Macrosialin, a Mouse Macrophage-restricted Glycoprotein, Is a Member of the lamp/lgp Family*  

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Macrosialin is a heavily glycosylated transmembrane protein of 87–115 kDa, highly and specifically expressed by mouse tissue macrophages, and to a lesser extent by dendritic cells. We have isolated cDNA clones encoding macrosialin from a thioglycollate-elicited peritoneal macrophage cDNA library by transient expression in COS cells and panning with the antimalcosialin monoclonal antibody FA11. A single 1.3-kilobase macrosialin transcript was detected in both untreated and phorbol 12-myristate 13-acetate-stimulated RAW cells. The cDNA sequence predicts a type I integral membrane protein of 326 residues with a heavily glycosylated extracellular domain of 306 residues containing nine potential N-linked glycosylation sites and numerous potential O-linked glycosylation sites. The extracellular domain consists of two distinct regions, separated by an extended 12 residue proline-rich hinge; a membrane-distal mucin-like domain of 89 residues containing short peptide repeats and consisting of 44% serine and threonine residues; and a membrane proximal domain of 170 residues, which has significant sequence homology to a family of lysosomal associated glycoproteins known as the lamp-1 group. Macrosialin is the murine homologue of the human macrophage glycoprotein CD68 (72% identity, 80% similarity). Both proteins are preferentially expressed by macrophages and share the same bipartite structure having a mucin-like domain and a domain common to the lamp family. Macrosialin and CD68 are the first examples of a lamp family protein with a restricted cell-type-specific expression. They may have evolved from the lamps to carry out specialized functions in dedicated phagocytic cells.

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Macrophages derive from the myeloid lineage and are believed to be the end-stage of differentiation of blood monocytes. Macrophages are professional phagocytic cells and play key roles in many immune functions, including phagocytosis of foreign and necrotic material and antigen processing and presentation (Gorden, 1986). Macrophages are resident in most organs and tissues but are particularly abundant in lymphoid organs (Gorden et al., 1992).  

Monoclonal antibodies specific for macrophages have been made both for human (CD68) (Parwaresch et al., 1986; McMichael et al., 1987; Micklem et al., 1988; Warnke et al., 1989; Pultz et al., 1990; Knapp et al., 1991; Smith et al., 1991) and murine macrophages (FA11 antigen (Smith and Koch, 1987), now termed macrosialin (Rabinowitz and Gordon, 1989, 1991)). Macrosialin is the major sialylated glycoprotein of elicited macrophages and is differentially glycosylated in response to inflammatory stimuli. On SDS-PAGE, it resolves as a broad band of 87–115 kDa. Resident peritoneal macrophages express low levels of a non-lectin-binding glycoform, whereas inflammatory stimuli lead to a 17-fold increase in macrosialin expression and acquisition of lectin binding properties (Rabinowitz and Gordon, 1991). The major remodeling occurs in O-linked glycans with increase in sialylation and appearance of poly-N-acetyllactosamine structures.

Macrosialin and CD68 share a number of common biochemical properties: macrophage-restricted expression, similar molecular mass (87–115 kDa), similar glycosylation patterns, being rich in both N-linked and especially O-linked sugars and bearing numerous terminal sialic acid residues, and mainly intracellular location in endosomes or lysosomes with only a small amount being expressed on the cell surface at any one time (Saito et al., 1991; Rabinowitz et al., 1992). We have recently cloned a cDNA encoding CD68β by transient expression in COS cells and found that it is a member of a family of lysosomal/endosomal associated glycoproteins known as the lamp/lgp family (human lamp-1, Fukuda et al. (1988); mouse lamp-1, Chen et al. (1988); mouse lamp-2, Cha et al. (1990); chicken lep100, Lippincott-Schwartz and Fambrough (1987), Fambrough et al. (1988) and Zot et al. (1990); and rat lgp120, Howe et al. (1988)). To better understand the structure and function of macrosialin, and more clearly establish the relationship of human CD68 to murine macrosialin, we report here the cloning of a cDNA clone encoding macrosialin by transient expression in COS cells and immunoselection (Seed and Aruffo, 1987) using the anti-macrosialin mAb FA/11 (Smith and Koch, 1987). Macrosialin is also a member of the lamp/lgp family and is the murine homologue of human...
Macrosialin cDNA

CD68 (72% identity). Thus CD68/macroisalin are lineage-restricted, lysosomal-associated glycoproteins that could play roles in the specialized phagocytic activities of tissue macrophages, both in intracellular lysosomal metabolism and extracellular cell-cell and cell-pathogen interactions.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—Thiglycollate-elicted peritoneal macrophages were obtained from BALB/c mice bred at the Sir William Dunn School of Pathology, 5 days after injection of 1.5 ml of Brewer's complete thiglycollate broth (Rabinowits and Gordon, 1973). RAW cells were obtained from the Sir William Dunn School of Pathology, Oxford. All cells were grown in RPMI, 10% fetal calf serum (FCS). RAW cells were maintained at a density of 5 x 10^5 cells/ml and induced with 25 ng/ml PMA for 24 h. The hybridomas secreting mAb FA/11, a rat IgG2a, was maintained in Iscove's modified Dulbecco's medium (Flow Laboratories, Rockmansworth, United Kingdom) with 10% FCS, 0.1 mM hypoxanthine, 16 mM thymidine, glutamine, and antibiotics. The FA/11 line was generously made available by Drs. M. Smith and G. Koch (Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom).

Library Construction and Screening—A cDNA library was constructed in the expression vector pCDM8 (Seed, 1987; Seed and Wondrak, 1991). cDNA prepared from thiglycollate-elicted peritoneal macrophages (BALB/c). Transfected COS cells transiently expressing macrosialin were isolated with the anti-macroisalin mAb FA/11 and panned on plastic Petri dishes coated with affinity-purified goat anti-rat IgG (Sigma). Episomal DNA was recovered from the panned cells, and the expression-panning cycle was repeated once more to obtain a cDNA clone designated pMS1. Expression of macrosialin on the surface of transfected COS cells was detected by indirect immunofluorescence using mAb FA/11 and fluorescein-conjugated goat anti-rat IgG antibody (Sigma).

Protein Labeling and Immunoprecipitations—J774 and RAW macrophage cell lines were grown in RPMI plus 10% FCS, 2 mM glutamine, penicillin, and streptomycin. COS cells and the B cell line N50 were grown in DMEM supplemented as above. All cells were washed in methionine-free DMEM (Gibco Laboratories), preincubated in the same medium containing 10% dialyzed FCS for 1 h, after which 50 mCi/ml Tran35-S-label (ICN, Irving, CA) were added and cells incubated for another 16 h. Cells were then washed 3 times with cold phosphate-buffered saline and extracted with 2% octyl glucoside in methionine-free DMEM (Gibco Laboratories), preincubated in the presence of E. coli ribonuclease and 1 mM leupeptin), for 1 h at 4°C for 1 min, 50°C for 1 min, 72°C for 1 min. PCR products were treated with Klenow and polynucleotide kinase and blunt-end-cloned into phage-plasmid-treated EcoRV-cut pBluescript. Individual recombinant colonies were sequenced with universal M13 sequencing primers.

DNA Sequencing—Double-stranded sequencing was conducted on the cDNA insert in pMS1 by dyeoxy chain termination (Sanger et al., 1977) using sequence-specific oligonucleotides. Both strands of the cDNA insert were sequenced entirely.

RESULTS AND DISCUSSION

To isolate a cDNA clone encoding murine macrosialin, a cDNA library was constructed from RNA prepared from thiglycollate-elicted peritoneal macrophages. The cDNA library was transiently expressed in COS cells, and cells expressing macrosialin cDNAs were isolated by panning with panning with FA/11 mAb. Episomal DNA was recovered from the adherent cells, amplified in Escherichia coli, and reintroduced into COS cells. After one additional round of expression and selection, 12 of 24 final round miniprep transfectants scored positive for staining with the FA/11 mAb. One type of cDNA insert was shown to have an insert of 1.2 kb, and one clone, pMS1, was pursued in greater detail.

mAb FA/11 immunoprecipitated a single protein of 85-110 kDa from 35S-labeled proteins prepared from J774 and RAW cells (Fig. 1). A slightly smaller protein of 75-100 kDa was immunoprecipitated from pMS1-transfected COS cells (Fig. 1). The smaller molecular mass of macrosialin expressed in COS cells is probably due to inefficient or incomplete glycosylation of the expressed proteins. mAb FA/11 did not immunoprecipitate any protein from the B cell line N50.

Northern blot analysis of poly(A)-selected RNA from RAW cells showed a single species of approximately 1.3 kb (Fig. 2) that was slightly increased on PMA treatment. Macrosialin transcripts appear to be rare, since a signal was only clearly seen in poly(A)-selected RNA and not in total RNA, where nonspecific binding of the pMS1.1 riboprobe to 28 and 18 S RNA is evident (Fig. 2).

The sequence of the cDNA insert in clone pMS1 (Fig. 3) consists of 1243 bp ending in a poly(A) tract, preceded by a potential polyadenylation site at 1207 bp (Fig. 3, AATAAA, underlined).

The predicted polypeptide sequence encoded by pMS1, consists of 318 residues, and has the typical features of a type I integral membrane protein. The sequence starts with an ATG at position 91 followed by a hydrophobic signal sequence.

**Fig. 1. Immunoprecipitation analysis of macrosialin.** 35S-Labeled proteins from the stated cell lines were immunoprecipitated with mAb FA/11-coupled Sepharose. Lane 1, molecular mass markers in kDa; lane 2, COS cells transfected with pMS1 cDNA clone; lane 3, RAW (macrophage); lane 4, J774 (macrophage); lane 5, N50 (B cell). Immunoprecipitates were resolved on 7.5% SDS-PAGE.
of 20 residues, which may be cleaved between glutamic acid 20 and aspartic acid 21 (von Heijne, 1986). The predicted mature form of macrosialin consists of an extracellular domain (271 residues), a hydrophobic transmembrane domain (25 residues), and a short cytoplasmic domain (2 residues).

There are nine potential N-glycosylation sites (Asn-X-Ser/Thr). The predicted mass of the polypeptide backbone in clone pMS.1 is only 35 kDa, whereas the observed mass of the mature glycoprotein is between 87 and 115 kDa. The extracellular domain has numerous contiguous runs of serine, threonine, and proline, which could act as sites for attachment to O-linked carbohydrate (Wilson et al., 1991). The largest contribution to the mass of the mature macrosialin comes from both O- and N-linked carbohydrate; approximately 40% from O-linked and 20–25% from N-linked sugars, with only about one third coming from the polypeptide backbone (Rabinowitz and Gordon, 1991).

Previous studies have defined the nature of the glycans borne by macrosialin on unstimulated and stimulated macrophages (Rabinowitz and Gordon, 1991). N-Linked sugars account for 21 kDa of the mass, and O-linked glycans account for 30–40% of the molecular mass of macrosialin. Both types of glycans show extensive remodeling upon activation of macrophages, acquiring numerous terminal sialic acid residues and poly-N-acetylglucosaminoglycans.

A search of the National Biomedical Research Foundation protein data base (Wilbur and Lipman, 1983) disclosed homology to mouse lamp-1 and lamp-2 (Table I) (Chen et al., 1988; Cha et al., 1990) and other members of the lamp family (Fig. 4). Members of the lamp/lgp family are ubiquitously expressed glycoproteins located in lysosomal membranes (Fukuda, 1991a). The lamp proteins show a high degree of homology across different species; for example, mouse lamp-1 is more homologous to human lamp-1 than to mouse lamp-2 (Table I). Macrosialin shows more homology to lamp-1 proteins than lamp-2 proteins (Table I). However, macrosialin is most closely related to human CD68, showing 72.0% identity and 80.6% similarity (Table I and Fig. 4).

The extracellular domain of the lamp/lgp family has a bipartite organization, the two domains being divided by an extended proline hinge (Fukuda, 1991a) (Fig. 5). Macrosialin and CD68 share this bipartite structure, divided by the proline hinge; in both molecules, the area of greatest conservation is from the hinge to the cytoplasmic tail. The membrane-proximal domain is compact and probably globular; it contains four regularly spaced cysteines (36–37 residues apart), and, in the lamp family, intradomain disulfide bonds are formed between the first and second and between the 3rd and 4th cysteines (Carlsson and Fukuda, 1988; Arturson et al., 1990). The equivalent domain of macrosialin also has 4 cysteines, all of which align with the equivalent cysteines in lamp-1 proteins (Fig. 4).

The N-terminal domain of macrosialin is unrelated to the equivalent domain of the lamp/lgp family but is homologous to the corresponding domain in CD68; being very dense in serine and threonine residues (43% Ser + Thr), characteristic of mucin-like molecules decorated with O-linked glycan chains (Jentoft, 1990; Fukuda, 1991b). CD68 has a region of short repeat motifs in this domain, 12-18-12-18 (A-B-A’-B’). The corresponding region of macrosialin has a similar run of repeats, although they are shorter (6-15-8-17) and less internally conserved (designated by arrows in Fig. 3; indicated in detail in Fig. 6).

The cytoplasmic tail in pMS.1 is only 2 residues (RR*) and differs markedly from the tails of CD68 and the lamp family, which are 10 residues and contain a key tyrosine residue preceded by a small side-group amino acid (alanine or glycine). This motif has been identified by in vitro mutagenesis studies.
TABLE I
Alignment of CD68, macrosialin, and mus-lamp1 with lamp proteins

Alignments were produced using the GAP program based on the algorithm of Needleman and Wunsch (University of Wisconsin Genetics Computer Group package; Devereux et al. (1984)). The percentage amino acid identities and the percentage similarities are tabulated, the percentage similarities being recorded in parentheses.

| CD68 | Macrosialin | mus-lamp1 |
|------|-------------|-----------|
| hum-lamp-1 | 26.6 (44.4) | 28.2 (51.5) | 67.8 (77.5) |
| mus-lamp-1 | 25.2 (45.7) | 27.6 (48.1) | |
| hum-lamp-2 | 22.2 (42.9) | 24.4 (46.7) | 35.7 (55.0) |
| mus-lamp-2 | 24.7 (46.9) | 24.0 (46.7) | 59.1 (80.0) |
| CD68 | 80.6 (70.2) | |

FIG. 4. Alignments of macrosialin with CD68 and the lamp family. A multiple alignments with representative members of the lamp-1 family are shown. Alignments were produced using the GAP program based on the algorithm of Needleman and Wunsch (University of Wisconsin Genetics Computer Group package; Devereux et al. (1984)). The proline hinge is boxed, and the transmembrane regions are underlined. Asterisks and/or boxes indicate the aligned cysteines that have structural significance for lamp-1. (I) is put between identical amino acids. (.) is put between similar amino acids whose comparison value is greater than or equal to 0.5. (:) is put between similar amino acids whose comparison value is greater than or equal to 0.10.

lography RNA used to construct the cDNA library, and by PCR from the macrophage cDNA library and from a RAW cDNA library. PCR products were cloned and sequenced.

Two types of cytoplasmic tail were seen; type 1, corresponding to the tail in pMs1 (RR*; ...CGC AGA TGA CAA...), was isolated from the original macrophage cDNA library and the RAW cDNA library, type 2, RRRQSTYQPL (...CGC AGA AGA CAA...), was isolated from the original macrophage library and from first strand cDNA. The type 2 tail is homologous to the 10-residue tail found in CD68 (RRRFSAYQAL) and the lamp/lgp family, and contains the key tyrosine residue. Four independent clones for the longer 10-residue tail were isolated from the macrophage cDNA library used for panning, and two independent clones were isolated from first strand cDNA.

The short 2-residue tail isolated by transient expression could have been an artifact introduced during cDNA synthesis or later during the two rounds of expression selection in COS cells. However, as the same RR* tail (resulting from a single base change; AGA to TGA) was re-isolated from a different cDNA library, it seems possible that this tail variant does indeed exist.

Macrosialin may therefore exist in two forms: one solely localized to the plasma membrane (RR*) and the other targeted to the lysosomes (RRRQSTYQPL). There are two possible ways in which this could arise. First, macrosialin may be encoded by two closely related genes with slightly different cytoplasmic tails; second, there may be a single gene with separate exons encoding the two tail variants and differential splicing gives rise to the two variants. A precedent exists for the first of these in the low affinity receptor for IgG, FcRIII, or CD16, which exists in two isoforms differing by only 1 nucleotide in the cytoplasmic domain (Ravetch and Perussia, 1989). The two isoforms are encoded by two nearly identical but separate genes that lie very close together in the genome. These genes are transcribed in a cell-type-specific manner and generate either a phosphatidylinositol-anchored receptor in neutrophils or a transmembrane-anchored receptor in natural killer cells, lymphocytes, and macrophages. For macrosialin, it will be interesting to determine the relative expression of these two tail variants in resting versus activated macrophages and whether this influences the amount of intracellularly expressed glycoprotein.

On the basis of the sequence and domain homologies presented here, macrosialin and its human homologue CD68 are new members of the lamp/lgp family of lysosomal associated glycoproteins. However, immunohistochemical studies reveal a difference between the expression profiles of macrosialin and CD68; FA/11 staining has only been detected in macrophages, Langerhans cells, and isolated dendritic cells (Gordon et al., 1992; Rabinowiz and Gordon, 1991), whereas CD68 is expressed at very low levels in most cell types but is abundant in macrophages and many tumor cell lines (Pullford et al., 1990). One explanation could be that the CD68 mAbs are detecting epitopes in other related proteins or lams, whereas the FA/11 epitope is absolutely specific for macrosialin. In addition, the CD68 studies were carried out with the more sensitive APAAP technique, whereas the macrosialin studies to date have utilized avidin-biotin-peroxidase. Alternatively, the expression profiles could indeed reflect a real difference between macrosialin and CD68; macrosialin may have a role only in macrophages, whereas CD68 may play a role in endocytosis or lysosomal traffic that is an essential, “housekeeping” activity in all cells, and this activity has been expanded to become a dominant part of the specialized function of phagocytes.
proposed as potential candidates. For example, the extended glycosylated glycoproteins listed above have been shown to bind leukocytes (Bradley et al., 1987). The selectins (E-, L-, and P-selectin) are a family of membrane glycoproteins that mediate cell-cell adhesion events, especially those involving the attachment of circulating cells with vascular endothelium (Moore et al., 1992). This glycoprotein is not restricted to leukocytes, but is also expressed on endothelial cells, where it may have a more specialized function associated with the phagocytic activities of macrophages such as binding foreign organisms (yeasts, viruses, bacteria, and protozoan parasites) and killing tumor cells. The expression of both macrosialin and CD68 in dendritic and Langerhans cells may point to a role for these molecules in antigen processing, which is a key function of these specialized cells. Domain swaps between the macrophages and CD68 can now be carried out to investigate these ideas.

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The intracellular role of the lamp family is unknown. It is proposed that the extensive O-glycosylation of these proteins protects the core protein and other closely associated membrane proteins from enzyme attack in the lumen of the lysosome (Jentoft, 1990; Rabinowitz and Gordon, 1992). The more restricted expression of macrosialin/CD68 suggests its involvement in specialized mononuclear phagocyte functions such as phagocytosis of pathogens and antigen processing and presentation. The overexpression of a mucin-protected lamp (macrosialin/CD68) could be essential for the survival of the macrophages and the survival of other associated glycoproteins, in the harsh hydrolytic environment of the lysosomes.

As CD68 and macrosialin also appear on the cell surface, they may also have extracellular functions. One possible extracellular role for these molecules could reside in their extensive glycan decoration. Recently, it has been proposed that one of the functions of heavily glycosylated hematopoietic membrane proteins such as CD43/leukosialin (Killeen et al., 1987; Shelley et al., 1989; Cyster et al., 1990), CD34 (stem cell antigen) (Sutherland et al., 1988; Simmons et al., 1992), and Sgp50 or GlyCAM-1 (the high endothelial ligand for L-selectin) (Lasky et al., 1992) is to present carbohydrate ligands to P-selectin on vascular endothelium (Moore et al., 1992). This glycoprotein is not lamp-1, lamp-2, or CD43, and we are currently investigating whether it could be macrosialin or CD68.

Macrosialin and CD68 are the first examples of the lamp family with a restricted cell-type-specific expression pattern. To date, all existing members of the family are constitutively and ubiquitously expressed in all cell types. Macrosialin/CD68 have clearly diverged from the main members of the family by replacing the N-terminal domain with a novel mucin-like domain. This may shed some light on the relative functions of the two distinct domains. The common globular membrane-proximal domain could play some general role in lysosomal metabolism, whereas the membrane-distal domain may have a more specialized function associated with the phagocytic activities of macrophages such as binding foreign organisms (yeasts, viruses, bacteria, and protozoan parasites) and killing tumor cells. The expression of both macrosialin and CD68 in dendritic and Langerhans cells may point to a role for these molecules in antigen processing, which is a key function of these specialized cells. Domain swaps between the lamps and macrosialin/CD68 can now be carried out to investigate these ideas.
