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*

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Background: Integrin αβ3-extracellular matrix interaction and/or αβ3 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 αβ3 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 αβ3 (i.e. the binding of extracellular matrix proteins) enhances signaling induced by IGF1 binding to IGF1R. We recently reported that IGF1 directly binds to αβ3 and induces αβ3-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 αβ3, 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 αβ3 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/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 αβ3 (i.e. the binding of extracellular matrix proteins such as vitronectin to αβ3) enhances signaling induced by IGF1 binding to IGF1R (1).
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Indeed, antagonists to αβ3 block IGF1 signaling. Anti-αβ3 mAb and echistatin, a snake venom disintegrin that specifically inhibits αβ3, 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 αβ3, 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 αβ3-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 αβ4-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 αβ1 (αβ1-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 αβ4 (αβ4-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 Cell Signaling Technology, Inc. (Danvers, MA). HRP-conjugated anti-β3 antibodies were pur chased from Qiagen (Valencia, CA). Anti-hamster β3 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 A11B2 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 MgCl2, 1 mM PMSF, 20 mM NaF, 1 mM Na3VO4, 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). polyHEMA-coated plates were prepared as described (12), except that the final polyHEMA concentration was 1.2 mg/cm2. 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 MnCl2, MgCl2, CaCl2, 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)PSYKSN sequence using oligonucleotide 5’-gtaagcttaggaacctggccaaacagcgacctcccccttgactacaaaat-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 pBl-1 vector together with the pNeo 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 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 MgCl2, 1 mM PMSF, 20 mM NaF, 1 mM Na3VO4, 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). polyHEMA-coated plates were prepared as described (12), except that the final polyHEMA concentration was 1.2 mg/cm2. Signaling assays were performed as described above, except that the cells were serum-starved for 3 h in DMEM without FCS.

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).
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RESULTS

**Integrin α6β4 Directly Binds to IGF1**—Integrin ανβ3, 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 ανβ3-IGF1-IGF1R ternary complex formation, whereas R36E/R37E is defective in this function, suggesting that direct binding of IGF1 to ανβ3 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 ανβ3 (17). Because α6β4 is overexpressed in many cancer cell types (18), like ανβ3, 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. 1a). We discovered that α6β4-CHO cells adhered to IGF1 better than β1-CHO cells (Fig. 1b). We confirmed that WT IGF1 and R36E/R37E (both His6-tagged) were coated onto plastic at an equal density using anti-His 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 ανβ3 (Fig. 1c). Because antibodies against α6 (GOH3) 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 extra-cellular 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. 1d). 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. 2a) (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. 2b). We found that β1-4-1-CHO cells adhered to WT IGF1 at a level comparable with α6β4 (Fig. 1b), and inhibitory anti-human β1 mAb AIIb2 suppressed the adhesion of β1-4-1-CHO cells to WT IGF1 (Fig. 2c). (Note that β1-4-1 is >99% integrin β1.) However, β1-4-1-CHO cells only weakly bound to R36E/R37E (Fig. 2d) 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 ανβ3 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. 3a), and the effect of the R36E/R37E mutation on IGF1-induced ERK1/2 activation was not clear (Fig. 3b). Also, the effect of R36E/R37E on IGF1-induced cell survival was not clear (Fig. 3c). 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 poly-HEMA-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 poly-HEMA-coated plates: WT IGF1 induced ERK1/2 and AKT activation in α6β4-CHO cells in poly-HEMA-coated plates, whereas WT IGF1 induced only weak ERK1/2 and AKT activation and the signals were quickly reduced in β1-CHO cells (Fig. 4a) 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. 4b). These results suggest that IGF signaling is dependent on α6β4 expression in poly-HEMA-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. 4c). 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 ανβ3 (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 poly-HEMA-coated plates (Fig. 5a). Also, IGF1 enhanced cell survival in β1-4-1-CHO cells, but only weakly in β1-CHO cells.
in polyHEMA-coated plates (Fig. 5b). Anti-β1 mAb AllB2 suppressed IGF1-induced cell survival of β1-4-1-CHO cells, suggesting that IGF1-induced signaling is specific to β1-4-1 (Fig. 5c). Also, WT IGF1 induced coprecipitation of β1-4-1 and IGF1R, whereas R36E/R37E was defective in this function (Fig. 5d). 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
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 /H92516/H92524 and are therefore suitable for testing the role of this integrin in IGF signaling. We stably expressed WT IGF1 or R36E/R37E in /H92516/H92524-CHO or CHO cells in the pSec-TagB secretion vector. Transfected cells secreted WT IGF1 and R36E/R37E at comparable levels (Fig. 6a). 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 /H92516/H92524-CHO cells (Fig. 6b), but not in CHO cells (Fig. 6c). 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 /H92516/H92524. CHO cells only weakly respond to WT IGF1 in anchorage-independent conditions, but CHO cells that express αβ3 (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. 6d) and Met-1 (Fig. 6e) cells in soft agar, whereas the integrin binding-
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 3. IGF signaling in CHO cells in regular tissue culture plates.](image)

![FIGURE 4. α6β4-dependent IGF1 signaling in polyHEMA-coated plates.](image)
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
did not detect an effect of WT IGF1 or R36E/R37E in regular tissue culture conditions in CHO, α6β4-CHO, H9251-CHO, or H9252-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 IGFR1, 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

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, antibodies (IGF1 has an N-terminal His, 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).
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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. 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, suggesting that R36E/R37E is a dominant-negative mutant by definition. Furthermore, R36E/R37E suppressed tumorigenesis in vivo. Why is the effect of R36E/R37E detected in the WPNSDP sequence? It is likely that the WPNSDP sequence (residues 157–185) directly binds to calcium-activated chloride channels 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-CHO mutant 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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