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The Structure of the β-Propeller Domain and C-terminal Region of the Integrin αM Subunit

DEPENDENCE ON β SUBUNIT ASSOCIATION AND PREDICTION OF DOMAINS*

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The αM subunit of integrin Mac-1 contains several distinct regions in its extracellular segment. The N-terminal region has been predicted to fold into a β-propeller domain composed of seven β-sheets each about 60 amino acid residues long, with the I-domain inserted between β-sheets 2 and 3. The structure of the C-terminal region is unknown. We have used monoclonal antibodies (mAbs) as probes to study the dependence of the structure of different regions of the αM subunit on association with the β2 subunit in the αM/β2 heterodimer. All of the mAbs to the I-domain immunoprecipitated the unassociated αM precursor and reacted with the αM subunit expressed alone on the surface of COS cells. By contrast, four mAbs to the β-propeller domain did not react with the unassociated αM precursor nor with the uncomplexed αM subunit expressed on COS cell surface. The four mAbs were mapped to three subregions in three different β-sheets, making it unlikely that each recognized an interface between the α and β subunits. These results suggest that folding of different β-propeller subregions is coordinate and is dependent on association with the β2 subunit. The segment C-terminal to the β-propeller domain, residues 599–1092, was studied with nine mAbs. A subset of four mAbs that reacted with the αM/β2 complex but not with the unassociated αM subunit were mapped to one subregion, residues 718–759, and five other mAbs that recognized both the unassociated and the complexed αM subunit were localized to three other subregions, residues 599–679, 820–882, and 943–1047. This suggests that much of the region C-terminal to the β-propeller domain folds independently of association with the β2 subunit. Our data provide new insights into how different domains in the integrin α and β subunits may interact.

The integrin family of adhesion molecules participate in important cell-cell and cell-extracellular matrix interactions in a diverse range of biological processes (1). Integrins are noncovalently associated α/β heterodimers, with each subunit consisting of a large extracellular domain (>100 kDa for α subunits and >75 kDa for β subunits), a single transmembrane region, and a short cytoplasmic tail (50 amino acids or less, except for the β4 subunit) (1). The adhesiveness of integrins is dynamically regulated in response to cytoplasmic signals, termed "inside-out" signaling (2–4). The leukocyte integrin subfamily consists of four members that share the common β2 subunit (CD18) but have distinct α subunits, αL (CD11a), αM (CD11b), αX (CD11c), and αd for LFA-1, Mac-1, p150, 95, and αd/β2, respectively (5–7). The leukocyte integrins mediate a range of adhesive interactions that are essential for normal immune and inflammatory responses (5).

Although the overall structure of integrins is unknown, several structurally distinct domains in the extracellular portions of both α and β subunits have been predicted or identified. The N-terminal region of the integrin α subunits contains seven repeats of about 60 amino acids each (8) and has recently been predicted to fold into a β-propeller domain that consists of seven β-sheets, with each β-sheet containing four anti-parallel β-strands (9). The leukocyte integrin α subunits (10), the α1 (11) and α2 (12) subunits of the β1 subfamily, and the αE subunit (13) of the β7 subfamily contain an inserted domain or I-domain of about 200 amino acids that is predicted to be inserted between β-sheets 2 and 3 of the β-propeller domain (9). The three-dimensional structure of the I-domain from the Mac-1, LFA-1, and α2β1 integrins has been solved and shows that it adopts the dinucleotide-binding fold with a unique divalent cation coordination site designated the metal ion-dependent adhesion site (14–17). The integrin β subunits contain a conserved domain of about 250 amino acids in the N-terminal portion. This domain has been predicted to have an "I-domain-like" fold (14, 18, 19). Very little is known about the structure of the C-terminal half of the extracellular portions of both α and β subunits. Electron microscopic images of integrins reveal that the N-terminal portions of the α and β subunits fold into a globular head that is connected to the membrane by two rod-like segments about 16 nm long corresponding to the C-terminal portions of the α and β extracellular domains (20–22). This would suggest that the C-terminal portions of both subunits are quite extended.

Previous studies using mAbs[1] as probes have shown that the structure of specific domains in LFA-1 requires association of the αL and β2 subunits. mAbs to the β2 subunit conserved domain do not react with the unassociated β2 subunit, whereas mAbs to the regions preceding and following this domain do, indicating that the structure of the conserved domain is dependent on association with the αL subunit (23). mAbs to the I-domain react with the unassociated αL subunit (24). This finding together with the fact that the I-domain can be expressed as an isolated domain (14, 16, 25, 26) show that the I-domain assumes a native structure independently of the β2

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[1] The abbreviations used are: mAb, monoclonal antibody; FBS, fetal bovine serum; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; hu, human; mo, mouse.

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subunit. By contrast, two mAbs (S6F1 and TS2/4) mapped to the N-terminal region of the β-propeller domain, and one mAb (G-25.2) that maps to a region of 212 amino acids with 159 amino acids located in the β-propeller domain and the remainder in the C-terminal region, do not recognize the αL subunit in the absence of association with the β2 subunit (24). Another mAb (CBRLFA-1/1) that maps to a region overlapping the I-domain and β-propeller domain reacts weakly with the uncomplexed αL subunit. These results indicate that at least one region in the β-propeller domain is dependent on association with the β2 subunit for mAb reactivity, and it has been suggested that the most likely explanation is that folding of the β-propeller domain is not completed until after association with the β subunit (24). Since mAbs specific for the region of the αL subunit C-terminal to the β-propeller domain have not been described, it is not known whether folding of this region is dependent on association with the β subunit.

In this study, we have used mAb probes to study the structure of the Mac-1 α subunit in the presence and absence of association with the β2 subunit. We have studied the β-propeller domain, the I-domain, and the extensive region C-terminal to the β-propeller domain. Compared with the previous studies on LFA-1, our studies on the β-propeller domain are more definitive, since mAb specificity is defined to individual amino acid substitutions between mouse and human, and mAb to epitopes that are widely separated in the predicted β-propeller structure all show a dependence on β subunit association for reactivity. Furthermore, we employ a panel of mAbs that defines four different subregions within the C-terminal region of the α subunit. The results show that epitopes in three of these regions have a native structure in the absence of β subunit association, whereas a fourth epitope is dependent on association with the β subunit. Thus, much of the C-terminal region of the αM subunit appears to assume a native fold independently of association with the β2 subunit.

MATERIALS AND METHODS

Cell Lines—U937, a human monoblast-like cell line, was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 50 μg/ml gentamicin, and 50 μM 2-mercaptoethanol (complete medium). COS cells (SV40-transformed monkey kidney fibroblasts) were maintained in RPMI 1640 supplemented with 10% FBS and 50 μg/ml gentamicin. Human embryonic kidney 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM glutamine, and 50 μg/ml gentamicin.

mAbs—The following murine mAbs against the αM subunit of human Mac-1 were previously described: OKM1, OKM9 (27), TGM-65 (28), CBRM1/1, CBRM1/2, CBRM1/29, CBRM1/20, CBRM1/32, CBRM1/10, CBRM1/16, CBRM1/17, CBRM1/18, CBRM1/23, CBRM1/25, CBRM1/26, and CBRM1/30 (29). All these mAbs were used as ascites except for CBRM1/29 that was used as concentrated hybridoma supernatant. CBRN1/6 and CBRN3/4 against the αM subunit of Mac-1[2] were used as hybridoma supernatant. TS1/18 and CBRLFA-1/2 against human leukocyte integrin β2 subunit were described previously (30, 31) and used as purified IgG.

DNA Constructs and Mutagenesis—The human wild-type αM subunit cDNA was subcloned in the expression vector pCDNA3.1+ (Invitrogen, Carlsbad, CA) as described.[3] For generating human-mouse αM chimeras, a *Sac*II site was created immediately after the stop codon (nucleotides 3532–3534). By specifically primed reverse transcription of murine spleen mRNA (CLONTECH, Palo Alto, CA) from approximately 50 nucleotides downstream of the stop codon, the first strand of the mouse αM cDNA (33) was generated with Moloney murine leukemia virus reverse transcriptase (Stratagene, La Jolla, CA). By using this as a template for PCR, a 2-kilobase pair mouse αM cDNA fragment covering nucleotides from the *Sfi*I site (nucleotide 1688) to the stop codon and having a *Sac*II site immediately after the stop codon was made. This mouse αM *Sfi*I-*Sac*II fragment was used to replace the corresponding human αM *Sfi*I-*Sac*II fragment to generate the initial chimeric αM cDNA encoding the N-terminal 529 residues of human sequence and the remaining C-terminal sequence from mouse. Using this initial chimeric construct as template, eight human-mouse αM chimeras with a variable mouse C-terminal portion were generated by overlap extension PCR (34, 35). Briefly, outer primers for overlap PCR were just 5′ to the *Sfi*I site and 3′ to the *Sac*II site, and the first set of reactions was carried out using the human wild-type αM and the initial chimeric construct as templates. After the overlap extension reaction, the chimeric products were digested with *Sfi*I and *Sac*II, and the *Sfi*I-*Sac*II fragments were swapped into the human wild-type αM in vector pCDNA3.1+. Human to mouse individual amino acid substitutions in the region from amino acids 718–759 of human αM were made by overlap extension PCR (34, 35). Briefly, the overlapping primers contained the desired mutations, and the outer primers were 5′ to the *Sfi*I site and 3′ to the *Nde*I site, respectively. The overlap extension PCR products were digested with *Sfi*I and *Nde*I and swapped into human wild-type αM in expression vector pEFpuro (36).

For mapping mAb epitopes in the β-propeller domain of the human αM subunit, 32 different chimeric αM subunits were made in which a short segment of mouse sequence comprising a predicted loop or a strand 4 was inserted in the human sequence. Mutagenesis was done by inverse PCR on plasmid pBluescript II containing Mac-1 αM cDNA fragments that included the *Not*I site 5′ to the coding region and the *Bsp*EI site at amino acid residue 180 or included the *Bsp*EI-*Bbs*I fragment from 180 to 672, as described elsewhere.[3] The mutated cDNA fragments were excised with *Not*I and *Bsp*EI or *Bsp*EI and *Bbs*I and swapped into wild-type αM cDNA contained in plasmid pCDNA3.1+. Mutants were named after the sheet (W) and the loop (L) or the strand (S) that was exchanged, *e.g.* hu(W7L3–4)mo has mouse sequence in the loop between strands 3 and 4 of W7, and hu(W1S4)mo contains mouse sequence in strand 4 of W1. In the following list, the amino acid segment or individual amino acid residue that was of murine origin is indicated for each mutant in the numbering system for the mature human α subunit. These mutants are as follows: hu(W7L3–4)mo, 7–8; hu(W7L4–1)mo, 16; hu(W1L1–2)mo, 26–29; hu(W1L2–3)mo, 38–44; hu(W1L3–4)mo, 55–56; hu(W1S4)mo, 58–61; hu(W1L4–1)mo, 66; hu(W2L1–2)mo, 82–84; hu(W2L2–3)mo a, 96–98; hu(W2L2–3)mo b, 104; hu(W2S3-I-domain)mo a, 115–120; hu(W2S3-I-domain)mo b, 127; hu(I-domain-W3S1)mo, 327; hu(W3L2–3)mo, 356; hu(W3L3–4)mo, 369–371; hu(W3S4)mo, 376; hu(W4L3–4)mo, 421–425; hu(W4S4), 428–432; hu(W4L4–1)mo, 435–439; hu(W5L1–2)mo a, 450–455; hu(W5L1–2)mo b, 457; hu(W5L2–3)mo, 469; hu(W5L3–4)mo, 484; hu(W5L4–1)mo, 495–500; hu(W6L2–3)mo, 531–534; hu(W6L3–4)mo a, 541; hu(W6L3–4)mo b, 543–550; hu(W6L3–4)mo c, 554; hu(W6S4)mo, 557–559; hu(W6L4–1)mo, 460–464; hu(W7L1–2)mo, 576; hu(W7S3-)-mo, 599–606.

All mutations were verified by DNA sequencing. At least two independent clones of each mutant were used for transfection, and identical results were obtained.

Transient Transfection—COS cells were transfected by the DEAE-dextran method (36) with the αM cDNA alone or were co-transfected with the wild-type or chimeric αM and β2 cDNA. The wild-type and chimeric αM cDNA were in plasmid pCDNA3.1+, and the β2 cDNA was contained in plasmid pEF-BOS (36). Three days after transfection, COS cells were detached with Hanks' balanced salt solution supplemented with 5 mM EDTA for flow cytometric analysis. 293 cells were transfected with the calcium phosphate method (37, 38). Briefly, 7.5 μg of wild-type or mutant αM cDNA in plasmid pEFpuro and 7.5 μg of β2 cDNA in plasmid pEF-BOS were used to transfect one 6-cm plate of 70–80% confluent cells. Two days after transfection, cells were detached with Hanks' balanced salt solution, 5 mM EDTA for flow cytometric analysis.

Flow Cytometry—COS cells and 293 cells were washed twice with L15 medium containing 2.5% FBS (L15/FBS) and resuspended to 1–2 × 10^6 cells/ml in the same medium. 50 μl of the cell suspension was incubated with an equal volume of the primary antibody (20 μg/ml purified mAb, 1:100 dilution of mAb ascites, or 1:2 dilution of hybridoma supernatant in PBS) on ice for 30 min. Cells were then washed three times with L15/FBS and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (heavy and light chain, Zymed Laboratories, San Francisco, CA) for 30 min on ice. For staining with mAb CBRM1/20 that requires Ca^{2+},[3] the primary and secondary antibodies were diluted in PBS supplemented with 1 mM Ca^{2+}. After washing, cells were resuspended in cold PBS and analyzed on a FACScan (Becton Dickinson, San Jose, CA).

Radiolabeling, Immunoprecipitation, and Gel Electrophoresis—For

[2] S. Q. Na and T. A. Springer, unpublished data.

[3] C. Oxvig and T. A. Springer (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4870–4875.

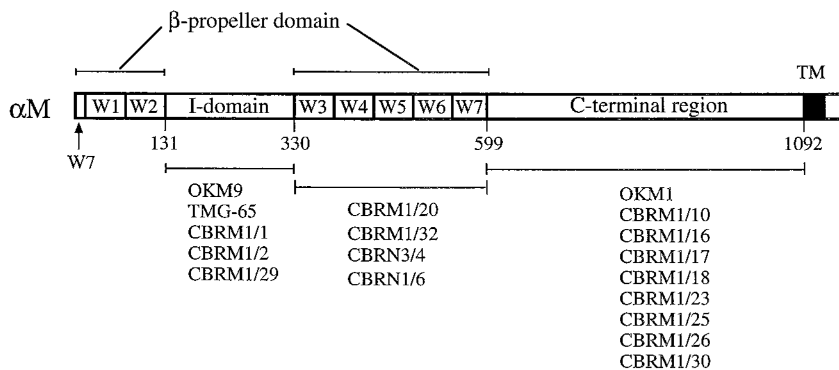

FIG. 1. **Schematic diagram of the integrin αM subunit and monoclonal antibody epitope localization.** *Numbers* are positions of amino acid residues at the putative boundaries of different regions. The *W*s are β-sheets of the β-propeller domain. The I-domain is inserted between β-sheets 2 and 3 of the β-propeller domain. The transmembrane domain (*TM*) is shown in *black*. mAbs and their epitope localization are shown *below* αM. Mapping of mAbs to different regions in the αM subunit has been described previously (29).[2]

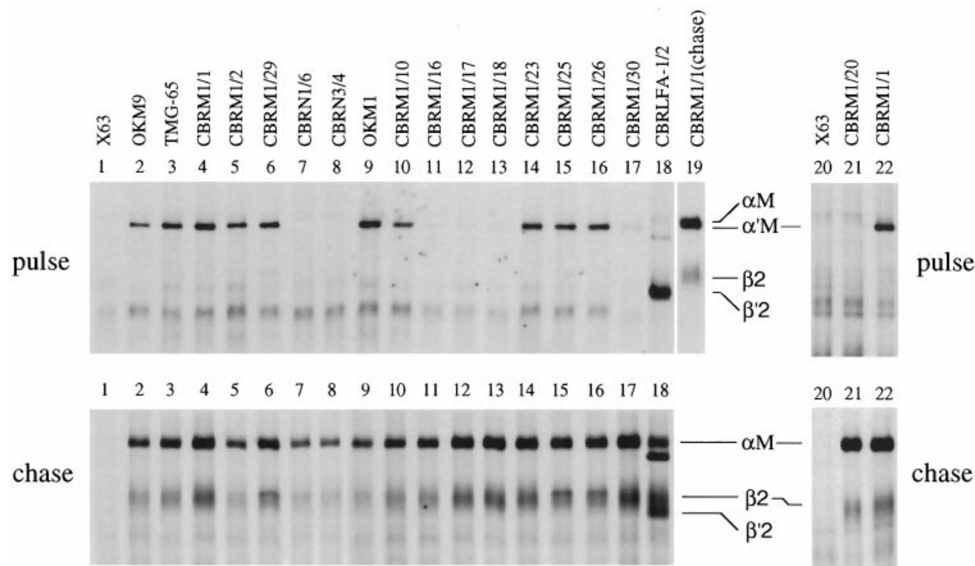

FIG. 2. **Immunoprecipitation of the unassociated αM precursor (α′M) and the αM/β2 complex.** PMA-induced U937 cells were pulse-labeled with [^{35}S]methionine for 30 min and chased with unlabeled methionine for 16 h. The αM and β2 precursors (α′M and β′2, respectively) and the αM/β2 complex were immunoprecipitated from lysates of pulse-labeled cells (*upper panel*) and pulse-chased cells (*lower panel*). Immunoprecipitates were subjected to 7.5% SDS-polyacrylamide gel electrophoresis and fluorographed. The nonbinding mAb, X63, was used as negative control. CBRLFA-1/2 is specific for the β2 subunit, and all other mAbs are against the αM subunit (see Fig. 1). In *lanes 20–22*, cell lysis and immunoprecipitation were carried out in the presence of 1 mM Ca^{2+}.

metabolic labeling, U937 cells were plated in four 10-cm Petri dishes and induced with PMA for 3 days as described previously (39). Cells in each dish were washed twice with methionine-free RPMI 1640 medium and labeled with 0.625 mCi of [^{35}S]methionine in 5 ml of methionine-free RPMI 1640 containing 15% dialyzed FBS. After incubation at 37 °C for 30 min, cells in two dishes were washed twice with cold PBS and lysed by addition of 3 ml of lysis buffer (1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.24 TIU/ml aprotinin, and 10 μg/ml each of pepstatin A, antipain, and leupeptin) and incubation for 30 min at 4 °C with gentle agitation. For chase labeling, 5 ml of complete medium supplemented with 100 μg/ml unlabeled methionine was added to each of the remaining dishes, and incubation at 37 °C was continued for 16 h. The chase-labeled cells were lysed identically to pulse-labeled cells, and lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4 °C.

For surface labeling, COS transfectants (2×10^6 cells) were washed three times with PBS and resuspended in 1 ml of PBS. The cells were surface-labeled with 1 mCi of $Na^{125}I$ using two IODO-BEADS (Pierce) following the manufacturer's instructions. The labeled cells were washed three times with PBS containing 10% FBS and once with PBS and lysed as described above.

For immunoprecipitation, cell lysates were precleared by addition of 1/10 volume of recombinant protein G agarose (50% suspension in PBS) (Life Technologies, Inc.) and incubation at 4 °C for 2–3 h with agitation. The precleared lysates were split into 250-μl aliquots, and to each aliquot, 2.5 μl of mAb ascites or 10 μg of purified mAb or 250 μl of mAb supernatant was added, and the final volume was adjusted to 500 μl with lysis buffer. After incubation overnight at 4 °C, followed by centrifugation at 12,000 rpm for 10 min at 4 °C to remove protein aggregates, the antigen/antibody mixture was incubated with 50 μl of protein G-agarose beads for 1.5–2 h at 4 °C with agitation. Beads were washed three times with lysis buffer and once with lysis buffer without detergent. For immunoprecipitation with mAb CBRM1/20, lysis buffer and wash buffer were supplemented with 1 mM Ca^{2+}. Bound proteins were eluted from beads with 50 μl of Laemmli sample buffer by heating for 5 min at 100 °C, and the immunoprecipitates were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis (40). The gels were processed for fluorography for [^{35}S]methionine-labeled proteins or autoradiography for ^{125}I-labeled proteins.

Secondary Structure Prediction—The amino acid sequences between the β-propeller domain and the transmembrane segment of 36 integrin α subunits (9) were aligned with ClustalW, and then the alignment was iteratively refined using default settings with PRRP and the Gonnet amino acid substitution matrix, and an evolutionary tree was prepared with PHYLP (41). The αM and αIIb subunits fall in different branches of this tree, each of which is well populated. One branch containing 11 subunits most closely related to human αM, *i.e.* murine αM, human αD, and αX, murine and human αL, human and rat α1, and bovine, human, and mouse α2, were realigned with one another using PRRP. They are 21–70%, $\bar{x}$ = 34% identical to human αM. Another branch containing the 17 subunits most closely related to human αIIb, *i.e.* hamster, human, and mouse α3, human and *Xenopus* α5, chicken and human α6, mouse α7, chicken and human α8, and chicken, human, mouse, and Pleurodes αV, and YMA1 of *Caenorhabditis elegans*, were realigned in a separate group. They are 20–38%, $\bar{x}$ = 28% identical to human αIIb. The alignments in MSF format, with gaps in human αM and human αIIb removed to increase prediction accuracy, were separately submit-

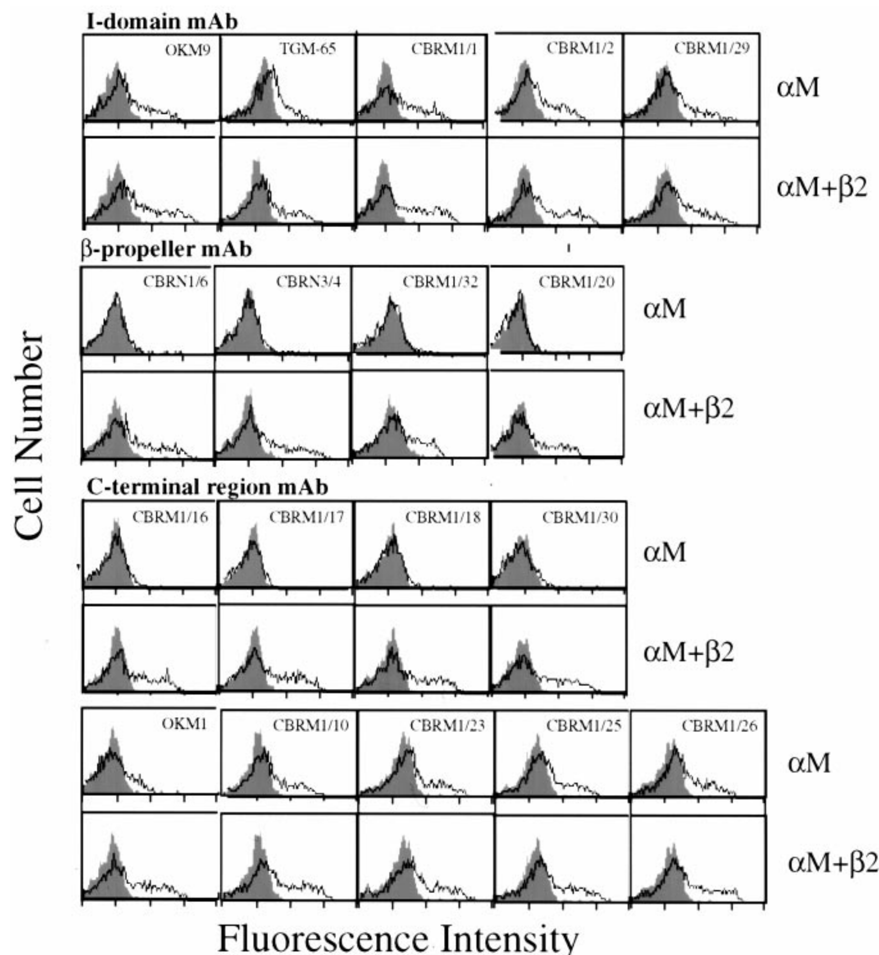

FIG. 3. **Flow cytometry of COS cells expressing the αM subunit or the αM/β2 complex.** COS cells were either mock-transfected, transfected with the αM cDNA alone, or co-transfected with the αM and β2 cDNAs. The transfectants were stained with mAbs to different regions in the αM subunit as indicated. The flow cytometry histogram of COS cells transfected with αM alone or co-transfected with αM and β2, as indicated on the *right*, was overlaid on that of the mock-transfected COS cells (shown in *gray*) stained with the same mAb.

ted for secondary structure prediction to PHD (42).[4] Smaller subgroups containing a higher degree of relationship to αM (6 α subunits, with 27–70% identity to αM) or to αIIb (9 α subunits, with 33–38% identity to αIIb) gave very similar predictions but with a slightly lower correlation between the αM and αIIb predictions.

RESULTS

mAbs to the β-Propeller Domain and a Subset of mAbs to the C-terminal Region Do Not React with the Unassociated αM Subunit—To study whether folding of the αM subunit is dependent on association with the β2 subunit, we examined the expression of mAb epitopes on the unassociated αM subunit. Eighteen mAbs that have previously been mapped to different regions in the αM subunit were used (29)[2] (Fig. 1). Previous studies on leukocyte integrin biosynthesis have shown that the α and β subunit precursors are initially unassociated in the endoplasmic reticulum and that transport to the Golgi apparatus and processing from high mannose *N*-linked carbohydrates to complex carbohydrates are dependent on the formation of α and β complex (39, 43, 44). We therefore examined whether mAbs to the I-domain, to the β-propeller domain, and to the C-terminal region immunoprecipitated the unassociated αM precursor (α′M). All mAbs immunoprecipitated the mature αM subunit with molecular size of about 170 kDa from the lysate of cells pulse-labeled with [^{35}S]methionine for 30 min and chased for 16 h (Fig. 2, *lower panel*). The αM subunit was complexed with the β2 subunit as shown by co-immunoprecipitation of the β2 subunit with the αM subunit. However, mAbs differentially precipitated the α′M precursor, which is slightly smaller than the mature αM subunit from the pulse-labeled cells (Fig. 2, *upper panel*). There was little or no α′M precursor associated with the β2 precursor (β′2) in the pulse-labeled cells, since no detectable β′2 over background was co-precipitated by mAbs to the αM subunit, but β′2 was precipitated with mAb CBRLFA-1/2 to the β2 subunit (*upper panel, lane 18*). All mAbs to the I-domain precipitated α′M (*upper panel, lanes 2–6*). By contrast, three mAbs (CBRN1/6, CBRN3/4, and CBRM1/20) to the β-propeller domain did not precipitate α′M (*upper panel, lanes 7, 8*, and *21*). mAb CBRM1/32 to the β-propeller domain did not precipitate the αM/β2 complex or α′M from cell lysates (data not shown), suggesting that its epitope is sensitive to detergent extraction. Five mAbs (OKM1, CBRM1/10, CBRM1/23, CBRM1/25, and CBRM1/26) to the C-terminal region precipitated α′M (*upper panel, lanes 9* and *10* and *14-16*), whereas four other mAbs (CBRM1/16, CBRM1/17, CBRM1/18, and CBRM1/30) precipitated no to very little α′M (*upper panel, lanes 11–13* and *17*). Thus, epitopes of mAbs to the I-domain are expressed on the unassociated αM precursor, whereas epitopes of β-propeller domain mAbs and a subset of mAbs to the C-terminal region are not.

To examine α subunit structure independently of maturation events occurring during biosynthesis, we examined mAb reactivity with the unassociated αM subunit expressed on the surface of COS cells. COS cells were transfected with cDNA for αM alone or for both αM and β2 subunits, and mAb reactivity with the uncomplexed αM or the αM/β2 complex expressed on the surface of COS transfectants was determined by immunofluorescent flow cytometry (Fig. 3). All mAbs to the I-domain (OKM9, TGM-65, CBRM1/1, CBRM1/2, and CBRM1/29) reacted with the unassociated αM subunit as well as with the

[4] Available on-line at the following address: http://www.embl-heidelberg.de./predict protein/.

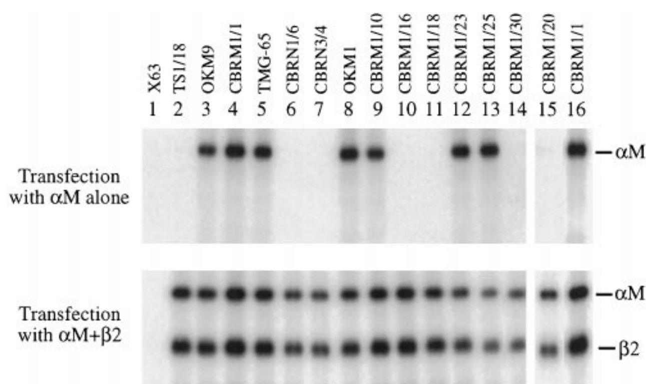

FIG. 4. **Immunoprecipitation of the αM subunit and the αM/β2 complex from surface-labeled COS transfectants.** COS transfectants expressing αM alone or the αM/β2 complex were surface-labeled with ^{125}I. Immunoprecipitates from lysates of COS transfectants were subjected to 7.5% SDS-polyacrylamide gel electrophoresis and autoradiography. *Upper panel*, COS cells transfected with the αM cDNA alone; *lower panel*, COS cells co-transfected with the αM and β2 cDNAs. The nonbinding mAb X63 was used as negative control. TS1/18 recognizes the complexed β2 subunit, and all other mAbs are against the αM subunit. In *lanes 15* and *16*, cell lysis and immunoprecipitation were carried out in the presence of 1 mM Ca^{2+}.

αM/β2 complex expressed on the COS cell surface. By contrast, all mAbs to the β-propeller domain (CBRN1/6, CBRN3/4, CBRM1/32, and CBRM1/20) reacted with COS cells expressing the αM/β2 complex but not with COS cells expressing αM alone. Four mAbs to the C-terminal region (CBRM1/16, CBRM1/17, CBRM1/18, and CBRM1/30) did not stain COS cells expressing the αM subunit alone, whereas five other mAbs did (OKM1, CBRM1/10, CBRM1/23, CBRM1/25, and CBRM1/26).

The data obtained by immunofluorescent flow cytometry were confirmed by immunoprecipitation. COS transfectants expressing the αM subunit alone or the αM/β2 complex were surface-iodinated, and the labeled proteins were immunoprecipitated from cell lysates. All mAbs precipitated the αM/β2 complex from COS cells co-transfected with αM and the β2 (Fig. 4, *lower panel*). mAbs to the I-domain precipitated the αM subunit expressed alone on the COS cell surface (*upper panel, lanes 3–5*). By contrast, β-propeller domain mAbs (*upper panel, lanes 6, 7* and *15*) and a subset of mAbs to the C-terminal region (*upper panel, lanes 10, 11* and *14*) failed to precipitate the uncomplexed αM subunit. Thus, expression of epitopes of the β-propeller domain mAbs and a subset of mAbs to the C-terminal region is dependent on αM and β2 heterodimer formation.

To test the possibility that the β2 subunit may directly contribute to the epitopes of the mAbs that did not react with αM in the absence of the β2 subunit, we expressed human αM in association with the mouse β2 subunit or the chicken β2 subunit on the surface of COS cells and human 293 cells. mAb reactivity with the transfectants was determined by immunofluorescent flow cytometry. mAbs CBRN1/6, CBRN3/4, CBRM1/20, CBRM1/32, CBRM1/16, CBRM1/17, CBRM1/18, and CBRM1/30 that did not react with the unassociated αM subunit reacted with the human αM/mouse β2 and human αM/chicken β2 complexes as well as with the human αM/human β2 complex (data not shown). These results suggest that the β2 subunit does not directly contribute to the epitopes of these mAbs.

Epitope Mapping of mAbs to the C-terminal Region and to the β-Propeller Domain of the αM Subunit—The finding that a subset of mAbs to the C-terminal region does not react with the unassociated αM subunit suggests that the structures of certain subregion(s) in this C-terminal 493-amino acid segment may be dependent on association with β2. To localize such subregion(s), as well as subregion(s) that fold independently of β2 association, epitopes of the nine mAbs to the C-terminal region were mapped using human-mouse αM chimeras. The chimeras were generated by progressively replacing the human sequences from the C terminus with the corresponding sequences from mouse αM (Fig. 5) and were co-expressed with human β2 in COS cells. mAb reactivity with chimeric αM/β2 was determined by immunofluorescent flow cytometry (Table I). All chimeras were expressed on the surface in association with the human β2 subunit, with levels of cell-surface chimeric αM/β2 complex comparable with that of wild-type αM/β2 complex. In addition, all chimeric αM/β2 complexes were stained with mAbs to the β-propeller domain (Table I and data not shown), showing structural integrity of the β-propeller domain despite the C-terminal region swapping. The results from epitope mapping are summarized in Table I and Fig. 6. A 41-amino acid sequence (residues 718–759) was required for epitopes of the four mAbs (CBRM1/16, CBRM1/17, CBRM1/18, and CBRM1/30) that did not react with the unassociated αM subunit. The epitopes of five mAbs that reacted with the unassociated αM subunit were mapped to three other subregions as follows: OKM1 to a region immediately following the β-propeller domain (residues 599–679); CBRM1/10, CBRM1/25, and CBRM1/26 to a region from residues 820 to 882; and CBRM1/23 to a region from residues 943 to 1047. Thus, mAb epitopes that map to one subregion (residues 718–759) require association of αM with β2, whereas epitopes localized in three other subregions (residues 599–679, 820–882, and 943–1047) are independent of the β2 subunit.

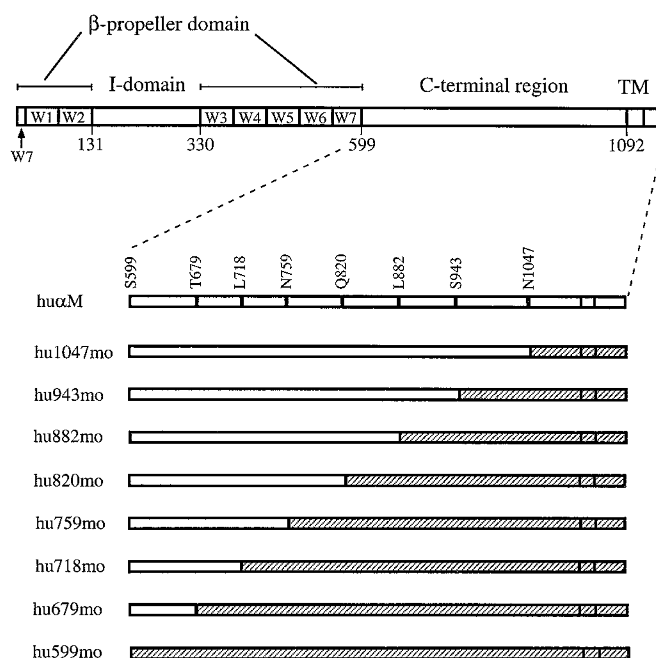

FIG. 5. **Schematic representation of human-mouse αM chimeras.** Human αM sequences (*open bar*) were progressively replaced from the C terminus with the corresponding sequences from the mouse αM subunit (*hatched bar*) as described under "Materials and Methods." Amino acid residues at the boundaries between human and mouse sequences are indicated above human αM (*huαM*).

The region from residues 718 to 759 contains eight amino acid differences between the human and mouse sequences (Fig. 7). To identify individual amino acid residues in this region that are required for epitopes of mAbs CBRM1/16, CBRM1/17, CBRM1/18, and CBRM1/30, single or double amino acid residues in the human αM sequence were replaced with corresponding residues from mouse αM. The mutants were co-ex-

TABLE I

mAb reactivity with COS cells expressing the human or chimeric αM subunit complexed with human β2

The human (hu) wild-type or human-mouse chimeric αM subunit was expressed in association with human β2 on the surface of transfected COS cells. mAb reactivity was determined by immunofluorescence flow cytometry. +, positive staining with mean fluorescence intensity comparable to human wild-type αM/β2 stained with the same mAb; −, staining was not significantly different from mock-transfected cells stained with the same mAb; ND, not determined. Epitopes of CBRM1/1 and CBRM1/32 were previously mapped (29). Of note, TS1/18 and CBRM1/32 are specific for complexed β2 and αM, respectively.

mAb	huαM	hu1047mo	hu943mo	hu882mo	hy820mo	hu759mo	hu718mo	hu679mo	hu599mo	Epitope
TS1/18	+	+	+	+	+	+	+	+	+	β2
CBRM1/1	+	+	+	+	+	+	+	+	+	I-domain
CBRM1/32	+	+	+	+	+	+	+	+	+	β-Propeller
OKM1	+	+	+	+	+	+	+	+	−	599–679
CBRM1/16	+	+	+	+	+	+	−	−	−	718–759
CBRM1/17	+	+	+	+	+	+	−	−	−	718–759
CBRM1/18	+	+	+	+	+	+	−	−	−	718–759
CBRM1/30	+	+	+	+	+	+	−	−	−	718–759
CBRM1/10	+	+	+	+	−	−	ND	ND	−	820–882
CBRM1/25	+	+	+	+	−	−	ND	ND	−	820–882
CBRM1/26	+	+	+	+	−	−	ND	ND	−	820–882
CBRM1/23	+	+	−	−	−	−	ND	ND	−	943–1047

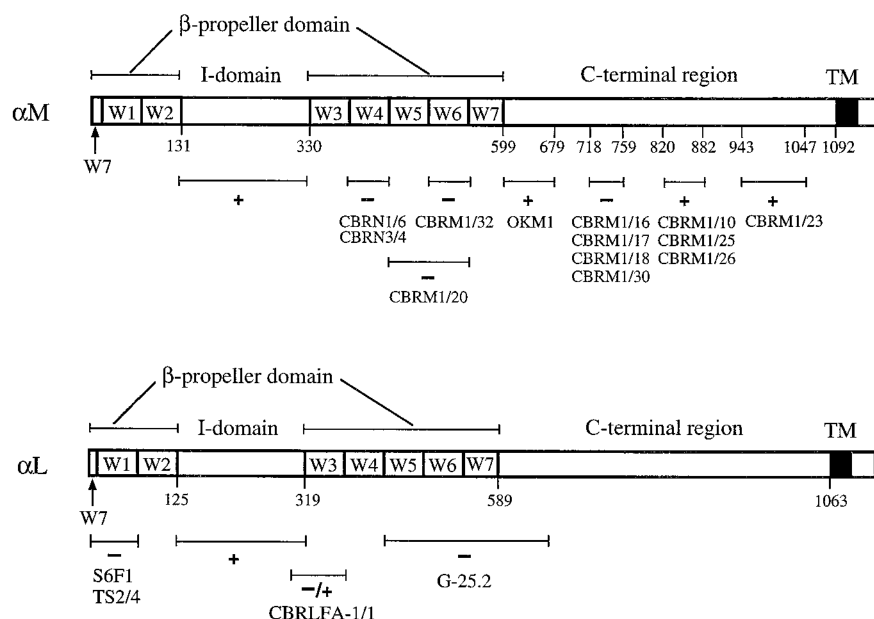

FIG. 6. **mAb reactivity with the αM and αL subunits in the absence of the β2 subunit.** Schematic diagrams of the αM and αL subunits are shown. W1 to W7 are β-sheets 1–7 of the β-propeller domain. *Numbers* are positions of amino acid residues at the boundaries between domains and between subregions in the αM C-terminal region. mAbs, except for those to the I-domain, and their epitope localization are shown under αM and αL. + indicates mAbs that react with the αM or αL in the absence of the β2 subunit; − indicates mAbs that do not react with the αM or αL in the absence of the β2 subunit; −/+ refers to weak reactivity with the unassociated α subunit compared with mAbs to the I-domain. All tested mAbs to the I-domain react with the unassociated α subunits. The original data on the αL subunit was reported elsewhere (24).

pressed with the β2 subunit in 293 cells, and mAb reactivity was determined by immunofluorescent flow cytometry (Table II). Substitution of Thr[725] to Glu (mutant T725E) completely abolished binding of all four mAbs, whereas substitution of Ser[728] and Ala[729] to Arg and Ser, respectively (mutant S728R/A729S), completely abrogated binding of mAbs CBRM1/16, CBRM1/17, and CBRM1/30 and decreased binding of CBRM1/18. All other substitutions did not affect binding of all four mAbs. Thus, mAbs CBRM1/16, CBRM1/17, CBRM1/18, and CBRM1/30 recognize overlapping epitopes.

To map mAb epitopes in the β-propeller domain, 32 different αM chimeras were constructed, in which a short segment of mouse sequence comprising a predicted loop or a strand was inserted in the human sequence, and mAbs were tested for reactivity with the αM chimeras co-expressed with the human β2 subunit in COS cells. Substitution of the 2–3 loop of W6 (mutant hu(W6L2–3)mo) or mutation of Arg[534] to Gln in this loop (mutant R534Q) completely abolished binding of mAb CBRM1/32, whereas binding of three other β-propeller domain mAbs (CBRN1/6, CBRN3/4, and CBRM1/20) and the I-domain mAb CBRM1/29 to these two mutants was not affected (Table III). Arg[534] is predicted to be on the upper, outer edge of the β-propeller domain in W6 (Fig. 8). Substitution of the loop 3–4 of W4 (mutant hu(W4L3–4)mo) completely abrogated mAb CBRN1/6 binding and decreased CBRN3/4 binding. This substitution did not affect CBRM1/32 and CBRM1/20 binding. The three human residues substituted in this mutant, Gln[421], Thr[423], and Met[425], are predicted to be in the lower outer edge of the β-propeller domain in W4 (Fig. 8). All other substitutions in 30 different segments of the β-propeller domain had no effect on binding of mAbs CBRM1/32, CBRN1/6, and CBRN3/4. mAb CBRM1/20 was mapped to five amino acid residues in the 1–2 loop of W5 and the 3–4 loop of W6[3] on the bottom of the β-propeller domain (Fig. 8).

DISCUSSION

By using mAbs as probes, we have examined the structure of different regions in the Mac-1 αM subunit during biosynthesis and αM/β2 heterodimer assembly and after expression on the cell surface. All five different mAbs to the I-domain reacted with the unassociated αM subunit, confirming that the folding of the I-domain does not require the β2 subunit. By contrast, four mAbs (CBRN1/6, CBRN3/4, CBRM1/20, and CBRM1/32) that map to three different subregions in the β-propeller domain did not react with the unassociated αM subunit (Fig. 6). CBRN1/6 and CBRN3/4 mapped to one or more of three residues in the 3–4 loop of W4 (residues 421–425) (Fig. 8). CBRM1/20 is specific for three amino acid residues in the 1–2 loop of W5 and two residues in the 3–4 loop of W6[3] (Fig. 8). The epitope for CBRM1/20 includes two residues, Asn[453] and

FIG. 7. **Sequence alignment and secondary structure prediction of the C-terminal regions of representative integrin α subunits.** The sequence alignments are condensed from a master alignment of 36 integrin α subunits ("Materials and Methods"). Cysteines known to be disulfide bonded in αIIb (48) are connected by *solid lines*; one disulfide that may differ in αM (see text) is shown with a *dashed line*. The *arrow* points at the main chymotryptic (*CT*) cleavage site (around Asn[570]) in αIIb (47, 48, 50). The region preceding the chymotryptic cleavage site and following the β-propeller domain that contains the OKM1 epitope is predicted to fold into a domain (see text). The regions to which mAb epitopes localize are shown *above* the human αM sequence. Two different sequence alignments containing separate branches of the integrin α subunit evolutionary tree were used to predict the secondary structure of human αM and αIIb with PHD (42). These predictions are independent of one another (see "Materials and Methods"). *E*, β-sheet; *H*, α-helix.

Asp[457], that are predicted to coordinate with Ca^{2+} in the 1–2 loop of W5, and binding of this mAb requires Ca^{2+} with an EC_{50} of 0.2 mM.[3] These mAbs did not immunoprecipitate the unassociated αM precursor or react with the αM subunit expressed alone on the surface of COS cells. mAb CBRM1/32 reacted with the αM/β2 complex expressed on the cell surface but did not react with the αM subunit expressed alone on the cell surface. The epitope of CBRM1/32 requires residue Arg-534 in the 2–3 loop of W6 (Fig. 8). One possible interpretation of our results is that all three epitopes in the β-propeller domain require the presence of the β subunit because the α and β subunits associate with one another in each of these regions, and each antibody binding site includes contacts with both the β subunit and α subunit. If so, the contacts with the β subunit do not include any antigenic residues, because all mAb reacted equally well whether the human or murine β subunit was associated with human αM. Furthermore, we tested the chicken β2 subunit, because 35% of the residues in the human and chicken β2 subunits differ, as opposed to only 18% between the human and the mouse (45). Amino acid differences between

TABLE II
mAb reactivity with human αM subunit mutants carrying human-to-mouse substitutions in the region from residues 718 to 759

293 cells were transiently co-transfected with cDNAs for human β2 and the wild-type or mutated human αM subunit containing human-to-mouse single or double amino acid residue substitution. mAb binding to the transfected cells was determined by immunofluorescence flow cytometry. +++, binding comparable to human wild type; +, binding decreased to less than 30% of human wild type; and −, binding completely abolished.

Mutation	TS1/18	CBRM1/1	CBRM1/32	CBRM1/16	CBRM1/17	CBRM1/18	CBRM1/30
F720Y/S721T	+++	+++	+++	+++	+++	+++	+++
T725E	+++	+++	+++	−	−	−	−
S728R/A729S	+++	+++	+++	−	−	+	−
E739M	+++	+++	+++	+++	+++	+++	+++
L744F	+++	+++	+++	+++	+++	+++	+++
L748M	+++	+++	+++	+++	+++	+++	+++

TABLE III
mAb reactivity with human αM subunit mutants containing human-to-mouse substitutions in the β-propeller domain

32 different chimeric αM subunits were made in which a short segment of mouse sequence comprising a predicted loop was inserted in the human sequence (see "Materials and Methods"). Mutants were named after the sheet (W) and the loop (L) that was exchanged, *e.g.* hu(W4L3–4)mo has mouse sequence in the loop between strands 3 and 4 of W4. The wild-type or mutated human αM subunit was transiently co-expressed in COS cells with human β2. mAb binding to the transfected cells was determined by immunofluorescence flow cytometry. +++, binding comparable to human wild type; +, binding decreased to less than 30% of human wild type; and −, binding completely abolished. Only the mutants that affected binding of mAbs CBRM1/32, CBRN1/6 and CBRN3/4 were listed in the table.

Mutation	CBRM1/29	CBRM1/32	CBRN1/6	CBRN3/4	CBRM1/20[a]
hu(W6L2–3)mo	+++	−	+++	+++	+++
R534Q	+++	−	+++	+++	+++
hu(W4L3–4)mo	+++	+++	−	+	+++
30 other mutants	+++	+++	+++	+++	+++[a]

[a] Mapping of mAb CBRM1/20 is described elsewhere[3]; binding of this mAb is affected only by chimeras hu(W5L1–2)mo and hu(W6L3–4)mo.

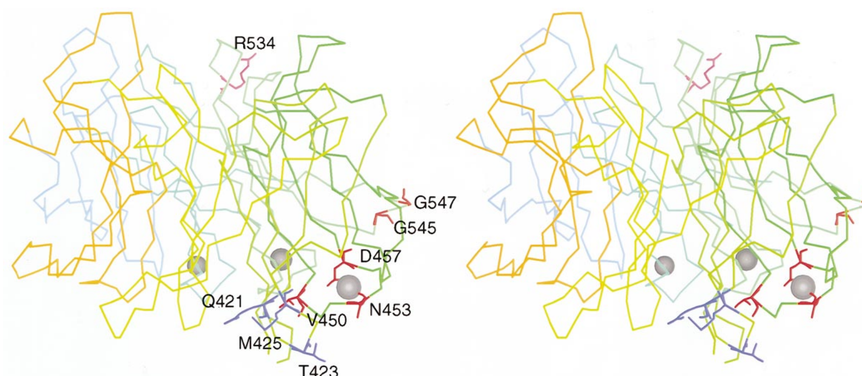

FIG. 8. **mAb epitope localization in the β-propeller domain of the αM subunit.** This stereoview of the side of the putative β-propeller domain with the upper surface on the top shows a C-α trace, with all atoms shown for residues involved in antigenic epitopes. They are *R534* for CBRM1/32 (*magenta*), *V450, N453, D457, G545,* and *G547* for CBRM1/20 (*red*), and *Q421, T423,* and *M425* for CBRN1/6 and CBRN3/4 (*purple*). All of the latter three residues were substituted together, and the epitope may require only a subset of these three residues. The β-sheets (*W*) are shown in different colors, with W1 in *cyan*, W2 in *orange*, W3 in *yellow*, W4 in *olive*, W5 in *green*, W6 in *aquamarine*, and W7 in *turquoise*. Ca^{2+} ions are *gray spheres*. The β-propeller domain of the Mac-1 αM subunit was modeled using the G-protein β subunit β-propeller domain as template, as described.[3] This figure was made with Look of GeneMine™ (Molecular Applications Group, CA).

species are preferentially found on the surface of proteins rather than buried. Although a substantial portion of surface residues are expected to differ on the chicken and human β2 subunits, whether the chicken or human β2 subunit was present did not affect mAb reactivity. The epitopes that were localized include some that are quite distant. The Arg-534 residue recognized by CBRM1/32 mAb is on the upper surface of the β-propeller, whereas residues recognized by the CBRM1/20 and the CBRN1/6 mAb are on the lower surface and point in opposite directions from one another. The C-α carbon of the Arg-534 residue is predicted to be 30 ± 3 Å and 41 ± 3 Å distant from residues recognized by the CBRM1/20 and the CBRN1/6 mAb, respectively, and the C-α carbons of residues recognized by the CBRM1/20 and the CBRN1/6 mAb are 23 ± 7 Å distant from one another. The probability that three out of three different epitopes would include surfaces from both the α and β subunits, even though some epitopes are quite distant from one another, would appear to be low. Because of this, and the lack of effect of the species origin of the β subunit on mAb reactivity, we favor the interpretation that association between the α subunit and β subunit is required for the β-propeller domain to assume its final three-dimensional structure, *i.e.* to assume the correct fold. Our data are consistent with the idea that there is an interface between the α subunit β-propeller domain and the β subunit, although we believe that the interface is not necessarily associated with any of the epitopes we have mapped. Conversely, a number of mAb to different epitopes in the conserved domain of the integrin β subunit are not reactive in the absence of the α subunit (23). Thus, the conserved domain of the β subunit is a candidate for association with the putative β-propeller domain of the α subunit. Analogously, the G-protein β subunit β-propeller domain is not properly folded in the absence of association with the G-protein γ subunit (46).

A previous study on the LFA-1 β-propeller domain used two mAbs (S6F1 and TS2/4) that map to the αL subunit N-terminal 57 amino acids, *i.e.* to part of β-sheets W7 and W1, and one mAb (G-25.2) that maps to a 212-amino acid region spanning W5–7 of the β-propeller domain and part of the C-terminal

region. These mAbs did not react with the unassociated αL subunit (24) (Fig. 6). Another mAb (CBRLFA-1/1) that overlaps the I-domain and W3 of the β-propeller domain showed weak reactivity in the absence of the β2 subunit. It is not known whether this mAb recognizes a boundary region between the I and β-propeller domains. Taken together, the findings on LFA-1 and Mac-1 demonstrate that multiple mAbs to different regions in the β-propeller domain do not react with the α subunit in the absence of the β subunit and suggest that the β-propeller domain folds as a unit and that this folding depends on association with the β subunit.

mAbs to the C-terminal region of the αM extracellular domain differentially reacted with the unassociated αM subunit. Five mAbs (OKM1, CBRM1/10, CBRM1/25, CBRM1/26, and CBRM1/23) reacted with both the unassociated and the complexed αM subunit and were mapped to three subregions. OKM1 mapped to a subregion immediately following the β-propeller domain, residues 599–679. CBRM1/10, CBRM1/25, and CBRM1/26 mapped to amino acids 820–882, and CBRM1/23 mapped to residues 943–1047. Within each of these subregions, there are multiple differences between the mouse and human sequences (Fig. 7). Whether the multiple mAbs that react with residues 820–882 recognize one or more epitopes within this subregion is not known. Minimally, these data show that three epitopes in three different subregions of the C-terminal segment are independent of the β2 subunit. By contrast, four other mAbs to the C-terminal region (CBRM1/16, CBRM1/17, CBRM1/18, and CBRM1/30) only reacted with the αM/β2 complex. These mAbs did not react with the unassociated αM precursor or with the uncomplexed αM subunit expressed on the COS cell surface. All four mAbs were mapped to residues Thr^{725} and, additionally, Ser^{728} and/or Ala^{729}. Although CBRM1/16, CBRM1/17, CBRM1/18, and CBRM1/30 did not react with the unassociated αM subunit, they reacted with the human αM/mouse β2 and human αM/chicken β2 complexes as well as with the human αM/human β2 complex (data not shown). Thus, association with the β2 subunit may be required for this region to assume its final structure. Although we believe that the interpretation that the α and β subunits both contribute to the antibody-binding site is less likely, either interpretation shows an important interaction with the β subunit for the region of residues 725–729. Overall, the results show that three out of four epitopes in the C-terminal region of Mac-1 αM subunit are intact in the absence of association with the β2 subunit. If these results are representative of the C-terminal region as a whole, our data would suggest that much of this region folds independently of the β2 subunit. This is in marked contrast to the β-propeller domain.

To place our results on the C-terminal region within a structural framework, we predicted its secondary structure using the PHD program (42) (Fig. 7). By using a phylogenetic tree based on an iteratively refined alignment (41) of 36 α subunit C-terminal region sequences, two subfamilies were identified. These subfamilies were large and contained members that were 1) sufficiently similar to one another to allow accurate alignment and to not be too divergent in tertiary structure, and 2) were sufficiently different from one another to contain a large amount of sequence information, and hence optimize prediction accuracy (42). An alignment of 11 subunits was used to predict the secondary structure of human αM, and an alignment of 17 other α subunits was used to predict the structure of human αIIb (Fig. 7). Since no sequences were shared between the two alignments, and between the two groups there is only 16–21% sequence identity, the predictions for αM and αIIb are largely independent of one another.

In the C-terminal segment, a total of 30–34 β-strands were predicted. Of these, 22 were independently predicted in both αM and αIIb. Only 5 α-helices were predicted, and in each case these were predicted in only one of the two α subunits. Thus, the C-terminal region is predicted to form domains of the all β class. In this respect, it is similar to the β-propeller domain (9) and different from the I-domain which is of the α/β class (14, 16).

The disulfide bond topology of αIIb has been chemically determined (47, 48). The conservation of cysteines suggest that 5 of 6 disulfide bonds are conserved in human αM, whereas one differs (Fig. 7). The first disulfide in this region, αIIb C473-C484, is confirmed by the sequence alignment of 36 integrin α subunits, since these two cysteines are selectively absent in the chicken α6 subunit, and the cysteines and the loop in between them are absent in α2 subunits and αE subunits. The cysteines corresponding to the last disulfide bond in αIIb, Cys^{885}-Cys^{890}, are missing from αL subunits. Otherwise, there is only one predicted difference between disulfide bonds in αIIb and the leukocyte integrin α subunits. The cysteine corresponding to αIIb Cys^{484} is missing in all leukocyte integrin α subunits, and all leukocyte integrin α subunits contain a cysteine with no equivalent residue in αIIb, *i.e.* Cys^{706} in αM. We predict that the cysteines at αIIb position 473, although aligned by sequence, are non-equivalent, *i.e.* that the cysteine in αM is involved in a different disulfide bond, to Cys^{706} (*dashed line* in Fig. 7).

Folds of the all-β class as a general rule contain anti-parallel β-sheets (49). The vast majority but not all of the predicted β-strands in the αIIb and αM C-terminal regions are markedly amphipathic with alternating hydrophobic and hydrophilic residues. We therefore predict that the C-terminal region folds into 2-layer, anti-parallel β-sheet structures, *i.e.* β-sandwich or β-barrel domains of which the Ig fold is one of many representatives. The total length of the C-terminal region of about 500 residues, the number of predicted β-strands, and the overall number and location of disulfide bonds are appropriate for approximately four to six β-sandwich domains.

In αIIb, a main chymotryptic cleavage site is located around Asn^{570} (47, 48). Cleavage of cell-surface αIIbβ3 releases a ligand binding complex containing an N-terminal fragment of αIIβ of 55 kDa ending at approximately Asn^{570}, and an 85-kDa N-terminal fragment of β3 (50). This suggests that the region around Asn^{570} is well exposed and may represent a domain boundary region. It is interesting that four mAbs dependent on β subunit association map to essentially the same site in αM (Fig. 7). The region preceding the chymotryptic cleavage site and following the β-propeller domain in αIIb contains one long range disulfide bond (Cys^{490}-Cys^{545}), and six predicted β-strands. In αM, the corresponding region contains two predicted long range disulfide bonds (Cys^{639}-Cys^{696}, Cys^{623}-Cys^{706}), and seven predicted β-strands. Based on these features, we predict that this region of about 120 amino acids following the β-propeller domain, residues 599 to about 718 for αM, and 450 to about 570 for αIIb, folds into a structurally independent domain. Consistent with this prediction, this region in αM appears to fold independently of association with the β subunit, as shown with the OKM1 mAb. This contrasts with the flanking N-terminal β-propeller domain and the flanking C-terminal region from residues 725 to 729, to which mAbs CBRM1/16, CBRM1/17, CBRM1/18, and CBRM1/30 map.

Our results together with other recent studies provide new insight into how different domains in the integrin α and β subunits may associate. The I-domain is predicted to be connected to the upper surface of the β-propeller domain (9). The α subunit β-propeller domain and the β subunit conserved domain may associate, since both are dependent on α and β

subunit association for folding (23, 24) (this study). The predicted β-sandwich/β-barrel domain that follows the β-propeller domain and contains the OKM1 epitope, residues 599–718, is connected to the C terminus of strand 3 of W7 of the predicted β-propeller domain and hence to the bottom of the β-propeller domain. The following subregion of the αM subunit, from residues 725 to 729, may directly associate with the β2 subunit, or its structure may be indirectly dependent on associations elsewhere with the β subunit. Other subregions in the C-terminal portions of α and β subunits might also participate in α and β subunit association as proposed for the $\alpha IIb\beta_3$ integrin (32, 51), while retaining similar conformations in the unassociated and complexed forms.

In summary, the results from this study suggest that proper folding of the β-propeller domain of the integrin αM subunit requires association with the β2 subunit, whereas the I-domain folds independently of the β2 subunit. Much of the region C-terminal to the β-propeller domain folds prior to β subunit association. Our results further advance the understanding of integrin structure and provide information that will be useful in guiding studies leading to the characterization of integrin three-dimensional structure.

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