The Direct Binding of Insulin-like Growth Factor-1 (IGF-1) to Integrin αvβ3 Is Involved in IGF-1 Signaling*

Jun Saegusa†‡, Satoshi Yamaji†§, Katsuaki Ieguchi‡, Chun-Yi Wu‡, Kit S. Lam‡, Fu-Tong Liu‡, Yoko K. Takada‡, and Yoshikazu Takada‡

From the Departments of †Dermatology and ‡Hematology/Oncology, University of California Davis School of Medicine, Sacramento, California 95817

It has been proposed that ligand occupancy of integrin αvβ3 with extracellular matrix ligands (e.g. vitronectin) plays a critical role in insulin-like growth factor-1 (IGF-1) signaling. We found that expression of αvβ3 enhanced IGF-1-induced proliferation of Chinese hamster ovary cells in serum-free conditions (in the absence of vitronectin). We hypothesized that the direct integrin binding to IGF-1 may play a role in IGF-1 signaling. We demonstrated that αvβ3 specifically and directly bound to IGF-1 in cell adhesion, enzyme-linked immunosorbent assay-type binding, and surface plasmon resonance studies. We localized the amino acid residues of IGF-1 that are critical for integrin binding by docking simulation and mutagenesis. We found that mutating two Arg residues at positions 36 and 37 in the C-domain of IGF-1 to Glu (the R36E/R37E mutation) effectively reduced integrin binding. Interestingly, although the mutant still bound to IGF1R, it was defective in inducing IGF1R phosphorylation, AKT and ERK1/2 activation, and cell proliferation. Furthermore wild type IGF-1 mediated co-precipitation of αvβ3 and IGF1R, whereas the R36E/R37E mutant did not suggest that IGF-1 mediates the interaction between αvβ3 and IGF1R. These results suggest that the direct binding to IGF-1 to integrin αvβ3 plays a role in IGF-1 signaling through ternary complex formation (αvβ3-IGF-1/IGF1R), and integrin-IGF-1 interaction is a novel target for drug discovery.

Integrins are a family of cell adhesion receptors that mediate cell-extraacellular matrix (ECM) interaction and cell-cell interaction (1). It has been proposed that signaling from inside the cells regulates the ligand binding affinity of integrins (inside-out signaling) (2). Each integrin is a heterodimer containing α and β subunits. At present 18 α and 8 β subunits have been identified that combine to form 24 integrins (3).

It has been reported that integrin αvβ3 plays a role in cancer proliferation and invasiveness. High levels of integrin αvβ3 correlate with growth and/or progression of melanoma (4, 5), neuroblastoma (6), breast cancer (7, 8), colon cancer (9), ovarian cancer (10), and cervical cancer (11). Moreover, individuals homozygous for the β3L33P polymorphism that enhances the ligand binding affinity of β3 integrins have an increased risk to develop breast cancer, ovarian cancer, and melanoma (12). However, it remains unclear whether and how increased levels of αvβ3 on tumor cells contribute to cancer development.

Insulin-like growth factor-1 (IGF-1) is a polypeptide hormone (75 kDa) that has a high degree of structural similarity to human proinsulin. IGF-1 acts through binding to the type I IGF receptor (IGF1R), a receptor tyrosine kinase. The IGF1R is a heterotetramer that consists of two α-subunits that contain the ligand-binding domains and two β-subunits that contain the tyrosine kinase activity. After ligand binding, the receptor undergoes a conformational change resulting in the activation of the tyrosine kinase, which results in transphosphorylation of the opposite β-subunit on specific tyrosine residues. These phosphotyrosines then bind to adapter molecules such as Shc and IRS-1. Phosphorylation of these proteins leads to activation of the phosphatidylinositol 3-kinase 3-kinase and mitogen-activated protein kinase (MAPK) signaling pathways (reviewed in Ref. 13).

IGF-1 has been implicated in cancer progression (14). One of the major actions of IGF-1 is to inhibit apoptosis. IGF-1 confers resistance to chemotherapy and radiation therapy. IGF-1 expression levels are increased in breast, lung, prostate, and many other cancers. Several strategies to target IGF-1 signaling have been extensively studied, including small interfering RNA and monoclonal antibodies for IGF1R and kinase inhibitors to inhibit the enzymatic activity of the receptor. The IGF-1 system is a therapeutic target for cancer, and elucidation of the IGF-1 signaling pathway should have a major impact in designing new therapeutic strategies.

It has been proposed that ligand occupancy of αvβ3 with ECM ligands such as vitronectin plays a critical role in enhancing IGF-1 signaling (14). It has been reported that inhibiting αvβ3-ECM interaction (“ligand occupancy”) of αvβ3 inhibited IGF-1 actions selectively in cell types that express αvβ3 (14). Inhibiting ligand occupancy of αvβ3 blocked IGF-1-induced cell migration (15), DNA synthesis, IRS-1 phosphorylation, and IGF1R-linked downstream signaling events, such as activation of phosphatidylinositol 3-kinase and ERK1/2 (16).

In the present study, we demonstrated that expression of αvβ3 enhanced proliferation of ovarian cancer cells in the pres-
ence of fetal bovine serum (FBS) and in serum-free conditions if IGF-1 was present. This suggests that IGF-1 is involved in enhanced proliferation of αββ3-expressing cells. We demonstrated that αββ3 bound to IGF-1 in several different binding assays. We found that two Arg residues at positions 36 and 37 in the C-domain of IGF-1 are critical for integrin binding by docking simulation and mutagenesis. Mutation of these Arg residues to Glu (the R36E/R37E mutation) effectively reduced integrin binding. Interestingly, the R36E/R37E mutant was defective in inducing cell proliferation and IGF-1 intracellular signaling, although it still bound to IGF1R. We demonstrated that wild type IGF-1 mediated co-precipitation of αββ3 and IGF1R, whereas the R36E/R37E mutant did not, suggesting that IGF-1 mediates the interaction between αββ3 and IGF1R. These results suggest that the direct binding to IGF-1 plays a role in IGF-1 signaling.

EXPERIMENTAL PROCEDURES

Materials

7E3 (anti-human integrin β3) and ALIB2 (anti-human integrin β1) hybridomas were obtained from ATCC. Chinese hamster ovary (CHO) cells that express human β3 and β1 and the β1-3-1 mutant have been described (17). The human IGF1R expression construct (18) was kindly provided by Rita Slaaby (Novo Nordisk A/S, Måløv, Denmark). Recombinant human soluble IGF1R (Met1–Asn932) was purchased from R & D Systems. Human fibroblast growth factor-1 (FGF1) (19) and regulin-1 (20) were synthesized as described in the cited references. Human recombinant IGF-1 (>97% pure) was obtained from R & D systems and used as a control.

Methods

Synthesis of IGF-1—A cDNA fragment encoding IGF-1 was amplified by PCR with synthetic oligonucleotides 5′-ccgagctcatcaagctgacgcaagttacagtctg-3′ and 5′-gttcggggtcgtcaggctgctcgg-3′ with human placenta cDNA library as a template. After Ncol/Xhol digestion, the cDNA fragment was subcloned into the Ncol/Xhol site of PET28a vector. The expression construct encodes IGF-1 (residues 49–118) with a His6 tag at the C terminus. MGPETLTCGAELVDALQFVC-GDGRGFYFNKTPGYGSSSSRRAAPQTGIVDECCFRSCDLRR-LEMYCAPLKPASKALEHHHHHH. Protein was expressed in Escherichia coli BL21 as insoluble proteins and purified using nickel-nitrioltriatomic acid affinity chromatography under denaturing conditions using the C-terminal His6 tag of the protein. To remove endotoxin, we washed the nickel-nitrioltriatomic acid resin with 1% Triton X-114 before eluting the bound protein. Purified proteins were refolded in vitro following the protocols (“Isolation of proteins from inclusion bodies” available from the Björkman laboratory). Briefly, purified proteins in 8 M urea were diluted into refolding buffer (100 mM Tris-HCl, pH 8.0, 400 mM l-Arg, 2 mM EDTA, 0.5 mM oxidized glutathione, 5 mM reduced glutathione, and protease inhibitors), kept for 8 h at 4 °C, and then concentrated by ultrafiltration. Refolded WT and mutant IGF-1 proteins were over 90% in a monomeric form and contain some polymerized forms in SDS-PAGE under nonreduced conditions. We obtained 2–5 mg of purified proteins from 1 liter of bacterial culture. Protein concentration of IGF-1 was determined by measuring A280.

Cell Proliferation—Cell proliferation was assessed based on the ability of the cells to convert MTS into formazan, using the Aqueous cell proliferation assay kit (Promega, Madison, WI). The cells were plated in 96-well plates (1 × 104 cells/well) and then incubated with different culture medium at 37 °C in 5% CO2 atmosphere.Twenty μl of MTS reagent was added to each well at the indicated time period. Relative cell number was measured based on increased absorbance at 490 nm.

Transfection of Human IGF1R to NIH 3T3 Cells—We transfected the human IGF1R expression construct (18) together with a pcDNA plasmid that contains a neo-resistant gene into NIH 3T3 cells using FuGENE (Roche Applied Science) following the instructions of the manufacturer. Stable transfectants were selected with G418 (1.2 mg/ml) and used for signaling experiments without further enrichment.

Western Blot Analysis—The cells were cultured to nearly confluent in DMEM supplemented with 10% FBS and then maintained in DMEM without FBS for 24 h. The starved cells were treated with WT or mutant IGF-1 (100 ng/ml) in DMEM without FBS for the time indicated. The cells were then solubilized in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin at 4 °C for 30 min. After centrifugation at 12,000 × g for 15 min, the supernatant was removed, and the protein concentration was determined using BCA protein assay reagent (Pierce). Samples containing 30 μg of proteins were boiled for 5 min in SDS sample buffer and resolved by SDS-PAGE under reducing conditions. The activation of ERK1/2 and AKT was determined by Western blot analysis using phosphorylation-specific antibodies (Cell Signaling Technology, Beverly, MA). The phosphorylation of IGF1R was determined by Western blotting using phosphorylation-specific antibodies (Cell Signaling Technology).

Immunoprecipitation—We treated serum-starved cells in 10-cm dishes with WT IGF-1 or R36E/R37E IGF-1 (100 ng/ml for 30 min). We generated cell lysates by suspending cells in 200 μl of lysis buffer containing 20 mM Hepes, pH 7.4, 100 mM NaCl, 1% glycerol, 1 mM MgCl2, protease inhibitor mixture (Sigma), 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM Na3VO4, and 1% Nonidet P-40, for 30 min on ice and centrifugation at 14,000 rpm for 20 min. We adjusted the protein concentration to 2.2 mg in 800 μl of lysis buffer and then incubated the cells in 20 μl of protein G-Sepharose (Invitrogen) conjugated with 10 μg of anti-mouse integrin β3 rat mAb (MBL, Nagoya, Japan) for 24 h at 4 °C. After washing with the lysis buffer without 0.5% Nonidet P-40 three times, we solubilized the immune complex by adding SDS sample buffer to the resin and analyzed with Western blotting.

Adhesion and Binding Assays—Cell adhesion to IGF-1 was measured as described (21). Briefly, IGF-1 was coated to wells of 96-well microtiter plates and incubated with cells for 1 h at 37 °C in Hepes/Tyroe buffer, 1 mM MgCl2 buffer. The bound cells were quantified after rinsing unbound cells by phosphatase assays. Enzyme-linked immunosorbent assay-type integrin binding assays were performed as described (22). Briefly, the
wells of 96-well microtiter plates were coated with IGF-1 and incubated with recombinant soluble αβ3 (5 μg/ml) in the presence of 1 mM MnCl₂ for 1 h at room temperature. Bound αβ3 was determined using anti-β3 mAb and peroxidase-labeled anti-mouse IgG.

FIGURE 1. αβ3 and β1-3-1 CHO cells proliferate faster than β1 CHO cells in vitro. a, uncloned β1, β3, or β1-3-1 CHO cells were plated in 96-well plates (1 × 10⁴ cells/well) and cultured in DMEM containing 10% FBS. Cell proliferation was measured by MTS assay. The data are shown as the means ± S.E. of triplicate experiments. *, p < 0.0001 between β1 and β3 and between β1 and β1-3-1 by two-way analysis of variance. b, cells (2.5 × 10⁵ cells/plate) were cultured in DMEM containing 10% FBS, and the number of cells was counted at the indicated time points. *, p = 0.0007 between β1 and β1-3-1, and p = 0.0005 between β1 and β3 by two-way analysis of variance. c, uncloned β1-3-1 or β1 CHO cells were cultured with or without 10 μg/ml of anti-β1 mAb AIIB2 in DMEM supplemented with 10% FBS for 24 h, and cell proliferation was measured by MTS assay. *, p = 0.046 between β1 and β3 CHO cells and 0.0382 between β1 and β1-3-1 CHO cells.

Competitive Binding of IGF-1 to Immobilized IGF1R—We immobilized soluble IGF1R (R & D Systems) by incubating 100 μl of 1 μg/ml IGF1R in 0.1 M NaHCO₃, pH 9.4, overnight at 4 °C in wells of 96-well microtiter plates. We incubated biotinylated WT IGF-1 (0.1 μg/ml) with immobilized IGF1R in the presence of increasing concentrations of nonlabeled WT IGF-1, the IGF-1 mutant, or irrelevant control ligand (WT FGF-1) in 100 μl of phosphate-buffered saline in wells of 96-well microtiter plates (for 3 h at room temperature). The bound biotinylated
FIGURE 3. Direct binding of IGF-1 to αvβ3 and αvβ1-3-1. a, adhesion of β3 and β1-3-1 CHO cells to IGF-1. IGF-1 was coated to wells of 96-well microtiter plates at 40 μg/ml coating concentrations and incubated with cells for 1 h at 37 °C in Hapes/Tyrode buffer, 1 mM MgCl2 buffer. The bound cells were quantified after rinsing unbound cells. The data are shown as the means ± S.E. of triplicate experiments. b, integrin antagonists inhibit β3 and β1-3-1 CHO cell adhesion to IGF-1. Anti-β1 mAb AIIB2 (10 μg/ml), anti-β3 mAb 7E3 (10 and 50 μg/ml), or cyclic RGFDN peptide (an antagonist specific to αvβ3, 10 μM) was included in the adhesion assays using 20 μg/ml coating concentration of IGF-1. mlgG, purified mouse lgG (10 μg/ml). *p = 0.051 and 0.0017 for 7E3 at 10 and 50 μg/ml, respectively, compared with purified mouse lgG. **, p = 0.008 compared with Me2SO. ***, p < 0.0001 compared with purified mouse lgG. c, binding of purified soluble αvβ3 to IGF-1. Wells of 96-well microtiter plate were coated with IGF-1 (WT and heat-treated, 80 °C, 10 min) and incubated with recombinant soluble αvβ3 (5 μg/ml) in the presence of 1 mM MnCl2 for 1 h at room temperature. Bound αvβ3 was determined by using anti-β3 mAb and peroxidase-labeled anti-mouse lgG. The data are shown as the means ± S.E. of triplicate experiments. d, specific binding of authentic IGF-1 to integrin αvβ3. We performed adhesion assays as described above except that we used commercial recombinant human IGF-1 (R & D Systems) at the coating concentration of 10 μg/ml. *, p = 0.0001 compared with the adhesion of β3 CHO cells to bovine serum albumin (BSA). **, p < 0.0001 compared with the adhesion in the presence of purified mouse lgG. ***, p < 0.0001 compared with the adhesion in the presence of Me2SO. wt, wild type.

WT IGF-1 was determined using horseradish peroxidase-conjugated streptavidin and peroxidase substrates at 580 nm.

Other Methods—Docking simulation, site-directed mutagenesis, and surface plasmon resonance analysis were performed as described (19).

RESULTS

Expression of αvβ3 or αvβ1-3-1 Enhances Cell Proliferation in the Presence of IGF-1 in Serum-free Conditions—To study the role of αvβ3 in tumor progression, we compared the rate of cell proliferation between CHO cells that express recombinant αvβ3 (β3 CHO cells) and those expressing αvβ1 (β1 CHO cells). We found that β3 CHO cells proliferated faster than β1 CHO cells by MTS assay (Fig. 1a) and by counting cell numbers (Fig. 1b) in DMEM supplemented with 10% FBS. These observations are consistent with the previous reports that αvβ3 expression plays a role in tumor progression (see Introduction).

We studied whether the difference in cell proliferation is related to the difference in ligand specificity between αvβ3 and αvβ1 integrins. The specificity loop is a disulfide-linked loop in the β l-domain-like domain and present in all β subunits except for β4, in which the loop is deleted and replaced with two remnant amino acid residues (3). The specificity loop is diverse in sequence and involved in ligand specificity and activation of integrins (3). We previously reported that when the specificity loop sequence CTSE-QNC (residues 187–193) of β1 is replaced with the corresponding CYDKMTTC sequence of β3 (residues 177–184), the ligand specificity of αvβ1 is altered. The mutant (αvβ1-3-1), like αvβ3, recognizes fibrinogen, von Willebrand factor, and vitronectin (a gain-of-function effect) (17). This suggests that the sequence of β3 (CYDKMTTC) is critical for ligand specificity of αvβ3 (17). Hence the loop was designated the “specificity loop.” Consistent with these observations, the loop is exposed to the surface in the ligand-binding site in the αvβ3 crystal structure (23). αvβ1-3-1 also recognizes viral surface proteins, another group of αvβ3 ligands (24). To test whether ligand recognition by the specificity loop of αvβ3 is involved in the enhanced cell proliferation, we used CHO cells that express β1-3-1 mutant (designated β1-3-1 CHO cells). We found that β1-3-1 CHO cells also proliferated faster than β1 CHO cells (Fig. 1a and b) and that anti-β1 mAb AIIB2 (function blocking) inhibited the enhanced proliferation of β1-3-1 CHO cells (Fig. 1c). The β1-3-1 mutant is still more than 99% human β1, and therefore its function is blocked by anti-human β1 mAb such as AIIB2 (17). These results suggest that ligand binding by the specificity loop is related to the enhanced cell proliferation.
of β3 and β1-3-1 CHO cells. We used uncloned β3, β1, and β1-3-1 CHO cells for these experiments, but we obtained essentially the same results with cloned β3, β1, or β1-3-1 CHO cells (data not shown).

We next tested whether serum adhesive proteins are responsible for the enhanced cell proliferation of β3 or β1-3-1 CHO cells. We found that the enhanced cell proliferation of β3 and β1-3-1 CHO cells occurred in commercial serum-free medium for CHO cells (CHO-A) (Fig. 1d). This suggests that αvβ3 can mediate enhanced cell proliferation in the absence of serum adhesive proteins, although the contents of proprietary CHO-A medium were not available. Notably, we found that β3 and β1-3-1 CHO cells proliferated better than β1 CHO cells in the presence of IGF-1 (100 ng/ml) in DMEM in the absence of FBS, but not in the presence of FGF-1 or neuregulin-1 (Fig. 2a). The effect of IGF-1 on cell proliferation was dose-dependent (Fig. 2b). These results suggest that IGF-1 mimics the effect of FBS in the proliferation of CHO cells.

Integrin αvβ3 Directly Interacts with IGF-1—In a current model of IGF-1 signaling, ligand occupancy of αvβ3 with ECM ligands such as vitronectin plays a critical role in enhancing IGF-1 signaling (14). We hypothesized that IGF-1 mimics the ECM ligands for αvβ3. We studied whether αvβ3 interacts with IGF-1 as a ligand using several different binding assays. We found that β3 CHO cells adhered to immobilized IGF-1 in a dose-dependent manner, whereas β1 CHO cells did only weakly (Fig. 3a). mAb 7E3 (anti-human β3) and cyclic RGDfV, a specific inhibitor of αvβ3 (25), reduced the adhesion of β3 CHO cells to IGF-1, suggesting that the interaction between αvβ3 and IGF-1 is specific (Fig. 3b). In addition, soluble recombinant αvβ3 integrin bound to IGF-1 in a dose-dependent manner in enzyme-linked immunosorbent assay-type assays, and heat treatment of IGF-1 markedly...
reduced the binding (Fig. 3c). This suggests that $\alpha{\nu}\beta{3}$ directly binds to IGF-1, and this interaction requires proper folding of IGF-1.

It has recently been reported that the specificity loop of $\beta{3}$ is critical for IGF-1 signaling, and antibodies specific to the specificity loop blocked IGF-1 signaling (through blocking ECM binding to $\alpha{\nu}\beta{3}$) (26). We found that $\beta{1}$-3-1 CHO cells bound better to IGF-1 than $\beta{1}$ CHO cells (Fig. 3a), and mAb A11B2 (anti-$\beta{1}$) reduced the adhesion of $\beta{1}$-3-1 CHO cells to IGF-1 (Fig. 3b). These results suggest that the specificity loop of $\beta{3}$ is critically involved in IGF-1 binding.

It is possible that these observations may be a unique property of the IGF-1 preparation used that has an extra sequence (His$_n$ tag) at the C terminus or because of impurity of the IGF-1 preparations we used. We repeated the adhesion experiments using highly purified commercial IGF-1 (R & D Systems) that has no His$_n$ tag. The results using the authentic IGF-1 were similar to those using our IGF preparation, suggesting that the integrin binding is the property of IGF-1. We used our IGF-1 preparation throughout subsequent experiments.

**Localization of the Integrin-binding Site in IGF-1**—To determine the biological role of the direct IGF-1 binding to $\alpha{\nu}\beta{3}$, we localized the integrin-binding site of IGF-1. We used docking simulation of the integrin $\alpha{\nu}\beta{3}$-IGF-1 interaction using AutoDock3 to predict the integrin-binding site in IGF-1. We performed 50 docking simulations, and poses were clustered at 2 Å root mean square deviation. We used a pose of IGF-1 in cluster 1 that provided the lowest docking energy ($-19.4 \text{ kcal/mol}$) (Fig. 4). We mutated Arg residues at positions 36 and 37 within the predicted binding interface of IGF-1 into Glu (designated the R36E/R37E mutant). The IGF-1 mutant was defective in supporting adhesion of K562 cells that express recombinant $\alpha{\nu}\beta{3}$ ($\alpha{\nu}\beta{3}$-K562) (Fig. 5a). Mock-transfected K562 cells only weakly adhered to WT or mutant IGF-1 (Fig. 5b). We performed surface plasmon resonance analysis to test the effect of the R36E/R37E mutation (Fig. 5, c and d). We demonstrate that WT IGF-1 bound to immobilized soluble $\alpha{\nu}\beta{3}$ at $K_a$ 31.3 nm, but the R36E/R37E mutant did not show significant binding to $\alpha{\nu}\beta{3}$. These results confirm that IGF-1 directly binds to $\alpha{\nu}\beta{3}$ and suggest that the Arg residues at positions 36 and 37 are part of the integrin-binding interface in IGF-1 as predicted by the docking simulation. These Arg residues are close to several amino acid residues (Asp$^{150}$, Tyr$^{178}$, and Asp$^{218}$) of αV, but not to the metal ion-dependent adhesive site (Asp$^{119}$, Ser$^{121}$, Ser$^{123}$, Glu$^{220}$, and Asp$^{251}$) (27) or the specificity loop of $\beta{3}$ (Fig. 4b).
Also, Arg^{36} and Arg^{37} are distinct from the IGF-binding protein (IGFBP)-4-binding site in IGF-1 (Fig. 4c), suggesting that these residues are not involved in IGFBP binding.

The R36E/R37E Mutant Is Defective in Inducing Intracellular Signaling and Cell Proliferation—We tested whether the integrin binding-defective mutation affects the ability of IGF-1 to induce cell proliferation. We serum-starved NIH 3T3 cells and cultured them in the presence of WT (wt) or mutant IGF-1 for 24 h. The results suggest that the R36E/R37E mutant is defective in inducing cell proliferation.

To test the effect of the R36E/R37E mutation on IGF intracellular signaling, we used NIH 3T3 cells that express human IGF1R (designated NIH 3T3-IGF1R) because nontransfected NIH 3T3 cells only generated weak signals (data not shown). We found that WT IGF-1 induced IGF1R phosphorylation, AKT phosphorylation, and ERK1/2 activation, whereas R36E/R37E was defective in these functions (Fig. 7a). We obtained similar results with C2C12 cells (Fig. 7b). These results suggest that R36E/R37E is defective in inducing IGF-1 intracellular signaling. It is likely that the direct integrin binding to IGF-1 is required at the initial step (transphosphorylation of IGF1R).

The R36E/R37E Mutation Does Not Affect the Binding of IGF-1 to IGF1R—It is possible that R36E/R37E is defective in IGF1R binding and cannot induce intracellular signaling. We tested the ability of the IGF-1 mutant to bind to IGFR in an enzyme-linked immunosorbent assay-type competitive binding assays (Fig. 8a). We found that excess WT IGF-1 and R36E/R37E suppressed the binding of biotinylated WT IGF-1 to immobilized recombinant IGF1R to the similar extent, suggesting that the IGF-1 mutation did not affect the binding of IGF-1 to IGFR. Control irrelevant ligand (WT FGF1) did not suppress the binding of WT IGF-1 to IGFR, suggesting that the interaction is specific.

If the integrin-binding site and IGF1R-binding site are distinct, it is expected that IGF-1 mediates ternary complex formation (IGF-1, integrin αβ3, and IGF1R) through simultane-
IGF-1, cells treated with R36E/R37E (Fig. 8). We treated cells with WT IGF-1 or R36E/R37E (100 ng/ml for 30 min) and immunopurified (IP) materials by Western blotting. We demonstrated that WT IGF-1 suppressed the binding of WT IGF-1 to IGF1R at similar levels. IGF-1 induced co-precipitation of R36E/R37E IGF-1 mutation does not affect the binding of IGF-1 to immobilized IGF1R. We immobilized soluble IGF1R to wells of 96-well microtiter plates and incubated with biotinylated WT IGF-1 (0.1 μg/ml) in the presence of increasing concentrations of nonlabeled WT IGF-1, the R36E/R37E mutant, or a control ligand WT FGF1 for 3 h at room temperature. The bound biotinylated WT IGF-1 was determined. The results suggest that the R36E/R37E mutant and WT IGF-1 suppressed the binding of WT IGF-1 to IGF1R at similar levels. WT IGF-1 induced co-precipitation of αvβ3 and IGF1R, but the R36E/R37E mutant did not. We treated cells with WT IGF-1 or R36E/R37E (100 ng/ml for 30 min) and immunopurified (IP) αvβ3 from cell lysates with anti-β3. We analyzed the immunopurified materials by Western blotting.

**DISCUSSION**

In the present study we establish that IGF-1 mediates enhanced proliferation of β3 CHO cells in serum-free conditions. These findings suggest that IGF-1 is one of the components that mediate enhanced proliferation of cancer cells that express αvβ3 and that serum adhesive proteins such as vitronectin are not important in this process. The ligand binding specificity of αvβ3 through the specificity loop plays a critical role in this function.

We demonstrated that αvβ3 interacts directly with IGF-1 in different binding assays. This interaction is specific because anti-β3 mAb 7E3 and cyclic RGDFV peptide blocked the interaction. It is still possible that these observations are unique to the IGF-1 preparation we used or are due to impurity of the IGF-1 preparations we used. We obtained similar results with highly purified IGF-1 from a commercial source (>97% pure), suggesting that the binding of IGF-1 to αvβ3 is the property of IGF-1. We used much lower coating concentrations (10 μg/ml) in Fig. 3d than in Fig. 3b (40 μg/ml). This will partly explain that the inhibitory effect of 7E3 and cyclic RGDFV peptide on adhesion β3 CHO cells in Fig. 3d is stronger than in Fig. 3b. We and other laboratories previously mapped the epitope of 7E3 to the specificity loop of β3 (28, 29). We demonstrated that the interaction involves the specific loop of the β3 subunit using the β1-3-1 mutant. Anti-β1 mAb AIIB2 effectively blocked IGF-1 binding to αvβ1-3-1. The epitope for mAb AIIB2 has been mapped to the non-ligand-binding site, and it appears that this mAb changes the conformation of the ligand-binding site of β1 integrins (30). It has been reported that the β3 mutant, in which the specificity loop of β3 is mutated, suppressed IGF-1 signaling, and antibodies specific to the specificity loop of β3 blocked IGF-1 signaling (26). Taken together it is highly likely that IGF-1 directly interacts with the specificity loop of β3.

We localized the integrin-binding site in IGF-1 by docking simulation and mutagenesis. The R36E/R37E mutation within the C-domain (residues 30–41) significantly reduced the ability of IGF-1 to interact with αvβ3 but did not affect its ability to bind to IGF1R. There is a possibility that IGF-1 indirectly interacts with αvβ3 and that the R36E/R37E mutation suppresses the indirect interaction. To rule out this possibility, we performed a surface plasmon resonance study using recombinant soluble αvβ3. We demonstrated that WT IGF-1 bound to immobilized αvβ3 at a high affinity, and the R36E/R37E mutation completely suppressed this interaction, suggesting that IGF-1 directly binds to αvβ3 and that the mutation suppressed this interaction. Notably, the mutant was defective in inducing cell proliferation, IGF1R phosphorylation, AKT phosphorylation, and ERK1/2 activation. These results suggest that the ability of IGF-1 to directly interact with integrins is critical for IGF signaling.

The Arg residues at positions 36 and 37 are not in the IGFBP4-binding site in IGF-1 in the IGF-1-IGFBP4 complex (Fig. 4c). It is thus unlikely that the R36E/R37E mutation or the binding of integrins to IGF-1 affects the binding of IGFBP4 to IGF-1. It has been reported that replacing the C-loop of IGF-1 with four Gly residues much reduced the binding affinity of IGF-1 to IGF1R (31), suggesting that the C-domain is involved.
in IGF-1-IGF1R interaction. Three Tyr residues at positions 24, 31, and 61 of IGF-1 are critical for IGF1R binding, because mutating these residues individually significantly reduced its affinity to IGF1R (32). IGF-1 binds to the hydrophobic patch in the L1 domain of IGF1R, and the three Tyr residues are expected to interact with the L1 domain (33). We generated a model in which the L1 domain of IGF1R and IGF-1 interact (not shown) according to the docking model of insulin-insulin receptor interaction (34). This model predicts that integrin and IGF1R-binding sites are distinct, and there will be no steric hindrance between integrin and the L1 domain if they bind to IGF-1 simultaneously. It is likely that soluble IGF-1 preferentially binds to IGF1R than to integrins, and αvβ3 may bind to receptor-bound IGF-1. Consistent with these predictions, we demonstrated that 1) the R36E/R37E mutation did not affect the binding of IGF-1 to IGF1R; 2) IGF1R co-precipitated with αvβ3 when WT IGF-1 was used for stimulating cells; and 3) IGF1R did not co-precipitate with αvβ3 when R36E/R37E was used, suggesting that ternary complex formation is dependent on the ability of IGF-1 to interact with αvβ3.

Is there any cooperativity between αvβ3 and IGF1R involved in IGF-1 binding? We showed that IGF-1 induced co-precipitation of IGF1R and integrin, suggesting that ternary complex formation is dependent on the ability of IGF-1 to interact with αvβ3. Taken together, the present results generate an alternative hypothesis of the role of integrin αvβ3 in IGF-1 signaling, in which IGF-1 directly binds to integrin αvβ3 in addition to IGF1R on the cell surface, and the interaction with this integrin plays a role in IGF-1 signaling. Because antagonists to αvβ3 are expected to block both αvβ3-ECM and αvβ3-IGF-1 interactions, it is unclear whether the reported inhibition of IGF-1 signaling by αvβ3 antagonists is due to the inhibition of the αvβ3-IGF-1 interaction and/or the αvβ3-ECM interaction. The IGF-1 mutant is expected to specifically block IGF-1-integrin interaction without affecting ECM-integrin interaction. Because IGF1R is widely expressed, it is likely that other integrins may also be involved in IGF signaling. Further studies of the role of integrins in IGF signaling through direct binding to IGF will be required.

Acknowledgment—We thank Rita Slaaby for IGF1R cDNA.

REFERENCES
1. Hynes, R. O. (2002) Cell 110, 673–687
2. Shattil, S. J. (2005) Trends Cell Biol. 15, 399–403
3. Takada, Y., Ye, X., and Simon, S. (2007) Genome Biol. 8, 215
4. Albelda, S. M. (1992) EXS 61, 188–192
5. Hsu, M. Y., Shih, D. T., Meier, F. E., Van Belle, P., Hsu, J. Y., Elder, D. E., Buck, C. A., and Herlyn, M. (1998) Am. J. Pathol. 153, 1435–1442
6. Gladson, C. L., Hancock, S., Arnold, M. M., Faye-Petersen, O. M., Castleberry, R. P., and Kelly, D. R. (1996) Am. J. Pathol. 142, 1432–1434
7. Sengupta, S., Chattopadhyay, N., Mitra, A., Ray, S., Dasgupta, S., and Chatterjee, A. (2001) J. Exp. Clin. Cancer Res. 20, 585–590
8. Pignatelli, M., Cardillo, M. R., Hanby, A., and Stamp, G. W. (1992) Hum. Pathol. 23, 1159–1166
9. Vonlaufen, A., Wiedle, G., Borisch, B., Birrer, S., Luder, P., and Imhof, B. A. (2001) Mod. Pathol. 14, 1126–1132
10. Liapis, H., Flath, A., and Kitazawa, S. (1996) Diagn. Mol. Pathol. 5, 127–135
11. Chattopadhyay, N., and Chatterjee, A. (2001) J. Exp. Clin. Cancer Res. 20, 269–275
12. Bojesen, S. E., Kjaer, S. K., Hogdaall, E. V., Thomsen, B. L., Hogdaall, C. K., Blaaker, J., Tybjærg-Hansen, A., and Nordestgaard, B. G. (2005) Endocr. Relat. Cancer 12, 945–952
13. Clemons, D. R., and Maile, L. A. (2005) Mol. Endocrinol. 19, 1–11
14. Clemons, D. R., Maile, L. A., Ling, Y., Tarber, J., and Busby, W. H. (2007) Growth Horm. IGF Res. 17, 265–270
15. Jones, J. L., Doerr, M. E., and Clemons, D. R. (1995) Prog. Growth Factor Res. 6, 319–327
16. Zheng, B., and Clemons, D. R. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 11217–11222
17. Takagi, J., Kamata, T., Meredith, J., Puzon-McLaughlin, W., and Takada, Y. (1997) J. Biol. Chem. 272, 19794–19800
18. Slaby, R., Schäffer, L., Lautrup-Larsen, I., Andersen, A. S., Shaw, A. C., Mathiasen, I. S., and Brandt, J. (2006) J. Biol. Chem. 281, 25869–25874
19. Mori, S., Wu, C. Y., Yamaji, S., Saegusa, J., Shi, B., Ma, Z., Kuwabara, Y., Lam, K. S., Isseroff, R. R., Takada, Y. K., and Takada, Y. (2008) J. Biol. Chem. 283, 18066–18075
20. Funes, M., Miller, J. K., Lai, C., Carraway, K. L., 3rd, and Sweeney, C. (2006) J. Biol. Chem. 281, 19310–19319
21. Eto, K., Puzon-McLaughlin, W., Sheppard, D., Sehara-Fujisawa, A., Zhang, X. P., and Takada, Y. (2000) J. Biol. Chem. 275, 34922–34930
22. Saegusa, J., Akakura, N., Wu, C. Y., Hoogland, C., Ma, Z., Lam, K. S., Liu, F. T., Takada, Y. K., and Takada, Y. (2008) J. Biol. Chem. 283, 26107–26115
23. Xiong, J. P., Stehle, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S. L., and Arnaout, M. A. (2002) Science 296, 151–155
24. Triantafillou, M., Triantafillou, K., Wilson, K. M., Takada, Y., and Fernandez, N. (2000) Hum. Immunol. 61, 453–459
25. Aumailley, M., Gurrath, M., Müller, G., Calvete, J., Timpl, R., and Kessler, H. (1991) FEBS Lett. 291, 50–54
26. Maile, L. A., Busby, W. H., Sitko, K., Capps, B. E., Sergent, T., Badley, M. A., Lovrecz, G. O., Cosgrove, L. J., Frenkel, M. J., and Ward, C. W. (2006) J. Biol. Chem. 281, 19794–19800
27. Xiong, J. P., Stehle, T., Diefenbach, B., Zhang, R., Dunker, R., Scott, D. L., Joachimiak, A., Goodman, S. L., and Arnaout, M. A. (2001) Science 294, 339–345
28. Puzon-McLaughlin, W., Kamata, T., and Takada, Y. (2000) J. Biol. Chem. 275, 7795–7802
29. Artoni, A., Li, J., Mitchell, B., Ruan, J., Takagi, J., Springer, T. A., French, D. L., and Coller, B. S. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 13114–13120
30. Takada, Y., and Puzon, W. (1993) J. Biol. Chem. 268, 17597–17601
31. Bayne, M. L., Applebaum, J., Underwood, D., Chichigi, G. G., Green, B. G., Hayes, N. S., and Cascieri, M. A. (1989) J Biol. Chem. 264, 11004–11008
32. Bayne, M. L., Applebaum, J., Chichigi, G. G., Miller, R. E., and Cascieri, M. A. (1990) J Biol. Chem. 265, 15468–15465
33. Garrett, T. P., McKern, N. M., Lou, M., Frenkel, M. J., Bentley, I. D., Lovrecz, G. O., Ellem, T. C., Cosgrove, L. J., and Ward, C. W. (1998) Nature 394, 395–399
34. Lou, M., Garrett, T. P., McKern, N. M., Hoyne, P. A., EPA, V. C., Bentley, J. D., Lovrecz, G. O., Cosgrove, L. J., Frenkel, M. J., and Ward, C. W. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 12429–12434