



Title	Cross-talk between Integrin $\alpha 6 \beta 4$ and Insulin-like Growth Factor-1 Receptor (IGF1R) through Direct $\alpha 6 \beta 4$ Binding to IGF1 and Subsequent $\alpha 6 \beta 4$ -IGF1-IGF1R Ternary Complex Formation in Anchorage-independent Conditions
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# Cross-talk between Integrin $\alpha 6 \beta 4$ and Insulin-like Growth Factor-1 Receptor (IGF1R) through Direct $\alpha 6 \beta 4$ Binding to IGF1 and Subsequent $\alpha 6 \beta 4$ -IGF1-IGF1R Ternary Complex Formation in Anchorage-independent Conditions<sup>\*[5]</sup>

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**Background:** Integrin  $\alpha \nu \beta 3$ -extracellular matrix interaction and/or  $\alpha \nu \beta 3$  binding to insulin-like growth factor-1 (IGF1; and integrin-IGF1-IGF1 receptor ternary complex formation) is critical for IGF signaling.

**Results:**  $\alpha 6 \beta 4$  directly bound to IGF1 and mediated IGF1 signaling through ternary complex formation.  $\alpha 6 \beta 4$  is required when cell-matrix adhesion is reduced or in three-dimensional culture.

**Conclusion:**  $\alpha 6 \beta 4$ -IGF1 binding is important for IGF signaling in anchorage-independent conditions.

**Significance:** The integrin-IGF interaction is a novel therapeutic target.

Integrin  $\alpha \nu \beta 3$  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  $\alpha \nu \beta 3$  (*i.e.* the binding of extracellular matrix proteins) enhances signaling induced by IGF1 binding to IGF1R. We recently reported that IGF1 directly binds to  $\alpha \nu \beta 3$  and induces  $\alpha \nu \beta 3$ -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  $\alpha \nu \beta 3$ , integrin  $\alpha 6 \beta 4$  is overexpressed in many cancers and is implicated in cancer progression. Here, we discovered that  $\alpha 6 \beta 4$  directly bound to IGF1, but not to R36E/R37E. Grafting the  $\beta 4$  sequence WPNSDP (residues 167–172), which corresponds to the specificity loop of  $\beta 3$ , to integrin  $\beta 1$  markedly enhanced IGF1 binding to  $\beta 1$ , suggesting that the WPNSDP sequence is involved in IGF1 recognition. WT IGF1 induced  $\alpha 6 \beta 4$ -IGF1-IGF1R ternary complex formation, whereas R36E/R37E did not. When cells were attached to matrix, exogenous IGF1 or  $\alpha 6 \beta 4$  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  $\alpha 6 \beta 4$ -dependent manner. Also IGF1 enhanced colony formation in soft agar in an  $\alpha 6 \beta 4$ -dependent manner. These results suggest that IGF binding to  $\alpha 6 \beta 4$  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  $\alpha \nu \beta 3$  plays a critical role in regulating insulin-like growth factor-1 (IGF1)<sup>2</sup> 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  $\alpha \nu \beta 3$  (*i.e.* the binding of extracellular matrix proteins such as vitronectin to  $\alpha \nu \beta 3$ ) enhances signaling induced by IGF1 binding to IGF1R (1).

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[5] This article contains supplemental Fig. S1.

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<sup>2</sup> 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)-2H-tetrazolium; ANOVA, analysis of variance.

Indeed, antagonists to  $\alpha \nu \beta 3$  block IGF1 signaling. Anti- $\alpha \nu \beta 3$  mAb and echistatin, a snake venom disintegrin that specifically inhibits  $\alpha \nu \beta 3$ , 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  $\alpha \nu \beta 3$ , 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  $\alpha \nu \beta 3$ -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,  $\alpha 6 \beta 4$ , which is overexpressed in many cancers, is involved in IGF1 signaling. We demonstrated that  $\alpha 6 \beta 4$  directly bound to IGF1, suggesting that this integrin plays a role in cancer progression and invasiveness through IGF signaling. WT IGF1 induced  $\alpha 6 \beta 4$ -IGF1-IGF1R ternary complex formation, but R36E/R37E did not. Notably, we demonstrated that  $\alpha 6 \beta 4$  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  $\alpha 6 \beta 4$  was synthesized as described (6). MCF-7 and CHO cells were obtained from American Type Culture Collection. CHO cells expressing human integrin  $\beta 1$  ( $\beta 1$ -CHO) or  $\beta 3$  ( $\beta 3$ -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  $\alpha 6 \beta 4$  ( $\alpha 6 \beta 4$ -CHO) have been described (9). Anti-phospho-ERK1/2 (Thr-202 and Tyr-204), anti-phospho-AKT (Thr-308), anti-phospho-IGF1R $\beta$  (Tyr-1135 and Tyr-1136), anti-integrin  $\beta 4$ , anti-ERK1/2, anti-AKT, anti-integrin  $\beta 1$ , and anti-IGF1R $\beta$  antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). HRP-conjugated anti-His tag antibody was purchased from Qiagen (Valencia, CA). Anti-hamster  $\beta 1$  mAb 7E2 (10, 11) was kindly provided by R. L. Juliano (University of North Carolina, Chapel Hill, NC). Anti- $\alpha 6$  mAb 135-13c and anti- $\beta 4$  mAb 439-9B were kind gifts from S. J. Kennel (University of Tennessee). Anti- $\alpha 6$  mAb G0H3 was a kind gift from A. Sonnenberg (Netherlands Cancer Institute). We obtained hybridoma of anti-human  $\beta 1$  mAb A1IB2 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<sub>2</sub>, 1 mM PMSF, 20 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 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<sup>2</sup>. Signaling assays were performed as described above, except that the cells were serum-starved for 3 h in DMEM without FCS.

**Coprecipitation of  $\alpha 6 \beta 4$ , IGF1R, and IGF1**— $\alpha 6 \beta 4$ -CHO or  $\beta 1$ -4-1-CHO cells were treated with WT IGF1 or R36E/R37E (100 ng/ml) for 15–30 min. We immunoprecipitated  $\beta 4$  or  $\beta 1$ -4-1 with anti- $\beta 4$  or anti- $\beta 1$  antibodies from cell lysates and analyzed the immunoprecipitated materials with antibodies specific to IGF1R,  $\beta 1$ , or  $\beta 4$  by Western blotting as described above.

**Binding of Soluble  $\alpha 6 \beta 4$** —We immobilized WT IGF1 or R36E/R37E (at a coating concentration of 20  $\mu$ g/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  $\alpha 6 \beta 4$  in HEPES/Tyrod's buffer containing 1 mM MnCl<sub>2</sub>, MgCl<sub>2</sub>, CaCl<sub>2</sub>, 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  $\alpha 6 \beta 4$  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  $\beta 1$  with the corresponding sequence of  $\beta 4$  was performed by site-directed mutagenesis as described (13). We replaced the CTSEQNCTS sequence of  $\beta 1$  (residues 187–195) with WPNSDP and generated -AKLRP( $\beta 1$ )(WPNSDP( $\beta 4$ ))( $\beta 1$ )PFSYKN sequence using oligonucleotide 5'-gctaagctcaggaacccttgccaaacagcgacccccatttagctacaaaaat-3' (designated the  $\beta 1$ -4-1 mutant). The presence of the mutation was confirmed by DNA sequencing. We transfected the  $\beta 1$ -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 ( $5 \times 10^4$  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  $\alpha 6 \beta 4$  Directly Binds to IGF1**—Integrin  $\alpha \nu \beta 3$ , 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  $\alpha \nu \beta 3$ -IGF1-IGF1R ternary complex formation, whereas R36E/R37E is defective in this function, suggesting that direct binding of IGF1 to  $\alpha \nu \beta 3$  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  $\alpha \nu \beta 3$  (17). Because  $\alpha 6 \beta 4$  is overexpressed in many cancer cell types (18), like  $\alpha \nu \beta 3$ , we hypothesized that  $\alpha 6 \beta 4$  is involved in IGF signaling in this cell type. To determine whether integrin  $\alpha 6 \beta 4$  directly interacts with IGF1, we used CHO cells expressing human  $\alpha 6 \beta 4$  (designated  $\alpha 6 \beta 4$ -CHO cells) by cotransfecting human  $\alpha 6$  and  $\beta 4$ . Cells stably expressing  $\alpha 6 \beta 4$  were cloned to obtain high expressers. The  $\alpha 6 \beta 4$ -CHO cells we used clonally expressed human  $\alpha 6$  and  $\beta 4$  (Fig. 1*a*). We discovered that  $\alpha 6 \beta 4$ -CHO cells adhered to IGF1 better than  $\beta 1$ -CHO cells (Fig. 1*b*). We confirmed that WT IGF1 and R36E/R37E (both His<sub>6</sub>-tagged) were coated onto plastic at an equal density using anti-His<sub>5</sub> tag antibody (data not shown). Interestingly,  $\alpha 6 \beta 4$ -CHO cells did not adhere to R36E/R37E, suggesting that  $\alpha 6 \beta 4$  binds to IGF1 in a manner similar to  $\alpha \nu \beta 3$  (Fig. 1*c*). Because antibodies against  $\alpha 6$  (G0H3) and  $\beta 4$  (439-9B) did not effectively block adhesion of  $\alpha 6 \beta 4$ -CHO cells to IGF1 (data not shown), we tested if recombinant soluble  $\alpha 6 \beta 4$  bound to IGF1 in ELISA-type assays. The soluble  $\alpha 6 \beta 4$  we used contains only the extracellular domains of  $\alpha 6$  and  $\beta 4$  and has been purified to homogeneity (6). We found that soluble  $\alpha 6 \beta 4$  bound to IGF1, but not well to R36E/R37E (Fig. 1*d*). Because only purified soluble  $\alpha 6 \beta 4$  and IGF1 were present in the assay system, we concluded that  $\alpha 6 \beta 4$  directly interacts with IGF1. We also tested if IGF1 is a cation-dependent ligand of  $\alpha 6 \beta 4$ , 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  $\alpha 6 \beta 4$  ligands in cation requirement.

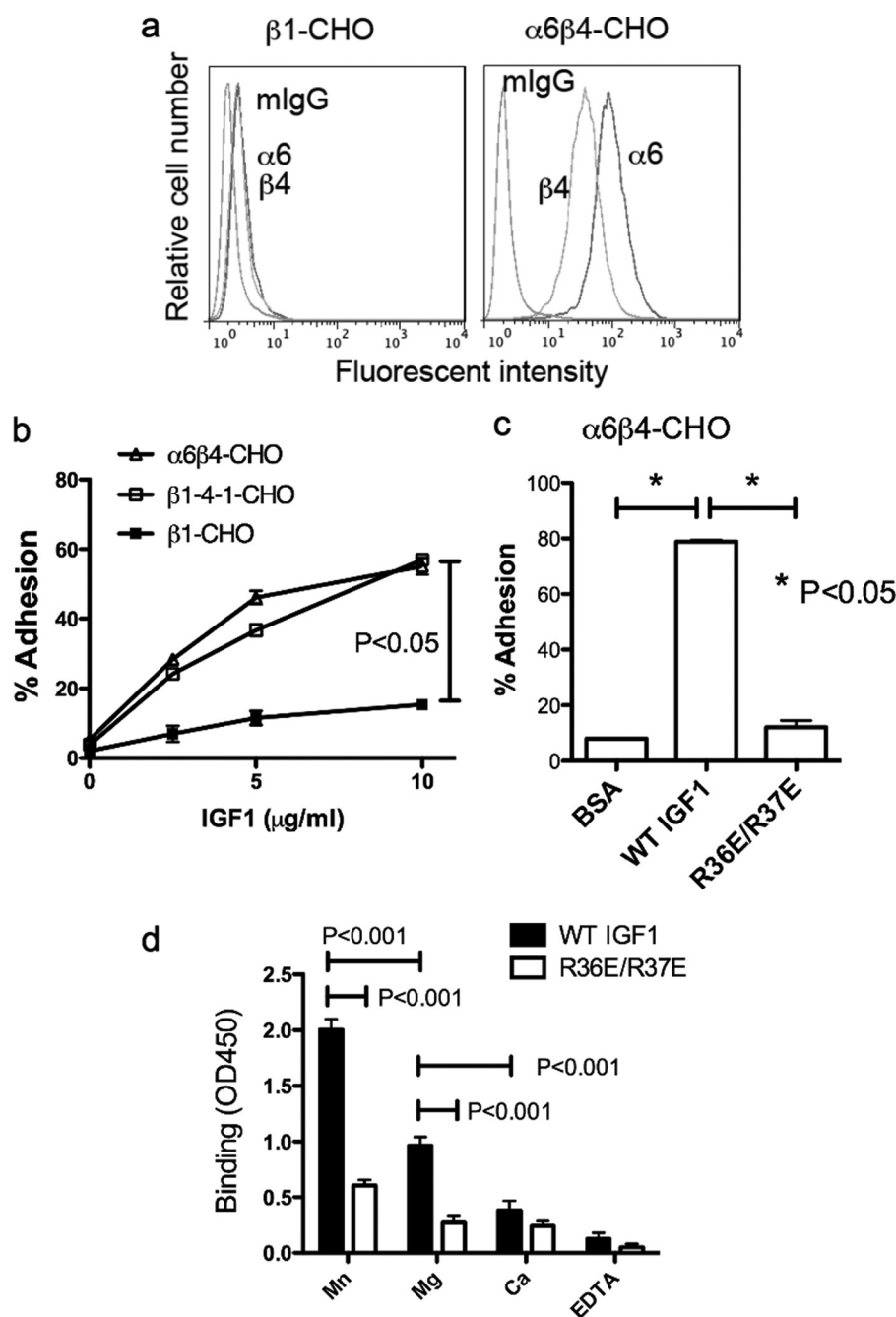
**Localization of IGF1-binding Site in  $\beta 4$** —We localized the IGF1-binding site in  $\beta 4$ . We previously reported that the disulfide-linked loop in the  $\beta 3$  subunit plays a role in recognizing IGF1 in  $\beta 3$  (5). The disulfide-linked specificity loop is not present in  $\beta 4$  and is replaced with remnant residues (Fig. 2*a*) (13). We hypothesized that IGF1 binds to the  $\beta 4$  sequence that corresponds to the specific loop in  $\beta 3$ . To test this hypothesis, we generated a  $\beta 1$  mutant in which the CTSEQNCTS sequence of  $\beta 1$  that contains the specificity loop was replaced with the corresponding WPNSDP sequence of  $\beta 4$  (designated the  $\beta 1$ -4-1 mutant). The  $\beta 1$ -4-1 mutant was stably expressed in CHO cells (designated  $\beta 1$ -4-1-CHO cells) and further cloned to obtain high expressers. In  $\beta 1$ -CHO and  $\beta 1$ -4-1-CHO cells,  $\beta 1$  and  $\beta 1$ -4-1 were expressed at comparable levels (Fig. 2*b*). We found that  $\beta 1$ -4-1-CHO cells adhered to WT IGF1 at a level comparable with  $\alpha 6 \beta 4$  (Fig. 1*b*), and inhibitory anti-human  $\beta 1$  mAb

AIB2 suppressed the adhesion of  $\beta 1$ -4-1-CHO cells to WT IGF1 (Fig. 2*c*). (Note that  $\beta 1$ -4-1 is >99% integrin  $\beta 1$ .) However,  $\beta 1$ -4-1-CHO cells only weakly bound to R36E/R37E (Fig. 2*d*) using  $\beta 3$ -CHO cells as controls. This suggests that the conserved Lys residues at positions 36 and 37 of IGF1 are involved in  $\alpha 6 \beta 4$  binding, as in  $\alpha \nu \beta 3$  binding. These results suggest that the region of  $\beta 4$  that corresponds to the specificity loop is involved in  $\alpha 6 \beta 4$  binding to WT IGF1.

**IGF Signaling Is Not Dependent on  $\alpha 6 \beta 4$  Expression in CHO Cells in Regular Tissue Culture Plates**—Integrin  $\alpha 6 \beta 4$  is a receptor for laminins, and thus, it is unclear if  $\alpha 6 \beta 4$  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  $\alpha 6 \beta 4$ -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  $\alpha 6 \beta 4$  expression, but may be dependent on  $\alpha 6 \beta 4$ -IGF interaction in anchorage-independent conditions in CHO cells.

**IGF Signaling Is Dependent on  $\alpha 6 \beta 4$  Expression in polyHEMA-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  $\alpha 6 \beta 4$  expression on IGF signaling in polyHEMA-coated plates: WT IGF1 induced ERK1/2 and AKT activation in  $\alpha 6 \beta 4$ -CHO cells in polyHEMA-coated plates, whereas WT IGF1 induced only weak ERK1/2 and AKT activation and the signals were quickly reduced in  $\beta 1$ -CHO cells (Fig. 4*a*) and parent CHO cells (supplemental Fig. S1). WT IGF1 enhanced cell survival in  $\alpha 6 \beta 4$ -CHO cells, whereas it had a negligible effect on cell survival in CHO and  $\beta 1$ -CHO cells (Fig. 4*b*). These results suggest that IGF signaling is dependent on  $\alpha 6 \beta 4$  expression in polyHEMA-coated plates, but not in regular tissue culture conditions. We found that WT IGF1 induced coprecipitation of IGF1R and integrin  $\beta 4$  in  $\alpha 6 \beta 4$ -CHO cells, whereas R36E/R37E was much less effective in this function (Fig. 4*c*). This suggests that  $\alpha 6 \beta 4$  binding to IGF1 is involved in the ternary complex formation in  $\alpha 6 \beta 4$ -CHO cells in a manner that is similar to  $\alpha \nu \beta 3$  (5). These findings are consistent with the idea that integrin  $\alpha 6 \beta 4$  is involved in IGF signaling.

**$\beta 1$ -4-1 Mimics  $\beta 4$  in Signaling Functions**—Although  $\beta 1$ -4-1-CHO cells adhered to IGF1 much better than  $\beta 1$ -CHO cells (at a level comparable with  $\alpha 6 \beta 4$ -CHO cells), it is unclear if the ability of  $\beta 1$ -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  $\beta 1$ -4-1-CHO cells than in  $\beta 1$ -CHO cells in polyHEMA-coated plates (Fig. 5*a*). Also, IGF1 enhanced cell survival in  $\beta 1$ -4-1-CHO cells, but only weakly in  $\beta 1$ -CHO cells

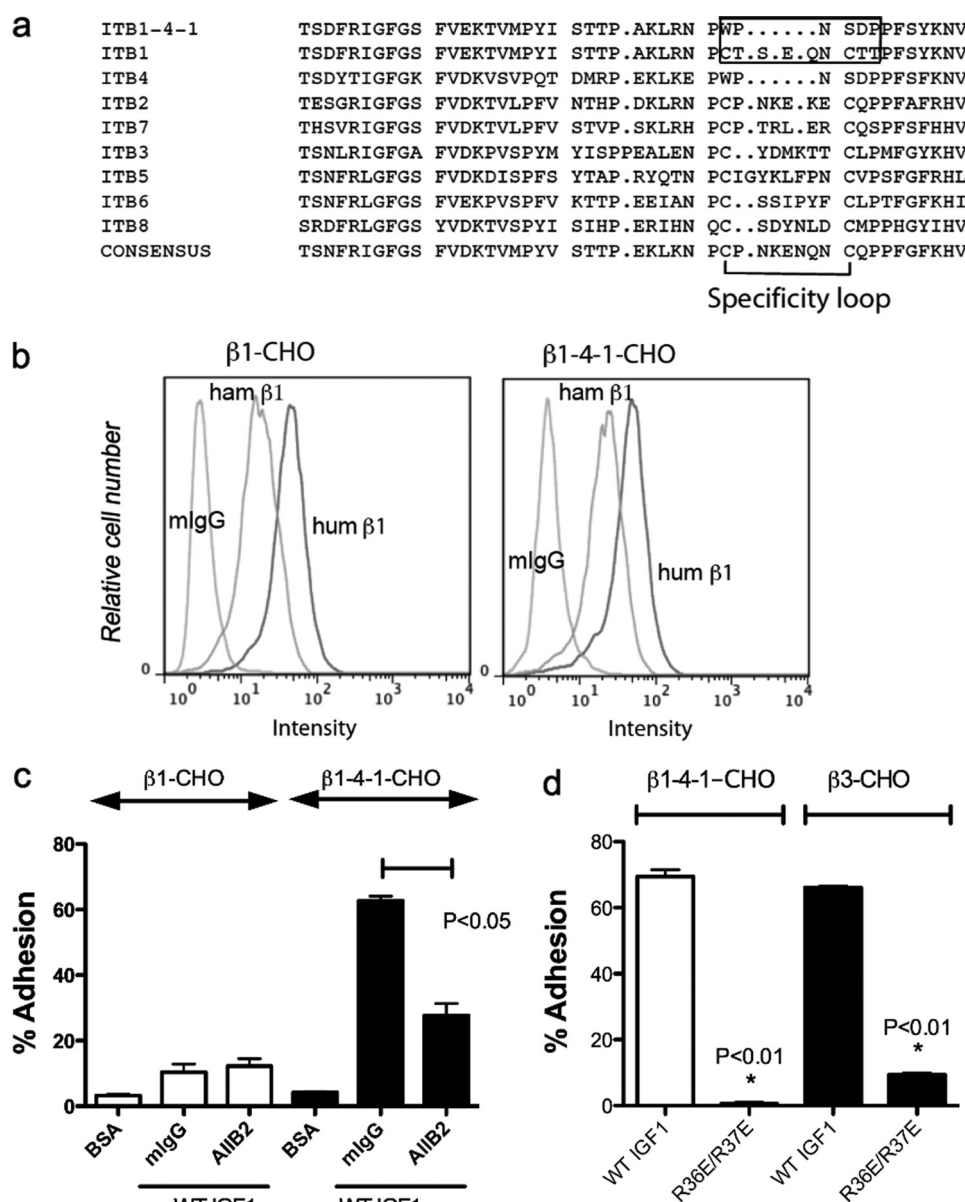


**FIGURE 1. Role of  $\alpha 6 \beta 4$  in IGF signaling.** *a*, expression of human  $\alpha 6$  and  $\beta 4$  integrins in  $\alpha 6 \beta 4$ -CHO cells. Expression of human integrin  $\alpha 6$  (mAb 138-13C) and  $\beta 4$  (mAb 439-9B) was analyzed by flow cytometry. The data show that  $\alpha 6 \beta 4$ -CHO cells clonally expressed  $\alpha 6$  and  $\beta 4$  integrins. *mlgG*, mouse IgG. *b*, adhesion of  $\alpha 6 \beta 4$ -CHO,  $\beta 1$ -4-1-CHO, and  $\beta 1$ -CHO cells to WT IGF1 and R36E/R37E. Adhesion assays were performed as described under "Experimental Procedures." Data are shown as means  $\pm$  S.E. ( $n = 3$ ). Statistical differences were tested by analysis of variance (ANOVA) and Tukey's multiple comparison test. *c*, adhesion of  $\alpha 6 \beta 4$ -CHO cells to WT IGF1 and R36E/R37E. WT IGF1 or R36E/R37E (20  $\mu\text{g/ml}$ ) was used for coating. Data are shown as means  $\pm$  S.E. ( $n = 3$ ). Statistical differences were tested by ANOVA and Tukey's multiple comparison test. *d*, binding of soluble  $\alpha 6 \beta 4$  to IGF1. WT IGF1 or R36E/R37E (20  $\mu\text{g/ml}$ ) was immobilized onto wells of 96-well microtiter plates. Data are shown as means  $\pm$  S.E. ( $n = 3$ ). Statistical differences were tested using *t* test.

in polyHEMA-coated plates (Fig. 5*b*). Anti- $\beta 1$  mAb A1B2 suppressed IGF1-induced cell survival of  $\beta 1$ -4-1-CHO cells, suggesting that IGF1-induced signaling is specific to  $\beta 1$ -4-1 (Fig. 5*c*). Also, WT IGF1 induced coprecipitation of  $\beta 1$ -4-1 and IGF1R, whereas R36E/R37E was defective in this function (Fig. 5*d*). Thus, the ability of  $\beta 1$ -4-1 to bind to IGF1 is directly related to enhanced IGF signaling. These results suggest that  $\beta 1$ -4-1 mimics  $\beta 4$  in IGF1 binding and signaling and that grafting the WPNSDP sequence of  $\beta 4$  to  $\beta 1$  dramatically changes the phe-

notype of  $\beta 1$ . Because the  $\beta 1$ -4-1 mutant does not contain the long cytoplasmic domain of  $\beta 4$ , it is likely that the  $\beta 1$  cytoplasmic domain is sufficient for mediating IGF signaling.

**Contribution of  $\alpha 6 \beta 4$  to IGF Signaling in Three-dimensional Culture**—Our results so far suggested that the contribution of integrin  $\alpha 6 \beta 4$  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



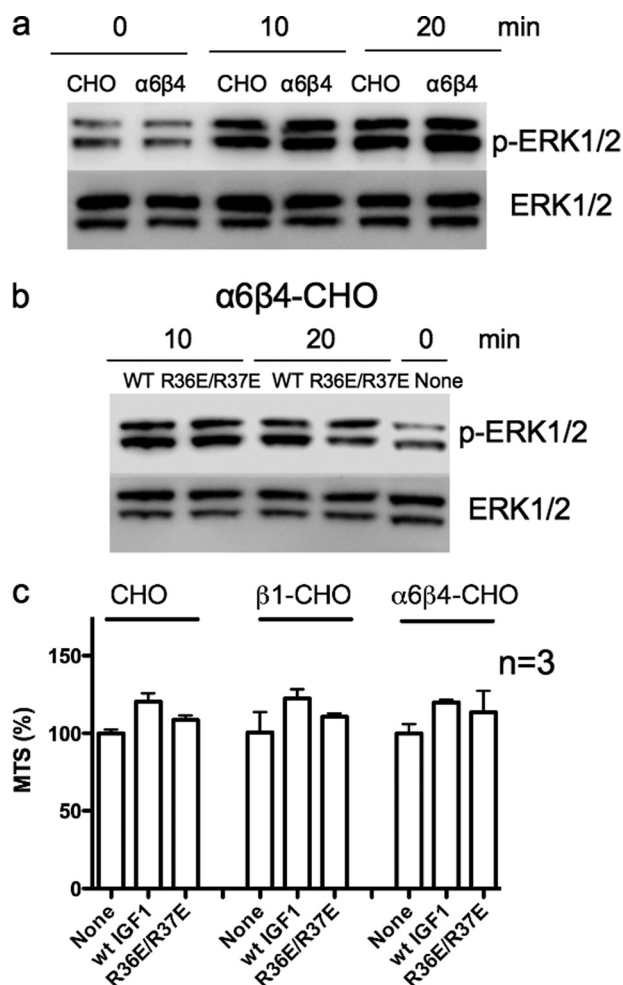
**FIGURE 2. Localization of IGF1-binding site in  $\beta 4$ .** *a*, alignment of the amino acid sequences around the specificity loop. *b*, expression of human integrin  $\beta 1$  on CHO cells.  $\beta 1$ -4-1- or  $\beta 1$ -transfected CHO cells were stained with anti-human (*hum*) integrin  $\beta 1$ , anti-hamster (*ham*) integrin  $\beta 1$ , and purified mouse IgG (*mlgG*) and analyzed by flow cytometry. *c*, effect of antibodies against  $\beta 1$  on cell adhesion to IGF1. WT IGF1 (20  $\mu\text{g}/\text{ml}$ ) was used for coating. Antibody concentrations were 10  $\mu\text{g}/\text{ml}$ . Data are shown as means  $\pm$  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  $\mu\text{g}/\text{ml}$ ) was used for coating. Data are shown as means  $\pm$  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  $\alpha 6\beta 4$  and are therefore suitable for testing the role of this integrin in IGF signaling. We stably expressed WT IGF1 or R36E/R37E in  $\alpha 6\beta 4$ -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  $\alpha 6\beta 4$ -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  $\alpha 6\beta 4$  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  $\alpha \nu \beta 3$  or  $\alpha 6\beta 4$ . CHO cells only weakly respond to WT IGF1 in anchorage-independent conditions, but CHO cells that express  $\alpha \nu \beta 3$  (5) or  $\alpha 6\beta 4$  (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-





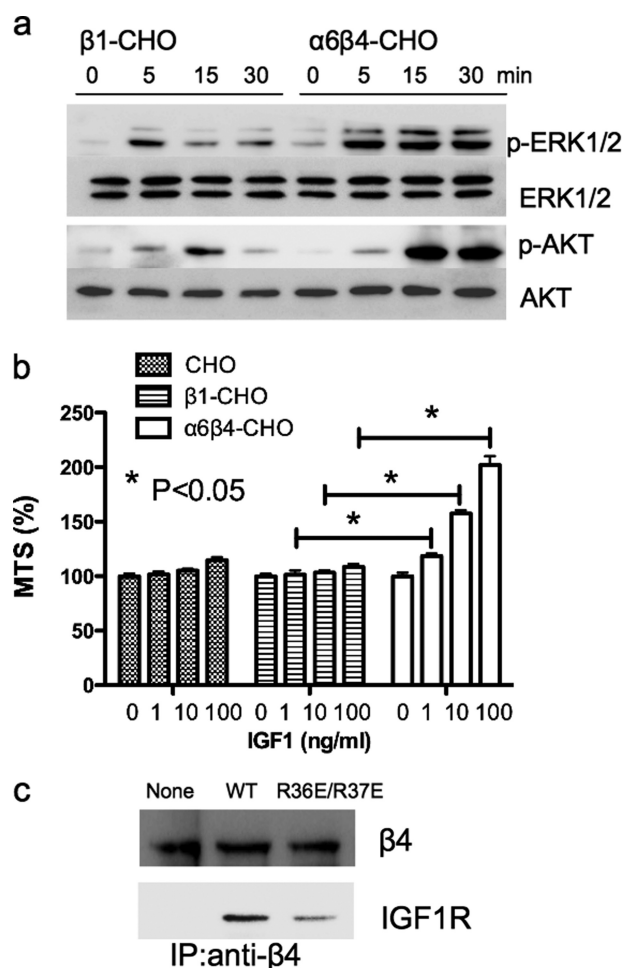
**FIGURE 3. IGF signaling in CHO cells in regular tissue culture plates.** *a*, time course of ERK1/2 activation by WT IGF1. We stimulated serum-starved (overnight) CHO or  $\alpha 6\beta 4$ -CHO cells with IGF1 (100 ng/ml) and analyzed cell lysates by Western blotting. *b*, effect of R36E/R37E on ERK1/2 activation in  $\alpha 6\beta 4$ -CHO cells. We stimulated serum-starved (overnight)  $\alpha 6\beta 4$ -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,  $\beta 1$ -CHO, or  $\alpha 6\beta 4$ -CHO cells ( $5 \times 10^3$  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  $\pm$  S.E. ( $n = 3$ ).

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

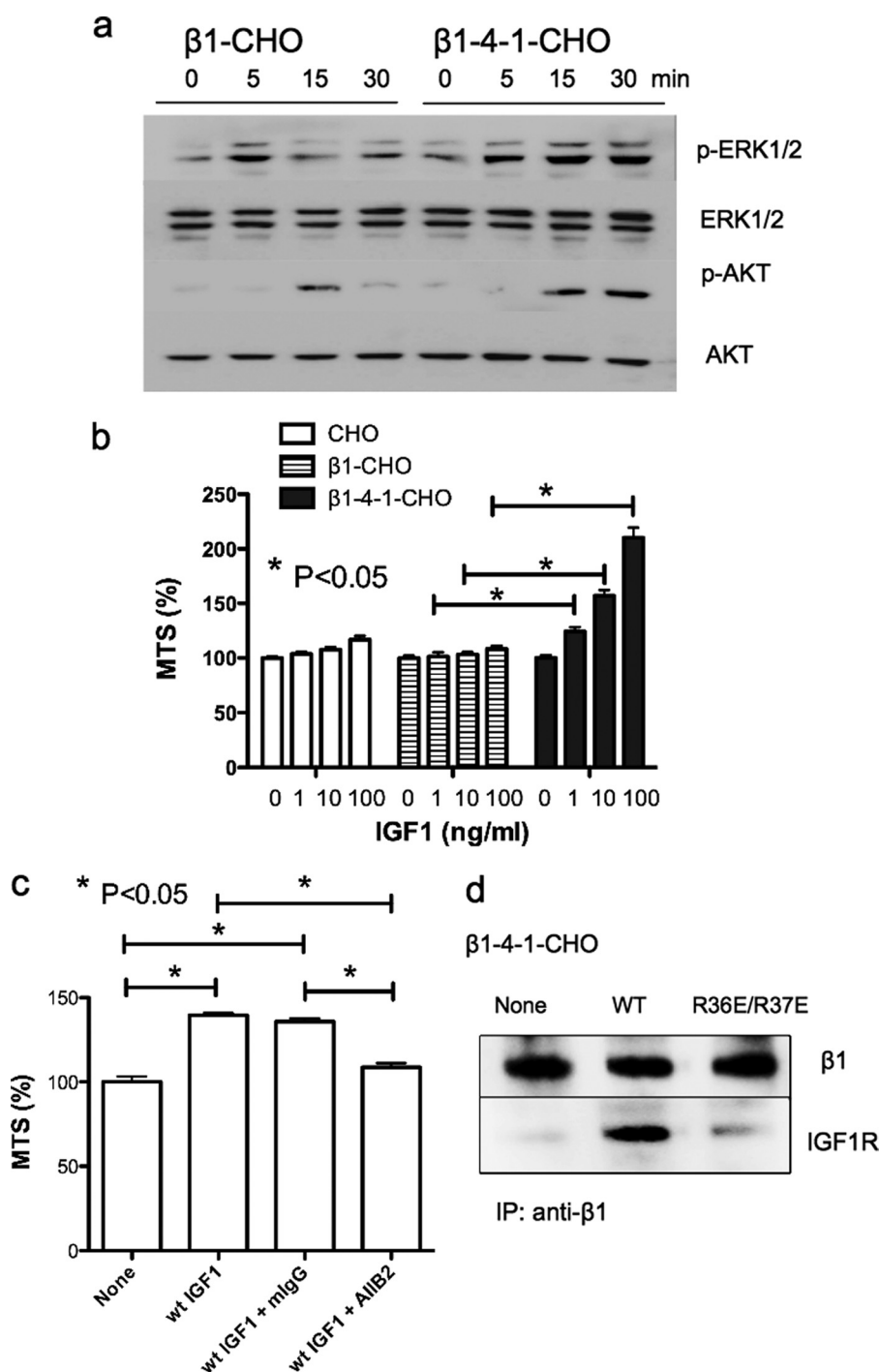
In this study, we established that integrin  $\alpha 6\beta 4$  plays a role in IGF signaling. WT IGF1 directly and specifically bound to integrin  $\alpha 6\beta 4$  and induced integrin  $\alpha 6\beta 4$ -IGF1-IGF1R ternary complex formation in  $\alpha 6\beta 4$ -CHO cells, whereas R36E/R37E was defective in these functions, as in the case of  $\alpha v\beta 3$  (5). It is thus highly likely that the ability of IGF1 to bind to  $\alpha 6\beta 4$  and to induce ternary complex formation is involved in IGF1 signaling.

The expression of  $\alpha 6\beta 4$  is associated with poor patient prognosis and reduced survival in a variety of human cancers (21, 22). The integrin  $\beta 4$  subunit was originally identified as a



**FIGURE 4.  $\alpha 6\beta 4$ -dependent IGF1 signaling in polyHEMA-coated plates.** *a*, IGF1 enhanced ERK1/2 and AKT activation in  $\alpha 6\beta 4$ -CHO cells much more strongly than in  $\beta 1$ -CHO cells. Cells ( $1 \times 10^5$  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*,  $\alpha 6\beta 4$ -CHO cells responded better to IGF1 compared with  $\beta 1$ -CHO cells on polyHEMA-coated plates. CHO,  $\beta 1$ -CHO, or  $\alpha 6\beta 4$ -CHO cells ( $2 \times 10^4$  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  $\beta 4$  with IGF1R in  $\alpha 6\beta 4$ -CHO cells. Serum-starved  $\alpha 6\beta 4$ -CHO cells were treated with 100 ng/ml WT IGF1 or R36E/R37E for 30 min, and integrin  $\beta 4$  was immunoprecipitated. Immunoprecipitated materials were analyzed by Western blotting. The data show that WT IGF1 induced coprecipitation of integrin  $\beta 4$  and IGF1R, whereas R36E/R37E was defective in this function. IP, immunoprecipitate.

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,  $\alpha 6\beta 4$  promotes motility and invasion in carcinoma cells (24). Moreover, suppression of  $\alpha 6\beta 4$  expression by siRNA diminishes invasive and tumorigenic potential (25, 26).  $\alpha 6\beta 4$  thus contributes to tumor progression, metastasis, tumor development, and primary tumor growth (27). Integrin  $\alpha 6\beta 4$  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  $\beta 4$  cytoplasmic domain revealed that integrin  $\beta 4$  signaling plays a role in mammary tumor progression (29). However, it has not been fully established how  $\alpha 6\beta 4$  is



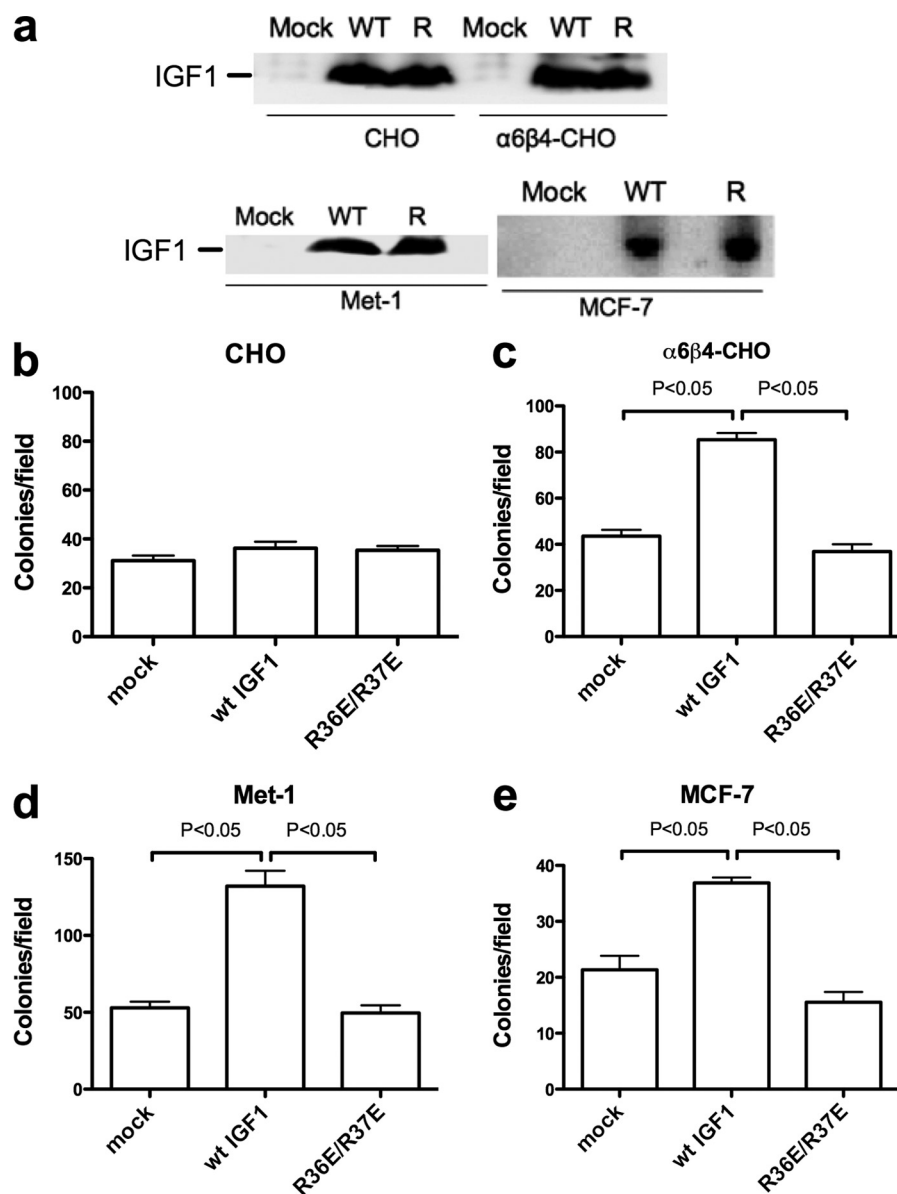
**FIGURE 5. Effect of  $\beta 1$ -4-1 mutation on IGF1 signaling.** *a*, IGF1 enhanced ERK1/2 and AKT activation in  $\beta 1$ -4-1-CHO cells much strongly than in CHO or  $\beta 1$ -CHO cells. Cells ( $1 \times 10^5$  cells) were plated in wells of 12-well plates, starved for 3 h, and stimulated with 100 ng/ml WT IGF1. Cell lysates were analyzed by Western blotting. *b*, WT IGF1 enhanced cell survival in  $\beta 1$ -4-1-CHO cells, but not in CHO or  $\beta 1$ -CHO, on polyHEMA-coated plates.  $\beta 1$ -4-1-CHO, CHO, or  $\beta 1$ -CHO cells ( $2 \times 10^4$  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  $\beta 1$  mAb AIB2 suppressed enhanced cell survival induced by WT IGF1. The mAb was used at 10  $\mu$ g/ml. Statistical differences were tested by ANOVA and Tukey's multiple comparison test ( $n = 6$ ). *d*, WT IGF1 induced coprecipitation of IGF1R and  $\beta 1$ -4-1, whereas R36E/R37E was defective in this function. Serum-starved  $\beta 1$ -4-1-CHO cells were treated with 100 ng/ml WT IGF1 or R36E/R37E for 15 min at 37  $^{\circ}$ C, and integrin  $\beta 1$  was immunopurified from cell lysates. Immunopurified materials were analyzed by Western blotting. *IP*, immunoprecipitate.

involved in cancer progression. We propose that  $\alpha 6\beta 4$  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  $\alpha 6\beta 4$ -CHO cells, but not in  $\beta 1$ -CHO cells, in anchorage-inde-

pendent conditions. However, in regular culture conditions, the effect of  $\alpha 6\beta 4$  expression on signaling was not detectable. This is probably because the effect of  $\alpha 6\beta 4$  on IGF signaling is masked by the massive signals from cell-matrix adhesion that enhance IGF signaling, as in the case of  $\alpha v\beta 3$  (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,  $\alpha 6 \beta 4$ -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<sub>6</sub> antibodies (IGF1 has an N-terminal His<sub>6</sub> 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,  $\beta 1$ -CHO, or  $\alpha 6 \beta 4$ -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. Con-

sistent with this idea, we detected a clear proliferative/anti-apoptotic effect of WT IGF1 in  $\alpha 6 \beta 4$ -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  $\alpha 6 \beta 4$  in IGF signaling because they express IGF1R, but not endogenous  $\alpha 6 \beta 4$ . 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  $\alpha 6\beta 4$  and  $\alpha \nu\beta 3$ , it is possible that more than one integrin is involved in IGF signaling. Indeed, we found that knockdown or overexpression of  $\beta 4$  in MCF-7 cells did not affect IGF signaling.<sup>3</sup> It is possible that integrins other than  $\alpha 6\beta 4$  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*,<sup>4</sup> suggesting that R36E/R37E is a dominant-negative mutant by definition. Furthermore, R36E/R37E suppressed tumorigenesis *in vivo*.<sup>4</sup> 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  $\alpha 6\beta 4$  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,  $\alpha 6\beta 4$  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,  $\alpha 6\beta 4$  cooperates with growth factor receptors and other surface molecules to amplify intracellular signaling pathways (28, 30, 31). We propose that  $\alpha 6\beta 4$  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  $\beta 1$ -4-1 mutation effectively induced the binding of the  $\beta 1$  integrins to WT IGF1, but not to R36E/R37E. This suggests that the WPNSDP sequence of  $\beta 4$  (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  $\beta 4$  that is common to other  $\beta 4$  ligands. Point mutations (K150A and Q155L) of  $\beta 4$  suppress binding of  $\alpha 6\beta 4$  to laminin-5 (32). Also, a GST fusion protein of the  $\beta 4$  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  $\beta 4$  (data not shown). The anti- $\alpha 6$

(G0H3, function-blocking) and anti- $\beta 4$  (439-9B, non-function-blocking) antibodies we used did not suppress IGF1 binding to  $\alpha 6\beta 4$ . Epitopes for these antibodies have not been well defined. One possible reason that G0H3 did not block IGF1 binding to  $\alpha 6\beta 4$  is that IGF1 is much smaller than laminins, known  $\alpha 6\beta 4$  ligands, and therefore, G0H3 did not block access of IGF1 to its binding site. In contrast, anti- $\beta 1$  mAb AIIB2 suppressed IGF1 binding to  $\beta 1$ -4-1-CHO cells. We reported that function-blocking (e.g. AIIB2) and activating (e.g. TS2/16) anti- $\beta 1$  mAbs recognize overlapping epitopes within residues 207–218 of  $\beta 1$  (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  $\beta 1$  in one way or the other and inhibits or activates  $\beta 1$  integrins rather than affecting the access of ligands to  $\beta 1$  integrins (33). This may explain why AIIB2 suppressed IGF1 binding to  $\beta 1$ -4-1-CHO cells, although AIIB2 may not directly block the access of IGF1 to the ligand-binding site (33).

$\alpha 6\beta 4$  is distinct from other integrin receptors because the  $\beta 4$  subunit contains a 1000-amino acid cytoplasmic domain (34). It has been proposed that this cytoplasmic domain is essential for coupling  $\alpha 6\beta 4$  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  $\beta 1$ -4-1-CHO cells, as in  $\alpha 6\beta 4$ -CHO cells, but not in CHO or  $\beta 1$ -CHO cells, although the  $\beta 1$ -4-1 mutant does not have the large cytoplasmic domain of  $\beta 4$ . 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  $\beta 4$  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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<sup>3</sup> M. Fujita, Y. K. Takada, and Y. Takada, unpublished data.

<sup>4</sup> M. Fujita, A. D. Borowsky, R. D. Cardiff, Y. K. Takada, and Y. Takada, submitted for publication.

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**Cross-talk between Integrin  $\alpha 6\beta 4$  and Insulin-like Growth Factor-1 Receptor (IGF1R) through Direct  $\alpha 6\beta 4$  Binding to IGF1 and Subsequent  $\alpha 6\beta 4$ -IGF1-IGF1R Ternary Complex Formation in Anchorage-independent Conditions**

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Fig. S1

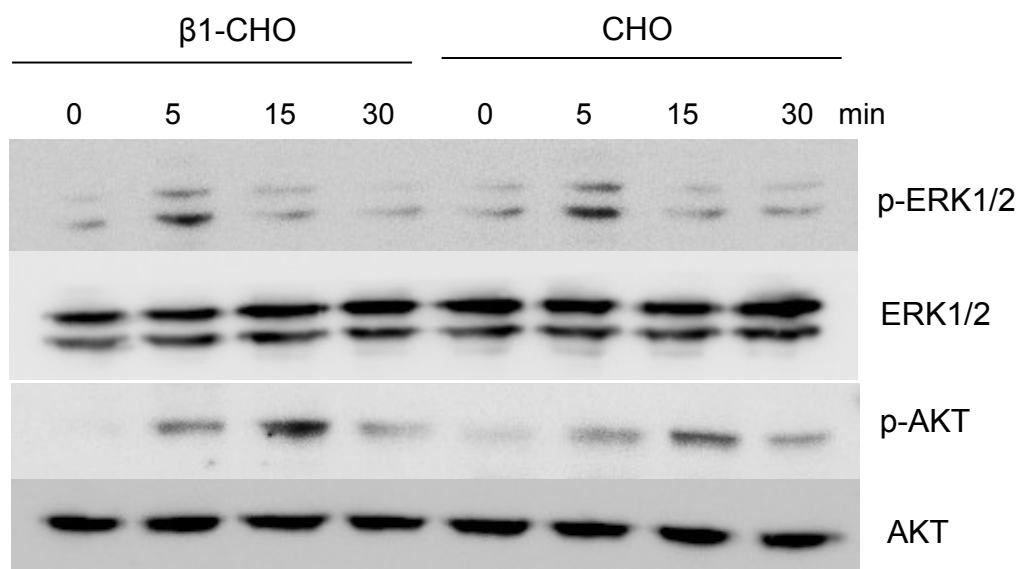


Fig. S1. IGF1 activates ERK1/2 and AKT in  $\beta$ 1-CHO and parent CHO cells at comparable levels on polyHEMA-coated plates. We plated cells ( $1 \times 10^5$  cells) in well of 12-well plate and starved for 3 h in polyHEMA-coated plates, and stimulated with 100 ng/ml wt IGF1. Cell lysates were analyzed in Western blotting.