Integrin receptors play essential roles in regulating cell adhesion, migration, survival, and differentiation by serving as bidirectional conduits for exchange of information between the intracellular and extracellular compartments (1). The initiation and propagation of the bidirectional signaling depend upon binding of intracellular constituents to the cytoplasmic tails (CTs) of integrin β subunits (2, 3). The integrin β CTs are relatively short (except β3), and most share several conserved regions, including two NxxY/F motifs. Among numerous β CT-binding partners, the talin head domain (talin-H) has been demonstrated to be an essential regulator of integrin activation (4–8). The activating function of talin-H depends on its direct association with the membrane-proximal NPxY motif and residues near the plasma membrane of the integrin β CTs (4, 9–12). The latter interaction leads to an unclamping of the intracellular α/β membrane-proximal complex and triggers unclamping of the transmembrane α/β association (13), which, in turn, generates conformational cues to the extracellular domain, rendering it competent to engage soluble ligands with high affinity. Recently, a solid body of evidence, ranging from analyses of model cell systems to in vivo analyses in mice and humans, has clearly demonstrated that talin alone is not sufficient to induce functionally significant integrin activation in intact cells; kindlins are required to support integrin activation and function (14–19). In contrast to the dependence of talin binding to the membrane-proximal NxxY motif, kindlin binding to the β CTs depends on the membrane-distal NxxY motif (14–16, 20).

The two NxxY motifs in the β3 CT are NPLY747 and NITY759, and they have been implicated in recognition of talin-H and kindlin, respectively (14). The tyrosine residues in these two motifs can be phosphorylated in cells, and such modifications influence integrin signaling (21). Even though tyrosine phosphorylation of the β3 CT has been clearly associated with integrin outside-in signaling (22–25), its functional regulation in inside-out signaling is less pronounced. Recent studies have demonstrated that Tyr747 phosphorylation in the β3 CT inhibited talin-H binding by both direct (decreasing the affinity of talin binding) and indirect (increasing the binding affinity of Dok1, a competitor of talin binding) effects (26, 27). These observations are compatible with the prior observation that tyrosine phosphorylation of the β3 CT by v-Src kinase abolished integrin β3-mediated cell adhesion (28). Here, we show that kindlin-2 binding to the β3 CT is abrogated by Tyr759 phosphorylation, and our functional analyses further establish that this phosphorylation event may be an important regulator of integrin activation.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction, Protein Preparation, and Peptide Synthesis**—For mammalian expression, the cDNAs of human αIIb and β3 subunits were inserted into pcDNA3.1. All regulators of integrin activation were cloned into either pEFGP or pDsRed-monomer vectors. GST-fused integrin β CTs and GST-kindlin-2 were expressed in Escherichia coli Rosetta™ 2(IDE3) cells (Novagen) and purified by glutathione chromatography. The purified proteins were quantified using Bio-Rad protein assays. All synthetic peptides were prepared, purified, and authenticated by tandem mass spectrometry in the Molecular Biotechnology Core of the Cleveland Clinic.

**Pulldown Assays and Western Blotting**—Pulldown assays were performed using GST fusion proteins. Equal amounts of GST-fused integrin β CT were added together with glutathione-Sepharose 4B (GE Healthcare) to aliquots of the cell lysates. In peptide inhibition experiments, the indicated pep-
tide was also added to the slurries at the selected concentrations. After overnight incubation at 4 °C, the precipitates were washed and boiled in Laemmli sample buffer. The eluates were analyzed on gradient acrylamide gels under reducing conditions, and interactions of the integrin αIIbβ3 with kindlin-2 were determined by Western blotting. In parallel, the gels were also stained with Coomassie Blue to verify that sample loadings were similar.

**Surface Plasmon Resonance (SPR)**—Real-time protein-protein interactions were analyzed using a Biacore 3000 (Biacore, Uppsala, Sweden). N-terminally biotinylated peptides were bound to SA5 sensor chips (Biacore) according to the manufacturer’s instructions. Experiments were performed at room temperature in 10 mM HEPES buffer (pH 7.4) at a flow rate of 25 μl/min. Analyte binding to the immobilized ligand was recorded by measuring the variation of the SPR angle, and the results are expressed in resonance units (RU).

**Integrin αIIbβ3 Activation Assays**—Integrin αIIbβ3 activation was evaluated by a PAC1 binding assay as described previously(14, 29). Mutant forms of integrin αIIbβ3 together with the regulators, tagged with either DsRed monomer or EGFP, were expressed in CHO-K1 cells by transient transfection using Lipofectamine 2000 (Invitrogen). PAC1 binding to the different transfectants (EGFP and DsRed double-positive cells) was analyzed by flow cytometry after incubating the transfected cells with anti-PAC1 mAb for 25 min at room temperature, followed by Alexa Fluor® 633-conjugated secondary antibody for 25 min on ice. Variations in integrin expression levels on the transfected cells were normalized based on reactivity with a mAb (2G12) reactive with αIIbβ3 independent of its activation status. Integrin activation was expressed in terms of relative median fluorescence intensities by defining the basal PAC1 binding to WT αIIbβ3 cells positive for EGFP and DsRed as 1.0.

**RESULTS AND DISCUSSION**

The NPLY747 and NITY759 motifs (Fig. 1A) in the integrin β3 CT are directly involved in recognition of talin-H and kindlin, respectively (14). This specificity of kindlin-2 binding was verified in assays in which the WT αIIbβ3 CT and various point mutant forms expressed as GST fusion proteins were added to lysates of endothelial cells, and their ability to pull down endogenous kindlin-2 was detected by Western blotting. In these assays, interaction of endogenous kindlin-2 with WT αIIbβ3 CT, and this binding was abolished by substitution of Ala for...
Phosphorylation Regulates Kindlin-2 Binding to Integrin

The strategy of using a charged residue, either Asp or Glu, to mimic a phosphorylation state has been used extensively. However, such substitutions do not entirely recapitulate the structure of a phosphotyrosine. Therefore, as a more direct approach, small β3 CT peptides containing the membrane-distal NITY759 motif with or without phospho-Tyr759 (Fig. 2A) were tested as inhibitors of kindlin-2 binding to the β3 CT (14). Pulldown assays similar to those described for Fig. 1B were performed using GST-fused β3 CT to precipitate endogenous kindlin-2 from human umbilical vein endothelial cell lysates, and the bound kindlin-2 was measured by Western blotting. As shown in Fig. 2B, when added to cell lysates, the WT β3 peptide significantly inhibited kindlin-2 binding. By densitometry, the inhibition was 72%. In contrast, when the Tyr residue was phosphorylated, the peptide produced no apparent inhibition. These peptide data strongly support a negative regulatory role of Tyr759 phosphorylation in kindlin-2 binding to the β3 CT.

To exclude the possibility that such an effect could be confounded by indirect interactions that might occur in cell lysates, we further analyzed the interactions using purified kindlin-2 protein in SPR experiments. The β3 CT was immobilized on the biosensor chips, and kindlin-2 was used as the soluble analyte in the presence or absence of the two peptides. As shown in Fig. 2C, kindlin-2 bound to the immobilized β3 CT, yielding a typical progress curve with a rapid association and a prolonged dissociation phase. Inclusion of the WT peptide prevented the interaction. However, the phosphorylated β3 peptide did not show a significant inhibitory effect under the same conditions, suggesting a direct involvement of Tyr759 phosphorylation in negatively regulating kindlin-2 binding to the β3 CT.

As an independent approach to verify the negative regulation of Tyr759 phosphorylation in kindlin-2 binding, we directly immobilized the β3 CT C-terminal peptide conjugated with biotin at its N terminus onto streptavidin SA5 biosensor chips for SPR analysis (Fig. 3A). As shown in Fig. 3B, kindlin-2 bound to immobilized WT peptide, and a Kd of 1.8 × 10−7 M was calculated. This value was similar to that obtained with immobilized full-length β3 CT (Kd = 1.36 × 10−7 M), demonstrating that the β3 CT C terminus is primarily responsible for kindlin-2 recognition. As a control, talin-H did not bind to the WT β3 C-terminal peptide-coated chips under the same conditions (data not shown), indicating binding specificity for kindlin-2. When the biotinylated phosphopeptide was coated onto the chips, negligible binding of kindlin-2 was detected (Fig. 3C). The failure of the phosphopeptide to bind kindlin-2 was not due to poor coating of the biosensor chip because the WT pep-

**FIGURE 2.** Phosphorylation of the NxxY759 motif in the β3 CT peptides disables its ability to block interaction of kindlin-2 and the β3 CT. A, the amino acid sequences of the β3 CT C-terminal peptide containing the NITY759 motif and a modified peptide with Tyr759 phosphorylation (β3C-pep-phos) are shown. B, kindlin-2 and β3 CT interaction was evaluated by pulldown assays in the presence of the indicated peptides. IB, immunoblot. C, kindlin-2 or a mixture of kindlin-2 and the indicated peptide at a 1:100 molar ratio was passed over an SA5 biosensor chip coated with biotinylated β3 CT peptides, and the binding curves were recorded over time. To block nonspecific binding of the peptide to the chips, 0.1% BSA was included in the running buffer. The failure of the phosphopeptide to bind kindlin-2 was not due to poor coating of the biosensor chip because the WT peptide data strongly supported a negative regulatory role of Tyr759 phosphorylation in kindlin-2 binding to the β3 CT.

To determine how modification of the NITY759 motif regulates kindlin function, we performed experiments in widely used CHO cells with stable expression of integrin αIIbβ3. The lack of effect of the Tyr-to-Phe substitution is consistent with the ability of kindlins to control activation of the β2 integrins (17, 18, 30), which have NxxF motifs rather than NxxY motifs.

**A**

β3C-pep-wt: YKEATSTFNITYRGTT

β3C-pep-phos: YKEATSTFNITYRGTT

**B**

IB: kindlin-2

GST-protein

GST

GST-β3CT

**C**

![Binding Curves](Image)
buffer (0.1% BSA) at a flow rate of 25 µl/min. The binding signals were recorded, and the results are expressed in RU. The RU values of the chips prior to introduction of kindlin-2 were similar (Fig. 3A, B). These results demonstrate that Tyr759 is a key residue in supporting kindlin-2 binding to the β3 CT with very precise structural requirements, suggesting that alteration of the local structural environment by Tyr759 phosphorylation would be unfavorable for kindlin-2 recognition.

In summary, our study demonstrates that tyrosine phosphorylation of the NITY759 motif in the β3 CT provides a regulatory mechanism for controlling the role of kindlin-2 in integrin function. Based on the conservation of the membrane-distal NxxY motif in the integrin β subunits, the regulatory role of tyrosine phosphorylation might apply to other integrin family members. In fact, we did observe that a β1 CT peptide homologous to the β3 CT peptide used in Fig. 2 did inhibit β3 CT and kindlin-2 interaction (Fig. 4), and as expected, direct phosphorylation of Tyr759 or a phosphomimetic mutation (Y795D) but not a structure conservative Y795F mutation in β1 CT membrane-distal NPKY795 motif ablated the inhibitory activities of these β1 CT peptides (Fig. 4). In platelets and endothelial cells, it is well established that the tyrosine residues of NxxY motifs in the β subunits are subject to phosphorylation, and when modified, integrin β subunit functions are altered (22, 23, 31, 32). Because talin-H binding to the β subunits can also be negatively regulated by tyrosine phosphorylation of the membrane-proximal NaxX motif (27), the two NxxY motifs in the β CTs might act as a dual switch in controlling interactions with cytoplasmic regulators via phosphorylation and dephosphorylation. It would appear that phosphorylation of Tyr759 or a phosphomimetic mutation (Y795D) might allow for rebinding and reactivation of the integrin. Cycles of phosphorylation/dephosphorylation of the integrin β CT may be part of a complex network that encompasses many regulated events, including phosphorylation of integrin regulators them-
selves, talin, and kindlin (33–35), and the susceptibility of the \( \beta_3 \) CT to calpain proteolysis (36), which would delete the kindlin-binding site altogether. Thus, post-translation modifications may be part of a complex network of events that occur spatially and temporally within the cell to control adhesive and migratory responses. It would appear that, once activated, talin and kindlin-2 binding to the \( \beta_3 \) CT may not be required to sustain activation. Although we anticipate that phosphorylation of Tyr\(^{759}\) would also influence kindlin-3 binding to the \( \beta_3 \) CT, its very low expression in the same bacterial system used for kindlin-2 expression has precluded direct experimental testing of this prediction and remains an open question for future studies.

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