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Focal adhesion kinase (FAK) activates and stabilizes IGF-1 receptor

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ABSTRACT

Recent studies have shown a direct association between IGF-1R and FAK, two important mediators of cell growth, survival and migration. However, the mechanism by which FAK affects IGF-1R function remains unknown. This study investigates the potential role of FAK in mediating activation and stability of IGF-1R. Autophosphorylation and phosphorylation capacities of wild type and mutant IGF-1R were studied. Surprisingly, we found that the mutant IGF-1R lacking the three core tyrosine residues in the activation-loop can be phosphorylated although it is unable to undergo autophosphorylation, suggesting that another kinase possesses the ability to phosphorylate IGF-1R. By using wild type MEFs and FAK−/− MEFs we could demonstrate that FAK mediates activation-loop independent phosphorylation, as well as Akt and ERK activation. Furthermore, the stability of IGF-1R was decreased upon FAK siRNA or inactivation. Taken together, our data suggest a role for FAK in phosphorylation, signaling and stability of the IGF-1R.

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Introduction

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that is frequently overexpressed in various human cancers [1–5]. It is activated through integrin mediated cell adhesion to extracellular matrix (ECM) and modulates the activity of several intracellular signaling pathways, such as phosphatidyl inositol-3 kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways. The activation of FAK may further be enhanced by the stimulation of growth factor receptors with growth factors, such as bFGF, EGF or PDGF [6]. FAK coordinates integrin and growth factor signaling and promotes cell migration, proliferation and survival [7,8]. Furthermore, FAK activation has been suggested to be mediated by the insulin-like growth factor-1 receptor (IGF-1R), a receptor tyrosine kinase with important roles in cancer biology such as cellular transformation, differentiation, protection from apoptosis and cancer progression [9,10]. Upon ligand binding, IGF-1R initially is autophosphorylated at the three core tyrosine residues Y1135, Y1131 and Y1136 which acts as a platform for the recruitment and activation of distinct intracellular signaling pathways, such as PI3K/Akt and RAS/MAPK pathways. It is generally believed that the a-loop autophosphorylation makes the receptor fully active and capable of triggering cascades of intracellular signaling events involved in the regulation of multiple biological actions such as cell growth and protection from apoptosis.

Previous studies have shown that IGF-1R signaling cooperates with integrin mediated signaling in promoting tumor cell migration *in vitro* [11,12] and *in vivo* [13]. Moreover, FAK and IGF-1R association has recently been demonstrated in pancreatic cancer cells, and simultaneous inhibition of the two kinases resulted in synergistic anti-cancer effects [14–16].

However, the molecular mechanisms behind the cooperation between FAK and IGF-1R are still unknown. Our data suggest that FAK, apart from the intrinsic receptor kinase activity, is involved in phosphorylation of IGF-1R and may influence downstream signaling reactions and receptor stability.

Materials and methods

Reagents. Polyclonal IGF-1R, phospho-FAK(Tyr925), FAK, phospho-Akt (Ser 473), Akt, phospho-ERK and ERK antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), monoclonal antibody against phosphotyrosine (PY99) and polyclonal antibody against GAPDH were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Protein G Sepharose was from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents unless stated otherwise were obtained from Sigma (St. Louis, MO, USA).

Cell cultures. R− cells, a gift from Dr. Renato Baserga (Thomas Jefferson University, Philadelphia, PA) have been described else-

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where [17]. Wild type, and FAK−/− mouse embryonic fibroblasts (MEFs) [18] were maintained in DMEM supplemented with 10% (v/v) fetal calf serum, 10 mM L-Glu and 5 mg/ml penicillin/streptomycin. FAK−/− MEF was a gift from Dr. S. Johansson (Dept. of Medical Biochemistry and Microbiology, Biomedical Center, Uppsala, Sweden). The human melanoma cell line DFB has been described elsewhere [19].

Plasmids and small interfering RNA (siRNA). The plasmids containing wild type (WT), ATP mutant (ATPM) or Y1131–35–36F (TM) human IGF-1R cDNA were kind gifts from Dr. Renato Baserga (Thomas Jefferson University, Philadelphia, PA). Chemically synthesized double-strand siRNA against human FAK (position 3008, target sequence 5′-GCCUGGUGAAAGCUGUCAUCGAGAU-3′) was purchased from Invitrogen (Carlsbad, CA, USA).

Transient transfections. R− cells were grown to 90% confluency in 10 cm cell culture dishes and transfected using polyethylenimine (PEI) and 2 μg DNA. Twenty-four hours after transfection, cells were seeded into six-well plates and cultured for 24 h in the presence of 0.6 mg/ml G418.

SDS/PAGE and Western blot analysis. Cells were lysed in lysis buffer (0.5% Triton X-100, 0.5% Doc Deoxycholic acid, 150 mM NaCl, 20 mM Tris (pH 7.5), 10 mM EDTA, 30 mM NaPyroPhosphate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin) and Western blot analysis was preformed as described elsewhere [20].

Immunoprecipitation. Cells were cultured to subconfluency in six-well plates. Cells were serum-depleted for 24 h, and then stimulated with IGF-1 (50 ng/ml) at indicated time points. For determination of IGF-1R phosphorylation, cell lysates were extracted with lysis buffer as described above. After preclearing of the cell lysates, 20 μl Protein G Sepharose and 1 μg of antibody were added to 1 mg of protein material. After overnight incubation at 4 °C the Sepharose pellet was washed three times with lysis buffer, dissolved in a sample buffer and further analyzed by Western blot analysis.

Co-immunoprecipitation. Cells were grown under basal conditions in six-well plates until subconfluency before lysed as described above. 500 μl of each lysate and 1 μg of antibody or rabbit IgG was used for immunoprecipitation using Catch and Release® Reversible Immunoprecipitation System (Upstate, Temecula, CA, USA) according to the manufacturer's protocol. The immunoprecipitates were further analyzed by SDS/PAGE and Western blot analysis.

Autophosphorylation assay. IGF-1R tyrosine autophosphorylation was analyzed by a sandwich ELISA assay, essentially as described [21]. Briefly, 96-well plates were coated overnight at 4 °C with 1 μg/well of an antibody to IGF-1R β-subunit. R− cells were transfected with the different constructs, cultured until subconfluency and 24 h serum-starved in six-well plates and lysed as described above. The plates were blocked with 5% BSA in PBST for 1 h, and 80 μg/well of total protein extracts were added to the coated wells and were incubated over night. As a negative control we used total protein lysate from the R− cell line. The investigated constructs were incubated in the tyrosine kinase buffer (50 mM Hepes buffer, pH 7.4, 200 mM $MgCl_2$, 0.1 mM $MnCl_2$ and 0.2 mM Na_3VO_4) with or without ATP at room temperature for 1 h. The phosphorylated polymer substrate was probed with a phosphotyrosine-specific monoclonal antibody conjugated to HRP, clone PT-66. Color was developed with HRP chromogenic substrate *O*-phenylenediamine dihydrochloride (OPD) and quantified by spectrophotometry (Fig. 2).

Degradation assay. IGF-1R degradation was assessed by cycloheximide-chase assay as described previously [22]. The protein levels were detected by Western blot analysis at 6, 16 and 24 h after treatment with cycloheximide (50 μg/ml). The experiment was performed in complete culture medium in order to investigate receptor degradation under physiological conditions.

Suspension culture. Suspension culture on polyhydroxyethylmethacrylate (poly-HEMA)-coated plates was performed according to the method of Frisch and Francis [23]. In brief, cells were seeded on poly-HEMA-coated plates with culture medium containing 1.5 g methylcellulose per 100 ml media in order to avoid cell adhesion. Twenty-four and 48 h after incubation, the cells were harvested and lysed as described above. Protein levels were detected by Western blot analysis following SDS/PAGE.

Results

Triple mutated IGF-1 R is phosphorylated in response to ligand stimulation

Phosphorylation of wild type IGF-1R (WT) and triple mutant IGF-1R (TM), in which the three tyrosine residues (Y1131P, Y1135P, Y1136) in the kinase activation-loop (a-loop) are replaced by phenylalanine, were investigated by transiently transfecting them into IGF-1R null mouse embryonic fibroblasts (R− cells), denoted R− WT and R− TM, or wild type mouse embryonic fibroblasts (MEF), denoted MEF WT and MEF TM, respectively.

Following 24 h of serum depletion, the cells were stimulated with 50 ng/ml IGF-1 for 5 and 10 min and analyzed for receptor tyrosine phosphorylation. As expected, WT-IGF-1R was phosphorylated after both, 5 and 10 min IGF-1 stimulation (Fig. 1, left panels). It has previously been shown that TM-IGF-1R has severely reduced ligand-induced autophosphorylation capacities upon 5 min IGF-1 stimulation [24]. To our surprise, we detected tyrosine phosphorylation of TM-IGF-1R after 10 min IGF-1 stimulation (Fig. 1, upper right panel). Even though TM-IGF-1R shows delayed phosphorylation upon ligand stimulation, it is apparent from our results that it can be phosphorylated in a manner independent of canonical IGF-1R autoactivation.

Triple mutated IGF-1 R is not autophosphorylated

Next we sought to determine the autophosphorylation capacity of TM-IGF-1R in comparison with WT-IGF-1R in a cell free system. A kinase inactive IGF-1R construct with a point mutation at the ATP binding site (K1003R) [25] served as a negative control. WT or mutated IGF-1R isolated from transfected R− were captured in anti-IGF-1R coated ELISA plates and were incubated with tyrosine kinase buffer and ATP (see Materials and methods for details). The specificity of the assay was confirmed using samples without ATP as negative controls. In contrast to WT-IGF-1R, which showed ex-

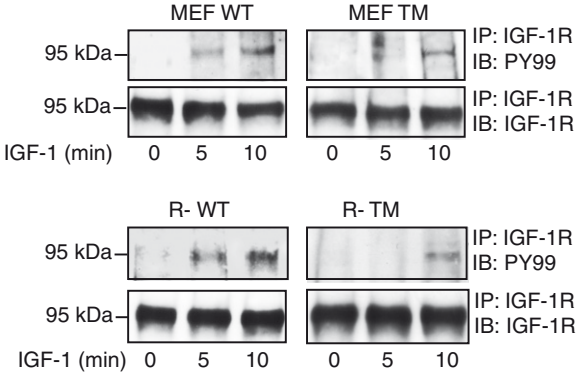

Fig. 1. IGF-1 induced phosphorylation of IGF-1R constructs. MEF and R− cells, transiently transfected with WT or TM-IGF-1R, serum-depleted for 36 h and stimulated with IGF-1 (50 ng/ml) for 5–10 min. IGF-1R was immunoprecipitated and immunoblotted for phosphotyrosine (upper panels) and total IGF-1R (lower panels).

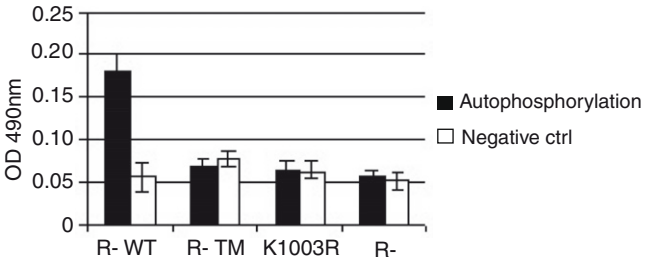

Fig. 2. Autophosphorylation of IGF-1R constructs. Autophosphorylation of WT, ATPM and TM-IGF-1R was measured as described in Materials and methods. R– cells were used as negative control. Means and standard deviations of triplicate determinations are shown. The experiment was repeated three times with similar results.

pected kinase activity due to autophosphorylation, the K1003R and TM-IGF-1R displayed no kinase activity. Even though disruption of the three tyrosines of the a-loop inhibits receptor autophosphorylation, TM-IGF-1R, in contrast to K1003R, can be phosphorylated in intact cells. This leads to the assumption that another/other kinase(s) may phosphorylate tyrosine residues in IGF-1R located outside of the a-loop.

FAK associates with and phosphorylates IGF-1R

Based on previously published data suggesting that IGF-1R is associated to FAK [15], we hypothesized that FAK might be a promising kinase candidate in phosphorylating IGF-1R. With this in mind, co-immunoprecipitation studies were firstly employed in order to confirm FAK-IGF-1R association in our cell systems. We immunoprecipitated R– cell lysates expressing WT or TM-IGF-1R with IGF-1Rβ antibody or rabbit IgG control followed by detection of FAK (Fig. 3A, middle and right panel). To confirm the physiological relevance of the interaction, the experiments were also performed in a human melanoma cell line (DFB), expressing endogenous IGF-1R (Fig. 3A, left panel). The results shown in Fig. 3A demonstrate that FAK co-immunoprecipitates with WT-IGF-1R in R– and DFB cells as well as with TM-IGF-1R. The expression levels of IGF-1R and FAK were compared in all cell lines. The transfected levels of WT and TM-IGF-1R were equal in R– cells

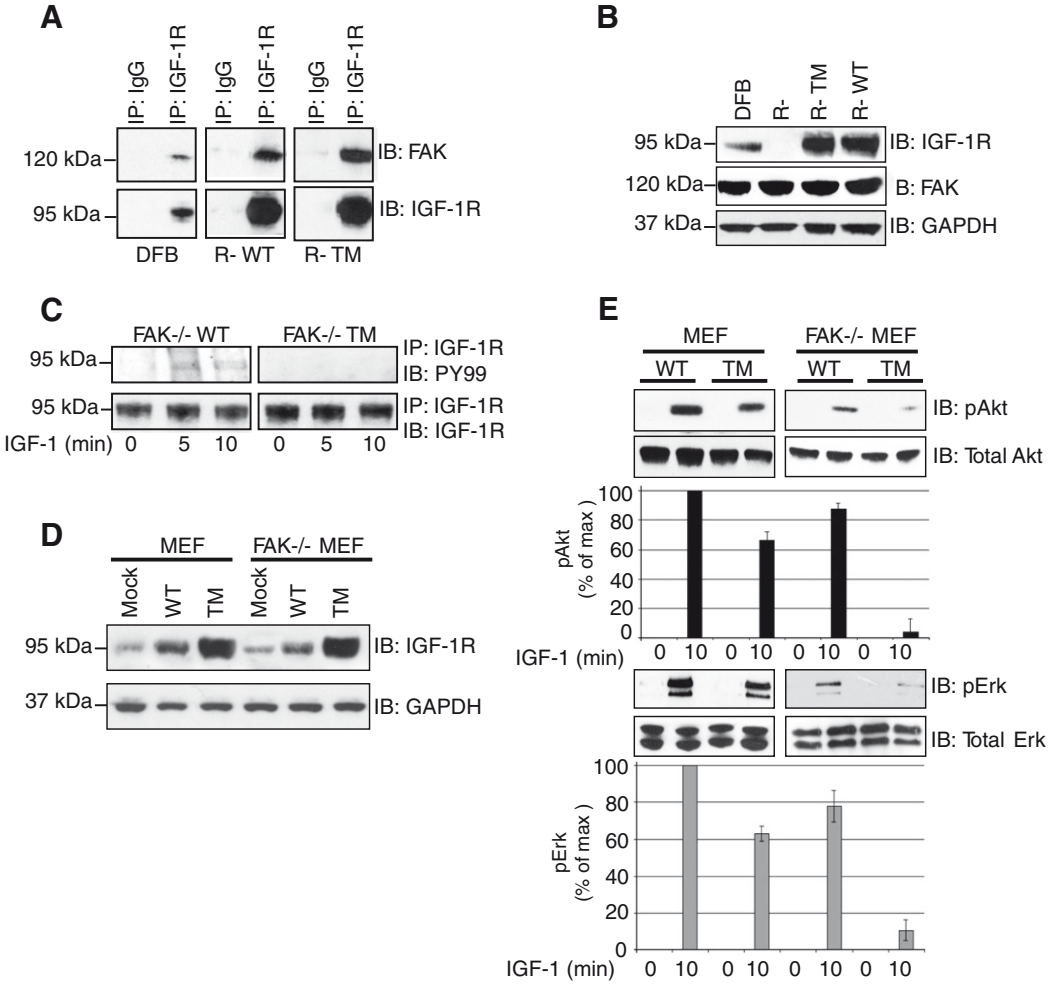

Fig. 3. FAK is associated with IGF-1R and necessary for a-loop independent phosphorylation of IGF-1R. Lysates of DFB cells and R– cells expressing either WT or TM-IGF-1R were subjected to immunoprecipitation with IGF-1R antibody and immunoblotted with FAK antibody (upper panel) or IGF-1R antibody (lower panel) (A). Total levels of IGF-1R (upper panel) and FAK (middle panel) of DFB, R– , R– TM and R– WT cells (B). FAK–/– MEF cells were transfected with WT or TM-IGF-1R constructs. Cells were serum-depleted for 36 h and stimulated with IGF-1 for 5–10 min. IGF-1R was immunoprecipitated and detected with anti-pY99 (C). Total levels of IGF-1R in MEF and FAK–/– MEF cells transiently transfected with mock, WT or TM-IGF-1R (D). MEF and FAK–/– MEF cells transiently transfected with WT or TM-IGF-1R were serum-depleted for 36 h and stimulated with IGF-1 (50 ng/ml) for 10 min. The levels of phospho-Akt and total Akt as well as phospho-ERK and total ERK were determined by SDS–PAGE and Western blotting (E, upper panels). The graphs represent quantified data of phospho-Akt and phospho-ERK levels, respectively, as normalized to GAPDH. Means and SDs of three separate experiments are shown. $P < 0.001$ (E, lower panels).

upon transient transfection and higher than the endogenous IGF-1R expression in DFB cells (Fig. 3B).

In order to investigate whether FAK may be responsible for the a-loop-independent phosphorylation of IGF-1R, wild type MEF and their FAK−/− counterparts were transfected with WT or TM-IGF-1R which allowed us to analyze the effect of the presence and absence of FAK on ligand-induced IGF-1R phosphorylation. As expected, WT-IGF-1R was phosphorylated in both FAK positive (Fig. 1, upper panel) and FAK negative MEFs (Fig. 3C, left panel) whereas TM-IGF-1R phosphorylation could not be observed in the absence of FAK (Fig. 3C, right panel). Taken together, the results suggest that FAK interacts with and phosphorylate IGF-1R, independently of the a-loop domain.

To determine if FAK also influences the IGF-1R signaling, we analyzed IGF-1 induced Akt and ERK phosphorylation as a measure of PI3K and MAPK activation, respectively. Therefore, we used wild type MEF cells and FAK−/− MEF cells transiently overexpressing WT or TM-IGF-1R. As can be seen in Fig. 3E, WT-IGF-1R activated both Akt and ERK upon ligand stimulation regardless of FAK expression. In cells overexpressing TM-IGF-1R both Akt and ERK phosphorylation upon IGF-1 stimulation was largely reduced in FAK negative cells compared to FAK positive ones, indicating that FAK also influences IGF-1R signaling.

Inactivation or downregulation of FAK leads to IGF-1R degradation

Since degradation of activated receptors is an important mode of deactivation and signal attenuation, we sought to determine whether FAK also affects IGF-1R degradation.

To address this issue, the turnover of endogenous IGF-1R was determined by CHX chase in human melanoma cell line DFB, after siRNA mediated FAK knockdown (Fig. 4A). The partial FAK depletion obtained by siRNA treatment reduced the overall level of IGF-1R considerably and also increased receptor degradation compared to untransfected controls (Fig. 4B).

Considering our finding that FAK activates the receptor independently of its intrinsic activity, we further studied the effect of FAK on TM-IGF-1R stability. Due to insufficient silencing of FAK in R− cells by various siRNA constructs, we instead downregulated FAK activity by culturing R− cells expressing WT or TM-IGF-1R under non-adherent conditions. Since integrin receptors are prevented from binding extracellular matrix proteins under non-adherent conditions, their association and activation of FAK is inhibited [26]. As can be seen in Fig. 4C, FAK phosphorylation is reduced upon 24 and 48 h growth in non-adherent conditions, whereas the level of total FAK is unaffected. Interestingly, the IGF-1R levels decrease under non-adherent conditions in both R− WT and R− TM cells. However, the effect is much stronger ($P < 0.001$) in cells expressing TM-IGF-1R, suggesting that the mutant variant of the receptor is more dependent on FAK activity than WT-IGF-1R (Fig. 4D). These data indicate that FAK activity stabilizes IGF-1R. Accordingly, the TM-IGF-1R is more dependent on FAK activity than the wild type variant. Taken together, our data suggest that FAK mediates the phosphorylation of IGF-1R and stabilizes the receptor.

Discussion

In this study we demonstrate that FAK, mainly known for its role in integrin signaling pathways, associates with and phosphorylates IGF-1R independently of IGF-1R's intrinsic tyrosine kinase activity. Several reports have shown a functional connection between IGF-1R and FAK. These studies demonstrated that IGF-R can induce either FAK phosphorylation [27,28] or dephosphoryl-

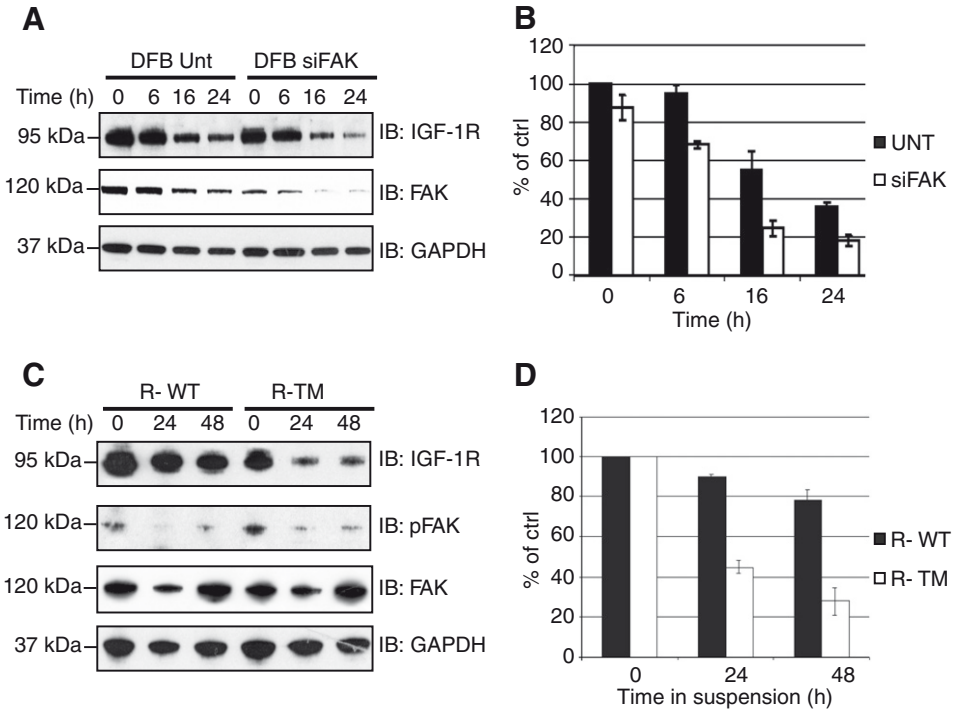

Fig. 4. FAK is necessary for IGF-1R stability. DFB cells were transfected with siRNA to FAK. Twenty-four hours after transfection, cells were treated with 50 μg/ml CHX for indicated time points. Total levels of IGF-1R and FAK were determined by SDS–PAGE and Western blotting (A). Quantified data of IGF-1R levels, normalized to GAPDH. Means and SDs of three separate experiments are shown. $P < 0.001$ (B). R− WT and R− TM cells were grown under non-adherent conditions as described in Materials and methods for 0, 24 or 48 h. Levels of total IGF-1R, pY FAK and total FAK were determined by SDS–PAGE and Western blotting (C). Quantified data of IGF-1R levels, normalized to GAPDH. Means and SDs of three separate experiments are shown. $P < 0.001$ (D).

ation [29–32] depending on the cellular architecture. In this way, IGF-1R activation triggers attachment–detachment signaling pathways necessary for cell motility [33]. IGF-1R and FAK were shown to interact via insulin receptor substrate 1 (IRS-1), an important molecule in IGF-1R signaling pathway, which associates to $\alpha_v\beta_3$ integrin and FAK [34]. Increasing evidence supports a role for IGF-IR in regulating focal adhesion molecules. Liu et al. showed a direct association between FAK and IGF-1R and that co-inhibition of FAK and IGF-1R results in a synergistic anti-cancer effect [14–16]. However, the molecular mechanism by which these synergic effects occur remains elusive.

Here, we describe an important role for FAK in IGF-1R activation by showing that FAK enables a-loop independent IGF-1R phosphorylation and Akt and ERK activation upon ligand stimulation. Even though ligand stimulation in itself cannot activate the mutated receptor, the ligand dependency of this process could be explained by previous studies showing the ability of IGF-1 to stimulate actin polymerization and ensuring optimal interaction of IGF-1R with β1 integrin and activation of focal adhesion signaling proteins [27,28].

Furthermore, our findings suggest that active FAK is necessary for IGF-1R stability. The decrease of IGF-1R levels upon FAK inhibition was stronger in cells expressing TM-IGF-1R than in those expressing WT-IGF-1R. A possible explanation is that the TM receptor is non-functional in cells lacking active FAK and therefore degraded at a higher extent than WT-IGF-1R. Furthermore, knockdown of FAK increased degradation of endogenous IGF-1R in the human melanoma cell line DFB upon CHX chase assay, which suggests that FAK stabilizes the receptor at the protein level. This could be explained by either FAK causing conformational changes of IGF-1R due to phosphorylating specific tyrosine residues important for receptor stability or that the interaction between IGF-1R and FAK itself restricts IGF-1R degradation. Our finding that FAK mediates IGF-1R stabilization could hence explain the synergistic anti-tumor effect of simultaneous inhibition of IGF-1R and FAK kinases [14–16]. The impact of FAK on the expression levels of IGF-1R could be multilateral; since FAK siRNA decreased the IGF-1R expression levels with approximately 20% 24 h post-transfection, indicating that FAK can affect *de novo* synthesis of IGF-1R.

In summary, our results provide further information on the functional connection between FAK and IGF-1R and their mutual role in cancer cell proliferation and survival. Studying the mechanism of the interplay between FAK and IGF-1R and their role in cancer therapy is therefore of great interest.

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