

Structure/Function Analysis of the Integrin β_1 Subunit by Epitope Mapping

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Abstract. Monoclonal antibodies (mAbs) have been produced against the chicken β_1 subunit that affect integrin functions, including ligand binding, α subunit association, and regulation of ligand specificity. Epitope mapping of these antibodies was used to identify regions of the subunit involved in these functions. To accomplish this, we produced mouse/chicken chimeric β_1 subunits and expressed them in mouse 3T3 cells. These chimeric subunits were fully functional with respect to heterodimer formation, cell surface expression, and cell adhesion. They differed in their ability to react with a panel of anti-chicken β_1 mAbs. Epitopes were identified by a loss of antibody binding upon substitution of regions of the chicken β_1 subunit by homologous regions of the mouse β_1 subunit. The identification of the epitope was confirmed by a reciprocal exchange of chicken and mouse β_1 domains that resulted in the gain of the ability of the mouse subunit to interact with a particular anti-chicken β_1

mAb. Using this approach, we found that the epitopes for one set of antibodies that block ligand binding mapped toward the amino terminal region of the β_1 subunit. This region is homologous to a portion of the ligand-binding domain of the β_3 subunit. In addition, a second set of antibodies that either block ligand binding, alter ligand specificity, or induce α/β subunit dissociation mapped to the cysteine rich repeats near the transmembrane domain of the molecule. These data are consistent with a model in which a portion of β_1 ligand binding domain rests within the amino terminal 200 amino acids and a regulatory domain, that affects ligand binding through secondary changes in the structure of the molecule resides in a region of the subunit, possibly including the cysteine-rich repeats, nearer the transmembrane domain. The data also suggest the possibility that the α subunit may exert an influence on ligand specificity by interacting with this regulatory domain of the β_1 subunit.

THE integrins represent a family of receptors involved in both cell-matrix and cell-cell adhesion (Albelda and Buck, 1990; Hemler, 1990; Hynes, 1992). They are heterodimers consisting of an α subunit non-covalently associated with a β subunit. Currently, 14 different α subunits and 8 different β subunits (not including alternatively spliced forms) have been described. They join in different combinations to form the 20 integrins presently recognized in vertebrates. Each heterodimer forms a transmembrane complex, its extracellular domain interacting with a ligand and its cytoplasmic domain interacting with elements of the cytoskeleton. Integrin-ligand interactions also serve to generate intracellular signals that result in cytoplasmic alkalization (Schwartz et al., 1991), protein phosphorylation (Guan et al., 1991; Kornberg et al., 1991; Guan and Shalloway, 1992), changes in calcium influx (Ng-Sikorski et

al., 1991; Pardi et al., 1989), and cell proliferation (Shimizu et al., 1990). In addition, integrin responses to second messages result in changes of ligand specificity or affinity (Shattil et al., 1985; Tanaka et al., 1992; Thornhill et al., 1991). Ligand binding and receptor activation are accompanied by conformational changes in integrins that result in new mAb binding sites and increased ligand affinity (Coller, 1985; Frelinger et al., 1990, 1991; Altieri and Edgington, 1988; Du et al., 1991). Thus integrins bind ligands, respond to intracellular and extracellular signals, coordinate cytoskeleton-membrane interactions, and transmit information into the cell resulting in changes in cell morphology and function (for review see Hynes, 1992).

To identify regions of the β_1 subunit that may be involved in several of these functions, we have mapped the epitopes of a panel of chicken β_1 -specific mAbs that interfere with a particular integrin function using a series of mouse/chicken chimeric subunits. The reagents employed in these studies include mAbs that perturb cell-substratum interactions, disrupt subunit association, or affect substratum specificity.

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Table I. PCR Primers for Chimeric Plasmid Construction

Chimeric construct	Sequence	Rest. Enzy. Site
MC-1	S 5'-AAGCCGAGGCCTGCCGACACCGACCCGAGACCC-3'	StuI
	AS 5'-AGCCAATTCGAAGTCTGAAGTAATCCTCTCA-3'	AsuII
MC-2	S 5'-CAGACTTTCGAATTGGCTTTGGC-3'	AsuII
	AS 5'-TCACTAAGCTTCTGAACAAGGTG-3'	HindIII
MC-3	S 5'-GTTCCAGAAGCTTAGTGAAAATAAT-3'	HindIII
	AS 5'-ACTTGGGATCCGTGCACTTACAA-3'	BamHI
MC-5	S 5'-CAGACTTTCGAATTGGCTTTGGC-3'	AsuII
	AS 5'-ACTTCATCTGTGCTACATTCAC-3'	
	S 5'- <u>G</u> TGAATGTAGCACAGATGAAGT-3'	
	AS 5'-TTTGGGATCCGTGCA-3'	BamHI

PCR primers used in the construction of four chimeric chicken/mouse cDNAs are shown. For each construct the cloning restriction enzyme site within the primer is highlighted and the enzyme listed in the right column. The complementary sequences of the primer pair used for splice overlap extension in the construction of MC-5 are underlined. S, sense primer; AS, antisense primer.

Among the antibodies that perturb cell substratum interactions are CSAT (Neff et al., 1982) and JG22 (Greve and Gottlieb, 1982), both of which have been extensively characterized. It is assumed that these antibodies interact with regions of the receptor involved in ligand binding. Another antibody, TASC (Neugebauer and Reichardt, 1991), has the ability to block the binding of neuronal cells to vitronectin, and also to stimulate their adherence to laminin. This antibody is assumed, therefore, to interact with regulatory domains of the integrin β_1 subunit.

Our results demonstrate that ligand-binding activity is blocked by mAbs that recognize epitopes widely separated on a linear amino acid map, and that there exist both ligand binding and regulatory regions that are distinct from one another.

Materials and Methods

Chimeric Plasmid Construction

We have previously described the construction and expression of the full length cDNA clone for chicken β_1 , pCINT β_1 RV (Solowska et al., 1991). The chicken β_1 cDNA was also cloned into the EcoRI site of the expression vector, pCMV-5 (Anderson et al., 1988), provided by Drs. Gary Cohen and Rosalyn Eisenberg of the University of Pennsylvania (Philadelphia, PA), that had been modified to eliminate the cloning sites between Kpn I and Sma I. The resulting expression plasmid was designated pCMV-5CINT β_1 RV. A 3.6-kb Hind III cDNA fragment containing most of the coding region of the mouse β_1 subunit in Bluescript (pBSM β_1) was kindly provided by Dr. V. Michael Hollers of Washington University (St. Louis, MO).

PCR-directed mutagenesis and PCR-driven splice overlap extension (SOE)¹ (Horton et al., 1990) were used to generate five chimeric mouse/chicken β_1 expression plasmids, designated MC-1 through MC-5, as described below. PCR reactions were performed in an automatic thermal cycler (Perkin-Elmer Cetus Instrs., Norwalk, CT). Each 100 μ l reaction mixture included 10 mM Tris-HCl, pH 8.0, 1.5 mM MgCl₂, 50 mM KCl, 0.1% gelatin, 10% DMSO, 0.06% 2-mercaptoethanol, 200 mM each dNTP and 2.5 U of Taq DNA polymerase (Perkin-Elmer Cetus Instrs.). Reactions were cycled 25 times through a program that included incubations at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min.

The primers used in the construction of each of the mouse/chicken chimeric cDNAs are shown in Table I.

Construction of MC1. Primers were designed to amplify the mouse β_1 subunit from bp 1 to bp 627 and contained Stu I (sense primer) and Asu

II (antisense primer) sites at their 5' ends. Because pBSM β_1 did not contain 395 bp of the 5' coding region, we used reverse transcription-PCR (RT-PCR) to amplify this fragment. Total cellular RNA was isolated from 3T3 cells by sequential extraction in RNazol (Biotecx, Houston, TX) and chloroform, followed by isopropanol precipitation. 5 μ g of total RNA was subjected to RT-PCR using the cDNA Cycle Kit (Invitrogen, San Diego, CA) according to the manufacturer's directions. First strand synthesis was accomplished using random primers and reverse transcriptase. This strand was used as template in the standard PCR reaction. The RT-PCR product was gel purified using Gene Clean II glass beads (Bio 101) according to the manufacturer's directions and cut with Stu I and Asu II. pCINT β_1 -RV was cut with Stu I and Asu II. The large piece of these digestion products was purified and ligated with the murine cDNA obtained above to yield the plasmid pMC1.

Construction of MC2. PCR primers were designed to amplify the region of mouse β_1 from bp 627 to bp 1077 of the cDNA and contained Asu II (sense primer) and Hind III (antisense primer) restriction sites at their 5' ends. Amplified DNA product was separated on a 1.0% agarose gel, visualized by ethidium bromide, purified, and digested with Asu II and Hind III. pCINT β_1 RV was cut with Asu II and Hind III and the digestion products were separated on an agarose gel, and the larger piece was cut out and purified. This larger piece was then ligated with the amplified mouse β_1 cDNA fragment. The resulting plasmid was designated pMC2.

Construction of MC3. Primers were designed to amplify the region of mouse β_1 from bp 1077 to 1967 from the cDNA and contained Hind III (sense primer) and BamH I (antisense primer) sites near their 5' ends. PCR product was purified and cut with Hind III and BamH I. pCMV-5CINT β_1 RV cut with Hind III and BamH I was resolved on an agarose gel. The larger piece was purified and ligated with the mouse sequence obtained above. The resulting plasmid was designated pMC3.

Construction of MC4. The plasmids pMC2 and pBSM β_1 were digested with Tth11 I and Xho I and resolved on an agarose gel. The larger fragments from both digests were purified and ligated together. The resulting plasmid was designated pMC4.

Construction of MC5. PCR primers were designed to amplify the mouse β_1 subunit from bp 627 to 1631 using mouse cDNA template and included an Asu II site at the 5' end of the sense primer. Additional primers were designed to amplify the chicken β_1 subunit from bp 1642 to 1980 using the chicken cDNA as template and included a BamH I site at the 5' end of the antisense primer. The mouse β_1 antisense and the chicken β_1 sense primers corresponded to a region that was identical between the species and were complementary to each other. The amplified mouse and chicken cDNA fragments were mixed together and subjected to a further round of SOE in the presence of the mouse sense and chicken antisense primers. The resulting spliced mouse/chicken cDNA was cut with Asu II and BamH I and gel purified. The coding sequence between Asu II and BamH I of pMC1 was cut out and replaced with the spliced cDNA to yield the plasmid pMC5.

Confirmation of Chimeric cDNA Sequence. The chimeric nature of each construct was confirmed by PCR amplification using one mouse-specific and one chicken-specific primer. Constructs were also sequenced across the chimeric junction by the dideoxy nucleotide method to further validate the nature of the insert.

1. **Abbreviations used in this paper:** RT-PCR, reverse transcription-PCR; SOE, splice overlap extension.

Transfection of 3T3 Cells

NIH 3T3 cells maintained in DMEM with 10% FBS were transfected as described previously (Solowska et al., 1989). Briefly, cells (10^5) plated the previous day in 100-mm dishes were cotransfected with 10 μ g of each chimeric construct plasmid, 2 μ g of pSV2-neo and 10 μ g of salmon sperm DNA as a calcium phosphate precipitate. After two days, the cells were split 1:15 into DMEM with 10% FBS and 1 mg/ml Geneticin (G418, GIBCO BRL, Gaithersburg, MD). Approximately 2 wk later, G418 resistant clones were picked and screened for expression of the mouse/chicken chimeric β_1 subunit by immunoblot assay using the chicken specific polyclonal antibody, Chickie (see below). The positive clones were further enriched by limited dilution subcloning and one clone of each construct was used for further characterization.

Protein Labeling

Cells were metabolically labeled by growing them overnight in methionine-free media supplemented with 250 μ Ci [35 S]methionine. Alternatively, cell-surface proteins were labeled with biotin. Briefly, confluent T25 flasks were washed with biotin washing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂) three times, and then labeled with 100 μ g/ml NHS-sulfo-biotin (Pierce Chem. Co., Rockford, IL) in biotin washing buffer for 30 min at 37°C with gentle rocking. After labeling, flasks were washed three times in PBS and the cells extracted with non-ionic detergent buffer (10 mM Tris-Acetate, pH 7.5, 0.5% NP-40, 0.5 mM CaCl₂) containing 2 mM PMSF for 20 min at 4°C. Cell extracts were clarified by centrifugation at 14,000 g for 20 min.

Antibodies

The following polyclonal antisera were used: Lennie (L) and anti-GPI40 were raised in rabbit and goat, respectively, against WGA affinity-purified extracts of rat L6A myoblasts and have been shown to react with a wide variety of mammalian integrin β_1 subunits, but have no reactivity with chicken β_1 (Solowska et al., 1991; Knudsen et al., 1981). Chickie (Ch) is a polyclonal rabbit antibody raised against the chicken integrin molecule (Damsky et al., 1985) that has minimal cross reactivity with mammalian integrins.

The following mAbs, each reacting exclusively with the chicken β_1

subunit, were used: CSAT (Neff et al., 1982), JG22 (Greve and Gottlieb, 1982), G (Buck et al., 1986), 2A10 (Chu and Grunwald, 1991), and TASC (Neugebauer and Reichardt, 1991). The WIB10, V2E9, V8B8, V8F5, V9C3, and W1G11 mAbs to the chicken β_1 subunit were prepared from integrins purified on a CSAT mAb column using 11 day chicken embryos. The properties of WIB10 and V2E9 have been described previously (Hayashi et al., 1990). Details about the mAbs are listed in Table II.

Immunofluorescence

Cells were plated in DMEM containing 2% FBS onto coverslips coated with human plasma fibronectin (10 μ g/ml). After 24 h, cells were fixed for 20 min with 3% buffered paraformaldehyde and permeabilized with an ice-cold solution of 0.15 M NaCl containing 1% NP-40 for 1 min. The coverslips were then incubated for 1 h at room temperature with the polyclonal anti-chicken integrin antibody, Ch, and a monoclonal anti-vinculin antibody (Sigma Chem. Co., St. Louis, MO), washed, and stained for 1 h at room temperature with fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG (Organon Teknica, Rockville, MD). The cells were examined using a Zeiss phase-epi-fluorescent microscope with 63 \times planachromat oil-immersion lens and photographed using Kodak Tri-X film.

Immunoprecipitation

For immunoprecipitation, the nonionic detergent cell extracts were preabsorbed with protein A-conjugated Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ) for 30 min at 4°C. One hundred microliters of this antigen solution were mixed with 50 μ l of antibody solution containing 1 μ g of immunoglobulin for 18 h at 4°C. Immune complexes were absorbed to protein A-conjugated Sepharose beads for 1 h at 4°C and were washed five times with a buffer containing 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5% deoxycholate, and 0.1% SDS. For sequential immunoprecipitation the beads were suspended in 1% SDS, boiled for 5 min, and the SDS diluted tenfold with 2.5% NP-40. The resulting released antigen was mixed with antibody and new protein A-Sepharose, and processed as described above. Immunoprecipitated complexes were then eluted in electrophoresis sample buffer (62.5 mM Tris base, 2% SDS, 10% glycerol, pH 6.8) and electrophoresed through 6% polyacrylamide gels. Biotinylated proteins were transferred from the gel to nitrocellulose paper using the Milli-

Table II. mAbs Used

mAb	Activity	Functional effect
CSAT (Neff et al., 1982)	Immunoprecipitation (IP)	Inhibits fibroblast, myoblast attachment to fibronectin (FN), laminin (LN), and collagen (COL)
JG22 (Greve and Gottlieb, 1982)	IP	
2A10 (Chu and Grunwald, 1991)	Immunoblot nonreduced (IB), IP	Inhibits retinal pigmented epithelial cell attachment to FN, LN
W1B10 (Hayashi et al., 1990)	IB, IP	Inhibits fibroblast attachment to FN; induces rounding of skeletal myoblasts
V8B8 (unpublished observations)	IB, IP	
TASC (Neugebauer and Reichardt, 1991)	IB, IP	Inhibits retinal neuron attachment to vitronectin; promotes retinal neuron attachment to LN and COL
G (Buck et al., 1986)	IB, IP	Disrupts $\alpha\beta$ heterodimer formation
V2E9 (Hayashi et al., 1990)	IB, IP	None
V9C3 (unpublished observations)	IB (also reduced), IP	
V8F5	IB, IP	
W1G11	IB, IP	

blot SDE system (Millipore Corp., Bedford, MA) according to the manufacturer's instructions. Biotinylated protein bands were detected by alkaline phosphatase conjugated-streptavidin (GIBCO BRL) using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chem. Co.) as substrate. [³⁵S]methionine-labeled protein containing gels were enhanced, dried, and exposed to X-omat film (Kodak).

Cell Adhesion Assay

Non-tissue culture 96 well plates were coated overnight with laminin (1 μg/well) at 37°C, and then coated with 4% BSA in PBS for 1 h. Cells, labeled with [³H]thymidine 24 h before use were trypsinized, washed three times in serum free DMEM, and plated at 5 × 10⁴ cells per well in the presence of specific antibodies. The cells were allowed to adhere for 2 h at 37°C. Unattached cells were removed by gentle washing and the remaining cells were collected on a glass fiber filter using the Tomtec 96 well cell harvester. Filters were air dried and counted in a Packard Matrix 96 counter. Percent adherence was determined by dividing the counts remaining by the total counts plated per well, and normalized to the condition of no added antibody. Each value represents the average of at least four different measurements, and is shown with standard error of the mean.

Results

Mouse-Chicken Chimeric β₁ cDNAs Encode Chimeric β₁ Subunits, Associate with Endogenous Mouse α Subunits, and Are Expressed on the Cell Surface

The chimeric β₁ subunits used throughout these investigations are schematically represented within each figure (e.g., Fig. 1 A). In all cases, within the schematic representation, the filled portions represent chicken sequences and open areas represent mouse sequences. The various constructs are designated C for chicken, M for mouse, and MC for mouse/chicken chimera.

The accuracy of the cDNA constructs was determined by PCR analysis and sequencing (see Materials and Methods). In each case, the PCR analysis and subsequent sequencing data confirmed the position of the inserted mouse sequences as well as the fact that no changes in nucleotide sequence occurred during the production of the chimeric cDNA (data not shown).

Fig. 1 demonstrates the specificity of the anti-chicken β₁ serum, the chimeric nature of each product, the association of each chimeric subunit with endogenous mouse α subunits and the surface expression of each chimeric subunit. Fig. 1 B shows that the chimeric subunits were found on the cell surface in association with mouse α subunits. To accomplish this, cell surface proteins were biotinylated, extracted with nonionic detergents, and subjected to immunoprecipitation followed by SDS-PAGE of the immuno complexes. The pair of SDS-PAGE lanes under the mouse schematic (M) shows the results of immunoprecipitating aliquots of an extract from nontransfected 3T3 cells with the anti-mammalian β₁ serum (L) and the anti-chicken β₁ serum (Ch). The endogenous β₁ integrins and accompanying α subunits were readily identified by the anti-mammalian β₁ serum but not by the anti-chicken β₁ serum. In contrast, 3T3 cells transfected with full-length chicken β₁ cDNA (C) or the three different mouse/chicken chimeric cDNAs (MC1, MC2, and MC3) all contained 110–120 kD β₁ subunits that could be recognized by the anti-chicken β₁ serum, plus coprecipitating proteins of ~140 kD representing the accompanying endogenous mouse α subunits. Thus, the products of the transfected

cDNAs all were recognized by the chicken-specific antiserum, all paired with endogenous mouse α subunits, and all were found on the cell surface.

Fig. 1 C illustrates the chimeric nature of the protein product of each cDNA. Transfected 3T3 cells were labeled with [³⁵S]methionine and extracted with NP-40. Each extract was first immunoprecipitated with the anti-chicken β₁ serum, Ch. The immunoprecipitate was then dissociated with SDS and divided into two portions. One was used directly for SDS-PAGE analysis and the other was reprecipitated with the anti-mammalian β₁ antibody, L. The first set of lanes under the schematic (M) representing endogenous mouse β₁, shows a typical immunoprecipitate of integrins from 3T3 cells. There are two non-specific bands noted, one in the vicinity of the 205 kD marker and the other at ~8 kD, typical of metabolically labeled extracts. The two bands at 110 and 120 kD represent the immature and fully glycosylated forms of the mouse β₁ subunit. The bands above this represent a mixture of mouse α subunits including α₃ and α₅ as shown previously (Solowska et al., 1989). No material was specifically immunoprecipitated from this extract by the anti-chicken β₁ antibody (Ch). The second set of controls is shown in the pair of lanes beneath schematic (C) representing chicken β₁. The SDS-PAGE profile of the initial immunoprecipitate (Ch) shows the immature (pre-β₁), fully glycosylated β₁ bands and accompanying α subunits. As expected, the anti-mammalian β₁ antibody (L) failed to reprecipitate any material. Similar immunoprecipitates from chimeric constructs are shown beneath each chimeric symbol (MC1, MC2, and MC3). In every case, material from the Chickie immunoprecipitate (Ch) could be reprecipitated with the antiserum that recognized mouse β₁ sequences (L). The immunoprecipitates in lanes designated L show only β or pre-β bands and little if any associated α subunit, as expected for integrins reprecipitated after dissociation with a denaturing agent. As judged by these criteria, the subunits encoded by the constructs are indeed chimeric proteins.

Chimeric β₁ Subunits Localize to Focal Contacts and Promote Cell-Substratum Adhesion

As a test of the functional integrity of the chimeric β₁ subunits, we evaluated their ability to localize in adhesive structures and to participate in cell-matrix adhesion. Transfected cells were plated onto coverslips coated with fibronectin, and 24 h later subjected to immunofluorescence analysis using the anti-chicken β₁ antibody. Fig. 2 shows, that in each case, the chimeric protein was found concentrated in focal contacts at the cell-matrix interface. That these were indeed focal contacts was confirmed by the fact that vinculin was localized to the same structures by double immunofluorescence.

The test for the ability of the chimeric subunits to promote cell adhesion was based upon the resistance of the chicken-mouse chimeric subunit to the adhesion disrupting activity of anti-GP 140, a polyclonal anti-mammalian β₁ subunit that has been previously used to perturb cell substratum adhesion (Knudsen et al., 1981; Damsky et al., 1982; Sutherland et al., 1988). A similar assay has been used to demonstrate the ability of chicken β₁ subunits to promote adhesion in mammalian cells (Hayashi et al., 1990; Solowska et al., 1991). For this purpose, cells were plated on

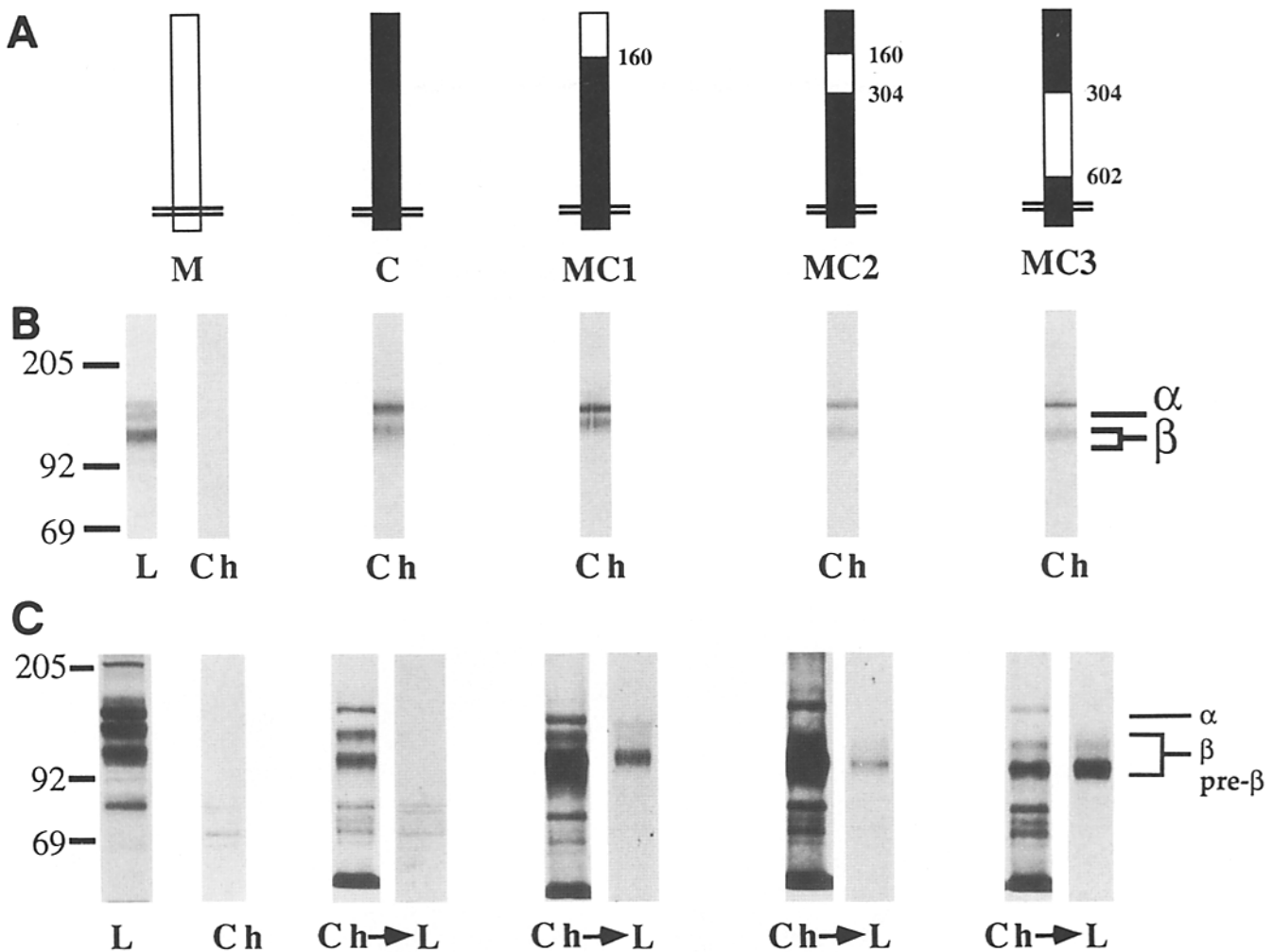


Figure 1. Immunological analysis of the products encoded by chimeric β_1 cDNAs. (A) Schematic representation of control and chimeric cDNA constructs. Open areas correspond to mouse sequences. Filled areas correspond to chicken sequences. Numbers correspond to amino acid residues bracketing inserted mouse cDNA fragments. *M*, mouse; *C*, chicken; *MC*, mouse/chicken chimera. (B) Immunoprecipitates of biotinylated cell-surface protein extracts from 3T3 cells expressing each construct are shown in SDS-PAGE lanes beneath the corresponding schematic symbol. Extracts from untransfected cells were immunoprecipitated with anti-mammalian β_1 (*L*) or anti-chicken β_1 (*Ch*) antibodies. The positions of integrin α and β subunits are designated on the right. The position of each *M_r* standard in kD is shown on the left. The data in the first two lanes represent immunoprecipitation of nontransfected 3T3 cells with both antibodies. These lanes show immunoprecipitation of integrin complexes (*M_r* 110–200) by *L* but not by *Ch* establishing the specificity of the antibodies with respect to endogenous β_1 integrins. The extracts containing intact chicken or chimeric β_1 subunit protein reveal surface-expressed integrin heterodimers. (C) Metabolically labeled extracts from 3T3 cells expressing wild-type or chimeric constructs were immunoprecipitated with the anti-chicken β_1 antibody (*Ch*). One half of the immunoprecipitate was dissociated with SDS and reprecipitated using the anti-mammalian β_1 antibody (*L*). Both precipitates were resolved on SDS-PAGE gels. Results are shown beneath the corresponding schematics. The arrow beneath the lanes indicates sequential immunoprecipitation. The positions of integrin α , pre- β , and β subunits are designated on the right. The pre- β subunit is the bottom band encompassed by the bracket. While all of the transfected cell extracts showed reactivity with *Ch*, only the chimeric subunits showed immunoreactivity with the anti-mammalian β_1 antibody on reprecipitation (*MC1*, *MC2*, and *MC3*).

dishes coated with laminin in the absence of blocking antibody or in the presence of either anti-GP140, CSAT, or anti-GP140 plus CSAT. The control cells used in this experiment were 3T3 cells transfected with full length chicken β_1 cDNA (*C*) or with cDNA encoding a chicken β_1 subunit missing the cytoplasmic domain (ΔC), and hence unable to promote cell-matrix adhesion, but capable of binding the CSAT mAb (Solowska et al., 1989). While CSAT alone had little or no effect on the ability of any of the cells to adhere to laminin,

anti-GP 140 reduced adhesion of the ΔC cells by $\sim 90\%$ indicating that adhesion was mediated by integrins containing the mammalian β_1 subunit. In contrast, cells expressing the intact chicken β_1 subunit or the chimeric mouse/chicken subunits remained adherent even in the presence of anti-GP 140. Furthermore, exposure of *C*, *MC2*, and *MC3* cells to both anti-GP 140 and CSAT prevented adhesion of 80–90% of the cells. However, $\sim 60\%$ of *MC1* cells remained adherent when CSAT was added to anti-GP140, suggesting a loss

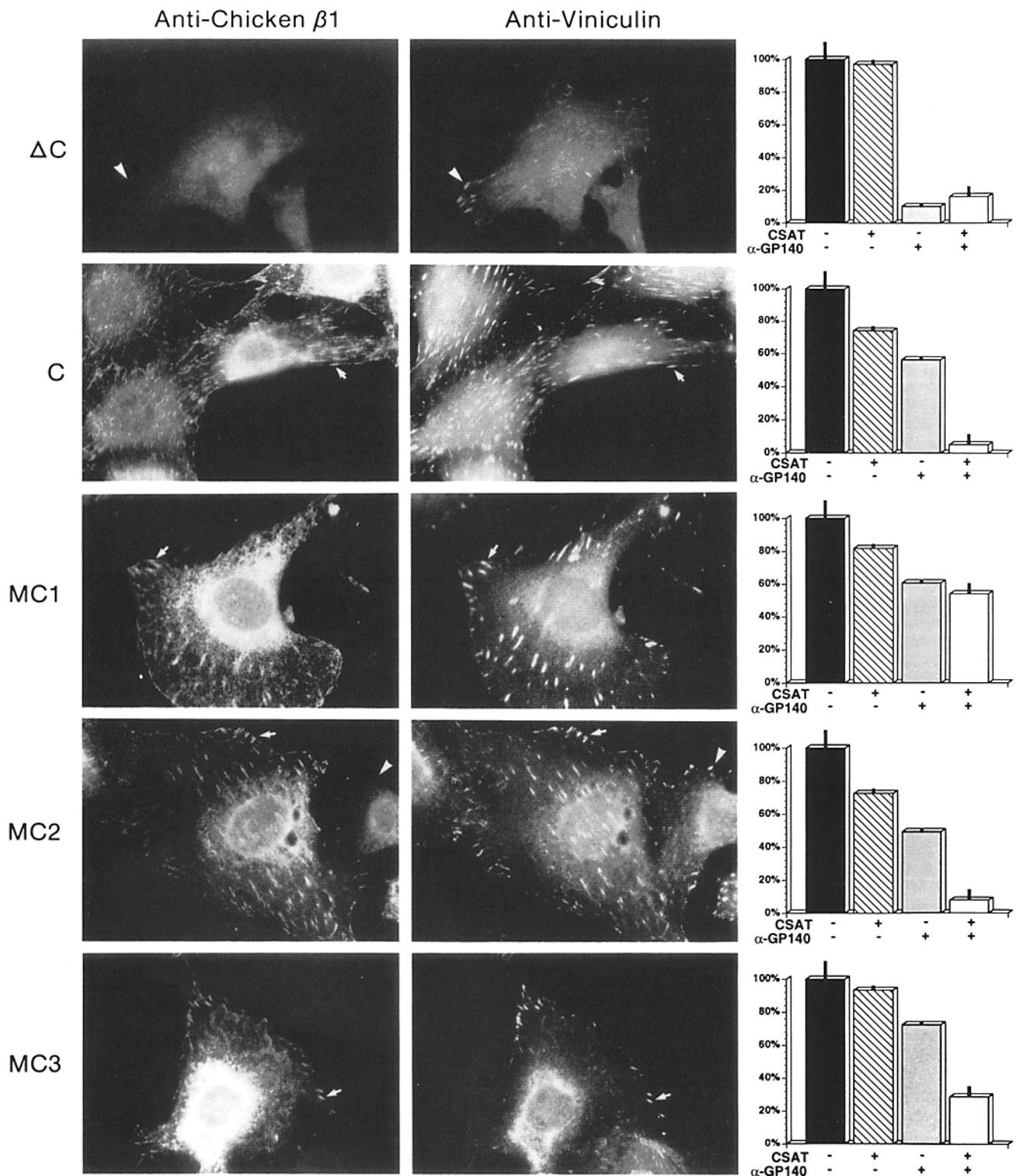


Figure 2. Chimeric β_1 subunits are found in focal contacts and participate in cell-substratum adhesion. Transfected 3T3 cells were plated onto coverslips coated with fibronectin and stained with a mixture of rabbit anti-chicken β_1 polyclonal antibody and mouse anti-vinculin mAb. The coverslips were counterstained with fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG. The photomicrograph pairs in each row depict cells expressing the construct indicated (*MC1*, *MC2*, and *MC3*) or control 3T3 cells transfected with a cytoplasmic deficient mutant of chicken β_1 (ΔC) or full-length chicken β_1 (*C*). Cells in the left column were photographed to show the chicken β_1 staining (fluorescein). The identical preparation was photographed to show the vinculin staining (rhodamine) in the central column. Arrows designate typical focal contacts showing both fluorescein and rhodamine staining. Arrowheads in the first pair of micrographs (ΔC) show rhodamine-stained focal contacts that contain no fluorescein staining, despite the fact that the cytoplasm of this cell is brightly fluorescent. Arrowheads in the photomicrographs of *MC2* show the appearance of rhodamine-, but not fluorescein-stained focal contacts in an untransfected cell further indicating the specificity of the Ch antibody for only those focal contacts containing subunits with chicken sequences. The right column shows antibody inhibition of adhesion of control and transfected cells labeled

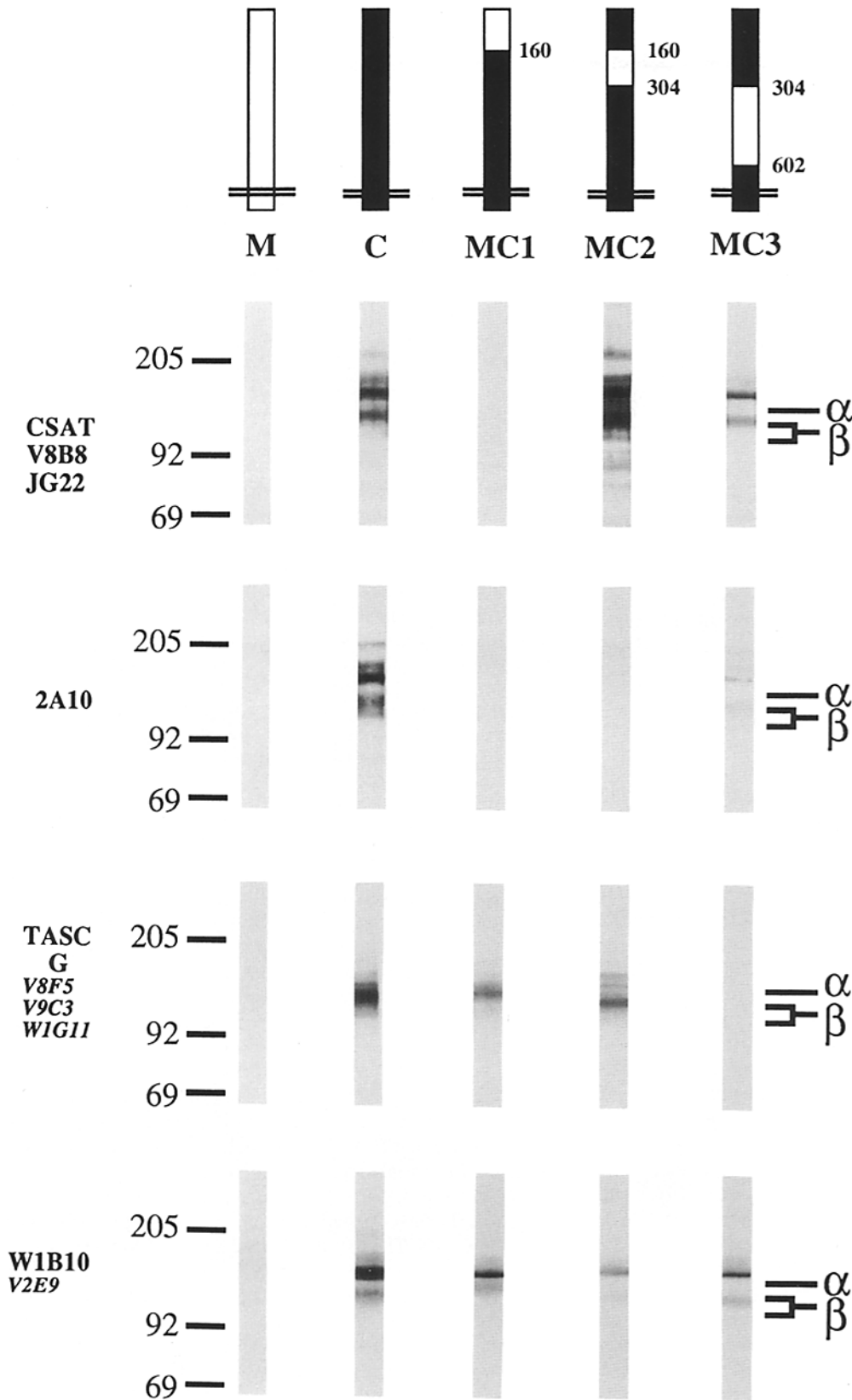


Figure 3. Position of epitopes for four representative mAbs. Immunoprecipitations using the indicated monoclonal anti-chicken β_1 antibodies of surface-biotinylated control and transfected 3T3 cell extracts were subjected to SDS-PAGE, transferred to nitrocellulose, and developed as described in Materials and Methods. The results are shown beneath the schematic of each construct. *M*, Mouse; *C*, Chicken; *MC*, Mouse/Chicken chimera. Open portions of symbols designate the position of mouse sequences; filled portions designate chicken sequences. The mAbs used for immunoprecipitation are shown at the left of each set of SDS-PAGE lanes. Where more than one antibody is specified, the results for each antibody were identical. The positions of α and β subunits are shown at the right of each set of lanes; *M*, standards in kD are shown on the left. Biotinylated mouse integrins were present in extracts of control 3T3 cells (*M*) as shown in Fig. 1. mAbs with no known biological activity are italicized.

with tritiated thymidine and plated in laminin-coated wells in the absence of antibody (*filled bars*); in the presence of CSAT (*hatched bars*), in the presence of an anti-mammalian β_1 subunit antibody (anti-GP140; *stippled bars*), or CSAT plus anti-GP140 (*open bars*). Control adhesion in the absence of added antibody was normalized to 100%. Note the resistance of all cells except for control (ΔC) to the adhesion disrupting effects of the anti-mammalian β_1 antibody, and the requirement for both antibodies for maximum inhibition of adhesion of cells transfected with chicken β_1 , MC2, and MC3, but not MC1 cDNA. See text for details.

of the CSAT reactive region in this chimeric subunit (see Fig. 3). Finally, the fact that MC1, MC2, and MC3 remain adherent in the presence of anti-GP140 suggests that the adhesion disrupting epitope recognized by this polyclonal antiserum is absent in all three chimeric subunits. The chicken sequence that is common to these three subunits contains the epitope for another adhesion blocking mAb, WIB10, as shown in Fig. 3. These results demonstrate that all the chimeric constructs actively promoted the adhesion of the cells to laminin.

The Epitopes for Adhesion Perturbing and Function Altering mAbs Map to Distinct Regions of the β_1 Subunit

The epitope mapping presented here takes advantage of the fact that each of the mAbs used reacts only with the chicken β_1 subunit. Therefore, the ability of any of these mAbs to recognize a chimeric mouse/chicken subunit requires the presence of the appropriate chicken peptide sequence folded into its native configuration. Since integrin function is easily disrupted as a result of genetic manipulation of subunit cDNA, the interpretation of these experimental results is dependent upon the functional integrity of the subunit under investigation (for review see Buck et al., 1990). The above experimental results suggest that the chimeric subunits maintained their functional integrity, and that the failure of any mAb to react with a particular mouse/chicken chimeric subunit should be due to replacement of the chicken sequence by the homologous mouse sequence, and not due to secondary structural alterations in the chimeric subunit. Therefore, loss of antibody reactivity constitutes evidence that at least a portion of the binding site for the particular mAb in question is within the replaced region of the molecule.

Chimeric constructs were prepared in which chicken sequences were systematically replaced with homologous mouse sequences between residues 1 and 602. The inserted mouse sequences ranged in size from 140 to 300 amino acids. Typical data for four different mAbs are presented in Fig. 3. Of the four mAbs used here, three disrupt cell substratum adhesion (CSAT, 2A10, and WIB10) and one blocks chicken cell adhesion to vitronectin but promotes adhesion to laminin (TASC). Transfected and control cells were biotinylated, extracted with NP-40, and the extracts immunoprecipitated with each of the mAbs. Immunoprecipitates were analyzed by SDS-PAGE. The SDS-PAGE profile of each construct is shown below its respective schematic presentation. All mAbs grouped together in Fig. 3 gave the same immunoprecipitation profiles. mAbs with no known biological activity are italicized.

Three of the mAbs that block cell substratum adhesion, CSAT, JG22, and V8B8 failed to immunoprecipitate chimeric subunit MC1 in which the first 160 amino acids of the chicken subunit were replaced by the homologous region from the mouse subunit. They did, however, immunoprecipitate each subunit in which the first 160 amino acids was chicken, suggesting that part of the epitope for these antibodies resides in this region of the molecule. 2A10, another adhesion-perturbing mAb, could only precipitate MC3, indicating that the epitope for this mAb resides on both sides of amino acid 160 of the chicken subunit. The fact that both the MC1 and MC2 chimeric subunits contained chicken amino

acid sequences from residues 602 to 779 indicates that the epitope for 2A10 does not lie in this region of the molecule.

Interestingly, the integrin activating mAb TASC could immunoprecipitate all the chimeric subunits with the exception of MC3. This suggests that the epitope for this mAb resides between amino acids 304 and 602. The epitopes for several other mAbs also mapped to this region of the β_1 subunit. These include the mAb "G," which has been previously shown to disrupt $\alpha\beta$ subunit association (Buck et al., 1986), as well as several mAbs with no known biological activity.

The mAb WIB10 is capable of disrupting cell-substratum adhesion. The epitope for this antibody, however, appears to rest outside the region of the subunit carrying the epitopes for other adhesion-disrupting mAbs. This mAb immunoprecipitated all the chimeric constructs. The only chicken sequence that is common to all of the chimeric subunits lies between residues 602 and 779. It is reasonable to assume that the antibody cannot react with residues 709 to 779 which include the transmembrane and cytoplasmic domain sequences of the subunit. At least part of the epitope for WIB10 must, therefore, rest between residues 602 and 709. The observation that MC1, MC2, and MC3 resist detachment by the anti-GP140 mammalian β_1 antiserum and in particular that MC1 is largely resistant to detachment by the combination of anti-GP140 and CSAT (Fig. 2), suggest that this polyclonal antibody is reacting primarily against the mouse homologue of the epitope seen by WIB10.

Insertion of Homologous Regions of the Chicken β_1 cDNA into the Mouse Subunit Confers Chicken Specific mAb Binding to the Chimeric Subunit

To confirm the location of two regions identified as antibody binding epitopes by the loss-of-binding studies described above, we tested two reciprocal constructs for the acquisition of mAb-binding activity. To accomplish this, we placed the chicken cDNA coding for the CSAT- or TASC-binding domains into the homologous regions of the mouse β_1 subunit. The sequences carrying the epitopes for these mAbs were chosen for two reasons. First, each antibody has distinctly different effects on integrin function. Second, their epitopes mapped to distinct regions of the molecule (i.e., CSAT epitope within the first 160 amino acids; TASC epitope within the cysteine-rich repeats, from amino acids 304 to 602). The two constructs are illustrated in Fig. 4. As would be predicted, MC4, in which the first 160 mouse sequences were replaced by chicken sequences, reacts with CSAT but not TASC. On the other hand, MC5, in which amino acids 493-779 are chicken, reacted with TASC, but not with CSAT. The fact that TASC did not react with MC3, which contains chicken sequence between amino acids 602 and 779, further limits its epitope to between amino acids 493 and 602. These experiments confirm the location of the epitopes and demonstrate that the loss of mAb-binding activity in the other chimeric subunits was not caused by secondary structural changes.

Discussion

To facilitate interpretation, the data presented here are schematically summarized in Fig. 5. The region of the β_1 subunit homologous to the suggested ligand-binding domain

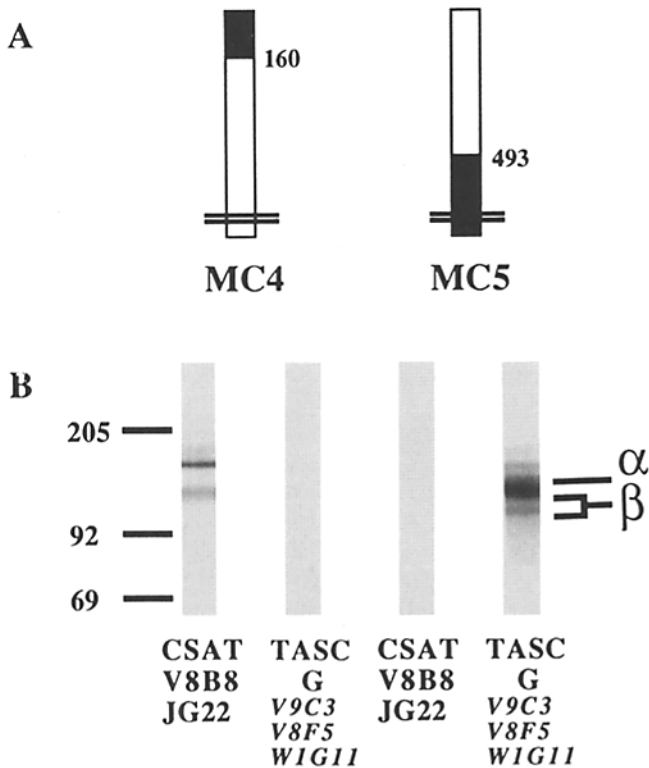


Figure 4. CSAT and TASC epitopes can be transferred to mouse β_1 cDNA. The regions of chicken β_1 cDNA carrying either the CSAT epitope (MC4) or the TASC epitope (MC5) were substituted into the homologous position on mouse β_1 cDNA. The resulting constructs were transfected into 3T3 cells. The transfected cells were biotinylated and extracted as described in Materials and Methods. The extracts were immunoprecipitated with CSAT or TASC as shown in the figure. Antibodies are listed as described in Fig. 3. In both cases, the substitution of the appropriate chicken sequence resulted in the chimeric subunit being immunoprecipitated by the corresponding mAbs.

of β_3 is indicated by shading. A 19-amino acid sequence within this region that is highly conserved among all β subunits is depicted as a solid bar. The cysteine residues that are homologous with those cross-linked in the β_3 subunit to form two long-range disulfide bonds (Calvete et al., 1991; Beer and Collier, 1989) are connected by lines. These include the first cysteine (C1) occupying position 7 paired with the 16th cysteine (C16) occupying position 445, and the 14th cysteine (C14) at position 415 paired with the 54th cysteine (C54) at position 672. Regions of the subunit bearing the epitopes for the various mAbs investigated here are bracketed by arrows. Numbers of important reference amino acid residues are indicated above the bar.

Integrin-ligand binding and ligand specificity can be altered as a result of long range structural perturbations by antibodies or by intracellular signals (Collier, 1985; Shattil et al., 1985; Brass and Shattil, 1987; O'Toole et al., 1990; Frelinger et al., 1991; Frojmovic et al., 1991; Sims et al., 1991; Chen et al., 1992; discussed in Ginsberg et al., 1992). The assignment of functional domains to an integrin subunit on the basis of epitope mapping, mutational analysis, deletion analysis or functional analysis of intersubunit chimeras must therefore take into account the possibility that any alterations in integrin function or, in this case, antibody binding, could be the result of secondary changes at other regions of the molecule. We therefore confirmed that the chimeric subunits under investigation here were fully functional and that the interchange of mouse and chicken domains did not, to the best of our knowledge, induce subtle secondary alterations in the structure. The chimeric β subunits paired with α subunits, were found on the cell surface, localized in adhesion plaques, and functioned as part of the cell-adhesive mechanism. In addition, the effects of mAb binding on substitution of mouse sequences into homologous regions of the chicken β_1 subunit were position specific and reciprocal. That is, if the substitution of a particular mouse domain on the chicken subunit resulted in loss of antibody-binding ac-

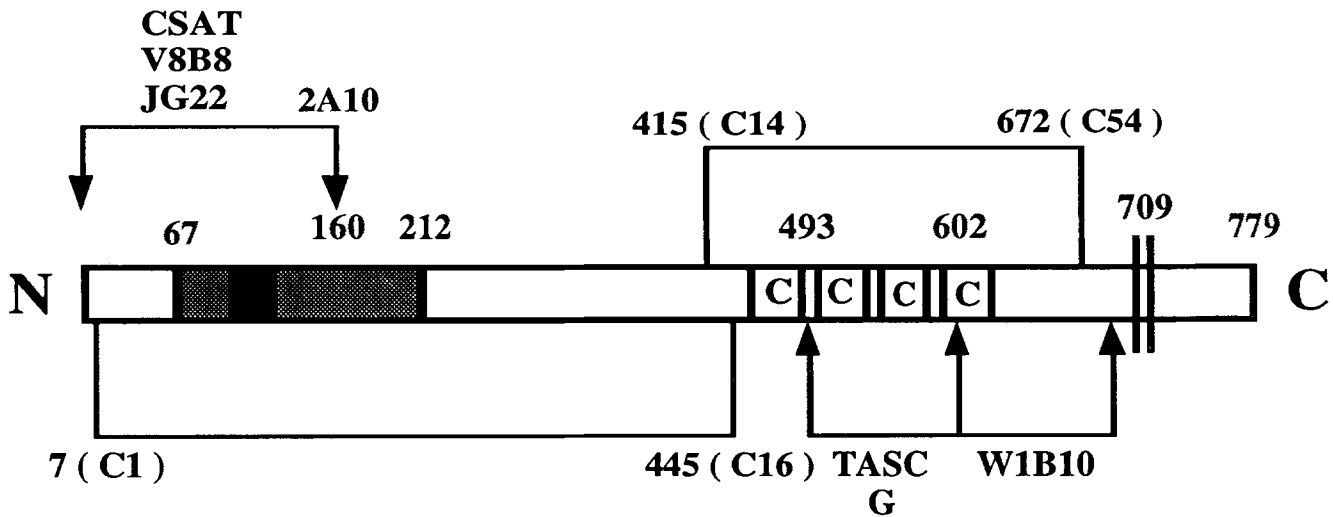


Figure 5. Linear schematic summary of epitopes for function-altering mAbs. The regions carrying the epitopes for each set of mAbs are bracketed by arrows. Relevant amino acid residue numbers are indicated along the top of the bar. Proposed long range disulfide bonds are joined linearly with the paired cysteines being designated at the ends of each line. Four cysteine rich repeats, C, are designated within the bar. Shaded and solid areas represent regions homologous to ligand-binding sites on the β_3 subunit as described in the text.

tivity, the replacement of the homologous domain of the mouse β_1 subunit with chicken amino acid sequences resulted in the acquisition of antibody reactivity.

Because of the apparent structural "plasticity" of integrin β subunits, the assignment of functional domains based solely on genetic manipulations may be misleading. Therefore, correlation of genetic analysis with direct chemical or physical determinations of molecular interactions greatly strengthens these structure/function assignments. The assignment of the ligand-binding domain of the β_1 subunit within the first 200 amino acid residues is consistent with several lines of evidence derived from independent analyses. Studies in which RGD-containing peptides have been chemically cross-linked to platelet $\alpha_{IIb}\beta_3$ (D'Souza et al., 1988) and the related vitronectin receptor, $\alpha_v\beta_3$ (Smith and Cheresch, 1988), have identified a homologous region within the first 250 amino acid residues of the β_3 subunit as a ligand-binding domain. Antibodies raised against the highly conserved sequence within this series of amino acid residues (see Fig. 5) can block ligand binding (Andrieux et al., 1991). A single amino acid substitution at position 119 within this region renders $\alpha_{IIb}\beta_3$ incapable of ligand binding (Loftus et al., 1991). A similar mutation in the homologous position of the β_1 subunit also prevents ligand binding (Takada et al., 1992).

The epitopes for other adhesion perturbing antibodies, WIB10 and TASC map between residues 493 and the transmembrane domain of the subunit, placing them almost at the opposite end of the extracellular portion of the molecule. Because there are no data implicating this region of any β subunit with direct ligand binding, we favor the idea that the activity of these mAbs is the result of their binding to regulatory domains of the subunit. The interaction of mAbs with such domains would change the ligand-binding potential of integrins even though the epitopes for these antibodies reside outside the actual ligand-binding site. In this case, antibody binding would result in the integrin losing its ability to recognize one ligand, and gaining the ability to bind another. This proposal is consistent with what is known concerning the effect of antibodies or ligand binding on the β_3 subunit. The binding of mAbs or RGD-containing peptides to the β_3 subunit will, by themselves, cause activation of $\alpha_{IIb}\beta_3$ and increased ligand affinity (O'Toole et al., 1990; Frelinger et al., 1991; Frojmovic et al., 1991). Similar examples of antibodies modifying the activity of integrins carrying the β_1 subunit have been documented. One stimulates the binding of $\alpha_4\beta_1$ on a myelomonocytic cell line to cells expressing recombinant VCAM-1 (Kovach et al., 1992). A second induces modification of ligand affinity on the fibronectin receptor $\alpha_5\beta_1$ (Faull et al., 1993). A third antibody changes the ligand specificity of $\alpha_2\beta_1$ to include both collagen and laminin (Chan and Hemler, 1993). The epitopes for these antibodies have not been identified, but likely reside in domains of the subunit that could be considered regulatory in nature. Assuming the mAb TASC and the ligand blocking mAb WIB10 define such regulatory domains in the β_1 subunit, our data would suggest that these domains will be found in the disulfide-rich region near the transmembrane domain of β subunits. Interestingly, the epitope for the mAb G, which dissociates α/β_1 heterodimers, also maps to this region of the molecule. This raises the possibility that the α subunit could influence ligand specificity both by providing part of

the structure of the actual ligand-binding domain (D'Souza et al., 1990) and by causing secondary changes in the β_1 subunit through interactions outside the actual ligand-binding site. This might help assure the ligand-binding specificity or affinity of integrins sharing a promiscuous α subunit such as α_v , that, depending upon the β subunit with which it associates, can bind to one or more ligands.

In summary, the epitope mapping data presented here are consistent with the idea (a) that the ligand-binding domain of the β_1 subunit is found within the first 200 amino terminal residues; (b) that there is a regulatory domain located more carboxy terminal between residue 493 and the transmembrane domain; and (c) that α subunit interactions may involve sequences also within this regulatory domain.

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