated incorporating the codon change for the new amino acid. The primers used for the first set of reactions were as follows: sense primer A, 5'-CCG CAA TTG ATG GCT GCG CCG AGG GCG-3'; antisense primer B, 5'-GGG ACC TCC GGC GTT TCC-3'. The primers used for the second set of reactions were as follows: sense primer C, 5'-TTT GGA AAC GCC GGA GGT C-3'; antisense primer D, 5'-GGG GTA CCT CAT TCT GTG ACC TCA GG-3'. The final PCR was performed using sense primer A and antisense primer D and the PCR products from the previous reactions as template. The final 1.370-kb DNA fragment encoding the mutant OmiS306A was digested with MfeI/KpnI and cloned into the EcoRI/KpnI sites of the pAcGHTL expression vector.

The fidelity of both cDNA clones encoding for wild type and mutant Omi was confirmed by DNA sequencing of both strands (United States Biochemicals). SF9 insect cells were co-transfected with the recombinant transfer vector and linearized BaculoGold baculovirus DNA (Pharmingen), according to the manufacturer's instructions. A high titer solution of recombinant baculovirus was used to infect SF9 cells. After 3 days of incubation at 27 °C, the insect cells were removed from the culture plates and recovered by low speed centrifugation. The cell pellets were stored at –80 °C until further processing.

Purification of Omi-GST Fusion Protein—Infected SF9 cells were resuspended into cell lysis buffer (Pharmingen) without protease inhibitors, using 1 ml of buffer for 2×10^7 cells. Cell lysis was performed on ice for 45 min and completed by Dounce homogenization. The cell lysate was clarified by low speed centrifugation at $40,000 \times g$ for 45 min. The supernatant was then added to glutathione-conjugated Sepharose beads (Sigma), and the slurry was incubated for 30 min at 4 °C on a rotating platform. The beads were recovered by low speed centrifugation and washed twice with a large excess of cold washing buffer solution (8 mM sodium phosphate buffer, pH 7.3, 1 mM EDTA, 150 mM sodium chloride, 1% (v/v) Triton X-100) followed by three washes with a large excess of 50 mM Tris-HCl, pH 7.6. The bound fusion protein was then eluted twice (20 min at room temperature) with 5 mM reduced glutathione in 50 mM Tris-HCl, pH 7.6, using a volume of reduced glutathione equal to the initial volume of packed beads. The presence of GST-Omi fusion protein was confirmed by immunoblot analysis using anti-Omi antiserum. The purified protein was used for proteolytic assays at a concentration of approximately 1 mg/ml.

FIG. 1. Amino acid sequence of Omi protein. A, the original cDNA clone isolated in the two-hybrid screen encoded 312 residues, corresponding to amino acids 147–458, shown here. Sure-RACE PCR (Origene) was used to obtain the complete 5' Omi cDNA sequence. Sequence (GNSGGPL) surrounding the active site of the catalytic domain with similarity to the consensus sequence of trypsin-like serine proteases is in *boldface*; the His, Asp, and Ser (catalytic triad) amino acids required for proteolytic activity are circled and in *boldface*; the SH3-binding sequence and the PRAXX-TXXTP motifs are boxed. The putative transmembrane region is *double underlined*, and the PDZ domain is *underlined*. B, schematic diagram of the domain structure of Omi. There is a catalytic domain (CD) localized at the carboxyl terminus of the protein with homology to serine proteases. Adjacent to the catalytic domain, there is a PDZ domain. The amino terminus of Omi contains the regulatory domain (RD) with unique sequence that includes three copies of a sequence motif (PRAXXTXXTP), a putative transmembrane domain (TM), and an SH3-binding motif (PPPASPR).



Assays for the Proteolytic Activity of Omi—The proteolytic activity of Omi was measured using a commercially available assay system (Athena Environmental Sciences, Inc.). The assay employs a universal substrate of a dye-protein conjugate cross linked to a matrix. Protease activity is determined spectrophotometrically by measuring the absorbance of the dye released from the matrix to the supernatant. The anti-Omi polyclonal antibody was prebound to protein G-agarose beads. Tissue lysate (500 μ g) prepared as from mouse kidneys as described below was added to 50 μ l of 50% suspension of beads and gently shaken for 2 h at 4 $^{\circ}$ C. The beads were washed four times with 1 \times STN buffer. The beads were then added as a suspension of 500 μ l of 10 mM Tris-HCl (pH 7.5) to the reaction vials containing the matrix-substrate and incubated for 3 h at 37 $^{\circ}$ C. The activity of Omi was measured at different incubation times, and reactions were terminated by adding 500 μ l of 0.2 N NaOH to each vial. The absorbance of the supernatant in each reaction vial was measured at 450 nm. The proteolytic activity of baculovirus made GST-Omi fusion was monitored using 10 μ l (approximately 10 μ g) of purified protein incubated with 5 μ g of β -casein (Sigma) in 50 mM Tris-HCl (pH 7.5) for 30 min, 1 h or 2 h at 37 $^{\circ}$ C. The proteolytic activity of GST-OmiS306A was monitored using 20 μ g of purified protein incubated with 5 μ g of β -casein (Sigma) in 50 mM Tris-HCl (pH 7.5) for 3 h. The reaction products were resolved by SDS-polyacrylamide gel electrophoresis and proteins visualized by staining with Coomassie Blue.

Immunocytochemistry—COS-7 cells were plated at 80% confluency on fibronectin (100 ng/ μ l)-coated coverslips. Cells were rinsed with PBS and fixed with 3.7% paraformaldehyde containing 0.1% Tween-20 in PBS at room temperature for 10 min. Incubation with primary and secondary antibodies was performed at room temperature for 1 h, followed by three 15-min washes in PBS. The following primary antibodies were used: rabbit polyclonal against Omi at a concentration of 0.8 μ g/ml and mouse monoclonal antibody against BiP (StressGen) used as an ER-specific marker at a dilution of 1:500. The secondary antibodies were fluorescein-conjugated anti-rabbit IgG (SantaCruz), used at a concentration of 15 μ g/ml, and rhodamine-conjugated anti-mouse IgG (Cappel), used at a dilution of 1:100.

Mouse Kidney Ischemia/Reperfusion—The left kidney of Balb/c mice was removed through a flank incision as described previously (19). Twenty-four h later, ischemia of the right kidney was induced for 30

min followed by reperfusion (19). Kidneys were harvested 1 or 24 h after reperfusion. The mouse kidneys were homogenized in 5 ml of lysis buffer (20 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM dithiothreitol, 1 \times Trasylol, 50 mM β -glycerophosphate) and sonicated on ice. Lysates were centrifuged for 10 min at 3000 rpm at 4 $^{\circ}$ C, and the collected supernatants were further centrifuged for 1 h at 100,000 \times g. The kidney supernatant was kept in a final concentration of 1% Triton X-100 and 1 mM Na₃VO₄. 150 μ g of total protein extracts was separated on an SDS-polyacrylamide gel, and the amount of Omi protein was detected by Western blot analysis using the Omi polyclonal antibody (1:1000).

RESULTS

Isolation and Sequence Characterization of Omi cDNA—In order to isolate Mxi2-interacting proteins, we used a modified yeast two-hybrid system to screen a HeLa cDNA library (14, 15). Of the 10⁶ primary yeast transformants screened, 88 colonies appeared to contain a potential interactor. Of these, only two had an unambiguous phenotype. The two plasmids were rescued and shown to represent the same cDNA, although one had a longer 3' untranslated sequence (results not shown). The partial cDNA encoded a polypeptide of 312 amino acids fused in-frame to the B42 activation domain of the pJG-4.5 vector. Sure-RACE PCR (Origene) was used to obtain the complete 5' Omi DNA sequence. The full-length Omi cDNA encodes for a protein of 458 amino acids with an estimated M_r of 50,000.

Based on homology with other known polypeptides, we arbitrarily assigned two domains to the protein: an amino-terminal regulatory domain and a carboxyl-terminal catalytic domain (Fig. 1B). The regulatory domain had a putative transmembrane region localized between amino acids 105–125 and a potential phosphorylation site (SPRS) for stress-activated kinases at position 142. Overlapping the phosphorylation site there was a SH3-binding domain (PPPASPR) (20, 21). Upstream of this site there was a sequence motif PRAXXTXXTP (where X can be any amino acid) that was present three times

HtrA	48	--VMP	SVVSINVEG	STTVN-----	TPRMP	RNFQOFF	GD----	DSPFC	QEGSP	FQS	91	
L56/hHtrA	142	-LHR	PPVIVL	QRGAC	QG-----	QEDPN	SLRH	KYNF	IADV	EKIAP	VVHIEL	191
Omi	110	ALG	AGAV	LLLL	WGGGR	PPAV	LA	AVP	SP	PPAS	PR	169
		SPFC	QGGQ	GGGG	QQQK	FAL	MG	SG	VI	I	DADK	151
		LPFS	K-----	REVP	VASG	S	GF	IV	SED-	GLIV	240	
		HPFL	G-----	REVP	ISNG	S	GF	V	V	AAD-	GLIV	218
		KMVG	KDPR	SDIAL	I	Q	IN	PK	NL	TA	IK	211
		KIKD	VDEK	ADIAL	I	K	ID	HQ	KL	PV	LL	300
		VVTA	VDPV	ADIAT	L	R	I	Q	T	K	E	278
		GRSG	--LNA	EN-	YEN	F	I	Q	T	DA	A	268
		QRGK	ELGL	RNSD	MDY	I	Q	T	DA	I	NY	355
		QRP	ADL	GLP	Q	T	N	VE	Y	I	Q	333
		PSNM	VKNL	TSQ	M	VE	Y	Q	V	K	R	328
		PS-----										381
		PS-----										360
		GIK	AGD	VIT	SL	NG	K	P	ISS	F	A	388
		-----										401
		-----										380
		DSS	SIF	NG	I	E	G	A	E	M	S	448
		-----										449
		-----										428
		KVLD	SKPS	V	L	A	L	N	I	Q	R	491
		DVI	KRE-	STL	N	M	V	R	R	G	N	480
		EAV	R	T	Q-	S	L	A	V	Q	I	458

FIG. 2. Comparison of Omi, L56, and HtrA amino acid sequences. The homology among these proteins extends through the catalytic domains shown here; no homology is found in the regulatory domains. Gaps introduced to optimize alignment are illustrated by dashes; identical residues are shaded; the PDZ domains of the three proteins are boxed. Protein sequences were aligned using the Genetics Computer Group (Madison, WI) GAP program.

in the regulatory domain (Fig. 1B). This was the first report on the identification of this motif. No function associated with this structure has been reported thus far.

The catalytic domain of Omi showed extensive homology to *E. coli* heat shock protease HtrA as well as with a mammalian protein called L56/hHtrA (8, 9) (Fig. 2). The catalytic domain of Omi had 51% identity and 68% similarity with the corresponding domain of L56/hHtrA and 36% identity and 58% similarity with bacterial HtrA. Like L56/hHtrA, Omi also has a single PDZ domain whereas the bacterial HtrA has two PDZ domains. These PDZ domains are involved in binding directly to the carboxyl termini of target proteins (6).

In Vivo Association of Omi with Mxi2—To study the interaction of Mxi2 or p38 and Omi *in vivo*, we performed co-immunoprecipitation experiments using HEK293 cells transiently transfected with plasmids expressing Mxi2-HA or p38-HA. We used a polyclonal antibody raised against Omi to precipitate the endogenous protein. After resolving the precipitated complex on an SDS-polyacrylamide gel, the presence of HA-Mxi2 or HA-p38 in the complex was detected using anti-HA antibody. These experiments showed that Omi can bind Mxi2 *in vivo*, and that the affinity of the interaction is strong enough to allow the proteins to co-immunoprecipitate. Omi was able to interact with Mxi2 when the transfected cells were treated with anisomycin (an activator of p38 stress-activated kinases) or SB205380 (a known inhibitor of p38 kinase). In the same experiment, no interaction was detected between HA-p38 and Omi (Fig. 3A).

Mapping Interaction Domain of Mxi2/Omi—To define the

amino acid sequence of Mxi2 responsible for binding to Omi, LexA-fusions corresponding to the full-length Mxi2 (aa 1–297) and three deletion mutants of Mxi2 (Δ 2Mxi2 aa 1–295, Δ 4Mxi2 aa 1–293, and Δ 6Mxi2 aa 1–291) were made. Also, full-length LexA-p38 (aa 1–360) and two chimeric fusion proteins, one containing the p38-specific carboxyl-terminal domain (PSD) fused to LexA-Mxi2 (LexA Mxi2+PSD) and another containing the Mxi2-specific carboxyl-terminal domain (MSD) fused to p38 (LexA p38+MSD), were used (Fig. 3B). In these experiments, Mxi2 but not p38 was able to bind to Omi and the carboxyl-terminal sequence of Mxi2 was required for interaction. Deletion of the last amino acid of Mxi2 abolished most of the binding with Omi (Fig. 3B). When the carboxyl terminus of Mxi2 was blocked by fusing the p38-specific, 80-amino acid sequence, the interaction with Omi was abolished. This indicated that the recognition sequence has to be at the end of the carboxyl terminus of the protein. To further explore the properties of the unique sequence of the carboxyl terminus of Mxi2, the 17-amino acid long Mxi2-specific carboxyl terminus was fused to the end of p38. This p38+MSD fusion protein was now able to bind Omi in yeast. Fig. 3C shows that the interaction between Mxi2 and the PDZ domain of Omi is highly specific. Mxi2 was not able to interact with the PDZ domain of L56, a homologue of Omi that has a PDZ domain with high similarity to the PDZ domain of Omi (see Fig. 2).

Expression of Omi mRNA in Normal Human Tissues and Transformed Cell Lines, and Chromosomal Localization—To investigate the expression of Omi, we performed Northern blot analysis using mRNA from different human tissues as well as

FIG. 4. **Expression of Omi in normal human tissues and cancer cell lines.** A, a membrane containing 2 μ g of mRNA from the indicated adult human tissues (CLONTECH) was probed with 32 P-labeled full-length Omi cDNA. B, a membrane with 2 μ g of mRNA from different tumor cell lines (CLONTECH) was also probed. Positions of molecular standards are indicated on the left. Two distinct mRNA transcripts were detected, a predominant mRNA of 2.1 kb and a less abundant mRNA of 4.5 kb.

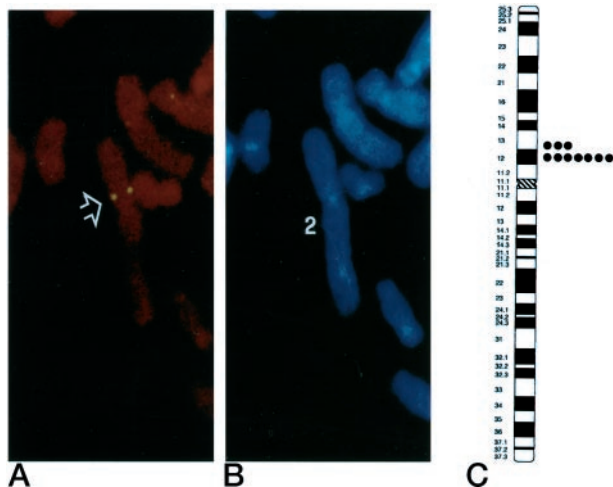
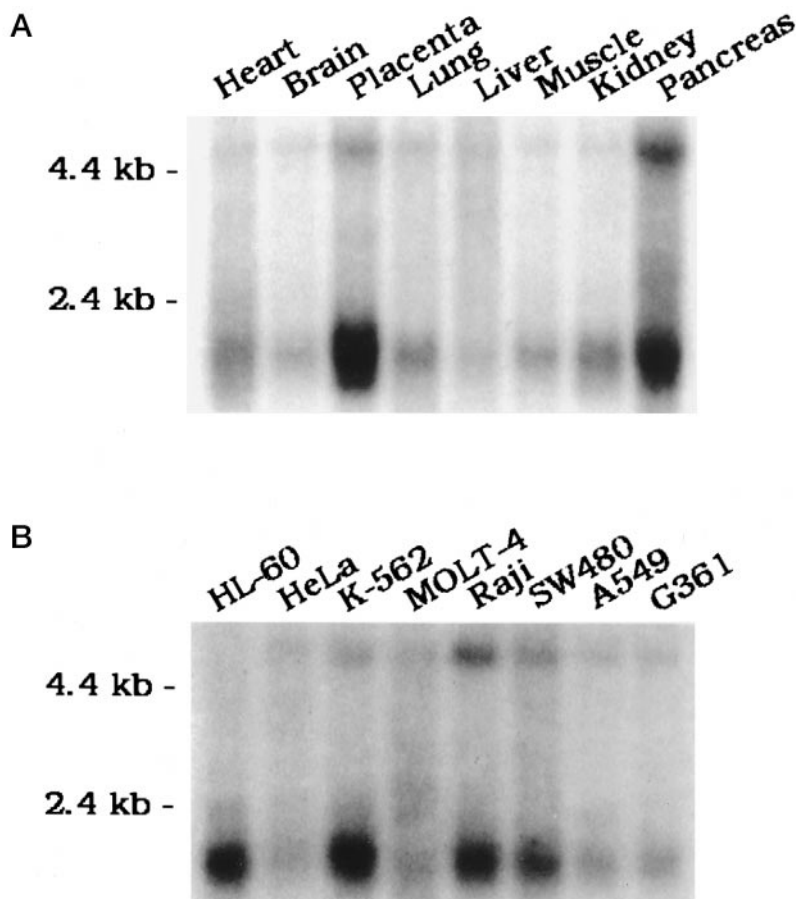


FIG. 5. **Chromosomal localization of Omi.** A, fluorescence *in situ* hybridization signal of Omi probe (arrow) on chromosome. B, same mitotic figure stained with 4,6-diamidino-2-phenylindole to identify chromosome 2 (2). C, diagram of fluorescence *in situ* hybridization mapping results for Omi probe. Each dot represents the double fluorescence *in situ* hybridization signal detected on human chromosome 2, region p12.

became apparent when it was learned that bacteria lacking this enzyme are unable to survive at 42 °C (24, 26). Furthermore, pathogenic bacteria that lack HtrA are less virulent, suggesting that Omi may have a role in counteracting and neutralizing host bactericidal mechanisms (3). Recently, it has been reported that bacterial HtrA has a dual role, acting as a chaperone at normal temperatures and as an active protease against a natural substrate (the MalS protein) at elevated

temperatures (7). The homology of HtrA and Omi is quite substantial and extends across the full length of the catalytic domain. Extensive search of the GenBank™ identified one more mammalian protein, L56, that has substantial homology to Omi and HtrA. L56 was isolated in a screen to identify differentially expressed genes when cells are transformed by SV40 virus (8). L56 is expressed in normal human fibroblasts, but its expression is inhibited when the same fibroblasts are infected with SV40 (8). The homology of L56 with Omi extends in the catalytic domain at the carboxyl termini of the proteins, and no homology is found in the regulatory domain present at the amino termini. L56 protein is secreted (9) whereas Omi is found in the ER. The different subcellular localization of Omi and L56 and their unique regulatory domains present at their amino termini suggest distinct functions for these two proteins. The proteolytic activity of *E. coli* HtrA, as well as that of L56 (human HtrA), has been shown to be completely eliminated by mutating the serine in the catalytic domain (GNSGGGA) or the histidine in position 105 (27). The serine at position 306 is an invariant amino acid in the “catalytic triad” of His, Ser, and Asp residues found in the catalytic domain of several serine proteases. When we made Omi in a baculovirus system that also has the serine in position 306 mutated to an alanine (S306A), this mutant Omi had no detectable protease activity against β -casein.

The amino terminus of Omi has a PPPASPR sequence that is identified as a SH3-binding domain (20, 21). It also has three copies of the sequence motif PRAXXTXXTP, where X is any amino acid. This sequence has not been reported before, and its function is unknown. We assume that the unique amino-terminal regulatory domain of Omi is involved in protein-protein interactions. Omi also has a PDZ domain at its carboxyl terminus. PDZ domains are named after the three

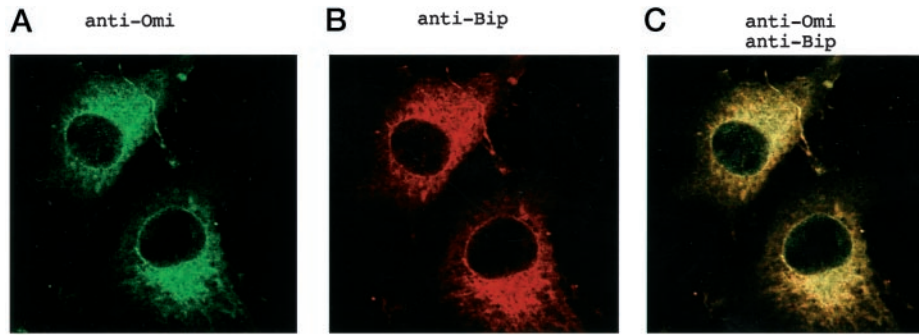


FIG. 6. Subcellular localization of Omi in COS-7 cells. Fluorescence images of COS-7 cells expressing endogenous Omi stained with the purified polyclonal Omi antibody followed by fluorescein-conjugated anti-rabbit IgG (A). The same cells were also stained with a mouse monoclonal antibody against the ER-resident protein (*BiP*) followed by rhodamine-conjugated anti-mouse IgG (B). C, merged images of A and B showing co-localization of Omi with the ER-resident protein BiP.

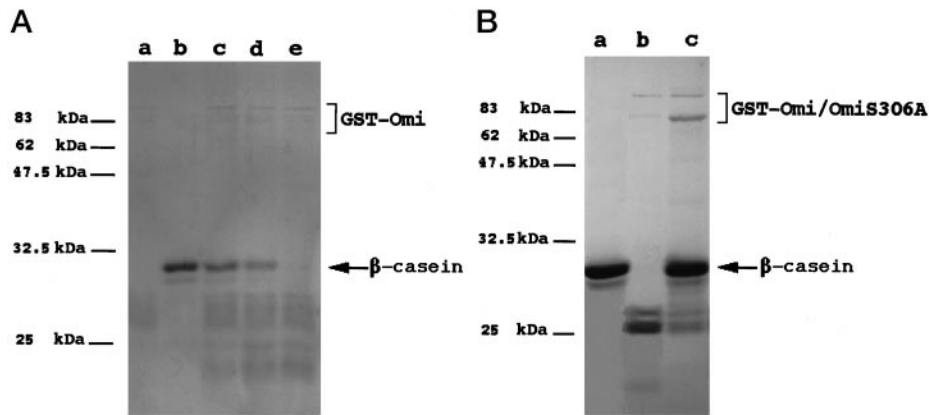
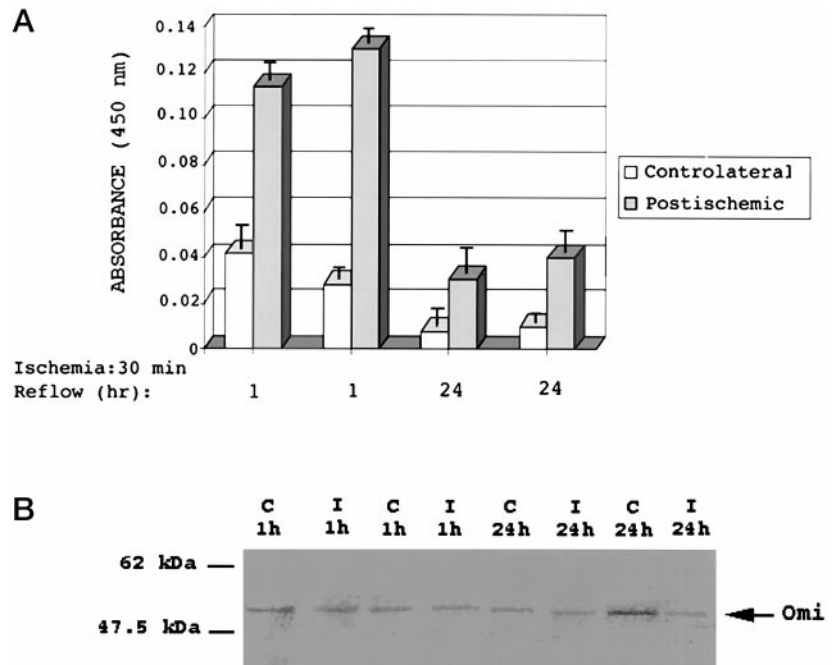


FIG. 7. GST-Omi protein expressed in baculovirus-infected SF9 cells is an active protease. A, Coomassie Blue-stained acrylamide gel of GST-Omi incubated at different times with β -casein. All incubations were performed at 37 °C. Lane a, GST-Omi, 2-h incubation; lane b, β -casein, 2-h incubation; lane c, GST-Omi + β -casein, 30-min incubation; lane d, GST-Omi + β -casein, 1-h incubation; lane e, GST-Omi + β -casein, 2-h incubation. Reactions were terminated by adding SDS-sample buffer, and proteins were resolved on a polyacrylamide gel and stained with Coomassie Blue. The bracket indicates the position of the intact GST-Omi; the arrow indicates the position of the β -casein substrate. B, Coomassie Blue staining of GST-OmiS306A incubated with β -casein. All incubations were performed at 37 °C. Lane a, β -casein alone, 3-h incubation; lane b, GST-Omi + β -casein, 3-h incubation; lane c, GST-OmiS306A + β -casein, 3-h incubation.

FIG. 8. Protease activity of Omi is regulated during kidney ischemia/reperfusion. A, Omi was precipitated from kidney extracts of control or ischemic mice, and the protease activity monitored using a commercially available kit. Activity is shown as the absorbance at 450 nm of substrate released. The activity shown is the average of three independent measurements using two mice for each measurement. Two independent experiments performed with mice are shown here, at 1 and 24 h following kidney ischemia/reperfusion. B, equal amounts of protein extracts from mouse kidneys that underwent ischemia/reperfusion (I) for 1 or 24 h or control (C) were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blot using Omi antibodies.



proteins in which they were first identified: postsynaptic density protein-95 (PSD-95) (28); *Drosophila* discs large tumor suppressor (Dlg) (29), and the tight junction protein,

(ZO-1) (30). The PDZ domain of Omi specifically recognizes the carboxyl terminus of Mxi2 and is responsible for its interaction with this kinase.

Omi mRNA is expressed ubiquitously, and increased levels were found in several tumor cell lines. Omi is localized on human chromosome 2p12 at a region reported to be rearranged in several human leukemias (22). Whether Omi overexpression plays a role in the development or progression of human cancer is not known. Because Omi might be involved in the cellular response to stress, its overexpression may render transformed cells more resistant to cellular damage caused by extracellular stress, hyperthermia, or chemotherapeutic agents.

To investigate any potential role of Omi in cellular stress, we used a mouse model system of kidney ischemia/reperfusion (19). Proteases, such as calpain (31) and caspases (32), have been found to be increased in activity and have been implicated in the pathophysiology of ischemic injury to the kidney and other organs. In the brain, for example, inhibition of calpain-mediated proteolysis protects hippocampal neurons against ischemic injury (33). We are proposing, however, that Omi may act similarly to heat shock proteins, which have been implicated in the protection of the kidney and other organs against ischemic injury (34).

We found that although the levels of Omi protein remained the same, the proteolytic activity of the enzyme substantially increased after ischemia/reperfusion. This suggests that the activity of Omi may be regulated by a post-translational mechanism such as phosphorylation. Alternatively, the protease activity of Omi might be regulated through protein-protein interactions at its amino-terminal regulatory domain. Whether this increase in the activity of Omi is necessary for the cellular changes that follow kidney damage and recovery remains to be investigated. Omi has numerous protein-protein interaction motifs that could target specific substrates for degradation after these proteins are denatured or damaged following stress. Alternatively, Omi could act as a chaperone to assist the proper folding and renaturation of substrates that bind to its numerous protein-protein interaction domains. The exclusive localization of Omi in the ER further reinforces the suggestion that its function could be to assist the proper folding of proteins and/or to degrade and remove damaged polypeptides. Whether Omi is a new member of the unfolded protein response pathway that originates in the ER and regulates the transcription of several target genes involved in protein folding remains to be elucidated. The presence of specific protein-protein interaction motifs at the amino terminus of Omi further suggests the proteolytic activity of Omi may be targeted to specific substrates. Isolation and characterization of these substrates will

assist the elucidation of the physiological function of Omi and its potential role in stress.

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Characterization of a Novel Human Serine Protease That Has Extensive Homology to Bacterial Heat Shock Endoprotease HtrA and Is Regulated by Kidney Ischemia

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