Identification of novel interaction partners for the conserved membrane proximal region of α-integrin cytoplasmic domains

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Abstract The α3Aβ1 integrin is a laminin receptor with a broad specificity for different laminin isoforms. Furthermore, it regulates the function of other integrins, like α2β1, α5β1 and α6Aβ1. In a yeast two hybrid screen of a human placenta cDNA library, we identified cDNAs coding for four different proteins that strongly interact with the conserved region of the cytoplasmic domain of the α3A integrin subunit. In addition to the cDNA for nucleotide exchange factor Mss4 and the putative tumour suppressor protein BIN1, two novel cDNAs were identified. Association analysis with different integrin subunits revealed them as cDNAs that encode binding proteins which react with a broad spectrum of α subunits. The conserved membrane proximal region of the α3A chain was identified as the binding site for all four proteins. They, therefore, may be involved in the regulation of general functions of integrins.

Key words: Integrin cytoplasmic domain; α3Aβ1 integrin receptor; Protein-protein interaction; Two hybrid system

1. Introduction

Integrins are transmembrane α/β heterodimeric receptors that provide a link between extracellular matrix proteins and the cytoskeleton [1–3]. The majority of the integrin molecules have large extracellular domains and small intracellular parts. The extracellular domains determine the specificity of ligand binding and provide the outside-in signalling, while the intracellular C-terminal sequences transfer the signal into the interior of the cell and further modulate the affinity of the receptor for its extracellular ligand in response to environmental and developmental factors (inside-out signalling) [1–4]. Both α and β cytoplasmic domains possess a stretch of highly conserved amino acids proximal to the cell membrane. Deletion of these amino acid sequences in either the α or the β chain results in a state of high affinity of the αIIbβ3 platelet receptor, while preservation of only the conserved region without the rest of the protein chain is enough to prevent this activation [5–8]. In other cells, in which integrins do not require activation for ligand binding, like αIIbβ3, deletion of the α cytoplasmic domain directly after the conserved KXGFFKR region, leads to their recruitment into focal adhesion complexes independent of ligand binding and to a diminished cell adhesion [9–11]. The presence of 3–4 amino acids after the conserved region restores the normal function. Interestingly, addition of just two alanine residues after the conserved motif also partially restores the function of the truncated receptor [10]. These data, together with results of numerous experiments with point and deletion mutants of the conserved α cytoplasmic domains, highlight the importance of this region in integrin-mediated signalling. The mechanism by which this region modulates integrin activity is, however, still unknown.

The ligand binding of integrins is not very specific. For example, α3β1 binds all laminin isoforms except laminin 1, having, however, the highest affinity for laminin 5 [12,13]. Furthermore, several integrins are able to bind the same ligands and often the same domains on the ligands. For example, α6β1, α6β4 and α7β1 all bind to the proteolytic fragment E8 of laminin 1 [12–14]. Nevertheless, integrins also seem to perform specific cellular functions as demonstrated by the developmentally and tissue restricted expression of certain integrins as well as by the results of numerous in vitro differentiation studies. For instance, myoblasts form myotubes on laminin, but not on fibronectin, for which the α5β1 integrin is the preferential receptor, although these cells express integrins for both ligands [15,16]. Similarly, neuronal differentiation of rat pheochromocytoma cells is facilitated by laminin rather than by fibronectin [17]. By contrast, differentiation of erythroblasts is supported by the activation of the fibronectin-specific α5β1 integrin [16].

Although both α and β cytoplasmic tails are involved in ligand binding and modulation of integrin function, they play clearly different roles. Overexpression of only the β cytoplasmic domain as a part of the transmembrane chimeric molecule, but not that of α, has a dominant negative effect, disrupting the integrin function and cell adhesion [8,18,19]. The exchange of cytoplasmic domains between α or β subunits does not abrogate the ligand binding, but the regulation of the affinity state of the integrins depends on the type of cells in which these chimeric molecules are expressed [19,20]. Further, depending on whether α3- or α6-, associated with β1, is activated, the morphology of focal adhesions is different, with the α3 chain regulating the function of the α6 subunit [21]. It has been shown also that α3β1 regulates the formation of focal adhesions of α2β1 and α5β1 integrins as well as cell migration mediated by these receptors [22].

As integrin α and β cytoplasmic domains have no enzymatic activities, they probably provide signals by recruiting and binding other proteins or messenger molecules. Interactions of integrins with other proteins are probably involved also in processes such as the transport of proteins to the Golgi complex, their redistribution on the cell surface and others. In an attempt to identify such intracellular proteins, we screened a human placenta cDNA library using the yeast two hybrid

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system with the cytoplasmic domain of the α3A chain as a bait.

2. Materials and methods

2.1. Plasmid construction

Total RNA from the human HBL100 cell line was isolated using the RNeasy kit (Qiagen, Hilden, Germany). cDNA fragments representing the cytoplasmic domains of alpha 2, alpha 3A, alpha 5, alpha 6A and beta 1A integrins were amplified by RT-PCR using the isolated RNA as template. The following primers were used: for alpha 2, 5′-CCGAATTCAAGCTCGGCTTCTTCAAAAG-3′ (upstream) and 5′-ATGGATCCTGGCAACTTTGGATGAGCAG-3′ (downstream): for alpha 3A, 5′-ACGGATCCGAATTCAAGTGCGGCTTCTTCAAGCG-3′ (upstream) and 5′-CCTGTGGACTGTCAGAGGC-3′ (downstream): for alpha 5, 5′-CCGAATTCAAGCTTGGATTCTTCAAACGC-3′ (upstream) and 5′-CAGGCAGAACCCAAATTCTGCAG-3′ (downstream): for alpha 6A, 5′-ACGGATCCGAATTCAAGTGTGGTTTCTTCAAGAAG-3′ (upstream) and 5′-GTTCCACTTTGTGATCCACTG-3′ (downstream): for beta 1A, 5′-CCGGAATTCAAGCTTTTAATGATAATTCATGAC-3′ (upstream) and 5′-TCCGCTCGAGGGATCCAACTAGCTAGCAGGACATTTAC-3′ (downstream). All upstream primers were designed to include an *Eco*RI restriction site. The downstream primers correspond to the 3′-untranslated regions of integrin sequences and include either a natural or an artificial *Bam*HI restriction site. The resulting PCR products were cloned into *Eco*RI/*Bam*HI restriction sites in the pAS2-1 vector (Clontech, Palo Alto, CA, USA) containing the GAL4 DNA binding domain.

cDNA fragments representing the complete cytoplasmic domains of the human alpha 3A* and -B*, the human alpha 6A* and -B*, and the murine alpha 7A* and -B* integrin receptors were amplified by PCR using as template full length cDNA constructs for alpha 3A* and -B* [23] and alpha 6A* and -B* [24] or a murine muscle lambda gt11 library (Clontech, Palo Alto, CA, USA) for alpha 7A* and -B*. The following primers were used: for alpha 3A* 5′-CTGTGGAAGCTTGGCTTCTTC-3′ (upstream), for alpha 3B* 5′-CTGTGGAAGCTTGACTTCTTTAAG-3′ (upstream) and 5′-ACTTCTAGATGCACTCGGCGTTGT-3′ (downstream) for both alpha 3A* and 3B*. For alpha 6A* and 6B* 5′-ATACTATGGAAGCTTGGTTTCTTC-3′ (upstream), 5′-CCGGAATTCCGCTATGAGTAG-3′ (downstream) for alpha 6A*, and 5′-GAAGAAACCAAGCTTCCATAGTAT-3′ (downstream) for alpha 6B*. For alpha 7A* 5′-CCCAAGCTTGGCTTCTTCCGTCGGAACAGTC-3′ (upstream), for alpha 7B* 5′-CCCAAGCTTGGATTCTTCAAGCGGGCGAAGC-3′ (upstream) and 5′-TGGAAAAGGGCGAAAGGAAC-3′ (downstream) for both alpha 7A* and 7B*.

All upstream primers for constructs marked with an asterisk were designed to include a *Hin*dIII site at the start of the DNA sequence encoding the cytoplasmic domain, resulting in the change of a Cys residue immediately in front of the conserved GFFKR domain to a Leu residue. Downstream primers corresponding to the 3′-untranslated regions of the integrin sequences included *Xba*I (for alpha 3), *Eco*RI (for alpha 6) or no (for alpha 7) sites. The resulting PCR products were subcloned into pBluescript II KS(+) (Stratagene, La Jolla, CA, USA), cut with the appropriate restriction enzymes and characterised by DNA sequencing. DNA fragments containing the complete integrin cytoplasmic domains were released from the pBluescript subclones by restriction digestion with *Cla*I and *Sma*I (5′ and 3′ of the integrin sequence, respectively), treated with Klenow DNA polymerase in the presence of dNTPs and subcloned into pAS2-1 cut with *Sma*I. This ensured cloning of the integrin sequences in frame with the GAL4 DNA binding domain protein. The alpha 1* integrin chain was cloned into the pAS2-1 vector after annealing of primers comprising the entire cytoplasmic domain of α1 and *Nde*I/*Eco*RI compatible overhangs. The primers were: 5′-TAT GAA GAT TGG ATT CTT CAA AAG ACC ACT GAA AAA GAA AAT GGA GAA ATG-3′ (upstream) and 5′-AAT TCA TTT CTC CAT TTT CTT TTT CAG TGG TCT TTT GAA GAA TCC AAT CTT CA-3′ (downstream).

The alpha 3A deletion mutants were generated by PCR using the pAS2-1/α3A plasmid DNA as a template. For alpha 3A (1015–1021) and alpha 3A (1015–1024) the primers were chosen in such a way that after their annealing, *Eco*RI and *Bam*HI compatible overhangs were created. The primers were: for alpha 3A (1015–1021), 5′-AATTCAAGTGCGGCTTCTTCAAGCGATGAG-3′ (upstream) and 5′-GATCCTCATCGCTTGAAGAAGCCGCACTTG-3′ (downstream) and for alpha 3A (1015–1024), 5′-AATTCAAGTGCGGCTTCTTCAAGCGAGCCCGCACTTGAG-3′ (upstream) and 5′-GATCCTCAAGTGCGGGCTCGCTTGAAGAAGCCGCACTTG-3′ (downstream): for alpha 3A (1022–1051) the upstream primer was 5′-CCGAATTCGCCCGCACTCGCGCCCTG-3′ and the downstream primer was the same as for wild-type alpha 3A. The PCR products were subcloned into *Eco*RI/*Bam*HI in the pAS2-1 vector. Point mutations were also generated by PCR, using the same primers as for obtaining the wild-type alpha 3A, except for nucleotide exchanges in the upstream primers for corresponding amino acid substitutions. All pAS2-1 subclones were analysed by DNA sequencing to confirm the correct in frame fusion of GAL4 and integrin sequences.

A human placenta cDNA library, cloned into the pACT2 vector fused with the GAL4 activation domain as well as the pAS2-1/LAM5′-1 construct, which was used as a negative control in interaction tests with proteins identified in a two hybrid screen, were purchased from Clontech (Palo Alto, CA, USA).

2.2. Yeast two hybrid library screening

Yeast cultures were grown under standard conditions in liquid or on solid media using YPD or minimal SD media. The yeast strain Y190 (Clontech, Palo Alto, CA, USA) was transformed sequentially with the pAS2-1 plasmid containing alpha 3A (aa 1015–1051) as bait and then with a pACT2 plasmid containing the cDNA library. Transformants were grown on SD medium lacking the amino acids leucine, tryptophan and histidine in the presence of 25 mM 3-amino-1,2,4-triazole. On day five, the grown colonies were tested for the activity of the LacZ reporter gene in a β-Gal filter assay.

2.3. Mating and transformation assay

To remove the bait cDNA, clones scored as positive during the screening, were further recultured on SD medium without tryptophan in the presence of 10 μg/ml cycloheximide. The cycloheximide resistant Y190 yeast clones were verified in a mating assay with yeast strain Y187 expressing the pAS2-1 plasmid with either alpha 3A, the unrelated protein lamin C, or the non-fused GAL4 DNA binding domain as baits. Clones were scored as positive when the HIS^+ and $LacZ^+$ phenotype of yeast cells was dependent on the coexpression of only alpha 3A as bait. The positivity of these clones was further retested in a cotransformation assay with purified plasmid cDNA using the same controls as in mating assays.

3. Results and discussion

3.1. Identification of proteins binding to the conserved region of the α3A cytoplasmic domain

Of a total of 1.4×10^7 clones screened, 159 appeared to interact with α3A, but only 84 of them had a HIS^+ and $LacZ^+$ phenotype after α3A was expressed, but not after coexpression of the unrelated lamin C protein or the non-fused GAL4 DNA binding domain. cDNA sequencing of the 84 α3A interacting clones revealed five genes. Two or three clones of each identified gene were used to map the binding site on the α3A integrin subunit. For this purpose, three different deletion mutants of α3A were created (Fig. 1). The peptides encoded by two cDNA clones interacted with the highly conserved KXGFFKR region just proximal to the membrane and for those encoded by the other two, three additional residues beyond this conserved motif were required for interaction (Fig. 1). The peptide encoded by the fifth clone did not interact with the conserved motif and will be described elsewhere. Here we report only clones whose products bind to the conserved region of α3A.

BLAST search in the NCBI data bank [25] revealed that two of these four clones encode known polypeptides, Mss4 (accession # S78873) and BIN1 (accession # AF068915), whereas the two others encode novel proteins. The most fre-

Constructs	Mss4	Clone 63	BIN1	Clone 80	DRAL
	His$^+$ and LacZ$^+$ expression				
1015 1022 1051					
α 3A (1015-1051) KCGFFKRARTRALYEAKRQKAEMKSQPSETERLTDDY-COOH	+++	+++	+++	+++	+++
α 3A (1015-1021) KCGFFKR- COOH	−	+++	++	−	−
α 3A (1015-1024) KCGFFKRART- COOH	+++	+++	+++	+++	−
α 3A (1022-1051) ARTRALYEAKRQKAEMKSQPSETERLTDDY- COOH	−	−	−	−	+

Fig. 1. The interaction of α3A deletion mutants with proteins identified in a two hybrid screen as potential α3A binding partners. The interaction was determined by growth on His$^-$ medium (growth results from activation of the first reporter gene) and evaluated further in a β-Gal filter assay for expression of the second reporter gene, LacZ. Yeast Y190 cells were transformed with pAS2-1 plasmid DNA expressing the integrin cytoplasmic domains along with the pACT2 vector bearing the identified proteins. The interaction was scored as negative (−) when no blue colonies were visible after 8 h: the interaction was scored as weak (+), intermediate (++) or strong (+++) when blue colonies became visible after 8 h, 4 h or 1 h, respectively. Numbers in parentheses refer to amino acid positions.

quent clone (48) always contained a cDNA with the entire open reading frame of the guanine nucleotide exchange factor Mss4 [26]. The encoded 14 kDa Zn^{2+} binding protein is found in all mammalian tissues, with the highest level of expression in brain, lung, liver, kidney and pancreas [27]. It binds to Rab3a GTPase, localised in the membrane of cytoplasmic vesicles, promoting GDP-GTP exchange, and it presumably plays a role in processes regulating the vesicular transport, facilitating, for example, the release of neurotransmitter [27,28]. The second isolated clone of known sequence was found twice as a 1.7 kb long insert. It represented the 3′ half of the cDNA for BIN1 including the 3′-untranslated region. BIN1 has recently been described as a protein strongly expressed in skeletal muscle and brain [29,30]. This protein has a SH3 domain and a Myc interacting site and is a putative tumour suppressor. Different splice variants of this protein were identified, and the variant encoded by cDNA containing the exon 10 has been reported to be upregulated in differentiated muscle cells [30,31]. Overexpression of BIN1 promotes myotube formation and upregulation of the myosin heavy chain [31].

The sequences of the other selected clones, 63 and 80, were novel. Clone 63 (accession # AJ131721) was identified as a 2.0 kb cDNA insert with an open reading frame which does not contain an obvious starting Kozak motif. Searching in data base banks revealed both 5′- and 3′ extending EST clones which suggests, that clone 63 does not contain the whole coding sequence. The second clone encoding a polypeptide with a novel sequence, the clone 80 (accession # AJ131720), occurred eight times. All eight clones had identical 3′ regions and contained a stop codon, while at the 5′ end they were differently extended, the largest of them containing about 2.5 kb of open reading frame. Searching in the data banks did not reveal any EST clones that extended clone 80 in both 5′ and 3′ direction. As the first methionine with a nearly optimal Kozak motif is localised about 600 bp downstream from the 5′ end of the available sequence, the 2.5 kb long clone 80 probably does not represent the full length cDNA sequence. Comparison of clones 63 and 80 with available cDNA and protein data banks did not reveal any similarity or homology to any of these sequences or motifs, except for the presence of numerous putative phosphorylation sites for PKA, PKC and CK2 kinases as well as putative myristoylation sites.

3.2. *Mapping of amino acids essential for protein binding in the conserved region of α3A*

The transmembrane regions of α and β integrins are thought to assume an α-helical structure that might be extended into the cytoplasmic domain [32,33]. The last residue of the transmembrane region is obligatorily an aromatic amino acid, either tryptophan or tyrosine, and the first cytoplasmic residue that follows is always a lysine. During the construction of GAL4/α3A clones, two chimeric constructs were generated. In the first construct the seven KCGFFKR amino acids of the conserved region were kept unchanged and an aromatic phenylalanine was inserted before the first lysine. In creating the second GAL4/α3A* fusion construct, another cloning strategy was used which resulted in the exchange of the aromatic amino acid for negatively charged aspartic acid and of the second cysteine (which is not conserved among α chains) for leucine. Surprisingly, the second α3A* chimeric protein did not bind to Mss4 or clone 63 in a direct two hybrid interaction assay (Fig. 2). To test whether the above two amino acids are essential for binding to the conserved region, α3A mutants were constructed, in which the cysteine at position +2 was exchanged for leucine and the phenylalanine at the position −1 for either aspartate or tryptophan (Fig. 2). As expected, exchange of the non-conserved cysteine at the position +2 had no influence on the binding capacity of

Table 1
Results of the β-galactosidase filter assay with cytoplasmic domains of integrin subunits

Proteins	α1*	α2	α3A	α3A*	α3B	α3B*	α5	α6A	α6A*	α6B*	α7A*	α7B*	β1A
Mss4	−	+++	+++	−	+	−	+++	+++	−	−	−	−	−
Clone 63	−	+++	+++	−	+++	−	+++	+++	NT	−	−	−	−
BIN1	+++	−	+++	+	++	++	−	−	NT	++	−	−	−
Clone 80	−	+++	+++	+++	−	−	−	++	NT	+++	+++	−	−

Integrin α chains marked with (*) possess either a methionine (for α1) or an aspartate before the first lysine of the conserved region which resulted from a different cloning strategy into the pAS2-1 vector. NT = not tested.

Constructs			His$^+$ and LacZ$^+$ expression			
			Mss4	Clone 63	BIN1	Clone 80
		1015 1022				
α 3A (C1016L)	F	KCGFFKR . . . -COOH	+++	+++	+++	+++
α 3A (F1014D, C1016L)	D	KLGFFKR . . . -COOH	−	−	+	+++
α 3A (C1016L)	F	KLGFFKR . . . -COOH	+++	+++	+++	+++
α 3A (F1014D)	D	KCGFFKR . . . -COOH	−	−	+	+++
α 3A (F1014W)	W	KCGFFKR . . . -COOH	+++	NT	+++	+++
α 3A (R1021D)	F	KCGFFKD . . . -COOH	−	+++	−	+++
α 3A (FF1019, 1020YY)	F	KCGYYKR . . . -COOH	−	+++	++	−

Fig. 2. The identification of amino acid residues in the conserved region of integrin α3A chain that are essential for the binding of the four identified interactive proteins. Numbers in parentheses refer to amino acid positions. The shaded letters in the α3A sequence represent amino acids that have been mutated. For simplicity only amino acids of the conserved region are presented. NT=not tested.

any of the four analysed proteins. However, Mss4 and clone 63, but not BIN1 and clone 80, failed to interact with α3A when the phenylalanine at position −1 was exchanged for the negatively charged aspartate. In contrast, all clones strongly bound to α3A when a tryptophan residue, that naturally occurs in α3A, was present instead of phenylalanine at position 1014 (Fig. 2). Thus, the aromatic amino acid before the first lysine of the conserved region which may be located in the membrane, either functionally belongs to the cytoplasmic domain, or substitution of this hydrophobic residue by a negatively charged one disrupts the proposed α-helical structure of the chain.

It has been shown that substitution of the first lysine or the arginine of the KXGFFKR region for negatively charged aspartic acid results in the loss of ligand-dependent focal contact localisation, whereas the exchange of these positively charged amino acids to a more hydrophobic alanine did not change the properties of the receptor [6,34–36]. Their replacement by aspartates alters the charge of the α chain, which may affect subunit association, transmembrane integrity of the α/β heterodimer and the function of the cytoplasmic domains [6,32–34]. The two hydrophobic phenylalanines in the KXGFFKR sequence have been shown to be also critical for the modulation of the affinity of integrins, as their replacement by alanines induced constitutive activation of the αIIbβ3 integrin [6]. Either exchange of phenylalanines for alanines, or their deletion in the α6A integrin sequence prevented the expression of this subunit at the surface of K562 cells, due to failure of association with the β1 integrin subunit. The mutated α6A chains were synthesised, but not transported to the Golgi apparatus [11]. To test whether positively charged arginine and two hydrophobic phenylalanines are essential for binding to α3A, these were exchanged with a negatively charged aspartate and with polar tyrosines, respectively. α3A-dependent binding of clone 63 was not affected by these point mutations. However, Mss4 did not interact with α3A when either the arginine or the two phenylalanines were replaced by other amino acids. For BIN1 and clone 80 these modifications had different effects (Fig. 2). Interestingly, the replacement of only the first phenylalanine by tyrosine had no influence on the binding of any of the four proteins, while the exchange of this residue with alanine produced a more pronounced effect, preventing binding of BIN1 and clone 80 (data not shown). We conclude from these data that while all four analysed proteins need the conserved region for binding to the α3A integrin subunit, their binding patterns are different, as the specific amino acids required by these proteins differ.

3.3. Binding specificity for the conserved region of α chains

To assess whether the identified proteins bind specifically to α3A, a direct two hybrid interaction assay was performed with cytoplasmic domains of several other integrin subunits. The α subunits of typical collagen binding receptors (α1 and α2), of a fibronectin binding receptor (α5) as well as α chains from additional laminin binding integrins (α3B, α6A, α6B, α7A and α7B) were used. As a non-α chain, the β1A subunit of these integrins was used (Table 1). None of the four isolated clones bound to the β1A subunit, which shows that they are specific for the α subunit. However, none of the proteins bound only α3A. Mss4 and the clone 63 bound all α chains that had an aromatic amino acid before the first lysine of the conserved region, while the binding pattern of BIN1 and clone 80 appeared to be more restricted (Table 1). This provides further support for the conclusion made above that the aromatic amino acid residue before the conserved region of integrin α chains is essential for their interaction with Mss4 and clone 63. Additionally, when the aspartate before the first lysine of the α3B cytoplasmic domain was replaced by phenylalanine, α3B showed a strong interaction with clone 63 and a moderate interaction with Mss4. As among the tested α subunits, α5 is the only non-laminin binding receptor, BIN1 and clone 80 proteins appeared to be laminin receptor-restricted interaction proteins. The restricted binding pattern of BIN1 and clone 80 may reflect the redundancy of laminin binding integrin receptors [12,36]. Although the binding sites for BIN1 and clone 80 were also mapped to the membrane proximal conserved region (−/+ the following three amino acids), the restricted manner in which they bind to different α chains indicates an influence of the unique C-terminal amino acids in these processes.

The membrane proximal conserved motif of integrin α chains represents the region that has been shown to be critical for the receptor function of integrins. If the GFFKR sequence is partially deleted or replaced by alanines the affinity of the

αIIbβ3 integrin shifts from low to high [37]. Despite sequence differences, several cytoplasmic domains of α chains can be interchanged without loosing their cell adhesion capacity (reviewed in [35]). However, deletion of unique sequences directly after the conserved KXGFFKR motif abolishes the constitutive ligand binding activity and the ligand-dependent recruitment into focal contacts of α1β1 [31], α2β1 and α4β1 [9,10] or α6β1 [11] integrins. Preservation of only 3–4 amino residues beyond the conserved region was sufficient to restore both properties. Surprisingly, addition of only two alanines to the C-terminal part of KXGFFKR also partially prevented the functional defects of receptors with truncated unique sequences [10]. The fact that the binding region on α3A for four proteins found in the two hybrid screen was mapped to the conserved region or to this region as well as the next three amino acids, raises the possibility that the proteins identified as α3A binding partners regulate processes common to several or even all integrins.

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