

## THE Mac-2 ANTIGEN IS A GALACTOSE-SPECIFIC LECTIN THAT BINDS IgE

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Mac-2 is a 32-kD murine macrophage cell surface protein first identified by Ho and Springer (1). In their original characterization of the antigen, they found that it was expressed on thioglycollate-elicited peritoneal macrophages at a much higher level than on other macrophages, and they suggested that it might be a subpopulation-specific marker. Subsequent work has demonstrated that the Mac-2 antigen is expressed to varying degrees in most macrophage populations, macrophage cell lines, interdigitating dendritic cells in various lymphoid organs, and in some epithelial cells (2, 3). Surface immunofluorescence studies indicate that the level of Mac-2 expression increases during the maturation of macrophage precursors, but that there is heterogeneity in expression among different macrophage subpopulations and in different macrophage cell lines (2, 4). It is not clear whether this variation correlates with differences in function. The elevated expression of Mac-2 on macrophages elicited by an inflammatory stimulus suggests that it may be involved in the recruitment of a specific subpopulation of macrophages to the site of inflammation or that it is induced during the process of activation by the inflammatory stimulus.

Despite these indications that the Mac-2 antigen may play an important role in macrophage biology, little is known about its structure and function. To learn more about this molecule, and as a part of our attempts to understand the early events in the inflammatory activation of macrophages, we have cloned cDNAs encoding the Mac-2 antigen by immunoscreening of a macrophage cDNA expression library. In the present report, we show that the nucleotide sequence of the Mac-2 cDNAs, apart from a short stretch at their 5' ends, is identical to that of carbohydrate binding protein 35 (CBP35)<sup>1</sup>, a galactose-specific lectin found in fibroblasts (5) and highly homologous to that of a rat IgE-binding protein (rIgEBP), originally described in a rat basophilic leukemia cell line (6). Furthermore, the *in vitro* synthesized Mac-2 protein binds to carbohydrate determinants on asialofetuin, a desialylated glycoprotein, and to IgE. The sequence of the Mac-2 cDNAs, like that of CBP35 and rIgEBP, does not contain a classical signal peptide or transmembrane domain. This finding is consistent with the intracellular location of the latter two proteins (7, 8); however,

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<sup>1</sup> *Abbreviations used in this paper:* CBP35, carbohydrate-binding protein 35; MBP, mannose-binding protein; PCR, polymerase chain reaction; rIgEBP, rat IgE-binding protein; RSB, reticulocyte saline buffer.

it is at odds with our observation that the Mac-2 protein, in addition to being present in the cytosol, is also secreted into the extracellular medium, and with the observations of several other groups that have demonstrated Mac-2 on the cell surface (2, 4).

These data raise questions regarding the function of the carbohydrate and IgE-binding properties of Mac-2 in inflammatory macrophages, and about the mechanism by which this protein reaches the cell surface.

### Materials and Methods

**Molecular Cloning of the Mac-2 cDNA.** A  $\lambda$ gt11-P388D1 expression library was obtained from R. A. B. Ezekowitz, Children's Hospital, Boston, MA. It was screened by the method of Snyder et al. (9), using M3/38, a rat mAb to the Mac-2 antigen (Boehringer Mannheim Biochemicals, Indianapolis, IN). A rabbit anti-rat antiserum (Zymed Laboratories, San Francisco, CA) followed by goat anti-rabbit antibody coupled to alkaline phosphatase (Promega Biotec, Madison, WI) was used as the detection system. 3 of the 10 positive plaques were purified, and the cloned phage (Mac 2.3, Mac 2.9, and Mac 2.10) were used to prepare DNA and lysates containing fusion protein (9). The lysates were subjected to Western blot analysis (10) using M3/38 and an isotype-identical control antibody to probe the blot. The bound antibody was detected using the enzyme-linked detection system described above. The insert DNAs from the  $\lambda$ gt11 clones were subcloned into the vector pBSK (Stratagene, La Jolla, CA). A radiolabeled (11) probe made from the insert DNA of Mac 2.3 was used to rescreen the library (12). Positive plaques were purified and the longest insert (from clone Mac 2.16) was subcloned into pBSK. Restriction maps of all pBSK clones were constructed and further subclones made so as to allow sequencing of both strands over the full length of the original inserts. Double-stranded sequencing was carried out with a kit from Boehringer Mannheim Biochemicals, using commercially available T3 and T7 primers (Stratagene).

**Northern Hybridization Analysis.** Total cellular RNA from various cells and cell lines was prepared by the guanidinium isothiocyanate method (13). The cell lines used were J774 and P388D1 (macrophage), A20.25 (B cell), EL4 (thymoma), 70Z/3 (pre-B cell), Swiss 3T3 (fibroblast) (all obtained from the American Type Culture Collection, Rockville, MD), and AJ9 (B cell; reference 14). 30  $\mu$ g of each sample was subjected to electrophoresis on a 1.2% formaldehyde agarose gel, transferred to nitrocellulose, and hybridized as described (12), using the radiolabeled (11) insert from Mac-2.16 as a probe. After washing, the filter was exposed for autoradiography.

**In Vitro Transcription and Translation.** Capped mRNA was synthesized in vitro from Mac-2 cDNA templates cloned in pBSK using the mCAP kit from Stratagene. In vitro translation of the RNA was then carried out using rabbit reticulocyte lysate (Stratagene), following the manufacturer's instructions.

**Binding to Immobilized IgE and Asialofetuin.** IgE (monoclonal mouse IgE anti-DNP; ICN Immunobiologicals, Lisle, IL), IgG, and IgM (Zymed Laboratories) were coupled to Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) at concentrations of 1 mg/ml packed beads, and asialofetuin (Sigma Chemical Co., St. Louis, MO) was coupled at a concentration of 10 mg/ml packed beads (15). Equal aliquots of the in vitro translation products were diluted in 1 ml of lysis buffer, i.e., 0.5% Triton X-100 in reticulocyte saline buffer (RSB; 10 mmol Tris-Cl, pH 7.4, 3 mmol MgCl<sub>2</sub>, 10 mmol NaCl) containing 2 mmol PMSF and mixed with 150  $\mu$ l of a 50% slurry of the immobilized ligand in PBS. For competitions, the in vitro translated product was mixed with 1 ml of lysis buffer containing the competing sugar (galactose, glucose, lactose, or maltose) at a final concentration of 100 mmol, just before addition of the immobilized ligand. N-glycanase treatment of the IgE-Sepharose and asialofetuin-Sepharose was carried out at 37°C overnight using 0.25 U of enzyme (Genzyme, Boston, MA) per 150  $\mu$ l of a 50% slurry of the immobilized ligand (16). Mock digestion was carried out in buffer without enzyme. After binding to the translated protein overnight at 4°C, the Sepharose beads were washed five times with wash buffer (50 mmol Tris-Cl, pH 8.2, 500 mmol NaCl, 5 mmol EDTA, 0.02% NaN<sub>3</sub>, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) and then boiled for 3 min in 50  $\mu$ l of 2 $\times$  SDS-PAGE sample buffer (125 mmol Tris-Cl, pH

6.8, 4% SDS, 20% glycerol, 10% 2-ME, 0.002% bromophenol blue). The released material was analyzed by SDS-PAGE.

**Labeling of Macrophages and Immunoprecipitations.** Inflammatory peritoneal macrophages were harvested by lavage with PBS from DBA/2 or BALB/c mice on the fourth day after intraperitoneal injection of 1.5 ml of sterile 3% brewer's thioglycollate (Difco Laboratories Inc., Detroit, MI), prepared according to manufacturer's instructions. Equal numbers of the exudate cells were distributed into 35-mm plastic tissue culture dishes (Corning Glass Works, Corning, NY) and allowed to adhere for 2 h at 37°C. After washing off nonadherent cells, the adherent cells were incubated in 1 ml of DME lacking methionine for 1 h at 37°C and then labeled with [<sup>35</sup>S]methionine (250 μCi/ml) for 1 h. At the end of the labeling period, the medium was removed, the cells were washed three times with ice-cold PBS, and then lysed in 1 ml of ice-cold lysis buffer containing 2 mmol PMSF. For the pulse-chase analysis, the cells were labeled for 1 h as above. After removing the medium and washing the cells, they were chased at 37°C in 4 ml/well of medium supplemented with 2 mmol methionine for different periods of time. At the end of the chase period, the wells were quickly chilled on ice, and the medium was collected and saved. The cells were then washed with 0.5 ml of ice-cold PBS, and the wash was added to the medium. The cells were lysed as described above. After the medium was centrifuged to remove contaminating cells, PMSF was added to a final concentration of 2 mmol and Triton X-100 was added to a final concentration of 0.5%.

Cell lysates were centrifuged at 600 g for 5 min to remove nuclei and then at 10,000 g for 20 min. They were then precleared overnight at 4°C with 2 μg of rabbit IgG (Zymed Laboratories) and 150 μl of a 3% slurry of protein A-Sepharose (Pharmacia Fine Chemicals) in PBS. Immunoprecipitations were carried out by adding 2 μg of M3/38 followed by 2 μg of rabbit anti-rat antiserum and 150 μl of protein A-Sepharose to the precleared lysate. Control immunoprecipitations used an isotype-identical rat mAb to IgM (Zymed Laboratories) in place of M3/38. Aliquots of the extracellular medium were precleared and immunoprecipitated in exactly the same way. As a control to check for passive leakage of cytosolic proteins into the extracellular medium, aliquots of the medium were immunoprecipitated with an antiactin antibody (Sigma Chemical Co.). After binding overnight at 4°C, the Sepharose beads were washed five times with wash buffer and then resuspended and boiled for 3 min in 50 μl of 2× SDS-PAGE sample buffer. Aliquots of the released material were analyzed by SDS-PAGE.

**Subcellular Fractionation.** Monolayers of thioglycollate-elicited macrophages were labeled and chased for 3 h as described above. At the end of the labeling period, the cells were washed with PBS and then with 0.3× RSB. The cells were then scraped with a rubber policeman into 1 ml of 0.3× RSB containing 2 mmol PMSF and then homogenized in a Dounce homogenizer. After spinning out the nuclei at 600 g for 5 min, the lysate was centrifuged at 400,000 g for 15 min in a TLA 100 rotor (Beckman Instruments, Inc., Palo Alto, CA). The supernatant was saved as the cytosolic fraction. The membrane pellet was washed once with 1× RSB and the wash was pooled with the cytosol. The final pellet was extracted in lysis buffer and, after spinning out insoluble material, the supernatant was saved as the membrane fraction. The cytosolic fraction was made up to the same detergent and salt concentrations as the membrane fraction before both fractions were immunoprecipitated. The validity of the fractionation protocol was checked by immunoprecipitating aliquots of the fractions with M1/70, a mAb to Mac-1, a known macrophage plasma membrane antigen (17).

**S1 Nuclease Protection Analysis.** The protocol used has been described previously (12). 5' end-labeled, double-stranded probes that encompassed the 5' ends of the cDNA were made from the Xba I-Xho I fragments of the pBSK clones Mac-2.16 and Mac-2.9 (see Figs. 1 A and 7 A). 40,000 cpm of the probe were hybridized with 10–20 μg of total cellular RNA or with tRNA (as a control) at 60°C overnight. Digestion with S1 nuclease (New England Biolabs, Beverly, MA), 400 U/ml, was carried out for 90 min at room temperature. The products were then analyzed on a 7 M urea/5% polyacrylamide gel.

**Anchored Polymerase Chain Reaction (PCR).** The protocol used was based on published procedures (18, 19). A 20-nucleotide oligomer (5' CCCTGCCCCAGGCTGGTTCC 3') designed to hybridize to Mac-2 mRNA 32 nucleotides downstream of the Eco RV site (Fig. 1 A) was used to prime first strand cDNA synthesis towards the 5' end of the mRNA using 5 μg of

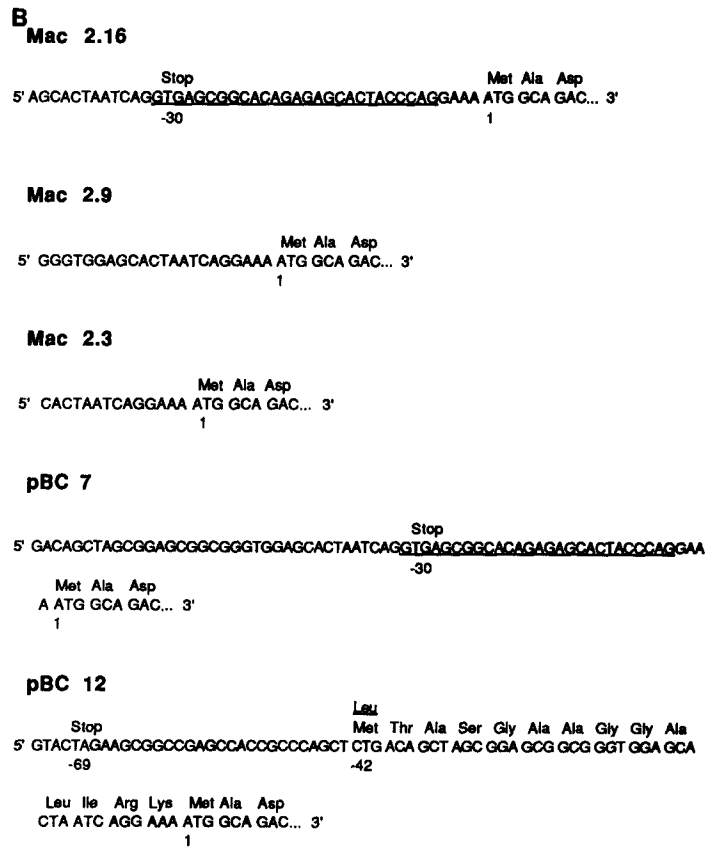
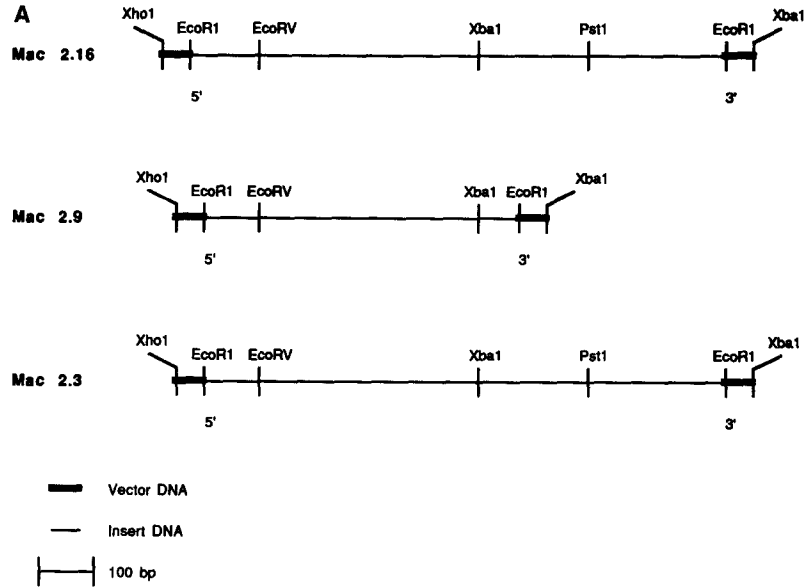
total cellular RNA from thioglycollate-elicited macrophages. A poly-G tail was then added to the 3' end of the cDNA. Amplification of the cDNA was then carried out using two oligonucleotide primers. A 28-nucleotide oligomer (5' GGCCCATGCGGCCGCCCCCCCCCCC-CCCC 3') that was complementary to the poly-G tail and contained a Not I restriction site allowed DNA synthesis to occur towards the 3' end of the mRNA. Synthesis in the reverse direction was achieved with an 18-nucleotide oligomer (5' ACCCGGATATCCTTGAGG 3') containing the Eco RV site (Fig. 1 A). The conditions of amplification were as follows: melting at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 3 min. After 40 cycles of amplification, the DNA product was cut with Not I and Eco RV, and analyzed on a 1% agarose gel. A ~150-bp band was visualized by ethidium bromide staining. This band was confirmed to be the specific amplification product by Southern analysis with a Mac-2 probe. It was isolated, cloned in pBSK, and sequenced.

**SDS-PAGE.** 12% polyacrylamide gels containing 0.1% SDS were used in all experiments and were run under standard conditions (20). After electrophoresis, gels (other than those used for Western analysis) were soaked in Autofluor (National Diagnostics, Inc., Somerville, NJ), dried, and exposed for autoradiography.

## Results

**Molecular Cloning of the Mac-2 cDNA.** We used the mAb M3/38 to clone the Mac-2 cDNA from a  $\lambda$ gt11-P388D1 expression library. 10 positive clones were obtained, of which three (Mac-2.3, Mac-2.9, and Mac-2.10) were purified. Lysogens of these three clones produced fusion proteins (with a molecular mass of ~150 kD in the case of Mac-2.3 and Mac-2.10, and ~135 kD in the case of Mac-2.9), which reacted on Western analysis with the ant-Mac-2 antibody M3/38, but not with an isotype-identical control antibody (data not shown). After subcloning the insert DNAs (sized 940, 570, and 940 bp, respectively) of the three clones into the Eco RI site of pBSK, restriction maps were constructed and are shown in Fig. 1 A. By restriction mapping and by subsequent sequence analysis, Mac-2.3 and Mac-2.10 were found to be identical. The insert DNA from Mac-2.3 was used to rescreen the library by DNA hybridization and, in this fashion, a clone with a slightly longer insert (970 bp) was obtained (Mac-2.16) (Fig. 1 A). The sequence of Mac-2.16 contained an open reading frame encoding a protein of predicted molecular mass of 27,482 daltons, consistent with the size of the Mac-2 protein on SDS-PAGE. The amino acid sequence consisted of two domains: an NH<sub>2</sub>-terminal domain containing a repetitive proline and glycine-rich motif, and a COOH-terminal domain containing the amino acid sequence HFNPRF . . . WGEXR (where the letters represent the standard one-letter amino acid code, X being any amino acid) found in S-type lectins (21). There were no potential N-glycosylation sites. When the sequence of the Mac-2 cDNA was compared with those in existing databases, it was found to be identical to that

FIGURE 1. (A) Restriction maps of the insert DNAs of the clones Mac-2.16, Mac-2.9, and Mac-2.3 in the vector pBSK. The lengths of the inserts of these clones are ~970, ~570, and ~940 bp, respectively. The scale is indicated by the 100-bp bar. (B) The nucleotide sequences of the 5' ends of Mac-2 cDNAs. Mac-2.16, Mac-2.9, and Mac-2.3 are  $\lambda$ gt11 clones, and pBC7 and pBC12 are clones obtained by PCR, pBC7 being the 5' extension of Mac-2.16, and pBC12 being the 5' extension of Mac-2.9 and Mac-2.3. The numbers below the sequence indicate nucleotide positions. The predicted amino acid sequence is indicated above the nucleotide sequence in the standard three-letter code. Stop, an in-frame stop codon. The underlined nucleotides in Mac-2.16 and pBC12 constitute the 27-bp sequence not found in Mac-2.9, Mac-2.3, and pBC7. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number X16834.



of the cDNA for a known galactose-specific S-type lectin, CBP35, and highly homologous to the cDNA for rIgEBP. Our sequence extended a little more 5' than the published sequences of CBP35 and rIgEBP, and included an ATG start codon and an in-frame stop codon upstream of it (Mac-2.16; only the 5' end of the sequence is shown, the complete sequence can be found in reference 5 and a comparison of CBP35 and rIgEBP in reference 22; Fig. 1 B). The predicted second amino acid of our sequence differs from the corresponding one of CBP35; the difference is probably attributable to a minor sequencing error. The nucleotide context of the putative start codon fits the Kozak consensus (23) in having an A residue at the -3 position and a G residue at the +4 position. To further confirm that we had indeed cloned the Mac-2 cDNA, the in vitro synthesized product of our clone was immunoprecipitated with M3/38. Fig. 2, lane 2, shows that the in vitro synthesized protein is specifically immunoprecipitated by M3/38 (and not by the control antibody; lane 1) and furthermore, comigrates precisely with the Mac-2 antigen expressed by macrophages (lane 3). We used the Mac-2 cDNA to assess its expression in various tissues and cell lines by Northern analysis. Fig. 3 shows that a ~1.2-kb transcript is identified by the Mac-2 probe. It is most highly expressed in thioglycollate-elicited macrophages and at moderate levels in the macrophage cell lines J774 and P388D1. It is also expressed at a low level in Swiss 3T3 fibroblasts and at a very low level in resident peritoneal macrophages (data not shown). There was no evidence of expression in any of the lymphoid lines examined, even on prolonged exposure of the filter, consistent with the presumed absence of the Mac-2 protein in lymphocytes (3).

*The Mac-2 Protein Binds to Carbohydrate Determinants and to IgE.* In view of the properties suggested by its sequence, we tested the carbohydrate- and IgE-binding properties of the in vitro synthesized Mac-2 protein using the desialylated glycoprotein asialofetuin, and IgE, IgG, and IgM, all coupled to Sepharose, as ligands. Fig. 4, lanes 1-5, show that asialofetuin binds to the Mac-2 protein and that galactose and the galactose-containing disaccharide lactose inhibit this binding, whereas glucose and maltose do not. Treatment of asialofetuin with *N*-glycanase to remove *N*-linked sugar residues completely abolishes its binding to the Mac-2 protein (Fig. 4, lanes 6 and 7). These findings suggest that Mac-2 binds to galactose-containing deter-

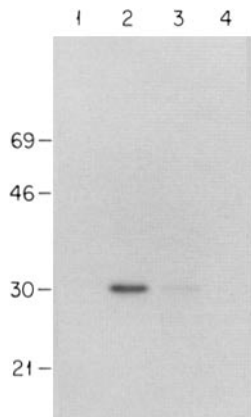


FIGURE 2. Autoradiograph of SDS-PAGE of immunoprecipitations of [<sup>35</sup>S]methionine-labeled in vitro synthesized Mac-2 protein (lanes 1 and 2) and lysates of thioglycollate-elicited macrophages (lanes 3 and 4). The anti-Mac-2 antibody M3/38 was used for the immunoprecipitations shown in lanes 2 and 3, and an isotype-identical control antibody to IgM for those in lanes 1 and 4. The numbers on the left of the figure indicate molecular mass in kilodaltons.

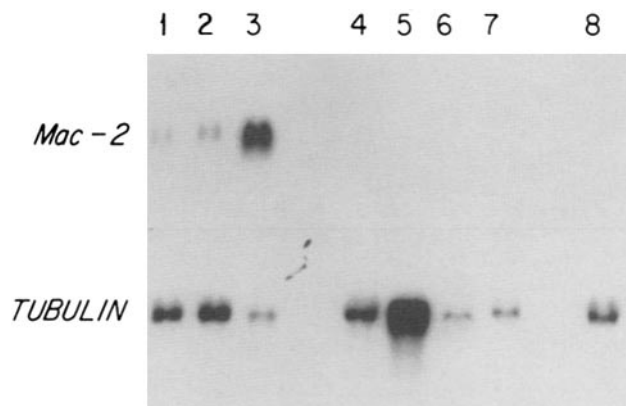


FIGURE 3. Expression of Mac-2 in various cell lines by Northern analysis. Lane 1, J774; lane 2, P388D1; lane 3, thioglycollate-elicited macrophages; lane 4, 70Z/3; lane 5, AJ9; lane 6, A20.25; lane 7, EL4; lane 8, Swiss 3T3 fibroblasts. (Top) The results of probing with the Mac-2 cDNA; (bottom) the results after stripping the filter and reprobing with a tubulin probe.

minants on the ligand. IgE also binds to Mac-2 and this interaction is inhibited by galactose and lactose (Fig. 4, lanes 8-12). This binding is IgE specific, since it is not displayed by IgG or IgM and is not abolished by treating the IgE-Sepharose with *N*-glycanase (data not shown). The latter finding suggests that if sugar residues on IgE are involved in binding to Mac-2, they may be *O*-linked.

*Mac-2 Is Present in the Cytosol, in the Extracellular Medium, and in the Membrane Fraction.* The results of initial experiments aimed at determining the subcellular location of the Mac-2 protein indicated that the protein was present in the extracellular medium as well as the cytosol. We then carried out a formal pulse-chase analysis, which showed that the amount of Mac-2 in the extracellular medium increased steadily with time up to 3 h (Fig. 5, top). Actin could not be detected in the medium at any time point, indicating that significant leakage of cytosolic proteins did not occur during the course of the experiment (data not shown). Though the amount of Mac-2 associated with the cell did not change dramatically with time, a slight decrease was discernible between 30 and 180 min (Fig. 5, bottom). When cells were fractionated into cytosolic and membrane fractions at the end of 3 h of chase, Mac-2 was found in both the cytosolic and membrane fractions, in approximately equal amounts (Figure 6, top). The latter could represent protein in transit to the extracellular medium or protein bound to the plasma membrane (see Discussion).

*An Alternatively Spliced Mac-2 cDNA.* In view of the fact that Mac-2 was appar-

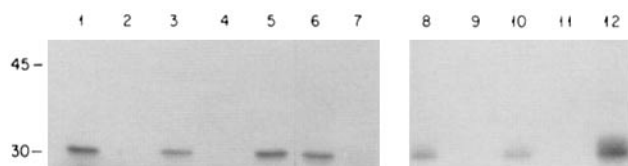


FIGURE 4. Autoradiograph of SDS-PAGE of precipitations of in vitro synthesized Mac-2 protein using immobilized ligand. Lane 1, asialofetuin-Sepharose; lane 2, asialofetuin-Sepharose plus 100 mmol galactose; lane 3, asialofetuin-Sepharose plus

100 mmol glucose; lane 4, asialofetuin-Sepharose plus 100 mmol lactose; lane 5, asialofetuin-Sepharose plus 100 mmol maltose; lane 6, mock-treated asialofetuin-Sepharose; lane 7, *N*-glycanase-treated asialofetuin-Sepharose; lane 8, IgE-Sepharose; lane 9, IgE-Sepharose plus 100 mmol galactose; lane 10, IgE-Sepharose plus 100 mmol glucose; lane 11, IgE-Sepharose plus 100 mmol lactose; lane 12, IgE-Sepharose plus 100 mmol maltose. The numbers on the left of the figure indicate molecular mass in kilodaltons.

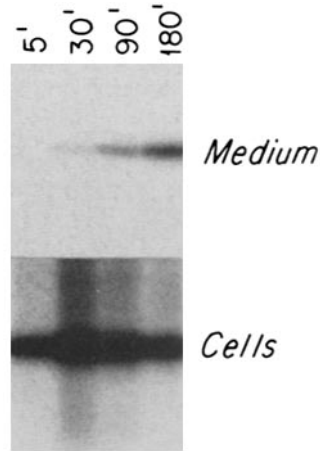


FIGURE 5. Autoradiograph of SDS-PAGE of the pulse-chase analysis of the Mac-2 protein in thioglycollate-elicited macrophages. The figure shows immunoprecipitations of the culture supernatant (*top*) or cell lysates (*bottom*) with the anti-Mac-2 antibody at 5, 30, 90, and 180 min of chase.

ently secreted into the medium, it was surprising to find that the Mac-2 cDNA did not encode a signal peptide (24), even though there are a few extracellular proteins that lack such a sequence (25, 26). Since we knew that Mac-2 was probably encoded by a single gene (J. Michaelson and M. Hanson, unpublished observations), we considered the possibility that an alternatively spliced cDNA might give rise to a signal peptide-containing protein. Two of the clones that we originally isolated from the  $\lambda$ gt11-P388D1 library, Mac-2.9 and Mac-2.3, did in fact differ at their 5' ends from Mac-2.16 (Fig. 1 B), though the rest of the sequence of the three clones was identical. To verify that this difference was not the result of a cloning artifact, we carried out an S1 nuclease protection analysis, using total cellular RNA and double-stranded end-labeled probes made from Mac-2.9 and Mac-2.16 (Fig. 7 A). Fig. 7 B shows the results of this experiment; lanes 5 and 10 show the 537-bp Mac-2.9 probe and the 558-bp Mac-2.16 probe, respectively, each corresponding to the Xho I-Xba I fragments shown in Fig. 7 A. Lanes 4 and 9 show the probes after digestion with Eco RI, which results in the 507-bp RI-Xba I fragment of Mac-2.9 and the 528-bp RI-Xba I fragment of Mac-2.16. Since the Xho I-R I fragments of each probe are derived from vector DNA, the RI-Xba I fragments represent the size of the full-length protected probe. Lanes 3 and 8 show that thioglycollate-elicited macrophages contain mRNA, which confers full-length protection on both the Mac-2.9

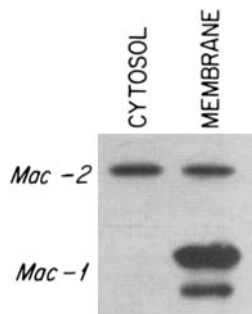


FIGURE 6. Autoradiograph of SDS-PAGE of sub-cellular fractions of thioglycollate-elicited macrophages. The figure shows immunoprecipitations of the cytosolic and membrane fractions with the anti-Mac-2 antibody (*top*) or the anti-Mac-1 antibody (*bottom*).



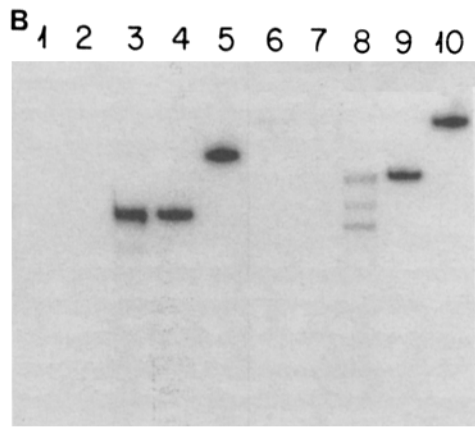
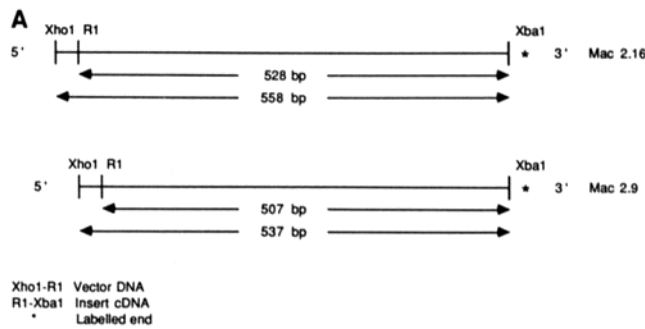


FIGURE 7. (A) Probes used for the S1 nuclease protection analysis. The probes were derived from the pBSK clones of Mac-2.16 and Mac-2.9 (see Fig. 1 A). R1 refers to Eco RI. (B) S1 nuclease protection analysis using Mac-2 probes and total cellular RNA. Lanes 1 and 6, tRNA; lanes 2 and 7, A20.25; lanes 3 and 8, thioglycollate-elicited macrophages; lanes 4 and 9, Eco RI cut probe; lanes 5 and 10, uncut probe. Lanes 1-5, Mac-2.9 probe; lanes 6-10, Mac-2.16 probe. Expected sizes of protected fragments: Mac-2.9, full-length protection, 507 bp; partial protection, 494 bp; Mac-2.16, full-length protection, 528 bp; partial protection, 494 bp.

and the Mac-2.16 probes. In addition to the fully protected fragments, shorter fragments representing partial protection of the probes are also seen. Lanes 1, 2, 6, and 7 are controls that indicate that the protected fragments are specific to RNA from macrophages. The fact that full-length protection of both probes is obtained strongly suggests that both Mac-2.9 and Mac-2.16 are authentic cDNAs derived from two distinct mRNAs present in macrophages. The presence of one fragment of 494 bp, representing partial protection of each of the two probes, is a corollary to this finding. The presence of more than one partially protected fragment (seen clearly in Fig. 7, lane 8) may indicate the existence of other Mac-2 transcripts that we have not yet identified as cDNA clones.

We obtained further 5' sequence for both Mac-2.9 and Mac-2.16 using the anchored PCR technique. The sequence of the two clones thus obtained are shown in Fig. 1 B, pBC7 being the 5' extension of Mac-2.16, and pBC12 being the 5' extension of Mac-2.9. Inspection of the sequences in Fig. 1 B indicates that the two types of clones are identical, except for a 27-bp insertion in Mac-2.16 and pBC7 (underlined in Fig. 1), which introduces an in-frame stop codon upstream of the initiator methionine codon. The 27-bp insert starts with GT, suggesting that it might represent an intron that is spliced out in the second type of clone (Mac-2.9 and pBC12). The 27-bp insert is too short to be an entire intron (27). It is theoretically possible that it might represent a partially deleted intron and that Mac-2.16 is, therefore,

an unspliced precursor of Mac-2.9 and Mac-2.3. This seems unlikely for two reasons. First, it is unlikely that identical deletions occurred independently during generation of the cDNA library as well as during the anchored PCR reaction. Second, the relative abundance of the fully protected species in the S1 nuclease protection experiment (Fig. 7 B, lane  $\beta$ ) when using a probe that completely spans the 27-bp insert strongly suggests that the insert cannot be a partially deleted intron, even though total cellular RNA was used for the analysis. Mac-2.16 must, therefore, be an alternatively spliced form of Mac-2.9 and Mac-2.3. A detailed analysis of the genomic region encoding these cDNAs would provide the most reliable confirmation of these predicted splicing events. In the type of cDNA represented by Mac-2.3, Mac-2.9, and pBC12, the alternative splicing event results in the open reading frame being continued upstream of the initiation codon till the stop codon at position -69. There are no ATGs in this 5' extension of the reading frame. However, there is a CTG codon at position -42 and CTGs have been reported to act as initiation codons in at least two other proteins in vivo and in one in vitro system (28-30). After the CTG codon, the sequence has the potential to encode a stretch of uncharged, predominantly hydrophobic amino acids that resembles a signal peptide.

#### Discussion

The most significant finding of the present study is that the cDNA sequence encoding the Mac-2 antigen, a protein that is highly expressed in inflammatory macrophages, is identical to that of CBP35, a galactose-specific S-type lectin found in murine fibroblasts (5), and highly homologous to that of rIgEBP, originally described in a rat basophilic leukemia cell line (6). Based on this information, we were able to demonstrate that the in vitro synthesized Mac-2 protein had the expected galactose-specific carbohydrate-binding activity as well as specific IgE-binding activity. Our data indicate that the binding to IgE is inhibited by galactose and lactose, suggesting that it involves the carbohydrate-binding site of the Mac-2 protein; however, we cannot rule out the possibility that Mac-2 also has a separate IgE-binding site. It is of interest in this connection that the low affinity IgE receptor is homologous to a galactose-specific lectin (31), although the role of the lectin domain in IgE binding has not been elucidated.

CBP35 and rIgEBP are presumed to be intracellular proteins (7, 8). Our data indicate that even though a large proportion of the Mac-2 protein is present in the cytosol, a significant amount is also present in the extracellular medium. It is not clear how the protein reaches the latter site since the cDNA sequence does not contain a classical signal peptide. This situation is not unique to Mac-2, however. Other S-type lectins and two other proteins, IL-1 and fibroblast growth factor, also share this property of being found in the extracellular medium and yet not having a signal peptide (21, 25, 26). As has been suggested in these cases, it is possible that Mac-2 reaches the exterior either by passive release from lysed cells or by a process of active secretion that does not require a signal peptide. In this connection, it is interesting that we have been able to demonstrate the existence of two alternatively spliced Mac-2 cDNAs, one of which has the potential to encode a colinear, NH<sub>2</sub> terminally extended Mac-2 protein containing a signal peptide-like sequence initiating with a CTG codon. Genomic clones of the Mac-2 gene are being analyzed to confirm the structural basis for the predicted splicing event and experiments to determine whether

the alternative splicing is in any way related to the existence of cytosolic and extracellular Mac-2 are currently in progress.

The Mac-2 antigen was originally described by Ho and Springer (1) as a cell surface protein based on immunofluorescence, cell surface iodination, and binding of antibody to intact cells. Based on their data, they calculated that there were  $1.7\text{--}3.4 \times 10^5$  surface molecules of Mac-2 per cell, making it a major surface component of thioglycollate-elicited macrophages. Immunofluorescence studies from a number of groups are consistent with surface expression of Mac-2 on macrophages (2, 4). The cDNA sequence suggests that Mac-2 is not a transmembrane protein. The most likely explanation for its being detected on the cell surface is that some of the protein that is released extracellularly binds to exposed galactose residues on plasma membrane glycoproteins or glycolipids. Preliminary experiments from our laboratory indicate that Mac-2 does indeed bind to a surface protein in macrophages. If Mac-2, like most other animal lectins (21), is multivalent, membrane-bound Mac-2 would still be able to bind external ligands and would, in effect, act as a surface receptor. An analogous process may explain the presence of a small proportion of rIgEBP on the surface of rat basophilic leukemia cells (8).

The identification of Mac-2 as a lectin provides some clues to its possible function in macrophage biology. Several recent reports have documented the lectin-like properties of adhesion molecules involved in leukocyte homing (32). An analogous function may be considered for Mac-2, especially in view of the fact that thioglycollate-induced inflammation appears to selectively recruit macrophages with a high level of Mac-2 expression. A number of macrophage lectins have also been described, namely, a mannose-fucose receptor on alveolar macrophages (33), a galactose-fucose receptor on Kupffer cells (34), and a sialic acid receptor on stromal tissue macrophages (35). A galactose-specific lectin on rat Kupffer cells has also been described that, interestingly enough, has a molecular mass of 30 kD (36). It is possible that Mac-2 is the murine homologue of this protein. The mannose-fucose receptor is one of the best studied of these lectins and has been shown to be involved in the binding and phagocytosis of leishmania (37) and possibly mycobacteria. It is likely that such lectin-mediated phagocytosis would require the cooperative action of multiple lectins of different specificity so as to engage different sugar determinants on the surface of the phagocytosed particle. Thus, galactose-specific lectins such as Mac-2 could contribute to such a process. One of the major surface determinants of the intracellular pathogen *Leishmania major* is a galactose-rich lipophosphoglycan that has been shown to be involved in the binding of this parasite to the macrophage (38, 39). Since this binding is competed by galactose, Mac-2 is a likely candidate as a receptor for this parasite glycoconjugate.

The human mannose-specific lectin, mannose-binding protein (MBP), is secreted by hepatocytes and is present in the serum at high levels after stress (40). Several functions of this protein have been recently demonstrated. It acts as an opsonin by binding to bacteria with high mannose surface determinants and facilitating their phagocytosis by macrophages (41). It has been shown to bind to gp120 of HIV and interfere with its binding to CD4 (42). Finally, a related rat mannose-binding protein has been shown to activate complement through the classical pathway (43). The structure of Mac-2 resembles that of MBP in some ways: both are  $\sim 30$  kD in size and both have a COOH-terminal lectin domain and an NH<sub>2</sub>-terminal domain with

a repetitive proline- and glycine-rich structure. Given this structural similarity, it is possible that Mac-2 will also share some of the functional properties of MBP.

Lymphocyte-derived IgE-binding factors have been described that are believed to be involved in the regulation of IgE expression (44). The IgE-binding property of Mac-2 raises the possibility that it may have similar functions and may implicate the macrophage in such regulatory processes. These and the other putative functions discussed can now be analyzed rigorously using the cloned Mac-2 molecule.

### Summary

A cDNA encoding the Mac-2 antigen, a surface marker highly expressed by thioglycollate-elicited macrophages, has been cloned by immunoscreening of a  $\lambda$ gt11-P388D1 expression library. The nucleotide sequence of the cDNA is identical to that of carbohydrate-binding protein 35, a galactose-specific lectin found in fibroblasts and highly homologous to a rat IgE-binding protein from basophilic leukemia cells. The in vitro synthesized Mac-2 protein displayed the expected carbohydrate- and IgE-binding properties. By pulse-chase analysis and subcellular fractionation studies, the Mac-2 protein was found in the cytosol but was also seen to accumulate in the extracellular medium. The latter finding was surprising in view of the fact that the cDNA did not encode a signal peptide or transmembrane domain. An alternatively spliced cDNA with the potential to encode a NH<sub>2</sub> terminally extended Mac-2 protein with a stretch of hydrophobic amino acids at its NH<sub>2</sub> terminus was also found, but it is not clear whether it is the source of the extracellular Mac-2. Possible functions for the Mac-2 protein based on its lectin- and IgE-binding properties are discussed.

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