Src Kinase Activity Is Required for Integrin αvβ3-Mediated Activation of Nuclear Factor-κB*

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Integrin adhesion to extracellular matrix proteins protects adhesion-dependent cells from suspension-induced apoptosis. Previous studies indicate that activation of the transcription factor nuclear factor-κB was necessary for the integrin αβ3 ligand osteopontin to protect endothelial cells from apoptosis caused by serum withdrawal. In this study, β3 integrins were overexpressed in smooth muscle cells. When plated on osteopontin, cells overexpressing wild-type β3 had enhanced cell adhesion, cell spreading, and nuclear factor-κB activation compared with vector control. Removal of four amino acids (759X) from the C terminus of β3 eliminated the ability of the integrin to promote these processes. Single amino acid substitutions indicated that phosphorylation at tyrosine 759 was not required for activation of the transcription factor, however this residue appeared to play a structural role, because mutation to alanine significantly inhibited nuclear factor-κB activation. The Src family of tyrosine kinases represents important transducers during integrin signaling, and the C terminus of β3 has been implicated as the binding site for Src. Immunoprecipitations demonstrated that Src associated with wild-type β3 integrins, but Src and integrins lacking the C terminus (759X) did not form a complex. Pharmacological inhibition with the Src inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-((t-butyl)pyrazolo[3,4-d]pyrimidine) or overexpression of kinase-dead c-Src blocked nuclear factor-κB activation. Mouse embryonic fibroblasts deficient for Src failed to activate nuclear factor-κB when plated on osteopontin, in contrast to control fibroblasts. Together, these experiments indicate that the C terminus of β3 and Src activity are required for integrin αvβ3-mediated nuclear factor-κB activation.

Integrins are heterodimeric receptors comprised of two type I transmembrane glycoproteins, α and β. Integrins typically contain a large extracellular domain, a transmembrane domain, and a short intracellular domain with no known enzymatic function. The integrin extracellular domain binds to extracellular matrix ligands or cell surface ligands, whereas the intracellular domain provides a link to adapter proteins and the cytoskeleton. Thus, integrins act as adhesion receptors, physically linking the extracellular matrix to the cytoskeleton. Inte-

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¶ The abbreviations used are: SFK, Src family of tyrosine kinase; SYF, cells deficient for c-Src, Yes, and Fyn; NF-κB, nuclear factor-κB; IκB, inhibitor of κB; OPN, osteopontin; WT, wild-type; RAEC, rat aortic endothelial cell; WKY, Wistar Kyoto rat smooth muscle cell; PBS, phosphate-buffered saline; FDL, poly-γ-lysine; KD, kinase-dead; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine.
Src Kinase Is Required for αβγ-Mediated NF-κB Activation

In the present study we provide evidence that the integrin αβγ/NF-κB pathway is found in multiple cell types, perform structure/function studies to establish the region of the integrin αβγ required for NF-κB activation, determine that SFKs bind to integrin βγ and the association requires the C terminus of the integrin, and identify that SFK kinase activity is required for integrin αβγ-mediated NF-κB activation.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Rabbit polyclonal anti-integrin βγ (Chemicon, Temecula, CA) and the mouse monoclonal antibodies anti-v-Src clone 327 (Oncogene Research Products, San Diego, CA), anti-Syk N-19 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-human integrin βγ clone SZ.21 (Beckman Coulter, Brea, CA), anti-human integrin βγ clone 1 and anti-rat integrin βγ clone F11 (BD Pharmingen) were commercially available. Anti-osteopontin (OPN) antibody OP199 has been previously described (9).

**Plasmid Constructs**—The retroviral vector PBMN-IRE5-eGFP and Phoenix packaging cell line were a gift from Gary Nolan (Stanford University, Palo Alto, CA) (10). The retroviral NF-κB reporter PMB-SIN-eB-luciferase was a gift from Elaine Raines (University of Washington, Seattle, WA) (11). The human integrin βγ cDNA construct was graciously donated by Mark Ginsberg (Scripps Research Institute, San Diego, CA) (12). Wild-type (WT) βγ was subcloned into the PBMN-IRE5-eGFP retroviral vector by cutting with XhoI and Xhel. Transfections 759X and 761X and amino acid substitutions Y759A and Y759F were produced through the Stratagene QuikChange site-directed mutagenesis kit according to manufacturer’s suggested protocol using the oligonucleotides listed. Modified plasmids were confirmed by DNA sequencing. 759X forward: 5′-GGCCGTGATATTGGTGAAGGTAG-3′; 761X forward: 5′-GGATG-CTACCTTCACCAATATCACGTTTCGGGGCACTTAATGG-3′; Y759F forward: 5′-CCATATGCAGCGAGGCCGTGATATTGG-3′; Y759A forward: 5′-CCATATGCCCCTGAGGCCGTGATATTGG-3′.

Chicken c-Src constructs (WT, K295R, Y527F, and K295R/Y527F retroviral plasmids) were a gift from Jonathan Cooper (Fred Hutchinson Cancer Research Center, Seattle, WA) (13). The mammary expression construct EMCV-Syk was a gift from Stanford Shattil (Schiller Research Institute) (14). Human βγ was subcloned into the PBMN-IRE5-eGFP retroviral vector by cutting with BglII and XhoI. The PBMN-IRE5-eGFP vector was also treated with calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA) to prevent self-ligation. Kinase-dead Syk (K402R) was generated with the Stra- gene QuikChange site-directed mutagenesis kit using the oligonucleotides listed. Mutated plasmids were confirmed by DNA sequencing. K402R forward: 5′-GGCCGTGATATTGGTGAAGGTAG-3′; 761X reverse: 5′-CCATATGCAGCGAGGCCGTGATATTGG-3′; Y759F forward: 5′-CCATATGCCCCTGAGGCCGTGATATTGG-3′; Y759A reverse: 5′-CCATATGCGGCCGTGATATTGG-3′.

**Cell Culture**—RAECs were purchased from VEC Technologies Inc. and grown in MCDB 131 media (Invitrogen) supplemented with 10 mM l-glutamine (Invitrogen), 10% fotal bovine serum (HyClone, Logan, UT) and 100 units/ml each of penicillin and streptomycin (Invitrogen). Mad- dle smooth muscle cells from Wistar Kyoto rat pups were isolated as previously described (15) and grown in Waymouth’s MB 752/2 media (Invitrogen) supplemented with 10% fetal bovine serum (HyClone) and 100 units/ml each of penicillin and streptomycin (Invitrogen). Cells were passaged by immunofluorescence microscopy.

**Retroviral Infections**—High titer amphotrophic retrovirus was prepared as previously described (16). Briefly, Phoenix packaging cells were transiently transfected with the retroviral plasmids. Medium was removed and 24 h post-transfection. At 24-h post-transfection the cells were moved to a 32 ºC incubator and virus was collected between 24 and 48 h after transfection. The virus-containing media was passed through a 0.45-μm filter to remove cellular debris.

**Western Blots**—Proteins were extracted from cell monolayers in Laemmli buffer containing protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 1 μM phenylmethylsulfonyl fluoride). After lysis and boiling, protein concentration was determined by MicroBCA assay. 30 μg of lysate was loaded onto 10% SDS-polyacrylamide gels. Identical gels were stained with Pro-Blue staining solution (Owl Separation Systems, Portsmouth, NH) to verify uniform loading. Samples were transferred to polyvinylidene difluoride membranes. Membranes were probed with the appropriate primary antibody, washed, and then horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). Bands were detected using the Western Lightning chemiluminescence kit (PerkinElmer Life Science).

**Immunoprecipitations**—WKY cells expressing c-Src and vector control or integrin βγ subunits, WT or 759X, were grown to confluence and lysed in lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, 1 mM sodium orthovanadate, 0.5 mM NaF and protease inhibitors. 800 μg of lysate was immunoprecipitated with rabbit polyclonal anti-integrin βγ antibody or rabbit IgG control antibody and Protein A-Sepharose beads (Sigma-Alrdrich). The immunoprecipitates or total cell lysate were subjected to Western blotting with the indicated detection antibodies.
Src Kinase Is Required for \( \alpha_{v}\beta_3 \)-Mediated NF-\( \kappa \)B Activation

TABLE I

| Name        | Cytoplasmic sequence |
|-------------|----------------------|
| Wild-type  \( \beta_3 \) | KLLITIHDRK EFAKFEERAAKMDTAANP LYKEATSTFT NITTYGOT |
| 761X        | KLLITIHDRK EFAKFEERAAKMDTAANP LYKEATSTFT NITTYR     |
| Y759F       | KLLITIHDRK EFAKFEERAAKMDTAANP LYKEATSTFT NIT2YR     |
| Y759A       | KLLITIHDRK EFAKFEERAAKMDTAANP LYKEATSTFT NIT7AROT   |
| 761X        | KLLITIHDRK EFAKFEERAAKMDTAANP LYKEATSTFT NIT2      |

FIG. 1. OPN-induced activation of NF-\( \kappa \)B in smooth muscle cells is integrin \( \alpha_{v}\beta_3 \)-mediated. Rat smooth cells expressing an NF-\( \kappa \)B-dependent luciferase reporter were pre-treated with soluble antibodies or vehicle and then plated on OPN- or PDL-coated surfaces in serum-free medium. After 7 h, cell lysates were harvested and luciferase activity was measured and normalized to total protein recovered. *, \( p < 0.01; \# \), \( p < 0.05 \).

FIG. 2. Surface expression of integrin \( \alpha_{v}\beta_3 \) in WKY cells overexpressing truncated \( \beta_3 \). Flow cytometry of WKY smooth muscle cells infected with vector control or integrin \( \beta_3 \) constructs: 759X, Y759A, Y759F, 761X, and WT. Histograms of integrin \( \beta_3 \) surface expression are shown. Percentage of cells positive for integrin \( \beta_3 \) surface expression are indicated.

Cells were incubated with an anti-human \( \beta_3 \) antibody to detect surface expression of the integrin. All \( \beta_3 \) constructs were efficiently expressed, with at least 80% of cells positive for surface expression of \( \beta_3 \). The level of surface expression was similar for each of the \( \beta_3 \) constructs, as shown in Fig. 2. Due to difficulties acquiring antibodies that recognize rat integrins, \( \beta_3 \) constructs were also expressed in human smooth muscle cells and surface expression of other integrins was detected by flow cytometry. Overexpression of \( \beta_3 \) did not significantly affect \( \alpha_{v}\beta_0 \), \( \alpha_{v}\beta_6 \), or \( \beta_3 \) integrin expression levels (data not shown). Assuming the same trend occurs in rat smooth muscle cells, then observed phenotypes in cells infected with the \( \beta_3 \) constructs are due to overexpression of \( \alpha_{v}\beta_3 \) integrins and not changes in the expression profile of other integrins.

To test functionality of the \( \beta_3 \) constructs, smooth muscle cells were seeded onto OPN-coated surfaces and allowed to attach for 2 h. Unattached cells were washed away, and phase contrast microscopy images of attached cells were acquired. All cell types attached to the OPN-coated surface (Fig. 3A), but substantial differences in post-receptor occupancy interactions were observed between the cell types. Overexpression of wild-type (WT) \( \beta_3 \) dramatically increased cell spreading compared with vector control. Truncation of four amino acids from the C terminus (759X) eliminated the ability of the integrin to promote cell spreading, whereas removal of two amino acids did not impair cell spreading (761X). Substitution of tyrosine 759...
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**Fig. 3.** Overexpression of \(\beta_3\) integrin enhances cell adhesion, cell spreading, and activation of NF-\(\kappa\)B.  

A, phase contrast microcopy images of rat smooth muscle cells overexpressing \(\beta_3\) integrins, 2 h after seeding onto OPN-coated surfaces (25 \(\mu\)g/ml). Scale bar, 50 \(\mu\)m. B, rat smooth muscle cells overexpressing \(\beta_3\) integrins (wild-type or mutated) were plated on OPN-coated surfaces. After 2 h non-adherent cells were removed, adherent cells were stained with toluidine blue, and absorbance was measured at 630 nm. C, rat smooth muscle cells overexpressing \(\beta_3\) integrins were plated on OPN-coated surfaces (1 \(\mu\)g/ml). Cell lysates were harvested, luciferase activity was measured, and activity was normalized to total protein recovery. *, \(p < 0.01\).

with phenylalanine (Y759F) did not reduce cell spreading, but mutation to alanine (Y759A) resulted in an intermediate level of cell spreading.

To quantify these post-receptor occupancy differences, adhesion assays were performed. Compared with vector control, overexpression of WT \(\beta_3\) significantly enhanced cellular adhesion by 3-fold or greater at all OPN concentrations tested (Fig. 3B). 759X significantly reduced adhesion compared with WT \(\beta_3\) (\(p < 0.001\)), whereas 761X did not impair the ability of the integrin to promote adhesion to OPN. Substitution of tyrosine 759 with phenylalanine (Y759F) did not reduce cell adhesion, indicating that phosphorylation of this residue was not required. Alanine substitution at this residue (Y759A) allowed for adhesion, but it was impaired compared with WT \(\beta_3\) especially at the lowest OPN concentration tested. Thus the adhesion assay results agreed with the differences in cell spreading seen in Fig. 3A.

To test the ability of the integrins to mediate NF-\(\kappa\)B activation, smooth muscle cells expressing the NF-\(\kappa\)B-dependent luciferase reporter and the integrin \(\beta_3\) constructs were plated on surfaces coated with the \(\beta_3\) ligand OPN. After 7 h, adherent cells were lysed, and luciferase activity was measured and normalized to protein recovered. For ease of comparison, vector control samples were then normalized to a value of one. Compared with vector control cells, overexpression of full-length \(\beta_3\) or 761X significantly increased NF-\(\kappa\)B activation (\(p < 0.01\)), whereas 759X was unable to increase NF-\(\kappa\)B activation when plated on OPN (Fig. 3C). Integrins with the Y759F mutation retained the ability to activate NF-\(\kappa\)B, but WKY cells overexpressing Y759A integrins have significantly reduced activation of NF-\(\kappa\)B compared with WT \(\beta_3\) (\(p < 0.01\)). These results indicated that tyrosine 759 of integrin \(\beta_3\) had a critical structural role in OPN-mediated NF-\(\kappa\)B activation, because truncation at position 759 or substitution to alanine significantly inhibited activation of the transcription factor, but tyrosine phosphorylation was not required, since the Y759F mutation retained the ability to signal.

**Pharmacological Inhibition of SFKs Reduced Integrin \(\alpha_v\beta_3\)-Mediated NF-\(\kappa\)B Activation**—Having identified that tyrosine 759 within the C terminus of \(\beta_3\) was required for NF-\(\kappa\)B activation, we performed a preliminary experiment to identify proximal downstream mediators of NF-\(\kappa\)B activation. The Src family of tyrosine kinases was recently reported to bind directly to \(\beta_3\) integrins through the C terminus, and its members are known proximal mediators of integrin signals (20). To test the role of SFKs in integrin-mediated NF-\(\kappa\)B activation, RAECs were pretreated with the Src family tyrosine kinase inhibitor PP2, the inactive analog PP3, or vehicle control and plated on OPN. Treatment with 10 \(\mu\)M PP2 significantly inhibited integrin-mediated NF-\(\kappa\)B activation (\(p < 0.01\)), whereas 10 \(\mu\)M PP3 or vehicle did not, as seen in Fig. 4. Similar results were seen when WKY smooth muscle cells were also treated with PP2 and PP3 (data not shown). Although PP2 inhibition is not complete at this dosage and higher doses of PP2 fail to yield further inhibition, 10 \(\mu\)M PP2 does lead to greater than 75% reduction in integrin-mediated activation of the transcription factor. These results indicate that NF-\(\kappa\)B activation required SFK activity in this model.

**Overexpression of Kinase-dead c-Src Blocked Integrin \(\alpha_v\beta_3\)-Mediated NF-\(\kappa\)B Activation**—To further characterize the role of SFKs, RAECs cells were infected with retroviral vectors encoding WT chicken c-Src (WT), kinase-dead c-Src (K295R), activated c-Src (Y527F), or the double mutant (K295R/Y527F) (13). As seen in Fig. 5A, lane 5, Western blots of total cell lysates indicated that the RAECs expressed endogenous SFKs (the antibody was unable to distinguish Src family members). Cells
was verified by Pro-Blue staining of identical gels. Expression was detected with v-Src clone 327. Equal protein loading was shown. These cells were plated on PDL- or OPN-coated surfaces. NF-κB activation was determined based on luciferase activity and normalized to total protein recovered.

* p < 0.01.

**Fig. 6. Fibroblasts deficient for SFKs are unable to promote integrin αβ3-mediated NF-κB activation.** A, mouse embryonic fibroblasts Sre8T and SYF cells expressing the NF-κB-dependent luciferase reporter were plated on surfaces coated with PDL, collagen I or OPN. Luciferase activity was normalized to total protein recovered. B, SYF cells overexpressing Src constructs or Sre8T cells were plated on PDL- or OPN-coated surfaces. NF-κB activity was determined based on luciferase activity and normalized to total protein recovered. *, p < 0.01.
rescue activation of the transcription factor, while expression of activated c-Src (Y527F) increased transcription factor activity regardless of the surface to which the cells were exposed. These results indicate that SFK kinase activity was necessary and sufficient for integrin αβ3-mediated NF-κB activation in fibroblasts.

**SFKs Associated with Wild-type β3 but Not 759X**—A recent study in Chinese hamster ovary cells determined that SFKs interact directly with the cytoplasmic tails of integrin β3 subunits and the Y759F mutation in the β3 subunit does not inhibit the interaction (20). Thus, we hypothesized that the 759X mutation inhibited SFK association with the β3 subunit, preventing SFK-mediated signal transduction following integrin binding to OPN. To test this hypothesis, integrin β3 subunits from WKY cells expressing c-Src and β3 integrin subunits, WT or 759X, were immunoprecipitated as described under “Experimental Procedures.” The lower panel of Fig. 7 demonstrates that human integrin β3 was recovered following immunoprecipitation with anti-β3 antibodies from cells expressing WT β3 or 759X. Endogenous rat integrin β3 was also immunoprecipitated but was not detected by the antibody used in the Western blot (data not shown). As predicted, SFKs associated with endogenous integrin β3 as seen in lane 2 of Fig. 7 (upper panel). Overexpression of WT β3 increased the intensity of the SFK band compared with vector control cells (lane 4 versus lane 2), whereas overexpression of 759X failed to enhance the SFK band (lane 6 versus lane 2). These results verified that removal of four amino acids from the tail of the β3 integrin subunit prevented SFK association.

**Syk Kinase Activity Was Not Required for Integrin αβ3-Mediated NF-κB Activation**—The non-receptor tyrosine kinase Syk also binds to the C terminus of the β3 subunit (21). To test the potential role of Syk in integrin αβ3-mediated NF-κB activation, RAECs and WKY smooth muscle cells were infected with retroviral vectors encoding WT Syk or kinase-dead Syk (K402R). Overexpression of Syk protein was verified by Western blot as seen in Fig. 8A; however, neither construct was able to modulate NF-κB activation in RAECs (Fig. 8B). Similar results were observed in WKY cells (data not shown). Therefore we conclude that Syk kinase was not involved in the integrin αβ3/NF-κB signaling pathway.

**DISCUSSION**

RAEC binding to OPN-coated surfaces leads to integrin αβ3 ligation and activation of the transcription factor NF-κB (2). In this study, we demonstrated that integrin αβ3-mediated NF-κB activation occurred in multiple cell types and required the C terminus of the integrin β3 subunit and SFK kinase activity. This was based on the findings that 1) smooth muscle cells activated NF-κB when plated on the α3β3 ligand OPN, 2) overexpression of the β3 subunit increased αβ3-mediated NF-κB activation, 3) mutations to the C terminus (759X or Y759A) of the β3 subunit eliminated the ability of the integrin to activate NF-κB, 4) SFKs associated with WT β3 but not 759X, and 5) pharmacological inhibition, overexpression of kinase-dead c-Src, or genetic ablation of SFKs inhibited activation of NF-κB.

In this study, adhesion of smooth muscles cells or fibroblasts to the α3β3 ligand OPN activated the transcription factor NF-κB, whereas adhesion to surfaces coated with the β3 ligand collagen or PDL did not. Inhibition of integrin αβ3 with a soluble neutralizing antibody for β3 prevented NF-κB activation in smooth muscle cells. These results, combined with the previous finding that endothelial cells activate NF-κB when plated on OPN, indicated that the OPN/integrin αβ3/NF-κB pathway exists in multiple cell types. Fibronectin, a ligand for αβ3 integrins, has also been reported to activate NF-κB in fibroblasts and smooth muscle cells (22). We found that overexpression of αβ3 in smooth muscle cells led to enhanced NF-κB activation on fibronectin (data not shown). Although these data did not rule out the potential role of αβ3 in fibronectin-mediated NF-κB activation, it indicated that the degree of NF-κB activation correlated with αβ3 expression and suggested that integrin αβ3-mediated NF-κB activation was not limited to just the ligand OPN.

We believe that β3 integrin-mediated NF-κB activation may be a common survival mechanism, particularly when cells adopt an invasive phenotype. Integrin αβ3 and the transcription factor NF-κB have already been linked to increased invasiveness and survival in melanoma cells. OPN binding to the integrin αβ3 activates NF-κB in melanoma cells (23, 24). Blockade of integrin αβ3 suppresses melanoma growth by inducing apoptosis of the tumor cells (25). Inhibition of NF-κB in melanoma cells also inhibits tumor growth (26). Combining these observations, a general picture emerges where ligation of integrin αβ3, a promiscuous integrin capable of binding multiple ECM components, leads to activation of NF-κB, which is necessary for cell survival during migration and invasion.

In the second part of this project, we overexpressed mutated β3 integrins to identify regions of the cytoplasmic tail that were required for NF-κB activation. The cytoplasmic tail of the β3 integrin is short (47 amino acids) and has no known enzymatic function. We found that removal of four or more amino acids from the C terminus (759X) of the β3 integrin subunit eliminated its ability to promote cell adhesion, cell spreading, and NF-κB activation in response to the β3 ligand OPN. Tyrosine
phosphorylation of residue 759 on integrin β3 was not required, because cells overexpressing β3 integrins with the Y759F mutation were able to adhere to OPN and activate NF-κB. The adhesion results were consistent with previous studies. In Chinese hamster ovary cells, overexpression of αIIbβ3 enhanced cell adhesion and spreading on fibrinogen, which required the cytoplasmic tail of β3 but not αIIb (27). 759X significantly reduced cell spreading and adhesion plaque formation, although complete loss of function required removal of six or more amino acids (757X) (12). The Y759A mutation to the β3 integrin subunit also inhibited cell spreading and adhesion plaque formation in this model. Previous studies investigating the αβ3 integrin observed similar findings. Overexpression of β3 in Chinese hamster ovary cells led to increased αβ3 surface expression, enhanced cell spreading, and elevated integrin-mediated signal transduction as demonstrated by FAK phosphorylation (28). The Y759A mutation significantly impaired these processes, whereas Y759F did not. These results agreed with our finding that β3 integrins required structural integrity of the NITY motif, rather than tyrosine phosphorylation of residue 759. Finally, overexpression of isolated integrin β3 cytoplasmic tails inhibited cell spreading in a dominant negative fashion, but an alternatively spliced form of β3 that lacked the NITY domain did not (29). These results are all consistent with the role of the NITY domain in cell adhesion and outside-in signaling.

In the final part of this project, we studied the role of SFKs during integrin αβ3-mediated NF-κB activation. SFKs associated with the integrin αβ3 and were activated following integrin ligation in multiple cell types, including osteoclasts (30), melanoma cells (31), and platelets (32). Likewise, we found that SFKs associated with WT β3 integrin subunits in smooth muscle cells. Truncation of the C terminus of the β3 integrin (759X) eliminated this interaction. These results agreed with the findings that SFKs associated directly with β3 integrins, but not β3 or β2 integrins, and removal of the cytoplasmic tail of β3 or competition with a C-terminal β3 peptide (748–762) eliminated the association (20). This group also observed that tyrosine phosphorylation of residue 759 in integrin β3 was not required for the interaction with SFKs. The need for SFK kinase activity in NF-κB activation was then tested by pharmacological inhibition of SFKs with PP2, overexpression of kinase-dead c-Src, or genetic ablation of SFKs. All three methods inhibited integrin αβ3-mediated NF-κB activation. Likewise, mutations that prevented SFK association with integrin β3 such as 759X, also eliminated integrin αβ3-mediated NF-κB activation, whereas mutations that did not affect SFK binding to β3 such as Y759F retained the ability to activate the transcription factor. Finally, overexpression of constitutively active c-Src activated NF-κB independently of the substrate, suggesting that SFKs were downstream of the integrin αβ3. These results indicated that SFK kinase activity was necessary and sufficient for integrin αβ3-mediated activation of NF-κB.

SFKs associated with αβ3 integrins through an interaction with the C terminus of the β3 subunit. The C terminus, including the NITY motif, is unique to the β3 integrin subunit, which may explain the specificity of the interaction of c-Src with β3 tails but not other integrin tails (20). This finding may also explain the specificity of β3 integrins for NF-κB activation. Overall, these results suggest a model in which integrin αβ3 ligation activates SFKs, leading to SFK kinase activity, which is essential for NF-κB activation. To support this model, ligation of β3 integrins with OPN has been shown to activate c-Src in melanoma cells (31) and breast cancer cells (32). The exact mechanism of SFK-mediated NF-κB activation is unknown at this time, although regulators of NF-κB, including IκBα, IκB kinases, and NF-κB-inducing kinase, have all been implicated as substrates for SFKs (34, 35). Future experiments will attempt to identify the mechanism of SFKs in integrin αβ3-mediated NF-κB activation.