A Selective Interaction between OS-9 and the Carboxyl-terminal Tail of Meprin β*

Received for publication, April 24, 2002, and in revised form, June 26, 2002
Published, JBC Papers in Press, July 1, 2002, DOI 10.1074/jbc.M203986200

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OS-9, a protein previously uncharacterized, was shown to interact specifically with the intracellular region of the membrane proteinase meprin β found in brush border membranes of kidney and small intestine. We have shown previously that this cytoplasmic region is indispensable for the maturation of meprin β, which included an endoplasmic reticulum (ER)-to-Golgi translocation. We characterized OS-9 and found that it is associated with ER membranes and that it is exposed to the cytoplasm. Consistent with the kinetics of maturation of meprin β, OS-9 associates with meprin β only transiently, coinciding with ER-to-Golgi transport of meprin β. The OS-9-binding site in the cytoplasmic domain of meprin β overlaps the region essential for this transport. We characterized alternatively spliced forms of rat and mouse OS-9, and we found that only the non-spliced form of OS-9 binds to meprin β, implicating the spliced out segment in the binding, and suggesting the possible mechanism of the regulation of OS-9 function. Taken together, our results indicated that OS-9 may be involved in the ER-to-Golgi transport of meprin β. Ubiquitous expression of OS-9 raises the possibility that it may interact with other membrane proteins that possess the cytoplasmic moiety homologous to that of meprin β during their ER-to-Golgi transition.

The kinase splitting membrane proteinase was discovered as an enzyme that specifically clips and inactivates protein kinase A in the preparations of the brush border membranes of the small intestine and kidney (1, 2). Subsequently, this proteinase was shown to be identical to a β subunit of meprin (3), a membrane metalloendoproteinase of the astacin family (4). We therefore refer to it here as meprin β. The physiological role of meprin is not established yet; however, it has been implicated in the degradation of the subset of biologically active polypeptide hormone gastrin (5, 6).

Peptides (meprin A, EC 3.4.24.18, and meprin B, EC 3.4.24.63) and oligomeric proteases are composed of two types of structurally similar subunits (α and β) that are targeted to the cell surface or are secreted (7, 8). Meprin A is composed of the disulfide-bridged dimers of α subunits, whereas meprin B is a heterodimer of α and β subunits. Higher multimeric structures formed by a non-covalent association of functionally active dimers of mouse meprin α were recently observed (9). Despite the high sequence homology and similar domain structure, the α and β subunits of meprin undergo different post-translational processing. Meprin α (but not meprin β) undergoes proteolysis in the endoplasmic reticulum (ER),† which results in the removal of its short carboxyl-terminal cytoplasmic tail as well as a transmembrane segment and an epidermal growth factor-like domain (cf. Fig. 1A) (10). The transmembrane and cytoplasmic domains of the immature α subunit of the human meprin mediate the retention of this subunit in the ER through an association with chaperones (11). Contrary to that, the cytoplasmic domain of rat meprin β is indispensable for its ER-to-Golgi transport (12). Although the cytoplasmic tail of meprin β does not contain any of the previously characterized ER export signals (13), its removal results in the entrapment of the truncated meprin β in the ER (12). This truncation did not affect the proteolytic activity and stability of meprin β, suggesting that this retention is not due to the incorrect folding of meprin β and is not mediated by ER chaperones. Mutant of meprin β, where the basic amino acids of the juxtamembrane region (682RRKYRKK688) were substituted with alanines, demonstrates a decreased rate of the ER-to-Golgi transport. A tyrosine-to-proline substitution (Y685P) in the middle of this cluster prevents the export of corresponding mutant from the ER, suggesting that this region of the cytoplasmic tail of meprin β is important for its recruitment into the transport vesicles that depart from the ER and may possess a novel ER export signal (12).

In this report we describe a protein that selectively interacts with the carboxyl-terminal tail of meprin β. This protein binds to the region 677TLISVYCTRRKYRKKA688 of rat meprin β in a yeast two-hybrid assay (14) and forms a transient complex with meprin β during its export from the ER when co-expressed in mammalian cells. This protein has a high degree of sequence identity with a product of a recently cloned human gene, OS-9 (15), and represents a rat homologue of human OS-9. The mRNA of human OS-9 (hOS-9) is ubiquitously present in human tissues and overexpressed in certain sarcomas, but to the best of our knowledge, no function has been assigned thus far to the OS-9 gene product. Moreover, OS-9 has no significant functional domains, and the deletion of OS-9 did not increase the release of meprin A.

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* This work was supported in part by The Israel Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

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1 The abbreviations used are: ER, endoplasmic reticulum; hOS-9, human OS-9; mOS-9, mouse OS-9; PK, proteinase K; RT, reverse transcriptase; PBS, phosphate-buffered saline; Endo-H, endo-β-N-acetylglucosaminidase H; DMEM, Dulbecco’s modified Eagle’s medium; MBP, maltose-binding protein; GFP, green fluorescent protein; ORF, open reading frame; HA, hemagglutinin; GST, glutathione S-transferase.
homology with any of the functionally characterized proteins, although the cysteine-rich amino-terminal domain of OS-9 is highly similar to the protein fragments predicted from genomic sequences of yeast Saccharomyces cerevisiae (YD9609.11) and Caenorhabditis elegans (F48E8.4) (15). Three alternatively spliced isoforms of hOS-9 mRNA were described (16) (Fig. 1B). We found that only the non-spliced OS-9 binds to the cytoplasmic tail of meprin β, suggesting the different functional role of the alternatively spliced isoforms. By using antibodies raised against the carboxyl terminus of rat OS-9, we demonstrated that OS-9 is a peripheral membrane protein associated with the cytoplasmic side of the ER. These results, together with the fact that OS-9 interacts with the region in meprin β that is essential for its export from the ER, raise the possibility that OS-9 may be involved in the ER-to-Golgī transport of meprin β as well as other membrane proteins containing a motif similar to the OS-9-binding site in meprin β.

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal antibodies against rat meprin β were prepared as described previously (5). Antibodies against the His6-tagged 339-amino acid carboxyl-terminal part of rat OS-9 (OS-9/L1 fragment, expressed in Escherichia coli) were raised in guinea pigs using the immunization protocol described previously (17). Anti-OS-9 antibody fragments were affinity-purified using a Sepharose column with an immobilized (His)6-OS-9/L1. Anti-GST antibodies were a gift of J. Blechman. Other antibodies were from commercial sources as follows: rabbit antibody against the cytoplasmic domain of calnexin and mouse monocular antibody against the immunoglobulin binding protein BiP (Stressgen Biotechnologies Corp.); rabbit antibody against Rab1 and Rab2 (Calbiochem-Novabiochem); mouse monocular antibodies against β-COP and the luminal domain of calnexin (Transduction Laboratories); mouse monoclonal antibody against the Golgi 58K protein (Sigma); and mouse monoclonal antibody against the HA epitope tag YPYDVPDYA (Covance). Secondary antibodies against guinea pig, rabbit, and mouse IgG (conjugated with horseradish peroxidase, Cy2, Cy3, or Cy5) were from Jackson ImmunoResearch.

Yeast Two-hybrid Library Screening—The cDNA of rat meprin β (coding carboxyl-terminal segment E57-Thr-Pho734, see Fig. 1) was amplified by PCR using primers Mγ-CTGGAATTCACCCTTATCAGCGTCT-3′ and 5′-TGCTCGGATCCAGTTAAP ATTCAAAACG-3′ (containing a 3′-untranslated region of mOS-9), and 200 units of SuperScript II RTase H-minus Reverse TranscriptaseTM (Invitrogen). PCR was carried out using DyNAzyme II DNA polymerase (Finnzymes Oy), and 5′-CGCCCATGTTGGGAAACAGGAGCTGTAAC-3′ (direct) and 5′-AGGCTCGAGTCAGAAGTCAAATTCATCC-3′ (reverse) primers supplied with the High GC content of OS-9 cDNA. PCR products were purified and either subcloned into pGEM-T Easy vector (Promega) or digested with the indicated enzymes for cloning into the pACT2 vector. In order to obtain the full-length isoform 1 of mOS-9, the BspMI–Av/P restriction fragment of the mouse 1050-bp non-spliced RT-PCR product (subcloned into the pGEM-T Easy vector) was inserted into the pDNA3 vector carrying the complete isoform 2 cDNA using the same unique restriction sites flanking the splice site.

For cloning of the 5′-end of rat OS-9, RT-PCR was performed on total RNA isolated from rat liver, as described above. The RT primer was designed to anneal with the bp 252–274 segment of the rat OS-9/L1 clone (5′-CATCCTCTTCTTCAGGAGCCACCC-3′). PCR primers were designed as follows: direct, 5′-CGGTTACGGCAGGAGATGCGGCGG-3′ (with KpnI site, derived from an identical region of human and mouse OS-9 gene preceding the ATG codon), and reverse primer 5′-AGACCC GCGGGTTCACCACCC-3′ (annealing with bp 248–269 in OS-9/L1 clone). The amplified product of 1.2 kb was purified, subcloned into pGEM-T Easy vector (Promega), and sequenced. Sequence analysis confirmed that the amplified cDNA fragment indeed contained the 5′-part of the rat OS-9 homologue (on the basis of the high homology with the mouse and human OS-9, and the identity of the 250 bp 3′-sequence of this cDNA with the 5′-part of the rat OS-9/L1 clone).

The 5′-end of mouse OS-9 cDNA coding for amino acids 1–300 was subcloned into pDNA3 vector with GFP fused in-frame at the carboxyl terminus (N-OS-9/GFP) and was used for fractionation studies.

In Vitro Binding Assay—The fragment of rat meprin β corresponding to the bait used in the two-hybrid screening (amino acids E57–Pho734) was expressed as a MBP fusion protein (designated as MBP-cyt). The rat OS-9 fragment corresponding to the L-1 clone was expressed as a GST fusion protein (GST-OS-9/L1). The fusion proteins were renatured and purified as recommended by the corresponding expression systems (New England Biolabs and Abersham Biosciences) and dialyzed against 20 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl and 5 mM MgCl2 (binding buffer). The MBP-β-galactosidase fusion protein (MBP-gal) was used as a control for nonspecific interaction. For the binding assay, 0.5 μg of MBP-cyt and MBP-gal proteins were incubated with the 0.1 μg of purified recombinant antibodies. The rat OS-9/L1 for 1 h at 4°C; then the amplified cDNA (10 μl of a 50% slurry) was added to the reaction mixture (50 μl) and further incubated for 1 h at 4°C. The resin was intensified washed with the binding buffer, containing 1% Nonidet P-40, prior to the elution with the binding buffer containing 10 mM maltose. The eluates were then analyzed by SDS-PAGE and Western blotting with anti-GST antibodies.

Transfections, Metabolic Labeling, and Immunoprecipitation—The DNA fragments of interest subcloned in the pDNA3 vector (Invitrogen) were used for transient and stable transfection of HER 293 cells by the LipofectAMINE™ reagent (Invitrogen). The expression of the proteins was analyzed by Western blot with specific antibodies or as specified in the text and in the legends to the figures.
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For metabolic labeling, subconfluent cells were incubated in a methionine-free DMEM for 1–3 h before the assay. [35S]Metionine (50 μCi/ml, Amersham Biosciences) was added to the same medium for 1 h. The cells then were rinsed with PBS and lysed in an IF buffer containing a 50 mM Tris buffer, pH 8.0, supplemented with 1% Brj 97, 10% glycerol, and protease and proteinase inhibitors mixture (Calbiochem-Novabiochem). Immunoprecipitation was carried out as described earlier (12).

**Kinetics of the OS-9 Interaction with Meprin β and the Endo-H Resistance Assay**—The HEK 293 cells (stably transfected by the pcDNA3 vector carrying the full-length isoform of mOS-9) were transiently transfected by meprin β using FAM-Fect® (Promega). Twenty four hours after transfection, the cells were re-plated onto four identical 10-cm dishes and grown for an additional 48 h. Then the cells were incubated in serum-free DMEM lacking methionine for 1 h and pulse-labeled by the addition of [35S]methionine (1 μCi/ml) for 15 min. The labeling medium was then replaced with complete DMEM containing 10% fetal calf serum, and the cells were further incubated for different times prior to lysis in the IP buffer (see above). The clarified cell extract were then subjected to immunoprecipitation as follows: 20% of each lysate was directly immunoprecipitated by anti-meprin β antibodies and used for Endo-H (Roche Molecular Biochemicals) assay, as described earlier (12). The remaining extracts were used to detect the meprin β associated with OS-9. The protein complexes were first immunoprecipitated with anti-OS-9 antibodies. The IPs were washed three times by PBS, and incubated for 15 min at 4 °C in a solution containing 50 mM Tris buffer, pH 8.0, supplemented with 1% Brij 97, 10% sucrose, adjusted to a 1.22 M sucrose, and loaded under a sucrose step gradient (1.15, 0.86, and 0.25 M sucrose). The gradient was centrifuged at 200,000 × g for 60 min. The supernatant was collected to represent the cytosol (S4) and the pellet (P4) to represent a light microsomal fraction. The heavy microsomal fraction was resuspended in 0.2 or 2 μg of PK (Roche Molecular Biochemicals). The reaction products were separated by SDS-PAGE and analyzed by immunoblotting with the appropriate antibodies.

**Subcellular Fractionation of Rat Liver**—Subcellular fractionation of rat liver was performed as described previously (24). The rat liver was homogenized in 5 volumes (v/w) of cold homogenization buffer (buffer H), containing 0.25 mM sucrose and proteinase inhibitors mixture (Calbiochem-Novabiochem) in 10 mM Hepes, pH 7.5. The homogenate was spun at 900 × g for 10 min in order to remove nuclei, mitochondria, and plasma membranes. The supernatant (S1) was centrifuged at 34,000 × g for 10 min; the pellet was discarded, and the supernatant (S2) was spun at 50,000 × g for 30 min. The resulting pellet (P5) containing the heavy microsomal fraction was collected, and the supernatant (S3) was centrifuged at 200,000 × g for 60 min. The supernatant was collected to represent the cytosol (S4) and the pellet (P4) to represent a light microsomal fraction. The heavy microsomal fraction was resuspended by gentle homogenization in 10 mM Hepes, pH 7.5, containing 52% sucrose, adjusted to a 1.22 M sucrose, and loaded under a sucrose step gradient (1.15, 0.86, and 0.25 M sucrose). The gradient was centrifuged for 3 h at 82,500 × g and carefully unloaded from the top.

**Protein Analysis and Secondary Structure Prediction**—Protein Identification and Analysis Tools available in the ExPASy Server (Geneva University, expasy.hcuge.ch/www/tools.html) were used to characterize proteins of interest. Multiple alignments of the DNA and protein sequences were performed using the Clustal method and MegAlign software (DNAStar Inc.). The proteins were washed and released from the beads with PBS and released into a homogenization buffer containing a mixture of proteinase and proteinase inhibitors (Calbiochem-Novabiochem), observed using a microscope equipped with an epifluorescence attachment EF3-3 (Nikon) and photographed. For double-staining experiments, the colored images were prepared using the SPOT video attachment (Diagnostic Instruments, Inc.) and processed using the Adobe® Photoshop® 5.5 software (Adobe Systems Inc.).

**Indirect Immunofluorescence**—Staining of GH3 cells (rat pituitary epithelial cell line), and transiently transfected COS-7 cells grown on glass coverslips was performed as described previously (12). In order to study the topology of OS-9, the plasma membrane of NIH 3T3 fibroblasts was selectively permeabilized using low concentration of digitonin (20). Briefly, cells were fixed by 4% paraformaldehyde for 20 min, washed three times with PBS, and incubated for 15 min at 4 °C with a solution containing 5 μg/ml digitonin, 0.1 M KCI, 2.5 mM MgCl2, and 10 mM Hepes, pH 6.9. The cells were then washed three times with PBS and incubated with 4% normal donkey serum in PBS for 30 min at 22 °C to block the non-specific binding sites. Control cells were treated as above, but 0.2% saponin was included in the blocking buffer to achieve complete permeabilization of the cell membranes. The cells were incubated for 1 h at 22 °C with anti-OS-9 (1:2000 serum or 1 μg/ml affinity purified antibodies), anti-BIP (mouse monoclonal, 1:200), and anti-calnexin cytoplasmic domain (rabbit polyclonal, 1:200) antibodies. Donkey anti-rabbit IgG-Cy2, anti-mouse IgG-Cy2, and anti-guinea pig IgG-Cy3 (all diluted 1:200 in PBS) were used to visualize cross-reacting mouse, rabbit, and guinea pig primary antibodies using the appropriate antibodies. The radioactively labeled proteins were analyzed by SDS-PAGE and fluorography using an AmplifiTM solution and a Hyperfilm™ MP autoradiography film (Amersham Biosciences).

**Characterization and Possible Function of OS-9**

The HEK 293 cells (stably transfected by the pcDNA3 vector carrying the full-length isoform of mOS-9) were transiently transfected by meprin β using FAM-Fect® (Promega). The labeling medium was then replaced with complete DMEM containing 10% fetal calf serum, and the cells were further incubated for different times prior to lysis in the IP buffer (see above). The clarified cell extracts were then subjected to immunoprecipitation as follows: 20% of each lysate was directly immunoprecipitated by anti-meprin β antibodies and used for Endo-H (Roche Molecular Biochemicals) assay, as described earlier (12). The remaining extracts were used to detect the meprin β associated with OS-9. The protein complexes were first immunoprecipitated with anti-OS-9 antibodies. The IPs were washed three times by PBS, and incubated for 15 min at 4 °C in a solution containing 50 mM Tris buffer, pH 8.0, supplemented with 1% Brij 97, 10% sucrose, adjusted to a 1.22 M sucrose, and loaded under a sucrose step gradient (1.15, 0.86, and 0.25 M sucrose). The gradient was centrifuged for 3 h at 82,500 × g and carefully unloaded from the top.

**RESULTS**

**OS-9 Specifically Interacts with the Intracellular Tail of Meprin β in the Yeast Two-hybrid System and in Vitro**—To elucidate the role of the intracellular (carboxyl terminus) moiety of meprin β, we attempted to identify proteins that might interact with this tail. Yeast two-hybrid screening of a rat cDNA library using the segment 674Thr–Lys688 of rat meprin β as a bait (Fig. 1A) revealed 11 specific interacting clones, 10 of which contained overlapping fragments of the same cDNA sequence. Data base search revealed that the protein encoded by this cDNA is highly homologous to the carboxyl-terminal part (positions 329–667) of the isoform 1 of human OS-9 (hOS-9) (GenBank™ accession number U146353) (Fig. 1B). Computer analysis of the hOS-9 protein did not detect any similarity with known functional domains or characterized proteins. The cysteine-rich amino-terminal region of hOS-9 has a significant homology with the ORFs deduced from the genomic sequences of S. cerevisiae (YD9609.11), Arabidopsis thaliana (U146353), C. elegans (F48E8.4), and fruit fly (AAF53149.1).

A secondary structure prediction identified a carboxyl-terminal region in OS-9 that has a high probability for a coiled-coil structure (Fig. 1B).

The interaction between the cytoplasmic fragment of meprin β and OS-9/L1 fragment was confirmed using an in vitro binding assay. The MBP-tagged fragment of meprin β corresponding to the bait used in the two-hybrid screening (MBP-cyt) and GST-tagged OS-9/L1 fragment was expressed in bacteria, purified, and used an in vitro binding assay (see “Experimental Procedures”). Purified MBP-gal fusion protein containing a 20-kDa fragment of β-galactosidase was used as a control. As shown in the Fig. 2A, GST-OS-9/L1 specifically interacts with MBP-cyt. The beads containing the control MBP-gal protein adsorbed a negligible amount of GST-OS-9/L1. This result indicates that the rat OS-9 fragment forms a complex with the cytoplasmic tail of meprin β in vitro and that additional components are required for this interaction to occur.

**Identification of the Region in the Cytoplasmic Tail of Meprin β That Is Involved in Binding to OS-9/L1**—To identify the region in the carboxyl-terminal tail of meprin β that is involved in binding to OS-9/L1, we prepared a series of mutant baits with deletions and alanine substitutions, and we compared them in the OS-9 binding assay. As shown in Fig. 2B, under the conditions of our experiment, the segment 674Thr–Lys688 in the
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Fig. 1. A, schematic structure of meprin α and β, and design of construct used as a bait in the two-hybrid assay. The predicted domain structure of meprin α and meprin β is taken from Ref. 4, and the amino acid sequence corresponds to the rat meprin β. S, signal peptide; P, prosequence; E, epidermal growth factor-like domain; TM, transmembrane domain; CYT, cytoplasmic region; GAL4 BD, the DNA binding domain in the pGBT9 vector. Arrow indicates the site of the proteolytic cleavage that occurs during the maturation of meprin α in the ER. B, schematic alignment of a 339-amino acid (a.a.) polypeptide translated from the DNA insert of the representative positive clone (OS-9/L1) obtained in yeast two-hybrid screening and the hOS-9 splice forms. Numbers on N to C axis of the figure show the positions of amino acid residues relative to the hOS-9 (isoform 1). Regions absent in the alternatively spliced variants of the hOS-9 (isoforms 2 and 3) are shown by thin lines. Dashed stretches correspond to regions with significant similarity to the F48E8.4 (C. elegans) and YD9609.11 (S. cerevisiae) ORFs; the gray boxes represent an acidic stretch found in human and rat OS-9. The putative coiled-coil region is shown as a black box.

Meprin β tail is both necessary and sufficient for the binding of OS-9. For example the mutant obtained by truncation at Ala^{689} (number 2) retains full (in fact somewhat enhanced) binding capacity, whereas the mutants with a truncation at Tyr^{679} (number 5) or with a 67–Thr–Lys^{688} deletion (number 3) have a negligible binding capacity. Furthermore, mutations in which the cluster of basic amino acids was substituted by alanines, in part (numbers 8 and 9) or completely (number 7), were found to have a reduced or a complete loss of binding capacity. Although these findings indicate an important contribution of this cluster to the binding of OS-9, they do not restrict the binding site to this segment, because the binding capacity of meprin β also is abolished upon substitution by alanine of either the 67–Thr–Val^{678} (number 10) or the 67–Thr–Val^{678} (number 11) segments. We conclude therefore that the entire 67–Thr–Lys^{688} segment in meprin β is involved in the binding of OS-9.

Cloning and Sequence Analysis of the Alternatively Spliced Mouse and Rat OS-9 cDNA—A partially sequenced mouse cDNA clone homologous to the 3′-terminus of the hOS-9 (GenBank™ accession number U41635) was identified using the TIGR Mouse Gene Index data base (The Institute for Genomic Research, www.tigr.org/db/mgi/mgi.html). This clone (GenBank™ accession number AA103675) was obtained from the I.M.A.G.E consortium collection and sequenced. The complete cDNA sequence of 2,610 bp was derived from 8 overlapping fragments corresponding to both the sense and the antisense DNA strands and was confirmed to represent a full-length mouse OS-9 (mOS-9) cDNA corresponding to the isoform 2 of hOS-9 described previously (16) (Fig. 1B).

We also found that the MGI data base contains three tentative sequences (TC31623, TC34772, and TC37764), identical to the fragments of mOS-9. Interestingly, the 5′-end of TC31623 contained a 42-bp fragment that did not align with the sequence of our clone but was similar to the corresponding region in the non-spliced hOS-9, indicating that like the human OS-9, the mouse OS-9 may also undergo alternative splicing. Indeed, an RT-PCR on mouse kidney RNA with the set of primers flanking the putative splicing site resulted in an amplification of the two products with an ~150-bp difference in their molecular weight. Sequencing analysis of these fragments confirmed that they represent isoforms 1 and 2 of mouse OS-9. RT-PCR analysis of RNA isolated from several rat and mouse tissues (liver, brain, and heart) and from cultured mouse fibroblasts revealed the presence of these isoforms in all analyzed samples (data not shown). Interestingly, we observed a somewhat different ratio between the two forms in the various tissues tested, but at this stage, we did not study this phenomenon systematically. In addition, we found that such splicing can occur also in the rat tissues, despite the fact that all 10 rat OS-9 clones (including OS-9/L1) obtained from the two-hybrid library screening, corresponded to the non-spliced variant (homologous to the isoform 1 of hOS-9). The splicing event identified in the rat and the mouse OS-9 isoforms occurs at exactly the same position (corresponding to exon 13), as in hOS-9 (16, 26), suggesting that the OS-9 gene in all three species might have a conserved exon/intron organization in this region.

The complete cDNA sequence of rat and mouse OS-9 (isoforms 1 and 2) was reconstituted from the sequenced overlapping RT-PCR products. The comparison of the protein sequences translated from the full-length rat and mouse cDNA with the reported sequence of hOS-9 (15) (Fig. 3A) revealed that OS-9 is highly conserved among these species (Fig. 3B), especially in the amino-terminal part (amino acid residues 1–250), which displays about 96% similarity between the human and the mouse protein sequences. Interestingly, this region was also found to have a significant homology with ORFs deduced from genomic sequences of S. cerevisiae (YD9609.11) and C. elegans (F48E8.4) (15) and may thus represent a conserved functional domain.

Characterization of OS-9 Using Antibodies against the Rat OS-9/L1 Fragment—In order to further characterize OS-9, we used polyclonal antibodies raised against the recombinant rat OS-9/L1 fragment. Western blotting of homogenates of different rat tissues with anti-OS-9 antibodies revealed two protein bands with apparent molecular masses of 88 and 97 kDa in all samples tested (Fig. 4A, upper panel). This result is in agreement with the observation reported previously (15) that hOS-9 mRNA is ubiquitously expressed in human tissues. The same cross-reactivity with the 88–97-kDa doublet was observed in various rat and mouse cell lines, such as NIH 3T3 (Fig. 4A, bottom panel), Swiss 3T3, GH3, and PC12 cells (data not shown). No immunoreactive material was found in human embryonic kidney fibroblasts (HEK 293, shown in Fig. 4A, bottom panel) and in monkey COS-7 fibroblast cell lysates (not shown). It is likely, however, that OS-9 is expressed in these cells and that the lack of detection is due to a low cross-reactivity of our antibodies with human or monkey OS-9 or due to a lower expression level of OS-9 in human cells. In fact, we were able to detect OS-9 mRNA in HEK 293 cells by an RT-PCR experiment using a set of primers specific to hOS-9 (16) (data not shown).

The specificity of our anti-OS-9 antibodies was confirmed by...
a Western blot analysis of the HEK 293 fibroblasts transiently transfected by the full-length mOS-9 cDNA (isoforms 1 and 2). Whereas the mock-transfected cells do not express any cross-reactive material when stained with anti-OS-9 antibodies, proteins with an apparent molecular mass of 97 and 88 kDa were detected by these antibodies in the cells transfected with isoforms 1 and 2 of mOS-9, respectively. The gel migration of transfected isoforms 1 and 2 is very similar to the migration of the upper and lower proteins bands present in the doublet detected in NIH 3T3 cells, respectively (Fig. 4A, compare lanes marked HEK 293 with lane marked NIH 3T3). We did not observe any difference between the apparent molecular weights of mOS-9 translated in vitro and that expressed in HEK 293 cells (Fig. 4A), compare lanes marked HEK 293 with lane marked NIH 3T3). We did not observe any difference between the apparent molecular weights of mOS-9 translated in vitro and that expressed in HEK 293 cells (Fig. 4A). Furthermore, the migration of OS-9 in the gel was not altered by pretreatment of the samples with the broad specificity endoglycosidase (peptide N-glycosidase F) or when the in vitro translation was carried out in the presence of canine microsomal membranes (data not shown), suggesting that OS-9 does not undergo post-translational glycosylation, despite the fact that it possesses an amino-terminal hydrophobic sequence (Met-Leu20) resembling the ER translocation signal, and a potential N-glycosylation site (177NGSK180).

Western blot analysis of the fractionated NIH 3T3 cells revealed that OS-9 is associated with the total membrane fraction and not with the cytosol (Fig. 4B, PBS), displaying the same distribution as calnexin, an integral membrane protein localized in the ER. In order to analyze the nature of the association of OS-9 with membrane, we tested the distribution of OS-9 between the lipid and aqueous fractions using a Triton X-114 extraction. As seen in Fig. 4B (TX-114), OS-9 is found in the aqueous fraction upon phase separation. A similar result was obtained upon alkaline extraction of the membrane fraction, where OS-9 was found in the soluble fraction and not in the membrane pellet, and an integral membrane protein calnexin remained associated with lipid fraction in both experiments (Fig. 4B, Na2CO3).

To test whether OS-9 is associated with membrane also under the overexpression conditions, we prepared a construct coding for a fusion protein consisting of an amino terminus of OS-9 (amino acids 1–300) and GFP (N-OS-9/GFP). This construct, as well as two control constructs (GFP alone and integral membrane protein CD147/GFP), was transfected into HEK 293 cells. When the transfected cells were fractionated into the membrane and cytoplasmic fractions, the N-OS-9/GFP construct was detected in both fractions, whereas GFP was found predominantly in the cytoplasm, and CD147/GFP was distributed exclusively into the membrane fraction (Fig. 4C). This result demonstrates that the membrane association of OS-9 depends on its level of expression, suggesting that OS-9 is unlikely to be translocated into the ER by means of its hydrophobic amino-terminal region.

**OS-9 Is Localized in ER by Immunocytochemistry and Subcellular Fractionation of Rat Liver**—Immunostaining of permeabilized rat pituitary cells (GH3 line) with anti-OS-9 antibodies

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**Fig. 2.** A, in vitro interaction of the rat OS-9 fragment and the cytoplasmic tail of meprin β. The purified recombinant GST-OS-9/L1 was incubated either with MBP-cyt or MBP-gal fusion proteins and precipitated by amylose-coated beads as described under “Experimental Procedures.” Protein complexes were eluted from the beads and resolved by SDS-PAGE. **Right panel** shows the bottom part of the gel stained with Coomassie Brilliant Blue (CBB) to detect MBP fusion proteins. **Left panel** shows the upper part of the gel that was Western blotted with anti-GST antibodies (WB: anti-GST). B, quantitative assay of β-galactosidase (β-gal) activity performed on yeast SFY526 cells co-expressing the rat OS-9 fragment (clone L1) and mutated carboxyl-terminal fragments of rat meprin β. The β-galactosidase activity was determined as described under “Experimental Procedures,” and the activity of an original bait (wild type) was taken as 100%. The data are presented as an average of three independent experiments, each performed in triplicates.
resulted in a specific detection of the vesicular structures around the nucleus that are extended to the cell periphery, resembling the distribution of the ER (Fig. 4D, anti-OS-9). No staining was detected in the cells incubated with the control guinea pig IgG (Fig. 4D, Control) and in non-permeabilized cells (data not shown). In COS-7 cells, transiently transfected by the mOS-9 (isoform 1 or 2), immunoreactive material was also observed in the perinuclear area (which may represent the Golgi or an intermediate ER-Golgi compartment) and in the fine ER network on the cell periphery (Fig. 4E, iso1 and iso2). The non-transfected COS-7 cells were did not display cross-reactivity with anti-OS-9 antibodies (data not shown). No difference in the pattern of distribution of OS-9 isoforms was observed.

To obtain biochemical support for the ER localization of OS-9, we carried out a fractionation of rat liver (see Experimental Procedures) and subsequent immunodetection of OS-9 alongside previously characterized proteins known to be localized in distinct membrane compartments. As shown in Fig. 5A, both OS-9 isoforms are found in the heavy microsomal fraction (sedimented at 50,000 \( \times g \)), with some minor amount present in the light microsomes (sedimented at 200,000 \( \times g \)). No OS-9 immunoreactivity was found in the cytosol (200,000 \( \times g \) supernatant), in agreement with the result obtained with fractionated NIH 3T3 cells (cf. Fig. 4). All fractions contained an equal amount of protein, as judged by the Ponceau Red staining of the nitrocellulose membrane before incubation with the antibodies (Fig. 5A, upper panel).

Immunoblot analysis of the heavy microsomes fractionated by floating upward in a sucrose gradient, according to a method described previously (27), revealed that OS-9 is associated with vesicles of a distinct floating density (Fig. 5B). Fractions containing OS-9 partially overlapped with those containing the ER.
chaperones calnexin, GRP 78 (BiP) and GRP 94, and the small GTPase Rab1, which is involved in the anterograde ER-to-Golgi vesicular transport (28). OS-9 did not co-fractionate with β-COP, a component of the COPI-coated vesicles (participating in the Golgi-to-ER retrograde transport (29, 30), with Rab2 and a 58K protein associated with Golgi compartment (31, 32).

Membrane Orientation of OS-9—Because the carboxyl-terminal tail of meprin β is facing cytosol, it was important to determine the orientation of the microsomal membrane-associated OS-9. First, we performed a PK digestion of the membranes prepared from NIH 3T3 cells, and we compared the protease accessibility of the luminal domain of ER chaperone calnexin (33) and OS-9. As shown in Fig. 6A, OS-9 was completely digested by increasing amounts of PK both in the absence and the presence of a non-ionic detergent, whereas calnexin was fully degraded only in the presence of Triton X-100. The change of the gel migration of calnexin upon PK treatment reflects the removal of its short (87 amino acids) cytoplasmic domain (33). These data suggest that OS-9 is associated with the cytosolic surface of the membranes, but because the result of such experiment might be strongly dependent on the protease sensitivity of the proteins in question, we also tested the OS-9 topology using the immunofluorescent detection of OS-9 in digitonin-permeabilized NIH 3T3 cells. This method is based on the fact that low concentrations of digitonin selectively permeabilize the plasma membrane because of its higher cholesterol content compared with intracellular membranes (34). Cells were simultaneously stained against OS-9 and one of the ER chaperones, BiP or calnexin. Staining against BiP (which is localized in ER lumen) was used to control the integrity of the ER membrane after digitonin treatment, and the detection of the cytoplasmic domain of calnexin was used to monitor the efficiency of the plasma membrane permeabilization. As shown in Fig. 6B, in digitonin-permeabilized cells, both OS-9 and the cytoplasmic tail of calnexin were accessible to the antibodies, whereas the luminal protein BiP was not detected under these conditions. Upon the complete cell permeabilization by saponin, the intensity of the BiP staining was dramatically increased, whereas the intensity of the OS-9 and calnexin staining was not changed. Taken together, the experiments described in this section clearly dem-

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**FIG. 4.** A, top, detection of the OS-9 protein in rat tissues. The proteins from 10 μg of rat tissue homogenates (brain, spleen, kidney, lung, small intestine (S. Int.), and large intestine (L. Int.)) were resolved by SDS-PAGE (10% gel), transferred onto nitrocellulose paper, and stained with anti-OS-9 antibodies. Bottom, expression of the mOS-9 isoforms in HEK 293 cells and in a cell-free system. The HEK 293 cells were transfected by pcDNA3 vector (mock) or by pcDNA3 carrying the isoforms in HEK 293 cells. B, detection of OS-9 in fractions collected and extracted by 1% Nonidet P-40 24 h after transfection. For the in vitro translation experiment, the same constructs were expressed using the coupled transcription and translation system. The proteins from 1 μg of the extracts of transfected HEK 293 cells or 5 μl of the translation mixture were analyzed by Western blotting with anti-OS-9 antibodies. The lysate of cultured mouse NIH 3T3 fibroblasts (5 μg) was run alongside as a control. C, membrane association of OS-9. Distribution of OS-9 between the membrane (mem) and cytosolic (cyt) fractions prepared from NIH 3T3 cells (PBS) or between membrane subfractions prepared by extraction with 1% Triton X-114 (TX-114) or 0.1 M sodium carbonate, pH 11.5 (Na2CO3) as described under “Experimental Procedures.” Upper panels show a Western blot with anti-OS-9 antibodies, and bottom panels show the same blot re-probed with anti-calnexin antibodies. D, sup, supernatant; pel, pellet, detergent; aq, aqueous. E, membrane association of OS-9 under the overexpression conditions. HEK 293 cells were transfected with either GFP, N-OS-9/GFP, or CD147/GFP, and used for the preparation of the membrane (M) and cytoplasmic (C) fractions. The proteins from 5 μg of the obtained fractions were analyzed by SDS-PAGE and Western blotting with antibodies against GFP. The same blot was then re-probed for calnexin and α-tubulin, markers of the membrane and cytoplasmic fractions. D, detection of OS-9 in rat pituitary epithelial cells (GH3). Cells were stained with either anti-OS-9 immune serum (OS-9) or with normal guinea pig serum (control). Cross-reacting material was visualized using anti-guinea pig IgG conjugated with Cy3, and the nuclei of the cells were stained by 4,6-diamidino-2-phenylindole (DAPI). Bar, 20 μm. E, COS-7 cells transiently transfected by isoform 1 or 2 of mOS-9 (iso1 and iso2) were stained for OS-9 as described above. Bar, 20 μm.

**FIG. 5.** Subcellular fractionation of rat liver. A, total homogenate (H), heavy microsomes (HM), light microsomes (LM), and cytosol (C) were prepared as described under “Experimental Procedures” and analyzed by Western blotting (WB) with anti-OS-9 antibodies (bottom panel). All fractions contained an equal amount of protein, as seen in the Ponceau Red staining of the nitrocellulose membrane prior to incubation with the antibodies (upper panel). B, the heavy microsomal fraction (enriched in OS-9) was subjected to centrifugation in the step sucrose gradient (details under “Experimental Procedures”). An equal amount of protein from the fractions was loaded on the gel (2.5 μg for the detection of OS-9, Golgi 58K protein, Grp 78 (BiP) and calnexin, and 25 μg for the detection of Rab1 and Rab2). The distribution of proteins in the density gradient was analyzed by Western blotting with indicated antibodies.
Characterization and Possible Function of OS-9

OS-9 fragments and the tail of meprin β is due to the deletion of the 55 amino acids in the spliced forms. However, this 55-amino acid fragment alone was not able to interact with the tail of meprin β when it was expressed as a GAL4 DNA-binding domain fusion protein (data not shown), suggesting that additional distal elements may be involved in the binding. Alternatively, it is possible that removal of the spliced out segment affects the conformation of OS-9 in a way that renders it incapable of interacting with the tail of meprin β.

In order to test whether the difference in the interaction of the OS-9 isoforms and meprin β occurs also in vivo, we expressed the full-length meprin β and the OS-9 isoforms in mammalian cells and performed a co-immunoprecipitation assay (see “Experimental Procedures”). We found that under our experimental conditions only a very minor amount of OS-9 and meprin β exist as a complex (<1% of the starting material), whereas the complex was predominantly detectable in the cells that co-express the non-spliced OS-9 (isoform 1) and meprin β (Fig. 7C). Because similar amounts of the OS-9 isoforms were present in the input material, we believe that this result reflects a difference in the ability of the two splice isoforms to bind meprin β. In most experiments, a significantly lower amount of meprin β was also detected in the anti-OS-9 immunoprecipitates obtained from the mock-transfected HEK 293 cells, as well as from cells transfected by the isoform 2 of OS-9. We presume that although the anti-OS-9 antibodies used in this assay do not detect hOS-9 on the Western blot, they might precipitate a small amount of endogenous OS-9 from HEK 293 cells, which may be associated with meprin β.

OS-9 Interacts with Meprin β During Its Export from the ER—The results described above suggest that OS-9 is a peripheral membrane protein localized at the cytosolic side of the ER. But does the interaction between OS-9 and meprin β indeed occur during the export of the latter from the ER? To answer this question, we performed a pulse-chase experiment, in which the amount of metabolically labeled meprin β bound to OS-9, and the susceptibility of meprin β to deglycosylation by Endo-H (which reflects the translocation step from ER-to-Golgi) was monitored simultaneously (cf. “Experimental Procedures” and the legend to Fig. 8). As seen in Fig. 8, the amount of radioactively labeled meprin β found in the protein complexes captured by anti-OS-9 antibodies and proteasome A beads is maximal at the beginning of the chase (15-min point) and decreases upon prolonged incubation. Such kinetics are very similar to the time course of the export of meprin β from the ER, as monitored by the decrease of the Endo-H-sensitive form of meprin β directly immunoprecipitated from the same cell lysates (Figs. 8A, right). The amount of directly immunoprecipitated metabolically labeled OS-9 and meprin β does not change during the chase. Therefore, we concluded that meprin β exists in a complex with OS-9 only transiently, most probably during its translocation from the ER to the Golgi.

**DISCUSSION**

Mapping the Interacting Sites in OS-9 and Meprin β Sets the Stage for Illustrating the Physiological Function of OS-9—This study stemmed from our attempt to elucidate the physiological role of the cytosolic tail of meprin β, using a yeast two-hybrid screening of a rat cDNA library to identify proteins that may interact with this tail. The results of this screening demonstrated that a 339-amino acid protein fragment, highly homologous to the carboxyl-terminal part of a previously uncharacterized human protein OS-9, specifically interacts with this tail.

To assess the functional significance of this interaction, we first cloned and characterized the full-length, alternatively spliced rat and mouse homologues of hOS-9. We then showed
that only the non-spliced form of rat or mouse OS-9 (isoform 1) is able to interact with meprin and that this interaction occurs both in vitro (with purified fusion proteins) and in vivo (when the full-length meprin β and the isoform 1 of OS-9 are co-expressed in mammalian cells). Because there is a distinct difference in binding of the alternatively spliced variants of OS-9 to meprin β, it is possible that OS-9 may be functionally regulated by alternative splicing. It is also possible that the isoform 2 of OS-9 has a different preference for binding or a different physiological assignment altogether. By testing the interaction between the fragment 328Gln–Phe667 of the rat OS-9 (isoform 1) and mutant fragments of the carboxyl terminus of meprin β with deletions or alanine substitutions, we found that the region 674Thr–Ala689 in meprin β is necessary and sufficient for the binding of OS-9. Because this segment in meprin β is also essential for its ER-to-Golgi transport (12), we propose that OS-9 may be involved in the ER-to-Golgi transport of meprin β. In support of this hypothesis we found that OS-9 does not bind to a mutant fragment of the tail if basic amino acids in the segment 682RRKYRKK688 are substituted by alanines, because this mutation significantly decreases the rate of the ER-to-Golgi transport of meprin β expressed in mammalian cells (12). These findings attribute an important role to the 682RRKYRKK688 segment in meprin β to the binding of OS-9, but it does not restrict the binding site to it, because the binding capacity of meprin β also is abolished upon substitutions by alanines in the segment 674TLISVYCT681 indicating that it is also important for OS-9 binding. Further studies are needed to obtain a detailed map of this interaction at the individual amino acid level because the substitution of the cluster of basic amino acid residues with alanines does not bring about a complete entrapment of the mutant meprin β in the ER, as in the case of the truncation mutants lacking
It should be noted that the S. cerevisiae ORF YD9609.11 that has a significant homology to the amino-terminal cysteine-rich domain of OS-9 (15) contains a carboxy-terminal HDEL motif characteristic for ER resident proteins. This hypothetical protein is the only one of the 11 HDEL proteins present in S. cerevisiae that is not functionally characterized yet. A study of the protein coded by YD9609.11 has been initiated in our laboratory in collaboration with Dr. Jeffrey Gerst, and our preliminary data suggest that this protein is indeed localized in the ER lumen of the yeast cells. Because the homology between the yeast YD9609.11 and mammalian OS-9 is limited only to the amino-terminal domain with no sequence similarity in the carboxy-terminal domain, it will be interesting to find out the function of this domain in yeast, and to see whether this function is also preserved in higher organisms.

**Is OS-9 a Cargo Adaptor?**—One of the fundamental questions in modern cell biology is the elucidation of the “traffic rules” controlling the intracellular transport and targeting of proteins. At this stage, it is not yet clear how the cargo selection machinery in the ER recognizes the integral membrane proteins destined to be delivered to the Golgi from those that should remain in the ER. It was recently shown that the vesicular stomatitis virus G protein is specifically recruited into the transport vesicles formed by purified recombinant COPII components, although it does not interact directly with these components (35). In this case, one would have to assume the existence of a membrane adaptor protein that could mediate this interaction. Such a cargo adaptor, directing the ER-to-Golgi transport of integral membrane proteins, has not been identified yet in mammalian cells.

Several lines of evidence suggest that isoform 1 of OS-9 may function as a cargo adaptor for meprin β: binding of OS-9 to the region in meprin β that is important for the export of meprin β from ER, coincidence of the OS-9/meprin β interaction with the export of meprin β from the ER, and localization of OS-9 is on the cytosolic side of the ER. It should be emphasized that this possibility is supported here only by indirect evidence. Additional experiments are needed to obtain direct and unequivocal proof for this suggestion. While this manuscript was in preparation, it was reported that the second C2 domain of N-copine interacts *in vitro* with the fragment of mouse protein, which has 90% identity to the carboxyl terminus of human OS-9 (amino acids 536–667) (36). N-Copine is expressed in the neuronal tissue, and it is a new member of the family of calcium-dependent, phospholipid-binding proteins called copines (37–39). Interestingly, a conserved C2 domain that defines copines is found in many proteins that play roles in membrane trafficking, such as synapticaptgam and Munc13 (40, 41), and human copine I was shown to bind secretory vesicles (38). Whether or not the interaction between OS-9 and copines has a physiological significance is a very intriguing question, and we hope that our data on OS-9 characterization will help to answer it.

The region in the meprin β tail, which is involved in OS-9 binding, is relatively extended and contains 15 amino acid residues. At this stage we did not systematically assess the contribution of each amino acid residue in this segment to the interaction with OS-9, but it is reasonable to assume that not all of them equally contribute to this binding. It is also possible that this leaves room for several variations within the segment accommodating the binding site that, in turn, could allow OS-9 to act as a multitarget adaptor. A search in the TrEMBL data base with a redundant pattern derived from the juxtamembrane sequence of rat meprin β (transmembrane domain -Y(F)CXXXR(K/R[K/R(K)]), where X indicates none or any amino acid using a PatScan program at www.msc.anl.gov/
Characterization and Possible Function of OS-9

This paper presents the first systematic characterization of OS-9 as a protein. Converging lines of evidence accumulated in the course of this characterization prompted us to propose that OS-9 is a member in the emerging team of proteins involved in the specific sorting of the cargo in the secretory pathway.

Acknowledgments—We thank Anton Chestukhin, Jeffrey Gerst, and Alexander Bershadsky for most helpful and stimulating discussions and J. A. DeCaprio for the valuable help in the preparation of this manuscript. We are grateful to Lior Soussan and Yosef Yarden for providing a cDNA library for yeast two-hybrid screening.

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A Selective Interaction between OS-9 and the Carboxyl-terminal Tail of Meprin β

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J. Biol. Chem. 2002, 277:34413-34423.
doi: 10.1074/jbc.M203986200 originally published online July 1, 2002

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