Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 752–756, February 1986
Immunology

A genomic clone encoding the α chain of the OKM1, LFA-1, and platelet glycoprotein IIb–IIIa molecules

(transfection/CR3 receptor/cell receptor/monoclonal antibody)

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Communicated by J. F. A. P. Miller, September 3, 1985

ABSTRACT **LFA-1, an antigen involved in cytolytic T lymphocyte-mediated killing, and Mac-1, the receptor for complement component C3bi, constitute a family of structurally and functionally related cell surface glycoproteins involved in cellular interactions. In both mouse and man, Mac-1 (OKM1) and LFA-1 share a common 95-kDa β subunit but are distinguished by their α chains, which have different cellular distributions, apparent molecular masses (165 and 177 kDa, respectively), and peptide maps. We report the isolation of a genomic clone from a human genomic library that on transfection into mouse fibroblasts produced a molecule(s) reactive with monoclonal antibodies to OKM1, to LFA-1, and to platelet glycoprotein IIb–IIIa. This gene was cloned by several cycles of transfection of L cells with a human genomic library cloned in λ phage Charon 4A and subsequent "rescue" of the λ phage. Transfection with the purified recombinant λ DNA yielded a transfectant that expressed the three human α chains of OKM1, LFA-1, and glycoprotein IIb–IIIa, presumably in association with the murine β chain.**

There is a family of at least three leukocyte surface glycoproteins, called Mac-1, LFA-1, and p150/95 (1, 2), which occur in both mouse and man and are identified by monoclonal antibodies. These molecules are related, in that they consist of α and β subunits, which are noncovalently associated in an αβ quaternary structure, of which the β chains are identical. The α subunits are different and vary in size, being 177, 165, and 150 kDa for LFA-1, OKM1 (Mac-1), and p150/95 molecular complex, respectively. The α chains also have different peptide maps and isoelectric points (3) and, recently, LFA-1 and Mac-1 α subunits have been shown to have homologous but different N-terminal amino acid sequences (4). These molecules also differ in their cellular distribution: the Mac-1 (OKM1) antigen is present on myeloid cells and absent from lymphoid cells, whereas LFA-1 is found on T and B cells, granulocytes, and monocytes. Monoclonal antibodies to human LFA-1 block helper-T-cell responses and the adhesion between effector and target cells both in cytolytic T lymphocyte-mediated killing and in natural killing (5). Anti-Mac-1 is believed to bind to an epitope on the receptor (CR3) for complement component C3bi, as it blocks binding of C3bi to CR3, as well as adhesion and phagocytosis of C3bi-coated particles by granulocytes and monocytes (6). Recently, it has been found that an antibody (25E11) to the platelet glycoprotein IIb–IIIa (gpIIb–IIIa) complex previously thought to be reactive only with megakaryocytes and platelets also reacts with monocytes, granulocytes, and natural killer cells (7). The platelet gpIIb–IIIa also consists of two chains, which we shall call α and β, of apparent molecular mass 140 kDa and 87 kDa, respectively, under reducing conditions (8); thus far there is no direct evidence linking platelet gpIIb–IIIa to the LFA-1/Mac-1 family. However, the gpIIb–IIIa complex is of central importance in platelet adhesion and aggregation, and upon activation with thrombin, ADP, or L-epinephrine, the complex undergoes a calcium-dependent rearrangement enabling it to bind to fibrinogen (9). We have found that OKM1 is present on platelets and megakaryocytes (unpublished observations). It is not unlikely that the p150/95 antigen is, in fact, "platelet" gpIIb–IIIa. On the basis of different tissue distributions of the three antigens and the recent description of N-terminal sequences of protein for the α chain of Mac-1 and LFA-1, it was considered that these three entities were encoded by different, yet related, genes—possibly as part of a gene family. However, we now report the isolation of a λ phage containing a 20-kilobase genomic fragment from a human genomic library, which, on transfection, leads to the expression of the OKM1, LFA-1, and platelet gpIIb–IIIa antigens.

MATERIALS AND METHODS

Human Genomic Library. The human genomic library used was made from human fetal liver DNA, partially digested with *Alu* I and *Hae* III and inserted into the *Eco*RI site of λ phage Charon 4A by the use of RI linkers (10). The recombinant λ Charon 4A clones were amplified by plating on a lawn of *Escherichia coli* LE392 cells, and phage DNA was isolated and purified by standard procedures. High molecular weight DNA was isolated from cultured cells by phenol extraction of $NaDodSO_4$-disrupted cells (11).

Cells. The thymidine kinase (TK)-deficient cell line LTA-5 was used as the recipient for DNA-mediated gene transfer and was maintained in Dulbecco's modified Eagle's medium (Flow Laboratories, Australia) supplemented with newborn calf serum (10%), glutamine (4 mM), penicillin (500 units/ml; Commonwealth Serum Laboratories, Melbourne, Australia), streptomycin (100 μg/ml), and 2-mercaptoethanol (0.14%). Transfected cells were maintained in the same medium containing hypoxanthine, aminopterin, and thymidine (HAT).

DNA-Mediated Gene Transfer. Either λ phage DNA or high molecular weight genomic DNA was introduced into LTA-5 cells by use of a modification of the calcium phosphate precipitation technique (12). LTA-5 cells (5×10^5, plated 24 hr earlier) were exposed for 24 hr to a precipitate of 10 μg of DNA and 75 ng of the herpes simplex virus TK gene cloned in pBR322 (HSV *TK*). HAT selection was applied 24 hr later and stable colonies were then tested by rosetting or fluorescence cytometry. The calcium phosphate precipitation technique was also used in a transient-expression assay, in which transfected L cells were washed in phosphate-buffered saline

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Abbreviations: HAT, hypoxanthine/aminopterin/thymidine; TK, thymidine kinase; HSV *TK*, herpes simplex virus thymidine kinase gene.

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(0.9% NaCl/20 mM phosphate, pH 7.4) and examined for antigen expression (see below) at 24, 48, and 72 hr after incubation with DNA.

Serological Assays and Monoclonal Antibodies. Cell surface binding of monoclonal antibodies was detected either by a rosette-forming assay [i.e., the formation of rosettes with sheep erythrocytes coated with sheep anti-mouse IgG (13)] or by fluorescence cytometry, using fluorescein isothiocyanate-conjugated anti-mouse IgG $F(ab')_2$. Platelets were tested by radioimmunoassay using ^{125}I-labeled sheep anti-mouse IgG (14). Monoclonal antibodies used were as follows. (*i*) Antibodies to the human LFA-1 α chain (TS1/22) and β chain (TS1/18) were obtained from T. Springer (15). (*ii*) Anti-Ly-15.2 (16) reacts with the murine β chain (unpublished observation). (*iii*) OKM1 (obtained from Ortho Diagnostics, Raritan, NJ) detects the α chain of the CR3 receptor in man; murine Mac-1 crossreacts with the human OKM1 antigen. (*iv*) Antibody to the Mac-1 antigen (M1/70) has been described (17). (*v*) Antibodies to the platelet gpIIb–IIIa complex were Hu-Pl-m1 (8) and 25E11 (7) (obtained from G. Burns, Walter and Eliza Hall Institute). (*vi*) Anti-Ly-2.1 (18) and anti-Ly-24.2 (produced in our laboratory) were used as control antibodies; L cells are Ly-2.1$^-$, Ly-24.2$^+$. (*vii*) Anti-mouse LFA-1 α chain (M7/14) was obtained from T. Springer (23).

Rescue of λ Charon 4A DNA. High molecular weight DNA from either the secondary transfectant or LTA-5 cells (used as control) was sheared by one passage through a 23-gauge needle prior to packaging. Recombinant Charon 4A phage was "rescued" from the transfectant by using 1 μg of sheared DNA that was packaged *in vitro* using a commercially available kit (Amersham, Melbourne, Australia) and then plated onto a lawn of *E. coli* LE392 cells (19).

RESULTS

The strategy for the cloning procedures was that used to clone the transferrin receptor and human lymphocyte T8 genes (20, 21); i.e., several cycles of transfection of murine TK$^-$ L cells with human DNA plus HSV *TK* DNA with selection of TK- and antigen-positive cells with monoclonal antibodies. To recover the human gene of interest, use was made of the properties of the recombinant λ phage to be packaged *in vitro* and yield infectious phage. Whole-transfectant DNA was used in this "rescue" procedure to isolate the gene coding for LFA-1 and other determinants.

Transfection of λ Charon 4A DNA into TK$^-$ L Cells. DNA was extracted from the pools of a library of human DNA (inserted into the *Eco*RI site of λ Charon 4A) and precipitated with calcium phosphate, together with HSV *TK* DNA. DNA (10 μg) was added to four Petri dishes, each containing 5×10^5 LTA cells, and after 24 hr the cultures were incubated in HAT medium. After 2 weeks, each dish contained 400–500 colonies, which were removed from the plates by use of 10 mM EDTA in phosphate-buffered saline at pH 7.4 or with a rubber policeman. Twenty anti-human monoclonal antibodies were used to test the transfectants, but only anti-platelet (Hu-Pl-m1) and OKM1 monoclonal antibodies reacted to any significant degree (20% of cells were positive by fluorescence cytometry). These results were confirmed using the rosetting assay and by radioimmunoassay using ^{125}I-labeled sheep anti-mouse IgG. The primary transfectant was cloned by the limiting-dilution technique, and the resultant clones were retested for their expression of platelet gpIIb–IIIa and OKM1 by flow cytometry and rosetting. With both Hu-Pl-m1 and OKM1, a distinct population of fluorescent cells stained clearly above background, as indicated by the shift in the channel showing peak fluorescence (Fig. 1) and represented 50–60% of positive rosetting cells in the population (Table 1).

Secondary Transfection of TK$^-$ L Cells. DNA was extracted from the cloned TK$^+$, gpIIb–IIIa$^+$, OKM1$^+$ transfected L cells, precipitated with calcium phosphate, and used to transfect TK$^-$ L cells as before, using additional HSV *TK* DNA. After 3 weeks, HAT-resistant, TK$^+$ cells appeared and were retested with the antibodies and found to be gpIIb–IIIa$^+$, OKM1$^+$. [It was noted that the frequency of gpIIb–IIIa$^+$, OKM1$^+$ clones had increased from 8/150 (5.3%) in the primary transfection to 25/230 (10.9%) in the secondary transfection.] This transfectant was cloned by the limiting-dilution method [15/49 (30.6%) positive clones with both antibodies] and the cells of the clones were examined by rosetting (Table 1). The secondary transfected, cloned cells were 80–85% OKM1$^+$ and gpIIb–IIIa$^+$; all positive clones were both gpIIb-IIIa$^+$ and OKM1$^+$, suggesting that the genes for the two antigens were closely linked in the transfectant, as they had remained together through several cycles of transfection. In these studies, nontransfected TK$^-$ L cells were never OKM1$^+$ or gpIIb–IIIa$^+$ (or Ly-2.1$^+$) but were fully capable of reacting with antibodies, as shown by the strong reaction with the control Ly-24.2 antibody (Table 1).

Presence of Human DNA in the Secondary Transfectant. The presence of gpIIb–IIIa and OKM1 human antigens on the surface of transfected L cells and their absence from normal L cells was a strong indication that the human genes had been transfected and were expressed in these cells. However, OKM1 and the mouse equivalent, Mac-1, are strongly cross-reactive (see below) and the possibility that the transfection procedure had led to the anomalous expression of murine genes could not be excluded. The DNA from the secondary transfectant was therefore subjected to dot blot hybridization with nick-translated probe complementary to the human repetitive *Alu* sequence; this analysis showed the transfectant to contain human DNA (data not shown). The next step was to obtain the human DNA by the rescue procedure and to determine whether one or more λ clones coded for the gpIIb–IIIa and/or OKM1 antigens.

Rescue of λ DNA Containing Human DNA Insert from the Secondary Transfectant. The initial transfections were performed with DNA obtained from a human genomic library, where the DNA (with 20-kilobase inserts) had been ligated into the λ Charon 4A phage so that the human DNA in the transfectant was likely to be still linked to the λ arms. If this were the case, the λ phage (containing the DNA of interest) should be able to be rescued by a simple packaging procedure wherein only λ arms containing the insert would be packaged. The *in vitro* packaging procedure was performed using DNA extracted from the secondary transfected L cells, and the λ phages were sought by plating on a lawn of LE392 bacteria. Only four plaques appeared, each of these then was amplified, λ phage DNA was isolated and purified, and the four DNA samples individually were transfected into TK$^-$ L cells with additional HSV *TK* DNA. After 2 weeks, stable HAT-resistant transfectants appeared, which were tested with antibodies and recloned as had been done for the primary and secondary cycles of transfection, and the cloned transfectants were tested serologically by both rosetting (Table 1) and fluorescence cytometry (Fig. 2).

Serological Analysis of L Cells Transfected with Rescued λ Phage DNA and Human Insert. An extensive serological analysis was performed on the transfectants, which were clearly still gpIIb–IIIa$^+$, OKM1$^+$—again suggesting that these were the products of very closely linked genes or of the same gene. (*i*) Antibodies to gpIIb–IIIa and OKM1 reacted with 50–70% of the cells, with reciprocal titers >4000 (Table 1), similar to those obtained with human granulocytes using the OKM1 antibody and with human platelets tested by radioimmunoassay (data not shown). (*ii*) Another anti-gpIIb–IIIa antibody, 25E11, which detects a unique epitope on platelets and has also been found on granulocytes, also

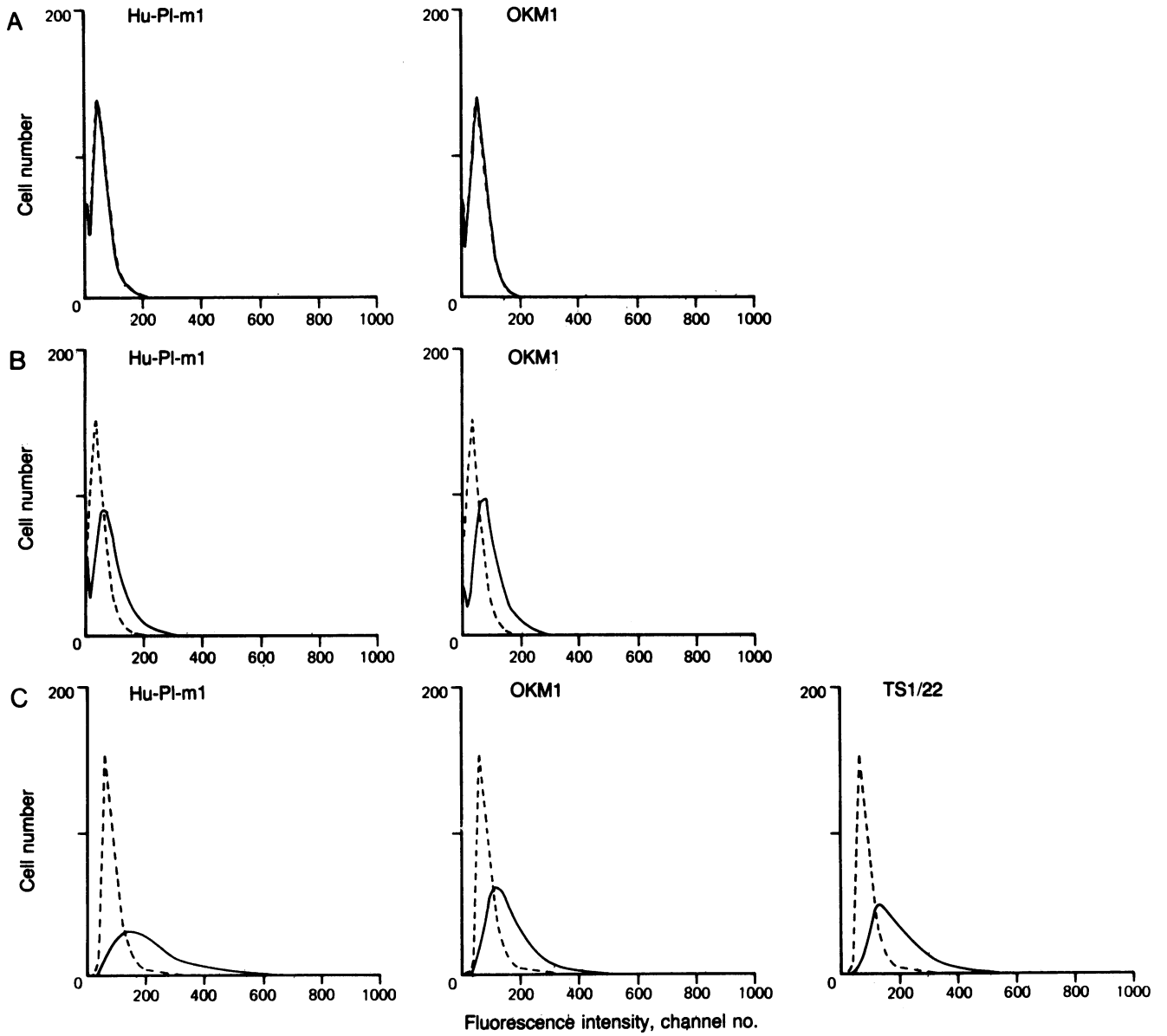

FIG. 1. Fluorescence profiles from L cells and primary and rescued clones of transfected L cells after reaction with test antibody (Hu-Pl-m1, OKM1, or TS1/22; solid curves) or with control antibody (anti-Ly-2.1; broken curves). (*A*) Nontransfected L cells. (*B*) Primary transfectant (cloned). (*C*) L cells after transfection with rescued λ phage DNA.

reacted with the transfectant. (*iii*) The antibody (TS1/22) to the α chain of human LFA-1 molecules was also found to be reactive with the transfectant (Table 1, Fig. 2). This antibody also reacted with normal human lymphocytes and granulocytes (data not shown) but not with L cells (Table 1, Fig. 1). Thus, the transfectant was $OKM1^+$, $LFA\text{-}1^+$, $gpIIb\text{–}IIIa^+$. (*iv*) Because of the likely insert size of 20 kilobases in the recombinant phage, it would have been unlikely for the cloned λ phage DNA to code for both human α and β chains, and further serological studies were performed to determine which β chain, if any, was expressed with the human α chains. Both L cells and the λ-DNA-transfected cells expressed the murine β chain Ly-15.2 (Table 1) but were not reactive with the antibody to the human equivalent (TS1/18), which detects the human LFA-1 β subunit. Transfected L cells presumably supply the murine β chain, a necessary requirement to permit expression of the α chains (3). In this regard we note that the antibody to the murine LFA-1 α chain is nonreactive (Table 1). In summary, the λ-phage-DNA-transfected cells have the α chain for the LFA-1, OKM1, and what we now call the α chain of platelet gpIIb–IIIa. There is no human β chain and no murine α-LFA-1 chain present on transfected or nontransfected cells (Table 1); murine gpIIb–IIIa cannot be measured.

Transient Expression of α Chains. The results presented in Table 1 were obtained by analysis of cloned transfectants that arose 18–21 days after transfection. To complement these findings, a transient-expression system was also used. In this system, the transfection procedure was identical, but the cells were examined after 48 hr by fluorescence cytometry. In addition, L cells were transfected with L-cell DNA as a control. A high percentage of cells expressing antigens detected by the OKM1, LFA-1, and gpIIb–IIIa monoclonal antibodies was found (Fig. 2), confirming the earlier results. The level of expression at 48 hr was far greater than that at 3 weeks, and the level at 24 or 72 hr was less than that at 48 hr. We therefore considered that the structure(s) present consisted of human α chain(s) associated with murine β chains; that is, transfection with a single λ phage led to the presence of surface molecules that reacted with all three antibodies.

Table 1. Serological reactions of cloned transfectants with monoclonal antibodies

	Antibody titer^{-1}	
	Transfected L Cells	TK$^-$ L Cells
Primary transfectant		
Anti-gpIIb–IIIa (Hu-Pl-m1)	>2048 (50)	<2 (0)
Anti-CR3 α-chain (OKM1)	>4000 (60)	<2 (0)
Anti-Ly-2.1	<2 (0)	<2 (0)
Anti-Ly-24.2	>2 × 10^6 (100)	>2 × 10^6 (100)
Secondary transfectant		
Hu-Pl-m1	>2048 (80)	<2 (0)
OKM1	>4000 (85)	<2 (0)
Anti-Ly-2.1	<2 (0)	<2 (0)
Anti-Ly-24.2	>2 × 10^6 (95)	>2 × 10^6 (95)
Transfected with rescued λ phage		
Hu-Pl-m1	>4000 (80)	<2 (0)
Anti-gpIIb–IIIa (25E11)	>320 (60)	<2 (0)
OKM1	>4000 (70)	<2 (0)
Anti-human LFA-1 α chain (TS1/22)	1.6 × 10^5 (60)	<2 (0)
Anti-human LFA-1 β chain (TS1/18)	<2 (0)	<2 (0)
Anti-mouse CR3 α chain (anti-Mac-1)	>64 (50)	>64 (90)
Anti-mouse LFA-1 α chain (M7/14)	<2 (0)	<2 (0)
Anti-mouse β chain (Ly-15.2)	>512 (30)	4096 (40)
Anti-Ly-2.1	<2 (0)	<2 (0)
Anti-Ly-24.2	>2 × 10^6 (95)	>2 × 10^6 (95)

The titer is the dilution giving 50% maximal rosette formation. Numbers in parentheses equal maximal percent of cells reacting with the antibody. All transfectant data are from cloned transfectants. All antibodies were tested on appropriate cells prior to use to confirm high titer and specificity.

DISCUSSION

We describe here the isolation of the gene coding for cell surface α-chain molecule(s) bearing determinants detected by the anti-LFA-1, OKM1, and platelet gpIIb–IIIa monoclonal antibodies. The method used was that of gene transfer by the transfection of murine L cells, a method commonly used to study the expression of cloned genes but that has also been used successfully to clone several cell surface molecules (19, 20). In our study, the key feature was to transfect with DNA obtained from a human genomic library in λ Charon 4A phages. Under these circumstances, all of the clones carry the λ arms, providing a convenient method of identifying and rescuing the required gene (21). Thus, after the initial transfection of TK$^-$ L cells with the human DNA cloned into λ phages, and selection using HAT and monoclonal antibodies to detect the appropriate gene, the clone was simply isolated from DNA of the transfected cells, by use of *in vitro* packaging (wherein only the λ arms containing a human DNA insert would be correctly packaged) followed by isolation of the λ phage. Upon retransfection, it was clear that one of the human genes had been obtained, and serological analysis (further discussed below) indicated this to be the α-chain gene of the gene family coding for LFA-1, OKM1, and platelet gpIIb–IIIa molecules.

The LFA-1, OKM1, and gpIIb–IIIa molecules have different α chains (1–4). Two, LFA-1 and OKM1, have a common β chain, as monoclonal antibodies to the β chain can precipitate both molecules (3). The relationship between the α and β chains is unknown, and in our study it appears that only the human α chain was expressed (Table 1). Using a variety of antisera that can detect human and mouse α chain or β chains, we found that only monoclonal antibodies recognizing the α-subunit of LFA-1, OKM1, or gpIIb–IIIa were reactive, whereas none of the antibodies to the human β chain were reactive. We presume that the molecular complex present consists of different human α chains complexed with murine β chains. Preliminary results (not shown) from immunoblot experiments suggest that separate α chains of gpIIb–IIIa and OKM1 molecules are present.

The two most important implications of this study are the relationship of gpIIb–IIIa to the LFA-1 and OKM1 and the genetic relationship of these gene products to each other. gpIIb–IIIa is one of the major cell surface glycoproteins of platelets and, until recently, had been found only on megakarocytes and platelets. Indeed, experiments with a number of monoclonal antibodies produced in various laboratories, including our own, have demonstrated this to be the case (8). However, a recent study (7) showed that one (but not all) of the epitopes present on gpIIb–IIIa molecules is found on monocytes and other cells. gpIIb–IIIa, like the OKM1 and LFA-1 molecules, is a two-chain structure with apparent subunit sizes of 140 kDa (α subunit) and 87 kDa (β subunit), but it has yet to be demonstrated that the β chain is identical to that found in OKM1 and LFA-1 molecules. It will clearly be of interest to determine the structural (and functional) relationships of platelet gpIIb–IIIa to LFA-1 and OKM1 and to determine the structure of the β chain of the platelet molecule. Clearly, the α chains of all three molecules are related genetically.

One of the surprising findings from this study is that a single gene could code for molecules that appear to have such different features when expressed on the cell surface. Thus, the α chains of LFA-1, OKM1, and gpIIb–IIIa have different tissue distributions, different molecular weights, and different peptide maps—evidence usually presented for the existence of separate genes. However, it had been suggested that LFA-1 and OKM1 are part of a "gene family" (i.e., a family of homologous, closely linked genes) and the recent demonstration of homology at the N-terminal ends of these molecules supports this concept. Whether gpIIb–IIIa can be considered to be part of this family requires further biochemical study. However, now we have a 20-kilobase fragment of DNA that codes for three different molecules in the transfected cells. Three possible explanations how the three molecules could arise from a 20-kilobase fragment are (*i*) the 20-kilobase fragment contains a single gene with the usual intron/exon structure and whose exons undergo genetic rearrangement on maturation (like immunoglobulin genes and the T-cell antigen-receptor gene) to give three different products; (*ii*) there is a single gene whose transcript is spliced differentially to give rise to three different molecules; or (*iii*) there is a gene family which *in toto* gives the 20-kilobase size of the insert. An alternative is that a regulatory rather than structural gene may have been cloned, but this explanation seems unlikely because a regulatory gene would also have caused the expression of mouse genes rather than human genes, and there is no evidence for this (Table 1). Final answers will not be obtained until the 20-kilobase insert is sequenced and the structures of the appropriate cDNAs are determined. Our prediction is that we have cloned a single gene that is spliced differentially to give the three moieties described. If this were so, it would be expected that the proteins have some structural homology, which is the case for LFA-1 and Mac-1, although the peptide maps are quite different (4). There may be a gene family present with few, if any, introns; of relevance to this is the recent description of the homology of LFA-1 and Mac-1 proteins to α-interferons (interferon genes lack introns) (22).

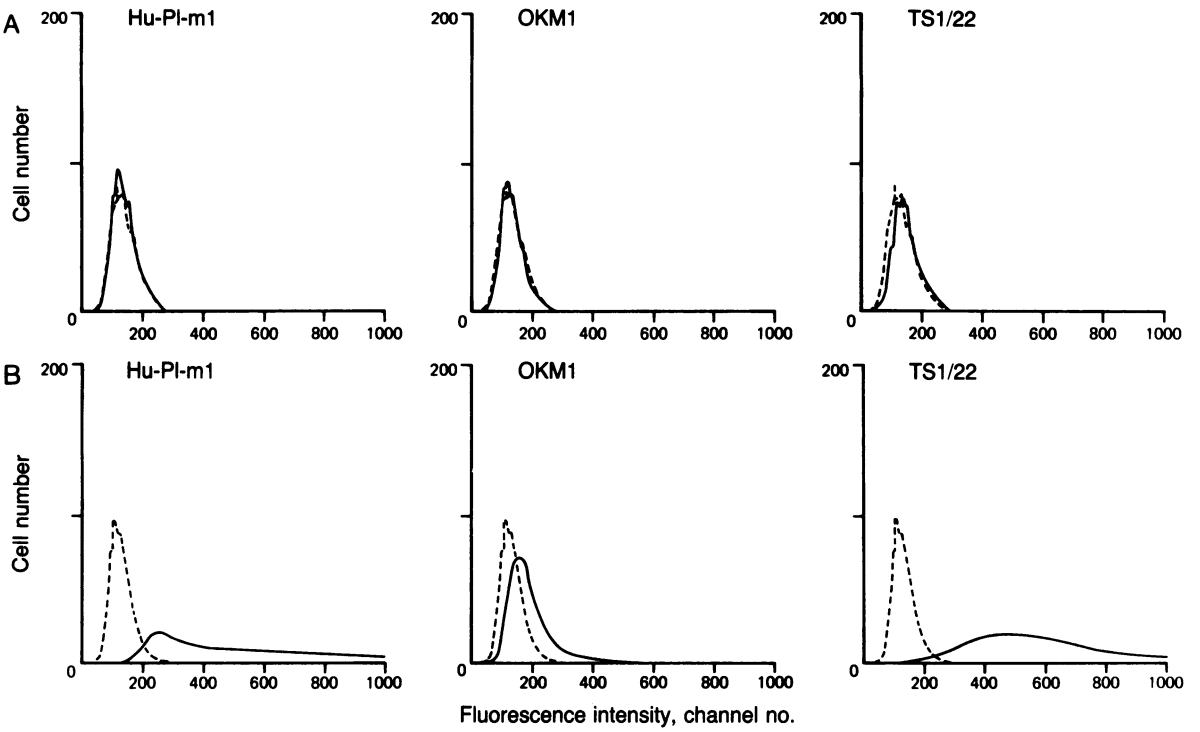

FIG. 2. Transient-expression analysis of LTA-5 cells. (*A*) L cells transfected with L-cell DNA. (*B*) L cells transfected with rescued λ phage DNA. Assays were performed 48 hr after transfection. ——, Test antibody; ----, control (Ly-2.1) antibody.

Our studies link three important cell surface molecules. LFA-1 is involved implicitly in the function of lymphocytes, presumably as a receptor for different target cells with which lymphocytes interact; OKM1 is the receptor for C3bi and thus of importance in the clearance of antigen–antibody complexes and thereby in the regulation of the immune response at both the T- and B-cell level. Finally, gpIIb–IIIa acts as a receptor for fibrinogen in a process intimately involved in blood coagulation. These three separate and discrete processes may not be so different as originally thought, and our results suggest that the three molecules may be encoded by the same gene.

We thank Dr. T. Maniatis and Dr. O. Bernard for the use of the λ library, Mr. Peter Collins for fluorescence cytometry, and Dr. Mark Hogarth for helpful discussion. Grant support was obtained from the National Health and Medical Research Council of Australia and from The University of Melbourne.

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