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Heterogeneous Mutations in the β Subunit Common to the LFA-1, Mac-1, and p150,95 Glycoproteins Cause Leukocyte Adhesion Deficiency

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Summary

Leukocyte adhesion deficiency (LAD) is a heritable disease involving deficient expression of three related leukocyte adhesion glycoproteins: LFA-1, Mac-1, and p150,95. These proteins are αβ heterodimers containing identical 95,000 dalton β subunits. Here we demonstrate that the primary defect in LAD is in the β subunit gene. We identified five distinct β subunit phenotypes in LAD patients: undetectable β subunit mRNA and protein precursor; low levels of β subunit mRNA and precursor; an aberrantly large β subunit precursor, probably due to an extra glycosylation site; an aberrantly small precursor; and a grossly normal precursor. Mutant β subunit precursors from LAD patients failed to associate with the LFA-1 α subunit. In family studies, inheritance of the aberrant precursors correlates with the known inheritance of the LAD defect.

Introduction

The leukocyte glycoproteins LFA-1, Mac-1, and p150,95 play a broad role in mediating cellular adhesion during virtually all phases of the immune response (reviewed in Springer et al., 1987; Anderson and Springer, 1987). These leukocyte adhesion proteins are evolutionarily related to the Arg-Gly-Asp receptors, which mediate adhesion to extracellular matrix components (Kishimoto et al., 1987; Hynes, 1987). The importance of the LFA-1/Mac-1/p150,95 family in immune function is underscored by the discovery of a human genetic disease called leukocyte adhesion deficiency (LAD) (reviewed in Anderson and Springer, 1987). Patients with LAD suffer from recurrent life-threatening bacterial infections, progressive periodontitis, lack of pus formation, and leukocytosis. Granulocytes, monocytes, and lymphocytes from these patients show profound defects, both in vivo and in vitro, in adhesion-related functions such as binding to endothelial cells, phagocytosis, cell-mediated cytolysis, and response to specific antigens. The molecular basis of this disease has been shown to be a deficiency in cell surface expression of LFA-1, Mac-1, and p150,95. LFA-1, Mac-1, and p150,95 are heterodimers consisting of a unique α subunit (180,000, 170,000, and 150,000 daltons, respectively) noncovalently associated with a common β subunit (95,000 daltons) (Sanchez-Madrid et al., 1983). These subunits are designated CD11a, b, and c, and CD18, respectively, in the international nomenclature. Studies with subunit-specific monoclonal antibodies (MAbs) have shown that the cell surface expression of all three α subunits and the common β subunit is deficient in leukocytes of patients with LAD (reviewed in Anderson and Springer, 1987). There is evidence for heterogeneity in the defect: severely deficient (expressing <1% of normal amounts of αβ complexes) and moderately deficient (expressing 5%–10% of normal levels) patient subgroups have been observed, and these subgroups differ in life expectancy and severity of symptoms (Anderson et al., 1985).

The molecular basis of LAD has been previously investigated in biosynthesis experiments and in human × mouse lymphocyte hybrids. Biosynthesis experiments have used Epstein-Barr virus (EBV)-transformed B lymphocyte and mitogen-stimulated T lymphocyte cell lines, which from healthy individuals synthesize the LFA-1 α subunit (αL) and the common β subunit, and express the LFA-1 αβ complex on cell surfaces. Early studies showed that patient cell lines synthesized apparently normal αL subunit precursor, but the αL precursor did not undergo carbohydrate processing and did not associate in an αβ complex, and neither subunit was expressed on the cell surface. In these studies, the use of available anti-β subunit MAbs did not allow for the immunoprecipitation of β subunit precursors from either control cells or LAD patient cells (Springer et al., 1984; Lisowska-Grospierre et al., 1986). Recently Dana et al. (1987) used a rabbit antiserum raised against the common β subunit and a β subunit cDNA probe and were able to identify normal quantities of the β subunit precursor and β subunit mRNA in four unrelated LAD patients. However, the β subunit precursors, as previously shown for LFA-1 α subunit precursors (Springer et al., 1984; Lisowska-Grospierre et al., 1986), did not undergo normal carbohydrate processing. Thus biosynthesis studies reported to date have not distinguished between a primary defect in the α subunits or a primary defect in the β subunit.

In human × mouse lymphocyte hybrids, human LFA-1 α and β subunits from normal cells have been shown to associate with mouse LFA-1 subunits to form interspecies αβ complexes. Surface expression of the α but not the β subunit of patient cells can be rescued by the formation of interspecies complexes (Marlin et al., 1986). These studies show that the LFA-1 α subunit in genetically deficient cells is competent for surface expression in the presence of an appropriate mouse β subunit. Additionally, they suggest that α subunits must associate with a β subunit in an αβ complex in order to mature and be stored in secretory granules (Todd et al., 1984; Miller et al., 1987a) or expressed on cell surfaces. Collectively these findings, and the additional observations that all three glycopro-

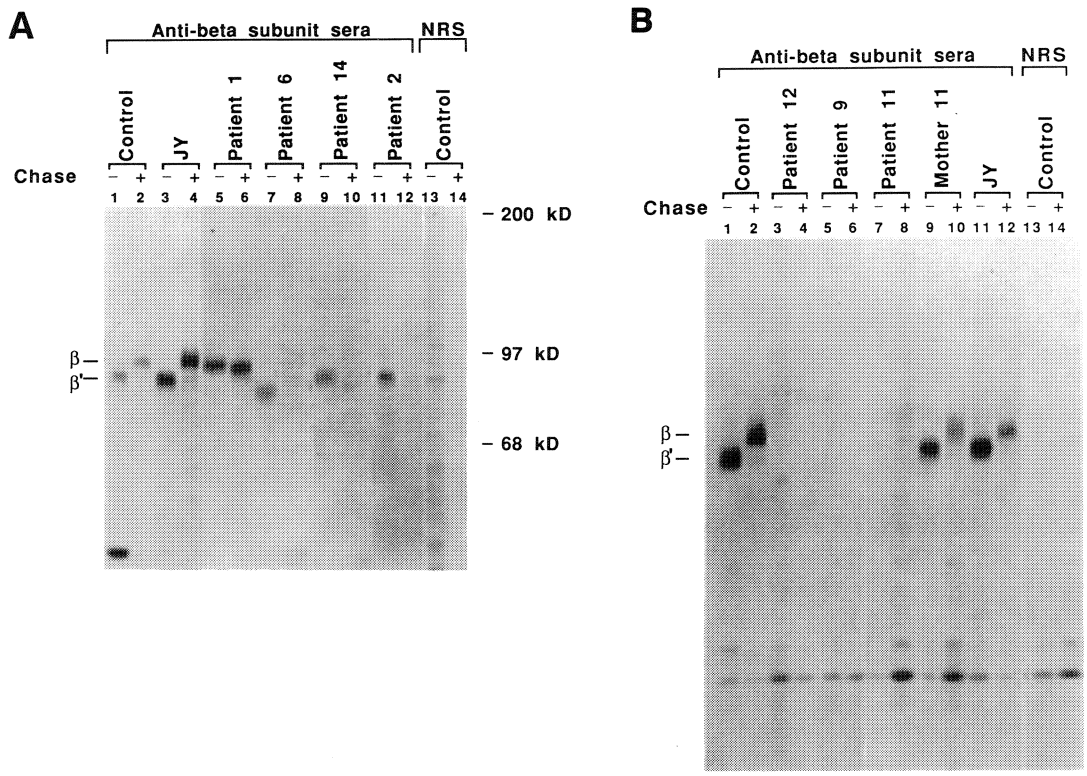

Figure 1. Immunoprecipitation of the β Subunit Precursor from the Cells of LAD Patients

(A) Distinct forms of the β subunit precursor. EBV-transformed cells from healthy controls, LAD patients, and the established cell line JY were pulse-labeled with [^{35}S]methionine for 1 hr with or without a 12 hr chase, as indicated. Cell lysates were denatured in SDS. The β subunit was immunoprecipitated with a rabbit anti-β subunit serum and protein A–Sepharose. Parallel aliquots from each cell lysate were incubated with normal rabbit serum and protein A–Sepharose. Representative control immunoprecipitations are shown in lanes 13 and 14. Samples were run on an 8% SDS–polyacrylamide gel and then subjected to fluorography. (B) LAD patients who are deficient in β subunit precursor synthesis. EBV-transformed cells were pulse-labeled with [^{35}S]cysteine with or without a 16 hr chase, as indicated. Immunoprecipitations with the anti-β subunit serum or normal rabbit serum were performed as described in (A). β′ indicates the β subunit precursor.

teins of the LFA-1/Mac-1/p150,95 family are deficient or absent in each of the 30 cases of LAD documented worldwide, suggest that LAD is secondary to a defect in the common β subunit. However, there has been no direct evidence to date that demonstrates this hypothesis.

Recently, the β subunit polypeptide has been purified to homogeneity, and the β subunit cDNA has been cloned and sequenced by us (Kishimoto et al., 1987) and, independently, by Law et al. (1987). In this study we have used the β subunit cDNA clone to analyze β subunit mRNA expression in seven unrelated patients. Furthermore, we have raised a rabbit antiserum against the purified denatured β subunit and have used it to immunoprecipitate the β subunit precursor from biosynthetically labeled cells from six unrelated LAD patients, and four related patients and kindred. We have identified four distinct classes of mutations that affect β subunit mRNA expression or the structure of the β subunit precursor, and have shown that the inheritance of an aberrant β subunit precursor correlates with the inheritance of the disease. These results provide the first evidence that mutations in the common β subunit cause LAD.

Results

Ten LAD patients, who have previously been characterized as deficient in their cell surface expression of LFA-1, Mac-1, and p150,95 (see Experimental Procedures and Table 1, below) were studied. EBV-transformed cell lines and T lymphoblastoid cell lines were established from these patients and, as expected, were deficient in cell surface expression of LFA-1, but had normal expression of HLA antigens and an unrelated 97,000 dalton adhesion protein, ICAM-1 (not shown; Rothlein et al., 1986). For immunoprecipitation studies, a rabbit antiserum was raised against the SDS–PAGE purified, denatured β subunit polypeptide. This antiserum immunoprecipitated all three leukocyte adhesion protein αβ complexes from Triton X-100 lysates of ^{125}I-surface-labeled granulocytes, and the β subunit alone when the lysates were denatured in SDS, thus confirming specificity of the antiserum for the β subunit (not shown). EBV-transformed cells and/or phytohemagglutinin (PHA)-stimulated lymphocytes from healthy controls and LAD patients were biosynthetically labeled for 1 hr with [^{35}S]methionine or [^{35}S]cysteine, and then

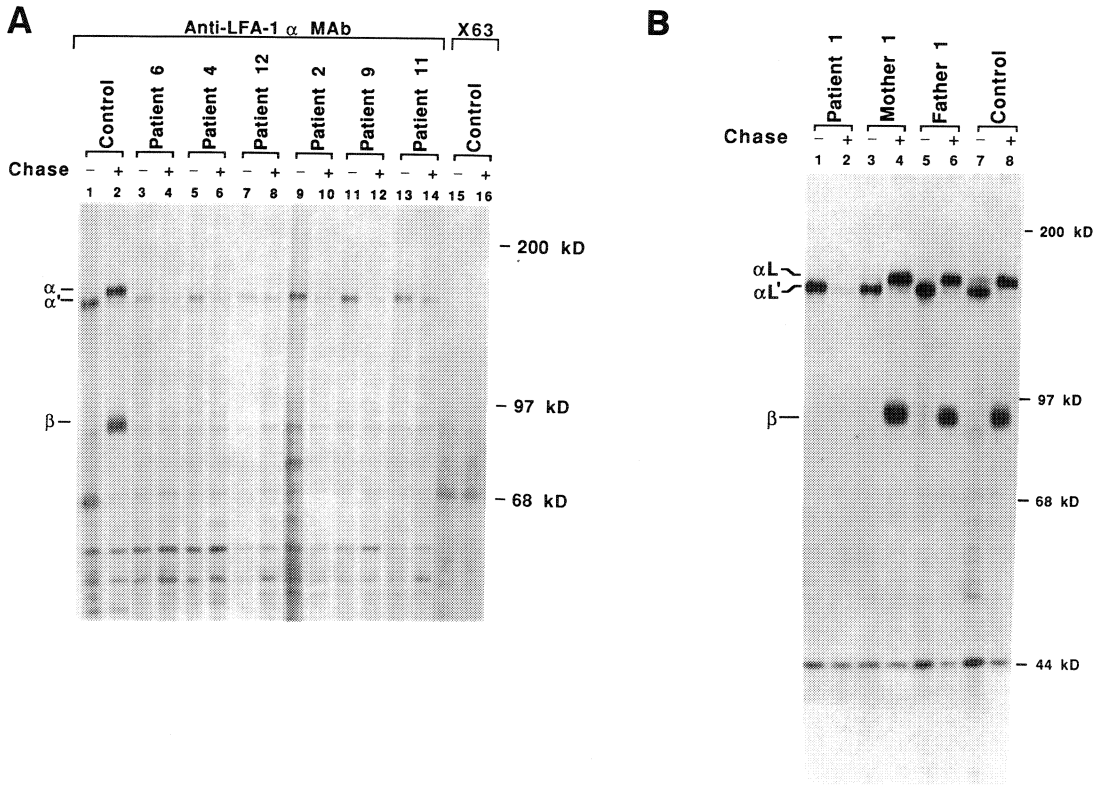

Figure 2. Biosynthesis of the LFA-1 α Subunit in the Cells of LAD Patients

EBV-transformed cells (A) or PHA blasts (B) were pulse-labeled with [^{35}S]cysteine for 1 hr with or without a 16 hr chase, as indicated. LFA-1 was immunoprecipitated with a LFA-1 α subunit–specific MAb coupled to Sepharose. Samples were subjected to 8% SDS–PAGE and fluorography. Parallel aliquots from each cell lysate were incubated with control X63 MAb–Sepharose. Representative control immunoprecipitations are shown in lanes 15 and 16. α′ (αL′) indicates the LFA-1 α subunit precursor.

chased for 12–16 hr with unlabeled methionine or cysteine as appropriate. The cell lysates were denatured by boiling in SDS, and after addition of an excess of Triton X-100, the β subunit was immunoprecipitated with the rabbit antiserum and protein A–Sepharose. A β subunit precursor of 89,000 daltons was precipitated from pulse-labeled control cells (Figure 1A, lane 1). Upon chase, the β subunit showed a characteristic shift in mobility to the mature form of 95,000 daltons (Figure 1A, lane 2).

Four of seven unrelated LAD patients produced a detectable precursor. Patient 1 synthesized an aberrantly large precursor of 92,500 daltons (Figure 1A, lane 5). Patient 6 produced an aberrantly small precursor of 84,000 daltons (Figure 1A, lane 7). Patients 2 (Figure 1A, lane 11) and 14 (lane 9) synthesized precursors of approximately normal size (89,500 daltons). The precursors from these patients failed to undergo processing to the higher molecular weight, mature form after chase. In patients 2, 6, and 14, the pulse-labeled precursor was degraded during the 12–16 hr chase (Figure 1A, lanes 8, 10, and 12). In cells that were chased for 6 hr, the β subunit was still detectable, but only in the precursor form, as confirmed by its sensitivity to endoglycosidase H (Endo H) (not shown). Only in patient 1 was the precursor still readily detectable after a 12 hr chase (Figure 1A, lane 6). The β subunit precursor of patient 1 was slightly reduced in size after the chase (also see Figure 4 below). This phenomenon, which is also seen for the α subunit precursor in patient cells (see Figure 2 below), has been shown for several proteins that reside for long periods of time in the rough endoplasmic reticulum to be due to removal of glucose and trimming of mannose from high-mannose oligosaccharides (Kornfeld and Kornfeld, 1985).

Patients 12, 11, and 9 were deficient in β subunit precursor synthesis (Figure 1B, lanes 3–8). Overexposure of the autoradiogram revealed trace amounts of β subunit precursor and mature β subunit in immunoprecipitates from patient 11, but not from patients 9 and 12 (not shown). Control cells (Figure 1B, lanes 1 and 2) and the mother of patient 11 (Figure 1B, lanes 9 and 10) produced the β subunit precursor, which was processed normally.

In additional experiments, cell lysates were not denatured in SDS prior to immunoprecipitation with the anti-β subunit serum. In control lysates, the LFA-1 α subunit coprecipitated with the β subunit. Whether or not the cells of LAD patients contained precipitable β subunit precursor, no association with the αL subunit was found (not shown).

The LFA-1 α (αL) subunit was further examined after immunoprecipitation from nondenatured lysates with an αL-specific MAb. Pulse-labeled control cells (Figure 2A, lane 1; Figure 2B, lane 7) and parental cells (Figure 2B, lanes

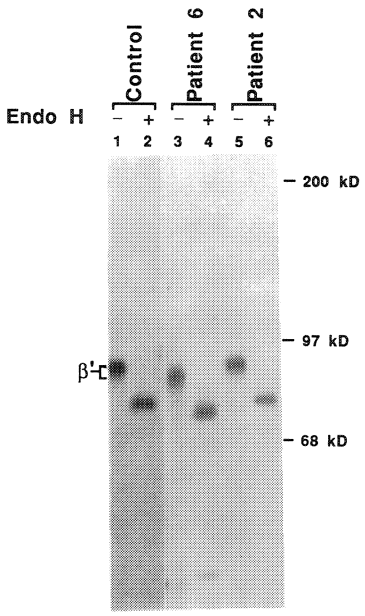

Figure 3. Endo H Digestion of the β Subunit Precursor

EBV-transformed cells from a healthy control and LAD patients 6 and 2 were pulse-labeled for 1 hr with [^{35}]cysteine, without chase. The β subunit precursor was immunoprecipitated from SDS-denatured cell lysates with the rabbit anti-β subunit serum and protein A–Sepharose (lanes 1, 3, and 5). Half of the samples were digested with 5 mU of Endo H for 16 hr at 37°C (lanes 2, 4, and 6). Samples were subjected to SDS–PAGE and fluorography.

3 and 5) produced the αL precursor (170,000 daltons), which was processed to the mature form (180,000 daltons) upon chase (Figure 2A, lane 2; Figure 2B, lanes 4, 6, 8). The mature β subunit was coprecipitated with the mature αL subunit, indicating the assembly of the αβ heterodimer. All cells from LAD patients also synthesized a normal-size αL precursor, but the precursor did not undergo processing to the mature form (Figure 2A, lanes 3–14; Figure 2B, lanes 1 and 2). Furthermore, the β subunit was not coprecipitated with the αL subunit, as previously shown (Springer et al., 1984). Cells from all LAD patients also synthesized normal quantities of ICAM-1, a single chain glycoprotein (not shown).

Endo H was used to examine whether the aberrancies in size of the β subunit precursor were due to gross differences in glycosylation or in the polypeptide chain. Endo H cleaves the immature, high-mannose form of N-linked oligosaccharides but not the mature, complex form. The normal β subunit precursor from control cells was cleaved by Endo H from an 89,000 dalton form (Figure 3, lane 1) to a 78,000 dalton form (lane 2). The β subunit precursors from patients 2 (Figure 3, lane 6) and 14 (not shown) were also reduced to 78,000 daltons. The aberrantly small precursor synthesized by patient 6 was cleaved from 84,000 daltons (Figure 3, lane 3) to 73,000 daltons (Figure 3, lane 4), showing that the defect in this case affects the polypeptide backbone.

Detailed studies with Endo H were carried out on the aberrantly large β subunit precursor of 92,500 daltons in cells from patient 1 and her parents. We examined both the inheritance of the aberrant precursor and whether the abnormality was due to differences in glycosylation or in the polypeptide chain backbone. The long half-life of this aberrant precursor also facilitated studies on β subunit processing in this family. The father and mother of patient 1 have previously been typed as heterozygotes, based on their expression of Mac-1 (52% and 63% of normal quantities, respectively; Springer et al., 1984). The aberrantly large precursor of 92,500 daltons synthesized by patient 1 (Figure 4A, lane 4) was reduced to 91,000 daltons after chase (Figure 4A, lane 6), which is probably due to oligosaccharide trimming in the endoplasmic reticulum, as noted above. The parents of this patient produced β subunit precursors that migrated as a doublet of 90,000 and 92,500 daltons, corresponding to the normal and abnormal precursors (Figure 4A, lanes 7 and 9). A doublet was also observed in the mother's cells after chase (Figure 4A, lane 8). The upper band (Figure 4A, lane 8) corresponds to the normal 90,000 dalton precursor, which was chased to the mature form of 95,000 daltons; the lower band corresponds to the aberrant 92,500 dalton precursor, which was trimmed to 91,000 daltons. Processing in cells from the father shows a similar pattern, but we cannot rule out the possibility of a second, distinct mutation (Figure 4A, lane 10). Endo H digestion of the aberrantly large precursor produced by patient 1 (Figure 4B, lane 1) cleaved it from 92,500 daltons (3,500 daltons larger than normal; compare to lane 9) to 79,000 daltons (lane 2) (1,000 daltons larger than normal; compare to lane 10). Since the error in these determinations is about ±1,000 daltons, this result suggests that much or all of the increased size of the aberrant precursor is due to an additional N-glycosylation site. Endo H cleavage of the mother's doublet β subunit precursor allowed an internal comparison of the size of the deglycosylated aberrant and normal precursors. The doublet (Figure 4B, lane 5) was reduced to a single band of 79,000 daltons after Endo H digestion (lane 6). These results suggest that the aberrancy is due to an additional N-glycosylation site rather than to gross differences in protein backbone size. After a 16 hr chase, the β subunit became almost completely Endo H–resistant in control cells (Figure 4B, lanes 11 and 12), whereas it remained completely Endo H–sensitive in cells from patient 1 (lanes 3 and 4), showing that the N-linked oligosaccharides had not been converted to the mature form. The chased product (Figure 4B, lane 7) from the mother was partially Endo H–sensitive and partially Endo H–resistant (Figure 4B, lane 8). These results are consistent with the mother being a heterozygous carrier of the LAD lesion.

The inheritance of the abnormally small precursor characterized above in patient 6 was examined in an extended family that includes three other LAD patients (Figure 5). All of the parents are from the same isolated rural region, and family records suggest some distant consanguinity (not shown). All four patients (closed symbols) present with similar clinical symptoms and are moderately deficient (5%–10% of normal quantities) in their cell surface expression of LFA-1, Mac-1, and p150,95. Ten mem-

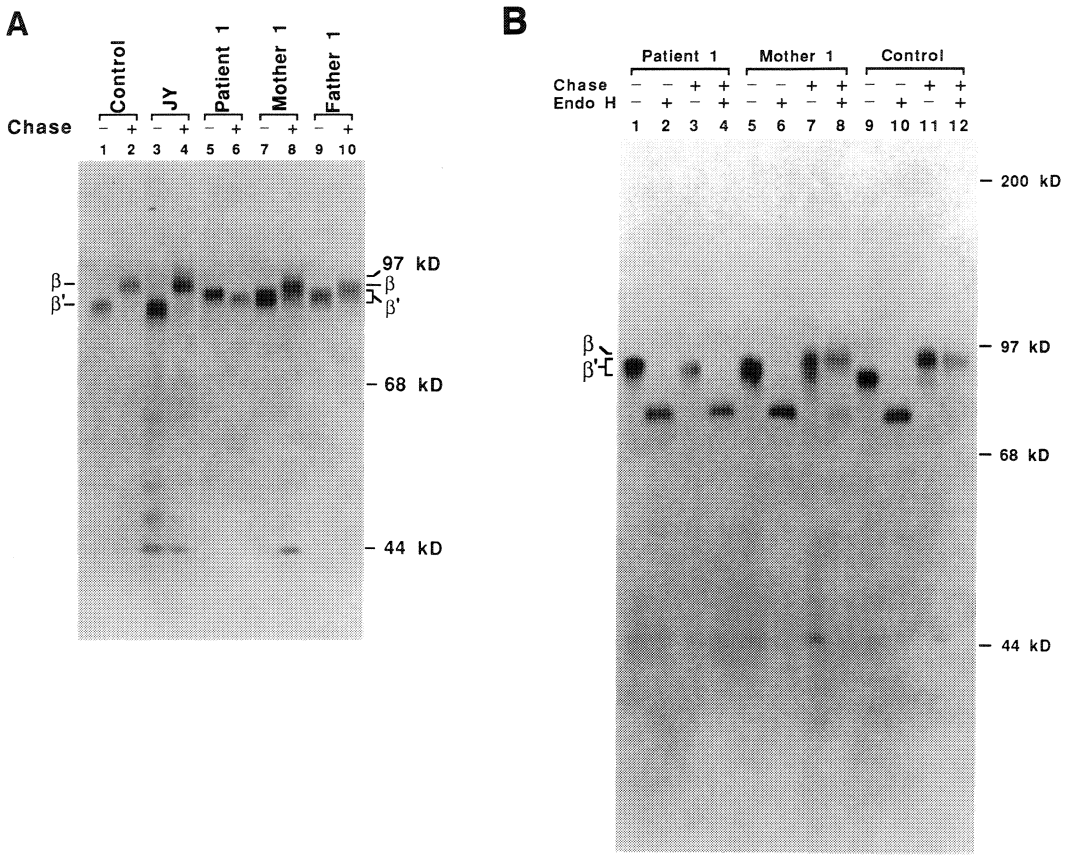

Figure 4. Analysis of the Aberrantly Large β Subunit Precursor

(A) The EBV line JY and PHA blasts from a healthy control, patient 1, and the parents of patient 1 were biosynthetically labeled with [^{35}S]cysteine for 1 hr with or without a 16 hr chase, as indicated. The β subunit was immunoprecipitated from SDS-denatured cell lysates with the rabbit anti-β subunit serum and protein A–Sepharose and was subjected to SDS–PAGE. (B) The β subunit was immunoprecipitated from biosynthetically labeled PHA blasts (odd lanes) as described in (A). Half of the samples were digested with 5 mU of Endo H for 16 hr at 37°C (even lanes). Samples were subjected to 8% SDS–PAGE and fluorography. β′ indicates the β subunit precursor.

bers of this family show no clinical symptoms; of these, nine have been previously shown to have half-normal expression of LFA-1, Mac-1, and p150,95 (heterozygous carriers, half-filled symbols), and one has normal expression (homozygous noncarrier, open symbol), as determined by quantitative flow cytometry (Anderson et al., 1985). EBV-transformed cell lines from 14 members of this family were pulse-labeled with [^{35}S]methionine. All four patients produced the aberrantly small β subunit precursor. The only family member typed as a noncarrier produced only the normal size precursor. The nine heterozygous carriers synthesized both the normal and aberrant forms of the β subunit precursor. The correlations between the presence of only the aberrant precursor and the disease, and between the presence of 0, 1, or 2 normal precursors and the quantity of cell surface expression were highly significant by Fisher's exact test ($p \leq .001$ and $p \leq .0001$, respectively). This result strongly suggests that the mutation that causes LAD in this kindred is a structural defect in the β subunit precursor.

The defect in LAD patients was examined at the mRNA level by Northern blot analysis. Total RNA from patient EBV-transformed cell lines was probed with a nick-translated β subunit cDNA clone (Figure 6). The normal control cells (Figure 6, lane 1) as well as cells from four of seven unrelated LAD patients expressed relatively normal quantities of β subunit mRNA (lanes 2, 3, 5, and 6). Patient 11 had a lower than normal level of β subunit mRNA expression (Figure 6, lane 7). Patients 12 and 9 produced no detectable message (Figure 6, lanes 4 and 8). These results are in agreement with the observation that patients 9, 11, and 12 had little or no immunoprecipitable β subunit precursor. As a control, the Northern blot was stripped and reprobed with a nick-translated actin cDNA probe (Figure 6, lanes 10–18). All patients expressed normal levels of the actin mRNA.

The defect was further studied by Southern analysis of restriction enzyme digests of high molecular weight DNA from LAD patients. PstI-digests were probed with 1.8 and 1.0 kb EcoRI fragment β subunit cDNA probes that span the entire coding region. Representative results are shown for patients 9 and 12, which do not synthesize any detectable β subunit mRNA. No gross deletions were detected in PstI-digested genomic DNA from these patients

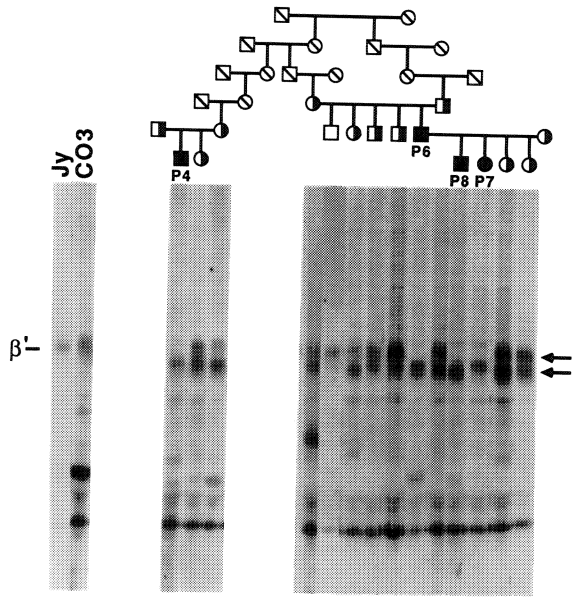

Figure 5. Inheritance of the Aberrantly Small β Subunit Precursor

Patients 4, 6, 7, and 8 and kindred were previously typed as homozygous deficient (closed symbols), heterozygous carriers (half-filled symbols), or homozygous noncarriers (open symbols) based on cell surface expression of the leukocyte adhesion proteins (Anderson et al., 1985). EBV-transformed cells from a healthy control (CO3), the JY cell line, and LAD patients and their kindred were pulse-labeled with [^{35}S]methionine without chase. The β subunit precursors were immunoprecipitated with rabbit anti-β subunit serum and protein A–Sepharose. Samples were subjected to SDS–PAGE and fluorography. The normal β subunit precursor (β′) and the aberrantly small β subunit precursor are indicated by arrows.

(Figure 7, lanes 1 and 2) or the other LAD patients (not shown). More extensive analysis with a panel of restriction enzymes and other family members would be required to define small deletions or restriction fragment length polymorphisms.

Discussion

In this study we have demonstrated that LAD is caused by defects in the gene for the β subunit common to LFA-1, Mac-1, and p150,95. The β subunit in 10 LAD patients and kindred was studied at the level of precursor synthesis, mRNA expression, and Southern DNA analysis. The study population represented approximately one-third of the LAD patients identified wordwide, and included individuals of Hispanic, Anglo-Saxon, Canadian, and Japanese origin (Anderson and Springer, 1987). Based on the quantity of LFA-1/Mac-1/p150,95 expressed on granulocyte surfaces, each of these patients has previously been categorized as severely deficient (<1% of normal expression) or moderately deficient (5%–10% of normal expression) (Table 1). The severity of the clinical symptoms correlates well with the surface expression phenotype. Patients with the severe form of the disease rarely survive beyond childhood. Moderately deficient patients suffer recurrent bacterial infections but can live to adulthood. However, within the severe and moderate phenotypes there are variations in clinical features suggesting further heterogeneity in the mutations causing LAD. We found five distinct classes of mutations (Table 1) that either affect the amount of β subunit precursor synthesized or affect the ability of the β subunit to associate with the α subunit in the endoplasmic reticulum after synthesis. The β subunit regulates the amount of the mature αβ complex expressed by cells, because, as previously shown, association of β with α appears required for processing and transport to the cell surface and to intracellular storage sites (Ho and Springer, 1983; Springer et al., 1984).

Two classes of mutations affect β subunit mRNA levels. Patients 9 and 12 (severely deficient) produce no detectable β subunit mRNA or protein precursor. Southern analysis of genomic DNA shows that the β subunit gene is present in both patients. Patient 11 (moderately deficient)

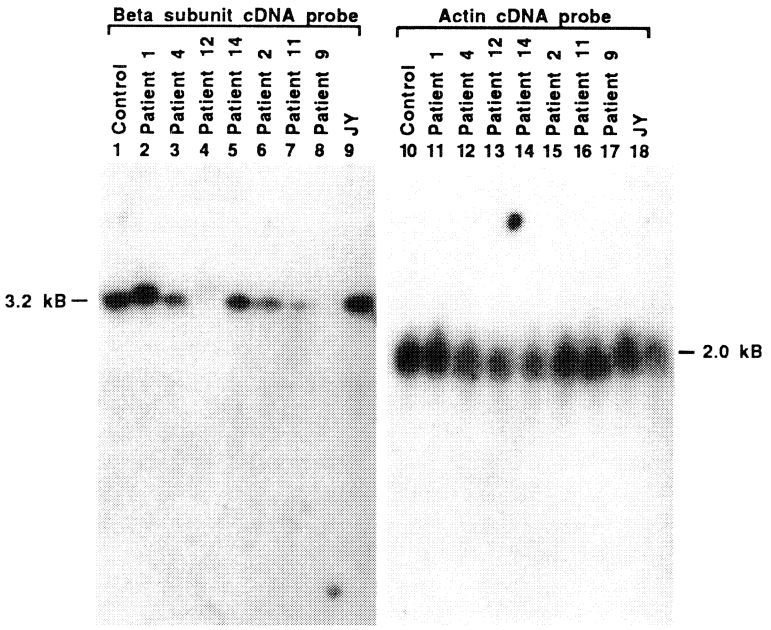

Figure 6. Northern Blot Analysis of β Subunit mRNA in the Cells of LAD Patients

RNA was isolated from EBV-transformed control cells, LAD patients' cells, and the established line JY. Twenty microgram aliquots of total RNA were denatured and then electrophoresed on a 1% agarose gel containing formaldehyde. The samples were transferred to nylon membranes and then probed with ^{32}P-labeled β subunit cDNA (lanes 1–9). The filter was washed and subjected to autoradiography. The filter was then stripped and reprobed with ^{32}P-labeled actin cDNA (lanes 10–18).

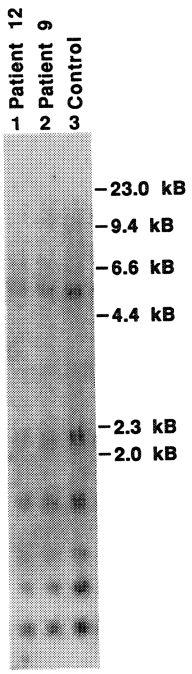

Figure 7. Southern Blot Analysis of Genomic DNA

Twenty microgram aliquots of high molecular weight DNA from LAD patients 12 (lane 1) and 9 (lane 2) and a healthy control (lane 3) were digested with PstI, electrophoresed on a 1% agarose gel, and transferred to nylon membranes. The filters were probed with ^{32}P-labeled β subunit cDNA.

has low levels of β subunit mRNA and synthesizes detectable but low quantities of β subunit precursor. Although the amount of β subunit synthesis in the latter case is diminished, it is sufficient to allow the ultimate expression of approximately 10% of normal amounts of mature αβ complexes on leukocyte surfaces. Mutations in these patients may affect either RNA transcription, processing, or stability.

A third class of mutation results in an aberrantly small β subunit polypeptide chain. Patients 4, 6, 7, and 8, who are members of a single moderately deficient kindred, synthesize a β subunit precursor that is aberrantly small (84,000 daltons). The deglycosylated mutant precursor was 5,000 daltons smaller than the deglycosylated normal precursor, demonstrating a defect in the polypeptide chain backbone. The β subunit mRNA from these patients appears normal in size and quantity by Northern blot analysis. However, more sensitive S1 nuclease protection studies have identified a gross deletion within the translated region of the β subunit mRNA (T. K. K., T. M. R., D. C. A., and T. A. S., unpublished). These results suggest that a deletion in the structural β subunit gene or a defect in RNA processing results in a deletion in the β subunit polypeptide precursor. Studies on the inheritance of this aberrant precursor in an extended family containing four patients, nine heterozygotes, and one noncarrier show perfect concordance with inheritance of the disease, strongly suggesting the presence of a structural mutation causing LAD. Inheritance of the aberrant β subunit precursor in this kindred is autosomal recessive, in agreement with mapping of the β subunit gene to chromosome 21 (Marlin et al., 1986). In biosynthesis studies, we failed to detect association of the aberrant β subunit precursor with the LFA-1 α subunit in any detectable amounts, and the β subunit precursor was rapidly degraded. Immunoprecipitation and immunofluorescence studies on these patients' granulocytes demonstrates 4.5% of normal cell surface amounts of the Mac-1 αβ complex (Anderson et al., 1985), and immunofluorescence shows 2% of normal quantities of the LFA-1 α and β subunits on EBV lines. Such small quantities do not appear detectable in biosynthesis experiments.

A fourth mutation apparently affects N-linked glycosylation of the β subunit precursor. Patient 1 synthesizes an aberrant β subunit precursor of 92,500 daltons, 3,500 daltons larger than normal. Endo H reduces this abnormally large β subunit precursor to a size comparable to that of the normal deglycosylated β subunit backbone. The mother of the patient synthesizes both the normal and aberrant β subunit precursors. Endo H digestion of these precursors yields a single polypeptide backbone of 79,000 daltons. These results suggest that a defect in N-glycosylation can account for the differences in the 90,000 and 92,500 dalton precursors. The 2,500 dalton difference correlates with the estimated size of an N-linked oligosaccharide moiety (Parham et al., 1977), and may reflect the presence of an extra glycosylation site on the 92,500 dalton precursor. The oligosaccharides of the aberrant precursor are apparently trimmed, but are not processed to an Endo H–resistant (mature) form during a 16 hr chase. Thus in this mutation, defective glycosylation may affect

Table 1. Classification of LAD Mutations

Class of Mutation	Patient	Phenotype	β Precursor	β mRNA Levels	References
I	9	Severe	None detectable	None detectable	Anderson and Springer, 1987
	12	Severe	None detectable	None detectable	Kobayashi et al., 1984
II	11	Moderate	Trace amounts	Low	Issekutz et al., 1979
III	4,6,7,8 (related)	Moderate	Aberrantly small	Normal	Anderson et al., 1985
IV	1	Severe	Aberrantly large	Normal	Anderson et al., 1985
V	2	Severe	Normal size	Normal	Anderson et al., 1985
	14	Moderate	Normal size	Normal	Ross et al., 1985

the tertiary structure of the β subunit and its ability to associate with the α subunit. In maternal cells, the normal β subunit precursor but not the aberrantly large precursor undergoes an increase in size associated with carbohydrate processing and appears to be converted to an Endo H–resistant form. Marlin et al. (1986) have shown that the LFA-1 α subunit in transformed lymphocytes from patient 1 is normal, since its expression can be rescued by an association with a mouse β subunit in interspecies cell hybrids. In contrast, human β subunit association with the mouse LFA-1 α subunit was obtainable only when the maternal, but not patient, cells were used in these hybrid cell studies. These results suggest that the 92,500 dalton precursor is structurally defective and is not merely a size polymorphism.

A fifth class of mutation has either a direct or indirect affect on the processing and association of the α and β subunit precursors. Patient 2 (severely deficient) and patient 14 (moderately deficient) synthesized β subunit mRNA and protein precursors of apparently normal size. Dana et al. (1987) have recently identified four patients, representing both severe and moderate phenotypes, that are consistent with this class of mutation. These patients may have subtle mutations within the β subunit that are not detectable by SDS–PAGE. However, since these patients also express normal levels of the LFA-1 α subunit precursor, which is not processed (this report; Springer et al., 1984), we cannot exclude the possibility that the mutation affects the α subunits or an unrelated protein involved in processing. There are probably several distinct mutations within this subclass, giving rise to either the severe or moderate clinical phenotypes.

The identification of five distinct β subunit phenotypes among the seven different patient kindreds suggests that the molecular basis of LAD is complex. Our findings further subdivide the moderate and severe deficiency phenotypes (Table 1), which is consistent with some clinical heterogeneity within these phenotypes (Anderson and Springer, 1987). With the exception of patients 4, 6, 7, and 8, the LAD patients represented here are of distinct ethnic and geographic origins and have no known genealogical relationship to one another (Anderson et al., 1985; Anderson and Springer, 1987). Thus it is not surprising to find a variety of lesions affecting the same gene, as is the case for other heritable diseases such as the β-thalassemias (Orkin and Kazian, 1984) and familial hypercholesterolemia (Goldstein et al., 1985).

The results presented here provide the first direct evidence that LAD is secondary to mutations in the common β subunit and provide insight into the molecular defects involved. There are several important areas for further study. For mutations resulting in defective mRNA expression, studies on mRNA transcription and stability could provide insights into regulation of the β subunit gene. For the mutant β subunit precursors that are synthesized in normal amounts, it is important to identify the exact amino acid changes responsible for the mutation. This can provide information on the regions of the β subunit that are critical for its association with the α subunit and its function. The ability of the transfected normal β subunit gene to restore cell surface expression of LFA-1 in patient cell lines could provide confirmation that the mutation is in the β subunit gene. Furthermore, since patient cell lines are defective in cell–cell adhesion (Rothlein and Springer, 1986), expression of the normal and altered forms of the β subunit gene would allow detailed structure–function studies.

Finally, LAD is a model candidate for gene therapy of a cell surface protein deficiency. Even low levels of expression attainable by current retroviral vector technology should be sufficient to reduce greatly the severity of the functional defects, since moderately deficient patients, who express 5%–10% of normal levels of LFA-1, Mac-1, and p150,95, have a much better prognosis than severely deficient patients.

Experimental Procedures

Cell Lines

EBV-transformed cell lines and PHA-stimulated T lymphocytes from 10 LAD patients, 12 heterozygous carriers, and four normal controls were studied. The clinical history and immunochemical characterization of patients 1, 2, 4, 6, 7, 8 (Anderson et al., 1985), 14 (patient 3 in Ross et al., 1985), 12 (Kobayashi et al., 1984), 9 (Anderson and Springer, 1987), and 11 (Issekutz et al., 1979) have already been described. The classification of phenotypes as severe or moderate is shown in Table 1. EBV-transformed cell lines from patients 1, 2, 4, 6, 7, 8, 9, and 14 were derived as described previously (Springer et al., 1984). EBV lines derived from patient 14, patient 11 and parents, and patient 12 were generous gifts of Dr. P. Lachmann, Dr. W. D. Biggar and Dr. K. Kobayashi, respectively. JY is an established EBV-transformed lymphoblastoid cell line. All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, in RPMI 1640 supplemented with 20% fetal calf serum, 2 mM glutamine, 50 μM β-mercaptoethanol, and 50 μg/ml gentamicin (complete medium). PHA blasts were derived as previously described (Springer et al., 1984). In brief, freshly isolated peripheral blood leukocytes were stimulated with PHA for four days. After day 4, PHA-stimulated lymphocytes were maintained in complete medium containing recombinant IL-2.

Biosynthetic Labeling

EBV-transformed cells or PHA blasts ($1–5 \times 10^7$) were pulse-labeled at a density of 5×10^6/ml for 1 hr at 37°C with 500 μCi of [^{35}S]cysteine in cysteine-free complete medium containing 20% dialyzed fetal calf serum. Half of the cells were harvested and lysed in 1% Triton X-100, 1% bovine hemoglobin, 0.14 M NaCl, 0.01 M Tris (pH 8.0), and freshly added 1 mM phenylmethylsulfonyl fluoride (lysis buffer) for 30 min at 4°C, and then centrifuged at 14,000 × g for 15 min. The remaining cells were chased for 12–16 hr by the addition of 250 μg/ml unlabeled cysteine. Cells were harvested and lysed as described above. In some experiments, cells were pulse-labeled at 5×10^6/ml with 250 μCi [^{35}S]methionine in methionine-free medium and subsequently chased with 100 μg/ml unlabeled methionine. The efficiency of incorporation was calculated after determining trichloroacetic acid–precipitable counts and was found to be similar for all cell lines.

MAbs and Polyclonal Antiserum

MAbs against the β subunit (TS1/18 [Sanchez-Madrid et al., 1983]; IB4 [Wright et al., 1983]), the LFA-1 α subunit (TS1/22 [Sanchez-Madrid et al., 1983]), p150,95 (S-HCL-3 [Schwarting et al., 1985]), HLA (W6/32 [Barnstable et al., 1978]), the adhesion protein ICAM-1 (RR1/1 [Rothlein et al., 1986]), and the control MAb X63 (Sanchez-Madrid et al., 1983) have been described.

The p150,95 protein was affinity-purified, using the S-HCL-3 MAb coupled to Sepharose, from hairy cell leukemia spleens as described previously (Miller et al., 1987b). A mixture of Mac-1, p150,95, and LFA-1 was purified from pooled human leukocytes with the β subunit–specific MAb IB4 coupled to Sepharose. The αβ complexes were eluted with 100 mM triethanolamine, 0.1% Triton X-100 buffer (pH 11.5). The β subunit from both types of preparations was purified to homogeneity

by preparative SDS–PAGE and electroelution as described previously (Kishimoto et al., 1987). Rabbits were immunized by intramuscular injection of 125 μg β subunit emulsified in complete Freund's adjuvant and were boosted three times with injections of 25–60 μg β subunit in incomplete Freund's adjuvant. Using the anti-β subunit serum, all three leukocyte adhesion proteins could be immunoprecipitated from ^{125}I-surface-labeled granulocyte lysates, and only the β subunit was immunoprecipitated from lysates denatured in SDS (data not shown). The antiserum reacted with higher affinity to the denatured β subunit.

Immunoprecipitation

Cell lysates were incubated with 5 μl of normal rabbit serum and 5 μl of rabbit anti-human immunoglobulin (2 mg/ml) for 3–6 hr at 4°C and were then extensively precleared with a Staphylococcus aureus bacterial suspension and a protein A–Sepharose slurry. For immunoprecipitation with the rabbit anti-β subunit serum, the cell lysates were denatured to minimize the effects that a mutation may have on the native tertiary structure. Aliquots of 3–5 × 10^6 cpm were denatured by the addition of SDS to 4% (vol/vol) and heated to 100°C for 10 min. Ten volumes of lysis buffer containing 2% Triton X-100 were added to each aliquot to form mixed micelles with the SDS. One microliter of either normal rabbit serum or the anti-β subunit serum was added to each aliquot. The samples were incubated at 4°C for 2 hr, and then 15 μl of a 50% protein A–Sepharose slurry was added to each sample. Samples were rotated at 4°C for 30 min and then centrifuged. The immune complexes were washed as previously described (Sastre et al., 1986). In some experiments the SDS denaturation step was omitted. Immunoprecipitations with monoclonal antibodies coupled to Sepharose were performed as previously described (Springer et al., 1984). Samples were subjected to 8% SDS–PAGE and fluorography.

Statistical Analysis

Statistical analysis was performed with Fisher's exact test (Pagano and Halvorsen, 1981).

Endo H Digestion

Endo H digestion of immunoprecipitated proteins was performed as previously described (Sastre et al., 1986). In brief, the SDS-denatured β subunit was digested with 5 mU Endo H (Genzyme) in 10 mM 2-(N-morphlino)ethanesulfonic acid (pH 5.5) and 0.14 M NaCl for 16 hr at 37°C. The samples were precipitated overnight with five volumes of acetone in the presence of 10 μg of tRNA and then resuspended in SDS–PAGE sample buffer.

Northern Analysis

Total RNA was isolated from EBV-transformed cell lines by the guanidinium isothiocyanate–CsCl method (Chirgwin et al., 1979). Twenty microgram aliquots were denatured and run in a 1% agarose gel containing 66 mM formaldehyde. Ethidium bromide staining showed comparable amounts and distribution of RNA and intact rRNA from each cell line. The RNA was transferred to nylon membranes and prehybridized as described previously (Kishimoto et al., 1987). The 1 kb EcoRI–EcoRI fragment from the 5′ end of the β subunit cDNA was nick-translated in the presence of [α-^{32}P]dCTP and [α-^{32}P]dGTP and hybridized to filters as previously described (Kishimoto et al., 1987). The filters were washed twice in 2× SSC, 0.5% SDS for 15 min at 25°C and twice in 0.3× SSC, 0.1% SDS for 20 min at 68°C. The filters were exposed to film overnight with an intensifying screen. The filters were stripped in 0.1× SSC, 0.1% SDS at 90°C and were then reprobed with nick-translated actin cDNA.

Southern Analysis

Twenty microgram aliquots of high molecular weight genomic DNA from healthy controls and LAD patients were digested with an excess of PstI for 16 hr at 37°C. The DNA was run on a 1% agarose gel and then transferred to nitrocellulose. The filter was probed with the nick-translated β subunit cDNA and washed at high stringency as previously described (Kishimoto et al., 1987).

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Note Added in Proof

Recently Dinanche et al. (Eur. J. Immunol. *17*, 417–419, 1987) analyzed three additional LAD patients for biosynthesis of the β subunit precursor. One patient synthesized a precursor of normal size, and the other two apparently synthesized β subunit precursors that were rapidly degraded.