FOCAL ADHESION KINASE (FAK) REGULATES INSULIN-STIMULATED GLYCOGEN SYNTHESIS IN HEPATOCYTES

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Running Title: FAK and hepatic insulin action

Keywords: glycogen synthase kinase-3β, protein kinase B, glycogen synthase, integrin, insulin receptor

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ABSTRACT

Experimental data support a role for FAK, an important component of the integrin signaling pathway, in insulin action. Insulin has been shown to regulate the tyrosine phosphorylation state of FAK in vitro and promotes the association of both insulin receptor substrate-1 (IRS-1) and phosphatidylinositol-3-kinase (PI3’-kinase) with FAK. We recently reported that intervention to reverse hepatic insulin resistance in an obese rat model was associated with markedly increased tyrosine phosphorylation of FAK in liver. To test the hypothesis that FAK plays a regulatory role in hepatic insulin action, we overexpressed wild-type (W/T), a kinase inactive (K/R) or a c-terminal focal adhesion targeting (FAT) sequence-truncated mutant of FAK, in HepG2 hepatoma cells. In control untransfected (NON), vector- (CMV2) and W/T-transfected cells, insulin stimulated an expected 54±13%, 37±4 %, and a 47±12% increase in U-14C-glucose incorporation into glycogen respectively. This was entirely abolished in the presence of either K/R (-1±7%) or FAT mutants (0±8%, N=5, p<.05 for K/R or FAT vs other groups) and this was associated with a significant attenuation of incremental insulin-stimulated glycogen synthase (GS) activity. In W/T FAK-transfected cells, the insulin receptor (IR) co-immunoprecipitated with FAK and this was unaffected by insulin stimulation or by overexpression of either FAK mutant. Insulin-stimulated tyrosine phosphorylation patterns of both the IR and IRS-1 were unaffected and PI3’-kinase activity was also unaltered by overexpression of either FAK mutant. However, insulin-stimulated serine phosphorylation of Akt/protein kinase B (Akt/PKB) was significantly impaired in mutant-transfected cells. Moreover, the ability of insulin to inactivate the glycogen synthase kinase-3β
(GSK-3β), the regulatory enzyme immediately upstream of GS, by serine phosphorylation (308±16, 321±41 and 458±34 densitometric units in NON, CMV2 and W/T respectively, p<.02 for W/T vs CMV2) was attenuated in the presence of either FAT (205±14, p<.01) or K/R (189±4, p<.005) mutants. FAK co-immunoprecipitated with GSK-3β, but only in cells overexpressing the K/R (374±254 optical densitometric units) and FAT (555±308) mutants was this association stimulated by insulin compared with NON (-209±92) CMV2 (-47±70) and W/T (-39±31 o.d.u.). This suggests that FAK and GSK-3β form both a constitutive association and a transient complex upon insulin stimulation, dissociation of which requires normal function and localization of FAK.

We conclude that FAK regulates the activity of Akt/PKB and GSK-3β and the association of GSK-3β with FAK to influence insulin-mediated glycogen synthesis in hepatocytes and thus may play an important role in hepatic insulin action. Insulin action may be subject to secondary regulation by the integrin signaling pathway, ensuring that these growth and differentiation-promoting pathways act in a coordinated and/or complimentary manner.
INTRODUCTION

Integrins constitute a cell-surface transmembrane receptor family that mediate growth and differentiation-promoting functions upon activation by engagement with basement membrane or other extracellular ligands (1). Clustering of a number of intracellular signaling proteins at the site of focal contact (focal adhesion complex formation) is a central feature of integrin activation (2,3). FAK is a 125kDa cytosolic protein tyrosine kinase, named for its predominant cellular localization within the focal adhesion complex, which is tyrosine phosphorylated and activated upon integrin engagement (4) and which interacts with PI3’-kinase, Shc and Grb2 to promote integrin signaling (5-7).

It has also been shown that the tyrosine phosphorylation state of FAK is regulated in a complex manner by growth factor stimulation and that FAK associates with both the IR and IRS proteins upon insulin stimulation (8-11). In the case of platelet-derived growth factor (PDGF) and insulin-like growth factor-I (IGF-I), FAK undergoes tyrosine phosphorylation at low ligand concentrations, but is dephosphorylated at higher concentrations (7). In addition, the regulation of the tyrosine phosphorylation state of FAK upon insulin stimulation is a function of the level of IR expression (4,12), cell type (13) and the adhesion state of the cell (9,14). It has also previously been reported that integrin engagement stimulates both IRS-1-associated PI3’-kinase activity and Akt/protein kinase B (Akt/PKB) activity (15), which are important steps in insulin’s action to stimulate glucose transport and glycogen synthesis. Integrin engagement appears to play a stimulatory role in insulin signaling (8) and specifically in insulin-stimulated mitogenesis (16), suggesting that the state of cell contact is an important regulator of insulin action.
In light of the above data, it is important to establish the role of FAK in the regulation of insulin action. Hitherto, the tyrosine phosphorylation state of FAK in response to insulin administration in vivo was unknown. However, we recently showed that in the liver of healthy Sprague-Dawley rats, FAK rapidly undergoes tyrosine phosphorylation upon insulin stimulation under euglycemic conditions in vivo (14). Moreover, we observed that when insulin resistance was ameliorated in obese Zucker rats by administration of an adenoviral construct containing cDNA encoding for a soluble inhibitor of tumor necrosis factor-α (TNF-i), this was associated with a consistent increase (> 4-fold) in the tyrosine phosphorylation state of a 125 kDa protein, the identity of which was subsequently confirmed to be FAK (14). Using HepG2 cells, a human hepatoma cell line, we further showed that pre-treatment with TNF-α abolished insulin-stimulated tyrosine phosphorylation of FAK, without altering IR abundance or IR tyrosine phosphorylation.

In light of the above data, we undertook the present study in order to test the hypothesis that FAK plays an important role in the regulation of hepatic insulin signaling and insulin action. In the present work, we employ mutant FAK constructs overexpressed in HepG2 hepatoma cells and show that both the focal adhesion targeting property and tyrosine kinase activity of FAK are essential for insulin-mediated stimulation of glycogen synthesis and that FAK acts to promote insulin action downstream of PI3'-kinase, by a specific interaction with GSK-3β and Akt/PKB.
EXPERIMENTAL PROCEDURES

Materials and supplies: Antibodies to FAK and GSK-3β were obtained from Transduction Laboratories (Lexington, KY). Anti-IR β-subunit, anti-IRS-1 and anti-p85 polyclonal antibodies were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Antibody to Akt and anti-phospho-specific Akt polyclonal antibody which recognizes serine\(^{473}\) phosphorylated Akt as well as anti-phospho-specific GSK-3β polyclonal antibody which recognizes serine\(^{9}\) phosphorylated GSK-3β were from Cell Signaling Technology (Beverly, MA). Antibody against phosphotyrosine (pY99) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Chemiluminescence kit, Protein A/G-conjugated agarose beads, HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were all purchased from Pierce (Rockford, IL). All cell culture reagents were obtained from GIBCO (Grand Island, NY). Nitrocellulose membrane was from Schleicher & Schuell (Keene, NH). [U-\(^{14}\)C]-d-glucose was from Amersham (Piscataway, NJ). [\(^{32}\)P]-ATP was obtained from NEN Life Science Products (Boston, MA). UDP-[U-\(^{14}\)C]-glucose (303 mCi/mM) was from ICN (Irvine, CA). The pFLAG-CMV2 vector, anti-FLAG antibodies and all other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Synthesis of pFLAG-CMV2 constructs with mutant FAK cDNAs: FAK wild-type (W/T), K\(^{454}\)R mutant of FAK, in which Lys\(^{454}\) is replaced by Arg (K/R), or the COOH-terminal deletion variant dl\(^{686-1011}\) of FAK (FAT) (17). cDNA was subcloned into the EcoRI site of pBluescript KS (pBS, Stratagene). The cDNAs encoding W/T and mutant forms of FAK were then excised from the pBS plasmid by SmaI and SalI digestion and directionally subcloned into EcoRV and...
SalI cut site of N-terminal flag-tagged pFLAG-CMV2 expression vector, since the C-terminal of FAK is required for location of FAK to the focal adhesion complex (17). Insert orientations were confirmed by direct sequencing and restriction fragment analysis (data not shown).

Cell culture, transfection and insulin stimulation: HepG2 is a cell line derived from a human hepatocyte carcinoma (Invitrogen, San Diego, CA). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 10 mM glutamine and 15 mM Hepes (pH 7.4). Cells were grown to 70-80% confluence in 100 mm dishes (Costar, Cambridge, MA) and were then transiently transfected using Tfx-20 reagent (Promega, Madison, WI) with pFLAG-CMV-expressing various FAK constructs as described above. Control cells were transfected with the pFLAG-CMV2 empty vector only. Transfection efficiency exceeded 50% (data not shown). Forty-eight hours post-transfection, cells were starved overnight in FBS-free DMEM before performing experiments. For insulin stimulation, recombinant human insulin was added to a final concentration of 100 nM. Following the indicated treatment times, cells were washed twice with ice-cold phosphate buffered saline (PBS) and liquid N\textsubscript{2} was added. 1 ml of RIPA lysis buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 0.5% deoxycholate, and 1% Nonidet P-40) containing aprotinin (20 µg/ml), leupeptin (20 µg/ml), 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfloride) was then added and the cells were scraped off and transferred to 1.5 ml microcentrifugation tubes. They were incubated on ice for 40 min and vortexed frequently. After centrifugation at 100,000 x g for 30 min at 4 °C, the clarified supernatants were aliquoted with RIPA lysis buffer to equal protein content (Bio-Rad).
**Determination of the rate of cell growth and cell morphology:** Cells were suspended at 0.5 x 10⁵ cells/ml in complete medium, plated on 6-well plates in triplicate samples (0.5 x 10⁵ cells/well) and transfected with FAK mutants. At 24, 48 and 72 hours after transfection, cells were harvested in 0.05% trypsin/EDTA and washed twice with PBS. Cells were then resuspended in 100 ul of PBS and mixed with an equal volume of 0.4% trypan blue (Sigma). 10 µl of suspended cells were then loaded onto a hemocytometer and viable cells were counted under a light microscope. Cell growth was expressed as the number of live cells per well. Cell morphology was quantified by determination of the cellular aspect ratio according to the method of Ridyard et al (18) after capturing the cell image on Image 1.61 software (NIH). Measurements were taken on 13 different cells in each culture. The aspect ratio was calculated as the long axis of the cell divided by its short axis.

**Immunoprecipitation and western blotting:** 500 µg of cell lysate protein was incubated with 10 µg of the respective antibody and agarose-conjugated Protein G at 4°C overnight. The immunoprecipitates were then washed three times in a buffer containing 50 mM Hepes, 100 mM Na₄P₂O₇, 100 mM NaF, 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na₃VO₄, 1 mM phenylmethylsulfonylfluoride (PMSF) and 0.1mg/ml aprotinin. The washed immunoprecipitates were boiled in 2X Laemmli buffer (19) and separated by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were electroblotted onto nitrocellulose membrane and the membrane was blocked in 5% non-fat dry milk in TBS containing 0.1% Tween-20. Membranes were blotted with various primary antibodies followed by appropriate HRP-conjugated secondary antibody. Results were visualized by Supersignal chemiluminescence kit (Pierce) according to the manufacturer’s instructions. In cases where
reblotting with a different primary antibody was required, the membrane was stripped by incubation in 50 mM Tris-HCL, pH 6.8, 100 mM 2-mercaptoethanol and 2% SDS for 30 min at 50 °C with constant agitation. The membrane was then washed thoroughly in Tris-buffered saline/ Tween 20 (TBST) and reprobed with the appropriate antibody.

**Co-immunoprecipitation studies:** Cell lysates were prepared as described above. Samples containing 500 µg of protein were precleared for 2hr at 4°C with protein G-sepharose that had previously been washed 3 times with lysis buffer, and were immunoprecipitated overnight at 4°C with 1 µg of monoclonal anti-GSK-3β or anti-Akt/PKB antibody. Protein G-sepharose (40 µl of beads in suspension) was added, and the incubation was continued for 3 hr. After washing the precipitates 3 times with lysis buffer, 20 µl of Laemmli stopping buffer was added to each pellet and the samples were placed in boiling water for 10 min before SDS-PAGE and immunoblotting. When the blots were probed with the monoclonal anti-FAK antibody, HRP-conjugated mouse antibody was used as secondary antibody. The blots were then stripped by incubation in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, at 50°C for 30 min with agitation, followed by thorough rinsing, blocking, and reprobing with monoclonal GSK-3β or polyclonal Akt/PKB antibody.

**Measurement of glycogen synthesis in HepG2 cells:** The determination of glycogen synthesis was made by measurement of the incorporation of [U-14C]-d-glucose into glycogen (20). Confluent monolayers of transfected HepG2 cells in 6-well plates were incubated overnight in FBS-free DMEM and then for 3 hr in FBS-free DMEM containing 5.5 mM glucose and 0.33 µCi/ml [U-14C]-D-glucose in the absence or presence of 100 nM insulin. The incubation was
terminated by removal of the medium and rinsing the cells 5 times in ice-cold PBS. Cells were solubilized with 20% KOH for 2 hr and an aliquot (100 µl) of the resulting lysate was removed for protein analysis. Lysates were extracted with 8% (w/v) tricarboxylic acid (TCA), neutralized with 2.0 M HCl, then boiled for 5 min, and 1 mg of glycogen was added as a carrier to each sample. Total glycogen was precipitated by addition of 80% ethanol (final concentration) for 2 hours at -20°C followed by centrifugation at 1100 x g for 10 min. This step was then repeated. After the pellets had been re-dissolved in distilled water, the samples were precipitated again as described above. The amount of incorporated radioactivity was determined by liquid scintillation counting.

**Measurement of glycogen synthase (GS) activity in HepG2 cells:** GS activity was determined by a modification of previously described methods (21,22). HepG2 cells transfected with FAK mutants were incubated in serum free medium overnight, followed by the addition of 100 nM insulin or saline for 10 min at 37 °C. After washing with PBS, cells were scraped into 500 µl of GS assay buffer (50 mM Tris-HCL, pH 7.8, 100 mM NaF, 20 mM EDTA, 0.5% glycogen, 1 mM PMSF, 1 mM benzamidine, 10 µg/ml leupeptin, 10 µg/ml aprotinin) and incubated on ice for 40 min with frequent mixing. After centrifugation at 100,000 x g for 30 min at 4 °C, the clarified supernatants were aliquoted with GS assay buffer to equal protein content (Bio-Rad). To measure GS activity, 50 µl of supernatant (4 µg protein per µl) were added to an equal volume of GS buffer containing 2 µCi/ml UDP-[¹⁴C]glucose and 15 mg/ml glycogen, in the presence or absence of 10 mM glucose-6-phosphate. After a 15 min incubation at 37 °C, assay tubes were chilled for a further 15 min on ice. Contents were then spotted on to labeled Whatman filter papers (GF/A; 2.4 cm), which were immediately immersed in 70% ethanol at 4°C, mixed for 40
min, then washed twice more in 70% ethanol (for 15 and 60 min respectively) to remove unincorporated substrate from precipitated glycogen. Filters were air-dried, and radioactivity was counted with 10 ml of liquid scintillation fluid.

**Measurement of PI3'-kinase activity:** PI3-kinase activity was measured by the technique of Backer et al (23). After incubation with insulin, cells were lysed at 4°C for 20 min in buffer containing 20 mM Tris-HCl, (pH 7.4), 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 10% (vol/vol) glycerol, 1% Nonidet P-40; 1 mM Na3VO4, and 1 mM PMSF. The resulting lysates were incubated with either anti-p85α or anti IRS-1 antibody for 2 hr and then for 1 hr with protein A-sepharose (4 µg/ml of lysate). Immunoprecipitates were washed twice with buffer A /1% Nonidet P-40, twice with 0.5 M LiCl/100mM Tris-HCl, pH 7.5, and twice with 10 mM Tris-HCL, pH 7.4/100 mM NaCl/1 mM EDTA and pellets were directly incubated with phosphatidylinositol (0.1 mg/ml) in medium containing 880 µM (30 µCi [γ32P]) ATP, 20 mM MgCl2, at room temperature for 10 min with constant shaking in a final volume of 80 µl. After addition of 20 µl of 8 M HCl, lipids were extracted with 160 µl CHCl3/CH3OH (1:1, by volume). The samples were centrifuged, and the lower organic phase was removed and applied to a silica gel thin-layer chromatography (TLC) plate (Merck Inc., St. Louis, MO) and coated with 1% potassium oxalate. TLC plates were developed in CHCl3/CH3OH/H2O/NH4OH (60:47:11.3:2), dried, and visualized by autoradiography.

**Statistical Analysis:** All statistical comparisons were conducted using Student’s 2-tailed t-test for paired or unpaired samples as appropriate, with appropriate correction for multiple
comparisons (SigmaStat statistical software version 2.0, SPSS Inc, Chicago, IL). Data are reported as mean ± SEM.
RESULTS

Overexpression of W/T and mutant FAK constructs in HepG2 cells: Recent studies have suggested several roles for integrin signaling via FAK in the regulation of cell survival (24,25), proliferation (26,27), spreading (28), and migration (26,29,30). Overexpression of FAK stimulated cell migration and mutation of tyr-397 to Phe abolished this property (31). To investigate the effect of various constructs of FAK on cell proliferation and morphology, we first measured the growth rate of cells transfected with various constructs of FAK: FAK wild-type (W/T), K454R mutant of FAK, in which Lys454 is replaced by Arg (K/R), or the COOH-terminal deletion variant d1686-1011 of FAK (FAT). As shown in Figure 1, the cell growth curves were similar among the 5 groups (Figure 1A) and 48 hours after transfection all 3 of constructs were overexpressed by approximately 4-5 fold. Analysis of cell shape showed that there were no significantly differences in the morphology of the transfected and non-transfected cells. The mean values for the aspect ratio were 1.23±0.04, 1.27±0.06 and 1.20±0.03 in W/T, FAT and K/R mutant transfected cells respectively. Mean value in non-transfected cells was 1.24±0.05.

In HepG2 cells transfected with the FAT mutant, the expression level of endogenous FAK was similar to that of non-transfected cells (Figure 1B, left), suggesting that transfection did not alter the cellular phenotype. Figure 1B, right: confirms the specificity of the anti-FLAG antibody for detecting exogenous FAK tagged with FLAG, since endogenous FAK is not visible.

Insulin induces a rapid and transient tyrosine dephosphorylation of FAK followed by phosphorylation in adherent HepG2 cells: To determine the effects of insulin stimulation on FAK tyrosine phosphorylation, HepG2 cells, either untransfected or transfected with W/T FAK,
were treated with 100 nM insulin for 0-60 min. Extracts were then immunoprecipitated with anti-FAK antibody and immunoblotted with anti-phosphotyrosine antibody, then stripped and reblotted with anti-FAK antibody to quantify the amount of FAK immunoprecipitated. After 5 min of insulin treatment, a significant decrease in FAK phosphotyrosine immunoreactivity was observed in both groups versus the non-insulin stimulated state (Figure 2A). The decrease in FAK phosphorylation in both groups was transient, as anti-phosphotyrosine immunoreactivity gradually increased with longer exposure to insulin, indicating that FAK was either subsequently re-phosphorylated or phosphorylated on tyrosine residues that are not phosphorylated in the non-insulin-stimulated state. In the basal state, as reported by Hildebrand et al (17), the kinase-defective variant K/R FAK showed significantly reduced tyrosine phosphorylation. After exposure to insulin for 10 min, tyrosine phosphorylation of K/R FAK was unchanged compared to basal levels (Figure 2B). This is an interesting observation indicating that the phosphorylation state of FAK is dependent upon its kinase activity. It has been reported that this protein may function in dominant negative fashion by occupying all of the potential binding sites for enzymatically active FAK (17). FAT-FAK showed an intermediate level of tyrosine phosphorylation both in the presence and absence of insulin stimulation. The tyrosine phosphorylation state of FAK in the FAT and W/T FAK groups was not significantly altered by insulin treatment (Figure 2B).

**Insulin fails to stimulate glycogen synthesis in HepG2 cells transfected with FAT and K/R FAK mutants:** As shown in Figure 3, overexpression of FAT and K/R FAK effectively abolished incremental insulin-mediated glucose incorporation into glycogen in HepG2 cells, indicating that correct cellular localization and intact kinase activity of FAK are required for
insulin stimulated glycogen synthesis. The mutants appear to act in a dominant negative manner, since mutant expression did not markedly diminish expression of endogenous FAK (Figure 1). However, overexpression of W/T FAK did not increase basal or incremental insulin-mediated glucose incorporation into glycogen compared with that seen in untransfected or vector-transfected cells, suggesting that parental endogenous expression levels of FAK were sufficient for maximum insulin action. The basal mean values were similar in all groups at 128±22, 127±21, 147±25, 140±26, and 142±24 pmol/hr/mg protein in NON, CMV2, W/T, FAT and K/R respectively.

**Insulin-stimulated GS activity is impaired in HepG2 cells transfected with FAT and K/R FAK mutants:** In order to determine the mechanism of the inhibition of insulin-stimulated glycogen synthesis in FAT and K/R mutant cells, GS activity was assayed. An *in vitro* assay in which C-14-UDP-glucose incorporation into glycogen was determined in the presence of GS precipitated from HepG2 cells overexpressing the W/T and mutant forms of FAK was employed. The data are shown in Figure 4 and demonstrate that incremental insulin-stimulated GS activity was significantly impaired in the presence of the 2 mutant FAK constructs. In NON, CMV2 and W/T preparations, insulin stimulation led to a 2.3, 2.1 and 2.1-fold increase in GS activity respectively. In contrast, in the preparations overexpressing FAT and K/R, GS activity increased by 1.7 and 1.3 fold respectively (p <0.05 versus NON, CMV2 and W/T). There was a significant difference between basal and insulin-stimulated GS activity in FAT (p <0.05), but not in K/R cells. Basal non-insulin-stimulated GS activity was similar in all preparations (not shown). Interestingly, the increment in insulin-stimulated GS activity in W/T cells was similar to that seen in control preparations, which paralleled the results for glycogen synthesis seen in Figure 3.
Overexpressing FAT and K/R FAK mutants impair insulin-mediated GSK-3β-ser⁹ phosphorylation in HepG2 cells: Activation of GS is a rate-limiting step in glycogen synthesis, and GS is regulated by both allosteric and phosphorylation-dephosphorylation mechanisms (32). There is evidence that inhibition of GSK-3β activity by insulin-mediated serine phosphorylation is a key regulatory mechanism of activation of GS upon insulin stimulation (33). Insulin treatment results in phosphorylation on Ser⁹ of GSK-3β, leading to enzyme inactivation, thus releasing GS from its inhibitory influence (34-36). To determine whether the observed effect of FAT or K/R FAK on glycogen synthesis was accompanied by changes in the activation state of GSK-3β, the effect of insulin on ser⁹ phosphorylation of GSK-3β was examined. Non-transfected HepG2 cells and cells transfected with FAK constructs were exposed to insulin for 10 min. GSK-3β was then immunoprecipitated and ser⁹-phosphorylation was analyzed using an anti-GSK-3β–ser⁹ antibody. As shown in Figure 5A, insulin stimulation led to a marked increase in GSK-3β ser⁹ phosphorylation in HepG2 cells transfected with W/T FAK (458±34) compared with non-transfected cells (308±16) or those transfected with vector alone (321±41). By contrast, insulin-stimulated phosphorylation of GSK-3β ser⁹ was significantly attenuated in HepG2 cells transfected with FAT (205±14) or K/R (189±4) FAK. These data are shown quantitatively in Figure 5B and demonstrate that the effect of FAT or K/R FAK on glycogen synthesis is accompanied by a decreased capacity for insulin to serine phosphorylate and inactivate GSK-3β. Interestingly, although overexpression of W/T FAK potentiated insulin-mediated serine phosphorylation of GSK-3β, insulin-induced glycogen synthesis in these cells was unchanged (Figure 3).
Association of FAK with GSK-3β in response to insulin is increased in HepG2 cells

**overexpressing FAT and K/R mutants:** The association of FAK with GSK-3β was then examined in HepG2 cells transfected or untransfected with FAK constructs. GSK-3β was immunoprecipitated from cell lysates with or without prior insulin stimulation, and immunoprecipitates were analyzed for the presence of FAK. As shown in Figure 6, in HepG2 cells transfected with FAT or K/R FAK, treatment with insulin for 10 min increased the amount of FAK that co-precipitated with GSK-3β. In the case of FAT mutant, association of endogenous FAK, rather than the truncated mutant itself, with GSK-3 appears to be stimulated by insulin treatment. In contrast, insulin treatment was associated with no significant increase in association between FAK and GSK-3β in HepG2 cells transfected with W/T FAK, empty vector, or untransfected cells (Fig. 6A). These data are shown quantitatively in Figure 6B and demonstrate that the effect of FAT or K/R FAK on insulin-mediated GSK-3β ser9 phosphorylation is also accompanied by increased insulin-stimulated association between FAK and GSK-3β. These findings suggest that a transient association between FAK and GSK-3β is necessary for insulin action to promote glycogen synthesis and that failure to terminate this association appropriately may lead to impaired insulin action, at least on glycogen synthesis.

**Insulin-mediated Akt/PKB-ser473 phosphorylation is impaired in HepG2 cells**

**overexpressing FAT and K/R mutants:** Since Akt/PKB is known to be the upstream regulator of GSK-3β in insulin signaling to glycogen synthesis (37), we analyzed the phosphorylation state of one key residue in Akt/PKB, namely ser473, using a phosphospecific antibody. Phosphorylation of ser473 of Akt/PKB occurs as a result of insulin stimulation (38). As shown in Figure 6A, there was a marked increase in phosphorylation of ser473 in response to exposure to
insulin in HepG2 cells transfected with either W/T FAK or vector alone, as well as in untransfected cells. However, similar to the effect on ser\(^9\) phosphorylation of GSK-3β presented above, the insulin-stimulated increase in Akt/PKB ser\(^{473}\) phosphorylation was significantly impaired in HepG2 cells overexpressing FAT or K/R- FAK mutants, compared with that seen with W/T FAK, vector alone, or in untransfected cells (Fig 7A). These data are shown quantitatively in Figure 7B. There was no immunoprecipitable association between FAK and Akt/PKB, either in the basal or insulin-stimulated state (data not shown).

IR-β and IRS-1 phosphorylation and PI3´-kinase activity are unaffected in cells overexpressing FAT or K/R mutants: Akt/PKB links insulin signaling to metabolic function through insulin-mediated activation of the IR and engagement of IRS proteins with PI3´-kinase (39). Since PI3´-kinase activation is central to realizing insulin’s metabolic effects (40), we also studied the regulation of IR-β/IRS-1 tyrosine phosphorylation or protein abundance and both total and IRS-1-associated PI3´-kinase activity by FAK mutant constructs in HepG2 cells in response to insulin. As shown in Figures 8 and 9, the level of IR-β or IRS-1 tyrosine phosphorylation and protein abundance was similar among 5 experimental conditions after insulin stimulation. In addition, as shown in Figure 10, after insulin stimulation the expected increase in docking of the p85α-subunit of PI3´-kinase with IRS-1 occurred, however this was similar among the 5 groups. Total PI3´-kinase activity in cell lysates was also similar both with and without insulin-stimulation in the 5 experimental groups (data not shown). Moreover, Figure 11 shows that insulin treatment resulted in the expected stimulation of specific IRS-1-associated PI3´-kinase activity, but that this was unaffected by the expression of W/T FAK or either of the mutant FAK species. These data suggest that the effect of FAK on
insulin-stimulated glycogen synthesis is localized downstream of IR, IRS-1 and PI3’-kinase.
Integrins are transmembrane receptors involved in interactions between cells and extracellular matrix (ECM) protein and which mediate both outside-in and inside-out signaling (41). FAK, a cytosolic tyrosine kinase, is a key component of the integrin signaling pathway (42,43).

Following integrin engagement by cell contact, FAK is activated and phosphorylated on tyrosine³⁹⁷, which serves as a docking site for c-Src (44). Src may regulate FAK activity by phosphorylating it on additional tyrosine residues and facilitating its interaction with other signaling proteins, including the p85α subunit of PI3´-kinase (6). Tyrosine phosphorylation of FAK requires that cells are adherent to the ECM and it has been reported that the level of tyrosine phosphorylation is reduced in suspended cells (1). When suspended cells are replated on ECM substrates, FAK becomes highly tyrosine-phosphorylated and shows enhanced kinase activity (45).

Insulin is a pleiotropic hormone which both effects and affects a broad range of biologic functions including regulation of glucose and fat metabolism, protein synthesis, cytoskeletal rearrangement, cell growth and differentiation (46). It is now accepted that insulin-mediated glycogen synthesis utilizes a signaling cascade involving PI3´-kinase (47,48) and the downstream effector Akt/PKB (49-51). The ability of insulin to regulate the activation state of FAK was, however, a recent observation (9,12). Most available information in the literature focuses on the role of FAK in insulin-mediated effects on the cytoskeleton (13,42). As a result, the precise role of FAK in insulin’s other metabolic actions remains poorly understood. Cross-talk between integrin and insulin signaling is suggested by the finding that insulin-stimulated
DNA synthesis is modulated by engagement of αVβ3 integrin (16,52,53). It has been shown that insulin promotes association of IRS-1 with integrin (16) and also that tyrosine phosphorylated IR rapidly associate with integrin at focal adhesion contacts (53). Furthermore, cell adhesion and integrin engagement promote insulin's ability to activate PI3´-kinase (7). Integrin also modulates the insulin and insulin-like growth factor-1 signaling pathways by regulating cellular IRS-1 mRNA expression and the pathway of FAK-mediated signaling to JNK appears to be involved in this process (11).

Insulin and IGF-1 induce either phosphorylation or dephosphorylation of FAK, depending upon whether cells are in the attached or suspended state (8-10,15). As also reported by others (9,17), we find that in attached cell preparations, insulin-induced dephosphorylation of FAK occurs in the presence of endogenous FAK and both W/T and FAT mutant FAK constructs. Whether or not FAK itself directly interacts with insulin signaling proteins and the site at which such an interaction occurs has not hitherto been fully addressed. The studies described here, which were carried out in an insulin-responsive cell line, demonstrate that FAK is likely to regulate insulin action on glycogen synthesis at a level downstream of IR, IRS-1 and PI3´-kinase, possibly via direct interaction with Akt/PKB and GSK-3β.

Evidence in the present work supporting a role for an interaction between FAK and GSK-3β in the regulation of glycogen synthesis by insulin includes the following: Firstly, overexpression of FAT or K/R mutant FAK abolished insulin-stimulated glucose incorporation into glycogen, was accompanied by significantly reduced serine phosphorylation of both Akt/PKB and GSK-3β, and
significantly increased the insulin-stimulated co-immunoprecipitation of FAK with GSK-3β. The regulation of the serine phosphorylation state of GSK-3β by FAK may thus be secondary to its ability to regulate Akt/PKB activation. Insulin-mediated serine phosphorylation of GSK-3β was upregulated in W/T overexpression, while both Akt/PKB phosphorylation and glycogen synthesis were unchanged. Possible explanations for this finding include additional regulation of GS by Akt/PKB through a mechanism other than via inhibition of GSK-3β alone, or that endogenous levels of FAK permit activation of Akt/PKB and inhibition of GSK-3β sufficient for maximum insulin-stimulated glycogen synthesis in this system. It has previously been reported that Akt/PKB and GSK-3β can be activated by PI3'-kinase-independent mechanisms (54,55).

Initially, the observed association between FAK and GSK-3β in the basal state was unexpected. However, Ding et al have reported that β-catenin is a substrate of GSK-3β in the Wnt signaling pathway (56). β-catenin is involved in mediation of cell adhesion and transcription and associates with focal adhesion proteins, including FAK (57,58). However, activation of the Wnt signaling pathway does not regulate GS activity, and does not lead to the phosphorylation of serine^9 of GSK-3β nor activation of Akt/PKB which occur following insulin stimulation. Thus, the association between FAK and GSK-3β may involve intermediary proteins in more than one signaling pathway.

Although the basal association between FAK and GSK-3β was increased, this was not further disproportionately stimulated by exposure to insulin in W/T cells. By contrast, the abolition of insulin-stimulated glycogen synthesis seen in cells overexpressing FAT and K/R mutants was associated in both cases with a clear insulin-mediated increase in association between FAK and
GSK-3β. Interestingly, in the case of FAT, the increased association involved only endogenous FAK and not FAT itself. This suggests that either localization to the focal adhesion complex is required for the subsequent insulin-stimulated association-dissociation to occur, or that FAT is acting as a dominant negative at a step outside the focal adhesion complex, thereby inhibiting the required dissociation of even normally localized FAK from GSK-3β.

Since the phosphate groups on GS turn over rapidly (59), it has been proposed that inhibition of GSK-3β serves as a physiologically relevant mechanism for regulating GS activity. The regulation of GSK-3β by insulin has been shown to be mediated by Akt/PKB (60,61). Upon insulin stimulation, the serine\textsuperscript{473} residue of Akt/PKB is phosphorylated and Akt/PKB is activated (62). Akt/PKB has been implicated as a mediator of metabolic responses to insulin, including GS activation (63). Subsequently, GSK-3β is phosphorylated on serine\textsuperscript{9}, which leads to a decrease in GSK-3β activity (61,64). Although this has usually been detected as a 50-70% drop, it is apparently sufficient to relieve the inhibition of GS and allow glycogen synthesis to proceed in an insulin-responsive manner (56). Therefore further potentiation in Akt/PKB serine phosphorylation might not have led to increased glycogen synthesis. Apparently, GSK-3β inhibition alone is not sufficient for the dephosphorylation and activation of GS (32). Our data suggest that FAK mutants may potentially inhibit insulin-stimulated phosphatase activity and that FAK plays a regulatory role in insulin-stimulated glycogen synthesis and that this is achieved through an interaction between FAK, Akt/PKB and GSK-3β.

The fact that incremental insulin-stimulated GS activity was partially, but significantly, attenuated in the presence of FAT and K/R mutants indicates that impaired regulation of GS
activity is at least partly responsible for the reduction in insulin-stimulated glycogen synthesis seen in the presence of these mutants.

The mechanism whereby these FAK mutants inhibit the ability of insulin to induce phosphorylation of Akt/PKB remains unclear. It is possible that endogenous FAK interacts with an unidentified substrate that is necessary for the interaction between PI3′-kinase and Akt/PKB and that mutant FAK exerts a dominant negative effect on endogenous FAK. A similar dissociation between PI3′-kinase activity and activation of Akt/PKB has previously been reported (65,66). Lin et al found that the protein kinase C (PKC) activator phorbol dibutyrate decreased the ability of insulin to phosphorylate both Akt/PKB and GSK-3β by 90% and 35% respectively in rat skeletal muscle, while PI3′-kinase activation was unaffected (65). Also, Matsumoto et al reported that in CHO cells and L6 myocytes, expression of kinase-defective PKC-δ inhibited insulin-mediated phosphorylation and activation of Akt/PKB, while having no inhibitory effect on phosphorylation or activity of PI3′-kinase (66). Whether PKC is involved in the downstream regulation of insulin signaling by FAK remains to be investigated.

We also found that the effect of overexpression of these FAK constructs on glycogen synthesis was unaccompanied by changes in the tyrosine phosphorylation state of either the IR-β−subunit or IRS-1, nor was the level of expression of either of these upstream proteins affected. Importantly, IRS-1-associated PI3′-kinase activity was also unaltered. In this respect, our findings differ from those of Lebrun et al (10), who reported that FAK interacts directly with IRS-1 and regulates IRS-1-associated PI3′-kinase activity. The following may be offered to explain this discrepancy: Firstly, the studies were performed in different cell systems, FAK−/−
cells and DA2 cells being of fibroblast lineage, while HepG2 cells are insulin-responsive human hepatoma cells (9). Secondly, Lebrun et al studied cells that were in the suspended state for 2 hours (11), while we utilized attached cells. Since the nature of the cross-talk between insulin/IGF-I receptors and FAK is dependent upon cell architecture, the interaction of the insulin/IGF-I signaling system with integrin will vary accordingly (9). Finally, Goode et al (67) have already reported that GSK-3β can be phosphorylated by certain isoforms of PKC in vitro. Bogdanovic et al (68) also reported that under conditions of insulin resistance PKC downregulates Akt/PKB and that this is unaccompanied by a change in PI3’-kinase activity. We hypothesize that the FAK mutants used in this study may also down regulate Akt/PKB activity. Moreover, the increased association of FAK mutants with GSK-3β upon insulin stimulation emphasizes this down regulation. Possibly, this downregulation is PKC-mediated, leading to reduced phosphorylation of GSK-3β. However, further studies will be required to address this issue.

The effect of these FAK mutants to inhibit insulin-mediated glycogen synthesis in this adherent cell system appeared to parallel their inhibition of the insulin-mediated dephosphorylation of FAK. It is unclear whether this dephosphorylation is necessary for insulin mediated glycogen synthesis to occur in hepatocytes. In studies using a mutant insulin receptor tyr1210phe overexpressed in CHO cells, Van der Zon et al found that insulin-mediated dephosphorylation of FAK was almost abolished, but that insulin-mediated glycogen synthesis was maintained, although somewhat diminished (69), suggesting that FAK dephosphorylation is not an essential component of insulin-mediated glycogen synthesis. Future studies with PTP inhibitors may also address this question.
We conclude that FAK regulates the activity of Akt/PKB, GSK-3β and GS to influence insulin-mediated glycogen synthesis in hepatocytes and thus may play an important role in hepatic insulin action. Insulin action may be subject to secondary regulation by the integrin signaling pathway, ensuring that these growth and differentiation-promoting pathways act in a coordinated and/or complimentary manner.
ACKNOWLEDGMENTS

This work was supported in part by a Merit Review Award from the Veterans Administration, and by a research grant from the American Diabetes Association to MBA. This work is based in part upon support provided by the Office of Research and Development (R&D) of the Department of Veterans’ Affairs. MBA received partial salary support under NIH-General Clinical Research Center Grant #RR00211 and from the UCLA Gonda (Goldschmied) Diabetes Center. The advice of Dr. Joan Guinovart on the assay for glycogen synthase activity is gratefully acknowledged.
REFERENCES

LEGENDS TO FIGURES

Figure 1: Expression of FAK and epitope-tagged FAK variants in HepG2 cells. Figure 1A: Cells were suspended at 1.0 x 10^5 cells/ml in complete medium, then transfected with FAK mutants. At the times shown, cells were resuspended and counted as described in Experimental Procedures. Cell growth was expressed as the number of live cells per dish ± SE for 4 separate experiments. Whole cell lysates were prepared and 30 µg of total cell protein was resolved by SDS-PAGE and western blotting with antibody to FLAG epitope to show exogenous FAK (Figure 1B, right) or antibody to FAK, to show both endogenous and exogenous FAK (Figure 1B, left). Figure 1C: Total FAK abundance was quantitated by densitometry. NON = untransfected cells; W/T = cells transfected with wild-type FAK; FAT = cells transfected with c-terminal focal adhesion targeting region deficient mutant FAK; K/R = cells transfected with kinase-incompetent mutant FAK; CMV2 = cells transfected with empty vector.

Figure 2: Insulin-induced tyrosine phosphorylation of exogenous FAK. HepG2 cells that were not transfected or transfected with FAK W/T were exposed to 100 nM insulin for different periods. Exogenous W/T FAK was immunoprecipitated with a monoclonal anti-FLAG antibody (Figure 1A, top right) and untransfected endogenous FAK was immunoprecipitated with a monoclonal anti-FAK antibody (Figure 1A, top left), and immunoblotted with PY99 monoclonal antiphosphotyrosine antibody. The membrane was then stripped and rebotted with monoclonal anti-FAK antibody (Figure 1A, lower panel). HepG2 cells that were not transfected or transfected with FAK constructs were exposed to 0 nM insulin ( - ), or 100 nM insulin ( + ) for 10 min. Exogenous FAK was immunoprecipitated with a monoclonal anti-FLAG antibody, and the precipitates were immunoblotted with PY99 antibody (Figure 1B, upper...
The membrane was then stripped and reblotted with monoclonal anti-FAK antibody (Figure 1B, lower panel). Representative immunoblots are shown.

**Figure 3:** Insulin-stimulated glucose incorporation into glycogen in HepG2 cells that were not transfected or transfected with FAK constructs. HepG2 cells were incubated for 16 hr in DMEM containing 5 mM glucose and then for 3 hr in fresh medium containing 15 mM [U-14C] glucose with or without 100 nM insulin. Values are mean±SE for 5 different experiments and represent percent change in glycogen synthesis versus non-insulin stimulated cells. * p< 0.05 relative to NON, CMV2 or W/T. Labels are as for Figure 1.

**Figure 4:** GS activity in HepG2 cells transfected with FAK constructs. HepG2 cells were incubated for 10 min with or without 100 nM insulin. GS activity was assayed in the supernatants of cell lysates as described in ‘Experimental Procedures’. GS activity is expressed as a percentage of the basal non-insulin stimulated value. Values shown are mean±SE for 3 different experiments. * p< 0.05 relative to NON, CMV2 or WT. Labels are as for Figure 1.

**Figure 5:** Insulin-induced Ser^9^ phosphorylation of GSK-3β. HepG2 cells that were not transfected or transfected with FAK constructs were exposed to 0 nM insulin ( - ), or 100 nM insulin ( + ) for 10 min. GSK-3β was immunoprecipitated with a monoclonal anti-GSK-3β antibody, and the Ser^9^ phosphorylation of GSK-3β was analyzed with anti-phospho-Ser^9^-GSK-3β antibody. **Figure 4A:** A representative immunoblot is shown. **Figure 4B:** Levels of GSK-3β Ser^9^ phosphorylation were quantitated by densitometry. Results are expressed as mean±SE of 3 different experiments. *p< 0.05 vs NON or CMV2.
Figure 6: Association between GSK-3β and FAK in response to insulin treatment in cells transfected or untransfected with FAK constructs. Lysates were prepared from cells incubated without ( - ) or with ( + ) 100 nM insulin for 10 min. GSK-3β was immunoprecipitated from each sample, and the precipitates were immunoblotted with anti-FAK antibody (Figure 5A, upper panel) then stripped and rebotted with anti-GSK-3β antibody (Figure 5A, lower panel). Figure 5B: The association of GSK-3β with FAK was measured by densitometry. Results are expressed as mean±SE for 3 different experiments. *p< 0.05 vs NON or CMV2.

Figure 7: Insulin-induced Ser473 phosphorylation of Akt/PKB. HepG2 cells that either were or were not transfected with FAK constructs were exposed to 0 nM insulin ( - ), or 100 nM insulin ( + ) for 10 min. Panel A, upper band: Akt/PKB was immunoprecipitated with a monoclonal anti-Akt/PKB antibody, and the Ser473 phosphorylation of Akt/PKB was analyzed with an anti-pSer473-Akt/PKB antibody. Panel A, lower band: The membrane was then stripped and rebotted with anti-GSK-3β antibody. Panel B: Levels of Akt/PKB Ser473 phosphorylation were quantitated by densitometry. Results are expressed as mean±SE for 3 different experiments. *p< 0.05 vs non-transfected, CMV2 or W/T-transfected cells.

Figure 8: The effect of FAK mutants on insulin-induced tyrosine phosphorylation of IR-β subunit. HepG2 cells that were not transfected or transfected with FAK constructs were exposed to 0 nM insulin ( - ), or 100 nM insulin ( + ) for 10 min. IR-β subunit was immunoprecipitated with a polyclonal anti-IR-β antibody, and the precipitates were immunoblotted with the
monoclonal anti-phosphotyrosine antibody (Panel A). The membrane was then stripped and rebotted with polyclonal anti-IR-β antibody (Panel B). Representative immunoblots are shown. The levels of IR-β tyrosine phosphorylation were similar among the 5 groups after insulin stimulation.

**Figure 9:** Effect of transfection of FAK mutants on insulin-induced tyrosine phosphorylation of IRS-1. HepG2 cells that were not transfected or transfected with FAK constructs were exposed to 0 nM insulin ( - ), or 100 nM insulin ( + ) for 10 min. IRS-1 was immunoprecipitated with a polyclonal anti-IRS-1 antibody, and the precipitates were immunoblotted with the monoclonal anti-phosphotyrosine antibody (Panel A), then stripped and rebotted with polyclonal anti-IRS-1 antibody (Panel B). Representative immunoblots are shown. The levels of IRS-1 tyrosine phosphorylation were similar among the 5 groups after insulin stimulation.

**Figure 10:** Effect of FAK mutants on association of IRS-1 with p85 of PI3’-kinase in HepG2 cells. Following overnight serum starvation, cells were stimulated with 0 nM insulin ( - ) or 100 nM insulin ( + ) for 5 min. IRS-1 was immunoprecipitated with a polyclonal anti-IRS-1 antibody, and the precipitates were immunoblotted with an antibody specific for the p85 subunit of PI3’-kinase (Panel A), then stripped and rebotted with polyclonal anti-IRS-1 antibody (Panel B). Representative immunoblots are shown. There was no difference in the amount of IRS-1-associated with p85 of PI3’-kinase among the 5 groups.
**Figure 11:** Effect of FAK mutants on IRS-1-associated PI3′-kinase activity. Following overnight serum starvation, cells were stimulated with 0 nM insulin (-) or 100 nM insulin (+) for 5 min. Lysates were prepared and PI3′-kinase activity was measured in IRS-1 immunoprecipitates. Activity was quantitated by densitometry. **Panel A:** a representative autoradiogram of thin layer chromatography together with quantitation of the results of 3 different experiments is shown. There was no significant difference in the values among the 5 groups either with or without insulin stimulation.
FIG. 1

A. IB: FAK

125
85

NON W/T FAT K/R CMV2

IB: FLAG

125
85

NON W/T FAT K/R CMV2

B. FAK abundance

% of control (Densitometric Units)

0 100 200 300 400 500

NON W/T FAT K/R CMV2

C. Cell Growth (cell number x 10^5)

10

0 2 4 6 8 10

0 24 48 72

Time (hours)
FIG. 2

A.

<table>
<thead>
<tr>
<th>Insulin (100nM)</th>
<th>IP: FAK</th>
<th>IP: FLAG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0’ 5’ 15’ 30’</td>
<td>0’ 5’ 15’ 30’</td>
</tr>
<tr>
<td>IB: PY-99 135</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IB: FAK 135</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NON W/T</td>
<td></td>
<td></td>
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</table>

B.

<table>
<thead>
<tr>
<th>Insulin (100nM)</th>
<th>IP: FLAG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>− + − + − + − + − + +</td>
</tr>
<tr>
<td>IB: PY99</td>
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</tr>
<tr>
<td>FAT</td>
<td></td>
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<tr>
<td>135</td>
<td></td>
</tr>
<tr>
<td>IB: FAK</td>
<td>95</td>
</tr>
<tr>
<td>NON CMV2 FAT W/T K/R</td>
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</tr>
</tbody>
</table>
FIG. 3

![Graph showing Insulin Stimulated Glycogen Synthesis Percent Change from Basal for different groups: NON, CMV2, W/T, FAT, K/R. The graph indicates variations in glycogen synthase activity (% of basal).]

FIG. 4

![Graph showing Glycogen synthase activity (% of basal) for different groups: NON, CMV2, WT, FAT, K/R. The graph indicates variations in glycogen synthase activity with asterisks indicating significant differences.]
FIG. 5

A. IP: GSK-3β

Insulin (100nM)

IB: Phospho-GSK-3β

IB: GSK-3β

NON  CMV2  W/T  FAT  K/R

B. GSK-3β Ser9-Phosphorylation

Change From Basal (Densitometric Units)

NON  CMV2  W/T  FAT  K/R

*
FIG. 6

A.

IP: GSK-3β

Insulin (100nM)  
- + - +  
- + - +  
- + - +  
- + - +

IB: FAK

131

95

IB: GSK-3β

36.4

NON  CMV2  W/T  FAT  K/R

B.

Insulin Stimulated GSK-3β Association with FAK (Densitometric Units)

-200  0  200  400  600  800  1000

*  *

NON  CMV2  W/T  FAT  K/R

*  *
FIG. 7

A. IP: Akt/PKB

Insulin (100nM)

IB: Phospho-Akt/PKB

IB: Akt/PKB

NON    CMV2    W/T    FAT    K/R

B. Ser473 phosphorylation of Akt/PKB (Densitometric Units change from basal)

NON    CMV2    W/T    FAT    K/R

*    *
FIG. 8

IP: IR-β

Insulin (100nM)

IB: PY-99 135

IB: IR-β 95

NON CMV2 W/T FAT K/R

FIG. 9

IP: IRS-1

Insulin (100nM)

IB: PY-99 215

IB: IRS-1 215

NON CMV2 W/T FAT K/R
FIG. 10

IP: IRS-1

Insulin (100nM)

IB: PI3'-Kinase

IB: IRS-1

Non

CMV2

W/T

Fat

K/R

+ + + + +
FIG. 11

IRS-1 Associated PI3'-Kinase Activity (Densitometric units/mg protein)

Insulin: 
-orig

PIP

Orig

NON CMV2 W/T FAT K/R

450
400
350
300
250
200
150
100
50
0

Insulin: 
- + - + - + - + - +

FIG. 11

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Focal adhesion kinase (FAK) regulates insulin-stimulated glycogen synthesis in hepatocytes
Danshan Huang, Anthony T. Cheung, J. Thomas Parsons and Michael Bryer-Ash

J. Biol. Chem. published online January 23, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M104252200

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