βig-h3 Interacts with α3β1 Integrin to Promote Adhesion and Migration of Human Hepatoma Cells

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βig-h3 is a TGF-induced extracellular matrix (ECM) protein. Our previous evidence suggests that βig-h3 may promote adhesion and invasion potential of human hepatoma cells, but the mechanism underlying this process is still unknown. The present study identifies a pivotal role for molecules of the βig-h3 signal transduction pathway. We demonstrated that βig-h3 co-immunoprecipitated with α3β1 integrin in human 7721 hepatoma cells. The addition of α3β1 integrin antibodies inhibited the elevated adhesion and migration in βig-h3-over-expressing 7721 cells, but not in βig-h3 siRNA transfected 7721 cells. The expression and activity of integrin downstream molecules FAK and paxillin show a positive correlation with βig-h3 expression in different human hepatoma cells. Levels of focal adhesions and stress fibers were decreased in βig-h3 siRNA transfected 7721 cells. We suggest that by interaction with α3β1 integrin, βig-h3 activates FAK-paxillin signaling linkage, leads to cytoskeleton reorganization, and thus enhances the metastatic potentials of human hepatoma cells. Exp Biol Med 234:35–39, 2009

Key words: βig-h3; hepatoma cells; metastasis; signal pathway; α3β1 integrin

Introduction

βig-h3 gene was first identified in the human A549 lung adenocarcinoma cell line by long-term treatment with TGF-β1. It encodes a 68-kDa protein with an amino-terminal signal peptide, and 4 internal FAS-1 domain repeats which are homologous to fasciclin-1 in Drosophila (1). Its carboxy terminal sequence can serve as a ligand recognition site for several integrins (2). As an ECM protein, βig-h3 is involved in cell growth, migration, apoptosis, wound healing and tumorigenesis (1, 3–5), and might function as either a promoter or an inhibitor of carcinogenesis, depending on cells and tumor types. The gain or loss of expression of βig-h3 might be involved in tumor formation and acquisition of a metastatic phenotype in human cancer. We previously reported that the expression of βig-h3 is positively correlated with the expression of HAb18G/CD147 which is upregulated in different human hepatoma cell lines and plays an important role in invasion and metastasis (6). Several reports suggest integrin α3β1 is associated with CD147 (7), but the precise mechanism of invasion and metastasis involving the interaction of CD147 with integrin is not as clear yet. βig-h3 does have the RGD motif which binds to integrins, but no investigations have been conducted on the exact signal transduction pathway of βig-h3 to promote adhesion and invasion of human hepatoma cells. Here we investigated the relative contributions of the integrin signaling pathway to βig-h3-elevated adhesion and invasion of human hepatoma cells. These studies demonstrate that by interaction with α3β1 integrin, βig-h3 increases the phosphorylation and the expression level of FAK and paxillin, and by FAK-paxillin signaling linkage, leads to cytoskeleton reorganization, thereby enhancing malignant behaviors of human hepatoma cells.

Materials and Methods

Cell Culture. Human SMMC-7721 cells (obtained from the Institute of Cell Biology, Academic Sinica, Shanghai, China) were maintained in RPMI 1640 medium (Gibco, New York, USA) supplemented with 10% FBS, 1% penicillin/streptomycin and 2% L-glutamin at 37°C in a humidified atmosphere of 5% CO_2.

Cell Transfection. The expression vector pEGFP-C2-βig-h3 was made in our laboratory (6). The recombinant

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plasmids were transfected into 7721 cells using LipofectamineTM2000 (Invitrogen) according to the manufacturer's instructions. The pEGFP-C2 empty vector was transfected into 7721 cells as a negative control. The expression of the EGFP was detected under a fluorescent microscope (Olympus, Nagoya, Japan).

Western Blot Analysis. Protein expression was analyzed by Western blotting as previously described (8). In brief, cells were lysed in 1% n-octyl-p-D-glucopyranoside(OG) buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% OG, 1 mM EDTA, 10 g/ml leupeptin, 2 g/ml aprotinin, 1 mM PMSF). BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) was employed to determine the total protein density and equal amounts of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then electransferred to polyvinylidene fluoride (PVDF) microporous membrane (Millipore, Boston, MA). After blocking with 5% non-fat milk, the membrane was incubated for 2 hrs at room temperature with the designated antibody. Immunodetection was performed by using the Western-Light chemiluminescent detection system (Applied Biosystems, Foster, CA).

Co-Immunoprecipitation (Co-IP). The interactions between βig-h3 and integrin α3β1 in native cells were detected by ProFoundTM Mammalian Co-Immunoprecipitation Kit (Pierce), according to the manufacturer's instructions. Briefly, 7721 cells were lysed by M-per reagent and then the lysate was collected onto Coupling gel which was pre-bound with βig-h3 monoclonal antibody, followed by four washes with the co-immunoprecipitation buffer. Immune complexes were eluted from Coupling gel with Elution buffer and resolved by 10% SDS-PAGE. Integrin α3β1 in the immune complexes was detected by Western blot using, respectively, anti-α3 and anti-β1 antibodies (BD, NY). HRP-conjugated goat anti-rabbit IgG (Amersham Pharmacia) was used as the negative control.

Gene Silencing. The sense sequence for βig-h3 small interfering RNAs (siRNAs) was 5′-CCUUUACGAGACCCUGGGATT-3′ and the anti-sense sequence was 5′-UCCCAGGGUCUCGUAAAGGTT-3′ (Ambion, Austin, TX). βig-h3 siRNA was suspended in serum-free DMEM with LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) for 20 mins. The mixture was then aliquoted into 6-well plates containing pre-plated FHCC-98 cells to a final concentration of 40 nM of siRNA per well. Forty-eight hours later, cells were lysed for Western blot analysis. Silencer negative control siRNA (snc-RNA) was used as a negative control under similar conditions (Ambion).

Adhesion Assay. The wells of a 96-well culture plate were coated with Matrigel (BD, NY) at a concentration of 5 mg/ml and incubated at 4°C overnight. The coated wells were blocked with PBS containing 2% BSA for 30 mins and then washed with PBS. Cells suspended in serum-free medium containing 0.1% BSA were added to the wells (2×104/well) and incubated at 37°C, 5% CO_2 for 30–60 mins with or without antibodies. After removing medium and non-attached cells, 0.2% crystal violet was added for 10 mins. The plate was gently washed with tap water and dried in air for 24 hrs. Then, 0.1 ml 5% SDS/50% ethanol was added for 20 mins and then the plates were read at 540 nm.

Invasion Assay. The chemotactic cell invasion assay was performed using 24-well transwell units with an 8-mm pore size polycarbonate filter (Millipore) according to the method described previously (9). Each lower compartment of the transwell contained 600 ml of 0.5% FBS as chemoattractant or 0.5% BSA as negative control in RPMI 1640. The upper side of a polycarbonate filter was coated with Matrigel (5 mg/ml in cold medium) to form a continuous thin layer. Prior to addition of the cell suspension, the dried layer of Matrigel matrix was rehydrated with medium without FBS for 2 hrs at room temperature. Cells (1×10^5) pre-incubated with antibodies were suspended in 0.1 ml RPMI 1640 containing 0.1% BSA and added into the upper compartment of the Transwell unit and incubated for 36 hrs at 37°C in a humidified atmosphere containing 5% CO_2. Cells remaining in the upper compartment were completely removed with gentle swabbing. The number of cells that had invaded through the filter into the lower compartment was determined using a colorimetric crystal violet assay.

Immunofluorescence. Cells were allowed to attach for 3 hrs to glass coverslips. They were then fixed in 3.7% formaldehyde in PBS, permeabilized with 0.5% Triton X-100 and blocked with 1% BSA (Fraction V) in PBS for 1 hr. Coverslips were incubated with the indicated antibodies at a 1:500 dilution, or with rhodamine-phalloidin (Molecular Probes, Eugene, OR) at a 1:40 dilution in PBS for 20 mins. Antibody-treated cells were washed in PBS and incubated with FITC goat anti-mouse or Texas red donkey anti-goat secondary antibodies (Pierce) at a 1:500 dilution in PBS for 1 hr. Cell nuclei were stained with DAPI (Vector Labs, Burlingame, CA). Finally, the cells were mounted using glycerol. Cells probed with rhodamine-phalloidin were washed and immediately mounted and observed by FV1000 laser scanning confocal microscopy (Olympus).

Results

Immunoprecipitation of βig-h3 with α3β1 Integrin in Human Hepatoma Cells. Integrins are cell surface adhesive receptors composed of α- and β-chain heterocomplexes, which mediate the physical and functional interactions between the cell and its surrounding ECM. Therefore, they serve as bidirectional transducers of extracellular and intracellular signals in processes such as cell adhesion, proliferation, differentiation, apoptosis and tumor progression (7). α3β1 integrin was found to co-immunoprecipitate with endogenous βig-h3 in 7721 cell lysates, indicating that βig-h3 and α3β1 integrin interact in their native conformations (Fig. 1).

Involvement of α3β1 Integrin in βig-h3-Induced Adhesion and Migration of Human Hepatoma Cells. To investigate the involvement of α3β1 integrin in

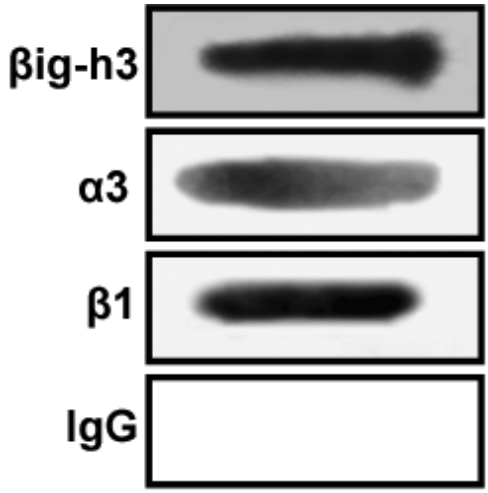

Figure 1. βig-h3 immunoprecipitation with α3β1 integrin in 7721 cells. Lysates of 7721 cells were subjected to immunoprecipitation with anti-βig-h3 antibody. The co-precipitated α3β1 integrin was identified by Western blot analysis. Mouse IgG was used as a negative control.

the βig-h3-induced adhesion and migration by human hepatoma cells, we incubated pEGFP-C2 empty vector transfected 7721 cells and βig-h3 transfected 7721 cells with antibodies respectively for α3 and β1 integrins individually, or in combination, to test the inhibitory effect on cell adhesion and invasion potential. The presence of antibodies for α3 and β1 integrins markedly reduced cell adhesion and adhesion in the empty vector transfected 7721 cells and completely blocked the enhancement of these effects of βig-h3 in the βig-h3 transfected 7721 cells ($P < 0.01$, Fig. 2). However, when the experiments were reproduced in βig-h3 siRNA transfected 7721 cells, no significant variations were observed between α3 and β1 antibody treated groups and the untreated group. The addition of α3 or β1 integrin antibodies reduced the adhesion and invasion of 7721 cells and βig-h3 transfected 7721 cells to the levels detected in the βig-h3 siRNA transfected 7721 cells ($P > 0.05$). To further rule out the nature of the integrins involved in βig-h3-mediated metastasis and migration, experiments were repeated with cells incubated with α3 antibodies and β1 antibodies together. No significant differences were observed in cell adhesion and invasion when comparing the groups treated with either antibodies or both ($P > 0.05$).

Changes in Expression and Phosphorylation of FAK and Paxillin in Response to βig-h3 Expression in Human Hepatoma Cells. Signaling pathways of integrins involve many cytoskeleton proteins and enzymes, of which FAK and paxillin are crucial components (10). To identify the molecules involved in the βig-h3-mediated and integrin-activated invasion and metastasis by human hepatoma cells, we examined the expressions of FAK, paxillin and their phosphorylated forms [p-FAK (pY397) and p-paxillin (pY118)] in cells with different expression levels of

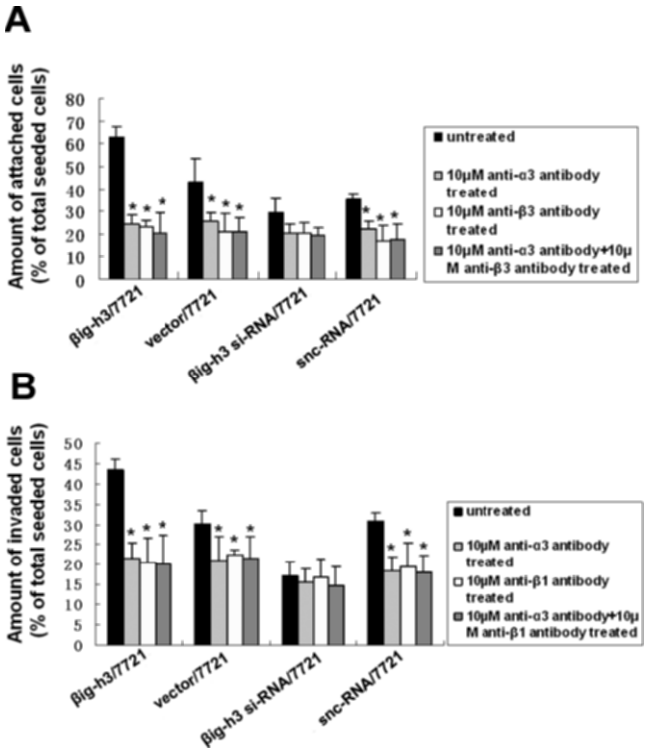

Figure 2. Adhesion (A) and invasive potential (B) of hepatoma cells incubated with or without antibodies specific for α3 or β1. The adhesion and invasion assays were performed as described in Materials and Methods. Bars represent the mean of triplicate samples; error bars represent standard deviation. Data are representative of three independent experiments. * $P < 0.01$ vs. no antibody treated cells (one-way ANOVA).

βig-h3. Expression levels of FAK and p-FAK increased 27.4% and 51.6% respectively in βig-h3 transfected 7721 cells compared with empty vector transfected 7721 cells. FAK and pFAK levels decreased by 43.5% and 58.2% respectively, in the βig-h3 siRNA transfected 7721 cells compared with snc-RNA transfected 7721 cells ($P < 0.01$, Fig. 3). Paxillin expression and phosphorylation patterns also demonstrated a positive correlation with βig-h3 expression. The expression levels of paxillin and p-paxillin increased 22.2% and 40.0% respectively in βig-h3 transfected 7721 cells compared with empty vector transfected 7721 cells, and were diminished 60.4% and 36.3% respectively in βig-h3 siRNA transfected 7721 cells compared with snc-RNA transfected 7721 cells ($P < 0.01$, Fig. 3).

Focal Adhesion Levels and the Organization of Cytoskeleton in βig-h3-Suppressed Human Hepatoma Cells. Cells were stained for FAK and paxillin to assess the relative quantity and cellular distribution of focal adhesions. Focal adhesions were significantly decreased and both FAK and paxillin were redistributed in βig-h3 siRNA transfected 7721 cells as compared with the snc-RNA transfected 7721 cells (Fig. 4). F-actin stress fibers labeled with rhodamine-phalloidin were reduced and disorganized specifically in βig-h3 siRNA transfected 7721 cells, while

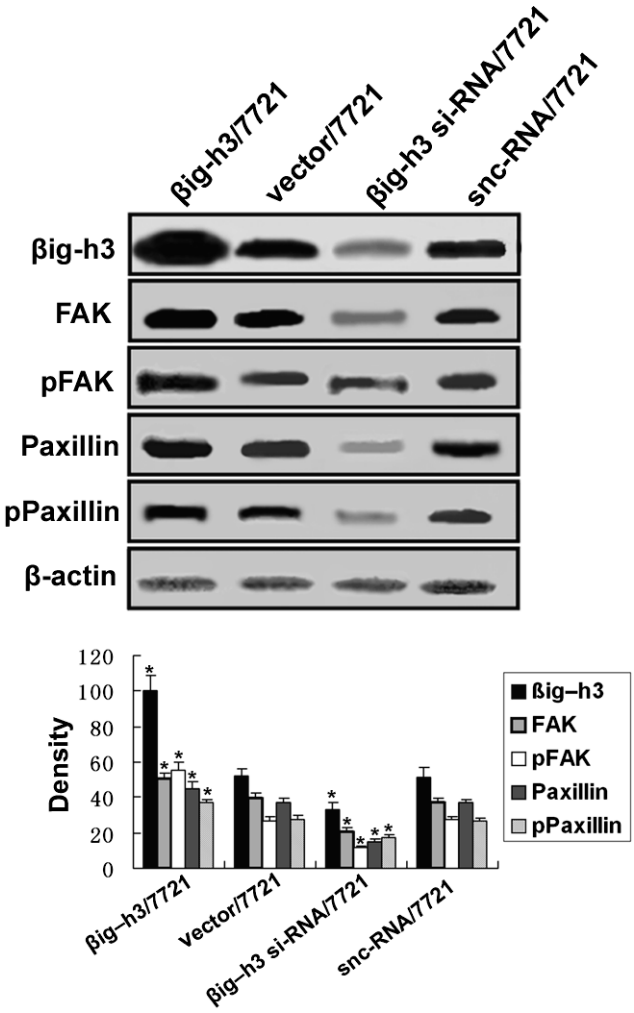

Figure 3. Expressions and activity levels of FAK and paxillin in βig-h3 overexpressing and βig-h3 silencing hepatoma cells. FAK, p-FAK(pY397), paxillin and p-paxillin(pY118) expression levels were analyzed in βig-h3 transfected 7721 cells and βig-h3 siRNA transfected 7721 cells. Empty vector transfected 7721 cells and snc-RNA transfected 7721 cells were used as negative controls respectively. Protein expressions levels were normalized to human β-actin expression. Bars represent the mean of triplicate samples; error bars represent standard deviation. Data are representative of three independent experiments. * $P < 0.01$ vs. the corresponding negative control cells (Student's *t* test).

those in snc-RNA transfected 7721 cells exhibited plentiful and thicker filopodial protrusions.

Discussion

Extracellular matrix (ECM) is involved in the morphology, migration, proliferation and differentiation of cells. As an ECM protein, βig-h3 influences cell attachment, migration and invasion (1, 11). The expression and functions of βig-h3 are diverse in different cells types, and it is still not clear whether it is a promoter or an inhibitor of tumorigenesis. We have, however, previously demonstrated that βig-h3 overexpression enhances invasion and metastasis potential in hepatoma cells (6).

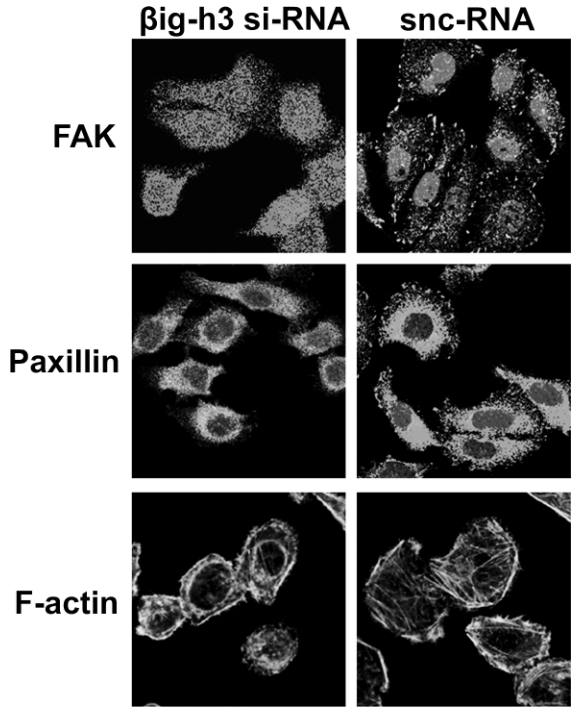

Figure 4. The disposition of focal adhesions in βig-h3 siRNA transfected 7721 cells. Top and center rows: cells were probed with either anti-FAK or anti-paxillin antibodies. Bottom row: cells were probed with rhodamine-phalloidin antibody. Snc-RNA transfected 7721 cells were used as negative controls (original magnification ×600).

βig-h3 contains four internal FAS-1 repeat domains and an RGD (Arg-Gly-Asp) motif which may interact with different integrins on various cell types to influence cell attachment and migration in various tumor cells (12–14). In human hepatoma cells, the integrins α3β1 and α6β1 are highly expressed and mediate adhesion and invasion processes (15–17).We previously proposed a hypothesis that integrins (especially α3β1) act as a bridge between HAb18G/CD147 and βig-h3 to form a trimer, which regulates metastasis of human hepatoma cells. However, direct evidence in support of this hypothesis has thus far been absent. In the present study, we have demonstrated that βig-h3 can be co-immunoprecipitated with α3β1 integrin and that this interaction enhanced βig-h3-induced adhesion and the invasive behavior of human hepatoma cells. Antibody-mediated blocking of integrin signaling completely reduced the βig-h3-mediated adhesion and migration by human hepatoma cells. We propose that βig-h3 induced signals are transduced through α3β1 integrins in these hepatoma cells. The sites for βig-h3 binding to α3β1 integrin have been recently identified. These are highly conserved amino acid sequences (aspartic acid and isoleucine) in the second and fourth FAS-1 domains of βig-h3 for binding to α3β1 integrin in human corneal epithelial cells (14). Whether βig-h3 may play roles in human hepatoma cell through its two α3β1 integrin-interacting motifs needs further investigation.

To gain insight into integrin signaling mechanisms by which βig-h3 promotes adhesion and invasion of human hepatoma cells, we investigated the expression and activation of integrin downstream molecules. It is known that integrins initiate intracellular signaling pathways by recruiting and activating protein kinases such as focal adhesion kinase (FAK) and paxillin. These signaling pathways are involved in the regulation of cell migration, proliferation, and survival (18). We demonstrated in the present study that not only the phosphorylation levels of FAK and paxillin, but also the expression levels of FAK and paxillin were enhanced by βig-h3 overexpression in human hepatoma cells. These results suggest that βig-h3 can facilitate FAK and paxillin activation through its interaction with α3β1 integrin.

As a direct downstream target molecule for integrin activation, FAK connects to integrins through C-terminal domain-mediated interactions. The FAK C-terminal region which contains the focal adhesion targeting (FAT) domain also contains binding sites for the integrin-associated proteins talin and paxillin. FAK activity is regulated by integrin-mediated cell adhesion as well as activation of growth factor and G-protein-linked receptors (19, 20). In many cells, FAK activation leads to the Src homology 2 (SH2) domain of Src family protein tyrosine kinases (PTKs) binding to the motif surrounding the FAK Tyr-397 phosphorylation site. It is the FAK-Src complex that promotes the tyrosine phosphorylation of substrates such as paxillin and p130Cas, and FAK-Src-associated signaling can lead to the activation of multiple protein kinase cascades (19–21).

FAK and paxillin are crucial components of focal adhesions, which are points for high-strength attachment of the cell to the ECM formed by the leading edge of a migrating or attaching cell (21). The level of focal adhesions represents cell adhesion and motion potentials (10). The studies reported here show that suppression of βig-h3 expression can reduce focal adhesions and filopodial protrusions in hepatoma cells. This is the first direct evidence for mechanisms by which tumor-associated molecule βig-h3 promotes adhesion and migration in human hepatoma cells.

In summary, this study demonstrates that βig-h3 interactions with α3β1 integrin, activates the FAK-paxillin signaling pathway, leading to cytoskeleton reorganization, thereby enhancing the metastatic potentials of hepatoma cells.

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