Cloning and Expression of a Mouse Macrophage cDNA Coding for a Membrane Glycoprotein of the Scavenger Receptor Cysteine-rich Domain Family*

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We have cloned from murine macrophages a cDNA coding for a new protein of the scavenger receptor family whose mRNA is increased very strongly by adherence and moderately by exposure to tumor necrosis factor and interferon-γ. The nucleotide sequence extends for 2168 bases and encodes a protein of 559 amino acids with six potential glycosylation sites. The first 100 NH2-terminal amino acids represent a single scavenger receptor cysteine-rich domain, whereas the COOH-terminal end of the molecule is compatible either with a transmembrane hydrophobic peptide followed by a very short intracytoplasmic sequence or a signal sequence for an anchoring via a glycosphatidylinositol. The protein is highly homologous to most of the very recently identified human MAC-2-binding protein and murine cyclophilin C-associated protein.

Macrophages have been described to be capable under certain conditions of releasing erythropoietin or a substance with a comparable activity on the erythroid lineage precursor cells (1-4). In the course of a study intended to explore the presence of erythropoietin mRNA in mouse macrophages of different origins and under a variety of conditions, we observed that a 3'-fragment, but not a 5' fragment of erythropoietin cDNA (5, 6), hybridized on Northern blots of total macrophage RNA with a mRNA of a size larger than that of the erythropoietin mRNA and was markedly increased when macrophages were obtained from anemic mice or activated in vitro following adherence. In the process of searching for the corresponding molecule in a cDNA library made from activated mouse macrophages, we identified a cDNA sequence, which happened to be unrelated to the one searched for. This cDNA was sequenced and found to direct the synthesis of an as yet unidentified glycoprotein, bearing a special motif characteristic of the scavenger receptor cysteine-rich (SRCR) domain family (7). The amount of the corresponding mRNA is modulated in macrophage by a variety of conditions. This mRNA is also expressed in cell lines of other origin, in particular fibroblasts. The protein may be the murine homologue to the very recently identified human MAC-2-binding protein (hMAC-2-BP) (8). MAC-2 is a bifunctional secreted protein (9) with a lectin domain capable of binding laminin (10) and which, in association with its ligand, MAC-2-BP, may serve as a bridge between the macrophages and the extracellular matrix, macroorganisms, or other cells bearing galactosylated proteins. One of the roles of this new cell membrane protein might then be related to macrophage adhesion and migration to the site of infection. With very minor differences, this protein is also homologous to the cyclophilin C-associated protein (CyCAP) (11), which makes it a candidate in the cascade of immunoregulatory events resulting from the absorption of the immunosuppressive drug cyclosporin A.

MATERIALS AND METHODS

cDNA Cloning and Sequencing—Poly(A)+ RNA from thioglycolate-induced peritoneal adherent macrophages obtained from BALB/c mice and selected after 6 h of adherence to plastic in vitro was prepared on an oligo(dT)-Sepharose column (Pharmacia, Uppsala, Sweden) and used to construct a cDNA library in pcDNAII vector (The Librarian, Invitrogen, San Diego, CA). The library, which contained about 95,000 clones, was split into 10 sublibraries whose plasmid DNA was explored by polymerase chain reaction, using as 5'-primer oligonucleotides corresponding to the Sp6 or T7 promoters contained in the pcDNAII vector and as the 3'-primer the gene-specific primer E6 (5' CACAGCGAGTTCCAAGGCC3') derived from the mouse erythropoietin sequence. Polymerase chain reaction cycles were 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C for a total of 40 cycles. The DNA of a sublibrary gave an amplified fragment of 1 kilobase pair using E6 and Sp6 primers. This fragment, digested with BamHI to eliminate the region between the Sp6 promoter site and the BstXI site of insertion, was used, after 32P labeling by random priming, to identify hybridizing clones from the positive sublibrary spread on agar plates. Two positive clones were identified. In order to sequence entirely the cDNA, restriction enzyme fragments were cloned in pGEM3Z (Promega, Madison, WI) or pBluescript SK(+) (Stratagene, La Jolla, CA) plasmids, and chain termination sequencing was carried out with the U. S. Biochemical Corp. sequencing procedure with Sp6, T3, or T7 promoter sequencing primers. The final sequence was determined from both strands.

Transfection Experiments—The cDNA was inserted at the XhoI site of the pcGGS expression vector containing the β-actin promoter (a kind gift of J. Miyasaki) (12). Three days after transfection, COS-7 cells were lysed with 1% Nonidet P-40 in phosphate-buffered saline, nuclei eliminated by a 1000 × g centrifugation, and the postnuclear supernatants processed for SDS-polyacrylamide gel electrophoresis in reducing conditions. The gel was stained with silver nitrate.

Computer Analysis—Searches for homologies in the DNA or protein sequences were done with the aid of the Genetics Computer Group (13) and IG (Intelligenetics, Mountain View, CA) programs. Translation, predictions of eucaryotic secretory signal sequence and transmembrane region, and detection of sites and signatures in the protein sequence were done with the PCgene programs (A. Bairoch, Yves Chicheportiche and Pierre Vassalli; Fax: 41-22-347-33-34.

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‡ The abbreviations used are: SRCR, scavenger receptor cysteine-rich; hMAC-2-BP, human MAC-2-binding protein; MAMA, murine adherent macrophage; CyCAP, cyclophilin C-associated protein; sper receptor, sea urchin spermatozoon receptor for peptides; GPl, glycosphatidylinositol; TNF, tumor necrosis factor.
Mouse Macrophage cDNA with a SRCR Domain

RESULTS

Cloning and Sequencing—a cDNA library was prepared in the pcDNAII vector from the poly(A) RNA obtained from mouse thioglycolate-elicited peritoneal macrophages selected after 6 h of adherence to plastic in vitro. This library was then split into 10 sublibraries, the plasmid DNAs of which were screened by polymerase chain reaction using as primers the oligonucleotide E6 (the erythropoietin-specific primer) and oligonucleotides corresponding to either the Sp6 or the T7 promoter. With one sublibrary, an amplified fragment of 1 kilobase pair was observed, excised from agarose gel, and used as a probe for screening the positive sublibrary, thus allowing the identification of two positive clones, both with an insertion of 2.2 kilobase pairs. Fig. 1 shows the complete nucleotide sequence together with the deduced amino acid sequence.

This cDNA extends for 2168 nucleotides. There is a long

Peter D. Allet, Glaxo, Geneva, Switzerland (10 ng/ml), lipopolysaccharide (Difco) at 0.5 ng/ml, and mouse recombinant interferon-y (Gene- nentec, South San Francisco, CA) at 50 units/ml.

RNA and DNA isolation and analysis—BALB/c mouse peritoneal macrophages that were fixed with 3% paraformaldehyde (17), and a pool of 25 pg of each coupled peptide was mixed in 0.1 M sodium acetate, pH 5.2, 20% glycerol, and revealed with a rhodamine-conjugated goat anti-rabbit IgG. (Statens seruminstitut, Copenhagen, Denmark) with glutaraldehyde (14). Agarose gel electrophoresis and transfer of RNAs onto Hybond X membranes (Amersham, Amersham, United Kingdom) were done after denaturing transfer onto Hybond N+ (Amersham, Amersham, United Kingdom) were done after repeated boosts, polyclonal antibodies were purified by affinity chromatography on a peptide-coupled CNBr-Sepharose column. Immunofluorescence studies with these antibodies were done on 24-h adherent cell cultures or organs was extracted according to Chomczynski and Sacchi (15). cDNA was labeled with [35S]dCTP by random priming (Megaprime labeling system, Amersham). Prehybridization and overnight hybridization were done at 65 °C in 5 × SSC, 5 × Denhardt's solution, 5 mM EDTA, pH 8.5, and 2% SDS. After two h washes in 2 × SSC, 0.1% SDS and two 1-h washes in 0.2% SSC, 0.2% SDS, the filters were exposed to x-ray film at −70 °C with a Dupont Cronex intensifying screen. 10 μg of genomic DNA purified from BALB/c mouse liver (16) were digested overnight with the indicated restriction enzymes and were run in 40 mM Tris acetate, 2 mM EDTA, pH 8.5, and 2% SDS. After denaturing transfer onto Hybond N+ (Amersham), the membrane was processed as for Northern hybridization.

Peptide Synthesis, Antibody Production, and Immunofluorescence—From the deduced amino acids sequence, we chose 4 peptides of 15 amino acids (A, 1–2 to +13; B, 90–104; C, 314–328; D, 403–417) identified as immunogenic with the aid of the PCgene program. The peptides, synthesized in the laboratory of Dr. R. Frank (University of Heidelberg, Germany), were coupled to purified protein derivative (Statens seruminstitut, Copenhagen, Denmark) with glutaraldehyde (17), and a pool of 25 μg of each coupled peptide was mixed in Freund's complete adjuvant and injected into a rabbit. After repeated boosts, polyclonal antibodies were purified by affinity chromatography on a peptide-coupled CNBr-Sepharose column. Immunofluorescence studies with these antibodies were done on 24-h adherent peritoneal macrophages that were fixed with 3% paraformaldehyde and revealed with a rhodamine-conjugated goat anti-rabbit IgG. Con- focal immunofluorescence analysis was done with the Bio-Rad MRC600 coupled to a Zeiss Axiophot fluorescence microscope.

...cDNA. The nucleotide sequence of the coding strand of the MAMA cDNA and the predicted amino acid sequence are shown. Amino acids are given in single-letter code. The signal sequence following the ATG initiation codon is underlined. Extracellular cysteines are circled. Asparagine-linked glycosylation site is in boldface letters.

**FIG. 1. Nucleotide sequence of MAMA cDNA.** The nucleotide sequence of the coding strand of the MAMA cDNA and the predicted amino acid sequence are shown. Amino acids are given in single-letter code. The signal sequence following the ATG initiation codon is underlined. Extracellular cysteines are circled. Asparagine-linked glycosylation site is in boldface letters. Black dots show the putative cleavage/attachment site for GPI proteins.
open reading frame starting from the initiation codon ATG at nucleotide positions 169–171 and ending at the termination codon TAA at position 1900–1902. The nucleotides surrounding the ATG codon match the consensus sequence for a translation initiation site (18). The next 18 amino acids have the characteristics of a signal peptide sequence (19). A putative transmembrane domain of 16 amino acids partitions the protein into a 531-amino acid extracellular domain, containing six consensus sequences for N-linked glycosylation and 15 cysteine residues and a very short intracellular domain of 12 amino acids. The COOH-terminal end sequence is also compatible with an anchoring of the protein via a glycosylphosphatidylinositol (see "Discussion"). The 3' non-coding region of 266 nucleotides contains one of the three possible polyadenylation signals, ATTTAAA, 21 bases upstream from the poly(A) tract. We gave to this cDNA the acronym MAMA, for murine adherent macrophage cDNA. The sequence corresponding to the oligonucleotide primer used for the anchored polymerase chain reaction is located between nucleotides 1017 and 1030.

Expression in COS-7 Cells—In order to demonstrate that this cDNA indeed directs the synthesis of a protein of the expected size, it was introduced in an eucaryotic expression vector containing the β-actin promoter (12). Two vectors, with the insert in opposite directions were used to transfect COS-7 cells. SDS-polyacrylamide gel electrophoresis analysis of the whole cell lysates after 3 days of culture showed the presence of a protein of 60–65 kDa only in the cells transfected with the vector containing the cDNA in the right direction (Fig. 2). This result is consistent with the expected molecular weight of the unglycosylated protein.

Confocal Immunofluorescence Analysis—Polyclonal anti-

![Fig. 2. Expression of MAMA in transfected COS-7 cells. COS-7 cells were transfected with MAMA cDNA cloned in the right (+) or opposite direction (−) in pCAGGS plasmid. The stained gel shows the presence of a 60–65-kDa protein in the + postnuclear cell lysate (arrow).](image)

Sequence Comparisons—Screening of data bases showed no overall homology in the total nucleotidic or amino acid sequences with other known sequences. When specific sites and signatures were screened with the Prosite program, it appeared that amino acids 6–107 of the MAMA protein match perfectly the consensus sequence of the cysteine-rich domain of the speract receptor (20), a domain also found in the macrophage scavenger receptor (7) and the T cell membrane protein CD6 (21) (Fig. 4). Another feature of the extracellular domain is found between amino acids 410 and 432 where 8 tyrosine residues are clustered. While this work was being prepared for publication, the sequences of a human protein described as the hMAC-2-BP and of the murine CyCAP were reported (8, 11). Except for very minor divergences, CyCAP appears to be the same protein as MAMA. hMAC-2-BP is 69% homologous to the MAMA protein, with all the cysteines and the N-glycosylation sites conserved, SRCR domains being 82% homologous (Fig. 5), but the proteins being divergent in two domains. The tyrosine-rich domain contains 23 amino

![Fig. 3. Immunofluorescence analysis with confocal microscopy. Fixed 24-h adherent macrophages were incubated with rabbit anti-peptide antibodies followed by a goat anti-rabbit Ig conjugated to rhodamine. Photographs represent the confocal analysis of two different fields of cells with slices of 0.9 μm, starting from the upper region of the cells. Control staining with nonimmune rabbit Ig showed no fluorescence.](image)

![Fig. 4. Amino acid sequence comparisons of selected SRCR domains. MAMA, the murine scavenger receptor (Mu SR), the bovine scavenger receptor (Bo SR), the speract cross-linking protein from sea urchin sperm (Speract-1), and the human CD6 (CD6-2) SRCR domains were aligned. The consensus positions are mentioned (top row). The amino acids most represented at each position are in boldface letters.](image)
acids in the MAMA protein and 31 amino acids in the hMAC-2-BP (amino acids 419-449). The postulated transmembrane hydrophobic domain of the MAMA protein is absent from hMAC-2-BP, and indeed the 97-kDa hMAC-2-BP is mainly found in supernatants of a variety of cultured cells and is a component of the human fluids.

**Genomic Analysis**—Southern analysis with various restriction enzymes, including enzymes cutting in exons, suggests that the corresponding gene is present as a single copy (Fig. 6).

**Modulation of MAMA mRNA Level in Macrophages and Constitutive Presence in Other Cell Types and Various Organs in Vivo**—As shown in Fig. 7A, MAMA mRNA progressively accumulates in time until 24 h when thioglycolate-elicited peritoneal macrophages are cultured in vitro; this is due to adherence (which also induces the increase of some other macrophage mRNA), since when macrophages were cultured in a Teflon vial, a condition that does not allow adherence, no increase in mRNA with time was observed (not shown).

Different inducers of macrophage activation were tested for their ability to modulate MAMA mRNA. Two of these, interferon-γ and TNF, increased the level of the transcript. This increase was apparent after 2 h with interferon-γ and after 24 h with TNF (Fig. 7B). In contrast, neither lipopolysaccharide, granulocyte-macrophage-stimulating factor, macrophage colony-stimulating factor, nor phagocytosis of opsonized red blood cells did modify the mRNA levels (not shown).

Cultures of other cell types or lines showed that MAMA mRNA is also detectable in fibroblasts, in some B cell populations, and in cells of epithelial origin from the mammary gland but not in keratinocytes (Fig. 8A). Finally, MAMA mRNA is present at variable levels in all the organs tested, with the exception of the brain (Fig. 8B).

**DISCUSSION**

The new protein identified by the cloning and sequencing of the cDNA described in this report is a membrane glycoprotein of 559 amino acids, containing at its NH2 terminus around 100 residues that display 48% homology with the SRCR of mouse macrophage and matching the consensus sequence of the domains found in the newly recognized family of SRCR proteins. Besides the scavenger receptor itself, this growing family is composed of the sea urchin spermatozoa receptor for peptides (speract receptor) involved in the stimulation of sperm metabolism and motility (20); the CD6 (21), CD5 (22), and WC1 (23) cell surface receptors present on T lymphocytes, and the serum protein CF1 synthesized by monocytes and involved in the complement cascade (24). As is the case for the scavenger receptor and CF1, the MAMA protein has only one SRCR domain, whereas other members

**Fig. 5. Amino acid sequence comparisons of MAMA and hMAC-2-BP.** The two sequences including the signal sequences were aligned. Identical amino acids at each position are marked by a vertical bar. Conserved amino acids are indicated by dots. Cysteine residues are circled.

**Fig. 6. Southern analysis of the MAMA gene.** Genomic Southern blot using DNA from BALB/c mouse liver. 10 μg of DNA were digested overnight with the indicated restriction enzymes cutting (Apal at bases 586 and 2052, Sphe at base 774) or not (NcoI and EcoRI) in the cDNA. The blot was hybridized to the 32P-labeled 2.2-kb MAMA cDNA.
Nucleotide sequence analysis shows that the MAMA protein is 69% homologous to the recently identified human MAC-2-BP (8), thus may represent its mouse equivalent, and, furthermore, is identical to the newly discovered mouse CyCAP (11). Indirect evidence exists that CyCAP is a membrane molecule (because cyclophilin C binds to the cell surface (11, 32), and our observation now establishes this point. Human MAC-2-BP, in contrast, has been mainly detected in extracellular mediums of a variety of cultured cells. However, in HT-29 cell lysate, this protein is found in a fully mature form and associated with its ligand MAC-2 (8), a galactose-specific lectin (33, 34) that is also detectable on the cell surface of thioglycolate-induced murine macrophages (35). Thus, this provides indirect evidence that MAC-2-BP can also be found in a membrane-associated form. On the basis of what has been discussed above, the possibility that it may be released from a GPI anchor, explaining its finding as a soluble molecule, has to be considered. A soluble form may also be generated as the result of alternate exon splicing. Thus, MAMA should also exist in a soluble form in mouse fluids if, as seems likely, it is the murine form of hMAC-2-BP. In light of this possibility, an intriguing point would be that the two ligands so far identified for this MAMA/CyCAP/MAC-2-BP protein, namely the MAC-2 protein and cyclophilin C, are also extracellular proteins that nevertheless lack a signal sequence. The possibility of a role for MAMA in the transport of these ligands to the cell surface remains conjectural, but in any case MAMA would appear to be essential in holding them on the outer cell membrane.

Is there a possible relationship between the capacity of binding two apparently so disparate ligands and the strong induction of MAMA by macrophage adhesion? It has been suggested that one of the possible roles of MAC-2 is related to cell adhesion to the extracellular matrix; MAC-2 bears a lectin domain (apparently responsible for its fixation to MAC-2-BP) that can also bind laminin, a major extracellular matrix component (9, 10). MAMA/MAC-2-BP could thus play the role of a loose link between the macrophage surface and the

![FIG. 7](image-url)  
**Induction by adherence, interferon-γ, and TNF of the MAMA mRNA in macrophages.** A, BALB/c mice peritoneal macrophages were incubated for the indicated times (in h) in Dulbecco's modified Eagle's medium. The macrophages were lysed and processed for RNA extraction. Note the slow induction of the transcript with a maximum at 24 h (exposition time, 15 h). B, 1-h adherent macrophages from C3H/HeJ mice were treated with interferon-γ (c), adherent macrophages were incubated for the indicated times (in h) in Dulbecco's modified Eagle's medium. The macrophages were lysed and processed for RNA extraction. Note the slow induction of the transcript with a maximum at 24 h (exposition time, 15 h).

![FIG. 8](image-url)  
**MAMA mRNA distribution in cells of different origin and in organs.** A, Northern blot of total RNA from the indicated cell population or cell lines. B, Northern blot of total RNA from liver (Li), spleen (Sp), kidney (Ki), lung (Lu), heart (He), intestine (In), thymus (Th), lymph node (LN), and bone marrow (BM) from BALB/c mice.
extracellular matrix, via MAC-2, being involved in macrophage migration during inflammation or, if the complex MAC-2-BP. MAC-2 is released from the cell, between the extracellular matrix and various cell or microorganism surfaces bearing glycoproteins. As for cyclophilin C, its role is presently unknown. Besides its cis/trans peptidyl prolyl isomerase activity, common to all cyclophilins and in the light of the possible involvement of MAMA in macrophage adherence and activation, it is very striking that cyclophilin A can be released in vitro by lipopolysaccharide-activated macrophage and can act, in a secreted form, as an inflammatory cytokine in vivo and as a factor chemotactic for polymorphonuclear leukocytes and monocytes in vitro (36). A comparable activity has not been tested for cyclophilin C. There is presently no evidence that the presence of one or other forms of cyclophilin on the surface of activated macrophages has not been explored. This suggests the very intriguing possibility that MAMA may be involved not only in the role of this protein in cell adhesion in a variety of target organs of the cytotoxicity of cyclosporin. The association of cyclophilin C to its binding protein, here identified as tumor-associated antigens in a melanoma (38), since this drug prevents the involvement of this protein in cell adhesion in a variety of cell types. With the antibodies used in the present study, we have, for instance, isolated from kidney lysates the MAMA protein; it is of interest to recall in this respect that the kidney is one of the target organs of the cytotoxicity of cyclosporin. The involvement of this protein in cell adhesion in a variety of normal tissues, or in tissue remodeling, needs to be explored. Since proteins probably identical to MAC-2-BP have been detected as tumor-associated antigens in a melanoma and in a lung tumor cell lines, MAMA may as well play a role in tumor cell adherence and spreading. Where the SRCR takes in place in MAMA functions remains also to be determined.

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