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Review

How integrin phosphorylations regulate cell adhesion and signaling

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Cell adhesion is essential for the formation of organs, cellular migration, and interaction with target cells and the extracellular matrix. Integrins are large protein α/β-chain heterodimers and form a major family of cell adhesion molecules. Recent research has dramatically increased our knowledge of how integrin phosphorylations regulate integrin activity. Phosphorylations determine the signaling complexes formed on the cytoplasmic tails, regulating downstream signaling. α-Chain phosphorylation is necessary for inducing β-chain phosphorylation in LFA-1, and the crosstalk from one integrin to another activating or inactivating its function is in part mediated by phosphorylation of β-chains. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus receptor angiotensin-converting enzyme 2 (ACE2) and possible integrin coreceptors may crosstalk and induce a phosphorylation switch and autophagy.

Highlights

Integrins are transmembrane proteins involved in cell–cell and cell–matrix communication.

Recent studies show how integrin phosphorylations regulate integrin activity.

Phosphorylation of both integrin α- and β-chains are emerging as being critical for activity.

Phosphorylation of integrin β-chains enables kindlin binding by the assistance of talin, resulting in cell adhesion.

Crosstalk between integrins and other receptors may occur by phosphorylation switches.

Cell adhesion functions by cooperation between extracellular and intracellular molecules

A variety of adhesion molecules bind cells to each other and mediate both cell–cell and cell–matrix communication. The adhesion molecules form molecular families, which include the integrins, cadherins, immunoglobulin superfamily molecules, and selectins. While earlier reviews dealt with integrins and their interactions with cytoplasmic interactors [1–8], recent studies have increased our understanding of integrin-mediated adhesion and signaling. This includes how specific integrin phosphorylations regulate cell adhesion and signaling by enabling specific and dynamic interactions between integrins and intracellular interactors.

Herein, we first briefly review what integrins are and their extracellular and cytoplasmic interacting proteins. We then focus on recent studies that show how specific phosphorylations of integrin cytoplasmic tails regulate their activity by mediating interactions with key intracellular proteins including 14-3-3ζ, talin, and kindlins, but also abrogating interactions with inhibitory proteins such as filamin A.

Integrins are important adhesion molecules regulated in a dynamic manner

The integrins are protein heterodimers consisting of α- and β-chains, which form integrin subfamilies. They are type I membrane glycoproteins with large extracellular domains, single transmembrane domains, and relatively short intracellular tails. Integrins can be activated by inside-out or outside-in activation. In inside-out integrin activation, signals originate from non-integrin receptors, which transmit signals to the integrins. In outside-in activation, ligands bind to the external integrin domains and signal into the cells. The most detailed information on integrin inside-out activation comes from leukocytes and platelets as model cells, but platelets have also provided important information on outside-in activation [2,3,7,8].

Some integrin α-chains contain an inserted I domain acting as the ligand-binding site, or the binding site may be formed by a combination of the α- and β-chains and form an integrin 'head.' In the

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resting state, the integrin ligand-binding head faces the membrane, and the intracellular tails are clasped. Upon activation, the integrin stalk extends, the ligand-binding site opens, and the intracellular integrin tails separate [2,9]. The different integrin conformations enable adjustments in cell adhesion and signaling.

Extracellular interactions

Integrin ligands include extracellular molecules such as fibronectin, laminins, fibrinogen, and collagens. Several integrins, including $\alpha_5\beta_1$, $\alpha_V\beta_3$, and $\alpha_{IIb}\beta_3$, recognize an RGD sequence found in fibronectin, fibrinogen, and several other proteins [10], but also in some virus proteins, among them the SARS-CoV-2 spike protein [11,12]. The major lymphocyte integrins, LFA-1 ($\alpha_L\beta_2$; CD11a/CD18) and VLA-4 ($\alpha_4\beta_1$; CD49d/CD29), bind to the cellular ligands intercellular adhesion molecules 1–5 (ICAM1–5) and the vascular cell adhesion molecule 1 (VCAM-1), respectively. Integrins are inhibited by antibodies, RGD, and other ligand-derived peptides and snake venom peptides such as echistatin. Del-1 is a natural inhibitor of LFA-1 [13].

Intracellular interactions

A number of cytoplasmic components regulate the interactions of integrins with the cytoskeleton, enabling changes in both ligand-binding **avidity** (see Glossary), due to integrin clustering, or allosteric alterations, affecting integrin ligand **affinity** [6]. Integrins are also regulated by mechanotransduction. Here, bonds are formed between integrins and extracellular or cellular ligands, which generate activation by inducing interactions between the integrin tails and cytoplasmic proteins [14,15].

The integrin β-chain cytoplasmic domains are homologous, containing conserved regions (Figure 1). The β-chains are important in integrin activity regulation by interacting with cytoplasmic proteins, some of which are shared by different β-chains, such as talin, and some are unique for a particular β-chain. By contrast, the α-chain tails show low homology. They contain a common GFFKR sequence close to the membrane, however, and its deletion activates integrins, probably due to unclasping of the integrin tails. The α-chain-binding proteins include calreticulin, RapL, paxillin, and SHARPIN [16], but they do not appear to be shared by all α-chains [8]. In fact, SHARPIN also binds to integrin β-chains [17].

Several proteins bind to the integrin cytoplasmic domains; most of those identified bind to integrin β-chains and a few to α-chains. LFA-1 is shown as an example in Figure 2. The best-known cytoplasmic binding proteins are filamin A, DOK1, talin-1, 14-3-3ζ, α-actinin, and kindlin-3. Many of the LFA-1 intracellular binding proteins also bind to other integrins.

Negative regulators of integrins

The filamins are large 280 kDa proteins containing two actin-binding domains followed by 24 immunoglobulin-like repeats [18]. Filamin A is an important negative regulator of integrins, among them LFA-1 and $\alpha_{IIb}\beta_3$, and its binding to integrins has been studied in detail [19]. It is phosphorylated on Ser2152 by the Ndr2 kinase, which promotes its dissociation from LFA-1 [20]. Filamin A seems to have many different functions, and mutations in it may result in cardiovascular malformations and skeletal dysplasia [21].

Like filamin A, Dok1 is an integrin inhibitor [22], and it may be more important in neutrophil and platelet adhesion than in T cell adhesion. It was observed bound with high affinity to the tyrosine phosphorylated proximal NPLY sequence in β_3 and competed with talin for binding [23]. Dok1 bound weakly to β_2 cytoplasmic peptides, but peptides phosphorylated at S756 showed stronger binding. The result suggests that in β_2-integrins, there may occur a phosphorylation

Glossary

Affinity: the binding strength of an individual molecule.
Avidity: increased avidity means that several interacting molecules together increase binding strength.
Constitutively phosphorylated: phosphorylation that is present without the preceding activation.
Crosstalk: the ability to connect some function from one molecule to another.
G protein: a protein regulated by GTP–GDP exchange.
Immunological synapse: an interface between an antigen-presenting cell and a lymphocyte.
LAD-I: leukocyte adhesion deficiency type I is a genetic disorder due to lack of functioning β_2-integrins.
LAD-III: leukocyte adhesion deficiency type III is a genetic disorder due to lack of kindlin-3 function.
Protein kinases: enzymes that phosphorylate proteins on tyrosine, serine, or threonine.
Protein phosphatase: enzyme that hydrolyses the linkage between a protein and phosphate.

αL KVGFFKRNLKEKMEAGRGVPNGIPAEDSEQLASGQEAGDPGGCLKPLHEKDSES(1140)GGGKD
αM KLGFFKRQYKDMMS(1126)EGGPPGAEPQ
αX KVGFFKRQYKEMMEEANGQIAPENGTQTPS(1158)PPSEK
αD KLGFFKRHYKEMLEDKPEDTATFSGDDFSCVAPNVPLS
αV RMGFFKRVRPPQEEQEREQLQPHENGEGNSET
αE KCGFFKRKYQQLNLESIRKAQLKSENLLEEEN
α1 KIGFFKRPLKKKMEK
α2 KLGFFKRKYEKMTKNPDEIDETTELSS
αIIb KVGFFKRNRPPLEEDDEEGE
α3 KCGFFKRARTRALYEAKRQKAEMKSQPSETERLTDDY
α4 KAGFFKRQYKSILQEENRRDS(988)WSYINSKSNDD
α5 KLGFFKRSLPYGTAMEKAQLKPPATSDA
α6 KCGFFKRNKKDHYDATYHKAEIHAQPSDKERLTSDA
α7 KCGFFHRSSQSSSFPTNYHRACLAVQPSAMEGGPGTVGWSSNGSTPRPPCPSTMR
α8 KCFFDRARPPQEDMTDREQLTNDKTPEA
α9 KMGFFRRRYKEIIEAEKNRKENEDSWDWVQKNQ
α10 KLGFFAHKKIPEEKREEKLEQ
α11 KLGFFRSARRRREPGLDPTPKVLE

β1 KLLMIIHDRREFAKFEKEKMNAKWDTGENPIYKSAVT(788)TVVNPKYEGK
β2 KALIHLSDLREY(735)RRFEKEKLKS(745)QWNNDNPLFKS(756)AT(758)TTVMNPKFAES
β3 KLLITIHDRKEFAKFEEERARAKWDTANNPLY(747)KEATS(752)T(753)FTNITY(759)RGT
β5 KLLVTIHDRREFAKQS(759)ERS(762)RARYEMASNPLYRKPISTHTVDFTFNKFNKSYNGTVD
β6 KLLSSFHDRKEVAKFEAERSKAKWQTGTNPLYRGSTSTFKNVTYKHREKQKVDLSTDC
β7 RLSVEIYDRREYSRFEKEQQQLNWKQDSNPLYKSAIT(782)TTINPRFQEADSPTL
β8 RQVILQWNSNKIKSSSDYRVSASKKDKLILQSVCTRAVTYRREKPREIKDISKLNAHETFRCNF

ACE2 KARSGENPY(781)AS(783)IDISKGENNPGFQNTDDVQTSF

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Figure 1. The sequences of the human integrin and the angiotensin-converting enzyme 2 (ACE2) severe acute respiratory syndrome coronavirus 2 receptor cytoplasmic tails. The known phosphorylation sites are marked in red. The important NPXY/F sequences are marked in blue. The β_4 tail is longer than the tails of the other β-chains, and is not shown.

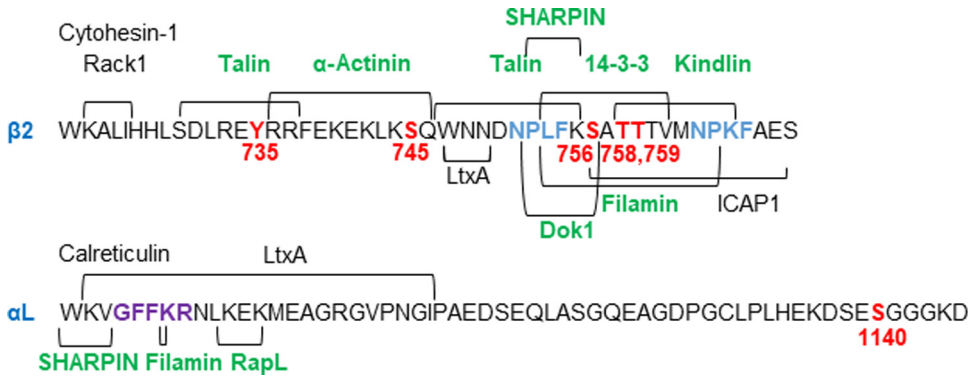

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Figure 2. Binding sites on the LFA-1 αL and β_2 cytoplasmic tails for important interacting proteins. Talin binds to two sites on the β_2 tail, one of which is the important proximal NPLF sequence. Filamin binds to a stretch covering part of the NPLF and NPKF sequences and the important TTT sequence between them. When T758 is phosphorylated, filamin is released, and 14-3-3ζ binds to the phosphorylated residue. In β_2, Dok1 binds to the phosphorylated S756, but when T758 is phosphorylated, it is released. Kindlin binds to the sequence from T758 to the end of the distal NPKF sequence. Its binding needs cooperation by talin and phosphorylation on T758. SHARPIN binds to the α-chain and probably to the proximal NPLF sequence in β-tails, as has been shown for β_1 (NPIY). The phosphorylation sites are marked in red. The most studied interacting proteins are marked in green.

switch that regulates Dok1 binding [24]. In the neutrophil $\alpha_M\beta_2$ integrin, the small **G protein** Rap1 bound to the phosphorylated S756 and may compete with Dok1 binding and release the inhibition [25].

SHARPIN is another negative regulator of some integrins, but less is known about how it regulates adhesion. This may be due to its interaction with the proximal NPXY/F sequence in integrin β-chains, where it competed with talin binding [17].

Positive regulators of integrins

The dimeric 14-3-3 proteins bind with high affinity to phosphorylated serine and threonine residues in proteins [26]. 14-3-3ζ is important in blood cell adhesion, and it binds to phosphorylated integrin β-chains [27].

Talin-1 and -2 are important components involved in integrin regulation [28]. Their 4.1-ezrin-radixin-moesin (FERM) domain binds to two sites on the β-chain cytoplasmic tails, including the proximal NPXY/F sequence (Figure 1). The talin rod binds to the cytoskeleton. Absence of talin is lethal [29].

Three kindlin molecules are present in mammals [30]. Kindlin-3 is expressed in hematopoietic cells, and lack of a functional molecule results in the leukocyte adhesion deficiency type III (**LAD-III)** syndrome, characterized by defective adhesion of blood cells [31–33]. Active kindlin-3 is a dimer [34–36], and its phosphorylation on S484 is induced by integrin-linked kinase (ILK)-stimulated **protein kinase** C α (PKCα), and it is required for activity [37].

Filamin A, 14-3-3ζ, kindlins, and talins together form an important integrin regulatory assembly, which depends on integrin phosphorylation as described later. Several other cytoplasmic proteins bind to the integrin cytoplasmic domains, but because the possible role of integrin phosphorylation on their activity is less understood, we do not focus on them. The reader is referred to reviews covering cytoplasmic proteins [5,8,38].

Site-specific integrin phosphorylations regulate integrin activity

In this review, we aim to explain how integrin activity is regulated by integrin phosphorylation. Protein kinases and phosphatases induce specific integrin phosphorylations and dephosphorylations, enabling the integrins to regulate dynamic interactions with cytoplasmic proteins to achieve changes in adhesion and signaling. A dramatic development in our understanding of integrin regulation has occurred during the past few years, and integrin phosphorylation has turned out to be of fundamental importance.

Integrins are phosphorylated at specific sites

Early work on integrin phosphorylation was initiated by the fact that PKC activation by phorbol esters induced integrin-dependent leukocyte adhesion [39]. Phosphorylation sites were first determined by labelling of cells with radioactive ^{32}P-phosphate, followed by immune precipitation with anti-integrin antibodies and Edman degradation of the isolated integrin chains [40]. Later phosphospecific antibodies have been used both against phosphorylated serine/phosphorylated threonine (pS/pT) sites and phosphotyrosine [41,42]. Antibodies are convenient to use, but their specificity must be carefully checked. Currently, mass spectrometry is the preferred technique to identify phosphorylation sites [43].

LFA-1 has been a favorite study object because of its importance in T cells. The α-chain of LFA-1 is **constitutively phosphorylated** in resting cells, but the phosphorylation is constantly turning

over. Activation results in β_2-chain phosphorylation. The integrin β_2-phosphorylation site was initially observed on S756, but the S756A mutation did not affect T cell adhesion [44]. Later work showed phosphorylation of the functionally important T758-T759 residues, but to observe it, it was necessary to inhibit **protein phosphatase** activity [40,45]. A similar finding was reported for T788-T789 in β_1-integrins [46]. The single α-chain phosphorylation sites of LFA-1, Mac-1 ($\alpha_M\beta_2$; CD11b/CD18), and $\alpha_X\beta_2$ (CD11c/CD18) were found to be essential for cell adhesion and cellular movement [47–49]. Importantly, α-chain phosphorylation enabled LFA-1 β-chain phosphorylation, including opening of the cytoplasmic clasp, allowing binding of cytoplasmic integrin regulatory proteins [41]. Structural and functional studies have now shown how molecular complexes are formed on the phosphorylated β-chain and how adhesion and signaling take place. The Mac-1, $\alpha_X\beta_2$, and $\alpha_D\beta_2$ integrins have not yet been studied in this respect.

$\alpha_{IIb}\beta_3$ is the major platelet integrin, and it is an important model for studies on outside-in integrin activation [50]. Important sites in the β-chains are the two NPXY sequences, which flank the conserved serine/threonines (Figure 1). The mutation Y747A in β_3 inhibited the uptake of fibrinogen-coated particles and cell spreading of $\alpha_{IIb}\beta_3$ transfected Chinese hamster ovary cells, and the Y759A mutation likewise inhibited fibrinogen uptake, but had less effect on cell spreading [51]. Sarcoma virus kinase (Src) phosphorylated both Y747 and Y759 in β_3 [52,53].

Inside-out signaling results in integrin phosphorylation

To get a more general understanding of integrin-dependent cell adhesion, we must compromise and combine results obtained with different cell, signaling, and adhesion models. The β_1-, β_2-, and β_3-integrins are best known, and many of the regulatory mechanisms appear similar in the different integrin families. A few protein kinases and phosphatases have been identified to be involved in integrin phosphorylation, but here much additional work is needed [27,54].

Activation of the T cell receptor (TCR) [55,56] and chemokine receptors results in β_2-integrin activation [41,57]. Figure 3 shows a simplified map of the signaling routes in inside-out activation of LFA-1. Signaling starts from the initial binding of an agonist to the TCR in the **immunological synapse**, activation of lymphocyte-specific protein tyrosine kinase (Lck), followed by activation of downstream signaling molecules.

An excellent example of integrin activation in T cells with large clinical implications is how the commonly used immunosuppressive drugs cyclosporine and FK506 inhibit the phosphorylation and the subsequent activation of LFA-1. Until recently, these drugs were only known to inhibit the dephosphorylation of nuclear factor of activated T cells (NFAT) proteins and transcription. Recent studies have shown, however, that they also inhibit T cell adhesion by inhibiting the phosphatase calcineurin [58]. Calcineurin normally dephosphorylates the inhibitory phosphorylated S59 on Lck kinase and activates it. This results in signaling to LFA-1 and β_2-chain phosphorylation [58]. Using a transgenic mouse model that expresses Lck-S59A, Otsuka *et al*. [59] found that the calcineurin inhibitors suppressed acute graft-versus-host disease via NFAT-independent inhibition of TCR signaling and T758 phosphorylation of β_2.

Like activation through the TCR, several chemokines activate leukocyte integrins. They bind to trimeric G protein receptors, which dissociate and activate phospholipase C β_2 (PLCβ_2) and PLCβ_3, which generate diacyl glycerol (DAG) and inositol trisphosphate. The chemokine induced activation also resulted in T758 phosphorylation [41]. ILK may be important for the activation of PKCα and its membrane targeting [60].

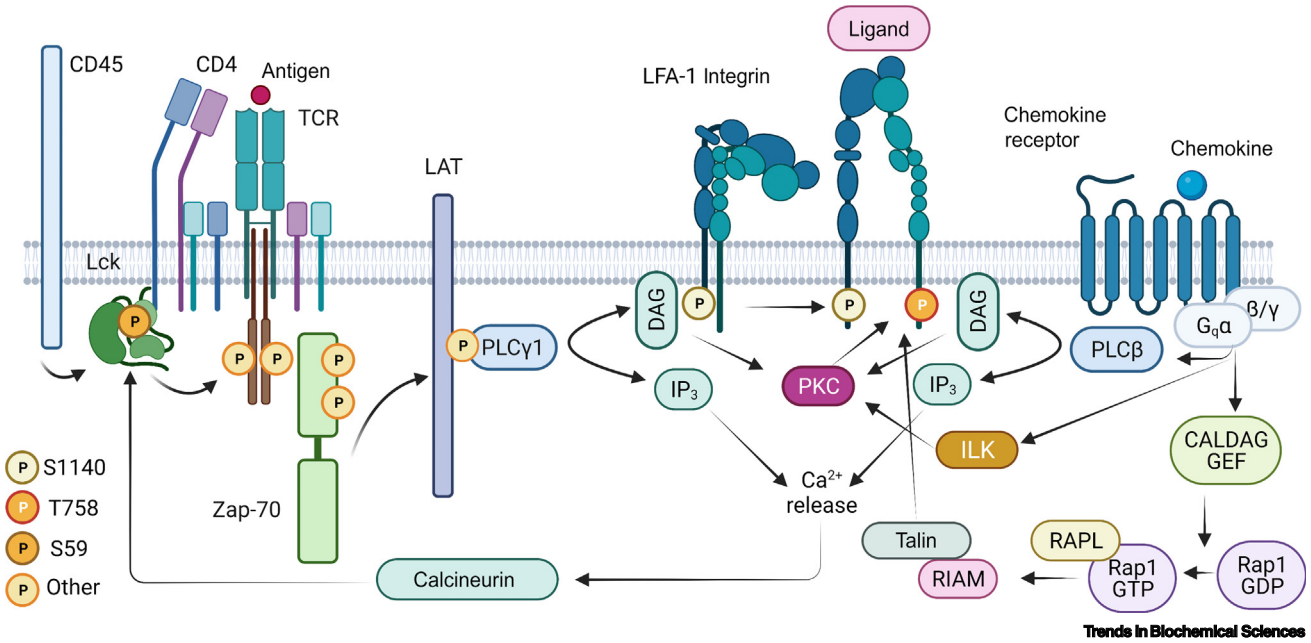

Figure 3. A simplified drawing of the inside-out signalling from the T cell receptor (TCR) and chemokine receptors to LFA-1. The tyrosine kinase Lck is activated after TCR activation by specific phosphorylations and dephosphorylation. The CD45 tyrosine phosphatase dephosphorylates the phosphorylated C-terminal tyrosine in Lck in the immunological synapse. The active Lck then phosphorylates the ZAP-70 kinase, which in turn phosphorylates the LAT adaptor protein. This activates phospholipase C γ1 (PLCγ1), resulting in the generation of diacyl glycerol (DAG) and inositol trisphosphate (IP_3). DAG activates protein kinase C (PKC) kinases, which phosphorylate the β_2 chain on T758 and less on T759. IP_3 stimulates Ca^{2+} release, which activates the calcineurin phosphatase, which removes the phosphate on S59 in previously inactive Lck molecules and further activates the kinase. Chemokine receptor activation results in activation of PLCβ and generates DAG and IP_3. This results in the activation of the small G protein Rap1. Rap1 in turn interacts with the Rap1-interacting adaptor molecule (RIAM), which binds to talin, and induces talin binding to the integrin β-chain [58]. PKC is activated downstream of DAG and integrin-linked kinase (ILK). The signalling results in integrin conformational changes, release and binding of cytosolic proteins, and integrin activation.

The interactions of cytoplasmic proteins with integrins are regulated by integrin phosphorylation

LFA-1 integrin as a model for adhesion studies

Let us now look in detail how integrin activity is regulated and use the T cell LFA-1 integrin as a model (Figure 4). The α-chain phosphorylation on S1140 is required for β-chain phosphorylation on T758 [41]. For example, when cells migrate towards a chemokine source, there must be a continuous adhesion and deadhesion to the substrate, and these events may be a consequence of α-chain phosphorylation turnover, which in turn regulates β-chain T758 phosphorylation and activates adhesion. The phosphorylation of T758 takes place by activated PKCs [27], but they must get access to their substrate in the β-chain. The Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has also been implicated in β-chain phosphorylation [61].

A separation of the clasped integrin tails upon activation was observed by electron microscopy and Förster resonance energy transfer (FRET) analysis [8]. α-Actinin binds to the cytoplasmic domain of LFA-1 with the intermediate-affinity extended conformation [62,63]. It bound well to the activated wild-type (WT) LFA-1 integrin, whereas the α-chain S1140A mutation inhibited binding, indicating steric hindrance in the clasped tail [41]. The separation of the cytoplasmic tails only occurred when both integrin chains were phosphorylated, the α-chain on S1140 and the β-chain on T758. The chain separation could be due to repulsion between the negatively charged α- and β-tails, but we cannot exclude that integrin tail binding proteins can be involved

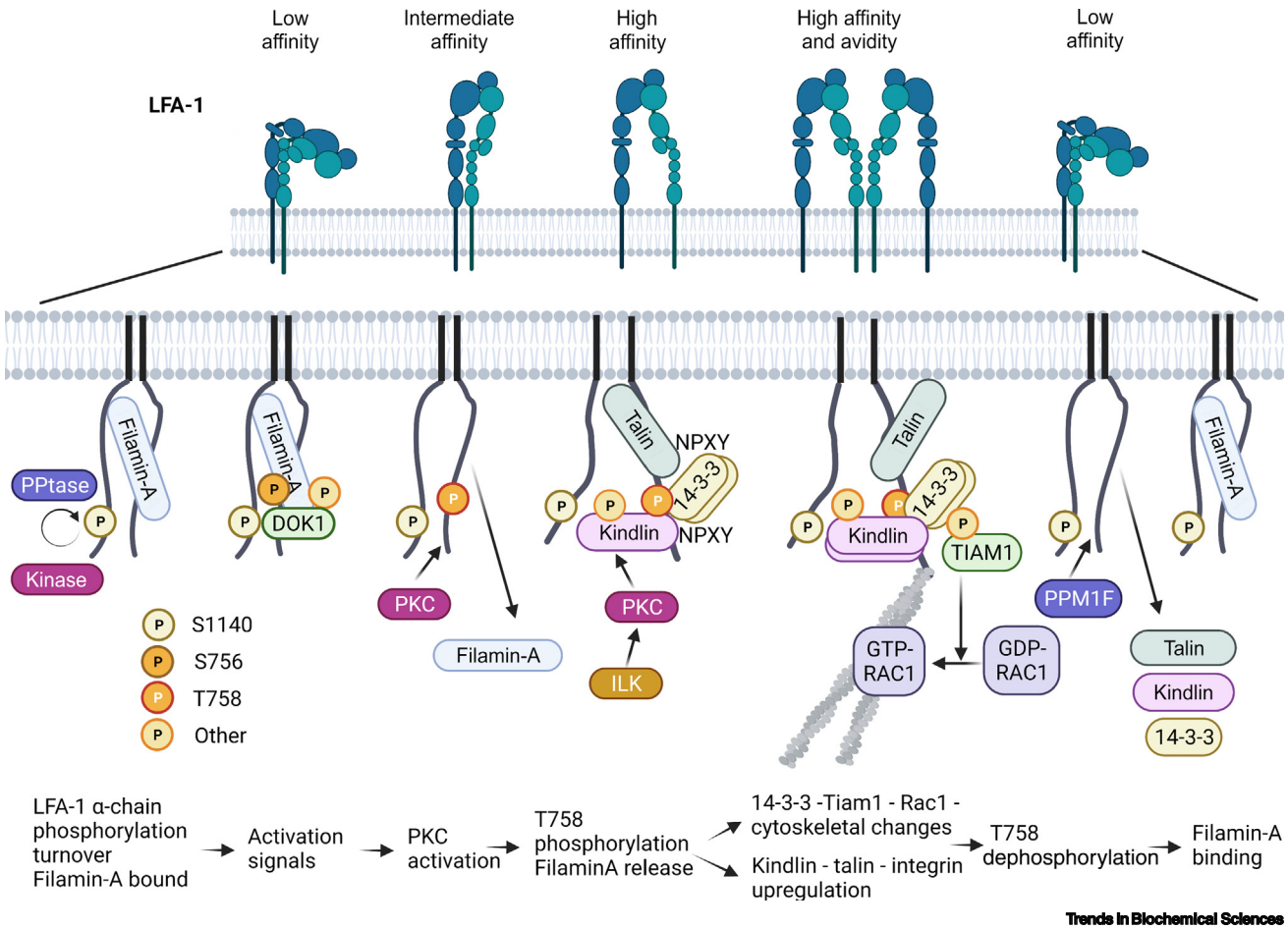

Figure 4. A detailed view of LFA-1 integrin activation. The upper part of the figure shows (from left) the inactive integrin with the head turned towards the lipid bilayer, the extended intermediate affinity integrin, the fully active integrin, the clustered high-affinity–high-avidity complex, and the inactivated integrin. The middle part shows that in the resting state, the LFA-1 α-chain is phosphorylated on S1140, but there is a turnover of the phosphate. The α-chain kinase(s) and phosphatase(s) [PPtase(s)] are not known. A filamin A molecule is bound to the β_2 tail. When the T cell is activated by phorbol esters, both S756 and T758 are phosphorylated. Dok1 can bind to the phosphorylated S756, but T758 phosphorylation outcompetes Dok1 binding because 14-3-3ζ binds to phosphorylated T758 with high affinity. Activation through the T cell receptor (TCR) targets T758 phosphorylation by protein kinase C (PKC). The S1140 phosphorylation is required for T758 phosphorylation. The phosphorylations facilitate binding of interacting proteins, and the tails move apart. Upon T758 phosphorylation, filamin A is released and replaced by 14-3-3ζ. Talin binds to two sites on β_2; one is close to the membrane, and the other is the proximal NPXY sequence. Integrin-linked kinase (ILK) is required for membrane targeting of PKC and stimulates the phosphorylation of kindlin-3, which dimerizes. The T758 phosphorylation enables kindlin-3 binding to the distal NPXY sequence with the help of talin, but a portion of the kindlin molecules could interact with the β_2-chain through 14-3-3ζ. These events upregulate LFA-1 affinity. The 14-3-3ζ/Tiam-1/Rac1 complex and α-actinin interact with the cytoskeleton and increase binding avidity by clustering the integrins on the plasma membrane. Finally, the PPM1F PPtase cleaves off the phosphate from T758 and releases the 14-3-3ζ/Tiam-1/Rac1 and talin/kindlin complexes, filamin returns to its binding site, and the integrin is inactivated. At bottom is a simplified drawing of the sequence of events. Note that the 14-3-3ζ-Tiam-1-Rac1 and kindlin–talin complexes can occur on the same β-tail, which enables both changes in integrin avidity and affinity.

in chain separation. The unclasping of the cytoplasmic domain then makes binding possible of the key components 14-3-3ζ, talin, and kindlin to the β tails.

Phosphorylation of LFA-1 β-chain promotes 14-3-3ζ binding

In resting cells, filamin A bound to the β_2-chain and inhibited cell adhesion [19]. When T758 was phosphorylated, 14-3-3ζ bound with high affinity and inhibited filamin A binding. The β_2-tail forms a loop, and whereas 14-3-3ζ readily fitted into the loop, the filamin A domain did not due to steric hindrance by pT758 [64]. The interaction with 14-3-3ζ did not affect talin binding. The dimeric

14-3-3ζ in turn bound to the Tiam1 adaptor protein, which further activated the small G protein Rac-1, which regulates the organization of the actin cytoskeleton [65].

Unclasping of LFA-1 cytoplasmic tails after β-chain phosphorylation

Talins and kindlins must cooperate to induce the integrin conformational changes, resulting in increased adhesion. In experiments with neutrophils, a portion of kindlin-3 molecules was recruited to the plasma membrane before the rolling-induced cellular arrest of leukocytes and the appearance of small clusters of highly active β_2-integrins [66]. Kindlin activity was induced before integrin-mediated adhesion was upregulated.

Kindlins bind to the TTTVMNPKF peptide sequence in β_2 (Figure 2); it covers both the important T758 site and the distal NPKF sequence. Indeed, when the T758-760/AAA mutated β_2 was expressed in mice, FRET analysis indicated a loss of kindlin-3 association with the β_2 chain [67]. The mice were healthy but showed an accumulation of leukocytes in the blood, fewer T cells in lymph nodes, decreased cell adhesion, and less spreading of T cells. The results support the concept that β_2-chain phosphorylation is important for leukocyte functions *in vivo*.

To further test if the phosphorylation of the threonine residues affects kindlin binding, various peptides were used, and the homologous integrin β_1 was used as a model [68]. In integrin β_1, residues T788-T789 correspond to T758-T759 in β_2. Kindlin-2 bound to the unphosphorylated WT β_1-peptide covering residues 762–798 *in vitro*, whereas a peptide containing pT788/pT789 showed no binding. Importantly, in the presence of the talin F3 domain, kindlin-2 bound to the integrin β_1 pT788/pT789 and required the intact distal NPKY motif for binding. The results are explained by the fact that although talin and kindlin do not directly interact with each other, talin reorients the integrin tail so that kindlin now can bind. A prerequisite for kindlin binding *in vivo* is thus that the T788/T789 residues in β_1 are phosphorylated [68]. The phosphorylated integrin outcompeted the binding of filamin A.

Further proof for the importance of T788/T789 phosphorylation for kindlin binding was obtained using GFP–kindlin-2. It strongly colocalized with the phosphomimicking T788D/T789D (threonines replaced by negative aspartic acids) β_1-integrin in intact cells [68]. The results support earlier results, which showed that T788/T789 must be phosphorylated for activation of β_1 [69]. Kindlin-2 has been shown to bind to the distal NITY sequence in β_3, and its tyrosine phosphorylation inhibited adhesion [70]. This finding strengthens the concept that an intact distal NPXY sequence is required to promote adhesion.

Very recent studies showed that kindlin-3 disrupted the association of the integrin β_2 tails and the subunit clasp and in this way contributed to integrin activation [71]. An interesting possibility is that a fraction of kindlin-3 molecules in hematopoietic cells could bind to the phosphorylated T758 residues in β_2 through 14-3-3ζ. It has been shown that 5–10% of the normal levels of kindlin-3 suffices for its basal functions in mice [72]. Therefore, the 14-3-3ζ-Tiam-1–Rac-1 pathway would not exclude binding of kindlin-3. The 14-3-3ζ proteins are dimers and could bind both phosphorylated T/S residues in integrin β-chains and phosphorylated kindlin-3. Kindlin-3 is phosphorylated at several sites, but the double-mutant T482/S484-AA in kindlin-3 inhibited adhesion of T lymphocytes, which shows that phosphorylation of kindlin-3 is functionally important [37,73].

Structural studies confirm that the β_2 phosphorylated cytoplasmic tail simultaneously can bind both the talin FERM domain and 14-3-3ζ [74]. Using single-protein analysis, it is now established that the β_1 tail, talin, and kindlin form a molecular complex and that the distances between the measured binding sites on individual integrins match the known binding sites [75]. These findings

indicate that single integrins can both cluster and change conformation by two different downstream events, originating from the threonines between the NPXY/F sequences. The talin/kindlin- and 14-3-3ζ/Tiam-1/Rac-1-induced structural changes greatly advance our understanding of how adhesion takes place.

Dephosphorylation of the β-chain allows filamin binding

The phosphorylated T788/T789 residues in β_1 are dephosphorylated by the PPM1F phosphatase, regaining filamin A binding and abrogating adhesion [68]. PPM1F-knockout cells, which express increased levels of the pT788/pT789 β_1-integrin, showed an accumulation of kindlin-2 with β_1. Overexpression of PPM1F impaired talin recruitment to the integrin tail. WT cells showed clustering of GFP-filamin A with β_1, whereas overexpression of inactive PPM1F showed no codistribution [70]. The results indicate that PPM1F activity towards T788/T789 is important for integrin/filamin A association. Currently, we do not know whether PPM1F is involved in the regulation of other integrins than β_1-integrins, but due to the high homology of β-chains, it is highly probable. We do not know how PPM1F is regulated, but it certainly plays an important role at least in the regulation of β_1-integrin activity.

Integrin serine/threonine phosphorylation has general significance

In addition to the β_1- and β_2-integrins, the possible involvement of serine/threonine phosphorylation for the activity of other integrins has been studied to some extent. For example, treatment of platelets with the phosphatase inhibitor calyculin A resulted in phosphorylation of T751 and T753 in β_3 [76]; the phosphorylation inhibited outside-in signaling. By use of a β_3 T753 phosphorylated cytoplasmic peptide, it was shown that the tyrosine phosphorylation by Src was not affected by the T753 phosphorylation. Rather, the T753 phosphorylation inhibited the binding of the adaptor protein SHC (SH2-domain-containing transforming protein C1). *In vitro* experiments showed that the PDK1 and/or Akt/PKB kinases could phosphorylate T753 [77]. Additionally, the β_5 chain in the $\alpha_V\beta_5$ integrin contains a SERS motif, and S759 and S762 in the motif were phosphorylated by the p21-activated kinase 4. Mutation of both residues to alanine abrogated cell migration [78].

Little is known about α-chain phosphorylation. The α-chain of VLA-4 was found to be phosphorylated on S988 by protein kinase A, and the phosphorylation displaced paxillin from the integrin, resulting in integrin activation [79–81].

Outside-in signaling through the platelet integrin $\alpha_{IIb}\beta_3$ involves both tyrosine and serine/threonine phosphorylations

The platelet integrin $\alpha_{IIb}\beta_3$ is an important model for outside-in integrin activation. Patients with Glanzmann thrombasthenia, who lack a functional $\alpha_{IIb}\beta_3$ integrin, develop serious bleeding due to absence or mutations in the integrin [82].

One should bear in mind that inside-out and outside-in activations are intimately connected. When integrins have been activated through inside-out activation, ligands bind and in turn may activate outside-in activation.

Src is the major tyrosine kinase in platelets, and it bound to the integrin β_3 chain C-terminal sequence RGT through its SH3-domain and phosphorylated it [83]. Integrin clustering enabled transphosphorylation of Src molecules and subsequent spleen tyrosine kinase (Syk) and focal adhesion kinase (FAK) tyrosine kinase activations. Like Lck, Src is autoinhibited by C-terminal tyrosine phosphorylation, and it is activated by tyrosine phosphatases such as protein tyrosine phosphatase IB [84]. Downstream of Src phosphorylation, Syk and FAK phosphorylate signaling molecules, among them PKCs and PLCs or α-actinin, respectively. Recent studies showed that,

upon activation with fibrinogen, a 14-3-3ζ/Src/β3 complex was formed through binding to the KEATSTF sequence of β_3 [85]. Interestingly, a myristylated KEATSTF peptide, which was taken up by cells, inhibited the β_3 outside-in signaling and platelet aggregation. The result indicates that it could be possible to develop drugs that interfere with functions associated with specific integrin phosphorylation sites.

Defects in integrin phosphorylation-mediated signaling could be less severe than total loss of integrins or adaptors

Deletion of the β_2-integrin gene results in leukocyte adhesion deficiency type I (**LAD-I)**, which is characterized by several deficiencies, including antibody production, T cell cytotoxicity, and chemotaxis [86]. To the best of our knowledge, no mutations of the human leukocyte integrin phosphorylation sites have been described in patients. Experimental results indicate that such mutations would be less severe than a total loss of integrin activity. Mutations in adaptor and signaling molecules may not wipe out all effects connected to a specific integrin, on the one hand, but could, on the other hand, affect the functions of several integrins. One example is the absence of a functional kindlin-3, which results in LAD-III [31–33]. In addition to defects in leukocyte cell adhesion-related functions, leukocyte development was impaired. Another example is deficiency in filamin function.

Phosphorylation is important in integrin crosstalk and may affect integrin/SARS-CoV-2 receptor ACE2 interaction

Crosstalk between integrins is well documented and depends on phosphorylation. For example, activated LFA-1 crosstalked with VLA-4 [87,88]. When LFA-1 was targeted with activating LFA-1 antibodies or ICAM-1, T758 in β_2 was phosphorylated, resulting in intracellular signaling, dephosphorylation of β_1, and inactivation of VLA-4 [89]. The two integrins have similar regulatory characteristics, and it is possible that the integrins simply exchange the β-chain-binding proteins, resulting in activation of one integrin and inactivation of the other. Monoclonal antibodies are used in the clinics to inhibit specific integrins, but we should now be aware of that by blocking the activity of one integrin, the activity of another integrin may change. Interestingly, T lymphocytes can migrate upstream of blood flow by binding of LFA-1 to ICAM-1, whereas they migrate downstream by VLA-4 interaction with VCAM-1 [90,91]. The ability to migrate upstream is preserved after the flow is terminated, due to integrin crosstalk between VLA-4 and LFA-1 [92].

The major receptor for the SARS-CoV-2 virus is angiotensin-converting enzyme 2 (ACE2) [93]. Its cytoplasmic domain contains short linear motifs with potential roles in endocytosis and autophagy [11]. ACE2 is present in the kidney and heart, but less in the lungs, where SARS-CoV-2 induces severe damage. This fact indicates that there may be alternative receptors for the virus in some tissues. One such receptor is neuropilin-1, which shows strong expression in the olfactory epithelium [94]. Integrins act as receptors for several viruses [95]. The SARS-CoV-2 virus spike protein contains an RGD sequence to which integrins expressed on lung epithelial cells can bind; these include $\alpha_V\beta_3$ and β_1 integrins such as $\alpha_4\beta_1$ [96–98]. β_1 and β_3 integrins could act as a coreceptor for SARS-CoV-2 [12,98], but they could also function as separate receptors in the lung and facilitate virus uptake by a phosphorylation switch [12,97]. In the immunological synapse, the binding specificity comes from the TCR–antigen interactions, but LFA-1–ICAM-1 interaction is important in strengthening the interaction [99]. In a corresponding way, integrins could strengthen the SARS-CoV-2–receptor interaction.

Previous work has shown a function for integrins and their NPXY motifs in both trafficking and autophagy [100,101]. The cytoplasmic tails of both ACE2 and integrin β_3 contain motifs that may participate in the internalization of the virus and its interactions with the autophagy

pathway (Figure 5). The β_3 cytoplasmic domain bound the autophagy-related protein 8 (ATG8) domains and required phosphorylation [14]. Phosphorylation of S752 in β_3 strengthened binding to all ATG8 domains, whereas T753 phosphorylation enhanced binding to one of the tested ATG8 domains, but not to the others. Phosphorylation of Y759 improved binding in all cases, and double phosphorylation on T753 and Y759 promoted ATG8 binding. The C-terminus of ACE2 is similar to integrin β-tails containing NPYA and NPGF sequences and serine residues between them (Figures 1 and 5). SH2 domain-containing tyrosine kinases showed binding to the ACE2 cytoplasmic domain, and pY781 disabled the interaction, while pS783 increased the affinity about twofold. The results indicate that the cytoplasmic domains of the ACE2 and integrins may regulate SARS-CoV-2 endocytosis and autophagy, but further work on the role of these proteins as links between pathogen infection and autophagy is required.

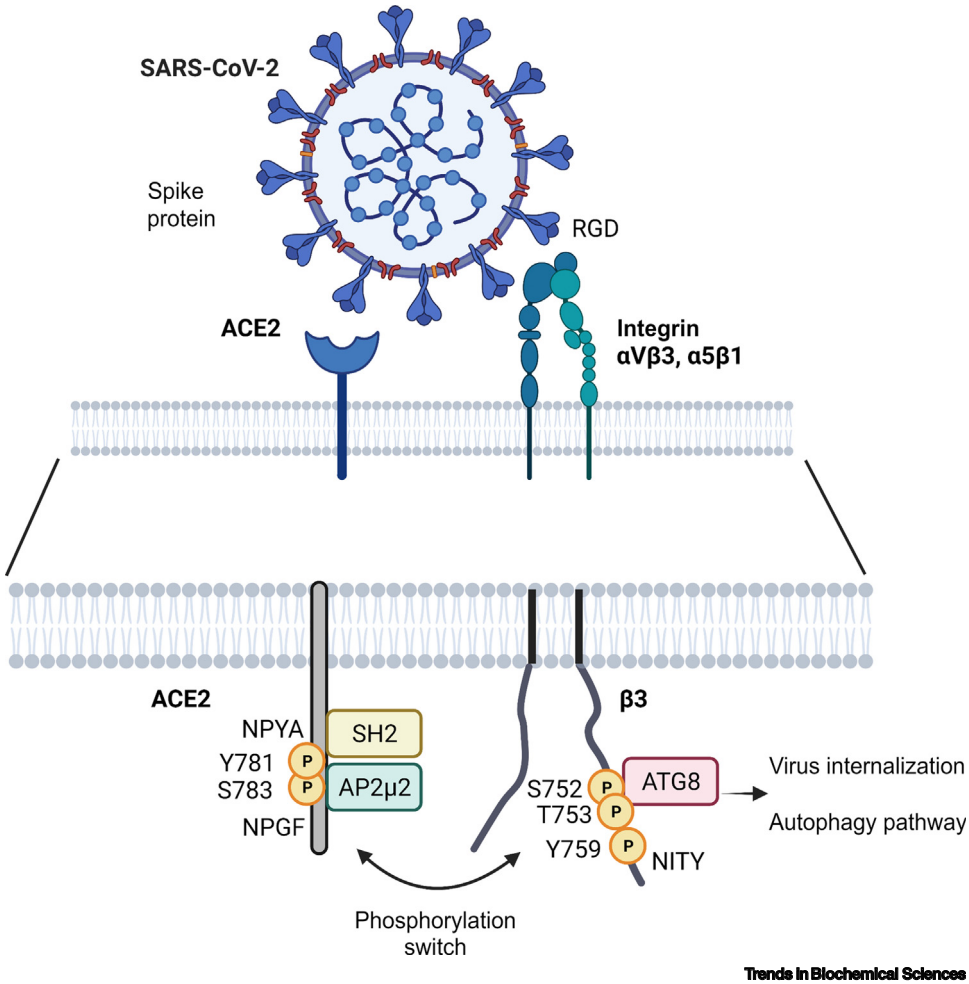

Figure 5. Binding of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to its receptors and possible crosstalk between integrins and the angiotensin-converting enzyme 2 (ACE2) receptor. The ACE2 receptor has been studied extensively and is strongly expressed in the upper airways, but not much in the lungs, where the most serious complications occur. Phosphorylated Y781 acts as a binding site for Src family kinases through their SH2-domains, and its phosphorylation inhibited the binding of the clathrin adaptor AP2μ2, whereas phosphorylation of S783 enhanced the binding [14]. It could act as a binding switch. β_1- and β_3-integrins are expressed in the lungs and can bind SARS-CoV-2 through the RGD motif in the virus spike. These integrins could act as coreceptors or as primary receptors, and they could bind autophagy-related protein 8 (ATG8) domains when phosphorylated on S752 or T753. The similarities between ACE2 and β_1- and β_3-cytoplasmic tails could induce phosphorylation switches such as those seen between LFA-1 and VLA-4 in T cells.

Concluding remarks

Cell adhesion is of pivotal importance for the development of organs, immunity against foreign microbes, metastasis from malignant tumors, restriction of excessive activity of T cells and macrophages towards own tissues, and clotting of platelets. Integrins constitute an important family of adhesion proteins, and their activity must be dynamically regulated to enable fast changes in activity. Several cytoplasmic proteins bind to the integrin cytoplasmic tails and affect their activity. Among them, 14-3-3ζ, talins, and kindlins are important activators, whereas filamin A and Dok-1 are inhibitory. Although relatively little is known about the protein kinases and phosphatases involved, they are responsible for specific integrin phosphorylations on both the α- and β-chains and regulate the interactions between the integrins and the cytoplasmic adaptors and thus adhesion. Monoclonal antibodies have been used to inhibit cell adhesion of leukocytes, but their clinical use has often been disappointing due to unwanted side effects. In the future, it should be possible to develop drugs that specifically target integrin phosphorylation sites and have more restricted effects (see Outstanding questions).

Outstanding questions

Which protein kinases and phosphatases, in addition to the few known, affect cell adhesion?

Are some integrins regulated in very different ways, or are the mechanisms for the most part similar?

What are the structures of integrin–adaptor complexes like? How are their dynamics regulated?

Will it be possible to develop drugs targeting the integrin phosphorylation sites?

Which additional cytoplasmic proteins will turn out to be important in adhesion?

Does cytoplasmic glycosylation regulate adhesion by competing with phosphorylation?

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Author contributions

Both authors wrote the text and planned the figures.

Declaration of interests

The authors have no conflict of interest.

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