

Insulin-like Growth Factor I Increases $\alpha_V\beta_3$ Affinity by Increasing the Amount of Integrin-associated Protein That Is Associated with Non-raft Domains of the Cellular Membrane*

Received for publication, August 30, 2001, and in revised form, November 7, 2001
Published, JBC Papers in Press, November 13, 2001, DOI 10.1074/jbc.M108380200

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Insulin-like growth factor I (IGF-I) stimulates an increase in $\alpha_V\beta_3$ ligand binding. Stimulation of smooth muscle cells by IGF-I requires $\alpha_V\beta_3$ ligand occupancy, and enhanced $\alpha_V\beta_3$ ligand occupancy augments IGF-I actions. Therefore, IGF-I-induced changes in $\alpha_V\beta_3$ ligand binding may act to further enhance IGF-I actions. Integrin-associated protein (IAP) has been shown to be associated with $\alpha_V\beta_3$ and is required for the binding of $\alpha_V\beta_3$ to vitronectin-coated beads. We therefore investigated whether IGF-I could stimulate IAP- $\alpha_V\beta_3$ association resulting in enhanced ligand binding. IGF-I stimulated an increase in IAP- $\alpha_V\beta_3$ association. This was due, at least in part, to an IGF-I-stimulated redistribution of IAP from the Triton-insoluble fraction of the cell to the Triton-soluble fraction of the cell, where most of the $\alpha_V\beta_3$ was located. Inhibition of the phosphatidylinositol 3-kinase pathway blocked both the redistribution of IAP and the increase in IAP- $\alpha_V\beta_3$ association, providing further evidence that the redistribution of IAP is essential for the increase in association. An anti-IAP monoclonal antibody, blocked both the IGF-I-stimulated increase in IAP- $\alpha_V\beta_3$ complex formation and cell migration. IGF-I-stimulated translocation of IAP and increase in IAP- $\alpha_V\beta_3$ association represent an important process by which IGF-I modulates $\alpha_V\beta_3$ ligand binding and cellular responses.

Insulin-like growth factor I (IGF-I)¹ is a potent stimulant of vascular smooth muscle cell migration (1–3). The $\alpha_V\beta_3$ integrin is an important cellular membrane receptor that is utilized by IGF-I to regulate vascular smooth muscle cell migration. Specific inhibitors of $\alpha_V\beta_3$ ligand occupancy block IGF-I-stimulated migration (4). Additionally, treatment of cells with IGF-I has been shown to increase ligand binding by $\alpha_V\beta_3$ (5). This IGF-I-induced change in $\alpha_V\beta_3$ affinity may be an important component of its ability to stimulate cell migration. The mechanism by which IGF-I stimulates the increase in ligand binding is unknown. IGF-I does not physically associate with $\alpha_V\beta_3$ (6),

which suggests that IGF-I may be acting via an intermediary protein to exert its effects on $\alpha_V\beta_3$ affinity.

Signals from within the cell can result in changes in integrin ligand affinity and avidity. One of the proteins implicated in these events is integrin-associated protein (IAP), also termed CD47. IAP was first identified due to its association with $\alpha_V\beta_3$ purified from placenta (7). IAP is expressed on many mammalian cells and consists of a N-terminal (extracellular) immunoglobulin variable (IgV)-type domain followed by five potential membrane-spanning hydrophobic helices and a cytoplasmic tail (8, 9). Its involvement in integrin signaling is suggested both by its physical association with integrins (7) and by experimental results showing that antibodies to IAP affect a large number of functions associated with $\alpha_V\beta_3$ (9, 10). More specifically, anti-IAP antibodies inhibit the binding of $\alpha_V\beta_3$ in a variety of cells lines (11) to vitronectin (Vn)-coated beads. Furthermore, it has been shown that the IAP-negative cell line OV10 (an ovarian carcinoma cell line) can only bind Vn-coated beads if these cells are made to express IAP (12).

Because IGF-I exposure enhances ligand binding to $\alpha_V\beta_3$, we determined whether IGF-I receptor activation altered the physical association of IAP with $\alpha_V\beta_3$ and thereby increased its ability to bind to ligands and whether inhibition of IAP- $\alpha_V\beta_3$ association resulted in inhibition of IGF-I-stimulated cell migration. To investigate the mechanism by which IGF-I stimulated IAP- $\alpha_V\beta_3$ association, we determined whether $\alpha_V\beta_3$ and IAP were associated with different lipid compartments of the cellular membrane and whether IGF-I could alter this distribution, thus allowing better access of IAP to $\alpha_V\beta_3$.

EXPERIMENTAL PROCEDURES

Materials—Human IGF-I was a gift from Genentech (South San Francisco, CA). Polyvinylidene difluoride filters were purchased from Millipore Corp. (Bedford, MA). Autoradiographic film was obtained from Eastman Kodak Co. Fetal bovine serum, Dulbecco's modified Eagle's medium, penicillin, and streptomycin were purchased from Invitrogen. Polyclonal anti- α_V and anti- β_3 antisera were purchased from Chemicon (Temecula, CA). The monoclonal antibody used to detect IAP (B6H12) was a kind gift from Dr. Eric Brown (Washington University, St. Louis, MO). Polyclonal antibody against IAP was raised in rabbit against amino acids 50–70 of the human IAP extracellular domain. The polyclonal antibody against β_3 was raised in rabbit against amino acids 60–85 of the porcine β_3 sequence. Biotinylated human Vn was a kind gift from Wayne Engleman (Amersham Biosciences, Inc.). All other reagents were purchased from Sigma Chemical Co. unless otherwise stated.

Porcine aortic smooth muscle cells (pSMCs) were isolated as described previously (13) and maintained in Dulbecco's modified Eagle's medium supplemented with glucose (4.5 g/liter), penicillin (100 units/ml), streptomycin (100 ng/ml), and 10% fetal bovine serum in 10-cm tissue culture plates. The cells were used between passage 5 and 16.

Determination of Soluble Vn Binding to $\alpha_V\beta_3$ —Cells were grown to 80% confluence in 48-well plates. The cells were then incubated overnight in serum-free Dulbecco's modified Eagle's medium H plus 0.01% bovine serum albumin (SFM) and then exposed to 100 ng/ml IGF-I for

* This work was supported by National Institutes of Health Grant HI-56580. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: IGF-I, insulin-like growth factor I; IAP, integrin-associated protein; Vn, vitronectin; pSMC, porcine aortic smooth muscle cell; SFM, serum-free Dulbecco's modified Eagle's medium H plus 0.01% bovine serum albumin; PI3K, phosphatidylinositol 3-kinase.

the appropriate length of time. The cells were then washed three times with SFM plus 20 mM HEPES and incubated with biotinylated Vn (10^{-8} M) in SFM containing 20 mM HEPES for 5 h at 4 °C. The cells were rinsed again in SFM before lysis at 4 °C in ice-cold buffer A (150 mM HEPES, pH 7.5, 50 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin A, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin). The lysates were clarified by centrifugation at $14,000 \times g$ for 10 min at 4 °C. Equal amounts of protein were then separated under nonreducing conditions by SDS-PAGE, 8% gel. The amount of biotinylated vitronectin in each sample was visualized as described below.

Preparation of Whole Cell Lysates—Cells were incubated overnight in SFM and then exposed to 100 ng/ml IGF-I as indicated in each figure. Cells were then rinsed three times with phosphate-buffered saline (pH 7.4) and lysed by the addition of 900 μ l of boiling 2 \times Laemmli buffer. Cell lysates were scraped from the dish into microcentrifuge tubes and boiled for 5 min. To reduce viscosity, samples were then sonicated for 10 s and centrifuged again for 5 min. Lysates were then separated under nonreducing conditions by SDS-PAGE, 8% gel. The level of each protein was then determined by Western immunoblotting as described below.

Determination of IAP-integrin Association—Cells were incubated overnight in SFM and then exposed to 100 ng/ml IGF-I for the appropriate length of time before lysis at 4 °C in ice-cold 0.2% Triton X-100 lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin A, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin (buffer B)). The lysates were clarified by centrifugation at $14,000 \times g$ for 10 min 4 °C. In some experiments, cells were preincubated with 4 μ g/ml B6H12 (an anti-IAP monoclonal antibody) before the addition of IGF-I. Immunoprecipitation was then carried out as described below.

Determination of the Distribution of Proteins between the Triton-soluble and -insoluble Fractions—Cells were incubated in SFM and then exposed to 100 ng/ml IGF-I for the appropriate length of time before lysis at 4 °C in buffer B. The lysates were clarified by centrifugation at $14,000 \times g$ for 10 min at 4 °C. The resultant supernatant was termed the Triton-soluble fraction. The remaining pellet was resuspended in lysis buffer C (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin A, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin). The resuspended pellet was re-centrifuged at $14,000 \times g$ for an additional 10 min at 4 °C, and the resulting supernatant was considered to be the Triton-insoluble fraction of the cell, excluding the insoluble cytoskeletal fraction. This method has been shown to separate proteins that are bound to membrane rafts (Triton-insoluble) from those that are not raft-associated (Triton-soluble), as verified by sucrose gradient density centrifugation (14–16). 10 μ l of 4 \times nonreducing Laemmli buffer was added to 50 μ l of each supernatant and heated at 65 °C for 10 min. The proteins were separated by SDS-PAGE (8% gel) and then visualized by Western immunoblotting as described below.

To test the effect of the various inhibitors on IGF-I-stimulated IAP-integrin association and IGF-I-stimulated redistribution of IAP, following overnight incubation in SFM, the cells were incubated with either 0.1% DMSO (vehicle) or the appropriate inhibitor (100 nM wortmannin or 1 μ M Ly294002) for 30 min before the addition of IGF-I. We have previously shown that wortmannin and Ly294002 at these concentrations specifically inhibit different physiological actions of IGF-I and that their inhibitory activity can be maintained for at least 24 h (18).

Immunoprecipitation—The supernatants were incubated overnight at 4 °C with the appropriate antibody (polyclonal anti- α_V antibody at 1:1000, polyclonal anti- β_3 antibody at 1:1000, or monoclonal anti-IAP antibody (B6H12) at 1:500). Immune complexes were then precipitated by the addition of protein A-Sepharose and incubation for an additional 2 h at 4 °C. The samples were then centrifuged at $14,000 \times g$ for 10 min, and the pellets were washed four times with lysis buffer. The pellet was suspended in 45 μ l of nonreducing Laemmli buffer and heated at 65 °C for 10 min, and the proteins were separated by SDS-PAGE, 8% gel.

Western Immunoblotting—After SDS-PAGE, the proteins were transferred to a polyvinylidene difluoride membrane. The membranes were blocked in 1% bovine serum albumin in Tris-buffered saline with 0.1% Tween (TBST) for 2 h at room temperature. The membranes were incubated with one of four primary antibodies (the IAP monoclonal antibody (B6H12) at 1:500, the IAP polyclonal antibody at 1:500, the β_3 polyclonal antibody at 1:500, or the α_V polyclonal antibody at 1:500) overnight at 4 °C and then washed three times in TBST before incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. To visualize the biotinylated vitronectin, membranes were

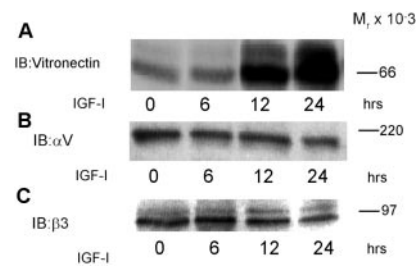


FIG. 1. A, IGF-I stimulates an increase in Vn binding to $\alpha_V\beta_3$. Cells were grown to 80% confluence and incubated with IGF-I for 6, 12, or 24 h. This was followed by a 5-h incubation with 10^{-8} M biotinylated vitronectin. After lysis, the amount of biotinylated vitronectin associated with the cells was determined by Western immunoblotting. The results are expressed as arbitrary scanning units: lane 1, 4315; lane 2, 4857; lane 3, 16,706; and lane 4, 18,451. B and C, IGF-I does not increase the amount of α_V or β_3 . Cell lysates from cells incubated with or without IGF-I were immunoblotted with an anti- α_V (B) or anti- β_3 (C) antibody.

incubated with extravidin conjugated to horseradish peroxidase (1:10,000). Binding of the peroxidase-labeled antibody was visualized using enhanced chemiluminescence following the manufacturer's instructions (Pierce).

Cell Wounding and Migration Assay—Cells were plated in 6-well plates and grown to confluence over a 7-day period with one media change. Wounding was performed as described previously (5). Briefly, a razor blade was used to scrape an area of cells, leaving a denuded area and a sharp visible wound line. The wounded monolayers were then rinsed three times in SFM, and then ten 1-mm areas along the wound edge were selected and recorded for each treatment. The wounded monolayers were then incubated with SFM (plus 2% fetal bovine serum) with or without 100 ng/ml IGF-I. To test the effect of B6H12 on IGF-I-stimulated migration, the wounded monolayers were incubated with B6H12 (4 μ g/ml) for 2 h before the addition of IGF-I. The cells were then fixed and stained (Diff Quick; Dade Behring, Inc., Newark, DE), and the number of cells migrating into the wound area was counted. At least five of the previously selected 1-mm areas at the edge of the wound were counted for each data point.

Our previous analysis of the wounded monolayer by [3 H]thymidine autoradiography demonstrated that the labeling index of pSMCs at the wounded edge was $7 \pm 4\%$ at the basal level and $18 \pm 7\%$ after treatment with IGF-I. Therefore, $<10\%$ of cells present in the denuded area at the end of the migration assay are considered to result from cell division rather than cell migration (5).

Student's *t* test was used to compare differences between treatments. Band intensities on autoradiographs were measured by scanning densitometry and analyzed using NIH Image, version 1.61. The results shown are representative of at least three similar experiments.

RESULTS

IGF-I Stimulates an Increase in the Binding of Soluble Vn to the $\alpha_V\beta_3$ Integrin—A 24-h incubation with IGF-I followed by measurement of biotinylated Vn binding to the cell surface showed that binding was significantly increased (6.1 ± 1.4 -fold, mean \pm S.E.; $n = 3$; $p < 0.05$). This effect required at least 12 h of incubation because no increase was observed at earlier time points. A representative experiment is shown in Fig. 1A. There was no significant change in the level of either α_V (Fig. 1B) or β_3 (Fig. 1C) during the time course of this experiment, and this confirms our previous observation that there was no increase in $\alpha_V\beta_3$ receptor number in response to IGF-I (5). This suggests that the increase in $\alpha_V\beta_3$ ligand binding that occurs after IGF-I receptor stimulation is not simply due to an increase in integrin receptor number and is probably due to a change in the affinity of $\alpha_V\beta_3$ for vitronectin. The IGF-I receptor does not physically associate with the $\alpha_V\beta_3$ integrin (6), which suggests that IGF-I exerts its effects on integrin ligand binding via an intermediary protein.

IGF-I Stimulates an Increase in the Association of IAP with $\alpha_V\beta_3$ Integrin—Previous reports have suggested that the direct physical association of the extracellular domain of IAP with the

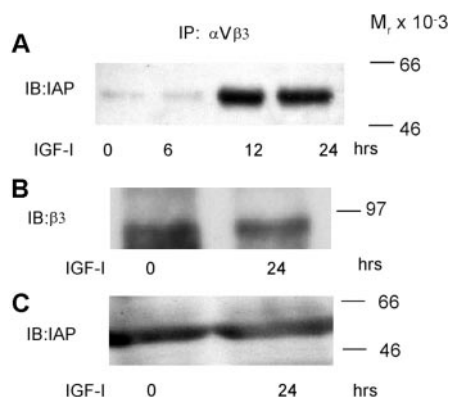


FIG. 2. IGF-I stimulates an increase in IAP- $\alpha_V\beta_3$ association. A, cells were grown to 80–90% confluence in 10-cm plastic dishes in growth medium and then incubated overnight in SFM. This was followed by a 6-, 12-, or 24-h incubation with or without IGF-I (100 ng/ml). After lysis and immunoprecipitation with an anti- α_V antibody, the level of IAP association was determined by Western immunoblotting with the anti-IAP monoclonal antibody. The results are expressed as arbitrary scanning units: lane 1, 3741; lane 2, 3954; lane 3, 22,754; and lane 4, 26,027. B, the amount of $\alpha_V\beta_3$ immunoprecipitated before and after 24-h treatment with IGF-I in the same experiment was determined by stripping the blot and reprobing with an anti- β_3 polyclonal antibody. The results are expressed as arbitrary scanning units: lane 1, 25,472; and lane 2, 23,210. C, the level of IAP in whole cell lysates before and after 24-h treatment with IGF-I was determined by immunoblotting with the anti-IAP monoclonal antibody. The results are expressed as arbitrary scanning units: lane 1, 52,866; and lane 2, 42,538.

$\alpha_V\beta_3$ integrin is necessary and sufficient for $\alpha_V\beta_3$ binding to Vn-coated beads (12). Because both IAP and $\alpha_V\beta_3$ are expressed on the cell surface, we investigated whether treatment of pSMCs with IGF-I increased the physical association of IAP with $\alpha_V\beta_3$, thus contributing to the enhancing effect of IGF-I on $\alpha_V\beta_3$ ligand affinity. We have shown previously that the majority of α_V on the surface of pSMCs is associated with β_3 (5); we therefore used an antibody against the α_V subunit to immunoprecipitate the $\alpha_V\beta_3$ integrin receptor and then immunoblotted for IAP to examine changes in complex formation. Fig. 2A shows that in pSMCs, minimal IAP was associated with the $\alpha_V\beta_3$ integrin in the basal state. In contrast, after a 12-h incubation with IGF-I, there was a 6-fold increase in IAP- $\alpha_V\beta_3$ association, and this increased still further after a 24-h incubation with IGF-I (6.5 \pm 1.75-fold, mean \pm S.E.; $n = 3$; $p < 0.05$). Importantly no increase in IAP- $\alpha_V\beta_3$ association was detected at the earlier 6 h time point. This time course is the same as that observed for the increase in vitronectin binding shown in Fig. 1. Because IAP forms a complex with the $\alpha_V\beta_3$ dimer but not with either subunit alone, to control for loading differences we immunoblotted for β_3 because this would be precipitated with the α_V , and we therefore believe it is a better index of the total amount of $\alpha_V\beta_3$ that was immunoprecipitated in each lane. Immunoblotting for β_3 shows that there was no change in the amount of $\alpha_V\beta_3$ immunoprecipitated between the different treatments that would account for the increase in IAP (Fig. 2B). Fig. 2C shows that there was no increase in the total cellular content of IAP during the time course of the experiment.

IGF-I Stimulates the Redistribution of IAP from the Triton-insoluble to the Triton-soluble Fraction of the Cell—It has been shown previously that increasing the expression of IAP or $\alpha_V\beta_3$ can increase complex formation (12). However, as shown in Figs. 1 and 2, there were no changes in the total amount of α_V , β_3 , or IAP during the time course of the experiment. It is known that plasma membrane proteins can be associated with different microdomains of the membrane, based upon the polarity of the lipid groups (14). Sucrose gradient density centrifugation

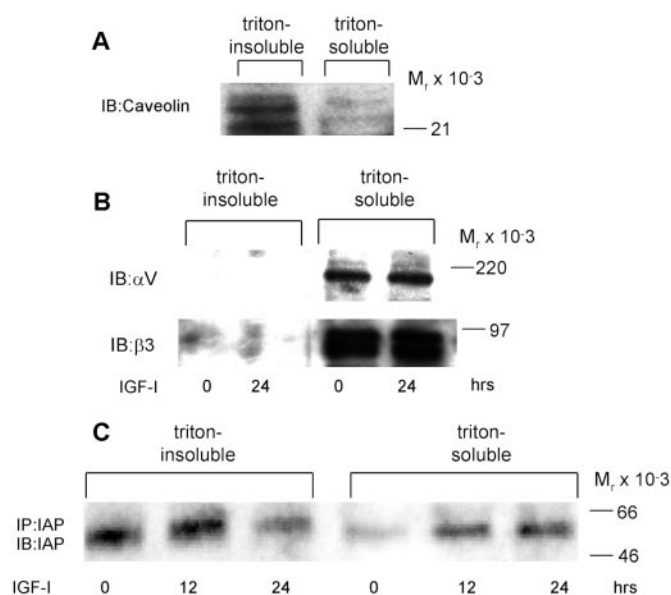


FIG. 3. IGF-I stimulates the redistribution of IAP from the Triton-insoluble fraction to the Triton-soluble fraction of the cell. A–C, after overnight incubation in SFM, cells were incubated in either SFM or IGF-I for the indicated length of time; Triton-soluble and Triton-insoluble cell lysates were either separated immediately by SDS-PAGE (A and B) or first immunoprecipitated with an IAP monoclonal antibody (B6H12) (C). Proteins were visualized after incubation with the indicated antibody. The results in C are expressed as arbitrary scanning units: lane 1, 8392; lane 2, 6136; lane 3, 3731; lane 4, 2141; lane 5, 5384; and lane 6, 6475.

has been used to separate membrane raft proteins from proteins associated with non-raft domains (14). Extraction of these membrane subfractions with Triton at 4 °C has shown that the Triton-insoluble fraction corresponds to proteins associated with membrane rafts as determined by sucrose gradient density centrifugation (14–16). IAP- $\alpha_V\beta_3$ complex formation can only be detected after lysis in a Triton buffer, which suggested that the increase in their association might be due to a specific increase in the levels of one or both of the complex components in the Triton-soluble fraction. We therefore investigated the distribution of complex components between the Triton-soluble and Triton-insoluble fractions of the cell before and after IGF-I stimulation following protein extraction at 4 °C. To first confirm that proteins from the Triton-insoluble fraction had been separated from proteins from the Triton-soluble fraction, we immunoblotted for caveolin, a protein known to be associated almost exclusively with membrane rafts (17) and therefore found in the Triton-insoluble fraction of the plasma membrane. Fig. 3A confirms that 82 \pm 6.5% (mean \pm S.E.; $n = 3$) of caveolin was detected in the Triton-insoluble fraction. In Fig. 3B, it can be seen that the majority of α_V (top panel; 94 \pm 6%, mean \pm S.E.; $n = 3$) and β_3 (bottom panel; 93 \pm 7%, mean \pm S.E.; $n = 3$) was associated with the Triton-soluble fraction of the cell, and there was no significant change in $\alpha_V\beta_3$ distribution after IGF-I treatment. In contrast, Fig. 3C shows that in the basal state, 85 \pm 7.7% (mean \pm S.E.; $n = 3$) of the total IAP was associated with the Triton-insoluble membrane fraction. Importantly, after incubation with IGF-I, there was a marked decrease (2.2-fold) in the amount of IAP in the Triton-insoluble fraction and an increase (3-fold) in IAP in the Triton-soluble fraction. After treatment with IGF-I, 79.7 \pm 10.5% (mean \pm S.E.; $n = 3$) of the total IAP was associated with the Triton-soluble fraction.

Inhibition of the PI3K Pathway Inhibits the IGF-I-stimulated Increase in IAP-Integrin Association—To provide further evidence that the IGF-I-stimulated redistribution of IAP is impor-

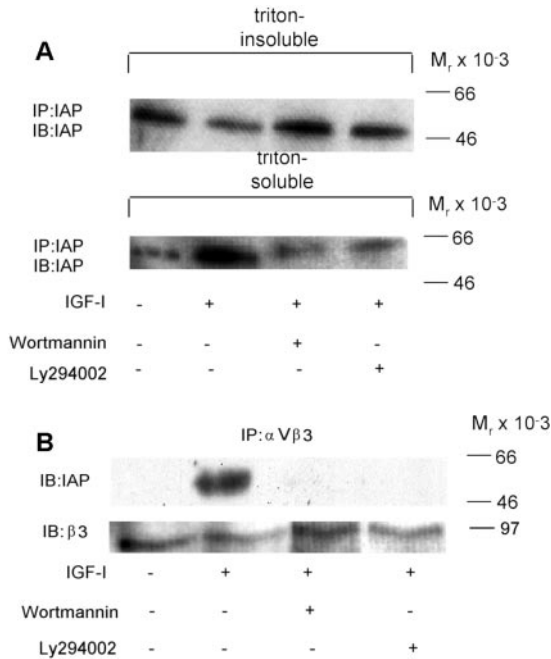


FIG. 4. A, effect of PI3K inhibitors on IGF-I-stimulated redistribution of IAP. Cells were incubated overnight in SFM, and the inhibitors (100 nM wortmannin or 1 μ M Ly294002) were added 30 min before the addition of IGF-I. After a 24-h incubation, the Triton-soluble and Triton-insoluble cell lysates were immunoprecipitated with the anti-IAP monoclonal antibody (B6H12). The level of IAP was then determined by Western immunoblotting with the polyclonal anti-IAP antibody. The results are expressed as arbitrary scanning units. *Top panel*: lane 1, 19,231; lane 2, 11,105; lane 3, 19,765; and lane 4, 16,882. *Bottom panel*: lane 1, 14,365; lane 2, 32,108; lane 3, 17,508; and lane 4, 11,964. **B**, effect of PI3K inhibitors on IGF-I-stimulated increase in IAP- $\alpha_V\beta_3$ association. Cells were incubated overnight in SFM, and the inhibitors (100 nM wortmannin or 1 μ M Ly294002) were added 30 min before the addition of IGF-I. After a 24-h incubation, cell lysates were then immunoprecipitated with α_V , and the amount of IAP associated with the $\alpha_V\beta_3$ complex was determined by immunoblotting with the anti-IAP monoclonal antibody (*top panel*). The results are expressed as arbitrary scanning units: lane 1, 2,797; lane 2, 28,643; lane 3, 2,396; and lane 4, 1,635. To control for loading inequities, the amount of $\alpha_V\beta_3$ immunoprecipitated in the same experiment is shown in the *bottom panel* by immunoblotting for β_3 . The results are expressed as arbitrary scanning units: lane 1, 1,811; lane 2, 1,545; lane 3, 2,282; and lane 4, 1,442.

tant for the increase in complex formation, we examined the effect of various inhibitors on both IGF-I-stimulated IAP redistribution and IAP-integrin complex formation. We have previously shown that inhibitors of the PI3K pathway effectively block IGF-I-stimulated migration (18). Fig. 4A shows, as before, that IGF-I stimulates a 1.7-fold decrease in IAP levels in the Triton-insoluble fraction (*top panel*) and a 2.2-fold increase in the Triton-soluble fraction (*bottom panel*). However, in the presence of two structurally distinct PI3K inhibitors, wortmannin and LY294002, the IGF-I-stimulated redistribution of IAP from the Triton-insoluble fraction to the Triton-soluble fraction was inhibited. This inhibition of IAP translocation from the Triton-insoluble fraction to the Triton-soluble fraction was associated with an inhibition in IGF-I-stimulated complex formation (Fig. 4B, *top panel*). Reprobing of the membrane with the β_3 antibody (Fig. 4B, *bottom panel*) showed that the reduction in IAP- $\alpha_V\beta_3$ association was not due to changes in the amount of $\alpha_V\beta_3$ immunoprecipitated under each condition. Neither of the inhibitors had any effect on basal IAP-integrin association (data not shown).

B6H12, the Anti-IAP Monoclonal Antibody, Blocks Both IGF-I-stimulated IAP-Integrin Complex Formation and Cell Migration—To determine whether the IGF-I-stimulated increase in IAP binding to $\alpha_V\beta_3$ was required for IGF-I-stimulated migra-

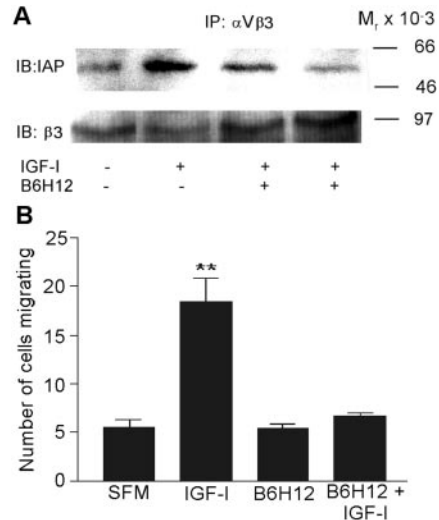


FIG. 5. A, effect of anti-IAP monoclonal antibody B6H12 on the IGF-I-stimulated increase in IAP- $\alpha_V\beta_3$ complex formation. Cells were grown to 80–90% confluence in 10-cm plastic dishes in growth medium and then incubated overnight in SFM. This was followed by a 2-h incubation with or without B6H12 (4 μ g/ml). This was then followed by a 24-h incubation with or without IGF-I. After lysis and immunoprecipitation with an anti- α_V antibody, the level of IAP association was determined by Western immunoblotting with the monoclonal anti-IAP antibody (*top panel*). The results are expressed as arbitrary scanning units: lane 1, 4,907; lane 2, 13,471; lane 3, 6,590; and lane 4, 4,164. To control for loading inequities, the amount of β_3 co-precipitating with α_V in the same experiment is shown in the *bottom panel*. The results are expressed as arbitrary scanning units: lane 1, 4,818; lane 2, 5,295; lane 3, 5,482; and lane 4, 6,286. **B**, effect of anti-IAP monoclonal antibody B6H12 on IGF-I-stimulated migration. Cells were grown to confluence over a 7-day period and then wounded as described under “Experimental Procedures.” Cells were preincubated with B6H12 (4 μ g/ml) for 2 h before the addition of IGF-I (100 ng/ml). The number of cells migrating across the wound at at least five preselected regions of the wound was counted. Each data point represents the mean \pm S.E. of three independent experiments. **, $p < 0.05$ when migration in the presence of IGF-I is compared with incubation in SFM alone.

tion, we determined the effect of inhibiting complex formation on this process. Fig. 5A (*top panel*) shows that the monoclonal anti-IAP antibody (B6H12) potently inhibited the IGF-I-stimulated increase in IAP-integrin complex formation. Reprobing of the membrane with the β_3 antibody (Fig. 5A, *bottom panel*) showed that the reduction in association was not due to changes in the amount of $\alpha_V\beta_3$ immunoprecipitated under each condition. Fig. 5B shows that although IGF-I stimulated a significant (3.3 \pm 0.1-fold, mean \pm S.E.; $n = 3$; $p < 0.05$) increase in cell migration, this increase was completely inhibited if cells were preincubated with B6H12.

DISCUSSION

Similar to some types of transmembrane receptors, integrins have the ability to modulate their affinity for extracellular ligands in response to various signals from within the cell. This change can result in a switch from an inactive to an activated state (22). Binding of the activated integrin to extracellular proteins then generates signals that regulate cell adhesion, spreading, and migration. Several studies have shown that IAP can modulate $\alpha_V\beta_3$ affinity for its ligand, Vn. Using both anti-IAP antibodies and IAP-negative cells, it has been shown that IAP is required for $\alpha_V\beta_3$ binding to Vn-coated beads (11, 12). Membrane-anchored IAP containing one IgG domain in the extracellular region of the molecule is required to detect a positive effect of IAP on integrin signaling (12). This suggests that a direct physical association between the extracellular domain of IAP and the extracellular regions of α_V and/or β_3 occurs. In previous studies, we have shown that IGF-I can

stimulate a significant increase (2.4-fold) in $\alpha_V\beta_3$ ligand binding to the disintegrin kistrin and that this change requires 12 h. In this study, we have extended that observation to show that IGF-I can stimulate a 7-fold increase in the binding of $\alpha_V\beta_3$ to soluble Vn and that this increase requires at least 12 h of incubation with IGF-I. The results from this study show that IGF-I stimulates a 6.5-fold increase in IAP association with $\alpha_V\beta_3$ over the same time course. In light of the clearly established role of IAP in modulating the binding of Vn to $\alpha_V\beta_3$ and the similar time course, we propose a model whereby IGF-I stimulates this increase in ligand binding by stimulating the physical association of IAP and $\alpha_V\beta_3$.

Both IAP and $\alpha_V\beta_3$ have been shown to be expressed on the cell surface independently of each other, and complex formation is believed to be in equilibrium with nonassociated IAP and $\alpha_V\beta_3$ (12). A previous study showed that increasing the level of expression of either $\alpha_V\beta_3$ or IAP in the Triton-soluble fraction led to increased complex formation and increased vitronectin-coated bead binding (12). Our results show that IGF-I treatment stimulates this increase in IAP- $\alpha_V\beta_3$ association, at least in part, by stimulating the redistribution of IAP from the Triton-insoluble fraction to the Triton-soluble fraction, the fraction with which >90% of the $\alpha_V\beta_3$ is associated.

Our conclusion that the increase in IAP association with $\alpha_V\beta_3$ requires translocation of IAP to the Triton-soluble fraction is supported by our findings using the inhibitors of PI3K. Blocking the PI3K pathway inhibited both redistribution of IAP to the Triton-soluble fraction and IAP- $\alpha_V\beta_3$ complex formation. Because we have shown that inhibiting PI3K inhibits the cellular migration response to IGF-I (18), these results support the conclusion that redistribution of IAP is required for IGF-I-stimulated increase in IAP- $\alpha_V\beta_3$ association and that this change could lead to stimulation of cell migration. It remains to be determined whether this is only one of several steps required for IGF-I to increase IAP- $\alpha_V\beta_3$ association or whether this is the only change required. Because both IAP and $\alpha_V\beta_3$ undergo conformational changes (19), these changes could also be necessary for the observed increase in complex formation to occur.

Previous studies have suggested that the cellular localization of IAP may be important in the regulation of its activity (20, 21). Plasma membranes contain specialized glycosphingolipids and cholesterol-rich domains (membrane rafts) that have been proposed to be regulators of cell surface signal transduction via the segregation of membrane constituents. The proteins associated with membrane rafts can be isolated because of their resistance to solubilization by nonionic detergents at 4 °C (14). It has been suggested that membrane rafts are efficient sites for the initiation of signal transduction due to the sequestration of specific components of signaling complexes within this membrane fraction. However, in some cases, signaling molecules are also translocated out of rafts. Previous studies have demonstrated that IAP is associated predominantly but not exclusively with membrane rafts (20, 21). It was shown in C32 melanoma cells that $\alpha_V\beta_3$ within the membrane raft was four times more likely to form a complex with IAP compared with $\alpha_V\beta_3$ in the Triton-soluble component and that cholesterol, which is necessary for stabilization of raft formation, is also required for complex assembly and signaling (20). In a different context, *e.g.* T-cell signaling, the association of IAP with membrane rafts was associated with its ability to stimulate an increase in f-actin-stimulated cell spreading and the association of protein kinase C with the cytoskeleton (21). However, it was also shown in the C32 melanoma cells that IAP in the Triton-soluble fraction formed a complex with β_3 and G proteins and that association with membrane rafts was not

necessary for the IAP enhancement of α_V integrin binding to Vn-coated beads (12). Similarly, in T cells, IAP participation in phospholipase C and Ca^{2+} signaling was not dependent on its raft association (21). Thus, these studies have demonstrated that IAP is capable of interacting with both integrins and signaling cascades both within and outside of membrane rafts. Because insolubility in Triton detergent at 4 °C is a characteristic of proteins associated with membrane rafts (14), and given that caveolin, a marker protein for membrane rafts (17), was also found almost exclusively in the Triton-insoluble fraction, it seems likely that in our experiments extraction with Triton at 4 °C accurately reflected both IAP and caveolin associated with membrane rafts. Because $\alpha_V\beta_3$ was associated almost exclusively with the Triton-soluble fraction, consistent with previous reports (23), and complex formation only occurred after redistribution of IAP into the Triton-soluble fraction, our studies suggest that the site of localization of both $\alpha_V\beta_3$ and IAP appears to be a major determinant of whether they will interact.

To our knowledge, this is the first report describing the stimulation of translocation of IAP from the Triton-insoluble to the Triton-soluble fraction, and it suggests that IGF-I may be an important regulator of IAP activity through its ability to regulate its cellular distribution. Exactly how IGF-I stimulates this redistribution remains to be elucidated. Possible mechanisms include the alteration of an abundant raft protein such as caveolin-1 or changes in potential posttranslational modifications within IAP that have been shown to account for raft association such as glycosylphosphatidyl inositol anchoring, palmitoylation, or myristylation (14).

In the absence of appropriate cells that do not express IAP, we cannot say definitively that the IGF-I-stimulated increase in IAP association with $\alpha_V\beta_3$ results in the increase in ligand binding. However, previous studies demonstrating such a role for IAP strongly support this interpretation. However, our study does show that IAP plays an important role in IGF-I-stimulated vascular smooth muscle cell migration because the anti-IAP monoclonal antibody can block both the IGF-I-stimulated increase in IAP association with $\alpha_V\beta_3$ and IGF-I-stimulated migration. This is consistent with a previous report that demonstrated that an IAP-integrin association was involved in stimulation of vascular smooth muscle cell migration in response to thrombospondin-1 (24). However, in that study, the integrin involved was $\alpha_2\beta_1$; because our cells express very low levels of $\alpha_2\beta_1$ (5), we cannot determine whether IGF-I can have a similar effect on IAP association with this integrin. Despite the differences in integrin expression, both studies suggest that the IAP regulation of integrin ligand binding is an important mechanism in the regulation of smooth muscle cell migration in response to different stimuli. Additional studies will be necessary to elucidate whether the IAP also has additional signaling effects contributing to IGF-I-stimulated migration independent of its effect on $\alpha_V\beta_3$.

In summary, we have shown that IGF-I can stimulate an increase in IAP-integrin association via a PI3K-dependent pathway. This increase appears to result from an increase in IAP association with the Triton-soluble fraction of the cell. Taken together with previous evidence showing that IAP can increase $\alpha_V\beta_3$ binding to Vn, the data presented here suggest that the IGF-I regulation of IAP cellular distribution may be involved in the ability of IGF-I to alter the ligand binding capacity of $\alpha_V\beta_3$. Because ligand binding by integrins triggers signals inside the cell necessary for cell migration, our findings suggest that the IAP-induced alteration in $\alpha_V\beta_3$ ligand binding is important in the regulation of IGF-I-stimulated cell migration.

Acknowledgment—We thank Laura Lindsey for help in preparing the manuscript.

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