It has been proposed that CD2 mediates both cell-cell adhesion and antigen-independent activation reactions. The former function is well-established[2,3,18], but the case for the latter still rests on the unique properties of antibodies such as 9-1 in triggering proliferation in the presence of either group I antibodies[7,14] or sheep erthrocytes[19-21]. The first epitope region we have identified is probably important in both the adhesion and activation functions of CD2. Binding of the first region by antibodies allows CD2 to respond to subsequent binding of antibody 9-1 and we predict that LFA-3 binding to CD2 would allow comparable response to 9-1. If so, the adhesion and activation mediated by CD2 are intimately related and not distinct functions.

Because 9-1 does not block erythrocyte rosetting, and because a CD2 variant which does not react with mAb 9-1 still binds erythrocytes, it is unlikely that LFA-3 binding alone can cause activation; further analysis of the region recognized by 9-1 antibodies is necessary for insight into the activation mechanism.

Recently the case for inclusion of CD2 in the immunoglobulin superfamily[22] has been strengthened by discovery of highly significant homologies between CD2 and non-immunoglobulin members of the family[23]. Alignment of the N-terminal 115 residues of CD2 with immunoglobulin κ variable sequences shows that the CD2 regions 1 and 2 correspond to the locations of light chain hypervariable (antibody-combining site) regions 2 and 3 (Fig. 1). This suggests that CD2 ligand-binding sites are phylogenetically related to variable region-combining sites, and supports the idea that adhesion interactions between members of the immunoglobulin superfamily can mimic antibody-antigen interaction.

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Anchoring mechanisms for LFA-3 cell adhesion glycoprotein at membrane surface

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The manner in which a membrane protein is anchored to the lipid bilayer may have a profound influence on its function. Most cell surface membrane proteins are anchored by a membrane-spanning segment(s) of the polypeptide chain, but another type of anchor has been described for several proteins: a phosphatidyl inositol glycan moiety, attached to the protein C terminus[1,2]. This type of linkage has been identified on membrane proteins involved in adhesion[3] and transmembrane signalling[4,5] and could be important in the execution of these functions. We report here that an immunologically important adhesion glycoprotein, lymphocyte function-associated antigen 3 (LFA-3), can be anchored to the membrane by both types of mechanism. These two distinct cell-surface forms of LFA-3 are derived from different biosynthetic precursors. The existence of a phosphatidyl-inositol-linked and a transmembrane anchored form of LFA-3 has important implications for adhesion and transmembrane signalling by LFA-3.

LFA-3 is a cell-surface glycoprotein found on erythrocytes, epithelial cells, endothelial cells, fibroblasts and most cells of haematopoietic origin[6]. LFA-3 interacts with the T lymphocyte CD2 membrane glycoprotein, and this ligand-receptor pair mediates intercellular adhesion between LFA-3$^+$ cells and thymocytes, natural killer cells, cytolytic T lymphocutes, and other mature T lymphocytes[6-11]. Cell surface LFA-3 and LFA-3 reconstituted into artificial membranes can both activate T lymphocytes in conjunction with other signals[12,13], which is consistent with the ability of pairwise combinations of anti-CD2 monoclonal antibody (MAb) to activate CD2$^+$ cells[6]. Recently, we have found that LFA-3 is deficient in affected erythrocytes in patients with paroxysmal nocturnal haemoglobinuria (PNH) (ref. 14), an acquired disorder affecting phosphatidyl inositol (PI)-linked proteins[15]. This suggests that LFA-3 is attached to the surface of human erythrocytes by a PI-glycan moeity.

We first obtained evidence for distinct forms of LFA-3 when studying its biosynthesis in the JY B lymphoblastoid cell line. Labelling of JY cells for one minute with [^{35}S]methionine, followed by a five minute chase and isolation with anti-LFA-3-Sepharose, revealed two distinct LFA-3 precursors of relative molecular mass (M_r) of 41,000 (41K) and 37,000 (37K) (Fig. 1*a*, lane 2 arrows). Chase for 10 and 20 minutes showed a gradual decrease in size of the precursors to 39K and 35K (Fig. 1*a*, lanes 3 and 4), which is probably due to trimming of glucose and mannose residues from high-mannose oligosaccharides[16]. There was no apparent interconversion of the two precursors, even during biosynthesis at 24 °C. After chase for 20-40 minutes, the precursors were converted to the mature form of LFA-3, which migrated as a broad band of mean size 65K (Fig. 1*a*, lanes 4 and 5) and corresponded to the form surface-labelled with ^{125}I (ref. 6).

Endoglycosidase H (Endo H) treatment of the 41 and 37K LFA-3 precursors resulted in two bands of corresponding intensity of 29K (p29) and 25.5K (p25.5) respectively (Fig. 1*b*, lanes 1 and 2), but had no effect on mature LFA-3 (Fig. 1*b*, lanes 5 and 6). Therefore each LFA-3 precursor contains 12K of high mannose *N*-linked oligosaccharides which are converted to endo H-resistant complex *N*-linked oligosaccharides during glycoprotein maturation. *N*-glycanase treatment converted both the precursor and mature forms of LFA-3 to two bands at 29K

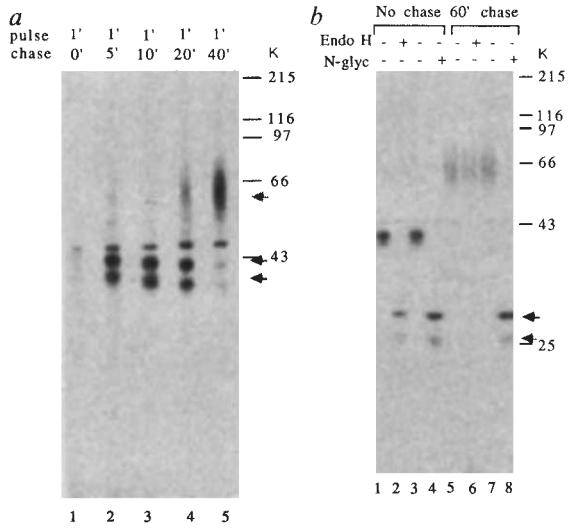

Fig. 1 Biosynthesis of LFA-3. *a*, JY cells were pulsed with ^{35}S-methionine and chased for the indicated times. LFA-3 samples were subjected to reducing SDS-9%-PAGE and fluorography. Solid arrows indicate LFA-3 precursors, hatched arrow indicates mature LFA-3. *b*, As in *a*, except JY cells were pulsed for 5 min and chased as indicated and SDS-denatured LFA-3 samples were incubated with or without 12 mU ml^{-1} Endo H (ref. 26) or 2.5U ml^{-1} *N*-glycanase (*N*-gly)[17] in 50 μl and run on reducing SDS-12%-PAGE. Solid arrow indicates p29, hatched arrow indicates p25.5. The band at 45K on both gels is consistent with actin and was present in MAb-Sepharose isolates of other membrane proteins.

Methods. 5 × 10^6 JY cells were labelled with 0.5 mCi ^{35}S-methionine and lysed[23]. Isolation with anti-LFA-3-Sepharose[11] was as described, except MAb-Sepharose was washed twice with buffer containing 1% Triton X-100, 1% deoxycholate and 0.1% SDS (ref. 24) after incubation with the lysate and before continuing with the washing procedure[25]. SDS-PAGE and fluorography were performed as described previously[23].

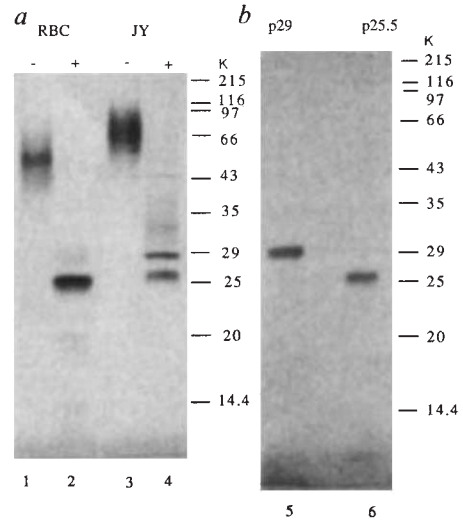

Fig. 2 Purification of LFA-3. LFA-3 was immunoaffinity purified[11] from red blood cells (RBC) (lanes 1 and 2) or JY cells (lanes 3 and 4) and treated with *N*-glycanase[17] (lanes 2 and 4). p29 and p25 from preparative SDS-PAGE of LFA-3, which had been purified from JY cells and treated with *N*-glycanase, were applied to lanes 5 and 6.

Methods. The purification procedure[11] was modified for JY in that 50 g of JY cells were lysed in 500 ml of 2% Triton X-100 in buffer[11] and centrifuged sequentially for 10 min. at 2,000*g* and 2 h at 150,000*g* before affinity chromatography of the supernatant[11]. Preparative PAGE and electroelution were as described[17]. SDS-12%-PAGE with silver staining[11] used 100–500 ng protein.

and 25.5K (Fig. 1*b*, lanes 3,4 and 7,8). These bands were essentially identical in size to those from Endo H treatment of the LFA-3 precursors, allowing for the *N*-acetylglucosamine residue left at *N*-glycosylation sites by Endo H, but not by *N*-glycanase. After the 60 minute chase, both forms of LFA-3 were fully sensitive to proteinase K (not shown). Thus, as shown by studies on their p29 and p25.5 backbones, both the 41K and 37K precursors yield distinct forms of mature, cell surface LFA-3.

The 41K and 37K precursors were seen in similar relative amounts in all cell lines examined, despite the wide size variation in the mature form of LFA-3: Reid-Sternberg line L428, 64K; plasmacytoma line U266B$_1$, 43K; cervical carcinoma line HeLa, 59K; EBV-transformed B lymphoblastoid line F2B, 76K; T lymphoma line Jurkat, 64K; concanavalin A T lymphoblasts, 68K and adherent peripheral blood mononuclear cell fraction, 55K (not shown). The size heterogeneity of mature LFA-3 was due to *N*-linked carbohydrate, as shown with *N*-glycanase; this heterogeneity caused the 2 forms of mature LFA-3 to partially overlap in SDS-PAGE. The size of LFA-3 determined in reducing and non-reducing gels is similar, so LFA-3 does not exist in a disulphide-linked complex on the cell surface (not shown). Immunoaffinity isolation after denaturation in SDS at 100 °C (Fig. 3*b* legend) showed that both p29 and p25.5 possess the TS2/9 (LFA-3 MAb) epitope.

LFA-3 purified[11] in 100 μg quantities from JY B lymphoblastoid cells yielded both p29 and p25.5 after *N*-glycanase digestion (Fig. 2, lanes 3 and 4), as predicted by our biosynthesis studies. In contrast, LFA-3 purified from erythrocytes yielded p25.5 after deglycosylation, and only a trace amount of material comigrating with p29 (Fig. 2, lanes 1 and 2). To determine the relatedness of p29 and p25.5 from JY cells, they were separated by preparative SDS-PAGE (Fig. 2, lane 5 and 6) and 50–80 pmol was subjected to gas phase *N*-terminal sequencing[17]. The *N*-terminal sequence FSQQIYGVVYGDVTFHVPSNVPL was obtained for both p29 and p25.5, and is identical to the *N*-terminal sequence previously described for *N*-glycanase-treated erythrocyte LFA-3 (ref. 17). Therefore, p29 and p25.5 are distinct forms of LFA-3 which share a common epitope and *N*-terminal sequence.

We determined whether one of these forms was selectively released from the surface of JY cells by phosphatidyl inositol-specific phospholipase C (PIPLC). Surface iodinated JY cells were treated with or without PIPLC. LFA-3 remaining on the cell surface was isolated with anti-LFA-3-Sepharose from detergent lysates of the cell pellets and LFA-3 released by PIPLC was isolated from the medium. A portion of cell surface LFA-3 was specifically removed by PIPLC (Fig. 3, lanes 1–4). To resolve which form(s) was released, aliquots of the isolated LFA-3 were digested with *N*-glycanase before SDS-PAGE (Fig. 3, lanes 5–8). PIPLC treatment resulted in no change in the amount of cell surface p29 and released no p29 into the supernatant. In contrast, PIPLC greatly decreased the amount of cell surface p25.5 and a strong band at 26K appeared in the supernatant. The slight change in the mobility of p25.5 after removal of phosphatidylinositol is consistent with reports on other PI-anchored proteins[15].

The PIPLC sensitivity of p25.5 shows that it is a PI-anchored form of LFA-3, but only indirectly indicates that p29 has a polypeptide membrane anchor. The deduced amino-acid sequence of an LFA-3 complementary DNA clone[17] predicts a signal sequence, an 187-amino-acid extracellular domain, a 23-amino-acid transmembrane domain, and a 12-amino-acid cytoplasmic domain. The cytoplasmic tail has potential trypsin cleavage sites 4, 8, and 11 amino acids from the C terminus. In microsomes, the LFA-3 C terminus is predicted to be external. Cells were labelled with [^{35}S]-methionine for 4 minutes, broken in a dounce homogenizer, and the trypsin sensitivity of the LFA-3 precursors in microsomes was assessed[18]. Trypsin treatment of intact microsomes resulted in a 0.5K decrease in p29

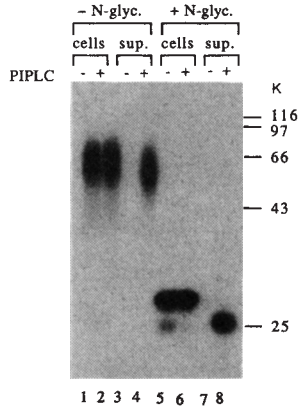

Fig. 3 PI-anchored form of LFA-3. LFA-3 immunoprecipitated from cells treated with or without PIPLC was treated with or without *N*-glycanase, as indicated. 'Cells' indicates LFA-3 from detergent-lysed cells; 'sup' indicates LFA-3 from the media in which the cells were treated with or without PIPLC.LFA-3 immunoaffinity isolated from Triton X-100 lysates of surface iodinated cells (lane 5) had a higher ratio of p29 to p25.5 compared to lysates of cells metabolically labelled for less than 2 h. Greater recovery of LFA-3 after solubilization with PIPLC could be due to more efficient solubilization or immunoaffinity isolation. Resistance of PI-linked proteins in the plasma membrane to solubilization with Triton X-100 has been reported[26] supporting the latter.
Methods. JY cells were iodinated, washed, and treated with *S. aureus* PIPLC (ref. 14). Immunoprecipitation was from lysates of washed cells of from 100,000*g* supernatants of the media, as described. Other methods as in Fig. 1, except that autoradiography was used instead of fluorography[14].

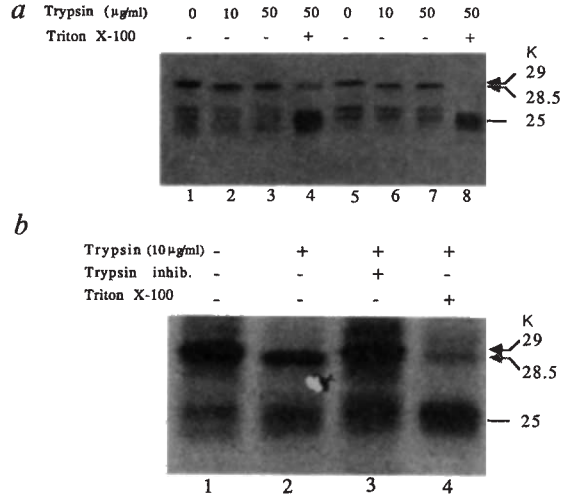

Fig. 4 Transmembrane polypeptide anchored form of LFA-3. *a*, and *b*, LFA-3 precursors, isolated by two cycles (*a*) or one cycle (*b*) of immunoprecipitation from microsomes treated as indicated, were subjected to SDS-12%-PAGE.
Methods. L428 cells (5×10^7) were pulsed for 4 min with 4 mCi of ^{35}S methionine and Dounce-homogenized in a hypotonic buffer[18] containing 2 mM dithiothreitol. The post-nuclear supernatant was treated with or without 10 or 50 μg ml^{-1} trypsin, 0.5 mg ml^{-1} soy bean trypsin inhibitor and/or 0.1% Triton X-100 for 1 h at 4 °C (*a*, lanes 1–4; *b*) or 24 °C (*a*, lanes 5–8) and the reaction was terminated with soy bean trypsin inhibitor. LFA-3 was subjected to a single cycle of isolation with MAb-Sepharose (*b*) or to an additional cycle of isolation after elution from MAb-Sepharose with 0.5% SDS and addition of a sevenfold excess of Triton X-100 (*a*) (ref. 27). The second immunoprecipitation step was to reduce background bands. Digestion with *N*-glycanase and SDS-12%-PAGE and fluorography as in Fig. 1.

(Fig. 4*a* and *b*, lane 2, compare lane 1). The apparent size decrease in p29 was blocked by prior addition of soy bean trypsin inhibitor to the post-nuclear supernatant (Fig. 4*b*, lane 3). When detergent was included, the bands at 28.5K and 25.5K were decreased in intensity or eliminated (Fig. 4*a*, lanes 4 and 8, Fig. *b*, lane 4) and a band of 24–25K appeared instead. There was no shift detected in p25.5 in the absence of Triton X-100. The trypsin sensitivity of the p29 form of LFA-3 in intact microsomes strongly suggests that it is a transmembrane protein.

We have found two cell-surface forms of LFA-3 which have distinct PI and polypeptide chain membrane anchors and are derived from distinct biosynthetic precursors. Our results are concordant with findings on LFA-3 in PNH. PNH, an acquired clonal defect in hematopoietic cells, leads to a selective absence of PI-anchored proteins[1,15]. Our result that p25.5 has a PI membrane anchor is in agreement with the finding that the erythrocyte form of LFA-3 corresponds to p25.5, and that LFA-3 is deficient on PNH erythrocytes[14]. In contrast, neutrophils and monocytes from PNH patients which are clonally affected, as shown by complete deficiency of decay accelerating factor, express only slightly reduced quantities of LFA-3 (M. E. Medof, P.S., M.L.D., D. G. Ayers, E. I. Walter, H. A. Stafford, R. Green, M. L. Tykocinski and T.A.S., unpublished). We predict that the form of LFA-3 on the surface of affected leukocytes from PNH patients is p29. p29 may correspond to LFA-3 exactly as encoded by a recently isolated LFA-3 cDNA (ref. 17), whereas p25.5 might be encoded by the same messenger RNA or a different mRNA, possibly generated by alternative splicing, as reported for neural cell adhesion molecule[19].

In addition to mediating intercellular adhesion by binding to CD2, LFA-3 might be able to transduce signals, as the binding of anti-LFA-3 MAb to monocytes, thymic epithelial cells and keratinocytes enhances the release of IL-1 (ref. 20). The presence of PI- and polypeptide-anchored forms of LFA-3 on the surface of nucleated cells raises the possibility of division of the adhesion and signalling functions between these two form of LFA-3. The PI-anchored form is predicted to have a significantly faster lateral mobility in the cell membrane[21], which would facilitate diffusion of LFA-3 to sites of adhesion with CD2$^+$ cells[22]. Expression of PI- and polypeptide anchored form of neural cell adhesion molecule has also been reported[3,19], suggesting that the expression of ligand binding domains of adhesion molecules with different types of membrane anchorage may be a general strategy for regulating adhesiveness or other functions.

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