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Cross-talk between Integrin α6β4 and Insulin-like Growth Factor-1 Receptor (IGF1R) through Direct α6β4 Binding to IGF1 and Subsequent α6β4-IGF1-IGF1R Ternary Complex Formation in Anchorage-independent Conditions*[S]

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Background: Integrin αvβ3-extracellular matrix interaction and/or αvβ3 binding to insulin-like growth factor-1 (IGF1; and integrin-IGF1-IGF1 receptor ternary complex formation) is critical for IGF signaling.
Results: α6β4 directly bound to IGF1 and mediated IGF1 signaling through ternary complex formation. α6β4 is required when cell-matrix adhesion is reduced or in three-dimensional culture.
Conclusion: α6β4-IGF1 binding is important for IGF signaling in anchorage-independent conditions.
Significance: The integrin-IGF interaction is a novel therapeutic target.

Integrin αvβ3 plays a role in insulin-like growth factor-1 (IGF1) signaling (integrin-IGF1 receptor (IGF1R) cross-talk). The specifics of the cross-talk are, however, unclear. In a current model, "ligand occupancy" of αvβ3 (*i.e.* the binding of extracellular matrix proteins) enhances signaling induced by IGF1 binding to IGF1R. We recently reported that IGF1 directly binds to αvβ3 and induces αvβ3-IGF1-IGF1R ternary complex formation. Consistently, the integrin binding-defective IGF1 mutant (R36E/R37E) is defective in inducing ternary complex formation and IGF signaling, but it still binds to IGF1R. Like αvβ3, integrin α6β4 is overexpressed in many cancers and is implicated in cancer progression. Here, we discovered that α6β4 directly bound to IGF1, but not to R36E/R37E. Grafting the β4 sequence WPNSDP (residues 167–172), which corresponds to the specificity loop of β3, to integrin β1 markedly enhanced IGF1 binding to β1, suggesting that the WPNSDP sequence is involved in IGF1 recognition. WT IGF1 induced α6β4-IGF1-IGF1R ternary complex formation, whereas R36E/R37E did not. When cells were attached to matrix, exogenous IGF1 or α6β4 expression had little or no effect on intracellular signaling. When cell-matrix adhesion was reduced (in poly(2-hydroxyethyl methacrylate-coated plates), IGF1 induced intracellular signaling and enhanced cell survival in an α6β4-dependent manner. Also IGF1 enhanced colony formation in soft agar in an α6β4-dependent manner. These results suggest that IGF binding to α6β4 plays a major role in IGF signaling in anchorage-independent conditions, which mimic the *in vivo* environment, and is a novel therapeutic target.

It has been well established that integrin αvβ3 plays a critical role in regulating insulin-like growth factor-1 (IGF1)[2] signaling (1). IGF1 is a polypeptide hormone that has a high degree of structural similarity to human proinsulin. IGF1 acts through binding to the IGF1 receptor (IGF1R), a receptor tyrosine kinase. IGF1 is involved in cell growth, and consequently, IGF1 inhibition is being pursued as a potential measure for treating and preventing cancer. Ligand binding induces phosphorylation of specific tyrosine residues of IGF1R. These phosphotyrosines then bind to adaptor molecules such as Shc and insulin receptor substrate-1. Phosphorylation of these proteins leads to activation of PI3K and MAPK signaling pathways (2).

IGF1 has been implicated in cancer progression (1). Many cancer cells secrete abnormally high levels of IGF1 and IGF2. Once released by cancer cells, both growth factors bind and activate IGF1R on their surface. This autocrine receptor activation causes the release of intracellular signals that are strongly anti-apoptotic, notably through their ability to activate the PI3K/AKT pathway. IGF1 thereby confers resistance to chemotherapy and radiation therapy. Several strategies to target IGF1 signaling have been developed, including siRNA and monoclonal antibodies for IGF1R and kinase inhibitors to inhibit the enzymatic activity of the receptor (1).

In a current model, "ligand occupancy" of αvβ3 (*i.e.* the binding of extracellular matrix proteins such as vitronectin to αvβ3) enhances signaling induced by IGF1 binding to IGF1R (1).

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[2] The abbreviations used are: IGF1, insulin-like growth factor-1; IGF1R, IGF1 receptor; polyHEMA, poly(2-hydroxyethyl methacrylate); MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; ANOVA, analysis of variance.

Indeed, antagonists to αvβ3 block IGF1 signaling. Anti-αvβ3 mAb and echistatin, a snake venom disintegrin that specifically inhibits αvβ3, block IGF1-induced cell migration (3). Also, echistatin blocks IGF1-stimulated DNA synthesis and insulin receptor substrate-1 phosphorylation and attenuates IGF1R-linked downstream signaling events such as activation of PI3K and ERK1/2 (4).

We recently discovered that IGF1 directly and specifically binds to αvβ3, and we generated an integrin binding-defective mutant (R36E/R37E) of IGF1 (5). R36E/R37E is defective in inducing cell survival and IGF signaling, although the mutant still binds to IGF1R (5). Also, WT IGF1 induces αvβ3-IGF1-IGF1R ternary complex formation, but R36E/R37E does not. This suggests that the direct binding of integrins to IGF1 is critical for IGF signaling and a potential mechanism of integrin-IGF1R cross-talk.

In this study, we discovered that another integrin, α6β4, which is overexpressed in many cancers, is involved in IGF1 signaling. We demonstrated that α6β4 directly bound to IGF1, suggesting that this integrin plays a role in cancer progression and invasiveness though IGF signaling. WT IGF1 induced α6β4-IGF1-IGF1R ternary complex formation, but R36E/R37E did not. Notably, we demonstrated that α6β4 mediated IGF signaling in anchorage-independent conditions in poly(2-hydroxyethyl methacrylate) (polyHEMA)-coated plates and in three-dimensional culture in soft agar. These results suggest that IGF signaling requires direct integrin IGF1 interaction in anchorage-independent conditions.

EXPERIMENTAL PROCEDURES

Materials—Recombinant WT IGF1 and R36E/R37E were synthesized as described (5). Recombinant soluble α6β4 was synthesized as described (6). MCF-7 and CHO cells were obtained from American Type Culture Collection. CHO cells expressing human integrin β1 (β1-CHO) or β3 (β3-CHO) have been described (7). Met-1 mouse breast cancer cells (8) were provided by A. D. Borowsky (University of California, Davis, CA). CHO cells expressing human α6β4 (α6β4-CHO) have been described (9). Anti-phospho-ERK1/2 (Thr-202 and Tyr-204), anti-phospho-AKT (Thr-308), anti-phospho-IGF1Rβ (Tyr-1135 and Tyr-1136), anti-integrin β4, anti-ERK1/2, anti-AKT, anti-integrin β1, and anti-IGF1Rβ antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). HRP-conjugated anti-His tag antibody was purchased from Qiagen (Valencia, CA). Anti-hamster β1 mAb 7E2 (10, 11) was kindly provided by R. L. Juliano (University of North Carolina, Chapel Hill, NC). Anti-α6 mAb 135-13c and anti-β4 mAb 439-9B were kind gifts from S. J. Kennel (University of Tennessee). Anti-α6 mAb G0H3 was a kind gift from A. Sonnenberg (Netherlands Cancer Institute). We obtained hybridoma of anti-human β1 mAb AIIB2 and mAb TS2/16 from American Type Culture Collection.

Signaling Assays—In regular tissue culture, we cultured cells to near confluence in DMEM with 10% FCS and then serum-starved them in DMEM with 0.4% FCS overnight. The starved cells were stimulated with WT IGF1 and/or R36E/R37E for 5–15 min. We solubilized cells in lysis buffer (20 mM HEPES (pH 7.4), 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM $MgCl_2$, 1 mM PMSF, 20 mM NaF, 1 mM Na_3VO_4, and protease inhibitor mixture (Sigma-Aldrich)). The cell lysates were analyzed by Western blotting using specific antibodies. Bound IgG was detected using HRP-conjugated second antibody and SuperSignal West Pico (Thermo Scientific). We analyzed images using a Fuji LAS 4000 mini luminescent image analyzer and Multi Gauge V3.0 software (Fujifilm, Tokyo, Japan). poly-HEMA-coated plates were prepared as described (12), except that the final polyHEMA concentration was 1.2 mg/cm^2. Signaling assays were performed as described above, except that the cells were serum-starved for 3 h in DMEM without FCS.

Coprecipitation of α6β4, IGF1R, and IGF1—α6β4-CHO or β1-4-1-CHO cells were treated with WT IGF1 or R36E/R37E (100 ng/ml) for 15–30 min. We immunopurified β4 or β1-4-1 with anti-β4 or anti-β1 antibodies from cell lysates and analyzed the immunoprecipitated materials with antibodies specific to IGF1R, β1, or β4 by Western blotting as described above.

Binding of Soluble α6β4—We immobilized WT IGF1 or R36E/R37E (at a coating concentration of 20 μg/ml) onto wells of 96-well microtiter plates in PBS for 1 h at room temperature and blocked the remaining protein-binding sites by incubation with 0.1% BSA in PBS for 1 h at room temperature. Recombinant soluble α6β4 in HEPES/Tyrode's buffer containing 1 mM $MnCl_2$, $MgCl_2$, $CaCl_2$, or EDTA was added to the wells, followed by incubation for 2 h at room temperature. After rinsing the wells to remove unbound proteins, we measured bound α6β4 using anti-Velcro antibody, HRP-conjugated anti-mouse IgG, and a substrate of HRP (3,3′,5,5′-tetramethylbenzidine) (6).

Mutagenesis—Swapping the specificity loop of β1 with the corresponding sequence of β4 was performed by site-directed mutagenesis as described (13). We replaced the CTSEQNCTS sequence of β1 (residues 187–195) with WPNSDP and generated -AKLRP(β1)(WPNSDP(β4))(β1)PFSYKN sequence using oligonucleotide 5′-gctaagctcaggaacccttggccaaacagcgacccccca-tttagctacaaaaat-3′ (designated the β1-4-1 mutant). The presence of the mutation was confirmed by DNA sequencing. We transfected the β1-4-1 mutant in the pBJ-1 vector together with the pFneo vector into CHO cells by electroporation and selected for stable transfectants with G418 as described (13). Stable transfectants were sorted for high expressers and cloned by flow cytometry.

Soft Agar Colony Formation Assays—Soft agar colony formation assays were performed as described previously (14). We cultured cells to near confluence in DMEM with 10% FCS and then serum-starved them in DMEM with 0.4% FCS overnight. The starved cells (5×10^4 cells) were suspended in DMEM with 0.4% FCS containing 0.3% agar and layered them onto a bottom layer of DMEM with 0.4% FCS containing 1.0% agar. We overlaid DMEM with 10% FCS and cultured the cells for 3 weeks at 37 °C. The medium was replaced twice a week. The number of the colonies were determined from the digital images of colonies using NIH ImageJ.

Other Methods—Cell adhesion assays (15), MTS assays (9), and flow cytometric analysis (16) were performed as described. Statistical significance was calculated using Prism 5 (GraphPad Software).

RESULTS

Integrin α6β4 Directly Binds to IGF1—Integrin αvβ3, which is overexpressed in cancer and implicated in cancer progression, directly binds to IGF1, and this interaction plays a role in IGF1 signaling because the integrin binding-defective mutant (R36E/R37E) of IGF1 is defective in inducing intracellular signaling, but it binds to IGF1R (5). WT IGF1 induces αvβ3-IGF1-IGF1R ternary complex formation, whereas R36E/R37E is defective in this function, suggesting that direct binding of IGF1 to αvβ3 and subsequent ternary complex formation are critical for IGF signaling.

MCF-7 human breast cancer cells, which are widely used for studying IGF signaling, express little or no αvβ3 (17). Because α6β4 is overexpressed in many cancer cell types (18), like αvβ3, we hypothesized that α6β4 is involved in IGF signaling in this cell type. To determine whether integrin α6β4 directly interacts with IGF1, we used CHO cells expressing human α6β4 (designated α6β4-CHO cells) by cotransfecting human α6 and β4. Cells stably expressing α6β4 were cloned to obtain high expressers. The α6β4-CHO cells we used clonally expressed human α6 and β4 (Fig. 1*a*). We discovered that α6β4-CHO cells adhered to IGF1 better than β1-CHO cells (Fig. 1*b*). We confirmed that WT IGF1 and R36E/R37E (both His_6-tagged) were coated onto plastic at an equal density using anti-His_5 tag antibody (data not shown). Interestingly, α6β4-CHO cells did not adhere to R36E/R37E, suggesting that α6β4 binds to IGF1 in a manner similar to αvβ3 (Fig. 1*c*). Because antibodies against α6 (G0H3) and β4 (439-9B) did not effectively block adhesion of α6β4-CHO cells to IGF1 (data not shown), we tested if recombinant soluble α6β4 bound to IGF1 in ELISA-type assays. The soluble α6β4 we used contains only the extracellular domains of α6 and β4 and has been purified to homogeneity (6). We found that soluble α6β4 bound to IGF1, but not well to R36E/R37E (Fig. 1*d*). Because only purified soluble α6β4 and IGF1 were present in the assay system, we concluded that α6β4 directly interacts with IGF1. We also tested if IGF1 is a cation-dependent ligand of α6β4, as are other known integrin ligands. The data suggest that interaction is cation-dependent: manganese, magnesium, and calcium supported the binding in this order, but EDTA did not, suggesting that IGF1 is similar to other known integrin α6β4 ligands in cation requirement.

Localization of IGF1-binding Site in β4—We localized the IGF1-binding site in β4. We previously reported that the disulfide-linked loop in the β3 subunit plays a role in recognizing IGF1 in β3 (5). The disulfide-linked specificity loop is not present in β4 and is replaced with remnant residues (Fig. 2*a*) (13). We hypothesized that IGF1 binds to the β4 sequence that corresponds to the specific loop in β3. To test this hypothesis, we generated a β1 mutant in which the CTSEQNCTS sequence of β1 that contains the specificity loop was replaced with the corresponding WPNSDP sequence of β4 (designated the β1-4-1 mutant). The β1-4-1 mutant was stably expressed in CHO cells (designated β1-4-1-CHO cells) and further cloned to obtain high expressers. In β1-CHO and β1-4-1-CHO cells, β1 and β1-4-1 were expressed at comparable levels (Fig. 2*b*). We found that β1-4-1-CHO cells adhered to WT IGF1 at a level comparable with α6β4 (Fig. 1*b*), and inhibitory anti-human β1 mAb AIIB2 suppressed the adhesion of β1-4-1-CHO cells to WT IGF1 (Fig. 2*c*). (Note that β1-4-1 is >99% integrin β1.) However, β1-4-1-CHO cells only weakly bound to R36E/R37E (Fig. 2*d*) using β3-CHO cells as controls. This suggests that the conserved Lys residues at positions 36 and 37 of IGF1 are involved in α6β4 binding, as in αvβ3 binding. These results suggest that the region of β4 that corresponds to the specificity loop is involved in α6β4 binding to WT IGF1.

IGF Signaling Is Not Dependent on α6β4 Expression in CHO Cells in Regular Tissue Culture Plates—Integrin α6β4 is a receptor for laminins, and thus, it is unclear if α6β4 is involved in IGF1 signaling through direct binding to IGF1, indirectly through adhesion to the extracellular matrix, or both. We studied the effect of IGF stimulation in CHO cells in regular tissue culture conditions. IGF1 induced ERK1/2 activation in α6β4-CHO and CHO cells to a similar extent (Fig. 3*a*), and the effect of the R36E/R37E mutation on IGF1-induced ERK1/2 activation was not clear (Fig. 3*b*). Also, the effect of R36E/R37E on IGF1-induced cell survival was not clear (Fig. 3*c*). This is in contrast to IGF signaling in non-transformed cells (*e.g.* NIH 3T3 and C2C12), in which IGF1 induced robust intracellular signaling, and the effect of the R36E/R37E mutation was detected (5). It has been reported that cell-matrix adhesion masks growth factor signaling in cancer cells (19). We thus hypothesized that IGF signaling in regular tissue culture conditions is not dependent on α6β4 expression, but may be dependent on α6β4-IGF interaction in anchorage-independent conditions in CHO cells.

IGF Signaling Is Dependent on α6β4 Expression in poly-HEMA-coated Plates—To address this hypothesis, we studied IGF signaling in CHO cell lines in polyHEMA-coated plates, which have been widely used to suppress cell-matrix adhesion (12, 20). We detected the effect of α6β4 expression on IGF signaling in polyHEMA-coated plates: WT IGF1 induced ERK1/2 and AKT activation in α6β4-CHO cells in polyHEMA-coated plates, whereas WT IGF1 induced only weak ERK1/2 and AKT activation and the signals were quickly reduced in β1-CHO cells (Fig. 4*a*) and parent CHO cells (supplemental Fig. S1). WT IGF1 enhanced cell survival in α6β4-CHO cells, whereas it had a negligible effect on cell survival in CHO and β1-CHO cells (Fig. 4*b*). These results suggest that IGF signaling is dependent on α6β4 expression in polyHEMA-coated plates, but not in regular tissue culture conditions. We found that WT IGF1 induced coprecipitation of IGF1R and integrin β4 in α6β4-CHO cells, whereas R36E/R37E was much less effective in this function (Fig. 4*c*). This suggests that α6β4 binding to IGF1 is involved in the ternary complex formation in α6β4-CHO cells in a manner that is similar to αvβ3 (5). These findings are consistent with the idea that integrin α6β4 is involved in IGF signaling.

β1-4-1 Mimics β4 in Signaling Functions—Although β1-4-1-CHO cells adhered to IGF1 much better than β1-CHO cells (at a level comparable with α6β4-CHO cells), it is unclear if the ability of β1-4-1-CHO cells to bind to IGF1 has any effect on IGF1 signaling. We found that IGF1 induced stronger ERK1/2 and AKT activation in β1-4-1-CHO cells than in β1-CHO cells in polyHEMA-coated plates (Fig. 5*a*). Also, IGF1 enhanced cell survival in β1-4-1-CHO cells, but only weakly in β1-CHO cells

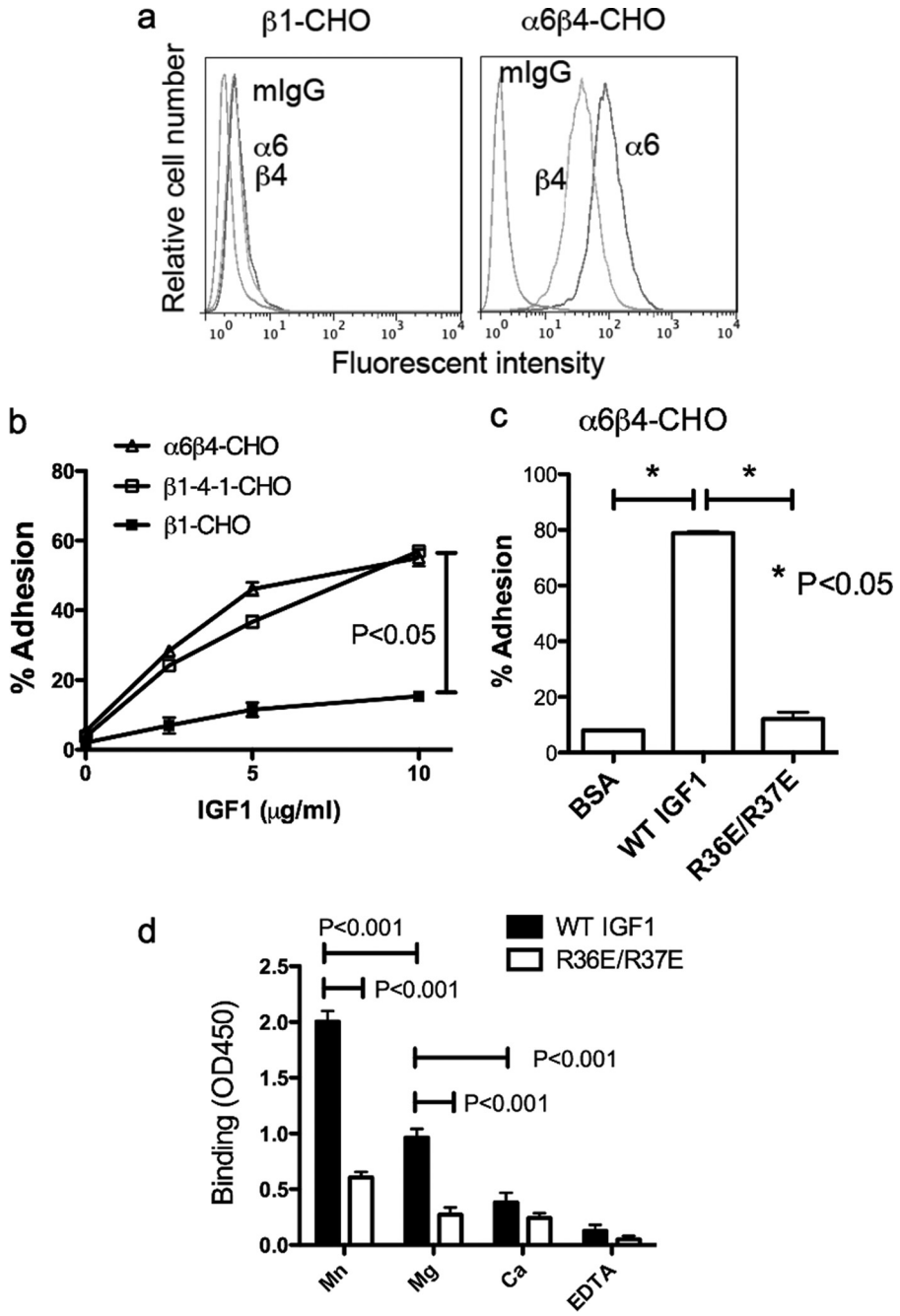

FIGURE 1. **Role of α6β4 in IGF signaling.** *a*, expression of human α6 and β4 integrins in α6β4-CHO cells. Expression of human integrin α6 (mAb 138-13C) and β4 (mAb 439-9B) was analyzed by flow cytometry. The data show that α6β4-CHO cells clonally expressed α6 and β4 integrins. *mIgG*, mouse IgG. *b*, adhesion of α6β4-CHO, β1-4-1-CHO, and β1-CHO cells to WT IGF1 and R36E/R37E. Adhesion assays were performed as described under "Experimental Procedures." Data are shown as means ± S.E. ($n = 3$). Statistical differences were tested by analysis of variance (ANOVA) and Tukey's multiple comparison test. *c*, adhesion of α6β4-CHO cells to WT IGF1 and R36E/R37E. WT IGF1 or R36E/R37E (20 μg/ml) was used for coating. Data are shown as means ± S.E. ($n = 3$). Statistical differences were tested by ANOVA and Tukey's multiple comparison test. *d*, binding of soluble α6β4 to IGF1. WT IGF1 or R36E/R37E (20 μg/ml) was immobilized onto wells of 96-well microtiter plates. Data are shown as means ± S.E. ($n = 3$). Statistical differences were tested using *t* test.

in polyHEMA-coated plates (Fig. 5*b*). Anti-β1 mAb AIIB2 suppressed IGF1-induced cell survival of β1-4-1-CHO cells, suggesting that IGF1-induced signaling is specific to β1-4-1 (Fig. 5*c*). Also, WT IGF1 induced coprecipitation of β1-4-1 and IGF1R, whereas R36E/R37E was defective in this function (Fig. 5*d*). Thus, the ability of β1-4-1 to bind to IGF1 is directly related to enhanced IGF signaling. These results suggest that β1-4-1 mimics β4 in IGF1 binding and signaling and that grafting the WPNSDP sequence of β4 to β1 dramatically changes the phenotype of β1. Because the β1-4-1 mutant does not contain the long cytoplasmic domain of β4, it is likely that the β1 cytoplasmic domain is sufficient for mediating IGF signaling.

Contribution of α6β4 to IGF Signaling in Three-dimensional Culture—Our results so far suggested that the contribution of integrin α6β4 to IGF signaling in regular tissue culture is masked by massive signals from cell adhesion and detected in polyHEMA-coated plates, which suppress cell adhesion. It was still unclear if direct integrin binding to IGF1 is involved in IGF

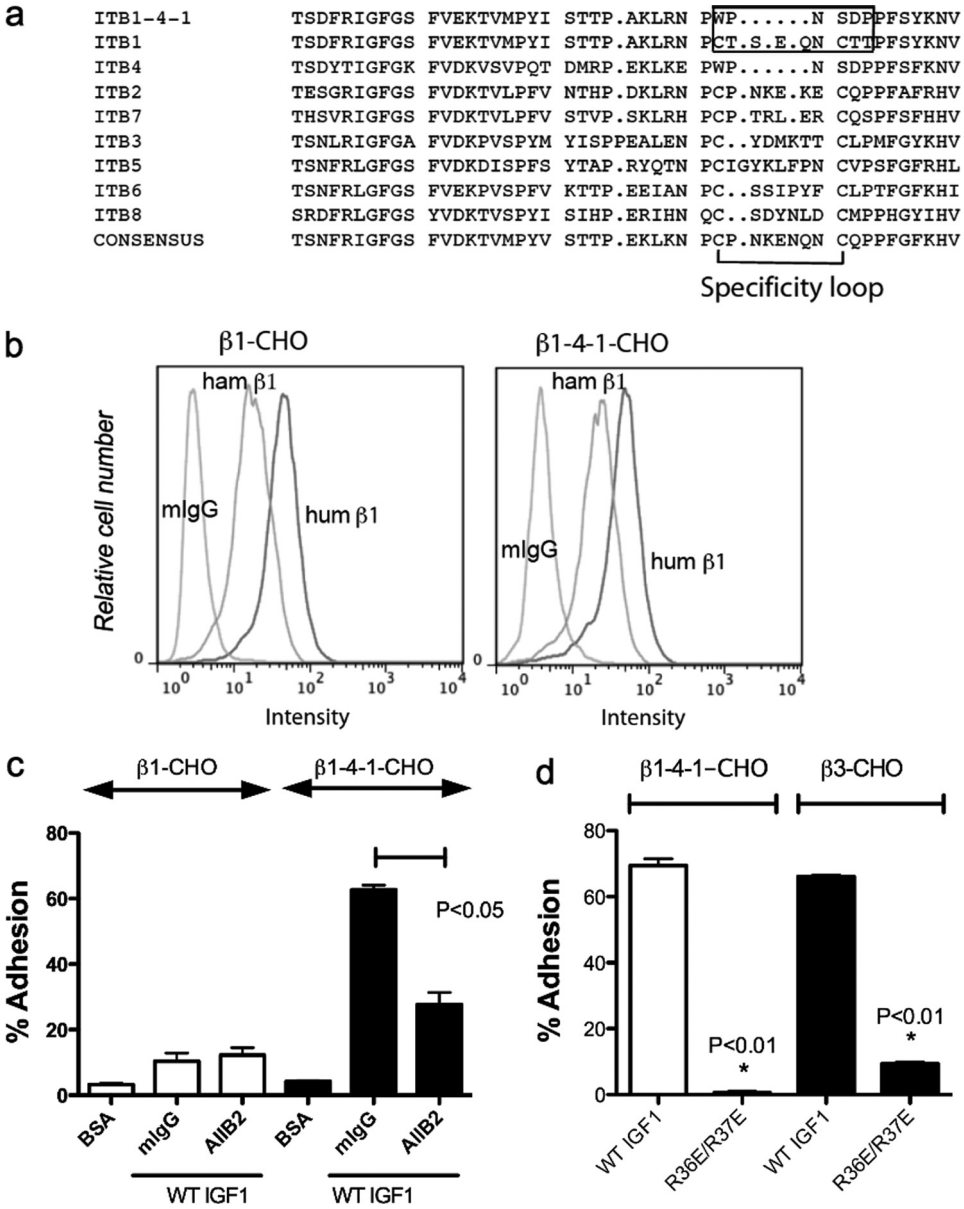

FIGURE 2. **Localization of IGF1-binding site in β4.** *a*, alignment of the amino acid sequences around the specificity loop. *b*, expression of human integrin β1 on CHO cells. β1-4-1- or β1-transfected CHO cells were stained with anti-human (*hum*) integrin β1, anti-hamster (*ham*) integrin β1, and purified mouse IgG (*mIgG*) and analyzed by flow cytometry. *c*, effect of antibodies against β1 on cell adhesion to IGF1. WT IGF1 (20 μg/ml) was used for coating. Antibody concentrations were 10 μg/ml. Data are shown as means ± S.E. ($n = 3$). Statistical differences were tested by ANOVA and Tukey's multiple comparison test. *d*, effect of the integrin binding-defective mutation of IGF1 (R36E/R37E) on cell adhesion to IGF1. WT or mutant IGF1 (20 μg/ml) was used for coating. Data are shown as means ± S.E. ($n = 3$). *, $p < 0.01$ (*t* test).

signaling in three-dimensional culture, which mimics *in vivo* cell growth. To address this question, we tested if WT IGF1 and R36E/R37E affect the growth of CHO cells in soft agar. Parent CHO cells do not express α6β4 and are therefore suitable for testing the role of this integrin in IGF signaling. We stably expressed WT IGF1 or R36E/R37E in α6β4-CHO or CHO cells in the pSec-TagB secretion vector. Transfected cells secreted WT IGF1 and R36E/R37E at comparable levels (Fig. 6*a*). We used cells stably secreting WT IGF1 or R36E/R37E without further selection. The cells were cultured in soft agar for 3 weeks, and the number of colonies was counted. We found that WT IGF1 markedly enhanced colony formation in α6β4-CHO cells (Fig. 6*b*), but not in CHO cells (Fig. 6*c*). These findings suggest that the enhancing effect of WT IGF1 is dependent on α6β4 expression in three-dimensional culture.

We used CHO cells to study the role of integrins in IGF signaling. CHO cells express IGF1R, but do not express αvβ3 or α6β4. CHO cells only weakly respond to WT IGF1 in anchorage-independent conditions, but CHO cells that express αvβ3 (5) or α6β4 (this study) robustly respond to WT IGF1. However, the contribution of integrins to IGF signaling may be specific to CHO cells. We thus tested if integrin binding to IGF1 is involved in anchorage-independent cell growth in MCF-7 cells and Met-1 mouse breast cancer cells. We found that WT IGF1 markedly enhanced colony formation in MCF-7 (Fig. 6*d*) and Met-1 (Fig. 6*e*) cells in soft agar, whereas the integrin binding-

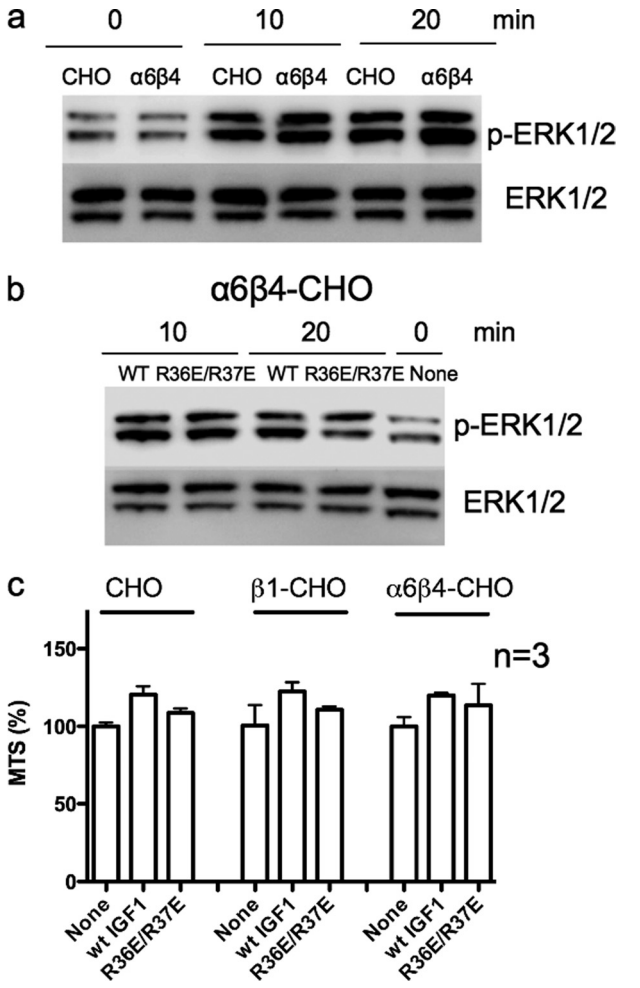

FIGURE 3. **IGF signaling in CHO cells in regular tissue culture plates.** *a*, time course of ERK1/2 activation by WT IGF1. We stimulated serum-starved (overnight) CHO or α6β4-CHO cells with IGF1 (100 ng/ml) and analyzed cell lysates by Western blotting. *b*, effect of R36E/R37E on ERK1/2 activation in α6β4-CHO cells. We stimulated serum-starved (overnight) α6β4-CHO cells with WT IGF1 or R36E/R37E (100 ng/ml). Cell lysates were analyzed by Western blotting. *c*, effect of WT IGF1 or R36E/R37E on cell survival. CHO, β1-CHO, or α6β4-CHO cells (5×10^3 cells/well) were plated in wells of regular 96-well plates and incubated with IGF1 for 24 h in DMEM. WT IGF1 and R36E/R37E had minimal effects on cell survival. Data are shown as means ± S.E. ($n = 3$).

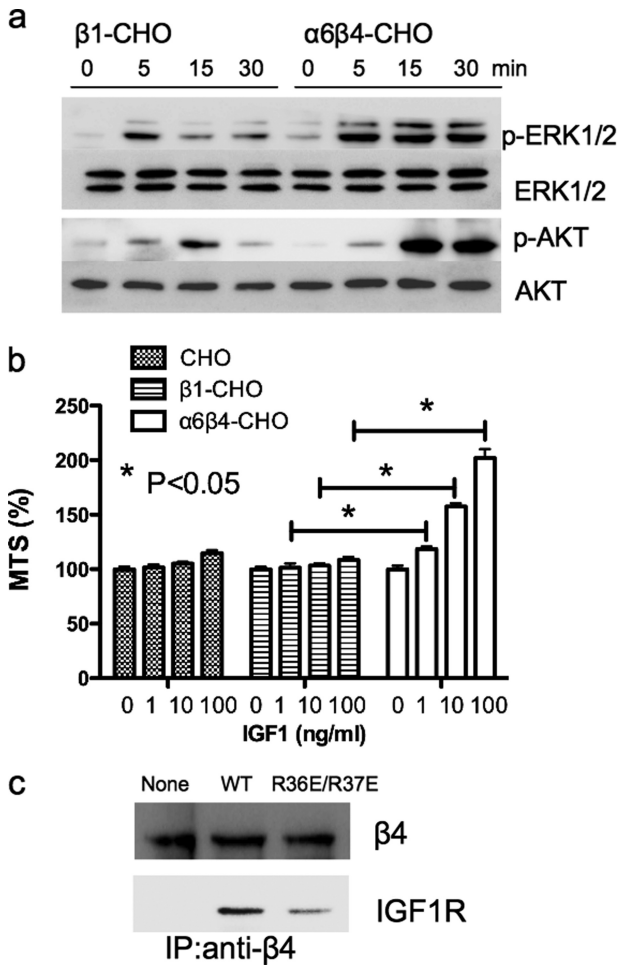

FIGURE 4. **α6β4-dependent IGF1 signaling in polyHEMA-coated plates.** *a*, IGF1 enhanced ERK1/2 and AKT activation in α6β4-CHO cells much strongly than in β1-CHO cells. Cells (1×10^5 cells) were plated in wells of 12-well plates, starved for 3 h in polyHEMA-coated plates, and stimulated with 100 ng/ml WT IGF1. Cell lysates were analyzed by Western blotting. *b*, α6β4-CHO cells responded better to IGF1 compared with β1-CHO cells on polyHEMA-coated plates. CHO, β1-CHO, or α6β4-CHO cells (2×10^4 cells/well) were plated in wells of polyHEMA-coated 96-well plates and incubated with WT IGF1 for 48 h in DMEM. Statistical differences were tested by ANOVA and Tukey's multiple comparison test ($n = 6$). Cell survival was measured by MTS assays. *c*, coprecipitation of β4 with IGF1R in α6β4-CHO cells. Serum-starved α6β4-CHO cells were treated with 100 ng/ml WT IGF1 or R36E/R37E for 30 min, and integrin β4 was immunopurified. Immunopurified materials were analyzed by Western blotting. The data show that WT IGF1 induced coprecipitation of integrin β4 and IGF1R, whereas R36E/R37E was defective in this function. *IP*, immunoprecipitate.

defective R36E/R37E mutant did not. Thus, these results suggest that direct binding of integrins to IGF1 is critical for IGF signaling in these cells in anchorage-independent conditions, whereas it is unclear which integrins are involved in these cells at this point.

DISCUSSION

In this study, we established that integrin α6β4 plays a role in IGF signaling. WT IGF1 directly and specifically bound to integrin α6β4 and induced integrin α6β4-IGF1-IGF1R ternary complex formation in α6β4-CHO cells, whereas R36E/R37E was defective in these functions, as in the case of αvβ3 (5). It is thus highly likely that the ability of IGF1 to bind to α6β4 and to induce ternary complex formation is involved in IGF1 signaling.

The expression of α6β4 is associated with poor patient prognosis and reduced survival in a variety of human cancers (21, 22). The integrin β4 subunit was originally identified as a tumor-related antigen expressed in metastatic cancer (23). In contrast with its function in regulating stable adhesion through the formation of hemidesmosomes in normal epithelial cells, α6β4 promotes motility and invasion in carcinoma cells (24). Moreover, suppression of α6β4 expression by siRNA diminishes invasive and tumorigenic potential (25, 26). α6β4 thus contributes to tumor progression, metastasis, tumor development, and primary tumor growth (27). Integrin α6β4 associates with ErbB2 in mammary cells and cooperates with ErbB2 to promote PI3K-dependent invasion and survival (28). In mouse mammary tumor virus-Neu mice, the introduction of a targeted deletion of the β4 cytoplasmic domain revealed that integrin β4 signaling plays a role in mammary tumor progression (29). However, it has not been fully established how α6β4 is

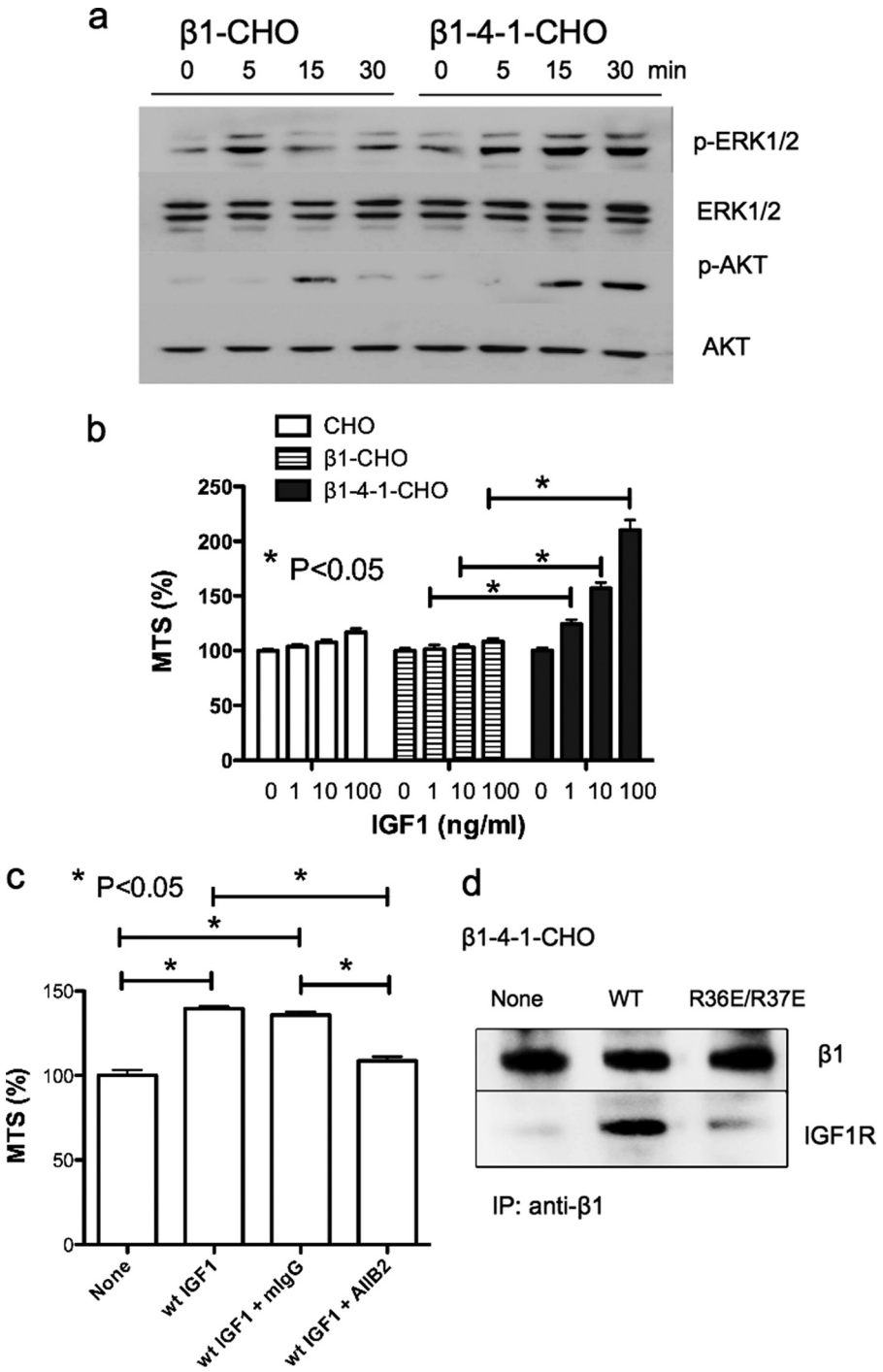

FIGURE 5. **Effect of β1-4-1 mutation on IGF1 signaling.** *a*, IGF1 enhanced ERK1/2 and AKT activation in β1-4-1-CHO cells much strongly than in CHO or β1-CHO cells. Cells (1×10^5 cells) were plated in wells of 12-well plates, starved for 3 h, and stimulated with 100 ng/ml WT IGF1. Cell lysates were analyzed by Western blotting. *b*, WT IGF1 enhanced cell survival in β1-4-1-CHO cells, but not in CHO or β1-CHO, on polyHEMA-coated plates. β1-4-1-CHO, CHO, or β1-CHO cells (2×10^4 cells/well) were plated in wells of polyHEMA-coated 96-well plates and incubated with IGF1 (0, 1, 10, and 100 ng/ml) for 48 h in DMEM. Statistical differences were tested by ANOVA and Tukey's multiple comparison test ($n = 6$). Cell survival was measured by MTS assays. *c*, anti-human β1 mAb AIIB2 suppressed enhanced cell survival induced by WT IGF1. The mAb was used at 10 μg/ml. Statistical differences were tested by ANOVA and Tukey's multiple comparison test ($n = 6$). *d*, WT IGF1 induced coprecipitation of IGF1R and β1-4-1, whereas R36E/R37E was defective in this function. Serum-starved β1-4-1-CHO cells were treated with 100 ng/ml WT IGF1 or R36E/R37E for 15 min at 37 °C, and integrin β1 was immunopurified from cell lysates. Immunopurified materials were analyzed by Western blotting. *IP*, immunoprecipitate.

involved in cancer progression. We propose that α6β4 expression in cancer cells increases tumorigenicity, invasiveness, and/or metastasis at least partly through direct binding to IGF.

We have also established that IGF1 signaling is robust in α6β4-CHO cells, but not in β1-CHO cells, in anchorage-independent conditions. However, in regular culture conditions, the effect of α6β4 expression on signaling was not detectable. This is probably because the effect of α6β4 on IGF signaling is masked by the massive signals from cell-matrix adhesion that enhance IGF signaling, as in the case of αvβ3 (1). Because we

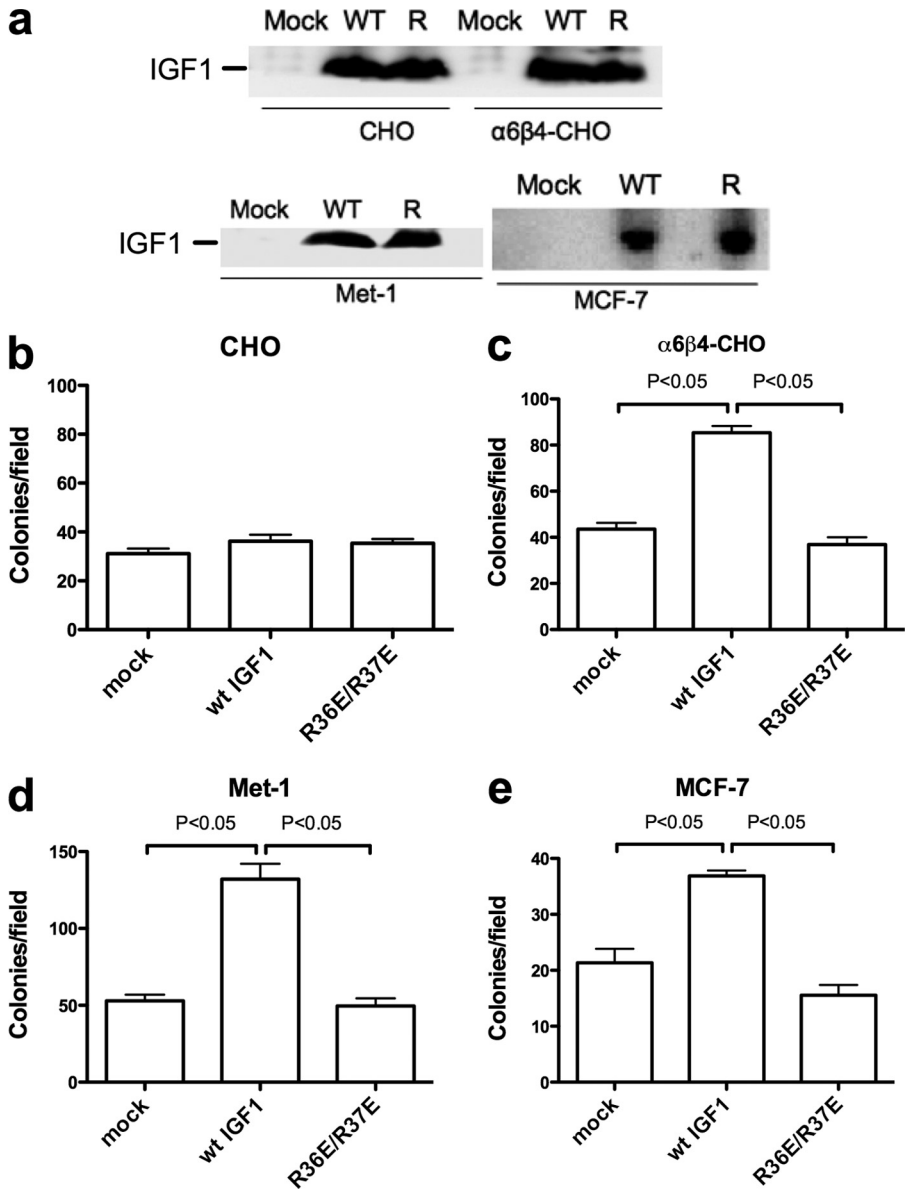

FIGURE 6. **Role of integrins in IGF signaling in three-dimensional culture (soft agar).** WT IGF1 or R36E/R37E was stably expressed in CHO cells, α6β4-CHO cells, Met-1 mouse breast cancer cells, and MCF-7 human breast cells. *a*, secretion of WT IGF1 or R36E/R37E (*R*). The culture medium was concentrated five times and analyzed by Western blotting using anti-His_5 antibodies (IGF1 has an N-terminal His_6 tag). *b–e*, the transfected cells were cultured in soft agar for 3 weeks, and the number of colonies was counted from digital images using ImageJ. Statistical differences were tested by ANOVA and Tukey's multiple comparison test ($n = 10$).

did not detect an effect of WT IGF1 or R36E/R37E in regular tissue culture conditions in CHO, β1-CHO, or α6β4-CHO cells, WT IGF1 or R36E/R37E is not important for cell proliferation in these conditions. This is probably because massive survival signals from the extracellular matrix mask signaling by extrinsic IGF. These findings are consistent with a previous report that cell-matrix adhesion masks the heparin-binding EGF signaling because cell-matrix adhesion provides cells sufficient proliferative signals through cell-matrix adhesion, but that it is possible to detect the proliferative effect of heparin-binding EGF on cancer cells *in vitro* in three- or two-dimensional culture, in which cell-matrix interaction is reduced (19). Cell proliferation in regular tissue culture is much faster than in anchorage-independent conditions, probably reflecting the amount of proliferative signals from cell-matrix adhesion. Consistent with this idea, we detected a clear proliferative/anti-apoptotic effect of WT IGF1 in α6β4-CHO cells in polyHEMA-coated plates and in three-dimensional culture (soft agar). Notably, the results in the *in vivo* xenograft model correlate well with those in these cultures, but not with those in regular tissue culture plates (19). Based on our results in IGF1 signaling in polyHEMA-coated plates and in soft agar, we propose that IGF signaling *in vivo* during tumorigenesis may be dependent on integrin-IGF interaction.

CHO cells are useful for studying the role of α6β4 in IGF signaling because they express IGF1R, but not endogenous α6β4. Thus, in most of our experiments, we used CHO cells. We have shown that the contribution of integrins to IGF signaling is not cell type-specific. We showed that R36E/R37E did not enhance the growth of two other cancer cell types (MCF-7

and Met-1) in soft agar, whereas WT IGF1 did. Because cancer cells express multiple integrins, including α6β4 and αvβ3, it is possible that more than one integrin is involved in IGF signaling. Indeed, we found that knockdown or overexpression of β4 in MCF-7 cells did not affect IGF signaling.[3] It is possible that integrins other than α6β4 are involved in IGF signaling in MCF-7 cells. At this point, it is unclear which integrins are involved in IGF signaling in these cells.

If ternary complex formation is critical for IGF signaling, it is predicted that the IGF1 mutant that is defective in this function (R36E/R37E) acts as an antagonist of IGF signaling. Consistently, we observed that R36E/R37E suppressed intracellular signaling induced by WT IGF1 in polyHEMA-coated conditions *in vitro*,[4] suggesting that R36E/R37E is a dominant-negative mutant by definition. Furthermore, R36E/R37E suppressed tumorigenesis *in vivo*.[4] Why is the effect of R36E/R37E detected in polyHEMA-coated plates but not in regular tissue culture conditions? One possibility is that IGF signaling plays a major role in cell proliferation/survival in anchorage-independent conditions (*e.g.* three-dimensional culture, polyHEMA-coated plates, and *in vivo*), and IGF signaling is dependent on integrin binding to IGF1 (and ternary complex formation).

It has been proposed that the function of integrin α6β4 is altered substantially as normal epithelia undergo malignant transformation and progress to invasive carcinoma and that the functions of this integrin contribute to the behavior of aggressive carcinoma cells (18). In response to signals that disrupt hemidesmosomes, α6β4 is released from interactions with the cytokeratin cytoskeleton and involved in *de novo* interaction with the actin cytoskeleton and exposed to signaling molecules in the apical region of the cells (18). In this signaling-competent state, α6β4 cooperates with growth factor receptors and other surface molecules to amplify intracellular signaling pathways (28, 30, 31). We propose that α6β4 in the apical region directly binds to IGF and induces intracellular signaling. This represents a drastic change in the biological roles of this integrin in cancer cells and migrating cells and is highly relevant to cancer initiation and progression.

In this study, we have demonstrated that the β1-4-1 mutation effectively induced the binding of the β1 integrins to WT IGF1, but not to R36E/R37E. This suggests that the WPNSDP sequence of β4 (residues 167–172) is involved in recognition of IGF1, but the disulfide linkage is not present in the WPNSDP sequence. Importantly, this suggests that IGF1 binds to the site in β4 that is common to other β4 ligands. Point mutations (K150A and Q155L) of β4 suppress binding of α6β4 to laminin-5 (32). Also, a GST fusion protein of the β4 region (residues 157–185) directly binds to calcium-activated chloride channels (32). The K150A and Q155L mutations are close to the WPNSDP sequence, and the GST fusion protein contains the WPNSDP sequence. It is likely that the WPNSDP sequence plays a role in these interactions as well because the WPNSDP sequence is exposed to the surface in the predicted ligand-binding site in the model of β4 (data not shown). The anti-α6 (G0H3, function-blocking) and anti-β4 (439-9B, non-function-blocking) antibodies we used did not suppress IGF1 binding to α6β4. Epitopes for these antibodies have not been well defined. One possible reason that G0H3 did not block IGF1 binding to α6β4 is that IGF1 is much smaller than laminins, known α6β4 ligands, and therefore, G0H3 did not block access of IGF1 to its binding site. In contrast, anti-β1 mAb AIIB2 suppressed IGF1 binding to β1-4-1-CHO cells. We reported that function-blocking (*e.g.* AIIB2) and activating (*e.g.* TS2/16) anti-β1 mAbs recognize overlapping epitopes within residues 207–218 of β1 (a regulatory region), which is outside the ligand-binding site (33). It has been proposed that the binding of antibodies to the regulatory region affects the conformation of β1 in one way or the other and inhibits or activates β1 integrins rather than affecting the access of ligands to β1 integrins (33). This may explain why AIIB2 suppressed IGF1 binding to β1-4-1-CHO cells, although AIIB2 may not directly block the access of IGF1 to the ligand-binding site (33).

α6β4 is distinct from other integrin receptors because the β4 subunit contains a 1000-amino acid cytoplasmic domain (34). It has been proposed that this cytoplasmic domain is essential for coupling α6β4 to the cytoskeleton and for its ability to activate intracellular signaling pathways (35). We have demonstrated that WT IGF1 enhanced cell survival and induced intracellular signaling in β1-4-1-CHO cells, as in α6β4-CHO cells, but not in CHO or β1-CHO cells, although the β1-4-1 mutant does not have the large cytoplasmic domain of β4. We propose that the ability of integrins to bind to IGF1 is required for inducing IGF signaling, but IGF signaling may not be specific to the β4 cytoplasmic domain. It will be necessary to determine the role of the integrin cytoplasmic domains in IGF signaling in future studies.

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[3] M. Fujita, Y. K. Takada, and Y. Takada, unpublished data.
[4] M. Fujita, A. D. Borowsky, R. D. Cardiff, Y. K. Takada, and Y. Takada, submitted for publication.

REFERENCES

1. Clemmons, D. R. (2007) Modifying IGF1 activity: an approach to treat endocrine disorders, atherosclerosis, and cancer. *Nat. Rev. Drug Discov.* **6,** 821–833
2. Clemmons, D. R., and Maile, L. A. (2005) Interaction between insulin-like growth factor-1 receptor- and αvβ3 integrin-linked signaling pathways: cellular responses to changes in multiple signaling inputs. *Mol. Endocrinol.* **19,** 1–11
3. Jones, J. I., Doerr, M. E., and Clemmons, D. R. (1995) Cell migration: interactions among integrins, IGFs and IGFBPs. *Prog. Growth Factor Res.* **6,** 319–327
4. Zheng, B., and Clemmons, D. R. (1998) Blocking ligand occupancy of the αvβ3 integrin inhibits insulin-like growth factor-1 signaling in vascular smooth muscle cells. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 11217–11222
5. Saegusa, J., Yamaji, S., Ieguchi, K., Wu, C. Y., Lam, K. S., Liu, F. T., Takada, Y. K., and Takada, Y. (2009) The direct binding of insulin-like growth factor-1 (IGF-1) to integrin αvβ3 is involved in IGF-1 signaling. *J. Biol. Chem.* **284,** 24106–24114
6. Nishiuchi, R., Takagi, J., Hayashi, M., Ido, H., Yagi, Y., Sanzen, N., Tsuji, T., Yamada, M., and Sekiguchi, K. (2006) Ligand-binding specificities of laminin-binding integrins: a comprehensive survey of laminin-integrin interactions using recombinant α3β1, α6β1, α7β1, and α6β4 integrins. *Matrix Biol.* **25,** 189–197
7. Takagi, J., Kamata, T., Meredith, J., Puzon-McLaughlin, W., and Takada, Y. (1997) Changing ligand specificities of αvβ1 and αvβ3 integrins by swapping a short diverse sequence of the β subunit. *J. Biol. Chem.* **272,**

19794–19800
8. Borowsky, A. D., Namba, R., Young, L. J., Hunter, K. W., Hodgson, J. G., Tepper, C. G., McGoldrick, E. T., Muller, W. J., Cardiff, R. D., and Gregg, J. P. (2005) Syngeneic mouse mammary carcinoma cell lines: two closely related cell lines with divergent metastatic behavior. *Clin. Exp. Metastasis* **22,** 47–59
9. Ieguchi, K., Fujita, M., Ma, Z., Davari, P., Taniguchi, Y., Sekiguchi, K., Wang, B., Takada, Y. K., and Takada, Y. (2010) Direct binding of the EGF-like domain of neuregulin-1 to integrins (αvβ3 and α6β4) is involved in neuregulin-1/ErbB signaling. *J. Biol. Chem.* **285,** 31388–31398
10. Brown, P. J., and Juliano, R. L. (1985) Selective inhibition of fibronectin-mediated cell adhesion by monoclonal antibodies to a cell-surface glycoprotein. *Science* **228,** 1448–1451
11. Brown, P. J., and Juliano, R. L. (1988) Monoclonal antibodies to distinctive epitopes on the α and β subunits of the fibronectin receptor. *Exp. Cell Res.* **177,** 303–318
12. Fukazawa, H., Mizuno, S., and Uehara, Y. (1995) A microplate assay for quantitation of anchorage-independent growth of transformed cells. *Anal. Biochem.* **228,** 83–90
13. Takagi, J., Isobe, T., Takada, Y., and Saito, Y. (1997) Structural interlock between ligand-binding site and stalk-like region of β1 integrin revealed by a monoclonal antibody recognizing conformation-dependent epitope. *J. Biochem.* **121,** 914–921
14. Stephenson, J. R., Axelrad, A. A., McLeod, D. L., and Shreeve, M. M. (1971) Induction of colonies of hemoglobin-synthesizing cells by erythropoietin *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **68,** 1542–1546
15. Puzon-McLaughlin, W., Kamata, T., and Takada, Y. (2000) Multiple discontinuous ligand-mimetic antibody-binding sites define a ligand-binding pocket in integrin αIIbβ3. *J. Biol. Chem.* **275,** 7795–7802
16. Kamata, T., Tieu, K. K., Tarui, T., Puzon-McLaughlin, W., Hogg, N., and Takada, Y. (2002) The role of the CPNKEKEC sequence in the β2 subunit I domain in regulation of integrin αLβ2 (LFA-1). *J. Immunol.* **168,** 2296–2301
17. Doerr, M. E., and Jones, J. I. (1996) The roles of integrins and extracellular matrix proteins in the insulin-like growth factor-1-stimulated chemotaxis of human breast cancer cells. *J. Biol. Chem.* **271,** 2443–2447
18. Lipscomb, E. A., and Mercurio, A. M. (2005) Mobilization and activation of a signaling-competent α6β4 integrin underlies its contribution to carcinoma progression. *Cancer Metastasis Rev.* **24,** 413–423
19. Mizushima, H., Wang, X., Miyamoto, S., and Mekada, E. (2009) Integrin signal masks growth promotion activity of HB-EGF in monolayer cell cultures. *J. Cell Sci.* **122,** 4277–4286
20. Folkman, J., and Moscona, A. (1978) Role of cell shape in growth control. *Nature* **273,** 345–349
21. Jones, J. L., Royall, J. E., Critchley, D. R., and Walker, R. A. (1997) Modulation of myoepithelial-associated α6β4 integrin in a breast cancer cell line alters invasive potential. *Exp. Cell Res.* **235,** 325–333
22. Lu, S., Simin, K., Khan, A., and Mercurio, A. M. (2008) Analysis of integrin β4 expression in human breast cancer: association with basal-like tumors and prognostic significance. *Clin. Cancer Res.* **14,** 1050–1058
23. Falcioni, R., Kennel, S. J., Giacomini, P., Zupi, G., and Sacchi, A. (1986) Expression of tumor antigen correlated with metastatic potential of Lewis lung carcinoma and B16 melanoma clones in mice. *Cancer Res.* **46,** 5772–5778
24. Borradori, L., and Sonnenberg, A. (1999) Structure and function of hemidesmosomes: more than simple adhesion complexes. *J. Invest. Dermatol.* **112,** 411–418
25. Lipscomb, E. A., Dugan, A. S., Rabinovitz, I., and Mercurio, A. M. (2003) Use of RNA interference to inhibit integrin α6β4-mediated invasion and migration of breast carcinoma cells. *Clin. Exp. Metastasis* **20,** 569–576
26. Bon, G., Folgiero, V., Bossi, G., Felicioni, L., Marchetti, A., Sacchi, A., and Falcioni, R. (2006) Loss of β4 integrin subunit reduces the tumorigenicity of MCF-7 mammary cells and causes apoptosis upon hormone deprivation. *Clin. Cancer Res.* **12,** 3280–3287
27. Yang, X. H., Richardson, A. L., Torres-Arzayus, M. I., Zhou, P., Sharma, C., Kazarov, A. R., Andzelm, M. M., Strominger, J. L., Brown, M., and Hemler, M. E. (2008) CD151 accelerates breast cancer by regulating α6 integrin function, signaling, and molecular organization. *Cancer Res.* **68,** 3204–3213
28. Gambaletta, D., Marchetti, A., Benedetti, L., Mercurio, A. M., Sacchi, A., and Falcioni, R. (2000) Cooperative signaling between α6β4 integrin and ErbB2 receptor is required to promote phosphatidylinositol 3-kinase-dependent invasion. *J. Biol. Chem.* **275,** 10604–10610
29. Guo, W., Pylayeva, Y., Pepe, A., Yoshioka, T., Muller, W. J., Inghirami, G., and Giancotti, F. G. (2006) β4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis. *Cell* **126,** 489–502
30. Chung, J., Yoon, S. O., Lipscomb, E. A., and Mercurio, A. M. (2004) The Met receptor and α6β4 integrin can function independently to promote carcinoma invasion. *J. Biol. Chem.* **279,** 32287–32293
31. Yang, X., Kovalenko, O. V., Tang, W., Claas, C., Stipp, C. S., and Hemler, M. E. (2004) Palmitoylation supports assembly and function of integrin-tetraspanin complexes. *J. Cell Biol.* **167,** 1231–1240
32. Abdel-Ghany, M., Cheng, H. C., Elble, R. C., Lin, H., DiBiasio, J., and Pauli, B. U. (2003) The interacting binding domains of the β4 integrin and calcium-activated chloride channels (CLCAs) in metastasis. *J. Biol. Chem.* **278,** 49406–49416
33. Takada, Y., and Puzon, W. (1993) Identification of a regulatory region of integrin β1 subunit using activating and inhibiting antibodies. *J. Biol. Chem.* **268,** 17597–17601
34. Hynes, R. O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell* **110,** 673–687
35. Murgia, C., Blaikie, P., Kim, N., Dans, M., Petrie, H. T., and Giancotti, F. G. (1998) Cell cycle and adhesion defects in mice carrying a targeted deletion of the integrin β4 cytoplasmic domain. *EMBO J.* **17,** 3940–3951

Cross-talk between Integrin α6β4 and Insulin-like Growth Factor-1 Receptor (IGF1R) through Direct α6β4 Binding to IGF1 and Subsequent α6β 4-IGF1-IGF1R Ternary Complex Formation in Anchorage-independent Conditions

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Fig. S1

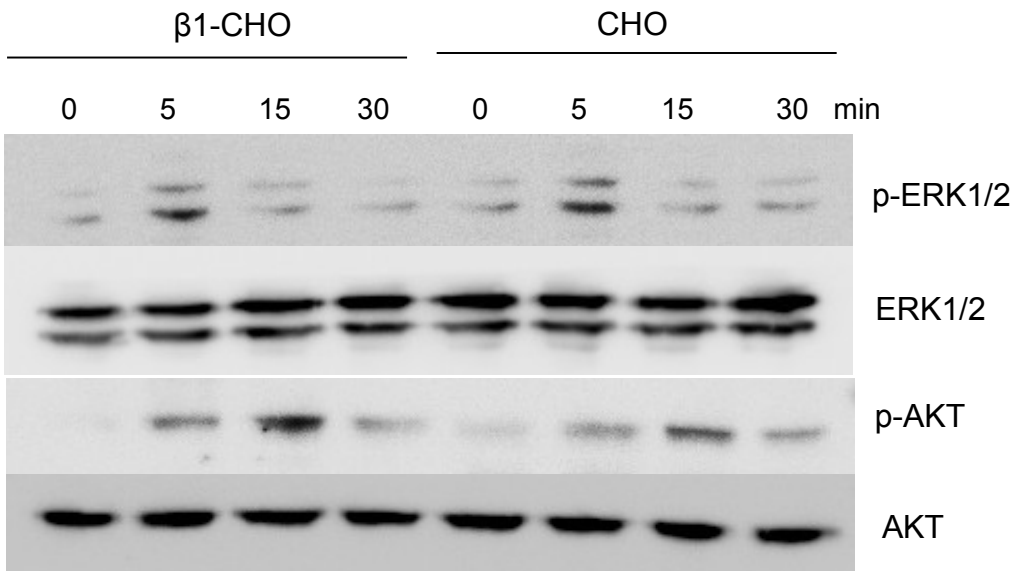

Fig. S1. IGF1 activates ERK1/2 and AKT in β1-CHO and parent CHO cells at comparable levels on polyHEMA-coated plates. We plated cells ($1x10^5$ cells) in well of 12-well plate and starved for 3 h in polyHEMA-coated plates, and stimulated with 100 ng/ml wt IGF1. Cell lysates were analyzed in Western blotting.