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Structural homology of a macrophage differentiation antigen and an antigen involved in T-cell-mediated killing

Konrad Kürzinger, May-Kin Ho & Timothy A. Springer

Laboratory of Membrane Immunochemistry, Sidney Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115, USA

Two distinct murine cell-surface differentiation antigens, Mac-1 and LFA-1 (lymphocyte function-associated antigen 1), are compared here and shown to be related at the molecular level. Mac-1, defined by the M1/70 rat anti-mouse monoclonal antibody (MAb), is expressed on macrophages, natural killer cells and 50% of bone marrow cells, but not on B or T lymphocytes[1-3]. In contrast, the LFA-1 antigen, defined by the M7/14 rat anti-mouse MAb, is expressed on B and T lymphocytes and 75% of bone marrow cells, but not on thioglycollate-induced peritoneal exudate macrophages[4,5]. MAb blocking studies suggest that LFA-1 participates in T-lymphocyte-mediated killing and T-lymphocyte antigen-specific responses[4,5]. Mac-1 and LFA-1 have α-polypeptide chains of 170,000 and 180,000 molecular weight (M_r), respectively, and both contain β polypeptides of 95,000 M_r. This similarity prompted us to investigate their relationship. Mac-1 and LFA-1 have distinct cellular distributions, MAb-defined antigenic determinants and α-subunits, but have highly homologous or identical β-subunits as shown by tryptic peptide mapping. Moreover, they share some common antigenic determinants recognized by a polyclonal antiserum. Cross-linking studies show that in each antigen the subunits are noncovalently associated in $\alpha_1\beta_1$ structures. Mac-1 and LFA-1 comprise a novel family of two-chain leukocyte differentiation antigens.

The distinct cellular distributions of the Mac-1 and LFA-1 antigens are emphasized by their reciprocal expression on spleen cells and thioglycollate-induced peritoneal exudate cell macrophages (PEC) (Fig. 1). Spleen cells are >93% LFA-1 positive but only 6% Mac-1 positive, while exudate macrophages showed no significant LFA-1 expression but are >94% Mac-1 positive. Expression of Mac-1 and LFA-1 on 50 and 75% of bone marrow cells, respectively[1,6], shows these antigens also have distinct myeloid distributions, and are co-expressed on a subpopulation of these cells.

Immunoprecipitation also confirmed that Mac-1 and LFA-1 are reciprocally expressed on concanavalin A-stimulated spleen cells (Con A blasts) and PEC, and that the MAb do not cross-react between them. Thus, M1/70 but not M7/14 precipitated material from PEC (Fig. 2*d*), while M7/14 but not M1/70 precipitated material from Con A blasts (Fig. 2*e*). No cross-reaction between M1/70 and M7/14 could be detected, even when autoradiography was prolonged by a factor of five. This was as expected from the differing specificity of the MAb for cells (Fig. 1).

Despite these differences in cellular distribution and antigenic determinants recognized by MAb, Mac-1 and LFA-1 have strikingly similar two-chain structures. The LFA-1 α-chain is slightly higher in M_r than the Mac-1 α-chain, while the β-chains are identical in M_r (Fig. 2*d*, *e*). Determination of M_r by co-electrophoresis with standards in nongradient SDS gels at three different polyacrylamide percentages showed that the Mac-1 and LFA-1 α-chains are 170,000 and 180,000 M_r, respectively, and the β-chains 95,000 M_r (data not shown). The α- and β-chains are not disulphide linked[1,6].

Precipitation by MAb of two distinct polypeptide chains could be because both chains express the antigenic determinant, or because only one chain expresses the determinant but is non-covalently associated with the other chain. To investigate the quaternary structure of Mac-1 and LFA-1, cross-linking experiments were carried out. Detergent-solubilized lysates were cross-linked with the cleavable reagent dithiobis(succinimidyl propionate) and subjected to immunoprecipitation and SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 3). Instead of α- and β-chains (Fig. 3, lane 2), a cross-linked Mac-1 product of M_r 235,000–275,000 was found (Fig. 3, lane 4), in good agreement with the M_r of 265,000 predicted for an $\alpha_1\beta_1$

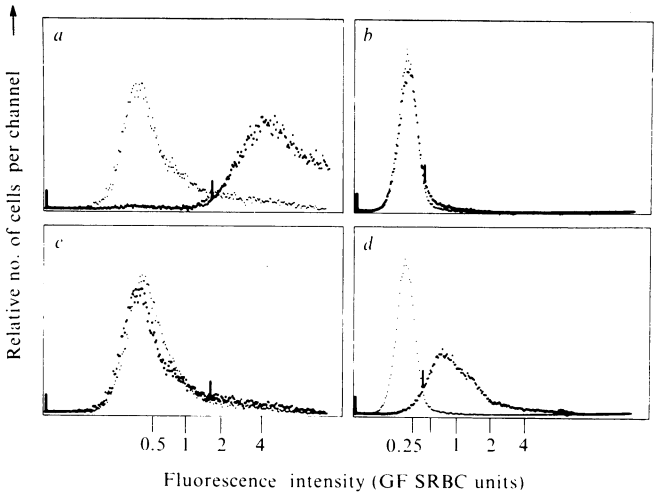

Fig. 1 Reciprocal immunofluorescent labelling of peritoneal exudate cell macrophages and spleen cells by M1/70 and M7/14 MAb. Cells (50μl, 5×10^7 ml in RPMI-1640, 5% fetal calf serum, 20 mM HEPES, 0.01 M NaN_3) were labelled with an equal volume of either M1/70 (*a*, *b*) or M7/14 (*c*, *d*) MAb-containing culture supernatants (dark dots) or NSI culture supernatant containing 50 μg ml^{-1} normal rat IgG as control (dim dots), washed, then labelled with affinity-purified fluorescein isothiocyanate rabbit $F(ab')_2$ anti-rat IgG absorbed with mouse IgG. Immunofluorescence flow cytometry was performed on a Becton Dickinson FACS II equipped with a Nuclear Data log amplifier which was calibrated with glutaraldehyde-fixed sheep red blood cells (GF SRBC). Four-day thioglycollate-induced PEC (*a*, *c*) or spleen cells (*d*) were scatter gated to exclude red cells and lymphocytes, or red cells, respectively.

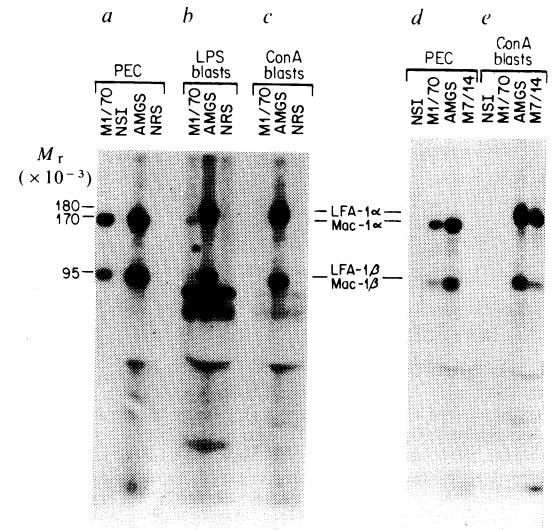

Fig. 2 SDS-PAGE of antigens precipitated from different cellular sources by M1/70 and M7/14 MAb and a polyclonal antiserum. Four-day Con A blasts (*c*, *e*), 4-day thioglycollate-induced PEC (>90% macrophages) (*a*, *d*) and LPS blasts (*b*) were prepared, surface labelled with ^{125}I, detergent solubilized, precleared, immunoprecipitated and subjected to SDS 5–15% gradient PAGE and autoradiography as previously described[1,6]. Two separate experiments (*a–c* and *d*, *e*) with independent cell preparations and SDS-PAGE were carried out. Cell lysates were immunoprecipitated with 25 or 50 μl of NSI culture supernatant containing 50 μg ml^{-1} normal rat IgG (NSI) or 1 μl of normal rat serum (NRS) as controls, 1 μl of AMGS, 25 or 60 μl of M1/70 MAb supernatant (M1/70), or 20 μl of 1 mg ml^{-1} M7/14 IgG (M7/14).

structure. Furthermore, the product was composed of α- and β-chains, as shown by reductive cleavage of the cross-links (Fig. 3, lane 6). A similar cross-linked product was obtained with LFA-1 (Fig. 3, lane 10). These results strongly suggest that both Mac-1 and LFA-1 contain α- and β-subunits which are noncovalently associated into $\alpha_1\beta_1$ quaternary structures.

One method of testing for structural homology between two proteins is by immunological cross-reactivity. MAb are not necessarily suitable, as they may recognize unique rather than shared antigenic determinants. Therefore, cross-reactivity was tested with a classical antiserum. A rat antiserum was raised against Mac-1 which had been partially purified from thioglycollate-induced macrophage membranes by *L. culinaris* lectin affinity chromatography and by MAb immunoadsorbent depletion of two other immunodominant antigens[7]. This anti-macrophage glycoprotein serum (AMGS) potently precipitated Mac-1 and little or nothing else from PEC (Fig. 2*a*, *d*). The AMGS also precipitated large amounts of material from lipopolysaccharide-stimulated spleen cells (LPS blasts) and Con A blasts with an M_r identical to that of LFA-1, while this material was not precipitated by M1/70 (Fig. 2*b*, *c*). The material precipitated by AmGS from PEC and Con A blasts was shown to be identical to Mac-1 and LFA-1, respectively, by co-migration in SDS-PAGE (Fig. 2*d*, *e*), and by Cleveland peptide mapping (data not shown).

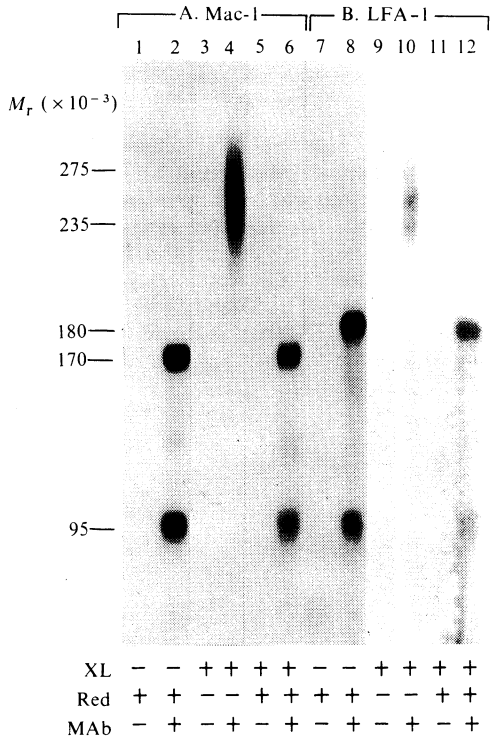

Fig. 3 Cross-linking of Mac-1 and LFA-1. PEC and EL-4 cells were surface labelled with ^{125}I using chloroglycoluril, and Triton X-100 cell lysates were prepared as previously described[6], except haemoglobin carrier protein was omitted. Lysates were dialysed against 0.1 M NaCl, 0.05 M sodium borate, *p*H 9, and 800-μl aliquots were either mixed with 16 μl of 5 mg ml^{-1} dithiobis(succinimidyl propionate) (Pierce) dissolved in dimethylformamide, or not treated. After 1 h at 21 °C, samples were dialysed against two changes of 0.14 M NaCl, 0.05% NaN_3, 0.01 M Tris HCl *p*H 7.5. Aliquots were immunoprecipitated with antibodies coupled to Sepharose and subjected to SDS-5% PAGE and autoradiography as previously described[6]. *a*, precipitates from PEC with M1/70 MAb- or normal rat IgG–Sepharose. *b*, Precipitates from EL-4 with M17/4 MAb- (anti-LFA-1, Sanchez-Madrid *et al.*, unpublished) or normal rat IgG–Sepharose. Samples were cross-linked (XL) and either reduced (Red) with 5% 2-mercaptoethanol or treated with 50 mM iodoacetamide before electrophoresis as indicated in the figure. Molecular weights were determined with standards as previously described[6]. Molecular weights >200,000 were determined by extrapolation and should be considered tentative. Cross-linked products were fuzzier than other bands, because the number and position of the cross-links formed affects hydrodynamic properties in SDS-PAGE.

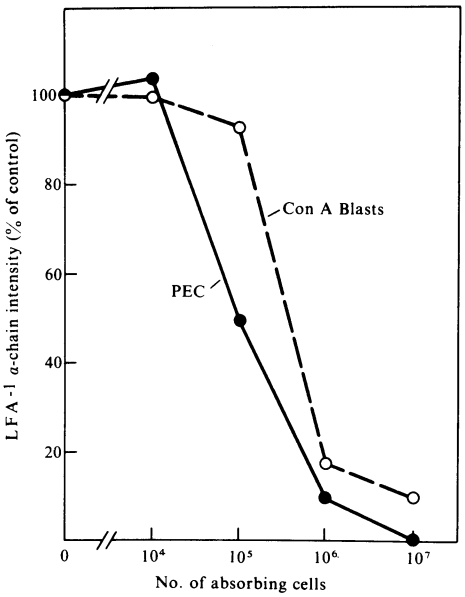

Fig. 4 Absorption by peritoneal exudate macrophages and Con A blasts of the ability of AMGS to precipitate LFA-1 from Con A blasts. AMGS (10 μl of a 1:100 dilution, which gave 70% maximal LFA-1 precipitation) was absorbed with PEC or Con A blasts in 100 μl of phosphate-buffered saline for 2 h at 4 °C. Cells were pelleted, and 80 μl of supernatant was used in indirect precipitation of ^{125}I-labelled Con A blast lysates and subjected to SDS-PAGE as described in Fig. 2 legend. After autoradiography with intensifying screens and hypersensitized Kodak XAR film, the film was scanned with a densitometer and LFA-1 α-chain was quantified as peak area.

To determine whether precipitation of LFA-1 by AMGS was due to a true cross-reaction of anti-Mac-1 antibodies with LFA-1, or to the presence of antibodies with independent specificities in the AMGS, it was important to test whether the anti-LFA-1 activity could be absorbed with thioglycollate-elicited macrophages, which are Mac-1$^+$ LFA-1$^-$. Therefore, AMGS was absorbed with either macrophages or Con A blasts, and the amount of LFA-1 precipitated from Con A blasts was measured by quantitative scanning of autoradiograms (Fig. 4). Despite their lack of LFA-1 expression, macrophages gave complete absorption. Furthermore, the macrophages were three-fold more effective than Con A blasts in absorbing anti-LFA-1 antibodies, showing absorption could not be attributed to contaminating lymphoid cells. The greater effectiveness of the macrophages was consistent with our previous findings that macrophages express 16×10^4 Mac-1 sites per cell while Con A blasts express 7.8×10^4 LFA-1 sites per cell[2,6]. It was concluded that antibodies to Mac-1 cross-react with LFA-1, and hence these molecules are structurally related.

The structural basis for this homology between Mac-1 and LFA-1 was investigated by peptide mapping. Mac-1 and LFA-1 were purified from the P388D$_1$ macrophage tumour line and the EL-4 T-lymphoma line, respectively, by MAb affinity chromatography, and labelled with ^{125}I. The α- and β-subunits were separated by SDS-PAGE (Fig. 5*a*), excised from gels and subjected to peptide mapping. Tryptic peptide mapping showed the Mac-1 and LFA-1 β-subunits share at least 10 tyrosyl peptides, and are thus highly homologous or identical (Fig. 5*e–g*). One peptide unique to the LFA-1 β-subunit and one increased in intensity (arrows) may be due to carbohydrate processing differences between the EL-4 and P388D$_1$ tumour lines, rather than to sequence differences. Cleveland peptide maps of LFA-1 and Mac-1 β-chains isolated from Con A blasts and PEC were identical.

The α-subunits had very different tyrosyl tryptic peptide maps (Fig. 5*b–d*). The α-chains from Mac-1 and LFA-1 showed 17 and 18 unique tryptic peptides, respectively. Co-migration of one peptide (marked by an arrow) was confirmed by a mixing

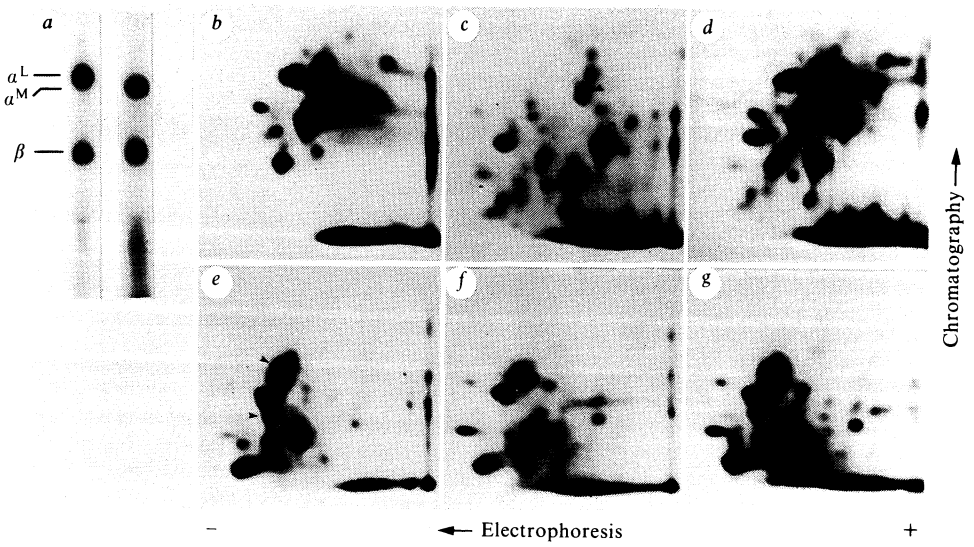

Fig. 5 Tryptic peptide maps of SDS-PAGE purified LFA-1 and Mac-1 α- and β-subunits. LFA-1 and Mac-1 were purified from EL-4 and P388D$_1$ cells, as will be described in detail elsewhere. Briefly, EL-4 or P388D$_1$ detergent lysates in the presence of protease inhibitors were purified by affinity chromatography on M7/14 or M1/70 coupled to Sepharose CL-4B, respectively, and eluted with 20 mM glycine-NaOH, *p*H 10, or 20 mM triethanolamine, *p*H 11, respectively, in 0.5% Triton X-100, 1 mM phenylmethylsulphonyl fluoride. Eluates were neutralized and repurified by a second cycle of affinity chromatography. Purified antigens were concentrated and dialysed against phosphate-buffered saline, and 10–15 μg was iodinated in a volume of 100 μl with 1 mCi ^{125}I and 6 μg chloramine-T (ref. 10) for 1 min, followed by addition in sequence of 6 μl of 1 mg ml^{-1} $Na_2S_2O_5$, 5 μl of 20 mg ml^{-1} KI and 5 μl of 20 mg ml^{-1} bovine serum albumin. Rat IgG was also iodinated and treated in parallel as a control. After dialysis, the α and β polypeptides were separated by SDS-7% PAGE and the wet gel was autoradiographed for 10 min (panel *a*). *a*, Lane 1, LFA-1; lane 2, Mac-1. Bands were excised and digested with TPCK-trypsin as described by Elder *et al.*[11]. Peptide aliquots (5–8 × 10^4 c.p.m. in 2–4 μl) were spotted on 10 × 11.5 cm cellulose TLC plates (EM Laboratories). Electrophoresis was in 15% acetic acid, 5% formic acid, chromatography was in *n*-butanol/pyridine/acetic acid/water(65 : 50 : 10 : 40), followed by autoradiography. Comparison to the control rat IgG peptides showed that none of the spots were due to free ^{125}I or simple adducts. *b*, LFA-1 α-chain; *c*, Mac-1 α-chain; *d*, mixture of α-chains; *e*, LFa-1 β-chain; *f*, Mac-1 β-chain; *g*, mixture of β-chains. The arrows in *b*, *c* and *d* are explained in the text.

experiment (Fig. 5*d*). These extensive differences strongly suggest that the Mac-1 and LFA-2 α-chains are products of distinct genes.

The large structural differences in the α-subunits suggest that the unique determinants recognized by the M1/70 and M7/14 MAb reside on these subunits. The β-subunits seem to be immunoprecipitated by virtue of their noncovalent association with the α-subunits. The α- and β-subunits do not appear structurally related, because they showed no tryptic peptide map identities. It seems likely that the common determinants on LFA-1 and Mac-1 revealed by polyclonal anti-Mac-1 antibodies are on the β-chain, although it is possible that the α-chains also cross-react. Definitive proof of where the determinants lie will require testing of antibodies on isolated chains.

Mac-1 and LFA-1 comprise a novel family of leukocyte differentiation antigens which contain noncovalently associated *a* and β polypeptides of 170,000–180,000 M_r and 95,000 M_r, respectively. Alternative forms of the α polypeptide can be associated with a common or highly homologous β polypeptide in $\alpha_1\beta_1$ structures. Only two other two-chain leukocyte cell-surface antigen families have previously been described, the major histocompatibility antigens[8] and immunoglobulins[9]. By analogy with these and other protein families such as the haemoglobins which have shared homologous or identical subunits, it seems likely that the Mac-1 and LFA-1 α-chains are also products of closely linked, homologous genes. The tryptic peptide differences in the α-subunits do not rule out homology, since amino acid sequence homologies of the order of 25–50% would be missed by peptide mapping. The selective expression of the Mac-1 and LFA-1 α-chains in the monocytic and lymphoid lineages is a particularly interesting feature of this differentiation antigen family. The structural relationships between these molecules suggest that the α- and β-chains may mediate specialized and general functions, respectively. LFA-1 plays an important part in antigen-specific T-lymphocyte-mediated killing of tumour cells and in T-helper cell responses[4,5]. Elucidation of the function of Mac-1 would provide a further comparison with LFA-1, and might provide important insights into the structure–function relationships of the α- and β-subunits.

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