Determination of N- and C-terminal Borders of the Transmembrane Domain of Integrin Subunits*

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Previous studies on the membrane-cytoplasm interphase of human integrin subunits have shown that a conserved lysine in subunits α5, α6, β1, and β2 is embedded in the plasma membrane in the absence of interacting proteins (Armulik, A., Nilsson, I., von Heijne, G., and Johansson, S. (1999) in J. Biol. Chem. 274, 37030–37034). Using a glycosylation mapping technique, we here show that α5, α6, and β2 subunits that deviate significantly from the integrin consensus sequences in the membrane-proximal region, were found to have the conserved lysine at a similar position in the lipid bilayer. Thus, this organization at the C-terminal end of the transmembrane (TM) domain seems likely to be general for all 24 integrin subunits. Furthermore, we have determined the N-terminal border of the TM domains of the α2, α5, α10, β1, and β2 subunits. The TM domain of subunit β2 is found to be 22 amino acids long, with a second basic residue (Arg684) positioned just inside the membrane at the exoplasmic side, whereas the lipid-embedded domains of the other subunits are longer, varying from 25 (α2) to 29 amino acids (α10). These numbers implicate that the TM region of the analyzed integrins (except β2) would be tilted or bent in the membrane. Integrin signaling by transmembrane conformational change may involve alteration of the position of the segment adjacent to the conserved lysine, To test the proposed “piston” model for signaling, we forced this region at the C-terminal end of the α2 and β1 TM domains out of the membrane into the cytosol by replacing Lys-Leu with Lys-Lys. The mutation was found to not alter the position of the N-terminal end of the TM domain in the membrane, indicating that the TM domain is not moving as a piston. Instead the shift results in a shorter and therefore less tilted or bent TM α-helix.

Integrins are heterodimeric receptors composed of an α subunit noncovalently associated with a β subunit. Each subunit has an N-terminal extracellular domain, a transmembrane (TM) region and a cytoplasmic domain. The human α- and β subunits constitute two unrelated protein families of 18 and 8 members, respectively (1, 2).

Integrins mediate cell adhesion to the pericellular matrix and to neighboring cells (1). In addition to the anchoring function, ligand binding may be important to detect cell adhesion required for several cellular processes, including cell migration and proliferation. The ability to bind ligands is regulated by mechanisms acting on the cytoplasmic part of the protein, an unusual receptor feature. Integrin activation by cytoplasmic signals has been shown to involve transmembrane conformational changes (3, 4). Subsequent ligand binding induces further structural rearrangements, as monitored by exposure of new epitopes, in the extracellular as well as in the intracellular domains (5, 6).

Recently, significant progress has been made in the elucidation of the mechanisms controlling integrin activation (“inside-out signaling”) and ligand-induced signaling (“outside-in signaling”). The cytoplasmic protein talin was found to bind to the membrane-proximal region of the β1, β2, and β3 subunits and thereby activates the integrins (7–10). Integrin activation has been shown to require separation of the α and β subunit cytoplasmic domains from each other (9, 11), and this is presumably the way by which talin activates integrins. In addition, recent reports have suggested that the TM domains of the subunits mediate integrin clustering after ligand binding (12). TM domains therefore appear to contribute to signaling in both directions across the membrane rather than serving merely to connect the intra- and extracellular domains. Evidence for the important functions of integrin TM domains is further provided by their high degree of conservation within the integrin α- and β-protein families and also between species for individual subunits.

Several models have been proposed to explain the transmembrane signaling of integrins. These are based on different types of movements of the TM domains, such as rotation, tilting, and piston movement (13–17). As a step toward the identification of the mechanisms used for outside-in and inside-out signaling, we have in the present study determined the boundaries of the TM domains from five selected integrin subunits. This information has allowed us to test the piston model for integrin α5β1.

MATERIALS AND METHODS

Enzymes and Chemicals—Unless stated otherwise, the enzymes were purchased from Promega, MBI Fermenta AB and New England Biolab. For PCR puReTaq™ Ready-To-Go™ PCR beads from Amer- sham Biosciences were used. PCR primers were from DNA Technology and TAG Copenhagen. DNA manipulations were made using the TOPO kit from Invitrogen, the Rapid Ligation kit from Roche Applied Sciences, and the QuikChange™ site-directed mutagenesis kit from Strat- agene. Ribonucleotides, the cap analogue m7G(5’ppp5’)G, and [35S]Met were from Amersham Biosciences. Dithiothreitol, bovine serum albumin, RNasin ribonuclease inhibitor, plasmid pGEM1, rabbit reticu- locyte lysate, and amino acid mixture without methionine were from Promega. Spermidine was from Sigma.

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The abbreviations used are: TM, transmembrane; aa, amino acids; MGD, minimal glycosylation distance.

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**DNA Manipulations**—The DNA sequence coding for the region containing the predicted TM domain of integrin subunits α2, α5, αv, β1, and β2, were amplified by polymerase chain reaction from corresponding cDNAs. The following primers were used: for α2, aT-ACCATGTTGTTCTCTAGG-3') and aT-CTCACTAGTGAATGTTTCTCCAGC-3'); for α5, aT-ACCATGAGCTGCAAAGGCAGCGC-3' and aT-ACCATGAGCTGCAAAGGCAGCGC-3'); for αv, aT-ACCATGAGCTGCAAAGGCAGCGC-3' and aT-ACCATGAGCTGCAAAGGCAGCGC-3'); and for β1, aT-ACCATGGAATGTTTCTCCAGC-3' and aT-ACCATGGAATGTTTCTCCAGC-3'). The sense primers introduced a BclI restriction site and the antisense primers an NdeI restriction site (marked in bold type). The pGEM1-based vectors encoding the protein Lep (ladder peptidase) with a glycosylation acceptor site at different positions have been previously characterized (21-24). The amplified TM regions were cloned into the Lep vectors, replacing a transmembrane region in the translated leader peptide (see Fig. 1). For α2, the amino acid residues 1029–1062 were inserted, for α5, residues 992–1029 were inserted, for αv, residues 1115–1154 were inserted, for β1, residues 722–760 were inserted, and for β2 residues 673–708 were inserted.

The mutations L1023K in α2 TM and L753K in β1 TM (marked in bold type) were introduced using the following primers: aT-ACATTCTCTAGAAGGGATTCCCTCAA-3', aT-ACATTCTCTAGAAGGGATTCCCTCAA-3', aT-ACATTCTCTAGAAGGGATTCCCTCAA-3', aT-ACATTCTCTAGAAGGGATTCCCTCAA-3', and aT-ACATTCTCTAGAAGGGATTCCCTCAA-3', respectively.

The Lep vector constructs were transcribed by SP6 RNA polymerase and translated in reticulocyte lysate in the presence and absence of dog pancreas microsomes as described (19). The proteins were analyzed by SDS-polyacrylamide gel electrophoresis, and the bands were quantitated by phosphorimaging on a Fluorescent Image Reader FLA-3000 using the Image Reader 1.1 software. The extent of glycosylation of a given construct was calculated as the quotient of the glycosylated band divided by the summed intensities of the glycosylated and nonglycosylated bands. The measured MGD values were used to estimate the positions of the chosen integral TM segments in the endoplasmic reticulum membrane by comparison with the 25LV model TM described above.

**RESULTS**

Glycosylation Mapping—The glycosylation mapping technique has previously been described in detail (18). Briefly, the assay is based on the ability of the luminal domain exposed to the water-lipid interface. The measured MGD values were used to estimate the positions of the chosen integral TM segments in the endoplasmic reticulum membrane by comparison with the 25LV model TM described above.

**Testing the Piston Model**—Several models for integrin-de-
ependent signal transduction across the membrane have been proposed (13–17), with special attention given to the highly conserved regions flanking the membrane-cytoplasm interphase. The C-terminal part of the TM domain has been suggested to move out of the membrane either by sliding of the TM helices in a piston-like motion (13), by changes in tilt (15), or by an uncoiling process (14) (see Fig. 6).

We have previously shown that the C-terminal end of the TM segment in \( \beta_1 \) shifts relative to the membrane if a second lysine is introduced next to the conserved membrane-embedded lysine, i.e. by mutating Leu\(^{753}\) to Lys in \( \beta_1 \) (15). In the present study we analyzed whether the N-terminal end of the integrin TM segment and the Asn in the engineered Asn-Ser-Thr glycosylation acceptor site. C, the amino acid sequence of the \( \alpha_\gamma \) TM region in three different B vectors (13–15). Note that the glycosylation acceptor site (NST, marked in bold type) is positioned closer to the TM insert in vectors with lower numbers.

DISCUSSION

The role of the cytoplasmic domains of \( \alpha \) and \( \beta \) subunits in integrin activation and signaling is well established (9, 21–23). Accumulating data indicate active roles also for the TM domains (12, 24), as well as for the membrane-proximal parts flanking the TM domains (8, 25). However, it is not yet known what kind of molecular movements of the TM domains are linked to these events. To better understand the role of integrin TM domains, we previously determined the membrane-cyto-

FIG. 2. Determination of the N-terminal borders of integrin TM segments. A, \textit{in vitro} translation of B vector constructs in the presence (+M) and absence (−M) of dog pancreas microsomes analyzed by SDS-PAGE. B, glycosylation efficiencies based on quantitation of the bands from the polyacrylamide gel as a function of the number of residues between the N-terminal end of the integrin TM segment and the Asn in the engineered Asn-Ser-Thr glycosylation acceptor site. C, the amino acid sequence of the \( \alpha_\gamma \) TM region in three different B vectors (13–15). Note that the glycosylation acceptor site (NST, marked in bold type) is positioned closer to the TM insert in vectors with lower numbers.
plasm interface for α2, α5, β1, and β6 using an in vitro glycosylation mapping assay (15). Unexpectedly, the transmembrane domain was found to include an additional 5–6 amino acids at the C-terminal end compared with earlier predictions; this result was subsequently confirmed by NMR studies of the TM and cytoplasmic domains in dodecylphosphocholine micelles (26). A basic amino acid, which is conserved in all human integrin subunits residue, Arg in α2 and α5 and Lys in all other subunits, is thus located in the plasma membrane in the absence of interacting proteins. A basic residue at this position is likely to influence interactions with membrane proteins and/or the orientation of the TM domain in the lipid bilayer.

In the present study, the characterization of integrin TM domains has been extended with (i) determination of the C-terminal border of β1-associated subunit α10; (ii) determination of the N-terminal borders of α2, α6, α10, and β1; (iii) determination of both ends of the strongly divergent β8 TM domain; and (iv) a test of the validity of the piston model (13) as a possible mechanism for propagating conformational changes across the plasma membrane.

The amino acid motif (K/R)xGFFKR is present at the membrane-cytoplasm interface in all 18 integrin α subunits except α8 (KGFFDR), α9 (KLGFFRR), α10 (KLGFFAH), and α11 (KLGFFRS). In view of the high degree of conservation of the motif, minor deviations such as those in α9 and α10 may be functionally significant. Analysis by the in vitro glycosylation assay showed that the α10 TM domain extends 1–2 amino acid...
residues further at the C terminus compared with the TM domain of other α subunits. This result is not unexpected considering the absence in α10 of the strongly charged dipeptide KR. Thus, the membrane-embedded lysine in α10 resides even deeper inside the membrane than in other α subunits.

It is not obvious from the primary sequences where the N-terminal borders of integrin TM domains are located. The border has usually been predicted to be located 23 amino acids or more upstream of the conserved membrane-embedded lysine (e.g. Lys1125 in β1) (27–31). However, not all integrin subunits may necessarily have TM domains of identical length, and the α subunits in particular have variable numbers of nonpolar amino acids upstream of the predicted 23 residues that may influence the length of the TM segment.

Applying the in vitro glycosylation method, the N-terminal borders for α5 and α10 were found to be located at the same distance upstream of the membrane-embedded lysine (Fig. 3). Thus, both these subunits have a tryptophan in position to influence the selection of the TM segment in and out of the membrane. In the coiled model (B) the two TM regions (red) are coiled around each other as a coiled coil. When uncoiled, the TM α-helices are too long to run perpendicular to the plasma membrane. Instead the C-terminal end of the TM region would move into the cytoplasm. In the tilting model (C) the TM regions adapt to the bilayer by tilting. Changes in the tilt angle will push the C-terminal end of the TM into the cytosol. The separation of the TM regions after talin binding is not included in these models because it is not clear in which conformation this occurs. If model B is correct, the coiled coil structure would correspond to a conformation before activation by talin.

Still, the TM segment in β8 is significantly shorter than that in β1. The β8 TM domain also exhibits several unique features. The sequence around the membrane-cyttoplasm interface, WKLXXX(I/F)/HDR/R/K/E, is conserved in β1, β2, β5, and β6, whereas significant deviations from the motif are present in β2, β3, and β8; β8 shows only weak similarity in this part of the protein, as well as in the cytoplasmic domain. Our measurement show that the β8 TM domain contains only four residues beyond the membrane-embedded lysine, compared with approximately 6 residues in β1. Other notable differences between β8 and β1 are the absence of Trp in front of the conserved C-terminal Lys, and the replacement of HDRRE with another polar sequence. Furthermore, the membrane-embedded arginine (Arg<sup>684</sup>) is only found in β8, whereas the β8 TM domain lacks both the glycine and alanine residues that are present at specific positions in most other β subunits.

Whether these structural features confer any particular function to β8 is presently not known. However, β8, as well as β1, β3, β5, and β6, associate with the αv subunit, and therefore the unusual structure of the β8 TM domain most likely does not influence the selection of the α subunit partner. Because β8 lacks key talin-binding residues in the membrane proximal and cytoplasmic domains (10), i.e. Ile-His<sup>706</sup>, Trp<sup>775</sup>, and Asn-
Ile-Tyr\textsuperscript{783} in human β1, αvβ3 may have a different mechanism of activation than other integrins. Possibly, this is reflected in the structure of the TM domain. Relatively little is yet known about the signaling properties of αvβ3 and further studies may clarify whether the β3 TM domain has any specific role in this context.

Under the conditions of the glycosylation assay, the α-carbon of Lys\textsuperscript{782} in the isolated TM domain of β3 and the corresponding lysine in other integrin α- and β-TM domains is clearly located in the lipid bilayer. A similar position for the lysine was found when a β1 fragment consisting of the TM and cytoplasmic domains was analyzed by NMR spectroscopy (26). However, the presence of a tryptophan or tyrosine at the position immediately preceding the conserved Lys/Arg in all integrin subunits except β3 suggests that the (W/Y)(K/R) motif may be found at the membrane-cytosol interphase in certain integrin configuration(s). The membrane proteins commonly have a tryptophan or a tyrosine at the ends of the TM segments where they can serve as anchors by interacting both with the fatty acid chains and the carbonyl group of the phospholipids via hydrophobic and hydrogen bonds, respectively (32). The basic residue (e.g. Lys\textsuperscript{782} in β1) may serve as a flexible anchor that can interact via its long side chain with the negatively charged phosphate groups of phospholipids even if the α-carbon moves a short distance in or out of the membrane.

It has been suggested that movement of the conserved C-terminal end of the TM domain in or out of the membrane could occur if the whole TM helix slides as a rigid piston through the membrane (13) (Fig. 6A). Because the extracellular region immediately outside of the TM domains analyzed in this study contains a short stretch of nonpolar or weakly polar residues, such a model appeared to be possible. However, we find that the position of the N-terminal end of the TM helix remains unaltered when the position of the C-terminal end is forced to shift from Phe-Lys\textsuperscript{1027} to Tyr-Lys\textsuperscript{1022} for α5 and from Ile-His\textsuperscript{758} to Trp-Lys\textsuperscript{752} for β1 by replacing a leucine with lysine at positions 1023 and 753, respectively (15). Therefore, the piston model seems unlikely for the α5 and β1 subunits. If the C-terminal end of the TM domain is induced to move into the cytoplasm by physiological stimuli, e.g. by a protein-protein interaction, altered tilting and/or uncoiling seem more likely mechanisms to account for the shortening of the membrane-spanning segment. Two schematic models for such shortening of the TM helix is pictured in Fig. 6 (B and C). Further experiments will be needed to test whether alterations in the orientation of the membrane-proximal region of one or both integrin subunits are linked to the active, inactive, or ligand-stimulated conformations.

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