0022-1767/86/1373-1060$02.00/0
THE JOURNAL OF IMMUNOLOGY
Vol. 137, 1060-1065, No. 3, August 1, 1986
Printed in U.S.A.

THE MOUSE LEUKOCYTE ADHESION PROTEINS Mac-1 AND LFA-1: STUDIES ON mRNA TRANSLATION AND PROTEIN GLYCOSYLATION WITH EMPHASIS ON Mac-1[1]

LEANDRO SASTRE, TAKASHI K. KISHIMOTO, CONNIE GEE,* THOMAS ROBERTS,* AND TIMOTHY A. SPRINGER

*From the Laboratory of Membrane Immunochemistry, *Division of Neoplastic Disease Mechanisms, Dana-Farber Cancer Institute, Boston MA 02115; and the Department of Pathology, Harvard Medical School, Boston, MA 02115*

Translation in vitro of mRNA and immunoprecipitation with specific rabbit antisera showed that the unglycosylated precursor polypeptides of the mouse Mac-1 and lymphocyte function associated antigen (LFA-1) α subunits are 130,000 M_r and 140,000 M_r, respectively. Furthermore, polysomes purified by using anti-Mac-1 IgG yielded a similar major product of translation in vitro of M_r = 130,000. The Mac-1 and LFA-1 α subunit translation products are immunologically noncross-reactive, showing that differences between these related proteins are not due to post-translational processing. Mac-1 and LFA-1 α subunits could only be in vitro translated from mRNA from cell lines the surfaces of which express the corresponding Mac-1 and LFA-1 α-β complexes, showing tissue-specific expression is regulated at the mRNA level. The glycosylation of Mac-1 was examined by both translation in vitro in the presence of dog pancreas microsomes and by biosynthesis in vivo and treatment with tunicamycin, endoglycosidase H, and the deglycosylating agent trifluoromethane sulfonic acid. High mannose oligosaccharides are added to the Mac-1 α and β polypeptide backbones of M_r = 130,000 and 72,000, respectively, to yield precursors of M_r = 164,000 and 91,000, respectively. The α and β subunit precursors are then processed with partial conversion of high mannose to complex type carbohydrate to yield the mature subunits of M_r = 170,000 and 95,000, respectively.

Mac-1 is a glycoprotein present on macrophages, monocytes, granulocytes, and large granular lymphocytes (1). Macrophage 1 molecule (Mac-1)[2] has been shown to be the complement receptor CR3 in the mouse (2) and human (2, 3). Macrophages are able to bind, phagocytose, and degrade particles coated with the complement component iC3b through this receptor (4). In addition, Mac-1 is also involved in adhesion of monocytes and granulocytes to a variety of surfaces including artificial substrates and endothelial cells (1). Mac-1 has been biochemically defined as a heterodimeric protein composed of two non-covalently associated subunits, αM (M_r = 170,000) and β (M_r = 95,000) (5).

Mac-1 is structurally related to two other leukocyte adhesion proteins, lymphocyte function-associated antigen 1 (LFA-1) and p150,95, which have identical β subunits and distinct α subunits (6, 7). LFA-1 is a general leukocyte cell-cell adherence protein (8). In contrast to Mac-1, LFA-1 is present on lymphocytes. LFA-1 is also present on granulocytes and monocytes, and on activated and primed but not resident or thioglycollate-elicited macrophages (1, 9). Thus the Mac-1 and LFA-1 α-β complexes, and hence their α subunits are expressed differentially on leukocytes.

Mac-1 and LFA-1 have been purified to homogeneity (10), and their α subunits have 33% amino acid sequence homology at their N-termini, suggesting that they could have evolved by gene duplication (11). These glycoproteins appear to play a central role in the immune response as shown in an immunodeficiency disease in which cell surface expression of Mac-1, LFA-1, and p150,95 is deficient. Granulocytes, monocytes, and lymphocytes from patients with Mac-1, LFA-1 deficiency disease have severe defects in adhesion and adhesion-dependent functions (1).

We report here the translation in vitro of the murine Mac-1 and LFA-1 α subunits, and studies on the glycosylation of Mac-1, both during translation in vitro and biosynthesis in vivo. The results show that the Mac-1 and LFA-1 α subunits have immunologically noncross-reactive polypeptide backbones of M_r = 130,000 and 140,000, respectively, and their leukocyte lineage-specific expression is regulated at the mRNA level. Rabbit IgG against Mac-1 has been used to obtain greatly enriched preparations of Mac-1 mRNA by polysome immunoselection. Furthermore, the steps in the glycosylation and carbohydrate processing of the Mac-1 α and β subunits have been defined.

MATERIALS AND METHODS

Rabbit antisera. Mouse Mac-1 and LFA-1 were purified to homogeneity by monoclonal antibody affinity chromatography (10). Rabbit antisera were raised by intramuscular injection of 75 μg of emulsified purified antigen in complete Freund's adjuvant. Rabbits were boosted intramuscularly after 2 and 4 wk with 25 μg of antigen in complete Freund's adjuvant.

RNA. Cells were cultured to a density of 10^6 cells/ml in RPMI 1640

Received for publication March 4, 1986.
Accepted for publication May 5, 1986.
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[1] This work was supported by National Institutes of Health Grants AI20316, CA31799, CA31798, an ACS faculty award (TAS), National Science Foundation predoctoral fellowship (TKK), and National Institutes of Health Fogarty fellowship (LS).
[2] Abbreviations used in this paper: Endo H, endoglycosidase H; LFA-1, lymphocyte function associated 1 molecule; Mac-1, macrophage 1 molecule.

medium containing 10% fetal calf serum (FCS) and were washed twice in phosphate-buffered saline (PBS) at 4°C. RNA was prepared by homogenization of the cells in guanidinium isothiocyanate followed by centrifugation over a cesium chloride cushion (12). Poly(A)$^+$ RNA was enriched by oligo(dT) cellulose chromatography (13).

In vitro translation and immunoprecipitation of translation products. Poly(A)$^+$ RNA was translated in vitro by using a rabbit reticulocyte system obtained as described by Pelham and Jackson (14). When indicated, in vitro processing and glycosylation of the translated polypeptides was performed by adding dog pancreas microsomes to the translation reaction (15). Both the reticulocyte lysate and the pancreas microsomes were pretreated with micrococcal nuclease (14). Immunoprecipitation of in vitro translation products was performed according to Anderson and Blobel (16). In summary, the in vitro translation reaction was stopped by adding sodium dodecyl sulfate (SDS) to a final concentration of 4%, and the translation products were incubated at 100°C for 5 min. Ten volumes of 10 mM Tris HCl (pH 7.8), 0.14 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) buffer containing 2% Triton X-100 to bind the SDS were added to each sample. Each sample was precleared twice with 30 μl of a 1:1 slurry of protein A-Sepharose in TSA buffer (10 mM Tris-HCl, pH 7.8, 0.14 M NaCl, and 0.01% NaN3) for 1 hr at 4°C with constant shaking. The protein A-Sepharose was removed by centrifugation, and 7 μl of rabbit serum were added to each supernatant. After 2 hr at 4°C, 50 μl of protein A-Sepharose slurry were added, and the samples were incubated for 1 hr at 4°C with constant shaking. Immune complexes adsorbed to the protein A-Sepharose were collected by centrifugation and were washed as described (16). The immunoprecipitated proteins were subjected to SDS 7% polyacrylamide gel electrophoresis (PAGE). The gels were soaked in PPO-dimethyl sulfoxide (DMSO) (17) and were fluorographed by using Kodak SB-5 film.

Metabolic labeling. P388D1 cells were washed and were incubated at 2×10^5 cells/ml for 2 hr in methionine-free RPMI 1640 media supplemented with 10% dialyzed FCS. [^{35}S]Methionine (NEG-009A, 800 Ci/mmol; New England Nuclear) was added (50 μCi/ml), and the cells were cultured for 1 hr at 37°C. The labeling was stopped by addition of 1 mM methionine, and the cells were either collected by centrifugation or were cultured for 6 additional hr. Cells were washed twice with PBS at 4°C and were lysed by incubation in 10 mM Tris-HCl (pH 7.8), 0.14 M NaCl, 1 mM PMSF, and 1% Triton X-100 at 4°C for 15 min at a concentration of 2×10^6 cells/ml. The lysate was centrifuged at 100,000 × G for 45 min, and the supernatants were used for immunoprecipitation experiments. When indicated, tunicamycin was included at 2.5 μg/ml during preincubation of the cells in methionine-free media and labeling. Immunoprecipitation from metabolically labeled cell lysates was according to the protocol for in vitro translation products, except that the final concentration of SDS in the samples was 1% (16), and only 1 μl of rabbit serum was used for each sample.

Endoglycosidase H digestion. Immunoprecipitated proteins were eluted from the protein A-Sepharose beads by boiling in 50 μl of 1% SDS and 0.1 M Tris-HCl (pH 8.0) for 5 min and were diluted with 9 vol of reaction buffer (10 mM 2-(N-morpholino)ethane sulfonic acid (MES) pH 5.5, 0.14 M NaCl). The reaction was carried out in the presence of 2 mU of endoglycosidase H and 1 mM PMSF at 37°C for 16 hr (18). The proteins were precipitated with acetone in the presence of 10 μg of tRNA as carrier and were resuspended in sample buffer before SDS-PAGE.

Trifluoromethanesulfonic acid treatment. Deglycosylation with trifluoromethanesulfonic acid was performed by a modification (19) of the method of Edge et al. (20). Immunoprecipitated Mac-1 was eluted from protein A-Sepharose beads, was trichloroacetic acid (TCA) precipitated, and was lyophilized. A 2:1 mixture of trifluoromethanesulfonic acid:anisole (200 μl) was added to the samples. The samples were flushed with nitrogen, were sealed, and were incubated 2 hr at 4°C and then at 2 hr at −20°C. The reaction was stopped by the addition of 500 μl of 10 mM triethanolamine (TEA), 2% Nonidet P-40 (NP-40) (pH 7.8), followed by 100 μl of 1 M TEA. The samples were then TCA precipitated and were reconstituted in SDS-PAGE sample buffer.

Polysome purification. Immunoselection of Mac-1-synthesizing polysomes was according to Shapiro and Young (21). Rabbit anti-Mac-1 serum was made RNase free by purification on a protein A-Sepharose column (22). P388D1 cells were washed with PBS containing 1 μg/ml cycloheximide and were lysed at 2×10^7 cells/ml in 50 mM Tris-HCl, pH 7.8, 150 mM potassium acetate, 10 mM $MgCl_2$, 1 μg/ml cycloheximide, 0.1 mg/ml heparin, and 0.35% NP-40 at 4°C for 15 min. Nuclei were pelleted at 7,000 × G for 15 min and microsomes were disrupted by making the supernatant to 0.35% Na deoxycholate. Polysomes were purified by centrifugation through a cushion of 65% sucrose (w/v) at 100,000 × G for 2 hr at 4°C. The sucrose layer containing the polysomes was diluted to 5 A_{260} units/ml with polysome buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 0.1% NP-40, 0.1 mg/ml heparin, and 1 μg/ml cycloheximide) and was incubated with RNase-free anti-Mac-1 IgG (0.3 mg/10^8 cell equivalents) for 16 hr at 4°C. The incubation mixture was passed first through a column of glycine-quenched CNBr-activated Sepharose CL4B to remove nonspecifically binding material, and then the IgG-polysome complexes were adsorbed to a protein A-Sepharose column. The column was extensively washed with polysome buffer. The adsorbed polysomes were then eluted with 25 mM Tris-HCl (pH 7.5) and 20 mM EDTA, were collected in 400 μl aliquots, and were quickly ethanol precipitated.

RESULTS

Identification of Mac-1 and LFA-1 in vitro translation products. Poly(A)$^+$ RNA was purified from the mouse T lymphoma line EL-4 that expresses LFA-1, and from the macrophage-like line P388D1, which expresses Mac-1 and in smaller amounts LFA-1 (10, 23). Aliquots of 1 μg of poly(A)$^+$ RNA from these cell lines were translated in vitro in a rabbit reticulocyte system. Translation products were heated at 100°C in 4% SDS and then were diluted with 2% Triton X-100 (16) to lower the background in immunoprecipitation. The translation products were then immunoprecipitated with rabbit antisera to Mac-1 or LFA-1, or with nonimmune serum (Fig. 1). The anti-Mac-1 serum specifically immunoprecipitated a major band of 130,000 M_r from translation products of P388D1 mRNA but not EL-4 mRNA, as expected for the Mac-1 α-chain polypeptide (Fig. 1, lane *1*). On the other hand, the anti-LFA-1 serum specifically immunoprecip-

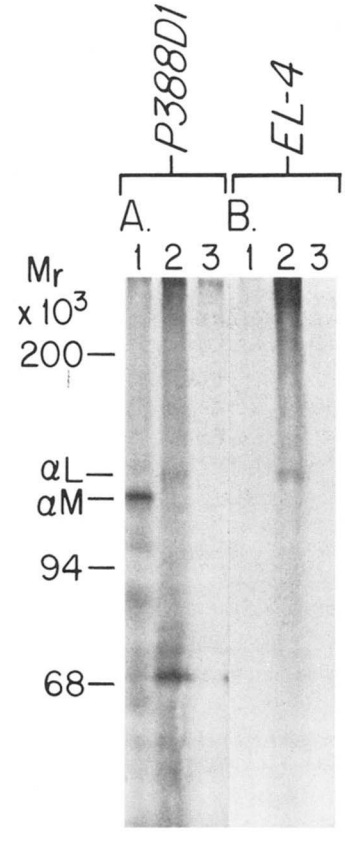

Figure 1. Immunoprecipitation of LFA-1 and Mac-1 α subunit in vitro translation products. Poly A$^+$ RNA from the mouse macrophage cell line P388D1 (*Panel A*) and the mouse T cell line EL-4 (*Panel B*) was translated in a rabbit reticulocyte system in the presence of [^{35}S] methionine. Translation products were immunoprecipitated with rabbit polyclonal antiserum raised against purified mouse Mac-1 (*Lane 1*), purified mouse LFA-1 (*Lane 2*), or preimmune rabbit serum (*Lane 3*), and were subjected to SDS-PAGE and fluorography. The position of the Mac-1 α subunit (αM) and LFA-1 α subunit (αL) products is indicated.

itated a polypeptide of 140,000 M_r from both P388D1 and EL-4 mRNA translation products as expected for an LFA-1 α-chain polypeptide (Fig. 1, lane *2*). The putative M_r = 130,000 Mac-1 and M_r = 140,000 LFA-1 α-chain polypeptides were consistently observed in many independent in vitro translation experiments. Neither α subunit was obtained after translation in vitro of mRNA from L cells, which are both Mac-1$^-$ and LFA-1$^-$ (not shown). Minor bands that were observed such as a 70,000 M_r polypeptide varied between experiments. No major band translated in vitro could be recognized as the β subunit polypeptide common to Mac-1 and LFA-1. Furthermore, no common band was immunoprecipitated from the translation products of P388D1 mRNA with the rabbit sera to LFA-1 and Mac-1. It is not clear whether this is due to poor precipitation of β by the antiserum, masking by background bands, or degradation of β mRNA. Monoclonal antibodies to the Mac-1 α, LFA-1 α, and common β subunit (7) did not immunoprecipitate products translated in vitro (not shown).

Immunopurification of Mac-1-synthesizing polyribosomes. We next examined whether anti-Mac-1 IgG could bind to the nascent Mac-1 polypeptide chain and be used to purify Mac-1-synthesizing polyribosomes. These experiments were designed as a second method of identifying the Mac-1 α chain translation product, and to examine the feasibility of immunologically purifying Mac-1 mRNA. Polysomes were purified from P388D1 cells and were incubated with purified, RNase-free anti-Mac-1 IgG. The mixture was passed through a pre-column to absorb nonspecific material and then through protein A-Sepharose to absorb specific Mac-1$^+$ polysomes. The polysomal nascent polypeptide chain-mRNA-ribosome complexes were dissociated with EDTA, and the mRNA and ribosomes were eluted from the column and were collected in fractions. One percent of the mRNA from each fraction was subjected to in vitro translation. The translation products were directly analyzed by SDS-PAGE (Fig. 2). The major translation product from the eluted mRNA is a 130,000 M_r polypeptide (Fig. 2, fractions 3 and 4) that can be immunoprecipitated by the Mac-1 antiserum and co-migrates with Mac-1 α translated from total poly(A)$^+$ mRNA (not shown). This band represents between 10 and 50% of the total translation products in different experiments. Quantitation of the eluted RNA has shown that it represents ~0.1% of the RNA applied to the column, which is in good agreement with the calculated percentage that Mac-1 represents of P388D1 total protein (10).

Comparison and glycosylation of Mac-1 products synthesized in vitro and in vivo. To show that the Mac-1 α-chain polypeptide synthesized in vitro is bona fide, two approaches were used. First, dog pancreas microsomes were added to in vitro translation systems to glycosylate the in vitro translation product, and it was compared with the in vivo glycosylated precursor; and second, the in vivo synthesized precursors were deglycosylated and were compared with the product translated in vitro in the absence of added microsomes. In the course of these experiments, we also examined the glycosylation in vivo of the Mac-1 β subunit. In all experiments, in vitro and in vivo synthesized products were heated in SDS at 100°C, then were cooled, and an excess of nonionic detergent was added before immunoprecipitation. Under

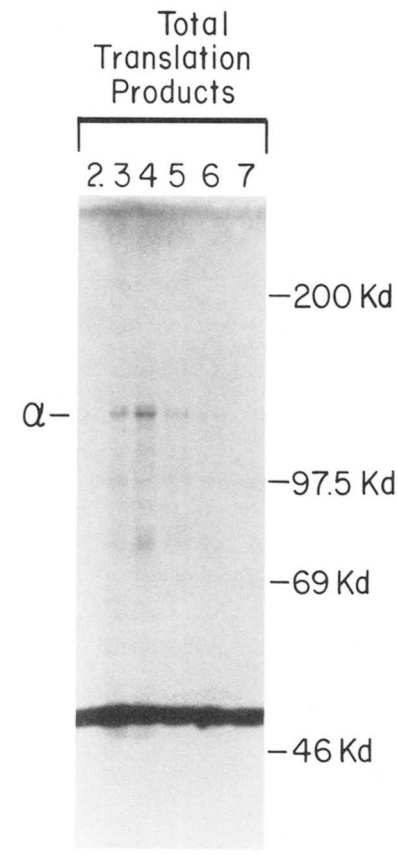

Figure 2. Immunoselection of Mac-1 α polysomes. Polysomes from P388D1 cells were immunoselected by incubation with anti-Mac-1 IgG and protein A-Sepharose chromatography as described in *Materials and Methods*. Purified mRNA was eluted from the column with EDTA, was collected in fractions, and was ethanol precipitated. One percent of the mRNA from each eluted fraction was translated in vitro. Samples were subjected to SDS-7.5% PAGE and fluorography. The position of the in vitro translated α subunit polypeptide is indicated.

these conditions, the α and β subunits are dissociated, and are independently precipitated by Mac-1 α subunit- and β subunit-specific antibodies present in the Mac-1 antiserum (7).

Dog pancreas microsomes added to in vitro translation systems have previously been shown to catalyze cleavage of the signal peptide from the polypeptide precursor and the addition of N-linked high mannose-type oligosaccharides (24). P388D1 mRNA was translated in vitro in the presence or absence of dog pancreas microsomes. Comparison of the products precipitated by anti-Mac-1 serum (Fig. 3, lanes *5* and *6*) showed that the microsomes converted approximately 50% of the polypeptide chain of M_r = 130,000 to a chain of M_r = 164,000. Furthermore, this 164,000 M_r chain, translated and glycosylated in vitro, migrated identically to the Mac-1 α-chain precursor synthesized in vivo (Fig. 3, lane *4*). Smaller amounts of lower M_r chains variably seen in the in vitro translation products (compare Fig. 1) were not glycosylated and did not appear related to the β subunit.

To isolate unglycosylated precursors synthesized in vivo, P388D1 cells were treated with tunicamycin, an inhibitor of N-linked glycosylation (25), and were labeled with [^{35}S]methionine. Two polypeptide chains of M_r = 130,000 and 72,000 were precipitated with anti-Mac-1 serum (Fig. 3, lane *2*). The 130,000 M_r deglycosylated chain synthesized in vivo migrated identically to the M_r = 130,000 product translated in vitro (Fig. 3, compare

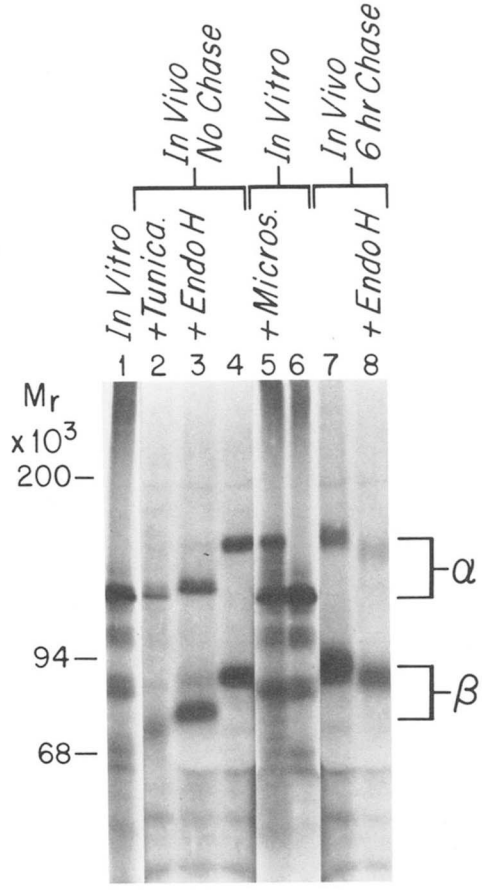

Figure 3. Comparison of in vitro translated and in vivo labeled Mac-1. Glycosylated and unglycosylated forms of Mac-1 were isolated from both in vivo and in vitro synthesized [^{35}S]methionine labeled molecules by immunoprecipitation with the anti-Mac-1 rabbit serum. Poly(A)$^+$ RNA from P388D1 cells was translated in vitro either in the presence (*Lane 5*) or absence (*Lanes 1* and *6*) of dog pancreas microsomes. The immature form of Mac-1 was immunoprecipitated from P388D1 cells that had been metabolically labeled 1 hr with [^{35}S]methionine (*Lane 4*). Endo H digest of this immature product is shown in *Lane 3*. Some P388D1 cells were also treated with the drug tunicamycin for 2 hr before and during the pulse labeling (*Lane 2*). The mature form of Mac-1 was isolated from cells that were pulsed-labeled for 1 hr and were chased for 6 hr with cold methionine (*Lane 7*). Mature Mac-1 digested with Endo H is shown in *Lane 8*. Samples were subjected to SDS-7% PAGE and fluorography. Control immunoprecipitates with normal rabbit serum had very little background (not shown).

lanes *2* and *1*), in agreement with their both being the unglycosylated Mac-1 α subunit. The second polypeptide of M_r = 72,000 appears to be the unglycosylated β subunit precursor, which for unknown reasons is not immunoprecipitated by the same antiserum from in vitro translation products.

We next examined the glycosylation of Mac-1 during in vivo biosynthesis. P388D1 cells were pulsed 1 hr with [^{35}S]methionine to obtain the precursor form of Mac-1, and then were chased with unlabeled methionine for 6 hr to obtain the mature form (26). After the 1 hr pulse labeling, precursors for the α-chain (164,000 M_r) and β chain (91,000 M_r) were immunoprecipitated (Fig. 3, lane *4*). Treatment of these precursors with endoglycosidase H (Endo H) yielded two polypeptides of M_r = 133,000 and 77,000 M_r, which are very similar in size to the unglycosylated α and β chain precursors (Fig. 3, lane *3*), and suggests that the Mac-1 α and β chain precursors contain exclusively high mannose type N-linked oligosaccharides. N-glycosylation is known to take place on the endoplasmic reticulum. The small difference in M_r between the polypeptides obtained by tunicamycin treatment and Endo H digestion (Fig. 3, compare lanes *2* and *3*) can be due to the presence of a molecule of N-acetylglucosamine, which is left at each glycosylation site after Endo H digestion (27), and would not be present in the molecules from tunicamycin-treated cells. The high-mannose oligosaccharide-containing precursor synthesized in vivo shows the same electrophoretic mobility as the molecule glycosylated in vitro (Fig. 3, lanes *4* and *5*, respectively), suggesting that both are Mac-1 α subunit precursors containing high-mannose oligosaccharides.

Trifluoromethanesulfonic acid treatment, which completely removes O-linked, as well as N-linked oligosaccharides (20), was used as a second means of removing carbohydrate from Mac-1 precursors synthesized in vivo. Trifluoromethanesulfonic acid-treated Mac-1 α and β precursors (Fig. 4, lane *4*) migrated identically with Mac-1 from tunicamycin treated cells (Fig. 4, lane *3*), and similarly to Endo H-treated Mac-1 (Fig. 4, lane *2*).

Mature Mac-1 was obtained by labeling cells for 1 hr with [^{35}S]methionine followed by a 6-hr chase period (26). Immunoprecipitation yielded the mature Mac-1 molecule composed of a 170,000 M_r α subunit and a 95,000 M_r β subunit (Fig. 3, lane *7*). Endo H treatment of the mature molecule decreased the apparent molecular mass to 158,000 for the α subunit and 91,000 for the β subunit (Fig. 3, lane *8*). Endo H cleavage of the mature α and β subunits (Fig. 3, compare lanes *7* and *8*) resulted in significantly less reduction in apparent molecular mass than Endo H cleavage of the immature α and β subunits (Fig. 3, compare lanes *4* and *3*). This suggests that the majority of the high mannose oligosaccharides on the

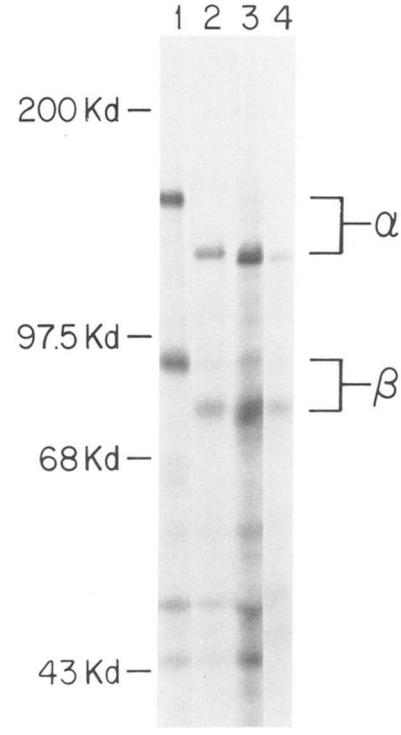

Figure 4. Deglycosylation of the Mac-1 precursor with Endo H, tunicamycin, and trifluoromethanesulfonic acid. The Mac-1 precursor was immunoprecipitated with rabbit anti-Mac-1 serum from P388D1 cells that were metabolically labeled 1 hr with [^{35}S]methionine. The immunoprecipitates were analyzed directly (*Lane 1*) or after treatment with Endo H (*Lane 2*) or trifluoromethansulfonic acid (*Lane 4*). Some cells were also treated with tunicamycin for the 2 hr prior to and during the pulse labeling (*Lane 3*.) Samples were subjected to SDS-7% PAGE and fluorography.

Mac-1 α and β subunits are converted to complex-type, Endo H-resistant oligosaccharides (27) during maturation in vivo.

DISCUSSION

Rabbit polyclonal antisera against purified mouse LFA-1 and Mac-1 antigens have been obtained that recognize the corresponding α subunits from products of mRNA translated in vitro. The Mac-1 antiserum immunoprecipitates an M_r = 130,000 polypeptide translated from mRNA of the macrophage line P388D1 but nothing translated from mRNA of the T lymphoma line EL-4, whereas the LFA-1 antiserum immunoprecipitates an M_r = 140,000 polypeptide from in vitro translation products of both mRNA. These in vitro translation results correspond with previous immunofluorescence, ^{125}I-labeling, and biosynthetic labeling studies, which have shown that P388D1 is Mac-1$^+$, whereas EL-4 is Mac-1$^-$, and both P388D1 and EL-4 are LFA-1$^+$ (10). Neither α subunit was translated from mRNA from Mac-1$^-$, LFA-1$^-$ L cells. This is the first demonstration that the tissue-specific expression of the Mac-1 and LFA-1 α subunits, and hence of the corresponding surface-expressed Mac-1 and LFA-1 α-β complexes, is regulated at the mRNA level. The presence of Mac-1 α subunit transcripts in P388D1 cells but not EL-4 or L cells has recently been confirmed with cloned DNA probes (28). No cross-reactivity between the mouse Mac-1 and LFA-1 α chain polypeptides translated in vitro was detected with the rabbit antisera. This is in agreement with the noncross-reactivity of the mature, glycosylated α subunits with rat antisera (7), and the distinct peptide maps and N-terminal sequences of the Mac-1 and LFA-1 α subunits (5, 11, 29). The findings support the notion that the Mac-1 and LFA-1 α subunits are products of distinct genes, and are not interrelated by processing.

Mac-1 mRNA has been purified by immunoselection of polysomes bearing nascent Mac-1 chains by using rabbit anti-Mac-1 IgG. Translation in vitro of the purified polysomal mRNA yields an M_r = 130,000 polypeptide as major translation product that can be immunoprecipitated by the anti-Mac-1 rabbit serum. The identification of a polypeptide of similar M_r by two different methods, immunoprecipitation of in vitro translation products and polysome immunoselection, additionally confirms that this polypeptide is the unglycosylated Mac-1 α subunit.

The in vitro translated Mac-1 and LFA-1 α subunits were both 40,000 g/mole lower in M_r than the mature α subunits of M_r = 170,000 and 180,000, respectively. This suggests that both mature α subunits are glycosylated to a similar extent and are 22 to 24% carbohydrate as estimated by SDS-PAGE.

We focused on Mac-1 to study the relationship of the Mac-1 α-chains synthesized in vitro and in vivo, and to study the glycosylation of the α and β subunits. Experiments that made use of pulse-chase metabolic labeling of P388D1 cells in the presence or absence of tunicamycin, Endo H, and trifluoromethanesulfonic acid treatments, and glycosylation in vitro were carried out. These experiments verified the authenticity of Mac-1 α translated in vitro. The correspondence of the Mac-1 α subunits synthesized in vivo and in vitro rules out a model in which the Mac-1 α and β subunits would be derived by cleavage from the same polypeptide chain precursor. Proteins that are cleaved into subunits by proteolytic processing in vivo, such as the C3 and C4 complement components, are synthesized as a single precursor polypeptide by in vitro translation systems (30).

In previous in vivo pulse-chase studies, the Mac-1 α and β subunits were found as separate precursors that after several hours became associated in an α-β complex, increased slightly in M_r, and were transported to the cell surface (26). The findings presented here allow us to propose the following glycosylation pathway for the Mac-1 antigen.

The Mac-1 α subunit precursor contains a polypeptide chain backbone of M_r = 130,000, which was identified both by translation in vitro and by tunicamycin treatment of cells and by deglycosylation of the in vivo precursor with trifluoromethanesulfonic acid. We have not been able to identify β subunit in vitro translation products, but tunicamycin treatment and chemical deglycosylation both yield an unglycosylated β precursor of M_r = 72,000. The α and β subunits are glycosylated in the endoplasmic reticulum to high mannose oligosaccharide-containing glycopolypeptides of M_r = 164,000 and 91,000, respectively. The 164,000 M_r Mac-1 α polypeptide can also be obtained by translation in vitro in the presence of dog pancreas microsomes. Endo H digestion of the in vivo α and β precursors reduces their M_r to 133,000 and 77,000, respectively. The number of N-linked oligosaccharides can be roughly estimated from the difference in molecular mass between unglycosylated and high-mannose containing peptides, assuming a M_r of 2500 for each oligosaccharide. These calculations predict the existence of 12 to 14 N-linked glycosylation sites for the α subunit and five to eight sites for the β subunit. Approximately five N-linked glycosylation sites on the β precursor were estimated by digesting with graded amounts of Endo H and counting the number of steps on the resulting "ladder". Partial Endo H digestion products of the α subunit were also obtained, but were not sufficiently well resolved to count (data not shown).

During a chase period of 6 hr, the Mac-1 α and β subunits are converted to the mature chains of M_r = 170,000 and 95,000, respectively (26), concomitant with a decrease in the amount their molecular mass was reduced by Endo H cleavage. The acquisition of Endo H resistance reflects the conversion of high-mannose oligosaccharides to the complex type, a step that is known to occur in the Golgi apparatus (25). Most but not all of the Mac-1 α and β subunit N-linked carbohydrate was converted to the Endo H resistant, complex type during maturation in vivo. From the decrease in molecular mass of the mature molecules after Endo H treatment, approximately five oligosaccharides for the α and two for the β subunit can be calculated to still remain as high mannose type. These studies do not exclude the possibility of the addition of O-linked polysaccharides or other covalent modifications of the glycoprotein subunits also taking place during processing.

These studies provide insights into the regulation of Mac-1 and LFA-1 α subunit expression and the steps in the biosynthesis and glycosylation of the Mac-1 α and β subunits. The characterization of the Mac-1 and LFA-1 α subunits translated in vitro from mRNA is also of use in confirming the identity of Mac-1 and LFA-1 cDNA clones. A Mac-1 α subunit genomic clone has recently

been shown to be authentic by hybrid selection of Mac-1 mRNA followed by in vitro translation, and by the correspondence of its nucleic acid sequence with the protein sequence (28). Northern blotting shows the Mac-1 α subunit mRNA is 6 kb, and its tissue-specific expression is in accordance with the findings here on mRNA translation. Furthermore, when a myelomonocytic cell line is stimulated with interferon-γ to differentiate along the monocytic pathway, both the 6 kb Mac-1 transcript and mRNA translatable in vitro into the Mac-1 α subunit are induced. Cloned Mac-1 and LFA-1 α subunit DNA will allow insights into how these genes are differentially regulated in the lymphoid and the monocytic/granulocytic lineages and how cytokines such as interferon-γ and colony-stimulating factors regulate their expression.

These studies also provide a model for understanding the α subunit processing defects in Mac-1, LFA-1 deficiency disease. Indirect evidence suggests that the primary defect in Mac-1, LFA-1 deficiency is in the β subunit (31). In normal human cells, α-chain and β-chain precursors appear to associate in an α-β complex and undergo an increase in M_r. Patient cells have an intracellular pool of the α-chain precursor that never undergoes the increase in M_r and never is expressed on the cell surface (31). This study indicates that this α subunit precursor form represents the high mannose glycosylated precursor, and processing to the higher M_r form is due to conversion of most of the oligosaccharide chains to an Endo H-resistant complex form. This suggests that in Mac-1, LFA-1 deficient patient cells, the α subunit is not transported to the Golgi apparatus, and the α-chain is highly dependent upon its association with the β chain for transport through the Golgi and eventual cell surface expression.

Acknowledgments. We thank Ms. J. Casaubon for excellent manuscript preparation and Dr. W. Bornstein, Massachusetts General Hospital, Boston, MA, for providing dog pancreas microsomes.

REFERENCES

1. **Springer, T. A., and D. C. Anderson.** 1986. The importance of the Mac-1, LFA-1 glycoprotein family in monocyte and granulocyte adherence, chemotaxis, and migration into inflammatory sites: insights from an experiment of nature. In *Biochemistry of Macrophages* (Ciba Symposium 118). Pitman, London. P. 102.
2. **Beller, D. I., T. A. Springer, and R. D. Schreiber.** 1982. Anti-Mac-1 selectively inhibits the mouse and human type three complement receptor. *J. Exp. Med. 156:1000.*
3. **Wright, S. D., P. E. Rao, W. C. Van Voorhis, L. S. Craigmyle, K. Iida, M. A. Talle, E. F. Westberg, G. Goldstein, and S. C. Silverstein.** 1983. Identification of the C3bi receptor of human monocytes and macrophages with monoclonal antibodies. *Proc. Natl. Acad. Sci. USA 80:5699.*
4. **Rothlein, R., and T. A. Springer.** 1985. Complement receptor type three-dependent degradation of opsonized erythrocytes by mouse macrophages. *J. Immunol. 135:2668.*
5. **Kürzinger, K., M. K. Ho, and T. A. Springer.** 1982. Structural homology of a macrophage differentiation antigen and an antigen involved in T-cell-mediated killing. *Nature 296:668.*
6. **Sanchez-Madrid, F., J. Nagy, E. Robbins, P. Simon, and T. A. Springer.** 1983. A human leukocyte differentiation antigen family with distinct alpha subunits and a common beta subunit: the lymphocyte function-associated antigen (LFA-1), the C3bi complement receptor (OKM1/Mac-1), and the p150,95 molecule. *J. Exp. Med. 158:1785.*
7. **Sanchez-Madrid, F., P. Simon, S. Thompson, and T. A. Springer.** 1983. Mapping of antigenic and functional epitopes on the alpha and beta subunits of two related glycoproteins involved in cell interactions, LFA-1 and Mac-1. *J. Exp. Med. 158:586.*
8. **Krensky, A. M., F. Sanchez-Madrid, E. Robbins, J. Nagy, T. A. Springer, and S. J. Burakoff.** 1983. The functional significance, distribution, and structure of LFA-1, LFA-2, and LFA-3: cell surface antigens associated with CTL-target interactions. *J. Immunol. 131:611.*
9. **Strassmann, G., T. A. Springer, and D. O. Adams.** 1985. Studies on antigens associated with the activation of murine mononuclear phagocytes: kinetics of and requirements for induction of lymphocyte function-associated (LFA)-1 antigen in vitro. *J. Immunol. 135:147.*
10. **Kürzinger, K., and T. A. Springer.** 1982. Purification and structural characterization of LFA-1, a lymphocyte function-associated antigen, and Mac-1, a related macrophage differentiation antigen. *J. Biol. Chem. 257: 12412.*
11. **Springer, T. A., D. B. Teplow, and W. J. Dreyer.** 1985. Sequence homology of the LFA-1 and Mac-1 leukocyte adhesion glycoproteins and unexpected relation to leukocyte interferon. *Nature 314:540.*
12. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry 18:5294.*
13. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA 69:1408.*
14. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem. 67:247.*
15. **Scheele, G., B. Dobberstein, and G. Blobel.** 1978. Transfer of proteins across membranes. Biosynthesis in vitro of pretrypsinogen and trypsinogen by cell free fractions of canine pancreas. *Eur. J. Biochem. 82:593.*
16. **Anderson, D. J., and G. Blobel.** 1983. Immunoprecipitation of proteins from cell-free translations. *Methods Enzymol. 96:111.*
17. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem. 46:83.*
18. **Sutherland, D. R., C. E. Rudd, and M. F. Greaves.** 1984. Isolation and characterization of a human T-lymphocyte associated glycoprotein (gp40). *J. Imunol. 133:327.*
19. **Oettgen, H. C., J. Kappler, W. J. M. Tax, and C. Terhorst.** 1984. Characterization of the two heavy chains of the T3 complex on the surface of human T lymphocytes. *J. Biol. Chem. 259:12039.*
20. **Edge, A. S., C. R. Faltynek, L. Hof, L. E. Reichert, and P. Weber.** 1981. Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Anal. Biochem. 118:131.*
21. **Shapiro, S. Z., and J. R. Young.** 1981. An immunochemical method for mRNA purification. *J. Biol. Chem. 256:1495.*
22. **Korman, A. J., P. J. Knudsen, J. F. Kaufman, and J. L. Strominger.** 1982. cDNA clones for the heavy chain of HLA-DR antigens obtained after immunopurification of polysomes by monoclonal antibody. *Proc. Natl. Acad. Sci. USA 79:1844.*
23. **Kürzinger, K., T. Reynolds, R. N. Germain, D. Davignon, E. Martz, and T. A. Springer.** 1981. A novel lymphocyte function-associated antigen (LFA-1): cellular distribution, quantitative expression, and structure. *J. Immunol. 127:596.*
24. **Anderson, D. J., and G. Blobel.** 1981. In vitro synthesis, glycosylation, and membrane insertion of the four subunits of Torpedo acetylcholine receptor. *Proc. Natl. Acad. Sci. USA 78:5598.*
25. **Kornfeld, R., and S. Kornfeld.** 1985. Assembly of asparagine-linked oligosaccharides. *Ann. Rev. Biochem. 54:631.*
26. **Ho, M. K., and T. A. Springer.** 1983. Biosynthesis and assembly of the alpha and beta subunits of Mac-1, a macrophage glycoprotein associated with complement receptor function. *J. Biol. Chem. 258:2766.*
27. **Robbins, P. W., S. C. Hubbard, S. J. Turco, and D. F. Wirth.** 1977. Proposal for a common oligosaccharide intermediate in the synthesis of membrane glycoproteins. *Cell 12:893.*
28. **Sastre, L., J. Roman, D. Teplow, W. Dreyer, C. Gee, R. Larson, T. Roberts, and T. A. Springer.** 1986. Isolation of a partial DNA genomic clone for the alpha subunit of the mouse complement receptor type 3 and adhesion molecule Mac-1. *Proc. Natl. Acad. Sci. USA In press.*
29. **Trowbridge, I. S., and M. B. Omary.** 1981. Molecular complexity of leukocyte surface glycoproteins related to the macrophage differentiation antigen Mac-1. *J. Exp. Med. 154:1517.*
30. **Goldberger, G., M. L. Thomas, B. F. Tack, J. Williams, H. R. Colten, and G. N. Abraham.** 1981. NH2-terminal structure and cleavage of guinea pig Pro-C3, the precursor of the third complement component. *J. Biol. Chem. 256:12617.*
31. **Springer, T. A., W. S. Thompson, L. J. Miller, F. C. Schmalstieg, and D. C. Anderson.** 1984. Inherited deficiency of the Mac-1, LFA-1, p150,95 glycoprotein family and its molecular basis. *J. Exp. Med. 160:1901.*