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Review

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β_3-Integrin cytoplasmic binding proteins

Renyi Zhao*, Alokkumar S. Pathak* and George A. Stouffer

Carolina Cardiovascular Biology Center, University of North Carolina, Chapel Hill, NC, USA

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Summary

Integrins are cell-surface adhesion receptors that play an important role in mediating numerous physiological processes, including inflammation, migration, adhesion, and proliferation. Integrin regulation by events within the cell has been termed "inside-out" signaling; this is a capacity that is unique to integrin receptors. As is typical of other cell-surface receptors, integrins can also transduce signals from outside the cell into the cytoplasm on binding extracellular ligands ("outside-in signaling"). Integrins are composed of an α and a β subunit, which form a heterodimer. The β_3-integrin family consists of $\alpha_{IIb}\beta_3$ found on platelets and megakaryocytes, and the more widely distributed $\alpha_v\beta_3$. β Subunits consist of a large extracellular domain, a single transmembrane segment, and a relatively short cytoplasmic tail. The cytoplasmic domains do not contain intrinsic tyrosine kinase activity, and therefore signaling occurs primarily via recruitment of intracellular signaling molecules. Integrins form transmembrane connections, and the interactions between integrin cytoplasmic domains, intracellular factors (cytoplasmic proteins and intracellular signaling pathways), and membrane-anchored proteins play an important role in integrin-mediated events. There are at least 21 proteins that associate with integrin β tails to regulate cell motility, proliferation, differentiation, and apoptosis. In this review, we will focus on 10 of these proteins and their function in integrin-mediated events.

Key words: **integrins • cytoplasmic domains • intracellular factors**

Abbreviations: **CHO** – Chinese hamster ovary, **FAK** – focal adhesion kinase, **ILK** – integrin-linked kinase, **NMHC-A** – non-muscle myosin heavy chain II-A, **PTB** – phosphotyrosine-binding domains, **RGD** – Arg-Gly-Asp, **SMC** – smooth muscle cells, **uPAR** – urokinase-type, plasminogen-activator receptor.

* Both of these authors made similar contributions.

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Author's address: George A. Stouffer, M.D., Division of Cardiology, University of North Carolina, Chapel Hill, NC 27599-7075, USA, tel.: +1 919 966-5141, fax: +1 919 966-1743, e-mail: rstouff@med.unc.edu

INTRODUCTION

Integrins are cell-surface adhesion receptors that play an important role in mediating numerous physiological processes, including inflammation, migration, adhesion, and proliferation. Integrins are composed of an α and a β subunit, which form a heterodimer. Eight β and 18 α subunits have been discovered to date and assort together to form 24 distinct integrins[89]. Each integrin performs specific functions depending upon the ligand-binding specificity. The system is complex in that different integrins can recognize the same ligand, whereas integrins with a common subunit exhibit different ligand-binding specificities.

The β_3-integrin family consists of $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa), found on platelets and megakaryocytes, and the more widely distributed $\alpha_v\beta_3$, found on endothelial cells, smooth muscle cells (SMC), platelets, lymphocytes, neutrophils, macrophages, and osteoclasts[10, 22]. Both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ have active and inactive conformations[5, 8, 9, 52, 80] and there are activation-dependent and activation-independent ligands. Ligand binding to $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ occurs primarily via Arg-Gly-Asp (RGD) sequences. Extracellular matrix proteins, particularly fibrinogen, vitronectin, fibronectin, von Willebrand factor, and thrombospondin, bind one or more conformations of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. Osteopontin binds $\alpha_v\beta_3$ but not $\alpha_{IIb}\beta_3$. These proteins exist in both soluble and immobilized states and are upregulated in injured blood vessels. Prothrombin binds both the activated and inactivated forms of $\alpha_{IIb}\beta_3$ [8], but only the activated form of $\alpha_v\beta_3$ [9]. α-Thrombin in soluble form binds $\alpha_v\beta_3$ on endothelial cells via an RGD site[3]. Immobilized α-thrombin binds purified $\alpha_v\beta_3$ and $\alpha_v\beta_3$ on endothelial cells[86].

β Subunits consist of a large extracellular domain, a single transmembrane segment, and a relatively short cytoplasmic tail (approximately 20-70 amino acids, except for β_4 integrin). The integrin cytoplasmic tails are the sites of interaction with cytoskeletal proteins and signaling molecules that transmit signals[25, 34]. Integrins form transmembrane connections with the intracellular contractile actin-microfilament system, linking it to the extracellular matrix[1, 7, 33]. The interactions between integrin cytoplasmic domains, intracellular factors (cytoplasmic proteins and intracellular signaling pathways), and membrane-anchored proteins have been studied intensively. In this review, we shall address the role of β_3 cytoplasmic-binding proteins and summarize recent experimental data that give us insight into integrin functioning.

INTEGRIN SIGNALING

Integrin regulation by events within the cell has been termed "inside-out" signaling; this is a capacity that is unique to integrin receptors. As is typical of other cell-surface receptors, integrins can also transduce signals from outside the cell into the cytoplasm on binding extracellular ligands ("outside-in signaling"). The relatively short cytoplasmic domains of integrins do not contain intrinsic tyrosine kinase activity, and therefore signaling occurs primarily via recruitment of intracellular signaling molecules. The importance of these protein-protein interactions was demonstrated by studies showing that in the absence of cytoplasmic tails, β_3 integrins failed to localize to focal adhesions, showed reduced activation of signaling molecules, and had reduced ligand-binding activity[50, 79]. The cytoplasmic tails of β_3 integrins have been shown to activate focal adhesion kinase (FAK) and to regulate cell cycle and actin cytoskeleton assembly[83]. There are at least 21 proteins that associate with integrin β tails[40] to regulate cell motility, proliferation, differentiation, and apoptosis. We will focus on 10 of these proteins and their function in integrin-mediated events.

The cytoplasmic domains of β_3 integrins have two NXXY motifs, which are putative binding partners for phosphotyrosine-binding (PTB) domains. Phosphorylation of the tyrosines in the β cytoplasmic domain is an important regulatory step in the binding of intracellular proteins. Of note, however, is that while it was originally thought that PTB domains would bind only phosphorylated motifs (hence the name), recently it has been shown that the PTB domains of proteins such as X11, FE65 and Numb can bind non-phosphorylated peptide motifs[55].

TALIN

Talin is a large, anti-parallel homodimer cytoskeletal protein of >2500 amino acids that contains two subunits of ~270 kDa. The amino-terminal of each subunit forms a globular head of ~50 kDa which contains an integrin-binding site. The carboxyl terminal of each subunit is rod shaped, ~220 kDa, and has actin-binding sites[13, 24]. Talin was the first cytoskeletal protein reported to bind directly to integrins[32]. Subsequent studies showed that talin binds to β subunit cytoplasmic tails and that over-expression of the talin-head-domain integrin-binding sites increased ligand-binding[13]. Conversely, reduced talin expression decreased integrin expression, focal adhesions, and cell migration[43, 61].

Talin is required for focal adhesion assembly in some systems[42]. Knocking out talin genes in undifferentiat-

ed mouse embryonic stem cells abolished the formation of focal adhesions and actin stress fibers[61]. Antisense mRNAs to talin reduced the cell adhesion and spreading of BALB/C3T3/Hela cells. Talin-deficient mice have a more severe embryonic-lethal phenotype than integrin β_3 knockout mice.

Talin plays a major role in integrin activation. Tadokoro et al.[82], utilizing small interfering RNAs to knockdown talin-1 expression in cultured Chinese hamster ovary (CHO) cells stably expressing $\alpha_{IIb}\beta_3$, provided insight into the mechanisms and function of talin binding to the β cytoplasmic tail, culminating in integrin activation. Talin knockdown did not affect the expression of integrin $\alpha_{IIb}\beta_3$ and focal adhesion proteins, but did reduce $\alpha_{IIb}\beta_3$ activation, as determined by PAC-1-binding. Integrin activation via extracellular domain-binding anti-LIBS-6 antibody remained intact. Studies with activated variants of $\alpha_{IIb}\beta_3$ and talin knockdown showed reduced energy-dependent integrin activation and no effect on energy-independent integrin activation, confirming the requirement of talin for the cellular activation of integrins[82].

Talin knockdown also inhibited conformational changes that augment integrin affinity in general (including $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins). Furthermore, talin knockdown inhibited platelet $\alpha_{IIb}\beta_3$ activation in response to agonists, and this effect was reversed only by an integrin-activating talin fragment and not by other integrin regulators (e.g. activated R-Ras, CD98 heavy chains). This indicates a pivotal role for talin as a downstream target for cellular signaling[82].

Binding of β cytoplasmic tails to talin has been localized to a PTB domain. The FERM domain of talin binds amino acids Trp^{739} to Lys^{748} of β_3 cytoplasmic tails by a variant of the classical PTB domain-NPxY interaction[14, 24]. Alanine substitutions of Trp^{739} or Leu^{746} of the integrin β tail inhibited talin binding specifically, without affecting filamin A or Syk binding[82]. In addition, point mutations in the talin PTB-like domain (R358A, W359A, and A360E) did not increase PAC-1 binding to $\alpha_{IIb}\beta_3$ integrin, indicating its inability to induce integrin activation.

INTEGRIN-LINKED KINASE

Integrin-linked kinase (ILK) is a 59 kDa serine-threonine kinase cytoplasmic protein that can interact with the cytoplasmic domain of β_1, β_2, and β_3 integrin subunits[90]. ILK contains a C-terminus kinase domain, 4 ankyrin-like repeats at its N-terminus, and a pleckstrin-homology-like domain between the ankyrin repeats and the kinase domain. The NH_2-terminal forms a complex with the LIM1 domain of PINCH, a focal adhesion protein. PINCH, in turn, binds to other adaptor proteins which are implicated in the regulation of signaling pathways and actin reorganization[13]. ILK localizes to focal adhesions and has been implicated in anchorage-dependent cell growth, cell adhesion, and fibronectin matrix assembly[30]. ILK activity is rapidly and transiently stimulated by insulin treatment, cell-fibronectin interactions, certain growth factors, and phosphatidylinositol (PI)-3 kinase[54]. ILK is inhibited by two phosphatases and PTEN (a tumor suppressor gene) which dephosphorylates PI-3,4,5 trisphosphate to PI-4,5 bisphosphate[84]. SiRNA knockdown or inhibition of ILK expression results in suppressed tumor growth and inhibition of vascular endothelial growth factor expression[39, 85]. Over-expression of ILK disrupts cell-extracellular matrix adhesion as well as cell-cell interactions, resulting in anchorage-independent cell survival and cell-cycle progression that induce tumor formation *in vivo*[62]. Over-expression of ILK also suppresses apoptosis and promotes hyperplasia[19, 56].

β_3-ENDONEXIN

β_3-Endonexin is a calcium-dependent membrane-binding protein located in the endoplasmic reticulum, initially identified using a yeast 2-hybrid system, that is expressed in various cells, including platelets and mononuclear leukocytes[78]. Three β_3-endonexin isoforms have been observed. The short isoform contains 111 amino acids and has the capacity to bind to the cytoplasmic tail of β_3 integrins. The long isoform contains 170 amino acids and does not interact with the integrin β_3 tail, but inhibits internalization of the integrin β subunit, suggesting a role in integrin recycling[21, 25]. The long isoform of β_3-endonexin interacts with cyclin A, and through this interaction, β_3-endonexin is phosphorylated and cyclin A-associated kinase activity is inhibited. This suppresses cell proliferation[49]. The 177-amino-acid isoform also binds to the ligand-binding domain of the thyroid hormone receptor α subunit and acts as a nuclear receptor co-activator[38].

The short isoform of β_3-endonexin selectively binds to the β_3 cytoplasmic C-terminal FTNITYR domains, but not to β_1 or β_2 integrins. The interaction of β_3 integrins with β_3-endonexin is independent of divalent cations or the connection of integrin α and β subunits. The N^{756} ITY^{759} motif in the β_3-integrin cytoplasmic domain is critical for this interaction[21]. Because of the NPXY turn, the FTNITYR domain is close to the β_3-integrin membrane-proximal region, which could possibly influence the conformation state of β_3 integrins. Mutation of the β_3-integrin cyto-

plasmic domains (S^{752} to P^{752}) reduces β_3-integrin interactions with β_3-endonexin by 64%[78].

Transient expression of β_3-endonexin in CHO cells increases the ligand-binding affinity of $\alpha_{IIb}\beta_3$. GFP--β_3-endonexin exists in both the cytoplasm and nucleus, but does not cluster in focal adhesions. Only weak staining was observed in focal complexes. This may imply a weaker or transient connection with β_3 integrins that allows other cytoskeleton proteins to interact with the β_3 tail[36]. Recent studies indicate that cell adhesion signals regulating the activity of cyclin A may be mediated by β_3-endonexin. The N-terminal RxL motif of β_3-endonexin is required for this interaction. Cyclin A is present in both the cytoplasm and nucleus and accumulates at sites of DNA replication during the S phase[48].

Both the 111- and 170-amino-acid isoforms of β_3--endonexin reduce urokinase-type plasminogen-activator receptor (uPAR) protein expression and inhibit its promoter activity in endothelial cells. This is mediated by integrin β_3 outside-in signaling. Binding of urokinase to uPAR increases the amount of plasmin formation, which in turn causes endothelial cell migration[6, 31].

CALPAIN

Calpain is an intracellular calcium-dependent protease. The calpains differ in the amount of calcium required for their activation[15, 17]. It has been shown that calpain cleaves the integrin β_3 cytoplasmic domain at the membrane-distal regions near the two NXXY motifs[20]. These motifs mediate signal transduction, focal adhesion formation, and integrin-cytoskeletal interactions, thereby regulating the affinity state of the receptor[51, 58, 65]. It has been reported that calpain cleaves β-integrin tails at sites between and adjacent to the conserved NPXY/NXXY motifs[57]. Cytoskeletal and regulatory proteins implicated in focal adhesions are substrates of calpains[16, 17, 46, 91]. Calpain activity is regulated by cytosolic inhibitor, calpastatin, plasma membrane-binding activator proteins, and certain phospholipids[68, 81]. It has been reported that calpain cleavage of intact talin releases the talin head domain, which has a multifold higher affinity for binding to the β_3 tail and increases the activation and clustering of integrins[92].

SKELEMIN

Skelemin is a ~200 kDa cytoskeletal M-band protein. It has repeats of two motifs, of the immunoglobulin superfamily (a 2-like motif and a fibronectin type-III--like motif)[59, 60]. A myosin-interacting protein[2], skelemin plays a role in linking integrin to the cytoskeleton. Skelemin binds the β_3 cytoplasmic domain at membrane-proximal residues 715-725[64]. Skelemin co-localized with β_3-integrin in calpain–induced integrin clusters, but was excluded from focal adhesions[63]. Cells transiently expressing skelemin with integrin-binding C_2 motifs failed to form integrin clusters or to exhibit cell spreading, indicating a role of skelemin in forming integrin clusters and cell spreading induced by signal transduction[63].

NONMUSCLE MYOSIN

There are three known isoforms of the non-muscle myosin heavy chain (NMHC) family. NMHC-A exists in platelets, lymphocytes, neutrophils, smooth muscle, and spleen. NMHC-B is highly expressed in neuronal tissue and testis[77]. The recently identified NMHC-C is found in skeletal muscle, heart, lung, brain, and abdominal organs[27]. All non-muscle myosins belong to the myosin-II family and share a high degree of homology. All isoforms are heterohexamers and composed of a pair of heavy chains and two pairs of light chains. They can be divided into head, neck, and tail domains. The head domain has actin- and ATP-binding sites. The neck domain binds light chains and calmodulins. The tail domain forms a thick filament and is required for filament assembly[77].

NMHC-A has been implicated in many cellular processes, including signal transduction, cytokinesis, granule exocytosis, cell motility, chemotaxis, and cell polarity in eukaryotic cells[88]. NMHC-A is regulated by SMC phenotype and expression is modulated during development and following injury. In humans[23] and rabbits[70], NMHC-A expression was prominent in the fetus and present at lower levels in adults. In the normal adult rabbit, 4–10% of SMC expressed detectable amounts of NMHC-A; however, expression increased following high cholesterol feedings[71] or balloon injury[70]. We subsequently showed that NMHC-A levels increased markedly following balloon injury of brachial arteries in baboons[69].

NMHC-A has been reported to bind peptides corresponding to 23 residues, i.e. 740-762, of the β_3-integrin cytoplasmic domain, and this binding was regulated by tyrosine phosphorylation in NXXY motifs of β_3-integrin[35]. In addition, NMHC-A co-immunoprecipitates with $\alpha_v\beta_3$ integrins upon thrombospondin stimulation of SMC[69]. NMHC-A has binding sites for signaling proteins, including protein kinase C, Grb2, PI3 kinase, and MAP-kinase, and its expression is regulated by cell-cycle transition[28, 37, 66, 67]. NMHC-A also associates with

FAK in thrombospondin-treated SMC[69] and binds to the tumor-suppressor proteins menin[45] and Mts-1[44].

TENSIN

Tensin is a 220 kDa cytoplasmic phosphoprotein localized to integrin-mediated focal adhesions[41]. Upon integrin ligation, tensin is rapidly translocated to focal adhesion sites[18]. Tensin, being a multi--domain protein, binds to several structural and signaling molecules. The PTB domain of tensin binds to the NPXY motifs in β-integrin cytoplasmic tails[11]. There has been emerging evidence about the role of tensin in connecting extracellular matrix, actin cytoskeleton, and signal transduction.

FOCAL ADHESION KINASE

FAK is an intracellular protein tyrosine kinase that binds numerous signaling molecules, including Shc, Grb2, and Src family kinases[75]. Integrin activation causes FAK to be recruited to focal adhesions, phosphorylate on tyrosine, and associate with signaling proteins such as GRB2/Sos[73]. FAK is part of multimeric protein complexes located at focal adhesions and binds, under various conditions, paxillin, $p130^{cas}$, Sos, dynamin, PLC-γ, and c-Src[29, 53, 74]. Interactions between these proteins and FAK are regulated by tyrosine phosphorylation, including autophosphorylation on Tyr-397 that mediates a direct interaction with v-Src by creating a high-affinity binding site for the SH2 domain on Src.

Schaller et al.[72] reported that FAK binds peptides derived from the cytoplasmic domain of β_3-integrin and speculated that this association resulted in tyrosine phosphorylation of FAK. Jenkins et al.[35], however, could not replicate this finding using non-phosphorylated, mono-phosphorylated, or di-phosphorylated peptides corresponding to residues 740-762 of the cytoplasmic domain. Furthermore, Tahiliani et al.[83] demonstrated that the NXXY motif in the carboxyl terminal segment of the β_3-integrin cytoplasmic domain was required for FAK phosphorylation, but that the putative focal adhesion kinase binding domain within the N-terminal segment of the β_3-integrin cytoplasmic domain was neither necessary nor sufficient for FAK phosphorylation.

FILAMIN

Filamin is a cytoskeletal actin-binding protein that stabilizes actin filaments. It has been demonstrated by kinetic studies that filamin has considerable binding affinity for β_3 ($\alpha_{IIb}\beta_3$) integrins[26]. Filamin also contributes as an adaptor protein for a number of signaling proteins[47]. Calderwood et al.[12] reported that filamin binding to integrin β_3 cytoplasmic tails inhibits cell migration.

INSULIN RECEPTOR SUBSTRATE-1

Vuori and Ruoslahti[87] reported that tyrosine phosphorylated insulin receptor substrate (IRS)-1 associated with $\alpha_v\beta_3$ integrins upon exposure of HIRcB cells to insulin and that insulin-treated cells expressing the $\alpha_v\beta_3$ integrin showed 2.5 times more DNA synthesis when plated on vitronectin (a ligand for $\alpha_v\beta_3$) than on other substrates. Moreover, treatment with $\alpha_v\beta_3$ integrin antagonists block insulin-induced IRS-1 phosphorylation in SMC[93] (and our unpublished data).

Another cytoplasmic protein has also been shown to associate with $\alpha_v\beta_3$ in growth factor-stimulated cells. Bartfeld et al.[4] showed that treatment of cultured fibroblasts with platelet-derived growth factor (PDGF)-BB resulted in phosphorylation of a 190 kDa protein which co-immunoprecipitated with $\alpha_v\beta_3$. The 190 kDa protein was associated with $\alpha_v\beta_3$ in quiescent cells, but only phosphorylated upon stimulation with PDGF. This protein is likely to be the PDGF receptor itself[76].

CONCLUSIONS

β_3 Integrins mediate "inside-out?" and "outside-in signaling". One of the primary mechanisms by which integrins transduce signals is via the recruitment of cytoplasmic proteins. Upon integrin activation, protein signaling complexes form which consist of cytoskeletal proteins, cytoplasmic signaling proteins, and membrane-anchored proteins. These protein complexes influence nuclear events (e.g. gene transcription) and also regulate the conformation of β_3 extracellular domains, thereby affecting ligand affinity.

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