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Targeting of the protein interaction site between FAK and IGF-1R

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Abstract

The interaction of focal adhesion kinase (FAK) and insulin-like growth factor-1 receptor (IGF-1R) plays an important role in cancer cell survival. Targeting this interaction with small molecule drugs could be a novel strategy in cancer therapy. By a series of pull-down assay using GST-tagged FAK fragments and His-tagged IGF-1R intracellular fragments, we showed that the FAK-NT2 (aa 127–243) domain directly interacts with the N-terminal part of the IGF-1R intracellular domain. Overexpressed FAK-NT2 domain was also shown to co-localize with IGF-1R in pancreatic cells. Computational modeling was used to predict the binding configuration of these two domains and to screen for small molecules binding to the interaction site. This strategy successfully identified a lead compound that disrupts FAK/IGF-1R interaction.

Introduction

Focal adhesion kinase (FAK) is a critical tyrosine kinase that modulates cell adhesion, migration, proliferation and survival in response to extracellular signals [1–6]. The C-terminal domain of FAK can bind to a number of proteins, such as paxillin, talin, phosphoinositide 3-kinases (PI3K) and the GTPase-activating protein Gaf, leading to the localization of FAK to focal adhesions and reorganization of the actin cytoskeleton [6–8]. FAK activation starts from autophosphorylation of tyrosine 397 (Y397) on the N-terminus [9]. The N-terminal FERM domain arranges into three lobes and regulates the kinase activity of FAK [10–12]. The N-terminal domain of FAK has been shown to associate with β subunit of integrin and growth factors [1,13,14], implying the important role of FAK in integrating diverse cellular signaling pathways. As a key player in regulating normal cellular activities such as adhesion, migration and survival, FAK is likely implicated in cancer cell invasion, metastasis and survival [15–19]. Upregulation of FAK has been documented in many kinds of tumors [20–26].

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The insulin-like growth factor (IGF) signaling system also plays an important role in the formation and progression of human cancer. Deregulation of the IGF system, such as the overexpression and over-activation of insulin-like growth factor-1 receptor (IGF-1R) is a common event in several malignancies [27–29]. Mature IGF-1R is a heterotetramer consisting of two α subunits and two β subunits. The α subunits reside entirely extracellularly and are responsible for ligand binding. The β subunit contains a transmembrane domain, a juxtamembrane domain, a tyrosine kinase domain and a C-terminal tail [30]. Initiated by ligand binding, IGF-1R undergoes conformational changes and autophosphorylation that trigger an intracellular signaling cascade through the insulin receptor substrates (IRSs) and Src homology and collagen domain protein (shc). The two main downstream signals of IGF-1R are the mitogen-activated protein kinase (MAPK) pathway and phosphatidylinositol 3 kinase (PI3) pathway [30]. IGF-1R signaling is required for cellular transformation by most oncogenes and facilitates the survival and spread of transformed cells [31]. Interruption of IGF signaling has been shown to inhibit tumor growth and block metastasis in a wide variety of tumor models [32,33].

Previous studies in our laboratory have shown that IGF-1R physically interacts with FAK to provide survival signals in human pancreatic cancer cells [34]. Further understanding of this mechanism may provide novel targets for drug treatment of pancreatic cancer. In the present study, using pulldowns with purified protein fragments, we demonstrate direct binding of a 116 amino acid fragment of the amino terminus of FAK with the intracellular domain of IGF-1R. Subsequently, computational molecular modeling was performed to predict the configuration of the binding between the amino terminus fragment of FAK and IGF-1R. Based on this information, small molecules were screened by high performance computing, using van der Waals charges and electrostatic interactions, for binding to FAK in the pocket of interaction between FAK and IGF-1R. A representative small molecule was selected and shown to disrupt binding of FAK and IGF-1R. This information will be utilized to select additional molecules that disrupt FAK and IGF-1R signaling and function by disrupting their protein-protein interactions.

Materials and Methods

FAK and IGF-1R cDNA constructs

FAK-NT (a.a. 1–415, N-terminal domain of FAK), FAK-NT1 (a.a. 1–126), FAK-NT2 (a.a. 127–243), FAK-NT3 (a.a. 244–415) and FAK-CD (a.a. 677–1052, C-terminal domain of FAK) were amplified by PCR using gene specific primers and cloned into the pGEX-4T1 GST vector (Amersham Biosciences, NJ). All sequences were confirmed by automatic sequencing (ICBR Sequencing Facility, University of Florida). cDNA for IGF-1R fragments consisting of the entire intracellular domain (a.a. 959–1367, IGF-1R-IC), N-terminal part of intracellular domain (a.a. 959–1266, IGF-1R-IC-N) and C-terminal of intracellular domain (a.a. 1267–1376, IGF-1R-IC-C) were amplified by PCR and cloned into pET200/D-topo vector (Invitrogen, CA) for expression of His-tagged proteins.

Production of GST-fusion proteins

The GST-fusion proteins (FAK fragments) were expressed in BL21 (DE3) *Escherichia coli* bacteria by incubation with 0.2 mM isopropyl β -D-galactopyranoside (IPTG) for 6 h at 37 °C. The bacteria were lysed by sonication, and the fusion proteins were purified with glutathione-sepharose 4B beads (GE Healthcare, NJ).

Production of His-tagged proteins

The His-tagged proteins (IGF-1R fragments) were expressed in BL21 (DE3) *Escherichia coli* bacteria by incubation with 0.2 mM (IPTG) for 6 h at 37 °C. The proteins were then isolated and purified using B-PRE 6xHis Spin Purification kit (Thermo Scientific, Rockford, IL).

Pull-down Assay

For the pull-down binding assay, His-tagged IGF-1R fragment protein (0.1 µg) were precleared with GST immobilized on glutathione-sepharose 4B beads by rocking for 1 h at 4 °C. The precleared His-tagged protein were incubated with 2 µg of GST fusion protein immobilized on the glutathione-sepharose 4B beads for 1 h at 4 °C and then washed three times with PBS. Equal amounts of GST fusion proteins were used for each binding assay. Bound proteins were boiled in 2x Laemmli buffer and analyzed by SDS-PAGE and Western blotting.

Antibodies used in Western blotting

Anti-FAK (C20) antibody (Santa Cruz Biotechnology. sc-558); anti-FAK antibody clone 4.47 (Upstate, 05-573); anti-IGF-1R β antibody (C20) (Santa Cruz Biotechnology. sc-713); anti-His antibody (QED, 18814-01) and anti-GST antibody (Sigma, G1160).

Cells culture and transfection

Panc-1 cells were obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1 µg/ml penicillin/streptomycin. To over-express FAK fragments, plasmids pEGFP-FAK, pEGFP-FAK-NT1, pEGFP-FAK-NT2 and pEGFP-FAK-NT3 were transfected into cells with Lipofectamine 2000 (Invitrogen, CA).

Immunostaining and Confocal Microscopy

Transfected Panc-1 cells were fixed in 4% paraformaldehyde in 1x PBS for 10 min and permeabilized with 0.2% Triton X-100 for 5 min on ice. After blocking with 25% normal goat serum in 1x PBS for 30 min, cells were washed in 1x PBS, and incubated with anti-IGF-1R antibody (Calbiochem, Cat. # GR11) diluted 1:200 in 25% goat serum in 1x PBS for 1 hour. Cells were then washed in 1x PBS for three times and incubated with Alexa Fluor 546 antibody (Invitrogen, A11003, 1:400 dilution in 25% goat serum) for 1 hour at RT. After washing, cells were incubated with Alexa Fluor 488 conjugated anti-GFP antibody (Invitrogen, A21311) for 1 hour at RT followed by staining with Hoechst 33342. Cells were examined under a fluorescent Zeiss microscope. Confocal microscopy was performed on a confocal laser inverted Leica microscope. Images were captured and analyzed with Leica CS software. Student's t-test was used to compare the co-localization rate of each group.

Structure-based in silico molecular modeling and computational docking

The crystal structures of the N-terminal domain of FAK (PDB code 2AL6) [35] and the kinase domain of IGF-1R (PDB 1P40A) [36] were utilized for *in silico* molecular modeling of their interaction. The molecular interaction program DOT (Daughter of Turnip) was used to predict the binding orientation of IGF-1R kinase domain to FAK-NT2 [37–38]. Briefly, DOT performed a systematic, rigid-body search of IGF-1R kinase domain translated and rotated about the interaction sites of FAK-NT2. 56,000 orientations of IGF-1R kinase domain were generated and scored against the FAK-NT2 domain. Note that all binding modes of IGF-1R were predicted to be with FAK-NT2 region (this was not specified as a constraint in advance). The intermolecular energies for all configurations generated by this search were calculated as the sum of electrostatic and van der Waals energies. These energy terms were evaluated as correlation functions, which were computed efficiently with Fast Fourier Transforms. Based

on these calculations, a small number of configurations were identified as the binding models for FAK/IGF-1R interaction. Further searching of the small molecules that might bind to FAK-NT2 and interrupt FAK/IGF-1R interaction was based on these binding models.

The three-dimensional coordinates of compounds from the database of the National Cancer Institute, Developmental Therapeutics Program (NCI/DTP) were docked onto the predicted interface of FAK-NT2/IGF-1R. All docking calculations were performed with the University of California-San Francisco DOCK 5.1 program, using a clique-matching algorithm to orient small molecule structures with sets of spheres that describe the target sites on FAK [39]. 100 orientations were created for each small molecule in the target site and were scored using the computer program grid-based scoring function. Docking calculations were performed on the University of Florida High Performance Computing supercomputing cluster using 16 processors (<http://hpc.ufl.edu>). A lead compound predicted to bind at high affinity was obtained from NCI for further analysis.

Results

Direct interaction of FAK-NT2 domain and IGF-1R intracellular domain in vitro

To determine the FAK and the IGF-1R domains that physically bind, we produced GST-tagged FAK fragments -- GST-FAK-CD, GST-FAK-NT, GST-FAK-NT1, GST-FAK-NT2, GST-FAK-NT3, and His-tagged IGF-1R intracellular domains -- His-IGF-1R-IC, His-IGF-1R-IC-N and His-IGF-1R-IC-C (Figure 1). Purified GST-tagged proteins were visualized by Coomassie staining and confirmed by Western blot analysis using specific anti-FAK antibody and anti-GST antibody (Figure S1). Using these tagged proteins, pull-down assays were performed to identify the interaction domains between FAK and IGF-1R. First, precleared His-IGF-1R-IC was incubated with GST-FAK-CD glutathione-agarose beads and GST-FAK-NT glutathione-agarose beads. The results showed that only the GST-FAK-NT beads pulled down the intracellular domain of IGF-1R (Figure 2A). Next, the three sub-domains of FAK-NT, GST-FAK-NT1, GST-FAK-NT2 and GST-FAK-NT3, were examined for their binding ability to His-IGF-1R-IC by pull-down assay. As shown in figure 2B, only the NT2 sub-domain of FAK-NT bound to the IGF-1R intracellular domain. Lastly, GST-FAK-NT2 beads were used for pull-down assay with C-terminal domain of IGF-1R, His-IGF-1R-IC-C, and the N-terminal part of the IGF-1R intracellular domain, His-IGF-1R-IC-N, which contains the juxtamembrane region and the kinase domain of IGF-1R. Only His-IGF-1R-IC-N bound to the NT2 domain of FAK (Figure 2C). These pull-down assays mapped the binding region to FAK-NT2 domain (a.a. 127–243) and IGF-1R-IC-N domain (a.a. 959–1266).

Co-localization of overexpressed FAK-NT2 domain with endogenous IGF-1R in pancreatic cancer cells

Plasmids encoding different FAK fragments tagged with GFP were transfected into Panc-1 cells. Endogenous IGF-1R and overexpressed GFP-FAK fragments were immunostained with specific antibody and visualized under the confocal microscope to assess the co-localization of IGF-1R and specific FAK fragments. The GFP-FAK-NT and GFP-FAK-NT2 both had over 93% of pixels overlapping with IGF-1R, while GFP-FAK-NT1 and GFP-FAK-NT3 had less than 21% of pixel overlapping with IGF-1R (Figure 3). These data support the *in vitro* pull-down assay showing the interaction of the NT2 domain of FAK with IGF-1R in living pancreatic cancer cells.

Molecular modeling of the interaction between FAK-NT2 and IGF-1R and screening of small molecules for interruption of this interaction

With the 3D structure of FERM domain of FAK (including NT2 domain) and kinase domain of IGF-1R resolved, computer-based molecular modeling program DOT was used to predict

the orientations of binding between FAK-NT2 and IGF-1R kinase domain. The DOT program returned three configurations that have the lowest free energy of interaction (Figure 4A, 4B and 4C), which were assumedly the most stable relative orientations between FAK and IGF-1R. These binding models were used as the platforms to screen for small molecules that may interrupt the interaction of FAK and IGF-1R by binding to the interaction site. Using DOT program, we were able to survey compounds from the database of Developmental Therapeutics Program for those that putatively bind to FAK-NT2 on the predicted FAK-NT2/IGF-1R interface. Compounds with high probability to bind to the interface were screened by MTT assay for their ability to suppress cancer cell growth. One of the compounds (designated Compound 32) showed strong suppression effect on pancreatic cancer cell line by MTT assay (data not shown). We then tested this compound for its ability to disrupt FAK-NT2/IGF-1R interaction. As showed in Figure 4D, 50 μ M of Compound 32 successfully blocked the pull-down of IGF-1R-IC by FAK-NT2. Presence of Compound 32 also decreased the co-localization of FAK-NT2 and IGF-1R in Panc-1 cells (Figure S2).

Discussion

Knowledge of specific protein-protein interactions is a key to understanding the fundamental problems of both normal and cancerous cellular activities. This information is also critical in developing new drugs for cancer therapy. While emerging data strongly suggests that FAK and IGF-1R are excellent targets for developmental therapeutics of cancer, specific small molecule kinase inhibitors of both proteins have been difficult to obtain. The main problem with kinase inhibitors is their lack of specificity. In addition, it appears that disruption of the kinase domain does not specifically interfere with the downstream signaling of FAK or IGF-1R and it is unclear whether the kinase function or the scaffolding function of FAK is more important [40–43]. Targeting protein-protein interaction sites represents a novel approach to FAK and IGF-1R inhibition with direct disruption of downstream protein signaling. Small organic molecules are particularly attractive as inhibitors of intracellular protein–protein interactions because of the ability to modify their structures to achieve optimal target binding, and because of their ease of delivery in *in vivo* systems.

Our present study has shown that the IGF-1R intracellular domain directly binds to the NT2 domain of FAK *in vitro* (Figure 2), and that the FAK-NT2 domain co-localizes with IGF-1R in pancreatic cancer cells (Figure 3). Targeting this interaction with small molecules is a novel strategy for treatment of pancreatic cancer or other cancers. The binding assays conducted in the present study defines the interaction site as the FAK-NT2 domain and N-terminal part of the IGF-1R intracellular domain, allowing the use of a highly efficient computer program DOT to predict the binding model based on the resolved crystal structures of the FAK-NT domain and IGF-1R kinase domain. DOT was able to find stable docked structures for FAK-NT2 and IGF-1R kinase domain by performing a systematic search over six degrees of freedom, producing the most favorable binding models. Since DOT is restricted to rigid molecules, and does not take solvation energy into account, the favorable points of interaction between FAK-NT2 and IGF-1 kinase may not be exact. But this approach does provide working models for screening of small molecules that effectively block the protein-protein interaction of interest, demonstrated by the successful identification of Compound 32 as a blocker of FAK/IGF-1R interaction (Figure 4D). Since the structure of the IGF-1R juxtamembrane domain is not available for DOT analysis, our present data could not rule out the binding of FAK-NT2 to this part of IGF-1R.

Dissection of the site of physical interaction between two biological macromolecules is a demanding physical task. Subsequently, screening for small molecules to disrupt this interaction experimentally is very time-consuming. The approach shown combines biochemical analysis of FAK and IGF-1R binding with computer-aided molecular modeling

and functional testing. This strategy could also be efficient in studying other protein-protein interactions and searching for specific drugs that target these interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Scheme for His-tagged IGF-1R and GST-tagged FAK domains

The scheme of His-IGF-1R intracellular domain constructs (A) and GST-FAK constructs (B) used for pull-down assays.

Figure 2. Identification of the FAK/IGF-1R binding domains by pull-down assay

2.0 μ g of GST-FAK protein was incubated with 0.1 μ g of precleared His-IGF-1R protein in total volume of 500 μ l at 4°C for 1 hour. His-IGF-1R protein associated with GST-FAK protein was analyzed by Western blotting. GST beads were processed in parallel for negative control. (A) IGF-1R intracellular domain binds to GST-FAK-NT, but not to GST-FAK-CD. (B) IGF-1R intracellular domain binds to NT2 domain of FAK, but not to NT1 or NT3. (C) N-terminal part of IGF-1R (IGF-1R-IC-N) intracellular domain binds to FAK-NT2, but not the C-terminal tail of IGF-1R (IGF-1R-IC-C).

Figure 3. Co-localization of the FAK-NT2 domain with IGF-1R in pancreatic cancer cells
 GFP-tagged FAK fragments were expressed in Panc-1 cells by transient transfection. Immunostaining was performed on fixed cells (see 'Materials and Methods'). GFP tagged FAK fragments were labeled green and endogenous IGF-1R red. The merged image was analyzed to calculate the co-localization rate as the percentage of dual-color pixels over all labeled pixels. Dual-color pixels were presented white in merged images. Five cells from each transfection group were analyzed. The co-localization rate in the GFP-FAK-NT1 and GFP-FAK-NT3 groups showed no significant difference compared to the control GFP group ($p>0.05$). GFP-FAK-NT2 showed significantly higher co-localization rates than the control group ($p<0.05$).

Figure 4. Molecular modeling of the FAK/IGF-1R interaction and disruption of the interaction by a small molecule inhibitor

The binding configurations with the lowest free energies of interaction, as calculated by DOT, are demonstrated in A, B and C. All three models involve the binding of the IGF-1R kinase domain to the NT2 domain of the FAK FERM domain. The energy for each model is recorded as the total energy: (electrostatic energy) + (van der Waals energy) in kcal/mol. (A) Model A has the lowest energy of -19.9197 : $(-3.2197) + (-16.7000)$. (B) Model B has the second lowest energy of -18.7027 : $(-6.8027) + (-11.9000)$. (C) Model C has the third lowest energy of -18.6312 : $(-9.1312) + (-9.5000)$. (D) $50\ \mu\text{M}$ of Compound 32 blocked the association of the FAK-NT2 and IGF-1R intracellular domains in a purified protein pull-down assay (see Materials and Methods).