Intrinsic Signaling Functions of the \( \beta_4 \) Integrin Intracellular Domain*

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A key issue regarding the role of \( \alpha 6 \beta 4 \) in cancer biology is the mechanism by which this integrin exerts its profound effects on intracellular signaling, including growth factor-mediated signaling. One approach is to evaluate the intrinsic signaling capacity of the unique \( \beta 4 \) intracellular domain in the absence of contributions from the \( \alpha 6 \) subunit and tetraspans and to assess the ability of growth factor receptor signaling to cooperate with this domain. Here, we generated a chimeric receptor composed of the TrkB extracellular domain and the \( \beta 4 \) transmembrane and intracellular domains. Expression of this chimeric receptor in \( \beta 4 \)-null cancer cells enabled us to assess the signaling potential of the \( \beta 4 \) intracellular domain alone or in response to dimerization using brain-derived neurotrophic factor, the ligand for TrkB. Dimerization of the \( \beta 4 \) intracellular domain results in the binding and activation of the tyrosine phosphatase SHP-2 and the activation of Src, events that also occur upon ligation of intact \( \alpha 6 \beta 4 \). In contrast to \( \alpha 6 \beta 1 \) signaling, however, dimerization of the chimeric receptor does not activate either Akt or Erk1/2. Growth factor stimulation induces tyrosine phosphorylation of the chimeric receptor but does not enhance its binding to SHP-2. The chimeric receptor is unable to amplify growth factor-mediated activation of Akt and Erk1/2, and growth factor-stimulated migration. Collectively, these data indicate that the \( \beta 4 \) intracellular domain has some intrinsic signaling potential, but it cannot mimic the full signaling capacity of \( \alpha 6 \beta 4 \). These data also question the putative role of the \( \beta 4 \) intracellular domain as an “adaptor” for growth factor receptor signaling.

The \( \alpha 6 \beta 4 \) integrin is a structural and functional anomaly among the integrin family of receptors. This integrin, which is expressed primarily on the basal surface of epithelia and in a few other cell types, is defined as an adhesion receptor for most of the known laminins (1–3). The distinguishing structural feature of \( \alpha 6 \beta 4 \) is the atypical intracellular domain of the \( \beta 4 \) subunit. Two pairs of fibronectin type III repeats separated by a connecting segment characterize this domain, and it is distinct both in size (\(~1000\) amino acids) and structure from any other integrin subunit (4). Although the \( \alpha 6 \beta 4 \) integrin provides a well-characterized adhesive function in normal epithelial cells by anchoring the epithelium to its underlying basement membrane, the carcinoma-associated functions of this integrin are becoming increasingly recognized (5). Importantly, the expression of this integrin is often maintained as epithelial structures dissociate during the initiation and progression of carcinomas, and, consequently, many carcinomas express \( \alpha 6 \beta 4 \) (6, 7). Numerous studies by our groups and others have revealed that \( \alpha 6 \beta 4 \) can facilitate the ability of carcinoma cells to migrate, invade, and resist apoptotic stimuli (8–16). More recently, \( \alpha 6 \beta 4 \) has been implicated in the genesis of squamous and breast carcinomas (17–19). The ability of \( \alpha 6 \beta 4 \) to impact these diverse functions results largely from its effects on multiple signaling pathways, including phosphatidylinositol 3-kinase/Akt and MAPK, a process that may result from its association with specific growth factor receptors, tetraspans, and possibly other molecules (5). The dichotomy of \( \alpha 6 \beta 4 \) function is summarized best by the hypothesis that \( \alpha 6 \beta 4 \) switches from a mechanical adhesive device into a signaling competent receptor during the progression from normal epithelium to invasive carcinoma (5, 20).

A key issue regarding the role of \( \alpha 6 \beta 4 \) in cancer biology is the mechanism by which this integrin exerts its profound effects on intracellular signaling. Given that \( \alpha 6 \beta 1 \) and \( \alpha 6 \beta 4 \) exhibit substantial differences in their known signaling functions, it is reasonable to postulate that the unique signaling properties of \( \alpha 6 \beta 4 \) derive largely from the \( \beta 4 \) intracellular domain (21). Despite its large size, however, the \( \beta 4 \) intracellular domain lacks intrinsic kinase activity. A current hypothesis argues that the \( \beta 4 \) intracellular domain functions as a “signaling adaptor” that facilitates signaling through growth factor receptors such as Met (22). Another viable, though not mutually exclusive, hypothesis is that the association of \( \alpha 6 \beta 4 \) with tetraspans and its localization in tetraspan-enriched membrane microdomains enhances its signaling capacity (23). These hypotheses are complicated by the finding that \( \alpha 6 \beta 4 \) signaling can be either dependent on engagement of its ligands (laminins) or independent of such ligation (22, 24).

One approach to understanding the nature of \( \alpha 6 \beta 4 \) signaling in more detail is to evaluate the intrinsic signaling capacity of the unique \( \beta 4 \) intracellular domain itself in the absence of con-
tributions from the α6 subunit and tetraspanins, and to assess the ability of growth factor receptor signaling to cooperate with this intracellular domain. To execute this approach, we generated a chimeric receptor composed of the TrkB extracellular domain and the β4 transmembrane and intracellular domains. Expression of this chimeric receptor in β4-null cancer cells enabled us to assess the signaling potential of the β4 intracellular domain either alone or in response to dimerization of the chimeric receptor using brain-derived neurotrophic factor (BDNF), the ligand for TrkB (25). The data obtained indicate that the β4 intracellular domain has some intrinsic signaling potential but that it cannot mimic the full signaling capacity of the intact α6β4 integrin. These data also highlight the need to re-evaluate the putative role of the β4 intracellular domain as an “adaptor” for growth factor receptor signaling.

**EXPERIMENTAL PROCEDURES**

**Cells**—The MDA-MB-435 cancer cell line, which has been reported to be derived from the melanoma cell line M14 (26), was obtained from the Lombardi Breast Cancer Depository at Georgetown University (Washington, D.C.) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 1 g/liter glucose, 5% fetal bovine serum, 1% penicillin-streptomycin, and 10 mM HEPES. MDA-MB-435 cells stably expressing the α6β4 integrin were described previously (21).

MDA-MB-435 cells stably expressing full-length TrkB, TrkBextra, and TrkBβ4 were generated as follows. For generation of a full-length TrkB retroviral expression construct, a 2.5-kb EcoRI/Sall fragment was removed from the rat TrkB expression region of the final 6 amino acids of the TrkB extracellular domain and the 4 intracellular domain has some intrinsic signaling potential. To execute this approach, we generated a chimeric receptor using brain-derived neurotrophic factor (BDNF), the ligand for TrkB (25). The data obtained indicate that the β4 intracellular domain has some intrinsic signaling potential but that it cannot mimic the full signaling capacity of the intact α6β4 integrin. These data also highlight the need to re-evaluate the putative role of the β4 intracellular domain as an “adaptor” for growth factor receptor signaling.

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To generate stable cell lines expressing full-length TrkB, TrkBextra, and TrkBβ4, the above retroviral expression constructs were transfected along with expression plasmids for the vesicular stomatitis virus glycoprotein envelope glycoprotein and Gag-Pol packaging proteins into 293T cells using Lipofectamine 2000 (Invitrogen). Three days post-transfection, viral supernatants were harvested, diluted in serum-containing media supplemented with 8 μg/ml Polybrene (Sigma), and used to infect MDA-MB-435 cells. Following 24 h of infection, cells were selected with Geneticin (2.5 mg/ml) to yield stable cell lines, and cells were then maintained in 1.0 mg/ml Geneticin. Immunoblotting was performed, as described below, to confirm protein expression in the stable cell lines.

For the generation of cell lines with stable knockdown of the SHP-2 protein, an shRNA cloned into the pSUPER retroviral expression construct was obtained from Ben Neel (Ontario Cancer Institute, Toronto), and viral production and infection were performed as described above. Puromycin (0.5 μg/ml) was used for cell selection.

To create the β4 shRNA-pFSIPPW expression plasmid, the following oligonucleotides (Invitrogen) were annealed and ligated into pFSIPPW (a gift of Andrew Kung, Harvard Medical School, Boston, MA) between the EcoRI and BamHI restriction sites: 5′-aatcccgAgctgGACGGGATGTGTTCTgaagagaGACACACTCGTGAGCTCGAGTGTGTTCTctctttcgagaGACACACTCGTGAGCTCGAGTGTGTTCTTGgg.

To generate stable HCC1937 cell lines expressing β4 shRNA-pFSIPPW, the above construct was transfecated along with the Viapower lentiviral packaging mix (Invitrogen) into 293T cells using Lipofectamine 2000 following the manufacturer’s instructions. Viral supernatants were harvested 3 days post-transfection, diluted in serum-containing medium supplemented with 8 μg/ml Polybrene, and used to infect HCC1937 cells. Stable cell lines were generated by selection with puromycin (1 μg/ml). Cells were maintained in 0.5 μg/ml puromycin. Suppression of β4 expression was confirmed by immunoblotting as described below.

A875 melanoma cells were a generous gift from Alonzo Ross (University of Massachusetts Medical School, Worcester, MA) and were maintained in DMEM containing 1g/liter glucose, 10% fetal bovine serum, 1% penicillin-streptomycin, and 10 mM HEPES.

**Reverse Transcription-PCR**—Total RNA was isolated from cells using an RNase mini kit (Qiagen). Gene-specific mRNA was then reverse transcribed into cDNA and amplified from 1 μg of total RNA using a OneStep reverse transcription-PCR kit (Qiagen). PCR amplification of cDNA was performed for 30 cycles using the following primers: p75NTR forward (5′-CGAGAACTCATCTCCCTGTCGT), p75NTR reverse (5′-ACTGCA-CAGACTCTCCACAG), glyceraldehyde-3-phosphate dehydrogenase forward (5′-ATCACATCTTCCAGGAGA), and glyceraldehyde-3-phosphate dehydrogenase reverse (5′-GCTCCACCACCTTCTTGAGTGT).
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Antibodies and Reagents—The following Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): polyclonal TrkB Aβ (H-181), SHP-2 mAb (B-1), phospho-tyrosine mAb (PY99), Src mAb (B-12), and actin Ab (C-11). Abs specific for phospho-SHP-2 (Tyr-542), phospho-Akt (Ser-473), Akt, phospho-p44/p42 Erk kinase (Thr-202/Tyr-204), and p44/p42 Erk kinase were obtained from Cell Signaling Technology (Beverly, MA). The Src (pTyr-418) and (pTyr-529) Abs were purchased from Invitrogen/BIOSOURCE (Carlsbad, CA). The 505 Ab against a peptide comprising the final 20 amino acids of the β4 C terminus (14) and α6 mAb (2B7) (28) were produced by our laboratory. An mAb against the extracellular domain of β4 (UMA9) was purchased from Ancell (Bayport, MN). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse Abs (Pierce) and a horseradish peroxidase-conjugated anti-goat Ab (Jackson ImmunoResearch, West Grove, PA) were used as secondary Abs. The streptavidin agarose-conjugate, calpeptin, calpastatin peptide, and ALLN were purchased from EMD Biosciences (San Diego, CA), and hepatocyte growth factor (HGF) and BDNF were purchased from Peprotech (Rocky Hill, NJ).

Biochemical Analyses—Biotinylation of cell surface proteins was performed using EZ-Link Sulfo-NHS-LC-Biotin (Pierce) following the manufacturer’s instructions with a few modifications, as follows. Cells were washed and suspended in PBS (pH 8.0) at a concentration of 25 × 10⁶ cells/ml and labeled with 0.5 mg/ml biotin for 30 min at room temperature. Following the labeling, cells were washed with PBS containing 100 mM glycine to quench and remove excess biotin and then extracted in a Triton X-100 buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) supplemented with 1 mM sodium fluoride, 1 mM sodium orthovanadate, and complete-Mini protease inhibitor mixture (Roche Applied Science).

For co-immunoprecipitation studies, cells were extracted with either the Triton X-100 buffer or an Nonidet P-40 buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and complete-Mini protease inhibitor mixture). Cell extracts were clarified by centrifugation at 16,000 × g for 10 min and pre-absorbed for 2 h using protein G-Sepharose beads. After centrifugation at 5,000 × g for 5 min to pellet the beads, extracts were incubated with primary Abs for 1 h, and immune complexes were then precipitated with protein G-Sepharose overnight. For precipitation of biotinylated proteins, pre-absorbed extracts were incubated with a streptavidin agarose-conjugate overnight. Precipitates were washed twice with lysis buffer, one time with PBS, and then eluted in 1 × reducing SDS sample buffer while boiling for 5 min.

For immunoblotting, cell extracts were prepared as described above, and protein concentrations determined using the Bradford assay (Bio-Rad). These extracts, or eluted immune complexes from the immunoprecipitations, were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were then blocked in TBS-T (Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk for at least 30 min, except for immunobLOTS for phosphorylated proteins, in which case TBS-T containing 5% BSA was used. Following blocking, membranes were incubated overnight with primary Ab in blocking buffer, washed 3 × with TBS-T, incubated with horseradish peroxidase-conjugated secondary Ab in blocking buffer, washed 3 × with TBS-T, and detected using SuperSignal West Pico Chemiluminescent substrate (Pierce).

For assays where BDNF was used to dimerize the TrkBβ4 chimera, cells were washed once with PBS and serum-deprived for 24 h in DMEM containing 1 g/liter glucose, 1% penicillin-streptomycin, 10 mM HEPES, and 0.1% BSA. Following serum starvation, cells were incubated with fresh serum-free medium supplemented with 100 ng/ml BDNF for the indicated time periods at 37 °C. Treatment of cells with HGF was performed in a similar manner, using HGF at a final concentration of 100 ng/ml.

Integrin Clustering—Cells were removed from their dishes with trypsin and washed twice with RPMI medium containing 1% BSA (RH/BSA). After washing, the cells were resuspended in the same buffer at a concentration of 10⁶ cells/ml and incubated for 30 min with either α6- or β4-integrin-specific Abs (2 μg/ml) or in buffer alone. The cells were washed once, resuspended in RH/BSA, and added to plates that had been coated overnight at 4 °C with anti-mouse IgG (100 μg/10-cm plate). The plates were blocked with RH/BSA for 30 min prior to the addition of the cells. Inhibitors were added to the cells for 15 min prior to plating the cells in the antibody-coated plates. After incubation at 37 °C for 15–60 min, the cells were washed twice with PBS and solubilized at 4 °C for 10 min in the Nonidet P-40 lysis buffer. Nuclei were removed by centrifugation at 12,000 × g for 8 min.

Migration Assays—Migration assays were performed using Corning (Corning, NY) Transwell chambers (8.0-μm pore size). Membranes were prepared by coating the upper and lower surfaces with 15 μg/ml collagen (Cohesion, Palo Alto, CA) overnight at 4 °C, and then blocking with DMEM containing 0.25% heat-inactivated BSA for 1 h at 37 °C. Cells were then trypsinized, counted, and resuspended in DMEM containing 0.25% heat-inactivated BSA. A total of 1 × 10⁶ cells was added to the upper chamber of the Transwell, and HGF (50 μg/ml) was added to the bottom wells as a chemoattractant. Migration was allowed to proceed for 2.5 h at 37 °C at which time non-migrating cells were removed mechanically from the upper chamber using a cotton swab. Cells that migrated to the lower surface of the Transwell membrane were fixed in methanol for 10 min at room temperature, and membranes were mounted on glass slides using Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Migration was quantified by counting the number of stained nuclei in five fields of view in each Transwell, in triplicate.

RESULTS

Generation and Characterization of a TrkBβ4 Chimeric Receptor—To assess the intrinsic signaling capacity of the β4 integrin intracellular domain, we generated an expression construct consisting of the extracellular domain of the neuronal TrkB receptor fused to the transmembrane and intracellular domains of β4 (Fig. 1A). We used this system as a model of β4 lateral clustering following adhesion of α6β4 to its extracellular matrix ligand by inducing dimerization of these chimeric mol-
The neurotrophin, BDNF, not only binds to the TrkB receptor but also binds to the non-selective p75 neurotrophic receptor (p75NTR) with a lower affinity (34). Expression of p75NTR has been observed in several non-neuronal cell types and cancers (35, 36), including melanoma cells (37). Given that the MDA-MB-435 cell line may possess properties of melanoma cells (26), we examined whether these cells express p75NTR, to exclude the possibility of signaling through p75NTR following BDNF treatment. Expression of p75NTR mRNA was examined by reverse transcription-PCR. Although the melanoma cell line, A875, expresses a significant amount of mRNA for p75NTR, the MDA-MB-435 TrkBβ4 cell line did not express mRNA for this receptor (Fig. 1E).

Dimerization of the β4 Integrin Intracellular Domain Induces SHP-2 Binding and Activation—A recent report demonstrated that signaling through the Met receptor can promote the association of the tyrosine phosphatase SHP-2 with the β4 intracellular domain (29). A key issue that has not been addressed, however, is whether clustering of the β4 intracellular domain by itself is sufficient to induce SHP-2 binding in the absence of growth factor stimulation. The TrkBβ4 chimera provided an ideal model system for testing this issue. SHP-2 was immu-
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A.

BDFN stimulation resulted in a significant increase in the amount of TrkB4 that co-immunoprecipitated with SHP-2. To verify this finding, we performed the reverse immunoprecipitation to determine whether SHP-2 co-immunoprecipitated with TrkB4. Indeed, SHP-2 co-immunoprecipitated with TrkB4, but the relative amount did not increase significantly upon BDNF stimulation. We attribute this result to the fact that the expression of TrkB4 is much higher than that of SHP-2 and that only a relatively small fraction of TrkB4 engages SHP-2. Together, the above studies demonstrate that the β4 intracellular domain, in the absence of any cooperative signaling from growth factor receptors, is able to bind SHP-2 and that this interaction is inhibited by binding of integrin to its ligand.

Subsequently, we assessed whether SHP-2 itself is activated following BDNF treatment of cells expressing TrkB4. Phosphorylation of SHP-2 on Tyr-542 activates the molecule by releasing it from its basal conformation that inhibits its phosphatase activity (38, 39). BDNF stimulation of cells expressing the TrkB4 chimaera resulted in an increase in SHP-2 (Tyr-542) phosphorylation that peaked at 15 min but remained higher than that observed for unstimulated cells for 60 min (Fig. 2B). No increase in SHP-2 phosphorylation was observed in cells expressing TrkBextra (Fig. 2B) or vector alone (data not shown) indicating that the observed effects could be attributed to the β4 intracellular domain and not to nonspecific effects of BDNF treatment. Interestingly, basal levels of phosphorylated SHP-2 (0-min BDFN) were slightly higher in the cells expressing TrkB4 as compared with cells expressing TrkBextra (Fig. 2B). This result suggests that expression of the β4 intracellular domain alone may allow for low levels of activation of SHP-2 and is consistent with the observation that some binding of β4 to SHP-2 occurs in the absence of BDNF (Fig. 2A). To demonstrate that the intact αβ4 integrin can activate SHP-2 in the absence of exogenous growth factor stimulation, we examined SHP-2 phosphorylation in MDA-MB-231 cells, which express significant amounts of endogenous αβ4. As shown in Fig. 2C, the phosphorylation of SHP-2 on Tyr-542 increased over time in response to αβ4 ligation. These results indicate that SHP-2 activity can be regulated by engagement of this integrin independently of growth factor signaling.

Fig. 2. Dimerization of TrkBβ4 with BDNF leads to increased association with SHP-2 and activation of this tyrosine phosphatase. A, extracts from MDA-MB-435 TrkBβ4 cells that had been treated (+) or not treated (−) with BDNF (100 ng/ml) for 30 min were immunoprecipitated with Abs specific for either SHP-2, the intracellular domain of β4 (S505), or an IgG control (mIgG, mouse IgG; rIgG, rabbit IgG), and immunoprecipitates were immunoblotted for β4 (S505, upper panels) or total SHP-2 (lower panels). An aliquot of whole cell extract (WCL) was also immunoblotted. B, MDA-MB-435 cells stably expressing TrkBextra or TrkBβ4 were treated with BDNF (100 ng/ml) for 0–60 min, as indicated, and equal amounts of total protein from extracts of these cells were immunoblotted for phosphorylated SHP-2 (Tyr-542, upper panel) or total SHP-2 (bottom panel), as described. C, MDA-MB-231 cells were clustered with a β4-specific antibody (UM-A9) for 30 min and either maintained in suspension (S) or allowed to adhere to anti-mouse IgG-coated plates for the time periods indicated. Equal amounts of total protein from cell extracts were immunoblotted for phosphorylated SHP-2 (Tyr-542, top panel) or total SHP-2 (bottom panel).

The Src family kinases (SKFs), proteins with molecular masses of ~60 kDa, are key mediators of integrin signaling (40, 41), including signaling through β4 (18, 29, 42, 43). Thus, we hypothesized that the 60-kDa phospho-protein detected in Fig. 3A could be an SKF. Activation of SKFs is coordinated by two tyrosine residues: dephosphorylation of the inhibitory Tyr-529, which allows for a conformational change in the molecule exposing the activation loop and phosphorylation of Tyr-418 within this loop that results in Src-kinase activation (44). BDNF treatment of cells expressing TrkB4 resulted in a time-dependent increase in phosphorylation of Tyr-418 and a concomitant decrease in the phosphorylation of Tyr-529 (Fig. 3B). No significant change in the phosphorylation of either site was observed in response to BDNF stimulation of TrkBextra cells (Fig. 3B) or cells expressing vector alone (data not shown), substantiating the conclusion that the observed increase in Src activation can be attributed to the β4 intracellular domain. As observed for SHP-2 (Tyr-542), the basal level of Tyr-418 phosphorylation (0 min BDNF) was higher in cells expressing TrkB4 than in the TrkBextra cells, but the basal
level of Tyr-529 phosphorylation was slightly higher in the TrkBextra cells (Fig. 3B). These results suggest that expression of the β4 intracellular domain by itself results in some activation of an SFK. Together, these findings imply that the β4 intracellular domain has the intrinsic ability to mediate the activation of an SFK, and that this activation is enhanced following its dimerization.

Previous work had suggested the involvement of SHP-2 in Src activation (45). To assess the potential involvement of SHP-2 in regulating Src activation by the β4 intracellular domain, we evaluated the ability of calpeptin, an inhibitor of the catalytic activity of SHP-2, to impede Src activation in response to dimerization of the TrkBβ4 chimera. Calpeptin was originally characterized as an inhibitor of the calpain proteases, but was more recently shown to also inhibit SHP-2 function (46–48). As shown in Fig. 3C, calpeptin prevented the phosphorylation of Tyr-418 on Src that is induced by BDNF stimulation of cells expressing TrkBβ4. To confirm that the effects of calpeptin were the result of inhibiting SHP-2 specifically, the BDNF-dependent activation of Src in these cells was also examined in the presence of ALLN, an inhibitor of calpain activity that also inhibits the proteasome (49). ALLN did not block phosphorylation of Tyr-418 on Src in response to dimerization of TrkBβ4 with BDNF (Fig. 3C).

We also confirmed the ability of the intact α6β4 receptor to activate Src. For this purpose, we examined Src activation in response to antibody-mediated ligation of either α6β1, in mock transfected MDA-MB-435 cells, or α6β1 and α6β4 in MDA-MB-435 cells that had been transfected with the full-length β4 integrin subunit. Although engagement of α6β1 with an α6 Ab stimulated Src activation, the level of activation in response to α6β4 ligation was markedly higher (Fig. 3D). In the presence of calpeptin, α6β4-dependent activation of Src was inhibited, whereas α6β1-dependent activation of Src was unaffected by the inhibition of SHP-2 (Fig. 3D). These results indicate that the mechanisms by which α6β1 and α6β4 activate Src are distinct. The α6β4-dependent activation of Src was also examined in the presence of ALLN and a peptide derived from calpastatin that is a calpain-specific inhibitor (50). Neither ALLN nor the calpastatin peptide inhibited Src activation in response to ligation of the α6β4 integrin, demonstrating that the reduction in Src activation observed using calpeptin resulted from SHP-2 inhibition (Fig. 3E). Furthermore, stable expression of an shRNA against SHP-2 to reduce SHP-2 protein levels also inhibited Src activation significantly in response to antibody-mediated ligation of α6β4 (Fig. 3F).
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A.

B.

Dimerization of the β4 Intracellular Domain Is Unable to Promote Activation of Key Downstream Signaling Pathways Associated with Ligation of the αβ4 Integrin—Ab-mediated ligation of αβ4 in MDA-MB-435 cells that express the intact αβ4 integrin resulted in a time-dependent increase in the activation of both Akt and Erk1/2 (Fig. 4A). This finding confirms previous studies, which concluded that the expression and ligation of the αβ4 integrin result in a robust activation of the PI3-K/Akt and MAPK signaling pathways (21, 51). As shown in Fig. 4B, however, dimerization of the TrkBβ4 chimera receptor with BDNF failed to induce activation of either Akt or Erk1/2 as assessed by phospho-immunoblotting. Dimerization of the full-length TrkB receptor in the same cells, however, resulted in activation of both pathways (Fig. 4A), in accord with previous studies (52, 53). These data indicate that expression and dimerization of the β4 intracellular domain are not sufficient to promote activation of Akt and Erk1/2.

The β4 Intracellular Domain Itself Does Not Amplify HGF Signaling and Function—The chimeric TrkBβ4 receptor provided an opportunity to evaluate the role of the β4 intracellular domain in amplifying growth factor-mediated signaling and function in carcinoma cells. For this purpose, we focused on HGF/Met signaling, because cooperation between Met and αβ4 signaling in carcinoma cells has been established and a significant amount of these data were derived from the use of MDA-MB-435 cells (29). Initially, we assessed the ability of HGF to stimulate tyrosine phosphorylation of the β4 intracellular domain in comparison to dimerization of this domain with BDNF. As shown in Fig. 5A, HGF stimulation resulted in a substantially greater increase in β4 phosphorylation than did BDNF stimulation. Interestingly, no synergic increase in phosphorylation was evident when both factors were added together (Fig. 5A). Based on these data, we compared the ability of HGF and BDNF to promote the association of β4 with SHP-2. The data shown in Fig. 5B reveal that HGF stimulation resulted in only a slight increase in β4/SHP-2 association in comparison to BDNF stimulation, and no synergy was evident when both factors were added together. These data argue that dimerization of the β4 intracellular domain is a much more potent inducer of SHP-2 association than is HGF stimulation. We
also assessed the ability of the TrkBβ4 chimera to amplify HGF-mediated activation of Akt and Erk1/2 using TrkBextra cells and cells that express the TrkBβ4 chimera. Although HGF was able to stimulate activation of both kinases in TrkBextra cells as determined by phospho-immunoblotting, expression of the β4 intracellular domain did not enhance this activation (Fig. 5C). Moreover, HGF-induced activation of Erk1/2 in HCC1937 breast carcinoma cells, which endogenously express β4, was similar in cells expressing an shRNA against β4 as compared with cells expressing a control shRNA against GFP (Fig. 5D).

The signaling data, as well as previous reports on the ability of β4 to enhance HGF-mediated migration (22, 54), prompted us to examine the ability of HGF to stimulate the chemotactic migration of cells that expressed either the TrkBβ4 chimera or TrkBextra. As shown in Fig. 5E, no difference was seen in the ability of HGF to stimulate the migration of TrkBextra cells as compared with cells that express the TrkBβ4 chimera. The conclusion from these results is that expression of the β4 intracellular domain itself is not sufficient to enhance HGF-mediated migration.

**DISCUSSION**

This study was predicated on numerous reports suggesting that the atypical intracellular domain of the β4 integrin subunit exhibits considerably more autonomy in signaling and function than other integrin intracellular domains (5, 55). The data reported in this study reveal that the β4 intracellular domain does indeed possess intrinsic signaling function but that it also is unable by itself to transduce all signaling events and functions that have been attributed to α6β4. The β4 intracellular domain can function independently of the α6 subunit, tetraspanins, and growth factor signaling to bind and activate SHP-2, as well as to activate Src. In contrast, expression and dimerization of this intracellular domain are not sufficient to activate two of the major kinases known to be involved in α6β4 signaling: Akt and Erk1/2 (21, 51). Also, expression of this intracellular domain does not enhance HGF-mediated activation of Akt and Erk1/2 or HGF-stimulated migration, as previously reported for the intact α6β4 receptor (22). One hypothesis that can be derived from these data is that the association of the β4 subunit with the α6 subunit and tetraspanins is necessary for complete α6β4 signaling and function.

Our data provide insight into the relationship between α6β4 and growth factor signaling. The ability of the β4 intracellular domain to associate with α6β4 in response to HGF signaling has been reported recently (29). In this study, the conclusions were drawn that SHP-2, when bound to β4, enhances Src activation, which then phosphorylates Gab1 leading to MAPK activation. This study examined the association between the β4 subunit and SHP-2 in response to HGF stimulation, but it did not address the independent functions of the β4 subunit. Our data using the chimeric TrkBβ4 receptor confirm the ability of SHP-2 to associate with β4. A critical difference, however, is our observation that dimerization of the β4 intracellular domain with BDNF promotes SHP-2 association to a greater extent than does transactivation of β4 by HGF stimulation. In fact, we detected only a slight increase in SHP-2/β4 association in response to HGF stimulation. These data argue that SHP-2 binding and activation are intrinsic functions of the β4 intracellular domain and that they can occur independently of growth factor signaling. Although the mechanism for SHP-2-mediated activation of Src has not been established in our model system, recent work suggests that SHP-2 may regulate Src indirectly via de-localization of Csk, which is responsible for tyrosine phosphorylation of Src on its inhibitory site, and not via direct dephosphorylation of this inhibitory site by SHP-2 itself (45).

Our data on β4 association with SHP-2, along with the failure of the intracellular domain of β4 to enhance growth factor-mediated activation of Akt and Erk1/2 in MDA-MB-435 cells, suggest that the role of the β4 intracellular domain as an adaptor for growth factor signaling needs to be evaluated more rigorously. This conclusion is substantiated by the inability of full-length β4 to enhance HGF-induced activation of Erk1/2 in HCC1937 cells.

An important question that arises from our study is why the β4 intracellular domain itself is unable to activate either Akt or Erk1/2, kinases that have been implicated in α6β4 signaling. In this respect, our data differ from other studies that have suggested that activation of these kinases is intrinsic to the β4 intracellular domain (11, 22). A likely explanation for our data is that the TrkBβ4 chimera is unable to associate with tetraspanins, integral membrane proteins that have been implicated in a wide variety of functions, including cancer progression (56, 57). The α6β4 integrin, as well as the α6β1 and α3β1 integrins, interact directly with several tetraspanins, and this interaction occurs through their α-subunits (57). Moreover, the concept of a “tetraspanin web” has been proposed, which is a membrane microdomain distinct from lipid rafts and formed by tetraspanin-tetraspanin interactions (23, 58). Such webs have been shown to contain not only tetraspanins and integrins, but other signaling molecules as well. Disruption of this tetraspanin web through suppression of tetraspanin expression significantly impairs the function of integrins, such as α6β4, that associate with these domains. For example, CD151 null mice have defects in both angiogenesis and epithelial wound healing, and cells derived from these mice are deficient in signaling in response to laminin adhesion (59, 60). Thus, the argument can be made that the incorporation of α6β4 into tetraspanin webs, or possibly other membrane microdomains, is necessary for realizing its full signaling potential. Interestingly, a previous study on endothelial cells using an interleukin-2 receptor/β4 intracellular domain chimera observed that the β4 intracellular domain can localize to “fibrillar” structures on the basal surface and associate with HD-1/nectin in the absence of α6 (61). It appears, therefore, that the localization and signaling properties of the β4 intracellular domain can be regulated differentially. The possibility also needs to be considered that the β4 extracellular domain, possibly through its ability to interact with α6, is required to allow conformational changes in the β4 intracellular domain that facilitate signaling. Finally, BDNF-induced dimerization of TrkBβ4 may not be sufficient to mimic the lateral clustering of α6β4 molecules completely following ligand binding. However, this possibility is diminished by our observation that Ab-mediated clustering of TrkBβ4 did not enhance Src activation to levels observed following Ab-mediated clustering of α6β4, or promote activation of Akt and Erk1/2 (data not shown).

Our data provide some insight into how growth factor receptor signaling impacts α6β4. A current hypothesis is that the β4...
intracellular domain functions as an adaptor for growth factor receptor signaling and that transactivation of β4 by such signaling results in β4 phosphorylation, the recruitment of signaling intermediates such as SHP-2 and the consequent activation of downstream signaling pathways (29, 62). In agreement with this hypothesis, we observed that HGF stimulated a marked increase in the tyrosine phosphorylation of the TrkB/β4 chimera. This increase in phosphorylation, however, was not manifested in a concomitant increase in SHP-2 binding or activation of either Akt or Erk1/2. Nor, was the expression of the β4 intracellular domain able to enhance HGF-mediated migration. These findings are in contrast to the reports that expression of the β4 intracellular domain tagged with c-myc in MDA-MB-435 cells is able to amplify HGF-mediated signaling and function (22, 29). Given that the c-myc/β4 receptor does not associate with α6, we cannot explain the discrepancy in the two sets of data. However, our data demonstrate clearly that the β4 intracellular domain is not simply a passive amplifier of growth factor signaling.

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