Determination of the Border between the Transmembrane and Cytoplasmic Domains of Human Integrin Subunits*

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In this study we have determined the position of the C-terminal end of the transmembrane domains of human integrin subunits (α2, α5, β1, β2) in microsomal membranes using the glycosylation mapping technique. In contrast to the common view, the transmembrane helices were found to extend roughly to Phe1129 in α2, to Phe1026 in α5, to Ile757 in β1, and to His728 in β2. The α-carbon of the conserved lysine present near the C-terminal end of the transmembrane helix (Lys1125 in α2, Lys1022 in α5, Lys754 in β1, and Lys724 in β2) is buried in the plasma membrane, and the charged amino group most likely reaches into the polar head-group region of the lipid bilayer. A possible role for the conserved lysine in integrin function is discussed.

Integrins are cell adhesive receptors composed of non-covalently linked α and β subunits. Each subunit consists of a large extracellular domain, a transmembrane helix (TMH), and a short cytoplasmic tail of usually less than 60 amino acids. Cell attachment to extracellular matrix via integrins is necessary for normal cell growth and differentiation. Integrins are also involved in cellular processes that require migration of cells, e.g. angiogenesis and extravasation of lymphocytes. Upon ligand binding, clustering of integrins leads to formation of focal contacts containing signaling complexes (1, 2).

The short cytoplasmic domains of integrin β subunits have multiple functions: to establish contact with the actin cytoskeleton, to start signaling cascades, and to regulate the conformation of the extracellular domain of the receptor and thereby the ability to bind extracellular ligands (3–6). All these functions depend on interactions with cytoplasmic proteins, some of which mediate outside-in signaling and some that regulate extracellular ligand binding affinity (so called inside-out signaling) (7). α subunits of integrins appear to have a regulatory role over the β subunits, possibly by hindering β subunits from binding to cytosolic proteins in the absence of bound extracellular ligand (8, 9). In addition, specific signals are also generated by cytoplasmic tails of α subunits (10–12). Thus, the cytoplasmic domains are indispensable for proper functioning of integrins as demonstrated by many studies.

However, for both integrin subunits, the border between the cytoplasmic domain and the C-terminal end of the transmembrane domain is unclear. Usually the cytoplasmic domain of integrins is assumed by most authors to start at the first charged residue after the continuous stretch of 23 hydrophobic amino acids. In a few cases the cytoplasmic domain of integrins has instead been suggested to be 4 and 5 amino acids shorter for the α and β subunits, respectively (see Fig. 1) (13–15). Interestingly, all presently known α and β subunits, except for β4, exhibit a similar pattern in this region: a single conserved, positively charged amino acid (Lys/Arg), a short stretch of hydrophobic amino acids, and highly polar sequence unlikely to be buried in the plasma membrane. The regions between and C-terminally adjacent to the predicted ends of the TMHs of α and β subunits (Fig. 1) are involved in affinity regulation and in dimerization of integrins (4, 16–20).

In this study, we have used a glycosylation mapping technique to determine the position of the C-terminal end of the TMHs of human integrin α subunits (α2, α5) and β subunits (β1, β2) in microsomal membranes. Our results establish that the α-carbon of the conserved lysine and the following short hydrophobic stretch of all tested subunits are buried in the membrane. Possible functional implications of this finding are discussed.

MATERIALS AND METHODS

Enzymes and Chemicals—Unless otherwise stated, all enzymes were from Promega and MBI Fermentas AB. Ribonucleotides, deoxyribonucleotides, dideoxyribonucleotides, the cap analog m7G(5′)ppp(5′)G, T7 DNA polymerase, and [32P]dATP were from Amersham Pharmacia Biotech. The plasmid pGEM1, dithiothreitol, bovine serum albumin, RNasein ribonuclease inhibitor, and rabbit reticulocyte lysate were from Promega. Spermidine was from Sigma. Oligonucleotides were from Kebo Lab (Stockholm, Sweden) and DNA Technology (Aarhus, Denmark).

DNA Manipulations—The pGEM1-based Lep vectors coding for a glycosylation acceptor site at different positions have been described (21). Introduction of the TMHs of human integrin subunits α2, α5, β1, β2 in Lep was performed by replacing the H2 segment (bracketed by BclI-NdeI restriction sites) by the appropriate fragments amplified by polymerase chain reaction from the corresponding cDNAs. For the amplification of the TMH of the integrin subunits, the following primers were used: α5, 5′-TTTTATGTTTACACCATGTTGCTT-CAAG-3′ and 5′-TTTATTACATATGACCTTCTGTCA-GG-3′; for α2, 5′-TTTTAATCATATGATCTTTTGAAGAA-CCG-3′; for β1, 5′-TTTTAATCATATGACCTTCTGTCA-GG-3′; for 5, 5′-TTTATTACATATGACCTTCTGTCA-GG-3′; for β2, 5′-TTTTAATCATATGACCTTCTGTCA-GG-3′; and for β2, 5′-TTTTAATCATATGACCTTCTGTCA-GG-3′. The primers for the TMHs of human integrin subunits were: α5, 5′-TTTTAATCATATGACCTTCTGTCA-GG-3′; for α2, 5′-TTTTAATCATATGACCTTCTGTCA-GG-3′; for β1, 5′-TTTTAATCATATGACCTTCTGTCA-GG-3′; for β2, 5′-TTTTAATCATATGACCTTCTGTCA-GG-3′; and for β2, 5′-TTTTAATCATATGACCTTCTGTCA-GG-3′. The primers for the TMHs of human integrin subunits were: α5, 5′-TTTTAATCATATGACCTTCTGTCA-GG-3′; for α2, 5′-TTTTAATCATATGACCTTCTGTCA-GG-3′; for β1, 5′-TTTTAATCATATGACCTTCTGTCA-GG-3′; for β2, 5′-TTTTAATCATATGACCTTCTGTCA-GG-3′; and for β2, 5′-TTTTAATCATATGACCTTCTGTCA-GG-3′.
type sequence), the flanking Pro residues were changed to Gln, since such flanking prolines reduce the efficiency of glycosylation (22–24). The mutations L753K (L-K753) and LLMII5753–757N (5N753–757) in β1 TMH were introduced by the polymerase chain reaction method using antisense primers β1TML-K (5′-TTATACATGACCTCTTCGAT-CAATTACATTTACACTTCTTCTTCAAATC-3′) and β1TM5N (5′-TTATACATGCCTCTTGATATTTATTTTCAATTTCCAAATC-3′), respectively, together with the sense primer β1TMu. The designed mutations were generated by the underlined nucleotides. The amino acids HDRR in mutations were generated by the underlined nucleotides. The amino acids adjacent to the N-terminal end of the TMHs are shown in bold. Four amino acids adjacent to the N-terminal end of the TMHs are shown in bold. Four amino acids adjacent to the N-terminal end of the TMHs are shown in bold. Four amino acids adjacent to the N-terminal end of the TMHs are shown in bold.

### RESULTS

**Glycosylation Mapping**—The glycosylation mapping technique has previously been described in detail (21). Briefly, the luminaly oriented active site of the endoplasmic reticulum enzyme oligosaccharyltransferase was used as a fixed point of reference against which the position of a TMH in the endoplasmic reticulum membrane could be measured (Fig. 2A). Based on the variation in glycosylation efficiency for a set of constructs differing only in the position of a glycosylation acceptor site, it was possible to define a MGD, i.e., the number of residues in the interspersed chain needed to bridge the distance between the end of the TMH and the oligosaccharyltransferase active site. By calibration of the MGD scale against TMHs whose position in the lipid bilayer had been determined by direct techniques such as x-ray crystallography, NMR, or fluorescence quenching measurements, the point where a TMH exited from the lipid environment could be determined to within less than ±1 residue.

Previously, we used this technique to study how the position of model TMHs in the endoplasmic reticulum membrane changed in response to single mutations such as the introduction of a proline or a charged residue (21, 22). We had also calibrated our measurements against two different TMHs of known position in the membrane, the TMH of the H-subunit from the photosynthetic reaction center and the TMH from the phage M13 major coat protein, as well as against model poly-Leu TMHs of varying lengths (21, 22). These studies showed that the MGD measured from the first residue after the hydrophobic region of a typical TMH is 9.5–10.5 residues.

**Determination of the Membrane-embedded Parts of Integrin Subunits**—For the studies of the TMH of α subunits, a segment encoding residues 989–1028 was amplified from the α5 and 1096–1131 from the α2 cDNAs and cloned into a series of previously constructed vectors based on the well-characterized protein leader peptidase (Lep) (Fig. 2A). The vectors differ only in the position of a single Asn-Ser-Thr glycosylation acceptor site downstream of the TMH and, thus, allow facile determination of the C-terminal MGD for any TMH.

The results of in vitro transcription/translation of three Lep-α2 and Lep-α5 constructs in the absence and presence of dog pancreas microsomes are shown in Fig. 2B, and the MGD-determination is shown in Fig. 2C. Essentially identical results were obtained for both tested α subunits. The glycosylation efficiency drops from 54% for the Asn82 construct to 6% for the Asn87 construct for α2 subunit and from 85% for the Asn85 construct to 6% for the Asn87 construct for α5 subunit. Since the expected MGD value for a TMH longer than ~23 residues is ~10 residues (21), this allows the C terminus of the TMHs of α2 and α5 to be positioned relative to the reference TMHs (Fig. 2D). Even with allowance for a rather wide margin of error, this clearly places the α-carbon of Lys1126 in α2 and Lys1022 in α5 more than one helical turn below the membrane-water interface, similar to the position of Lys46 in M13 coat protein (26, 27) and to single lysine mutations in a poly-Leu TMH (22).

Similarly, for the studies of β subunits, a segment encoding residues 723–761 from the β1 and 695–933 from the β2 cDNAs was amplified and cloned into the Lep vectors. Very similar results were obtained (Fig. 2B and C), placing the α-carbon of Lys752 in β1 and Lys724 in β2 well below the membrane-water interface (Fig. 2D).

We conclude that the membrane-embedded parts of the TMHs of the α subunits extend roughly to Phe1129 in α2 and to Phe1026 in α5. For the β subunits, the TMH of β1 extends to Ile757, and the TMH of β2, to His723. As proposed in the so-called snorkel model (28–30), the long, aliphatic side of the side chain of the membrane-embedded Lys in integrin α and β subunits most likely extends toward the membrane surface, placing the positively charged terminal amino group in the polar head-group region of the lipid bilayer.

**Mutations Near the C-terminal End of the Transmembrane Segments Cause a Shift in Membrane Location**—To confirm our interpretation of the glycosylation mapping data, we constructed two derivatives of the β1 TMH: one where the entire hydrophilic segment between Lys752 and His758 was replaced by Asn residues (β1Δ5N), and one where Lys753 was replaced by Lys (β1ΔL-K) (Fig. 3A). Since both Asn62 constructs were fully glycosylated (data not shown), the four residues HDRR near the lipid-water interface were deleted to facilitate the MGD determination. For both β1Δ5N and β1ΔL-K, the MGD measurement indicated a substantial shift in the position of the TMH in the membrane (Fig. 3B). Although we have only determined the MGD to within ±1 residue in this case, it is nevertheless clear that Lys752 is positioned much closer to the membrane-water interface in
these constructs (Fig. 2D). This demonstrates that the hydrophobic segment between Lys$^{752}$ and His$^{758}$ in wild type β is embedded in the membrane and that it is pushed out of the membrane when its hydrophobicity is reduced.

**DISCUSSION**

In this study the glycosylation mapping technique has been used to determine the position of the TMHs of two human integrin α and β subunits in the microsomal membrane. The TMHs of the α subunits were found roughly to Phe$^{1129}$ in α2 and to Phe$^{1026}$ in α5, and the TMHs of the β subunits were found to extend to Ile$^{757}$ in β1 and to His$^{728}$ in β2. Interestingly, the α-carbon of the single positively charged amino acid present near the C-terminal end of the TMHs (Lys$^{1125}$ in α2, Lys$^{1022}$ in α5, Lys$^{752}$ in β1, and Lys$^{724}$ in β2) was buried in the membrane in all cases. Thus, the same result was obtained, irrespective of whether tryptophan or tyrosine is N-terminally adjacent to the conserved sequence KXXGFFKR of the α subunits. Reductions in the hydrophobicity of the short hydrophobic stretch downstream of Lys$^{752}$ in the β1 TMH caused a shift in the position of Lys$^{752}$ relative to the membrane, confirming that the hydrophobic segment between Lys$^{752}$ and His$^{758}$ is indeed buried in the membrane in the wild type protein. These results were obtained using microsomal membranes; however, the integrin TMHs can be accommodated in the same way also in the plasma membrane, which actually is thicker than microsomal membranes.
The highly conserved motif KXGFFKR at the C-terminal end of the α subunit TMH is known to be critically important for integrin function, although the phenotypic effects of modification in this region vary between different integrins. Deletion of the motif from αIib results in a constitutive high affinity state of the αIibβ3 integrin. Similarly, deletion of LLTIHD in β3, the opposing region of KXGFFKR motif in αIib, also leads to a high affinity state of the receptor. It has been suggested that the amino acids Arg and Asp in these motifs form a salt bridge and that breaking the bridge locks the integrin in a conformation corresponding to a high affinity state (16, 17). This idea is further supported by molecular modeling on integrin subunits αIib and β3 showing that the LLTIHD sequence in β3 and the KXGFFKR motif in αIib are likely to be associated (31). Similarly, deletion of VGFFK in αL increases the affinity of the αLβ2 integrin, but in this case the mutation also interferes with post-ligand binding events dependent on the cytoskeleton (19, 32). In addition, the KXGFFKR motif has been shown to promote the assembly and/or stabilization of the αLβ2 heterodimer (18, 19) as well as of several β1 integrins (4, 20).

These regions have been demonstrated to interact with a variety of different proteins. The intracellular calcium-binding protein calreticulin was reported to bind the sequence KXGFFKR in α subunits, and this interaction may be required for integrin activation (33, 34). A recent report by Coppolino and Dedhar shows that interaction between α subunits and calreticulin is ligand-specific and transient, occurring shortly after ligand binding (35). The synthetic peptide KLLMIHHDREF derived from the β1 sequence was found to interact with focal adhesion kinase in vitro (36). Focal adhesion kinase is known to be involved in integrin-mediated signaling (37) but appears not to be required for integrin activation (38). Recently, the proteins Rack1 and skelemin were shown to bind to the membrane-proximal region of β-subunits in yeast two-hybrid assay (39, 40). Rack1 binding to β1 and β2 integrins requires activation of protein kinase, which is one of the early events after ligand binding of integrins (41, 42), and Rack1 may recruit protein kinase C to adhesion complexes by its ability to bind integrins. However, the functional role of Rack1 in integrin signaling remains to be elucidated. Muscle skelemin was found to bind β1 and β3 subunits but not to β2. In Chinese hamster ovary cells the skelemin-like protein colocalized with integrins at early stages of cell spreading (40).

Our results show that the KLGFF sequence in α2 and α5, KLLMII in β1, and KALIH in β2 are buried in the membrane in the absence of interacting proteins. Why, then, is a transmembrane lysine conserved in all known integrin subunits (17)? One possibility is presented in Fig. 4A. This model is based on the studies discussed above, demonstrating that the regions between arrows 1 and 2 in Fig. 1 are involved in interactions with intracellular proteins and, thus, are likely to be exposed to the cytoplasm. The charged residue may facilitate a transfer of this region out of the hydrophobic

**Fig. 3.** A. *in vitro* translation of the β1Δ5N and β1ΔL-K constructs in the absence (−) and presence (+) of rough dog pancreas microsomes (RM). Unglycosylated and glycosylated forms are indicated by white and black circles, respectively. The position of the acceptor Asn in Lep is indicated, cf. Fig. 2D. B. Glycosylation efficiencies based on quantitation of the gels shown in panel B (β1Δ5N, white circles; β1ΔL-K, black circles). The limits of the positions from which to calculate the MGD are estimated by extrapolation from the Asnα5 and Asnα6 points as shown, using the data for the wild type β1 construct (crosses) as a guide for the extrapolations. The arrow indicates the limits for the β1Δ5N construct (position 84.3 ± 1), as derived from the extrapolations to the line representing 40% glycosylation. Note that the glycosylation efficiency has only been measured for the β1Δ5N and β1ΔL-K constructs with glycosylation sites Asnα5 and Asnα6.

**Fig. 4.** Hypothetical models for the role of the conserved Lys/Arg in integrin function. A. Activation of integrins is suggested to involve a movement of the “mobile region” (black areas in the TMHs) out of the plasma membrane. In the inactive state this segment of both subunits may associate with each other. In this situation the extracellular domain is in a conformation that is incapable of ligand binding, and the cytoplasmic tail of the α subunit masks the cytoplasmic tail of β subunit. In a fully active integrin the mobile regions are exposed to interacting proteins in the cytoplasm, the transmembrane domains are 4–5 amino acids shorter, and the extracellular domain is able to bind to a ligand. It should be noted that this represents one of several similar possible scenarios; integrin activation may be obtained by moving the TMH of one or both of the integrin subunits in or out of the membrane. B. The positively charged residues of the integrin TMHs are indicated to participate in interactions with other membrane proteins (striped). Gray areas in the plasma membrane indicate polar head-group regions.
environment as a result of binding to intracellular proteins. Such a movement could trigger the conformational changes associated with integrin activation and/or ligand binding.

The possibility that the position of the conserved lysine and the following stretch of hydrophobic amino acids in the plasma membrane may contribute to the transmembrane signaling of integrins was first discussed by Williams et al. (43). Our study provides supporting evidence for this view. We suggest that this region in one or both of the integrin subunits is positioned differentially relative to the plasma membrane depending on the affinity state of the receptor. For example, in the inactive conformation, the $C_s$ of the lysines could be buried in the plasma membrane. In this situation the TMHs would probably be tilted in the membrane due to their length, and the $\alpha$ and $\beta$ subunits could associate close to the cytoplasm, e.g. via the Asp-Arg bridge. Upon integrin activation, the TMHs would be shortened by 4–5 amino acids, possibly induced by binding of cytosolic proteins to the cytoplasmic tails of integrin subunits. The tension exerted by actin cytoskeleton toward integrin clusters may also have a role in preventing backsliding of the mobile region (Fig. 4A).

Only a few studies have directly addressed the role of the conserved TMH Lys (e.g. Lys$^{765}$ in human $\beta$1 subunit) in integrin function. Mutation of the Lys in $\alpha$1 (Lys to Asp) did not impair the $\alpha$1$\beta1$-mediated adhesion to collagen IV but resulted in localization of $\alpha1$ to focal contacts also on a fibronectin substrate (44). The phenomenon of ligand-independent clustering of integrins in focal contacts has been suggested to reflect a substrate (44). The phenomenon of ligand-independent clustering of integrins in focal contacts has been suggested to reflect a substrate (44). The phenomenon of ligand-independent clustering of integrins in focal contacts has been suggested to reflect a substrate (44). The phenomenon of ligand-independent clustering of integrins in focal contacts has been suggested to reflect a substrate (44). The phenomenon of ligand-independent clustering of integrins in focal contacts has been suggested to reflect a substrate (44). 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