Focal adhesion kinase and Src mediate integrin regulation of insulin receptor phosphorylation

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Abstract We show here that phosphorylation of the insulin receptor and insulin receptor substrate-1 is increased when suspended cells are replated on fibronectin. This is not due to decreased numbers of cell surface receptors, alteration of insulin binding, or stimulation of a phosphatase activity in non-adherent cells. Expression of Src together with focal adhesion kinase (FAK) in suspended cells restores insulin-induced receptor autophosphorylation to levels observed in fibronectin-attached cells. Conversely, expression of dominant-negative mutants of either Src or FAK abolishes potentiation of insulin receptor phosphorylation by cell adhesion. The results suggest that both Src and FAK participate in integrin-mediated regulation of insulin receptor signal. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Insulin receptor; Integrin; Src kinase; Focal adhesion kinase; Adhesion

1. Introduction

Integrins are primary mediators of cell adhesion and regulators of cell growth, differentiation, survival and migration. They also coordinate growth factor responses and it is well known, for example, that integrin-mediated adhesion is required for efficient signaling of tyrosine kinase receptors, including the insulin receptor (IR) (reviewed in [1–3]).

IR is an oligomer consisting of two extracellular α-subunits and two transmembrane β-subunits with tyrosine kinase activity. Upon ligand binding, IR autophosphorylates several tyrosine residues, then interacts with and phosphorylates a number of cytosolic substrates, among which the insulin receptor substrate (IRS) family and Src homology collagen (Shc). Subsequently, IRS and Shc interact with SH2 domain-containing proteins, leading to activation of various signaling pathways such as the phosphatidyl inositol 3 (PI3)-kinase and mitogen-activated protein kinase pathways (reviewed in [4–8]).

There is evidence that integrin regulation of IR signaling occurs at several steps. For example, both IR and IRS-1 associate with $\alpha_v\beta_3$ integrins following hormone stimulation of fibroblasts plated on vitronectin. This association correlates with enhanced insulin-induced cell proliferation [9,10]. It was shown recently that cell adhesion regulates IRS-1 expression levels by controlling the synthesis of the corresponding mRNA [11]. Thus, integrins may alter the intensity of IRS-1-mediated responses. Another study showed that insulin induces focal adhesion kinase (FAK) dephosphorylation in attached cells, whereas it induces FAK phosphorylation in suspended cells, indicating that the effects of insulin on FAK phosphorylation are modulated by cell adhesion [12]. Finally, fibronectin or antibody-induced activation of β_1 integrin in Chinese hamster ovary (CHO) cells and in isolated adipocytes potentiates the effects of insulin on IR and IRS-1 phosphorylation and on IRS-associated PI3-kinase activity [13,14]. In another study however, IR phosphorylation was found to be independent of integrins in mammary cells, although IR signaling was not. Indeed, phosphorylation of IRS-1 and IRS-2 and their association with PI3-kinase in response to insulin was impaired in mammary cells cultured on collagen, as opposed to cells cultured on laminin-rich basement membrane. In contrast, IR phosphorylation was similar in both conditions [15]. Thus, whether IR phosphorylation is regulated by integrins or whether this regulation occurs downstream is an issue that remains to be clarified. We show here in three different cell lines that optimal IR phosphorylation is achieved only in attached cells. Moreover, we give evidence that FAK and Src mediate the potentiation of IR phosphorylation by cell adhesion.

2. Materials and methods

2.1. Reagents

Human recombinant insulin (actrapid) was from Novo Nordisk. Peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch Labs. Bovine plasma fibronectin was purchased from Sigma-Aldrich. [^{125}I]protein A (30 μCi/μg) was from ICN.

Constitutive active Src mutated on tyrosine 527 (Y527F) and kinase-deficient (kd) Src mutated on lysine 295 (K295M) in pSG vector were gifts from S. Courtneidge [29]. They were subcloned into pCEP vector as previously described [28].

2.2. Antibodies

Anti-myc and anti-phosphotyrosine antibodies were mouse monoclonal clones 9E10 and 4G10, respectively. These and rabbit polyclonal anti-IRS-1 antibodies (06–248) used in Western blots were from Upstate Biotechnology, Inc.

The antibodies used to immunoprecipitate IRS-1 were prepared in our laboratory and were raised against a synthetic peptide corresponding to the C-terminal sequence 1223–1235 of rat IRS-1 [16]. Rabbit polyclonal anti-Src (N16) was purchased from Santa Cruz Biotechnology. Production of monoclonal antibodies to human IR

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Abbreviations: FAK, focal adhesion kinase; IR, insulin receptor; IRS, insulin receptor substrate; CHO, Chinese hamster ovary; PI3-kinase, phosphatidyl inositol 3-kinase; PDGF, platelet derived growth factor; EGF, epidermal growth factor

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has been described previously [17]. In this study, B6 antibody was used for immunoprecipitation, whereas E9 antibody was used for cell surface detection of IR. For Western blots, we used the rabbit polyclonal antibody directed to IR C-terminus (C-19) from Santa Cruz Biotechnology.

2.3. Cell culture

NIH-3T3 cells that stably express human IR were produced in our laboratory as described previously [18]. Hep G2 are derived from a human hepatocyte carcinoma (Invitrogen). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and 10 mM glutamine. CHO cells were grown in F-12 medium supplemented with 10% (v/v) fetal calf serum and 10 mM glutamine.

All cells were cultivated at 37°C in 5% CO_2. They were depleted overnight in culture medium containing 0.2% (w/v) bovine serum albumin (BSA) before use.

2.4. Cell transfection

CHO cells were plated the day before use. Transfection was performed using fuGENE® reagent according to instructions (Roche Molecular Biochemicals). Briefly, 3 μl of reagent was mixed with 100 μl of serum-free medium for 5 min at 22°C and then added to 1–2 μg of DNA. After 30 min incubation, the mix was added dropwise to the cells. Experiments were performed 2 days later.

2.5. Cell suspension and replating

Starved cells were washed with phosphate-buffered saline (PBS) before addition of 1 mM EDTA for 15 min at 22°C. Cells were detached from dishes by gentle scraping. They were washed twice with PBS before suspension in 3 ml of culture medium containing 0.2% (w/v) BSA for 2 h at 37°C in 5% CO_2 atmosphere with occasional agitation to prevent clumping. Suspended cells were either used directly or were replated on fibronectin. For coating, 1 ml of PBS containing 20 μg fibronectin was poured into a 60 mm dish and left overnight at 4°C. Coated dishes were rinsed with PBS and blocked using 10 mg/ml (w/v) heat denaturated BSA for 10 min at 22°C.

Note that using EDTA to detach cells had no effect per se on receptor kinase activity (data not shown).

2.6. Detection of cell surface receptors

Hep G2 cells were left attached or were suspended as described above for 2 h before incubation with E9 monoclonal antibody or normal mouse ascites, in culture medium containing 0.2% BSA, 30 mM HEPES, pH 7.4, for 1 h at 4°C. The cells were washed with PBS and incubated with rabbit anti-mouse antibodies for 1 h at 4°C. After several washes, they were incubated with [^{125}I]protein A (1 μCi/2×10^6 cells) for 1 h at 4°C. They were washed again and lysed in 200 mM NaOH. Cell-associated radioactivity was determined in a γ counter.

2.7. IR and IRS-1 phosphorylation

Experiments were performed on attached cells, cells that had been maintained in suspension for 2 h, or cells replated on fibronectin for the indicated periods of time. The cells were left untreated or were treated with insulin (0.1 μM) for 10 min at 37°C, then chilled on ice and washed twice with ice-cold buffer consisting of 50 mM HEPES, pH 7.5, 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM $NaVO_4$. The cells were solubilized for 45 min on ice in the above buffer containing 1% (v/v) Triton X-100 and protease inhibitors (10 μg/ml aprotinin; 20 μM leupeptin; 1 mM phenylmethylsulfonyl fluoride). Lysates were then clarified by centrifugation at 13 000×*g* for 15 min at 4°C. Protein concentration was determined using the BCA® protein assay reagent (Pierce). 100 μg protein-samples were subjected to immunoprecipitation using antibodies against IR or IRS-1 that were preadsorbed on protein-A Sepharose (Pharmacia). Immunoprecipitations were conducted at 4°C for 3 h, pellets were washed four times in ice-cold stop buffer containing 0.1% (v/v) Triton X-100 and resuspended in Laemmli sample buffer.

2.8. Western Blot analysis

Samples were subjected to electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore) using standard procedures. Membranes were blocked in saline buffer (25 mM Tris–HCl, pH 7.4; 140 mM NaCl; 0.1% (v/v) Tween 20) containing 4% (w/v) BSA for 3 h at 22°C before addition of the antibodies for an overnight incubation at 4°C. Several washes were performed in saline buffer, and peroxidase-conjugated antibodies against mouse or rabbit immunoglobulins were added at a concentration of 1 μg/ml for 1 h at 22°C. After washes, the membranes were revealed using the Super-Signal® chemiluminescent substrate (Pierce), followed by autoradiography.

3. Results and discussion

Evidence that cell adhesion is required for full activation of IR came from experiments in which CHO cells expressing human IR were first detached and maintained in suspension for 2 h, then replated on fibronectin for increasing periods of time. Cells were incubated without or with insulin for 10 min before lysis.

As shown in Fig. 1, ligand-induced receptor autophosphorylation was low in suspended cells and cells replated on fibronectin for 15 min. However, receptor autophosphorylation increased after 30 min of replating, reaching a maximum level at 45 and 60 min. We also compared IR and IRS-1 phosphorylation in attached and suspended NHIR and Hep G2 cells, which are mouse NIH-3T3 fibroblasts stably expressing human IR and human hepatocyte carcinoma cells naturally expressing high levels of IR, respectively. In both cell lines, insulin-induced receptor autophosphorylation was dramatically reduced when cells were detached, compared to adherent cells (Fig. 2). IRS-1 phosphorylation was decreased the same extent as receptor autophosphorylation.

In time-course experiments, we observed that the decrease in receptor activity occurred as soon as cells were suspended, suggestive of a rapid regulatory mechanism (data not shown).

We conclude that optimal insulin-induced receptor autophosphorylation depends on cell adhesion, irrespective of the cell line. Similar potentiation of IR autophosphorylation has been described in CHO cells and in adipocytes [13,14]. There are many examples of tyrosine kinase receptor regulation by cell adhesion, such as receptors for insulin-like growth factor-I, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor and macrophage-stimulating protein [19–24]. Interestingly, all of these receptors, as well as IR, are localized at focal

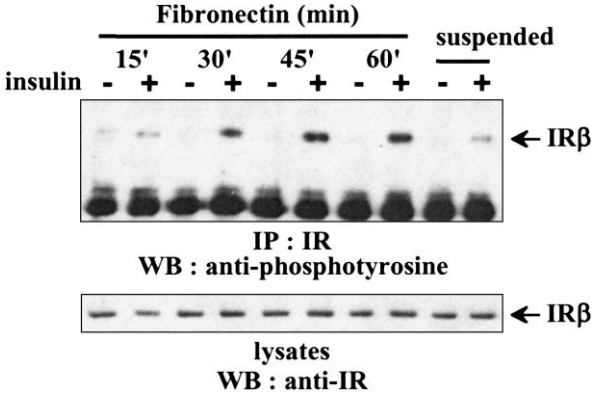

Fig. 1. CHO cells transiently expressing IR were suspended for 2 h before replating on fibronectin for the indicated time periods. Cells were treated or not with insulin (0.1 μM) for 10 min. After lysis, IR was immunoprecipitated using specific antibodies, and samples were analyzed by Western blotting with antibodies to phosphotyrosine. IR amounts were checked by Western blotting using antibodies to IR.

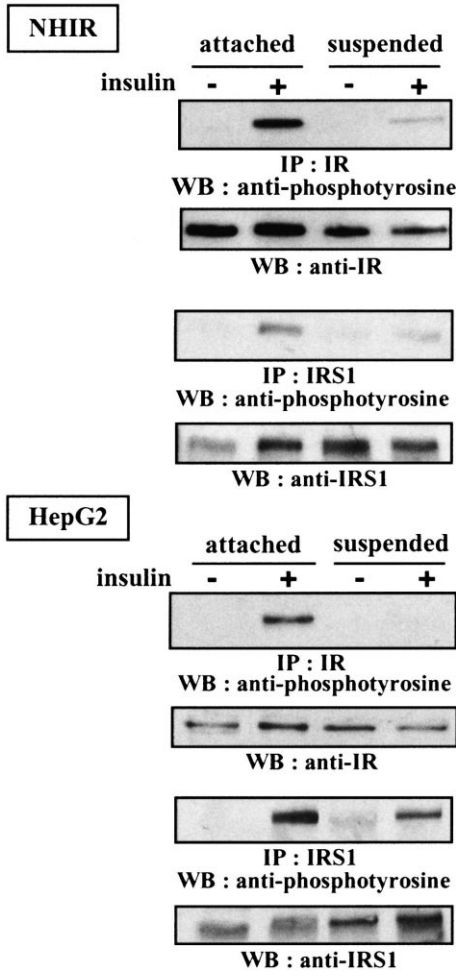

Fig. 2. Suspended or attached cells were stimulated or not with insulin (0.1 μM) for 10 min before lysis. IR and IRS-1 were immunoprecipitated from cleared lysates using specific antibodies. Phosphorylation was analyzed by Western blotting with antibodies to phosphotyrosine. Membranes were reprobed with antibodies to IR or IRS-1 as indicated. NHIR fibroblasts express 10^6 IR/cell, whereas Hep G2 cells express about 2×10^5 IR/cell.

adhesions or associated with integrins upon ligand binding [10,21,23,25].

Distinct mechanisms seem to account for the regulation of tyrosine kinase receptors by integrins. For example, PDGF-R β is rapidly degraded by the proteasome after cells are detached from the extracellular matrix [26]. However, this does not occur with IR since receptor levels are not altered in suspended cells (Figs. 1 and 2). Moreover, we observed no modification in cell surface levels of IR, as shown in Fig. 3A. In these experiments, a monoclonal antibody specifically directed against the extracellular domain of human IR was added to attached or suspended cells. Results indicated that the amount of bound antibody, which directly reflects IR numbers at the cell surface, were similar in all conditions.

In other experiments, we observed that increasing the hormone concentration used to stimulate the receptor did not lead to higher levels of phosphorylation. As shown in Fig. 3B, receptor autophosphorylation was very low in non-adherent cells, even at the highest concentration of insulin. Thus, decreased receptor response could not be explained by a decreased sensitivity to insulin.

Finally, vanadate was used to test the possible involvement of tyrosine phosphatases. Previous studies have shown that a tyrosine phosphatase deactivates receptors for EGF and prolactin upon disruption of adhesion [22,27]. This phosphatase activity can be blocked by vanadate, which restores activation of these receptors in suspended cells [27]. As shown in Fig. 3C, vanadate increased insulin-induced IR autophosphorylation in both conditions, consistent with the known role of tyrosine phosphatases in down-regulating insulin signals. However, vanadate failed to restore IR autophosphorylation in suspended cells compared to attached cells, indicating that a vanadate-sensitive tyrosine phosphatase is unlikely to play a role (Fig. 3C).

Another intriguing possibility would be that tyrosine kinases potentiate IR and IRS-1 phosphorylation upon cell adhesion. Indeed, integrin engagement activates several tyrosine kinases such as FAK and Src, which are key players in adhesion-induced signals [2]. These kinases are inactive in suspended cells, which would explain the defect in IR and IRS-1 phosphorylation compared to attached cells. Moreover, FAK has been found to interact with IRS-1 [28].

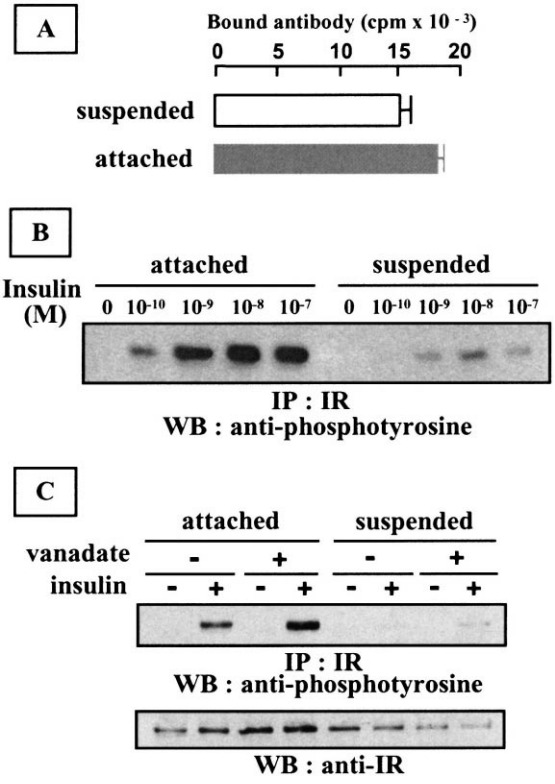

Fig. 3. A: Detection of cell surface receptors. Suspended or attached cells were incubated with monoclonal antibody E9 or with normal mouse serum, then with rabbit anti-mouse antibodies and finally with [^{125}I]protein A. After lysis in NaOH, cell-associated radioactivity was measured in a γ counter. B: Insulin dose–response. Cells were left attached or suspended for 2 h before stimulation with the indicated concentrations of insulin for 10 min. IR was immunoprecipitated and phosphorylation was analyzed by Western blotting using antibodies to phosphotyrosine. C: Effect of vanadate. Cells were treated with 100 mM $NaVO_4$ for 30 min before being suspended. Attached cells were treated similarly. Insulin was added or not for 10 min and cells were lysed. Immunoprecipitated IR was analyzed by Western blotting with antibodies to phosphotyrosine. Membranes were reprobed with antibodies to IR.

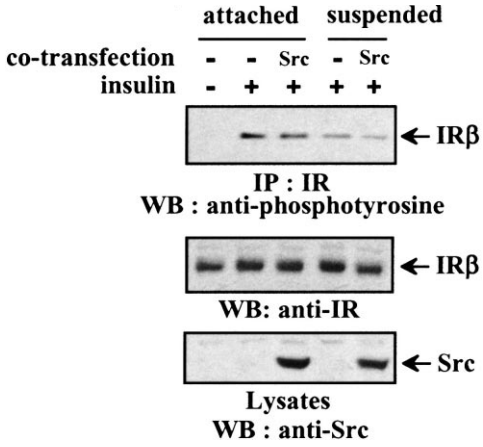

Fig. 4. CHO cells transiently expressing IR with or without Src, were left attached or suspended for 2 h before stimulation with insulin for 10 min. IR was immunoprecipitated from cleared lysates and samples were analyzed by Western blotting with antibodies to phosphotyrosine. Membranes were reprobed with antibodies to IR. A fraction of each cell lysate was analyzed by Western blotting using antibodies to Src as a control for expression.

Involvement of Src was tested by co-transfecting CHO cells with plasmids encoding IR and the constitutive active form of Src [29]. In these experiments, cells were left attached or suspended for 2 h before insulin stimulation for 10 min. As shown in Fig. 4, Src overexpression did not alter the lower insulin-induced phosphorylation of IR in suspended cells compared to attached cells. Similar experiments indicated that overexpression of FAK did not restore IR phosphorylation either (data not shown).

We then tested the combined effects of Src and FAK in similar experiments where suspended cells were compared to attached cells. All cells were transfected with IR either alone or with the indicated forms of FAK and Src. As shown in Fig. 5, insulin-stimulated IR phosphorylation was decreased by suspension (lane 4 compared to lane 2). Co-expression of both Src and wt-FAK restored insulin-induced IR phosphorylation in suspended cells (lane 5 compared to lane 4). Expression of kd-Src together with wt-FAK (lane 6), or Src with a mutant of FAK that does not interact with Src, i.e. FAK(Y397F) (lane 7) failed to restore IR phosphorylation in suspended cells. A fortiori expression of mutated kd-Src with FAK(Y397F) did not re-establish IR phosphorylation (lane 8). However, IR phosphorylation was slightly higher whenever the kinases were expressed compared to IR alone (lanes 6–8 versus lane 4) even when both Src and FAK were mutated, suggesting that defective mutants may still have some biological effects, perhaps acting as scaffolding proteins.

Taken together, our results suggest that forced expression of either Src or FAK in suspended cells is not sufficient to

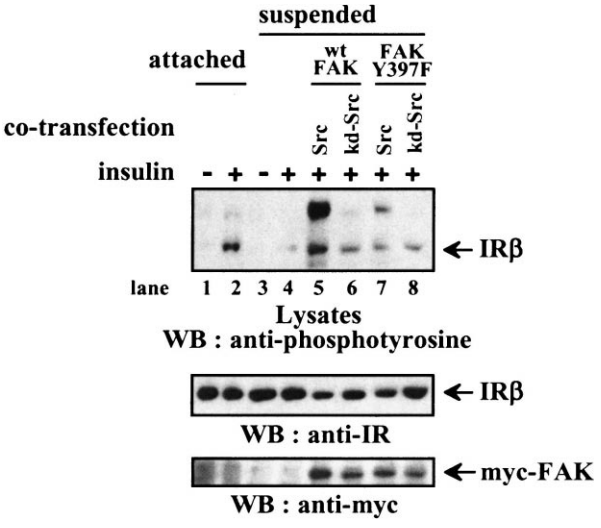

Fig. 5. CHO cells were transiently transfected to express IR (lanes 1–4), or IR together with the indicated forms of Src and FAK (lanes 5–8). Attached or suspended cells were stimulated or not with insulin for 10 min. Total cell lysates were analyzed by Western blotting with antibodies to phosphotyrosine. Membranes were reprobed with antibodies to IR, then with antibodies to myc.

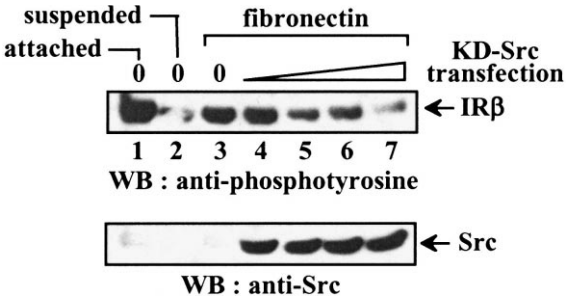

Fig. 6. CHO cells were transfected to express IR (1–3) or IR and increasing amounts of kd-Src (4–7). Specifically, cells were transfected with 0.25, 0.5, 1, 2 μg of plasmid encoding kd-Src (lanes 4–7 respectively). Cells were left attached, suspended for 2 h or suspended for 2 h and replated on fibronectin for 45 min. Insulin (0.1 μM) was added to all cells for 10 min before lysis. Whole cell lysates were analyzed by Western blot with antibodies to phosphotyrosine and to Src.

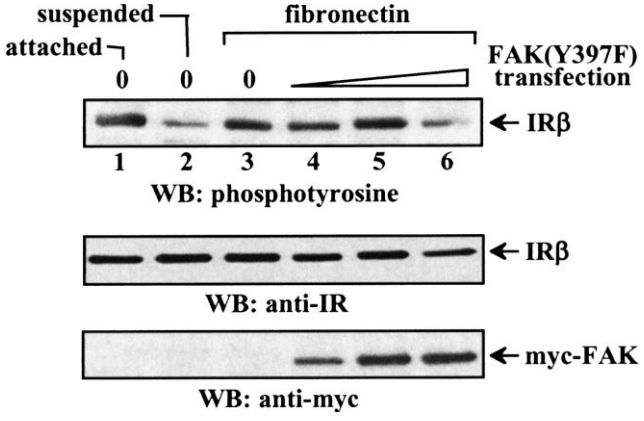

Fig. 7. CHO cells were transfected with the plasmid encoding IR alone (lanes 1–3) or with IR-encoding plasmid together with increasing amounts of a plasmid encoding FAK(Y397F) (0.5, 1, 2 μg of plasmid, lanes 4–6 respectively). Cells were left attached (lane 1), suspended for 2 h (lane 2) or suspended for 2 h and replated on fibronectin (lanes 3–6). All cells were stimulated with insulin (0.1 μM) for 10 min. Whole cell lysates were analyzed by Western blot with antibodies to phosphotyrosine. Membranes were reprobed first with antibodies to IR, then with antibodies to myc.

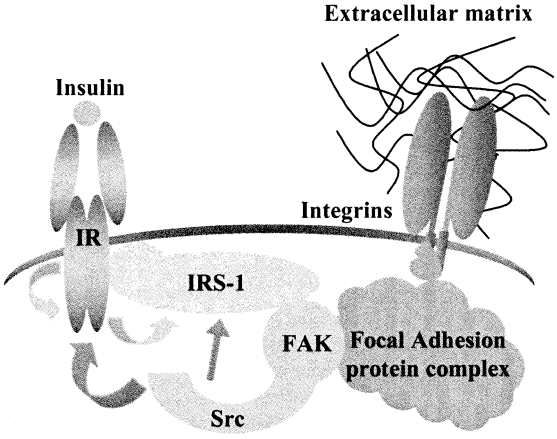

Fig. 8. Proposed mechanism of crosstalk between IR and integrin signaling pathways. Arrows indicate tyrosine phosphorylation.

promote full activation of IR, but expression of both kinases is required. Moreover, association of Src and FAK seems to be important since expression of FAK mutated at the Src-binding site (Y397) failed to restore the IR signal. Interestingly, we also observed a strong phosphorylation of a 185 kDa protein, which could correspond to IRS-1. Indeed, IRS-1 has been shown to be associated with FAK and strongly phosphorylated by the complex Src–FAK [28].

Involvement of Src and FAK is based on the observation that their co-expression mimics the effects of fibronectin. If this model is true, then inhibition of endogenous Src or FAK activities should abolish the effect of replating. To test this, CHO cells were transfected to express IR alone, or IR with increasing amounts of kd-Src. We compared attached cells and cells suspended for 2 h with cells replated on fibronectin for 45 min. In these experiments, all cells were stimulated with insulin for 10 min before lysis. As shown in Fig. 6, IR phosphorylation was lower in suspended cells (lane 2) compared to attached cells (lane 1). Replating restored IR phosphorylation to optimal levels (lane 3 versus lane 2). Expression of the kd-Src mutant abolished the effect of fibronectin on IR phosphorylation (lanes 4–7 compared to lane 3).

The effect of dominant-negative FAK(Y397F) expression was tested similarly. Cells were transfected to express IR alone (Fig. 7, lanes 1–3) or IR together with increasing amounts of FAK(Y397F) (lanes 4–6). Cells were left attached, suspended for 2 h, or replated on fibronectin for 45 min. All cells were stimulated with insulin for 10 min. As expected, IR phosphorylation was lower in suspended cells (lane 2) compared to attached cells (lane 1). Replating restored IR phosphorylation to optimal levels (lane 3). High-level expression of the FAK(Y397F) mutant abolished potentiation of IR phosphorylation by fibronectin (lane 6 versus lane 3).

As a whole, these experiments indicate that both FAK and Src are required for fibronectin-induced effects on IR and that association between FAK and Src is important.

Additional experiments were realized to detect direct interactions between proteins in the two-hybrid system. There was no association between IR and FAK, IR and Src, or Src and IRS-1, but interactions between FAK and IRS-1, IR and IRS-1, FAK and Src were detected (data not shown).

Therefore, we propose the following model as indicated in Fig. 8. Upon ligand binding, IR autophosphorylates and associates IRS-1 through its phosphotyrosine-binding domain [30]. Meanwhile, in attached cells, FAK and Src are activated and form a stable complex at focal contacts [2]. Constitutive association of FAK and IRS-1 [28] brings these complexes together, mediating the potentiation of cell adhesion on IR signaling. This may also explain why IR and IRS-1 localize to focal adhesions upon insulin binding.

4. Conclusion

These results, together with previously published observations, strongly support the notion that cell adhesion and insulin signals cooperate to promote full activation of hormone-induced signals. We propose that increased IR phosphorylation in attached cells is mediated by the FAK–Src complex.

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