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Cloning of the β Subunit of the Leukocyte Adhesion Proteins: Homology to an Extracellular Matrix Receptor Defines a Novel Supergene Family

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Summary

We have isolated cDNA clones encoding the β subunit of the human LFA-1, Mac-1, and p150,95 family of leukocyte adhesion proteins. The deduced 769-amino-acid sequence defines a cysteine-rich polypeptide with the characteristic features of an integral membrane protein. Peptide sequence data, Northern blot analysis, and Southern blot analysis suggest that a single gene encodes the β subunit of all three leukocyte adhesion proteins. There is 45% homology between the β subunit sequence and band III of integrin, a chick fibronectin and laminin receptor. This homology defines a new supergene family of cellular adhesion proteins.

Introduction

Cellular adhesion is a critical function for guiding development and maintaining the integrity of the body. In the immune system, antigen-specific cell–cell interactions are required for the induction and regulation of the immune response and for effector cell function. Leukocytes must also interact in an antigen-independent manner with vascular endothelial cells to be able to migrate to the site of infection or inflammation. These cellular adhesion reactions are mediated in part by a family of structurally related glycoproteins, LFA-1, Mac-1, and p150,95.

LFA-1, Mac-1, and p150,95 are composed of noncovalently linked α:β heterodimers, which have distinct α subunits with molecular weights of 180,000, 170,000, and 150,000, respectively. The β subunit, M_r = 95,000, has been shown to be identical in all three proteins by physicochemical and immunochemical studies (Kürzinger et al., 1982; Sanchez-Madrid, 1983a, 1983b; Hildreth and August, 1985). LFA-1, which is expressed by most leukocytes, enhances antigen-specific helper and cytolytic lymphocyte function and acts as a general adhesion protein in antigen-independent cellular interactions (Springer et al., 1982; Martz, 1987; Springer et al., 1987). Mac-1 and p150,95 are expressed on the cell surface of monocytes and granulocytes. Inflammatory mediators induce rapid mobilization of a large intracellular pool of Mac-1 and p150,95 to the cell surface (Springer et al., 1984; Todd et al., 1984; Anderson and Springer, 1987). This up-regulation event results in increased adhesiveness to endothelial cells, and mediates localization of leukocytes to inflammatory sites (Springer and Anderson, 1986; Anderson and Springer, 1987). In addition, Mac-1 and p150,95 also appear to bind the iC3b fragment of complement (Beller et al., 1982; Micklem and Sim. 1985).

The importance of the LFA-1/Mac-1/p150,95 family in immune function is underscored by the existence of the human genetic disease termed leukocyte adhesion deficiency (LAD). Patients with LAD are deficient in their cell surface expression of LFA-1, Mac-1, and p150,95 (Springer and Anderson, 1986; Anderson and Springer, 1987) and suffer from recurrent life-threatening bacterial infections. Leukocytes from these patients show profound defects, both in vivo and in vitro, in virtually all adhesion-related functions (Springer and Anderson, 1986; Anderson and Springer, 1987).

The common β subunit is of particular interest for several reasons. First, the β subunit appears to have an important role in the function of all three proteins. Monoclonal antibodies (MAb) directed against the β subunit are among the most potent inhibitors of adhesion-related functions mediated by Mac-1 and LFA-1 (Sanchez-Madrid et al., 1983a; Hildreth and August, 1985; Anderson et al., 1986; Martz, 1987; Springer et al., 1987). Second, the primary genetic lesion in LAD disease appears to be a defect in either the expression or structure of the common β subunit (Springer et al., 1984; Marlin et al., 1986).

Adhesion mechanisms are important in other systems for guiding cell migration and localization. The receptors for components of the extracellular matrix, such as fibronectin, laminin, and vitronectin, mediate cellular adhesion during morphogenesis and wound healing. The human extracellular matrix receptors are also composed of high molecular weight α:β complexes (Pytela et al., 1985, 1986; Leptin, 1986; Ruoslahti and Pierschbacher, 1986). The relationship between the Mac-1/LFA-1/p150,95 family and these other adhesion proteins has been unclear.

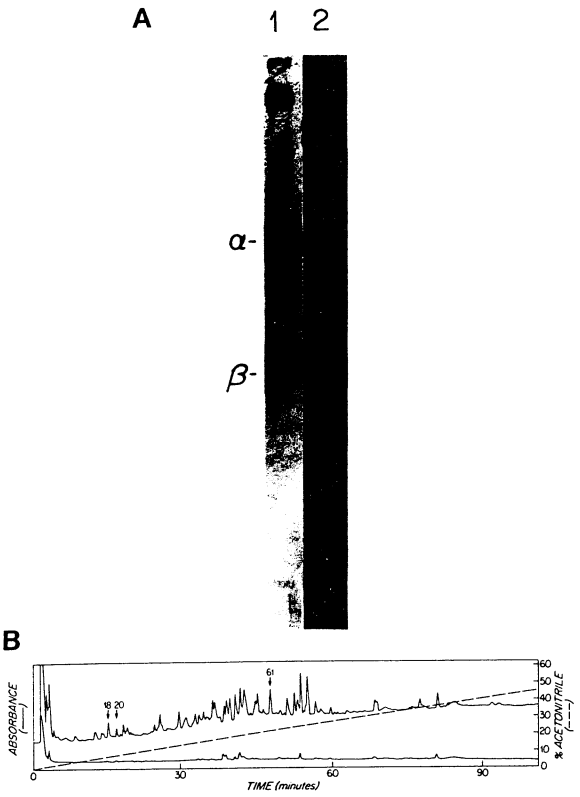
We report here the isolation and analysis of cDNA clones for the β subunit of the Mac-1/LFA-1/p150,95 family. The deduced amino acid sequence defines a cysteine-rich protein with the characteristic features of an integral membrane protein. The β subunit shows strong homology to band III of integrin, a chick fibronectin and laminin receptor (Tamkun et al., 1986). This homology defines a new supergene family of cellular adhesion proteins.

Results and Discussion

Partial Protein Sequence from the β Subunit

p150,95 was purified from hairy cell leukemia spleens by MAb affinity chromatography (Miller et al., 1987). Analysis of the purified protein by SDS–PAGE and silver staining revealed the characteristic α and β subunit, with no significant amounts of contaminating proteins (Figure 1A, lane 1). Following preparative SDS–PAGE, the β subunit band was excised and electroeluted (Figure 1A, lane 2).

The β subunit was partially sequenced to allow the construction of oligonucleotide probes. No sequence could be derived from the intact β subunit, suggesting that the



C

p150,95 β Subunit

P-61 sequence			L	Y	E	N	N	I	Q	P	I	F	A	V	T	S
Deduced sequence	K	L	A	E	N	N	I	Q	P	I	F	A	V	T	S
P-20 sequence			(T/C)	D	T	G	Y	I	G	K						
Deduced sequence	R		C	D	T	G	Y	I	G	K						
P-18 sequence			S	S	Q	E	L	E	G	S	(T/C)	(R)				
Deduced sequence	R	S	S	Q	E	L	E	G	S		C	R				

D

Mac-1 β Subunit

M-58 sequence			L	L	V	F	A	T	D	D	G	F	H	F
Deduced sequence	R	L	L	V	F	A	T	D	D	G	F	H	F	
M-52 sequence			X	A	V	G	E	L	S	E	X	(S)	X	N
Deduced sequence	K	S	A	V	G	E	L	S	E	D	S	S	N	

LFA-1 β Subunit

L56a sequence			E	C	Q	P	P	F	A	F	R			
Deduced sequence	K	E	C	Q	P	P	F	A	F	R				
L56b sequence			L	I	Y	G	Q	Y	C	E	(C)	D	T	I
Deduced sequence	K	L	I	Y	G	Q	Y	C	E	C	D	T	I	
L-65 sequence			V	F	L	D	H	N	A	L	P			
Deduced sequence	R	V	F	L	D	H	N	A	L	P				

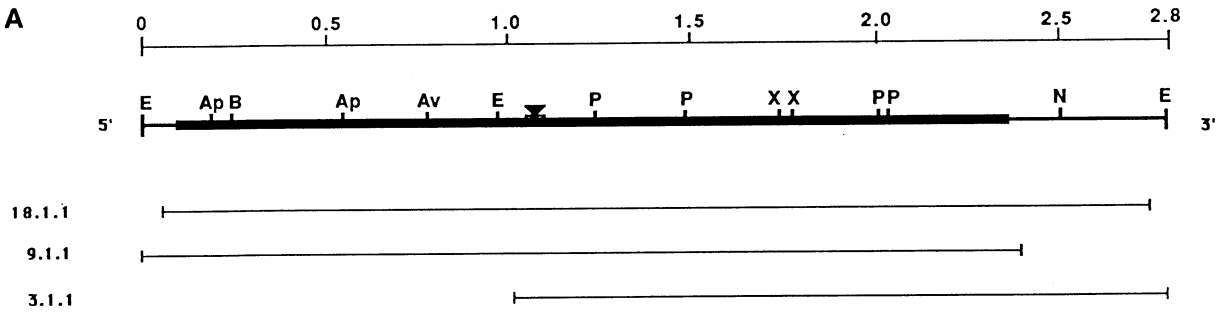
Figure 1. Purification of the β Subunit and Amino Acid Sequence Analysis

(A) Silver-stained SDS–polyacrylamide gel showing a sample of the affinity-purified p150,95 protein (lane 1) and the electrophoretically purified β subunit (lane 2). Both lanes were intentionally overloaded to show any traces of contaminating material. The samples were run on separate gels. (B) Reverse-phase HPLC profile of the β subunit tryptic peptides. Peaks P-18, P-20, and P-61 were subjected to amino acid sequence analysis. (C) Amino acid sequence of tryptic peptides from the p150,95 β subunit and comparison with the deduced amino acid sequence of the β subunit cDNA clone. Parentheses denote some uncertainty in the amino acid assignment. The location of the peptides within the β subunit polypeptide is shown in Figure 2. (D) Amino acid sequence of tryptic peptides from the β subunit of Mac-1 and LFA-1. An X signifies that no assignment could be made.

N terminus was blocked. To obtain internal amino acid sequence, the β subunit was digested with trypsin, and the tryptic peptides were resolved by reverse-phase HPLC (Figure 1B). Peaks were collected and applied to a gas phase microsequenator. Sequences for the P150,95 β subunit peptides P-61, P-20, and P-18 are shown in Figure 1C.

cDNA Cloning

Based on the peptide sequences, both unique sequence and mixed sequence oligonucleotide probes were synthesized. A unique sequence 39mer, designed according to human codon usage frequency (Lathe, 1985), and a 20mer of 96-fold redundancy were derived from peptide P-61. A 17mer of 192-fold redundancy was derived from an independent peptide, P-20 (Figure 1C). The 39mer and the mixed sequence 20mer were used to probe a Northern blot of poly(A)$^+$-selected RNA from U937 cells activated by phorbol myristyl acetate (PMA). Both probes identified a band of approximately 3 kb. The 39mer gave a much stronger signal (see Figure 3A) and was chosen for the primary screening of the cDNA library.



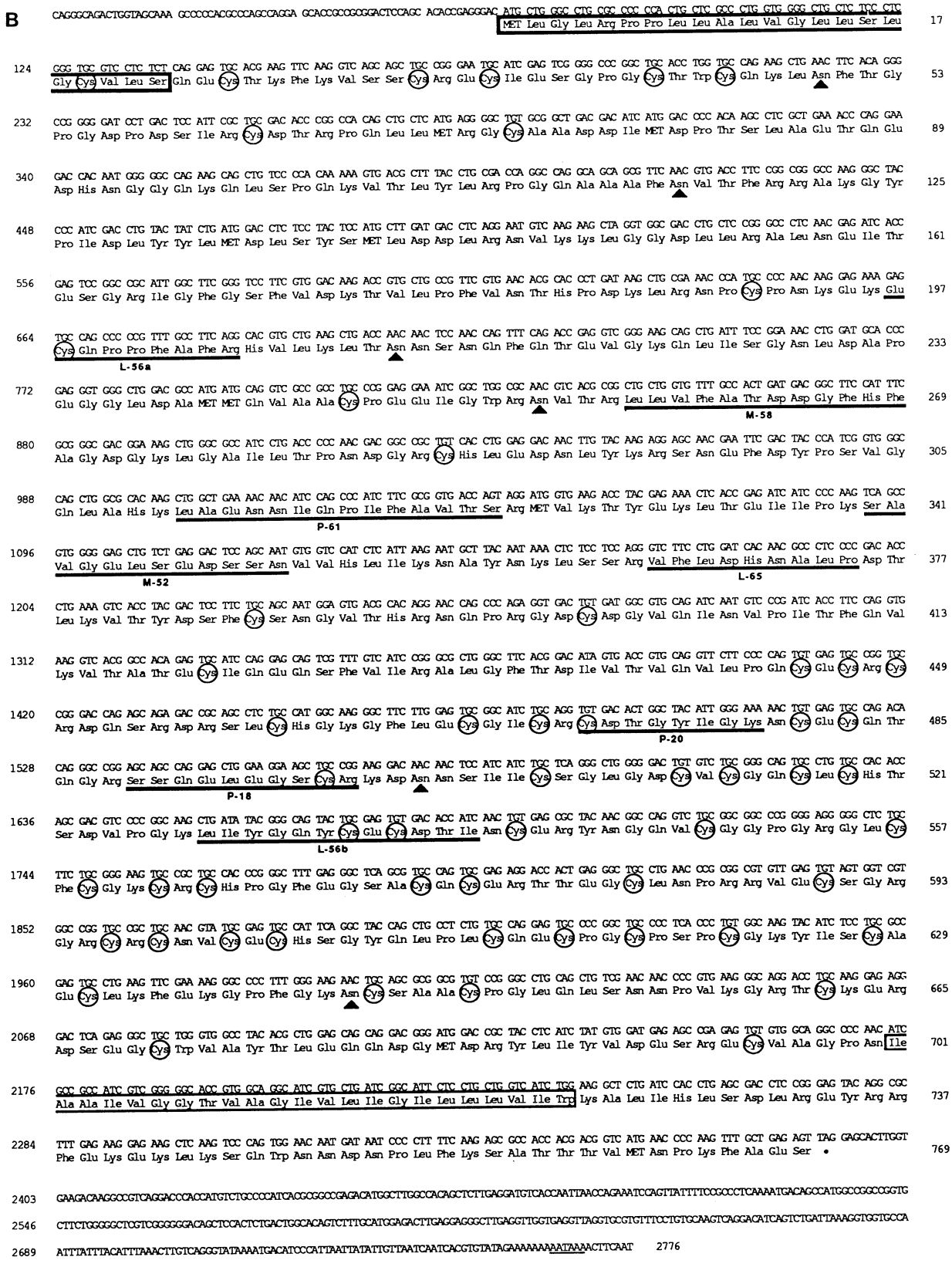


Figure 2. Isolation and Sequence of the cDNA Clones

(A) A partial restriction map for the β subunit cDNA clones (E = EcoRI, Ap = ApaI, B = BamHI, Av = AvaI, P = PstI, X = XmaI, and N = NaeI). The open reading frame is shown as a thick line. The sequence corresponding to the 39mer oligonucleotide is marked by a triangle. Three β subunit cDNA clones, 18.1.1, 9.1.1, and 3.1.1, are represented below the restriction map. (B) The complete nucleotide sequence and deduced amino acid sequence of the β subunit cDNA. The signal sequence is boxed with thick lines and the transmembrane domain is boxed with thin lines. The predicted amino acid sequences that correspond to the sequence of the tryptic peptides are underlined. The potential N-glycosylation sites are denoted with triangles. The cysteine residues are circled. A potential polyadenylation sequence is underlined.

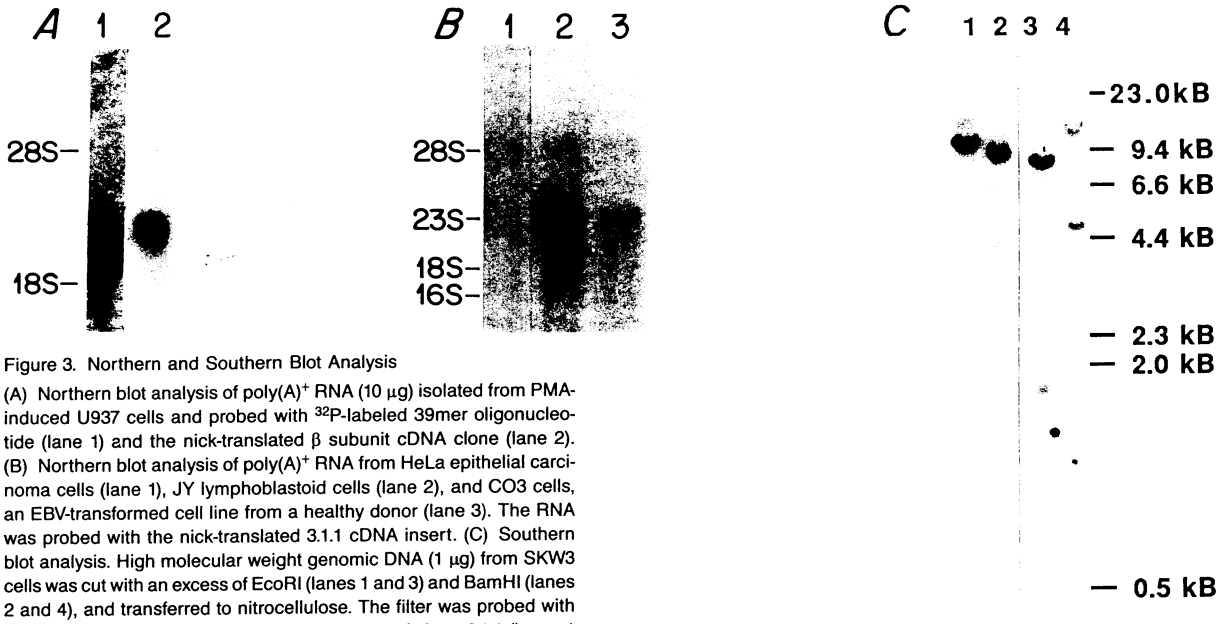


Figure 3. Northern and Southern Blot Analysis

(A) Northern blot analysis of poly(A)$^+$ RNA (10 μg) isolated from PMA-induced U937 cells and probed with ^{32}P-labeled 39mer oligonucleotide (lane 1) and the nick-translated β subunit cDNA clone (lane 2). (B) Northern blot analysis of poly(A)$^+$ RNA from HeLa epithelial carcinoma cells (lane 1), JY lymphoblastoid cells (lane 2), and CO3 cells, an EBV-transformed cell line from a healthy donor (lane 3). The RNA was probed with the nick-translated 3.1.1 cDNA insert. (C) Southern blot analysis. High molecular weight genomic DNA (1 μg) from SKW3 cells was cut with an excess of EcoRI (lanes 1 and 3) and BamHI (lanes 2 and 4), and transferred to nitrocellulose. The filter was probed with the nick-translated 1 kb EcoRI/EcoRI fragment of clone 9.1.1 (lanes 1 and 2). The same filter was reprobed with the nick-translated 1.8 kb EcoRI/EcoRI fragment of clone 3.1.1 (lanes 3 and 4).

Two hundred thousand recombinants from a size-selected human tonsil cDNA library (Wong et al., 1985) were screened with the 39mer oligonucleotide, and 15 positive clones were picked. Eight of the clones cross-hybridized with each other and gave positive signals with the 20mer mixed sequence probe and the independent 17mer mixed sequence probe (data not shown). Three of these clones were chosen for further analysis (Figure 2A). A 263-bp PstI/EcoRI restriction fragment that hybridized to the 39mer was sequenced by the dideoxy chain termination method (Sanger et al., 1977), and the deduced amino acid sequence confirmed the identity of the clone. The unique sequence oligonucleotide was 87% homologous to the cDNA sequence.

The β subunit nucleotide sequence and the deduced amino acid sequence are shown in Figure 2B. We have assigned the initiation codon to the ATG at position 73, which is flanked by a sequence fitting Kozak's criteria for a translation initiation site (Kozak, 1984). The initiation codon is followed by an open reading frame of 2304 bp. An in-frame stop codon (TAG) at position 2380 is followed by a 3′ untranslated region of 394 bp. The poly(A) tail was not found, although a consensus polyadenylation signal (AATAAA) is located 9 bp from the 3′ end.

The cDNA Clone Encodes the β Subunit of Mac-1, LFA-1, and p150,95

Additional peptide sequence data from the β subunit of p150,95 agreed with the sequence translated from the cDNA and confirmed the identity of the cDNA clones (Figure 1C). Mac-1 and LFA-1 were also affinity purified from different cell sources. Mac-1 was isolated from a lysate of pooled human leukocytes with a Mac-1 α subunit-specific MAb. LFA-1 was purified from the SKW3 T cell line with a MAb against the LFA-1 α subunit. The β subunits of Mac-1 and LFA-1 were purified, and tryptic peptides were prepared and sequenced (Figure 1D). The peptide sequence data, representing over 10% of the entire amino acid sequence, correlates with the predicted amino acid sequence and strongly suggests that the cDNA clone encodes the β subunit for all three proteins. A single amino acid substitution was detected in peptide P-61 (Figure 1C), which may reflect normal polymorphism.

β Subunit mRNA and Genomic DNA

The cDNA clones hybridize to a single mRNA species of approximately 3.0 kb (Figure 3A). This message is present in the promonocytic cell line U937 induced with PMA (LFA-1$^+$, Mac-1$^+$, p150,95$^+$) (Figure 3A), and the EBV-transformed lymphoblastoid cell lines JY and CO3 (LFA-1$^+$, Mac-1$^-$, p150,95$^-$) (Figure 3B). As expected, no β subunit message was detected in HeLa cells, an epithelial carcinoma cell line (LFA-1$^-$, Mac-1$^-$, p150,95$^-$) (Figure 3B). The longest cDNA clone, 18.1.1 (Figure 2A), is approximately 2.8 kb in length. Thus it is close to the estimated size of the RNA message minus its poly(A) tail.

Southern blot analysis of genomic DNA at high stringency indicates an upper limit 32 kb for the size of the β subunit gene (Figure 3C). The 1.0 kb EcoRI fragment that contains an internal BamHI site hybridized to only two bands in BamHI-digested DNA (Figure 3C, lane 2), which suggested that a single gene encodes the β subunit of the leukocyte adhesion proteins. Weakly hybridizing bands suggested the possibility of more distantly related genes (Figure 3C, lanes 1, 3, and 4). These data, together with the cDNA and peptide sequence data, support previous biochemical evidence (Kürzinger et al., 1982; Sanchez-Madrid et al., 1983a, 1983b; Hildreth and August, 1985) that the β subunit is identical for LFA-1, Mac-1, and p150,95.

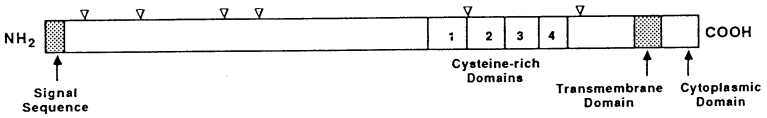


Figure 4. Primary Structure of the β Subunit of LFA-1, Mac-1, and p150,95

The potential N-glycosylation sites are marked with triangles.

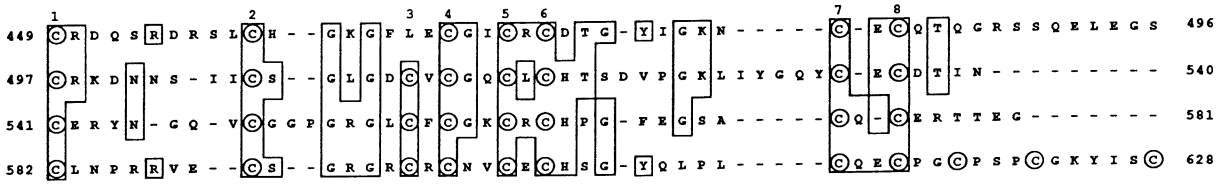


Figure 5. Homologous Repeats of an 8-Cysteine Motif

Tandem repeats were aligned with the Microgenie DNA analysis program (Beckman). Dashes denote gaps introduced to maintain optimal alignment. Homologous residues are boxed. Cysteine residues are circled. The cysteine residues that comprise the 8-cysteine motif are numbered.

Predicted Primary Sequence and Structure of the β Subunit

The cDNA encodes a 769-amino-acid polypeptide, which contains a putative signal sequence and transmembrane domain (Figures 2 and 4). The putative signal sequence consists of a hydrophobic stretch of 22 amino acids. Cleavage of the signal sequence during intracellular processing is predicted to yield a glutamine at the N-terminal position (von Heijne, 1984). N-terminal glutamine is often cyclized to pyroglutamic acid, which would be consistent with the N-terminal blockage of the β subunit. Between the signal sequence and the putative transmembrane domain is a sequence of 677 amino acids that contains all six potential N-glycosylation sites (Asn-X-Ser, Asn-X-Thr). The known glycosylation of the β subunit defines this region as the extracellular domain (Sastre et al., 1986). A second hydrophobic domain of 23 amino acids is located near the carboxyl terminus and has the features of a transmembrane domain (Figures 2 and 4). This region is followed by a putative cytoplasmic domain of 46 amino acids, which contains several charged, basic amino acids.

A striking feature of the β subunit is the high cysteine content (7.4%). A cysteine-rich (20%) region from amino acids 445 to 631 includes four tandem repeats of an 8-cysteine motif (seven in the first repeat) (Figure 5). All 56 cysteines of the mature β subunit polypeptide are located in the extracellular domain. As a general rule, extracellular cysteines are disulfide bonded; since there are no disulfide links between the α and β subunits of the leukocyte adhesion proteins, then these cysteines must be involved in intrachain disulfide bonds. This would give the cysteine-rich domain a very rigid structure. A high degree of intrachain disulfide bonds would be consistent with the observation that after digestion of whole cells with trypsin, the β subunit is cleaved but remains cell-bound and intact under nonreducing conditions (Selvaraj and Springer, unpublished data).

The predicted weight of the β subunit polypeptide after removal of the signal sequence is 82,562 daltons. This size is consistent with the polypeptide backbone of approximately 77,000 daltons, estimated by endoglycosidase H digestion of the in vivo synthesized β subunit precursor from mouse Mac-1 (Sastre et al., 1986) and human p150,95 (L. Miller and T. Springer, unpublished data). The actual difference may be due to anomalous migration of the cysteine-rich β subunit in SDS–PAGE. Assuming a weight of 2500 daltons per oligosaccharide moiety (Parham et al., 1977), the addition of six N-linked carbohydrates to the β polypeptide backbone would bring the weight to 97,500 daltons, close to the observed value of 95,000 daltons for the mature β subunit.

Homology to Integrin

A search of the Genbank national data base and the National Biomedical Research Foundation protein data base revealed no significant homologies to the β subunit. The gross structural similarity of the leukocyte adhesion proteins to extracellular matrix receptors led us to compare the β subunit sequence to the recently published sequence for a subunit of integrin (Tamkun et al., 1986), an avian fibronectin and laminin receptor isolated from embryonic fibroblasts. Integrin (also known as CSAT) was originally defined by MAb that inhibit cell-substrate attachment (Neff et al., 1982). It is thought to be composed of three polypeptide chains of 155,000, 135,000 and 120,000 daltons (Hasegawa et al., 1985). A family of extracellular matrix receptors has been defined functionally by the recognition of ligands that contain the sequence Arg-Gly-Asp (RGD) (Leptin, 1986; Ruoslahti and Pierschbacher, 1986). Small synthetic peptides containing this sequence are able to inhibit binding of these receptors to extracellular matrix components (Ruoslahti and Pierschbacher, 1986). These receptors include the avian integrin complex, the human receptors for fibronectin and vitronectin, and the platelet IIb/IIIa protein, which promiscuously recognizes fibronectin, vitronectin, fibrinogen, and von Willebrand factor (Leptin, 1986; Ruoslahti and Pierschbacher, 1986). The latter three receptors are heterodimers that have β subunits of approximately 90,000–115,000 M_r and α subunits that range from 130,000 to 160,000 M_r under nonreducing conditions (Pytela et al., 1986; Leptin, 1986; Ruoslahti and Pierschbacher, 1986). The similarities in

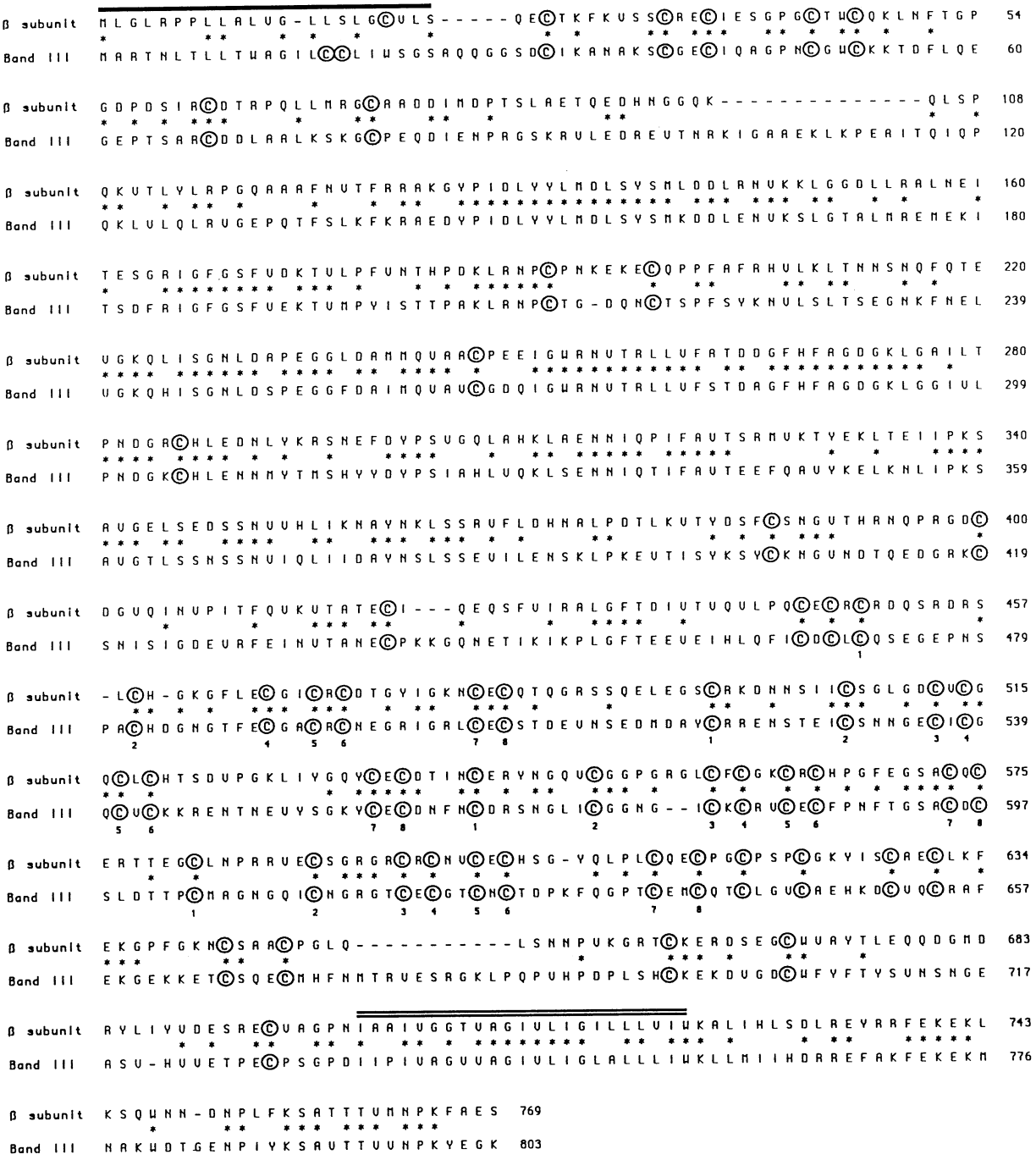


Figure 6. Homology of the β Subunit to Band III of Integrin

Dashes denote gaps introduced to maintain maximal homology. Homologous residues are marked with asterisks. Cysteine residues are circled. The eight cysteine residues of the tandem repeats are numbered. The signal sequence and the transmembrane domain are overlined.

function and in gross structure suggest that the RGD receptors may be homologous to each other (Leptin, 1986; Ruoslahti and Pierschbacher, 1986).

Comparison of the β subunit of the leukocyte adhesion proteins to the 120,000-dalton subunit (band III) of the avian matrix receptor, integrin, shows 45% amino acid sequence identity (Figure 6). This homology is particularly striking since we are comparing across species functionally distinct proteins that are expressed by different cell types. Homology is found throughout the entire molecule, with many of the mismatches being conservative amino acid changes. The signal sequence and the first 121 amino acids of the extracellular domain are only moderately conserved (32% and 33%, respectively). This is followed by a highly conserved domain of 241 amino acids (64% homology), which includes stretches of 15 of 15 and

25 of 27 amino acid identity. The high conservation of this domain suggests that it is of great functional importance and may be a ligand-binding domain. The 82 amino acids between this region and the cysteine-rich domain are 30% conserved between the two polypeptides.

The 190-amino-acid cysteine-rich domains are 42% homologous. The 37-cysteine residues in the cysteine-rich domains are 100% conserved, while the remaining amino acids in the domains are only 27% conserved. All 56 cysteines throughout the mature β and band III subunits are conserved; the only cysteine residues that do not correspond are found in the signal sequence. The tandem repeats of an 8-cysteine motif are found both in the β subunit and in band III of integrin (Tamkun et al., 1986) (Figure 6). The fact that the tandem repeats correspond in both polypeptides, and that the homology is weaker between tandem repeats than it is between the two polypeptides, suggests that the tandem duplication occurred prior to the divergence of these two proteins. The unusually high content and conservation of cysteine residues also suggests that these two polypeptides have a similar tertiary structure.

The 80-amino-acid stretch between the cysteine-rich domain and the putative transmembrane domain is only 26% conserved. In contrast, the putative transmembrane domains of the human Mac-1/LFA-1/p150,95 β subunit and band III of integrin are 70% identical. This high conservation suggests that the evolution of the transmembrane domain has been constrained, perhaps by interaction with the transmembrane segments of the various α subunits. It will be interesting to see if the transmembrane domains of the α subunits of both families of receptors are similarly conserved.

The cytoplasmic domains are also highly conserved (47%). This domain may be important in the interaction with components of the cytoskeleton. Immunofluorescence analysis of fibroblasts shows that fibronectin fibrils and actin microfilaments are aligned, suggesting that they are linked by the fibronectin receptor (Hynes and Destree, 1978; Heggeness et al., 1978). Recently, integrin has been found to bind directly to talin, a cytoskeletal protein that accumulates at adhesion foci (Horwitz et al., 1986). Talin and LFA-1 co-accumulate at adhesion foci between helper T cells and accessory cells (S. J. Singer, personal communication), and LFA-1 also accumulates at the site where natural killer cells bind to their target cells (Carpen et al., 1986). LFA-1 and Mac-1 have not been shown to directly interact with the cytoskeleton. However, cytochalasin B, a drug that disrupts microfilament organization, inhibits LFA-1-related functions (Pattaroyo et al., 1983; Rothlein and Springer, 1986; Martz, 1987; Springer et al., 1987). The β subunit, which is common to all three leukocyte adhesion proteins, is a good candidate for cytoskeletal interactions.

Tamkun et al. (1986) identified a region of the cytoplasmic domain of band III that is homologous to the tyrosine phosphorylation site of the epidermal growth factor (EGF) receptor. Recently, the band III polypeptide has been shown to be phosphorylated at tyrosine in oncogene-transformed cells (Hirst et al., 1986). However, both tyrosines present in the cytoplasmic domain of band III are replaced by phenylalanines in the β subunit sequence. There is a tyrosine in the cytoplasmic domain of the β subunit that is not found in integrin. This site is not homologous to any known tyrosine phosphorylation site, although many phosphorylation sites are yet uncharacterized. In addition, there are four serine and three threonine residues in the cytoplasmic domain that may serve as the targets for phosphorylation of the β subunit, as observed in PMA-activated peripheral blood mononuclear leukocytes (Hara and Fu, 1986).

Although the human leukocyte adhesion proteins and the chicken integrin proteins have subunits that are structurally related, they are not functional homologues. Both the integrin protein and the cDNA were isolated from embryonic fibroblasts. LFA-1, Mac-1, and p150,95 are restricted to the leukocyte lineage. Furthermore, the human leukocyte adhesion proteins are structurally and functionally distinct from known RGD receptors (Sanchez-Madrid et al., 1983a; Pytela et al., 1985, 1986). Genetic deficiencies do not overlap; LAD patients who are deficient in LFA-1, Mac-1, and p150,95 expression do not show any apparent defect in fibroblast or platelet function (Anderson and Springer, 1987), while deficiency of IIb/IIIa in Glanzmann's thrombasthenia does not affect leukocyte function (Ginsberg et al., 1983; Ruggeri et al., 1982). Band III of chick integrin appears to be much more homologous to the β subunit of the human fibronectin receptor than to the β subunit of Mac-1/LFA-1/p150,95 (E. Ruoslahti and R. O. Hynes, personal communication).

Antibodies to integrin immunoprecipitate three polypeptides; however, subunit stoichiometry has not been biochemically defined. Based on the homology of the β subunit to band III of integrin, we would predict that integrin is actually composed of two heterodimers that share a common (band III) subunit. This would be consistent with the two apparent functions of integrin as a receptor for fibronectin and laminin. The extracellular matrix receptors from human and other mammals are thought to have a similar heterodimeric structure (Pytela et al., 1986; Leptin, 1986).

A Novel Supergene Family of Adhesion Receptors

Recently, the N-terminal sequences of the α subunits of human Mac-1 and p150,95 (Miller et al., 1987) and mouse Mac-1 and LFA-1 (Springer et al., 1985) have been shown to be homologous to the α subunits of the human vitronectin receptor (Suzuki et al., 1986) and platelet IIb/IIIa protein (Charo et al., 1986; Ginsberg et al., submitted). This shows that the α subunits as well as the β subunits of the leukocyte adhesion proteins and the extracellular matrix receptors are evolutionarily related. The α subunits may confer much of the ligand-binding specificity. Current efforts in the laboratory are directed toward cloning the α subunits of Mac-1, LFA-1, and p150,95, so that this question can be approached.

The homology described here suggests that the leukocyte adhesion proteins may recognize ligand sequences similar to RGD. It is now important to investigate the interactions of the leukocyte adhesion proteins with RGD-like

sequences on ligands such as iC3b and ICAM-1 (Rothlein et al., 1986), a putative LFA-1 ligand.

Cellular adhesion and recognition mechanisms are the most basic requirements for the evolution of multicellular organisms. The homology of the β subunit of Mac-1/LFA-1/p150,95 to band III of integrin defines a new supergene family of adhesion proteins. Limited N-terminal homology of the α subunits of the two families and the similarity in overall structure and function support this conclusion. Recent data suggest that there may be two subfamilies of RGD receptors, each with a distinct β subunit that can associate with multiple α subunits. One subfamily consists of chicken integrin, the human fibronectin receptor, and human VLA antigens, which are immunologically cross-reactive (Takada, Huang, and Hemler, submitted). The VLA antigens constitute a widely distributed family of five heterodimers, which have distinct α subunits and a common β subunit and have been shown to mediate binding of cells to fibronectin and laminin (Takada, Huang, and Hemler, submitted). The second subfamily consists of the human vitronectin receptor and platelet IIb/IIIa protein, which share a β subunit of 90,000 M_r (Ginsberg et al., submitted). The N-terminal sequence of this β subunit is nonhomologous to band III of integrin and our β subunit, and is antigenically distinct from the β subunit of the human fibronectin receptor (Charo et al., 1986; Ginsberg et al., submitted). We propose that a series of gene duplication events gave rise to the multiple α and β subunits of this supergene family prior to the divergence of mammals and birds from reptiles 140–180 million years ago. The recent suggestion that the position specific (PS) antigens of Drosophila (Wilcox and Leptin, 1985) are structurally related to the extracellular matrix receptors (Leptin, 1986) would place the origin of this gene family prior to the divergence of higher invertebrates and vertebrates 700 million years ago.

The extracellular matrix receptors are thought to be important in both guiding morphogenesis and maintaining tissue organization. The PS antigens in Drosophila are thought to control cell segregation and localization in embryogenesis and metamorphosis (Wilcox and Leptin, 1985). It is possible that the leukocyte adhesion proteins evolved in invertebrates to guide phagocyte localization in inflammation and that this is analogous to the role of matrix receptors in guiding embryogenesis.

The cDNA clone for the β subunit of the Mac-1/LFA-1/p150,95 family provides a molecular probe for tracing the evolution of this supergene family as well as for analyzing the contribution of the β subunit in ligand specificity, transmembrane signaling, and interaction with the cytoskeleton. The β subunit cDNA will also enable us to study the genetic basis of LAD. The primary defect in LAD is in the β subunit (Springer et al., 1984; Marlin et al., 1986; T. K. Kishimoto, D. C. Anderson, and T. A. Springer, unpublished data). Expression of the β subunit cDNA in patient cells should allow normal expression of all three α:β complexes of the leukocyte adhesion glycoprotein family. Mouse β subunit introduced into patient cells by somatic cell fusion associates with patient α subunits in α:β complexes, allowing cell surface expression (Marlin et al., 1986). LAD appears well suited for gene therapy and is an attractive model system, since gene expression can be monitored by screening for α:β complex expression on the cell surface. LAD is currently treated with bone marrow transplantation (Anderson and Springer, 1987). Future efforts will be directed toward treating LAD patients by introducing the β subunit gene with retroviral vectors into bone marrow cells.

Experimental Procedures

Protein Purification and Sequencing

MAb directed against the α subunits of p150,95, Mac-1, and LFA-1 were used to affinity purify their respective proteins from different cell sources. The p150,95 protein was affinity purified from hairy cell leukemia spleens with the S-HCL-3 MAb, as described in Miller et al. (1987). Mac-1 was purified from pooled human leukocytes with the LM2/1 MAb (Miller et al., 1987). LFA-1 was purified from the SKW3 T cell line with the TS1/22 MAb (Sanchez-Madrid et al., 1983a; Larson and Springer, unpublished data). The β subunits of all three proteins were independently isolated by preparative SDS–PAGE. One-tenth mM of sodium thioglycolate was added to the upper chamber to scavenge free radicals in the gel (Hunkapiller et al., 1983). Bands were visualized by soaking of the gel for several minutes in 1 M KCl followed by excision. The β subunit was electroeluted as described by Hunkapiller et al. (1983). The purified protein was reduced with 2 mM DTT in the presence of 2% SDS and was alkylated with 5 mM iodoacetic acid in the dark. The sample was precipitated with four volumes of ethanol at −20°C for 16 hr. The protein pellet was redissolved in 30–50 μl of 0.1 M NH_4CO_3 containing 0.1 mM $CaCl_2$ and 0.1% zwittergent 3-14 (Calbiochem). The sample was digested with 1% w/w trypsin for 6 hr at 37°C. At 2 and 4 hr during the incubation, additional trypsin (1% w/w) was added. The tryptic peptides were resolved by reverse-phase HPLC (Beckman Instruments) with a 0.4 × 15 cm C4 column (Vydac). The peptides were eluted by a 2 hr linear gradient from 0% to 60% acetonitrile in 0.1% TFA. The peaks were monitored at both 214 and 280 nm and collected into 1.5 ml polypropylene tubes. The fractions were concentrated to 30 μl or less on a speed vac concentrator (Savant). Selected peptides were subjected to sequence analysis on a gas phase microsequenator (Applied Biosystems).

cDNA Isolation

A human tonsil cDNA library (generous gift of L. Klickstein) was size selected for inserts of 2 kb or greater and was constructed in λgt11 as described previously (Wong et al., 1985). The original library of 4 × 10^6 recombinants was amplified once. Two hundred thousand recombinants were plated at a density of 7500 plaques per 100 mm plate. The plaques were amplified in situ on duplicate nitrocellulose filters, as described by Woo (1979). A unique sequence 39mer oligonucleotide (5′-GGTCACAGCAAAGATGGGCTGGATGTTGTTCTCATACAG-3′) and a mixed sequence 20mer of 96-fold redundancy were designed based on the peptide sequence P-61. A mixed sequence 17mer of 192-fold redundancy was derived from peptide sequence P-20. Oligonucleotide probes were labeled with γ-^{32}P-ATP using polynucleotide kinase. The filters were prehybridized for at least 2 hr at 42°C in 6× SCC, 1× Denhardt's, 0.5% SDS, 0.05% sodium pyrophosphate, and 100 μg/ml of salmon sperm DNA. Hybridization with the 39mer was carried out overnight at 42°C in prehybridization solution containing 20 μg/ml tRNA. The filters were washed at 53°C to 55°C with 6× SSC, 0.1% SDS, and 0.05% Pi/PPi. The damp filters were covered with plastic wrap and exposed to film with an intensifying screen. Phage that gave positive signals on duplicate filters were plaque purified and rescreened with the 39mer at a higher wash temperature (60°C) and with the 20mer and 17mer mixed sequence probes.

DNA Sequencing

The cDNA clones were restriction mapped by single and double restriction digests and by end labeling and partial restriction digests (Maniatis et al., 1982). Restriction fragments were subcloned into M13 cloning vectors (Maniatis et al., 1982), and the nucleotide sequence of both strands was determined by the dideoxy chain termination method

of Sanger et al. (1977) using ^{35}S-dATP. All restriction sites were crossed in at least one direction.

Northern Blot Analysis

U937, JY, HeLa, CO3, and SKW3 cells were grown in RPMI 1640 containing 10%–15% fetal calf serum in a humidified atmosphere of 5% CO_2 and 37°C. The U937 cells were activated with 2 ng/ml PMA for three days prior to harvesting. The cells were lysed in a 4 M guanidium isothiocyanate solution, and the RNA was isolated on a 5.7 M CsCl gradient (Chirgwin et al., 1979). Poly $(A)^+$ mRNA was selected with oligo (dT)-cellulose columns (Maniatis et al., 1982) or oligo (dT)-affinity paper (Amersham). RNA was denatured and sized on a 1% agarose gel containing formaldehyde and was transferred to nylon membranes (BioRad) in 20× SSC (Maniatis et al., 1982). A lane containing 28 S and 18 S ribosomal RNA from human cells or 23 S and 16 S rRNA from E. coli was run as molecular weight standards. The filters were hybridized with a nick-translated cDNA insert at 42°C for 18 hr in 5× SSPE, 50% formamide, 10% dextran sulfate, 1× Denhardt's, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. The filters were washed at high stringency (65°C) in 0.2× SSC and 0.1% SDS.

Southern Blot Analysis

One-microgram aliquots of high molecular weight DNA from SKW3 cells were digested with 10 units of EcoRI or BamHI at 37°C for 16 hr. The samples were run on a 0.7% agarose minigel in TAE. The gel was soaked in 0.25 M HCl for 15 min, rinsed briefly, and transferred directly to nylon membrane (BioRad) in 0.4 M NaOH (Reed and Mann, 1985). The filters were prehybridized and then hybridized with the nick-translated probe for 16 hr at 68°C in 6× SSC, 0.5% powdered milk, 1% SDS, 0.01 M EDTA, and 100 μg/ml denatured salmon sperm DNA. The filters were washed twice for 15 min at 25°C in 2× SSC per 0.5% SDS and twice for 1 hr at 68°C in 0.3× SSC per 0.1% SDS.

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