Distinct Mutations in Two Patients with Leukocyte Adhesion Deficiency and Their Functional Correlates

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Summary

Two patients with leukocyte adhesion deficiency (LAD), one with a moderate phenotype (patient 14) and one with a severe phenotype (patient 2) who had been shown to have a normal sized β subunit protein precursor, were analyzed in an attempt to determine the molecular basis for their disease. RNase mapping located possible mutations to two distinct but adjacent regions of the β subunit cDNA. Sequencing of patient-derived cDNA clones in this region revealed a C for T difference at amino acid 149 in patient 14 which resulted in the substitution of a leucine for a proline, and an A for G substitution at amino acid 169 in patient 2 which mutated a glycine to an arginine. The mutated amino acids are in a region of the cDNA that is highly conserved between the β subunits of the integrin family and are identical in all known integrin β subunits. Co-transfection of the β subunit cDNA containing the patient 2 mutation with the wild-type α subunit of LFA-1 in a mammalian expression system resulted in no expression of LFA-1. In the case of the mutation in patient 14 there was markedly diminished expression of LFA-1 with loss of function and loss of the epitope for a number of anti-β mAbs. Normal half-life of the mutant β subunits, and previous demonstration of a lack of α/β complex formation during biosynthesis in patient cells, suggest a defect in association with the α subunit. Association with β is required for expression of the α subunit of LFA-1. Loss of functional expression with both of these β subunit mutations suggests that they lie in a site critical for association with the α subunit.

L eukocyte adhesion deficiency (LAD)[1] is a rare, inherited, autosomal recessive, immunodeficiency disease caused by the combined loss of expression on the surface of leukocytes of the leukocyte integrins LFA-1, Mac-1, and p150,95 (reviewed in reference 1). The disease is characterized by a marked granulocytosis, lack of neutrophil and monocyte mobilization to inflammatory sites as seen in biopsies and manifested in lack of pus formation, severe gingivitis and recurrent or progressive necrotic soft tissue infections. About 30 patients have so far been described with the disease. Based on degree of expression the disease was subdivided into severe (<1% expression) and moderate (3–10% expression) phenotypes (2). Degree of expression correlated with the clinical severity of the disease and the impairment of a number of in vitro immune functions such as granulocyte adhesion and chemotaxis and antibody-dependent cytotoxicity. Patients with the severe form of the disease tend to succumb early in life to overwhelming infection, whereas those with a moderate form of the disease often survive through to adulthood.

The three leukocyte integrins are noncovalently linked het-erodimers composed of a common β subunit (CD18) and homologous α subunits (LFA-1α, CD11a; Mac-1α, CD11b; p150,95α, CD11c) (3). They play a key role in a wide range of immune functions (reviewed in reference 4). They are part of a family of related receptors termed the integrins (4–6). Three integrin subfamilies are defined by distinct β subunits that can associate with multiple α subunits. The CD11/CD18 family is otherwise known as the leukocyte or $\beta2$ integrin family. The leukocyte integrins mediate cell–cell adhesion by binding to counter-receptors on other cells. The adhesion receptor LFA-1 binds to the counter-receptors ICAM-1 and ICAM-2 (7, 8). ICAM-1 expression is strongly induced in tissues by inflammatory stimuli (9). Avidity of LFA-1 for ICAM-1 is transiently increased by TCR stimulation and this is mimicked by phorbol esters (10). The $\beta1$ and $\beta3$ families are primarily receptors for extracellular matrix proteins (11). The $\beta1$ (VLA) family includes the fibronectin receptor (12) and receptors for collagen and laminin (13, 14). The $\beta3$ family includes the vitronectin receptor and the platelet glycoprotein IIb/IIIa (15). $\beta2$ shares 45% overall identity with $\beta1$ and 37% with $\beta3$ (16–19). There is, however, a highly conserved region in the extracellular domain of 241 amino acids in which the three β subunits are 65% identical. Contained

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[1] *Abbreviation used in this paper:* LAD, leukocyte adhesion deficiency.

within this region is an RGD binding site for platelet glycoprotein IIb/IIIa (20).

Several lines of evidence have indicated that the defect in LAD is in the common β subunit of the leukocyte integrins. All the patients so far described have loss of each of the three leukocyte integrins. In mouse/human lymphocyte hybrids it was possible to recover expression of the human α subunit of LFA-1 in combination with the mouse β subunit; recovery of the human β subunit in combination with the mouse α subunit was seen in hybrids with healthy but not with patient cells (21). Biosynthetic studies of the α subunit of LFA-1 in patient EBV-transformed B cells demonstrated that apparently normal synthesis of the LFA-1 α subunit occurred in LAD patients but that it failed to be processed to the mature form and was degraded without being expressed (22). Similarly using a rabbit antisera against the β subunit and a β subunit cDNA probe it was demonstrated that in a number of LAD patients the β subunit precursor was made in normal amounts but like the α subunit was degraded (23, 24). Studies of mRNA expression and biosynthesis of the β subunit in six unrelated patients and a family of four patients revealed five patterns of abnormality (24). Definitive evidence for a defect in the β subunit was found in two patients that had no mRNA or protein precursor, one patient that had trace mRNA and protein, and a family of patients that had a small protein precursor. One patient had a large precursor, probably due to an extra glycosylation site, and two patients had grossly normal mRNA expression and β subunit protein precursor. Whether these patients had point mutations in the β subunit was not determined. Heterogeneity in the defect has also been reported in a group of patients in France (25). The defect in the family with the small protein precursor was due to a single base pair substitution which resulted in aberrant splicing and the deletion of a 90-bp exon in 95% of the mRNA (26). The 5% of normally spliced mRNA allowed some expression of normal protein on the cell surface and explained the moderate phenotype in this kindred.

The molecular basis for LAD in patients who have a β subunit precursor of normal size and amount is not known and location of the defect in the β subunit remains presumptive. If the defect is in the β subunit in these patients it is likely to be subtle and due to a single amino acid substitution. Definition of the defect in these cases would help to confirm that all forms of LAD are due to defects in the β subunit as well as defining regions of the β subunit which are of importance in associating with the α subunit. In addition it may shed further light on the reasons for the different clinical phenotypes.

We therefore analyzed, by RNAse mapping, the RNA from two patients, both of whom expressed grossly normal mRNA and β subunit protein precursor but who had different clinical phenotypes. This revealed possible sites of mismatch in the region highly conserved among integrin β subunits. Sequence analysis of cDNA clones from this region revealed single amino acid substitutions in each of the two patients. Both mutations caused defective expression and function of the β subunit in COS cells.

Materials and Methods

Monoclonal Antibodies. The mAbs TS1/22 (LFA-1 α mAb, IgG1), TS1/18 (β mAb, IgG1), W6/32 (HLA-A,B mAb, IgG2a), RR1/1 (ICAM-1 mAb, IgG1), and X63 (myeloma IgG1), have been described (10). All other mAbs were obtained through the 4th International Leukocyte workshop myeloid and lymphocyte panels.

Cell Lines. Previously established EBV-transformed cell lines were maintained in RPMI 1640 medium supplemented with 20% FCS, 2 mM glutamine, and 50 μg/ml gentamicin (complete medium) at 37°C in a humidified atmosphere of 5% CO_2. Patients 2 and 14 are unrelated and had nonconsanguineous, Caucasian parents (2). Patient 2 had a severe phenotype and patient 14 a moderate phenotype. Both patients expressed grossly normal β subunit mRNA and precursor protein. Patients 1 and 15 investigated in the RNase protection assay had been shown to have normal RNA and precursor protein (24 and unpublished observation, respectively). JY is a long-established EBV-transformed B cell line. Patient cell lines and the CO3 healthy control line were transformed in parallel with EBV (22, 24). Patient numbers refer to reference 24. COS cells are SV40 transformed monkey kidney cells.

Oligonucleotides. Oligonucleotides were synthesized on a 380B DNA synthesizer (Applied Biosystems, Foster City, CA). Nomenclature was according to direction (sense βS, antisense βAS) followed by the number of the first oligonucleotide 5' base in the cDNA followed by m if encoding a mutation. Priming of cDNA libraries: Patient 14, 17mer βAS1138 (5'-ATTAAGAATGCTTA-CAA-3'); Patient 2, 26mer βAS2468 (5'CCTCAAGAGCTGTG-GCCAAGCCATG-3'). PCR: sense oligonucleotide encoding BamHI site, 20mer βS233 (5'-GGGGGATCCTGACTCCATTC-3'); antisense 20mer βAS780 (5'-GCCCACCCTCGGGTGCATCC-3'). Site-directed mutagenesis: 41mer βS490m, (5'-TTGATGAC-CTCAGAAATGTCAAGAAGCCAGGTGGCGACCTG-3'). Sequencing: 20mers βS40, βS233, βS375, βS508, βS570, βS800, βAS203.

Ribonuclease Protection Assay. This was performed as described (27). Total RNA was prepared from patient and normal EBV-transformed cells by the method of Chirgwin et al. (28). To prepare single-stranded RNA probes four fragments of ~700 bp covering most of the coding region of the β subunit cDNA (Fig. 1 A) were each subcloned into a plasmid vector (pT7T3 18u; Pharmacia Fine Chemicals, Piscataway, NJ) that contained T7 and T3 promoters flanking the polylinker. The plasmid template was transcribed in the presence of 100 μCi of α-[^{32}P] UTP (10 Ci/ml, 800 Ci/mmol; New England Nuclear, Boston, MA) and 10 U of T7 or T3 RNA polymerase (Pharmacia). The reaction mixture was incubated for 45 min at 37°C. The template was then digested with RNase-free DNase (Boehringer Mannheim Biochemicals, Indianapolis, IN) and unincorporated nucleotides removed by ammonium acetate precipitation. The probe was dissolved in hybridization buffer and 5 × 10^5 cpm added to 10 μg of patient or control RNA and incubated overnight at 45°C. The RNA was digested with ribonuclease A and T1 for 45 mins at 30°C. In some experiments RNase U_2 (Boehringer Mannheim) and RNase M (Bethesda Research Laboratories, Gaithersburg, MD) (50 U/ml) were added. Protected RNA was analyzed on a denaturing polyacrylamide/urea sequencing gel.

cDNA Library. Patient mRNA was obtained from total RNA (prepared as above) by passing through an oligo(dT)-cellulose type 3 column (Collaborative Research Inc., Lexington, MA). The cDNA was synthesized by the method of Gubler and Hoffman (29) using a specific β antisense oligonucleotide as a primer and avian myeloblastosis reverse transcriptase (24 units/μl; Life Sciences, St. Petersburg, FL). Second strand synthesis was achieved using

DNA Polymerase I (*Escherichia coli*) (New England Biolabs, Beverly, MA), and RNase H (Boehringer Mannheim). The cDNA was ligated to λgt10 (Stratagene, La Jolla, CA). 5 × 10⁴ colonies were screened with a nick-translated BamHI/EcoRI fragment of the β subunit cDNA. After two rounds of plaque purification the cDNA clones were subcloned into pGem7 (Promega Biotech, Madison, WI) using the EcoRI cloning site.

Taq Polymerase Reaction. This was performed as described (30). First strand synthesis with reverse transcriptase using a 20mer antisense oligonucleotide β AS780 as a primer was performed on 15 μg of total RNA from patient 14. A 20mer sense oligonucleotide βS233 encoding the BamHI site was then added together with 5 U of Taq polymerase (5 U/μl; U.S. Biochemical Corp., Cleveland, OH). 35 cycles of denaturation (94°C, 60 s), annealing (37°C, 2 min), and polymerization (68°C, 3 min) were undertaken with a further 5 U of Taq polymerase added after the 25th cycle. After the 35th cycle the mixture was incubated at 68°C for 10 min. Of the reaction mixture, 1/10th was analyzed on a 1% agarose gel to confirm the presence of the amplified cDNA fragment. The remainder of the mixture was blunt ended with the large fragment of *E. coli* DNA polymerase I, heated to 70°C for 15 min, digested with BamHI, and the 550 bp band excised from a low melting point agarose gel. The DNA was subcloned into pGem7 using the BamHI and SmaI cloning sites. Competent JM83 *E. coli* were transformed and plasmids containing the correct size insert were sequenced.

Generation of Mutant and Revertant Forms of β Subunit cDNA in pCDM8: (a) Patient 14. The mutation found in patient 14 was introduced by site-directed mutagenesis (31–33) into the wild-type β subunit cDNA cloned into pCDM8 (34), using oligonucleotide βS490m which encoded the single base pair substitution as well as a silent mutation which erased the unique Bsu36 I site. Two tests confirmed that defective expression was due to the mutation, and not to an incidental change in the plasmid. First, a fragment bounded by a vector EcoRI site upstream of the 5' untranslated region and the internal EcoRI site at 965 bp was sequenced after site-directed mutagenesis to confirm that no other substitution had occurred. Second, the mutant β cDNA in pCDM8 was reverted to wild type by subcloning a 1.0 kb wild-type EcoRI fragment into the mutant β.

(b) Patient 2. The mutation present in the patient 2 cDNA was introduced into the wild-type β subunit cDNA by exchanging a 530-bp Bsu36I/BstEII restriction fragment (nucleotides 500–1,030). This 530-bp region was sequenced to confirm that it encoded only one amino acid substitution. The mutant was reverted to wild type by subcloning the wild-type Bsu36I/BstEII fragment back into the patient 2 mutant β subunit cDNA.

Expression of Mutant Forms of LFA-1. Wild-type, mutant, and revertant forms of the β subunit were expressed in COS cells by co-transfection with the wild-type α subunit of LFA-1 by DEAE-Dextran transfection (35) using 12 μg of each of the α and β subunits cDNAs. 3 d after transfection the cells were detached with 10 mM EDTA in HBSS and then either prepared for flow cytometric analysis, ¹²⁵I labeling, or binding to ICAM-1–coated plates.

Flow Cytometric Analysis. Immunofluorescence flow cytometry was performed on an EPICS V analyzer after staining cells with mAb-containing supernatants or a 1/200 dilution of ascites followed by FITC-conjugated anti-mouse antibody (Zymed Laboratories, San Francisco, CA) as described (36). Cells were analyzed either unfixed or fixed in 1% paraformaldehyde.

Surface Iodination. ¹²⁵I labeling of COS cells was performed as described (26). COS cells were harvested, labeled with ¹²⁵I (0.5 mCi of 100 mCi/ml Na ¹²⁵I; Amersham Corp., Arlington Heights,

IL) and lysed for 1 h in 1 ml of ice cold lysis buffer (10 mM Tris pH 8.0, 0.15 M NaCl, 1% TX-100, 1% hemoglobin, 1 mM iodoacetamide, 1 mM PMSF, 0.25 TIU/ml Aprotinin, 0.025% NaN₃). After pelleting the nuclei, the lysate was precleared overnight with 100 μl of packed human IgG coupled to Sepharose. The lysate was then immunoprecipitated with 20 μl of packed mAb bound to Sepharose for 2 h. The beads were washed three times in 25 mM Tris pH 8.0, 150 mM NaCl, 0.1% TX-100, twice in 25 mM Tris pH 8.0, 150 mM NaCl, and once in 50 mM Tris, pH 8.0, and analyzed by SDS-PAGE under reducing conditions followed by autoradiography.

Biosynthetic Labeling. COS cells transfected with either the wild-type β subunit, patient 2 mutant β subunit or patient 14 mutant β subunit were pulsed-labeled at a density of 2–4 × 10⁵ cells/10 cm dish for 1 h at 37°C with 500 μCi of [³⁵S]cysteine/methionine in cysteine/methionine-free complete media containing 5% dialyzed FCS. The cells were chased for either 0, 2, 4, or 8 h in media containing 250 μg/ml cysteine and 100 μg/ml methionine. Cells were harvested with HBSS/10 mM EDTA and lysed as described above for surface iodination. Cell lysates were precleared overnight with 5 μl normal rabbit serum and 50 μl packed protein A–Sepharose, then immunoprecipitated for 2 h with either 2 μl normal rabbit serum or 2 μl rabbit anti–denatured β subunit serum (24) and 10 μl packed protein A–Sepharose. Immunoprecipitates were washed as described in the preceding section and subjected to 7% SDS-PAGE and fluorography.

Purification of ICAM-1. The procedure was modified from Marlin and Springer (7). ICAM-1 was purified by affinity chromatography on RR1/1-Sepharose from lysates of hairy cell leukemia spleens as described (34).

Binding of Transfectants to ICAM-1. Purified ICAM-1 was absorbed to wells of a PVC microtiter plate (Flow Laboratories, McLean, VA) as described (10) at a previously determined concentration of 4,000 sites/μm². ICAM-1 site numbers were determined using saturating amounts of ¹²⁵I anti-ICAM-1 mAb and calculated assuming bivalent binding of the mAb. Adherence of 10⁴ Na₂⁵¹CrO₄-labeled transfectants to ICAM-1–coated plates was assayed as described (34).

DNA Sequencing. All sequencing was performed on double-stranded DNA by the dideoxy chain termination method (37) using the protocol recommended by the manufacturer for the use of modified T7 DNA polymerase (Sequenase; US Biochemical Corp.).

Secondary Structure. Predictions used the University of Wisconsin genetics computing group software (38).

Results

Screening Strategy. The strategy of Myers et al. (39) was used to define sites of mutations in patient β subunit RNA. Radiolabeled antisense RNA probes spanning essentially the entire β subunit coding region (Fig. 1 *A*) were hybridized with patient total RNA and sites of mismatch, as evidenced by lack of protection from RNase digestion, were defined by gel electrophoresis. This method has the disadvantage that only certain mismatched bases are recognized by the available RNases, and thus only a subset of mutant alleles will be identified by this technique. However, it has the advantage that identification of the region of mismatch simplifies subsequent nucleotide sequence analysis. Of four patients studied initially, two were found to have sites of cleavage not
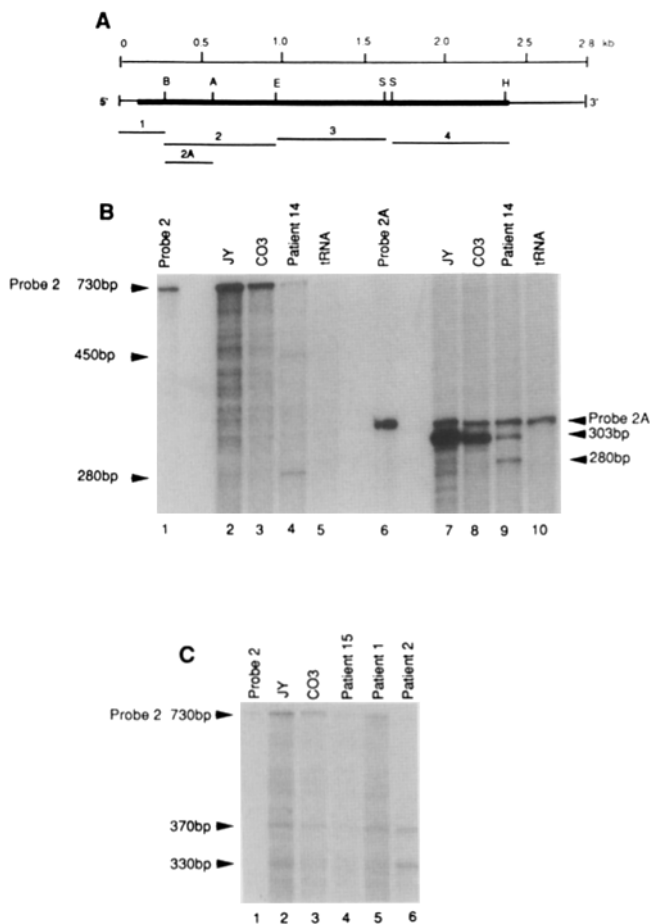
Figure 1. RNase protection assay. (A) Restriction fragments of the β subunit cDNA were subcloned into the plasmid pT7/T3 18U which contains diametrically opposed T3 and T7 promoters. Probes 1–4 span the entire coding region apart from a 42-bp region between the two SmaI sites. Probe 2A is a subfragment of Probe 2. B, BamHI; A, ApaI; E, EcoRI; S, SmaI; H, HgiAI. (B) Single-stranded, radiolabeled probe 2 RNA (lane 1) or probe 2A RNA (lane 6) were hybridized overnight to total RNA from JY (lanes 2 and 7), a control EBV-transformed B cell line CO3 (lanes 3 and 8), an EBV-transformed B cell line derived from patient 14 (lanes 4 and 9), and tRNA as a control for complete digestion (lanes 5 and 10). RNA was digested with ribonucleases A and T1 and protected RNA analyzed by gel electrophoresis. (C) Single-stranded, radiolabeled probe 2 RNA (lane 1) was hybridized overnight to total RNA from JY (lane 2), COS (lane 3), EBV-transformed B cells from patient 15 (lane 4), EBV-transformed B cells from patient 1 (lane 5), and EBV-transformed B cells from patient 2 (lane 6). RNA protected after digestion with ribonuclease was analyzed by gel electrophoresis.

Figure 2. Sequencing of mutations in patient-derived cDNA clones. (A) Patient 14. Using total RNA as a template and oligonucleotides βS233 and βAS780 as primers, a 547-bp fragment of the β subunit cDNA between 233 and 780 bp was amplified by PCR, subcloned into pGEM7, and 10 plasmids were sequenced in the region of the putative point mutation. Representative sequencing ladders are shown for the five patient clones that were wild type in this region and the five clones that encoded substitution of a T for a C at nucleotide 517, which changes a leucine to a proline at amino acid 149. (B) Patient 2. A representative sequencing ladder for the five independent β subunit cDNA clones derived from patient 2 that contain a substitution of a G for an A at nucleotide 577 which changes a glycine to an arginine at amino acid 169 is compared to a wild-type sequencing ladder.

present in controls. We therefore focused on these two patients, numbers 14 and 2.

Patient 14. In patient 14, probe 2 was partially digested to two bands of 280 bp and 450 bp which together added up to the size of the fully protected probe (Fig. 1 B, lane 4). This pattern was confirmed by using the shorter probe 2A, which also gave a 280-bp fragment and localized the defect to the region of nucleotide 513 (Fig. 1 B, lane 9). The probe contained vector sequences and thus protection with wild-type RNA yielded a 303-bp fragment (Fig. 1 B, lanes
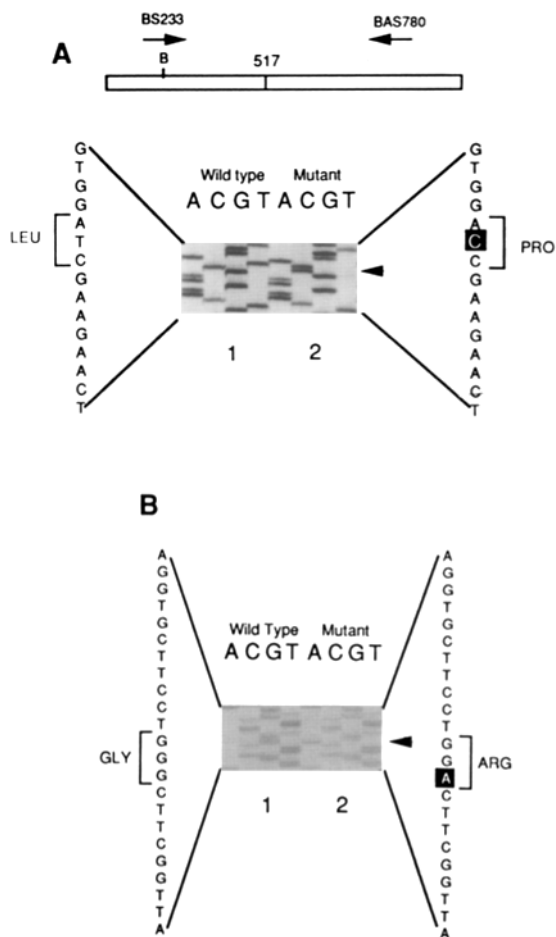
7 and 8); note equal amounts of the 303 and 280 bp fragments were yielded by patient 14 RNA (Fig. 1 B, lane 9).

A classical λgt10 library was constructed from patient 14 mRNA after specific priming with an antisense oligonucleotide at nucleotide 1188 of the β subunit. Six independent, hybridizing clones were plaque purified, subcloned, and sequenced in the region of nucleotide 513. In one clone a substitution of a T for a C was found at nucleotide 517; the other five clones were of wild-type sequence. One of the clones with the wild-type sequence at nucleotide 517 was sequenced from the ATG initiation codon to the internal EcoRI site at nucleotide position 965; no deviations from wild type were observed.
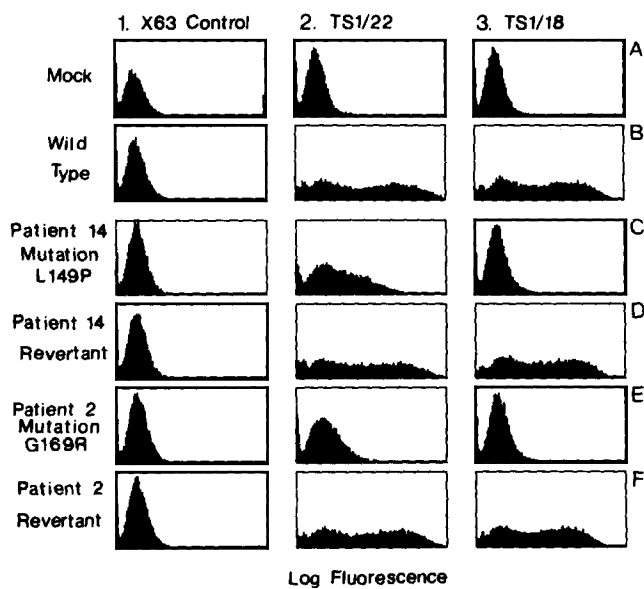
1. X63 Control	2. TS1/22	3. TS1/18	
Mock			A
Wild Type			B
Patient 14 Mutation L149P			C
Patient 14 Revertant			D
Patient 2 Mutation G169R			E
Patient 2 Revertant			F

Log Fluorescence

Figure 3. Flow cytometric analysis of wild-type, mutant, and revertant β subunit cDNAs expressed in COS cells. The indicated β subunit cDNAs in pCDM8 were analyzed for their ability to support expression of LFA-1 in COS cells after co-transfection with the α subunit of LFA-1. Mock transfectants in which no DNA was used in the transfection procedure were used as negative controls and wild type β subunit as a positive control. X63, Control antibody; TS1/22, anti-LFA-1 α mAb; TS1/18, anti-β mAb.

To further study the mutation, patient 14 mRNA was subjected to reverse transcriptase and polymerase chain reaction to amplify a 548-bp fragment encoding the region of interest. 10 plasmids containing the correct size insert were sequenced. Five were of wild type and five encoded the T to C transition at nucleotide 517 (Fig. 2 A). This mutation results in the substitution of a leucine for a proline at amino acid 149. Thus one of the β subunit alleles in patient 14 contains a mutation at nucleotide 517. The other allele appears normal in the region 5' to nucleotide 965, and we hypothesize a mutation 3' to nucleotide 965.

Patient 2. With the addition of RNase M and U2 into the RNase assay digestion mixture two bands of 370 and 330 bp appeared, suggesting a mismatch in the region of nucleotide 535 to 635 (Fig. 1 C, lane 6). No full-length band was seen. A full-length classical cDNA library was constructed in λgt10 from patient RNA, priming first strand synthesis with an antisense oligonucleotide at position 2468 in the 3' untranslated region. Five independent hybridizing colonies whose inserts extended beyond nucleotide 500 were plaque purified, subcloned, and sequenced in the region of interest. In each of the five clones a base pair substitution of a G for an A was observed at nucleotide 577 which resulted in the substitution of a glycine for an arginine at amino acid 169 (Fig. 2 B). These results, together with lack of a wild-type sized fragment in protection assays with probe 2, suggest either homozygosity of this allele or presence of this allele together with a second allele which does not give rise to a β subunit transcript.

Expression of Patient Mutations in COS Cells. Previous studies have shown that LFA-1 is expressed and functionally active in binding ICAM-1 in COS cells which are cotransfected with expression vectors containing the α and β subunit cDNAs (40). To examine the functional consequence of the base substitutions, and confirm that they were not normal polymorphisms, they were introduced into the wild type β subunit cDNA in the mammalian expression vector pCDM8. For the patient 14 mutation, site-directed mutagenesis was used; for the patient 2 mutation, a 530-bp fragment from the patient cDNA was subcloned into wild-type β subunit cDNA. The mutated forms of the β subunit were expressed in COS cells by co-transfection with wild type LFA-1 α. Cell surface expression was determined by immunofluorescence flow cytometry (Fig. 3). Expression of wild-type LFA-1 gave a high mean fluorescence with 65% of cells positive for both LFA-1 α subunit (TS1/22 mAb) and β subunit (TS1/18 mAb) (Fig. 3, B compared to mock transfected cells in A). A low level of expression of the LFA-1 α/β heterodimer with the β subunit containing the patient 14 mutation was detected with TS1/22 (anti-LFA-1α); however, no expression was seen when the same cells were stained with TS1/18 (anti-β) (Fig. 3, panel 3C). COS cells transfected with the α subunit of LFA-1 and the β subunit containing the patient 2 mutation showed very low levels of LFA-1 expression with TS1/22 and no expression with TS1/18 (Fig. 3, E). Very inefficient expression of the α subunit of LFA-1 was seen in cells transfected with the α subunit of LFA-1 alone (data not shown; 40).

To confirm that the mutations defined in patients were the cause of the defective expression, and that incidental mutations had not been introduced, the mutated β subunits in pCDM8 were reverted to wild type by exchanging wild-type fragments with the mutated region and the mutated region was sequenced. Both the patient 14 and the patient 2 revertants expressed normally as assessed by staining with both TS1/22 and TS1/18 (Fig. 3, D and F, respectively).

Analysis of Expression of the Patient 14 Mutant LFA-1 Using a Panel of Anti-LFA-1 Antibodies. To further explore the differential staining of COS cells transfected with the α subunit of LFA-1 and the β subunit encoding the patient 14 mutation noted above, transfectants expressing the patient 14 mutant LFA-1 were screened with two anti-LFA-1 α and six anti-β mAbs (Fig. 4). Both anti-LFA-1 α mAbs stained the patient 14 mutant COS cell transfectants (Fig. 4 B, panels 1, 2), however expression was significantly lower than that observed for wild type transfectants (Fig. 4 A, panels 1, 2). Three of the anti-β mAbs gave the same negative or weak staining pattern as TS1/18 (Fig. 4 B, panels 3–5, 8). Expression of the mutant β subunit was observed with two anti-β mAbs which stained with the same mean fluorescence as the anti-LFA-1 α mAbs (Fig. 4 B, panels 6, 7). The β subunit can be expressed in COS cells without an appropriate leukocyte integrin α subunit, however not all anti-β subunit mAb epitopes remain structurally intact (40, 41; Fig. 4 C). No expression of the patient 14 mutant β subunit was observed when transfected into COS cells in the absence of the LFA-1 α subunit (Fig. 4 D). Thus in patient 14 both the α subunit and the mutant β subunit were expressed on the surface of
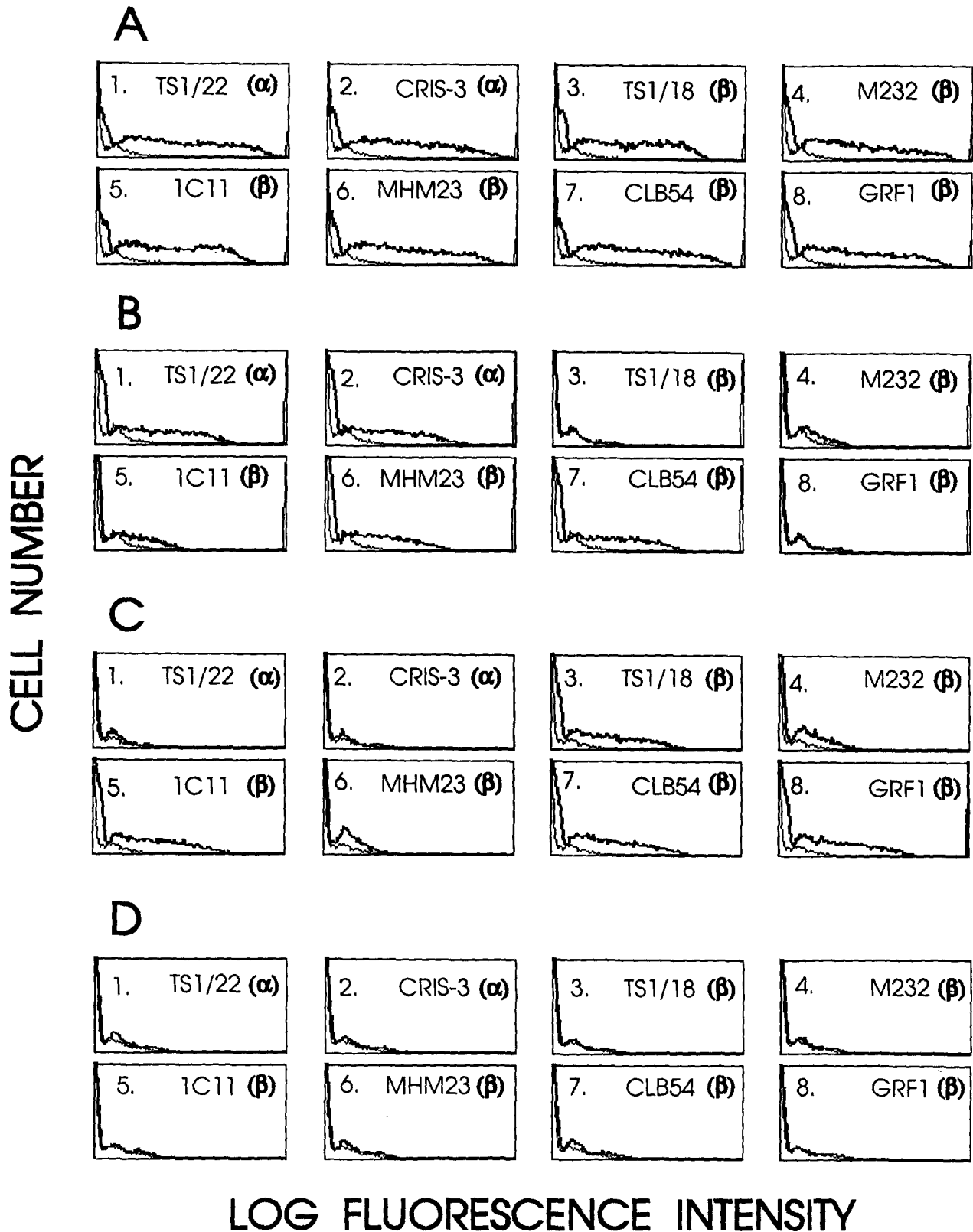
A

1. TS1/22 (α)	2. CRIS-3 (α)	3. TS1/18 (β)	4. M232 (β)
5. 1C11 (β)	6. MHM23 (β)	7. CLB54 (β)	8. GRF1 (β)

B

1. TS1/22 (α)	2. CRIS-3 (α)	3. TS1/18 (β)	4. M232 (β)
5. 1C11 (β)	6. MHM23 (β)	7. CLB54 (β)	8. GRF1 (β)

C

1. TS1/22 (α)	2. CRIS-3 (α)	3. TS1/18 (β)	4. M232 (β)
5. 1C11 (β)	6. MHM23 (β)	7. CLB54 (β)	8. GRF1 (β)

D

1. TS1/22 (α)	2. CRIS-3 (α)	3. TS1/18 (β)	4. M232 (β)
5. 1C11 (β)	6. MHM23 (β)	7. CLB54 (β)	8. GRF1 (β)

CELL NUMBER

LOG FLUORESCENCE INTENSITY

Figure 4. Flow cytometric analysis of COS cells transfected with (A) wild-type LFA-1 α and β subunits, (B) wild-type LFA-1 α and patient 14 mutant β subunits, (C) wild-type β subunit alone and (D) patient 14 mutant β subunit alone. Transfected COS cells were labeled with either the indicated anti-β and anti-LFA-1 α subunit mAbs (*thick lines*) or a nonbinding control mAb X63 (*thin lines*).
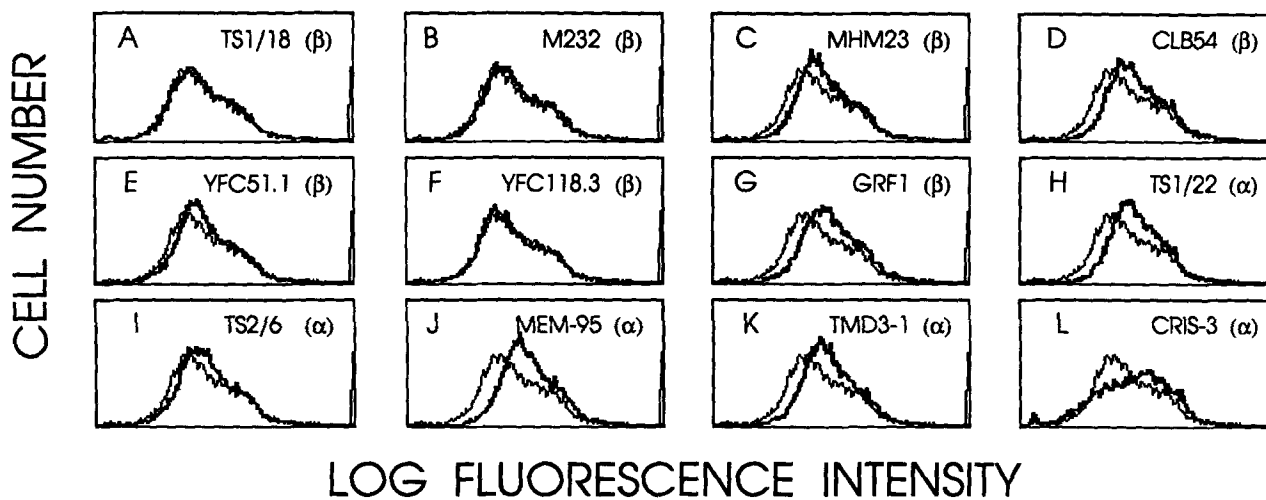
Figure 5. Flow cytometric analysis of patient 14 EBV-transformed B cell lines. Patient 14 EBV-transformed B cells were labeled with either the indicated anti-β and anti-LFA-1 α subunit mAbs (*thick lines*) or a nonbinding control mAb X63 (*thin lines*).

COS cells but with considerably reduced expression and, in the case of the β subunit, loss of the epitope for a number of anti-β mAbs.

Expression of LFA-1 on the surface of the patient 14 EBV-transformed B cells was also investigated using a similar panel of antibodies. These cells had low levels of expression of LFA-1 when stained with the five anti-LFA-1 α subunit mAbs and compared with a nonbinding control antibody, consistent with the moderate phenotype of this patient (Fig. 5, H-L). No expression was observed with TS1/18 (Fig. 5 A) or two other anti-β mAbs (Fig. 5, B and F). In contrast, a low level of expression was observed with four other anti-β mAbs (Fig. 5, C, D, E and G). One anti-β mAb that stained the patient 14 EBV-transformed B cells showed no reactivity with patient 14 mutant COS cell transfectants (Fig. 5 G compared with Fig. 4 B, panel 8). Positive staining with all the mAbs tested was confirmed by staining the CO3 control EBV cell line (data not shown).

Biosynthesis of Normal and Mutant β Subunits. Deficiency in surface expression of the β subunits of patients 2 and 14 could be because they were more rapidly degraded than the wild-type β subunit or because they associated less efficiently than wild-type β subunit with the α subunit. Previous experiments on the biosynthesis of mutant β subunits have been carried out in patient lymphoblastoid cell lines (24). The half-lives of some patient β subunits were shorter than wild-type, but this may have been because association of α with wild-type β resulted in transport to the cell surface and a longer half-life. The COS cell transfection system provided the opportunity to study β subunit half-life in the absence of the α subunit. COS cells transfected with either wild-type β subunit or mutant β subunits were pulse-labeled with [³⁵S]cysteine/methionine and chased for varying times. An immature β subunit of ~89 kD was immunoprecipitated from pulse-labeled cells expressing the wild-type β subunit (Fig. 6, lane 4). No change in mobility was observed upon

chase indicating that the β subunit is not being processed to the higher molecular weight, mature form in the absence of the LFA-1 α subunit (Fig. 6, lanes 10, 16, 22). As has been previously demonstrated (24), patients 2 (Fig. 6, lane 5) and 14 (Fig. 6, lane 6) synthesized precursors of approximately normal size. In both patients, the precursor was still readily detectable after an 8-h chase. Indeed, there was no significant difference in the amount of β subunit precipitated from cells expressing either wild-type or mutant β subunits after a 2-, 4-, or 8-h chase. These results indicate that the mutant β subunits are not being degraded more rapidly than the wild-type β subunit.

Analysis of the Functional Capacity of the Patient 14 Mutant LFA-1. To investigate if the patient 14 mutant LFA-1 could bind to its counter-receptor we measured binding of COS cells transfected with wild type, mutant and revertant LFA-1 to microtiter wells coated with ICAM-1 (Fig. 7). In the presence of W6/32 as a nonblocking antibody we observed 10% binding to ICAM-1 for wild-type and revertant transfectants compared with a background of 1-2% with mock transfectants or binding to human serum albumin (HSA). No binding was seen with COS cells transfected with the patient 14 mutant LFA-1. Binding of the wild-type and revertant transfectants was specific as shown by inhibition with the anti-ICAM-1 antibody RR1/1. Testing of the CLB54 mAb, one of the anti-β mAb that stains patient 14 COS cell transfectants, showed that it is able to block interaction of wild-type LFA-1 with ICAM-1.

Immunoprecipitation of ¹²⁵I Surface-Labeled Wild-Type and Patient 14 COS Cell Transfectants. To investigate if the α and β subunits of patient 14 LFA-1 were associated on the cell surface, COS cells transfected with wild-type and mutant LFA-1 were labeled with ¹²⁵I and cell lysates immunoprecipitated with anti-LFA-1 mAbs bound to Sepharose. In wild-type transfectants, the TS1/22 anti-α mAb and TS1/18 anti-β mAb each precipitated the mature forms of the α and
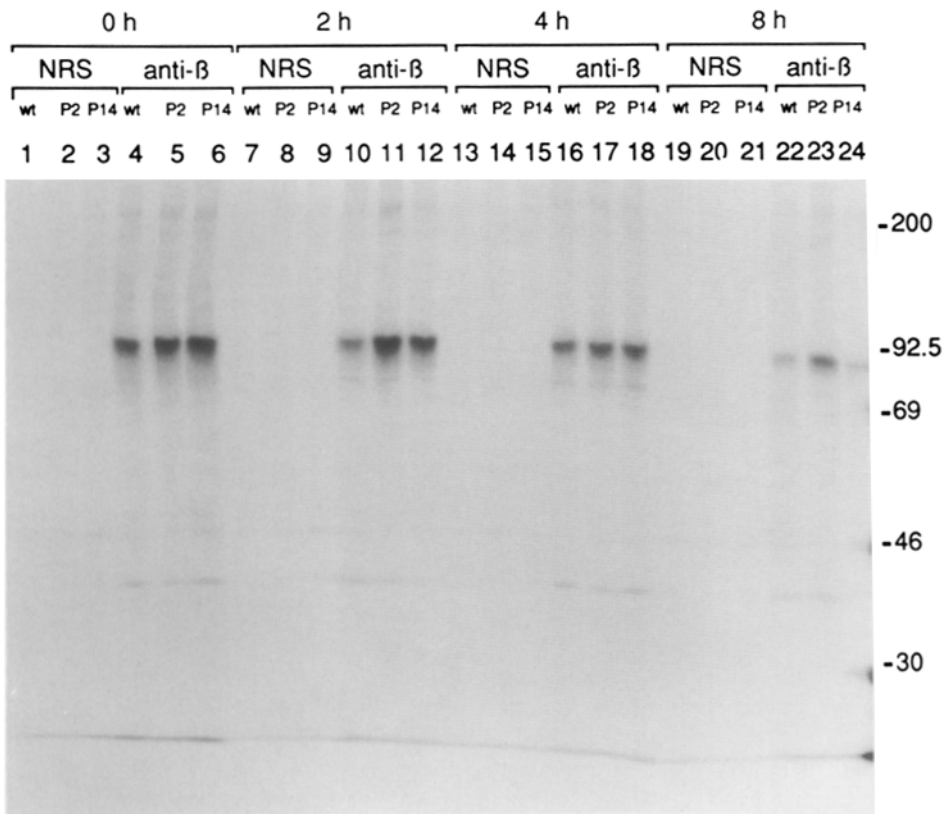
Figure 6. Biosynthesis of normal and mutant β subunits. COS cells transfected with either the wild-type β subunit, patient 2 mutant β subunit, or patient 14 mutant β subunit were pulse labeled with [³⁵S] cysteine/methionine for 1 h and then chased for either 0, 2, 4, or 8 h as indicated. Lysates were immunoprecipitated with either normal rabbit serum (NRS) or anti-β subunit serum as indicated and subjected to reducing SDS 7% PAGE and fluorography.

β subunits of LFA-1 at 180 and 95 kD, respectively (Fig. 8, lanes 2 and 3). In cells transfected with the LFA-1 α subunit and the β subunit containing the patient 14 mutation, TS1/22 mAb precipitated an α but no β subunit (Fig. 8, lane 5),
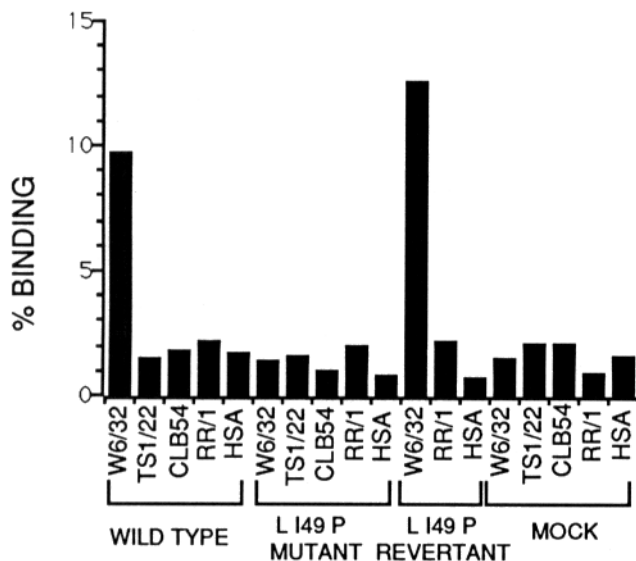
Figure 7. Binding of COS cells expressing wild-type, patient 14 mutant and revertant LFA-1 to ICAM-1. ⁵¹Cr-labeled transfectants were bound to ICAM-1-coated plates in the presence of W6/32, anti-HLA A/B; TS1/22, anti-LFA-1; CLB54, anti-LFA-1; RR1/1, anti-ICAM-1; or bound to human serum albumin (HSA)-coated plates.

suggesting that the α and β subunits were either not associated or that the association was weak and the subunits became dissociated during cell lysis. The α subunit appeared smaller than the wild type α subunit suggesting that it may not have been fully processed. TS1/18 anti-β mAb did not precipitate any LFA-1 from the mutant cells, as would be expected from the flow cytometry data and its previously described inability to precipitate the β subunit in its unassociated state (22).

Discussion

We have analyzed the molecular basis of LAD in two patients, one with a moderate clinical phenotype (patient 14) and one of the severe phenotype (patient 2). The RNase protection assay was used to localize possible sites of base pair mismatch. When examining cleavage of RNA:RNA duplexes with RNase A, Myers et al. (39) found that, depending on the surrounding bases in the probe, a C:A (probe:template) mismatch as in patient 2 cleaved in all cases tested, a A:C mismatch as in patient 14 cleaved in some cases, whereas a G:U mismatch never cleaved. This may explain why mismatches were detected in only two of the patients that were examined. In these patients, distinct defects were detected that mapped to adjacent regions of the β subunit cDNA.

Sequencing of cDNA clones from patient 2 in the region indicated by the RNase assay revealed a base pair substitution in all five clones examined, resulting in the substitution of a glycine for an arginine. Together with the total loss of protection of the full-length RNA in the RNase assay this
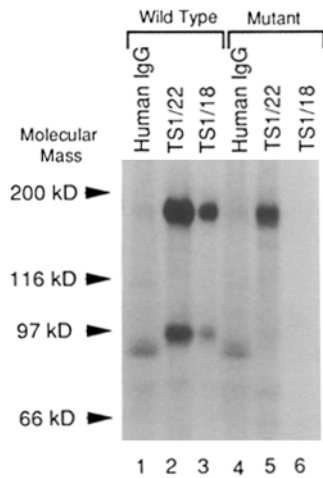
Figure 8. Immunoprecipitation of wild-type and patient 14 mutant LFA-1 expressed in COS cells. Lysates of ^{125}I-labeled COS cells co-transfected with wild-type LFA-1 α and either wild type β subunit or the β subunit containing the patient 14 mutation were immunoprecipitated with human Ig-Sepharose, anti-LFA-1 α subunit mAb-Sepharose or anti-β subunit mAb-Sepharose as indicated. Molecular weight standards are marked.

suggests that only one allele is expressed in this patient. In patient 14, in half the clones sequenced there was a single base pair substitution at nucleotide 517 that would result in the substitution of a proline for a leucine. In one of the clones which did not have this substitution no deviations from wild type were seen in the 5' end of the cDNA up to the internal EcoRI site, suggesting that the second allele in patient 14 carried a mutation in the cDNA 3' to the internal EcoRI site.

Both mutant β subunits were deficient compared with wild type in ability to be expressed in association with the α subunit on the surface of transfected COS cells. This was not due to decreased half-life of the mutant β subunit precursors as shown by pulse-chase experiments. Therefore, the defect is due to inefficient association of the mutant β subunits with the α subunit; previous biosynthesis experiments have demonstrated a lack of formation of an α/β complex in the endoplasmic reticulum of patient cells (24). No expression of LFA-1 was observed following cotransfection of the β subunit containing the patient 2 mutation with wild-type LFA-1 α subunit. This lack of expression is consistent with the severe clinical phenotype and lack of expression on the leukocytes of this patient. In contrast to patient 2, diminished although clearly detectable expression was observed following co-transfection of the β subunit containing the patient 14 mutation with the wild-type LFA-1 α subunit. With the majority of anti-β subunit mAbs no expression was observed; however, two anti-β mAbs detected expression comparable to that seen with the anti-LFA-1 α mAbs. The α subunit of LFA-1 cannot be efficiently expressed unless it first associates with the β subunit since it is expressed very poorly when transfected alone into COS cells (40). This suggests that in COS cells transfected with the β subunit containing the patient 14 mutation, association between the mutant β subunit and the wild-type α subunit must be taking place. However, this association is defective. The α subunit immunoprecipitated from the transfectants expressing the mutant LFA-1 appeared to be smaller than that precipitated from the wild-type transfectants, suggesting that it had not undergone full carbohydrate processing. Expression of both subunits was impaired

and the β subunit was not immunoprecipitated with the anti-LFA-1 α mAb. Dissociation of the two subunits could have occurred either during transport of the protein to the cell surface, on the cell surface, or during preparation of the cell lysates. If dissociation occurred on the cell surface this might explain the loss of reactivity of some of the anti-β mAbs in immunofluorescence flow cytometry, since the epitope for some anti-β antibodies is dependent on α/β association. Alternatively, α/β complexes may be present on the cell surface but the mutation may directly alter the epitope recognized by certain β mAb or indirectly disrupt conformation. Chou-Fasman prediction of secondary structure and the Garnier-Osguthorpe-Robson prediction of antigenicity suggest that the region of the mutation in patient 14 is an α helix of high antigenicity in which a turn is introduced by the substitution of the proline for the leucine at position 149.

The inability of the patient 14 mutant LFA-1 transfectants to bind to ICAM-1–coated plates is most readily explained by the diminished level of expression of LFA-1 compared to wild type. We have found that in EBV-transformed B cells the level of LFA-1 expression has a marked effect on binding (34). A contributory factor may be the altered structure and conformation of the α and β subunits on the cell surface.

Expression in COS cells of the Leu149Pro mutation was much higher than in the EBV-transformed B cells from patient 14. This is probably because the COS cell system is designed to overexpress and therefore drives the weak association between the α subunit and the mutated β subunit. Patient 14 was of the moderately deficient phenotype, having succumbed to septicemia in early adulthood. It is not clear whether the Leu149Pro mutation or the other allele expressed in patient 14 is responsible for the moderate phenotype. The other allele may account for reactivity of the GRF1 β mAb with the patient 14 cell line and not with COS cells transfected with the Leu149Pro mutant β subunit.

The two mutations that we have identified, which are located within 20 amino acids of each other, define a region of crucial importance for α/β association in the leukocyte integrins in vivo, although clearly Leu149Pro gives a moderate phenotype in COS cells in vitro. This appears not to be the only region of β important in association with α, as evidenced by the other allele in patient 14 which appears to have a mutation somewhere between residues 277 and 747. However, the two mutated residues we have defined are of particular interest. They are conserved in all known integrin β subunits (Fig. 9), including β3, the β1 subunits of human, chicken, and Xenopus, and the PS antigens of Drosophila. These two residues lie within a highly conserved region of 241 amino acids in the β subunit (20). In addition, both amino acids are in the 63 amino acid region that in the β3 subunit of platelet gpIIb/IIIa crosslinks to RGD peptides and thus appears to be a ligand binding site (Fig. 9). The close structural and functional relationships between the members of the integrin superfamily suggests that this region of the leukocyte integrin β subunit (β2) may also be involved in ligand binding. Disruption of association with α by the mutations described here suggests that this region may be one where
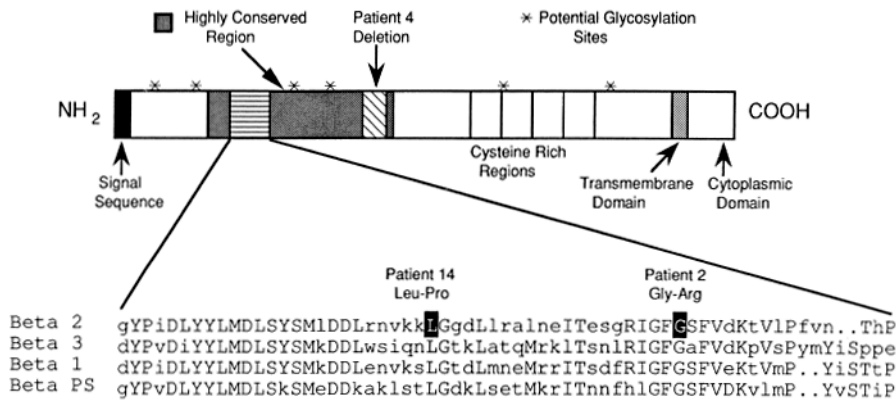
Figure 9. Schematic representation of the β subunit showing the location of the mutations in relation to the highly conserved region and the putative RGD binding region in platelet gpIIIa. The sequences of the three integrin β chains in the region to which RGD peptides cross-link is from D'Souza et al. (20). The original description of the sequences of β_2, β_3, β_1, and the PS integrin β subunit are found in (16–19, 42–43), respectively.

```
Beta 2   gYPiDLYYLMDLSYSMlDDLrnvkkLGgdLlralneITesgRIGFGSFVdKtVlPfvn..ThP
Beta 3   dYPvDiYYLMDLSYSMkDDLwsiqnLGtkLatqMrklTsnlRIGFGaFVdKpVsPymYiSppe
Beta 1   dYPiDLYYLMDLSYSMkDDLenvksLGtdLmneMrrITsdfRIGFGSFVeKtVmP..YiSTtP
Beta PS  gYPvDLYYLMDLSkSMeDDkaklstLGdkLsetMkrITnnfhlGFGSFVDKvlmP..YvSTiP
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the α and β subunits come together to form a ligand-binding pocket.

We have recently shown that stable expression of LFA-1 in EBV-transformed B cells from LAD patients with a variety of patterns of defect can be recovered following trans-fection with a normal β subunit gene subcloned into a mammalian expression vector (34). Together with the present study, this suggests that even in patients with LAD that express a β subunit that migrates normally in SDS-PAGE, that a mutation in this protein is the cause of the disease.

Note added in proof: Point mutations at β subunit residues 196 and 593 have been defined in a further LAD patient (44).

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