Internalization of the insulin receptor (IR) is a highly regulated multi-step process whose underlying molecular basis is not fully understood. Here we undertook to study the role of extracellular matrix (ECM) proteins in the modulation of IR internalization. Employing Chinese hamster ovary cells that overexpress IR (CHO-T cells), our results indicate that IR internalization proceeds unaffected even when Tyr phosphorylation of IR substrates, such as IRS-1, is impaired (e.g. in CHO-T cells overexpressing IRS-1 whose pleckstrin-homology domain has been deleted or in CHO-T cells that overexpress the PH/PTB domain of IRS-1). In contrast, IR internalization is affected by the context of the ECM proteins to which the cells adhere. Hence, IR internalization was inhibited 40–60% in CHO-T cells adherent onto galectin-8 (an ECM protein and an integrin ligand of the galectin family) when compared with cells adherent onto fibronectin, collagen, or laminin. Cells adherent to galectin-8 manifested a unique cytoskeletal organization, which involved formation of cortical actin and generation of F-actin microspikes that contrasted with the prominent stress-fibers formed when cells adhered to fibronectin. To better establish a role for actin filament organization in IR endocytosis, this process was assayed in CHO-T cells (adherent onto fibronectin), whose actin filaments were disrupted upon treatment with latrunculin B. Latrunculin B did not affect insulin-induced Tyr phosphorylation of IR or its ability to phosphorylate its substrates; still, a 30–50% reduction in the rate of IR internalization was observed in cells treated with latrunculin B. Treatment of cells with nocodazole, which disrupts formation of microtubules, did not affect IR internalization. These results indicate that proper actin, but not microtubular, organization is a critical requirement for IR internalization and suggest that integrin-mediated signaling pathways emitted upon cell adhesion to different extracellular matrices and the altered cytoskeletal organizations generated thereof affect the itinerary of the insulin receptor.

Receptor tyrosine kinases (RTKs) rapidly internalize following ligand binding. Internalized receptors are then sorted to distinct subcellular pathways that lead either to degradation or recycling to the cell surface (1–3). Similarly, internalization of the insulin receptor is a multi-step process (4). Following surface redistribution (5, 6) the receptor-insulin complex progressively concentrates in clathrin-coated pits that represent the internalization gates (cf. Ref. 4, for review). The internalized receptor undergoes sorting, which determines whether it will be subjected to degradation in lysosomes or whether it will recycle back to the plane of the membrane (7). Stimulation of the intrinsic Tyr kinase activity of the insulin receptor (IR) following insulin binding is a prerequisite for surface redistribution of receptor-insulin complexes; accordingly, mutations of IR that abolish its kinase activity or mutations that replace amino acids that constitute putative “internalization signal” motifs inhibit IR internalization (8–13).

The need for IR kinase (IRK) activity to promote receptor internalization raises the question of whether IR substrates regulate this process. Several proteins were identified as potential IR substrates. Most studied are the Shc proteins, Gab-1, Cbl, and the family of insulin receptor substrates (IRS-1 to IRS-4) (reviewed in Refs. 14 and 15). IRS proteins share a highly conserved amino terminus, which contains a pleckstrin homology (PH) domain that serves to bind phosphoinositide lipids and localize the IRS proteins to cellular membranes. A phosphotyrosine-binding (PTB) domain flanks the PH domain and binds IRS proteins to the N-terminal motif within the juxtamembrane (JM) domain of the insulin receptor (15). The carboxyl terminus of IRS proteins contains several Tyr residues that, upon phosphorylation, act as docking sites for the downstream effectors that mediate the metabolic and growth-promoting functions of insulin (14, 15).

Agents and conditions that modulate cell adhesion also regulate insulin receptor internalization. For example, carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) serves as an IR substrate and up-regulates receptor-mediated insulin endocytosis (16). Still, the interplay between receptor endocytosis and cell adhesion is poorly understood. In the present study we undertook to address this question and investigated the effects of insulin receptor substrates and extracellular matrix proteins (ECMs) on IR endocytosis. Our results indicate that, although IR internalization proceeds unaffected even when Tyr phosphorylation of IRS protein is impaired, this process depends upon the context of ECM proteins to which the cells adhere. Hence, signaling pathways emitted...
upon cell adhesion to different matrices and the altered cytoskeletal organizations generated thereof affect the itinerary of the insulin receptor.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human insulin was a gift from Novo-Nordisk (Copenhagen, Denmark). Protein A-Sepharose CL-4B, poly-l-lysine, fibronectin, laminin, t-histidinol, purumycin, and TRITC-labeled phalloidin were purchased from Sigma. Monoclonal PY-20, annexin II, and polyclonal IR β-subunit antibodies were obtained from BD Transduction Laboratories. Polyclonal IR antibodies (α subunit) were purchased from Santa Cruz Biotechnology. LipofectAMINE was obtained from Invitrogen. The polyclonal IRS-1 antibody was prepared as described (17). Sulfo-NHS-LC-biotin and immobilized NeutrAvidin were purchased from Pierce. Recombinant galectin-8 was generated as described (18).

Cell Lines—CHO-T cells that overexpress IR (CHO-T cells) and CHO-T cells that co-express either wild-type mouse IRS-1 or IRS-1 whose PH domain has been deleted were generated as we described previously (19). To generate CHO-T cells that co-express the Myc-tagged PH-PTB domain of wild-type IRS-1, the sequence encoding Myc-PH-PTB was amplified by PCR using the pCDNAIII-Myc-tagged IRS-1 (20) as a template. The 5′ primer (5′-GCGGTCGACGAATTCCTCTCTCAGGACATTG-3′) was located at nucleotide −56. The 3′ primer (5′-TTGCGAATTCCTCTCTCAGGACATTGCTGAGGCGACCT-3′) was located at nucleotide 1095 of the mouse IRS-1 gene. The PCR product was ligated into a pGEM-T plasmid (Promega), according to the manufacturer’s instructions. The Myc-tagged PH-PTB construct was excised from pGEM-T with EcoRV and NotI, gel-purified, and ligated into pcDNAIII/Amp (Invitrogen) to generate pcDNAIII-Myc-PH-PTB. CHO-T cells were co-transfected with PH-PTB, purumycin and the pcDNAIII-Myc-PH-PTB plasmid (at a 1:10 ratio) using LipofectAMINE as described previously (20). Following transfection, cells were incubated in non-selective medium and, 48 h thereafter, purumycin (10 μg/ml) was added for selection of stable colonies. Purumycin-resistant clones that overexpressed Myc-PH-PTB were isolated and further propagated.

Immunoprecipitation—Cells were solubilized at 4 °C in buffer A (25 mM Tris-HCl, 2 mM sodium orthovanadate, 0.5 mM EGTA, 10 mM NaF, 10 mM sodium pyrophosphate, 80 mM β-glycerophosphate, 25 mM NaCl, 1% Triton X-100 and protease inhibitor mixture, 1:1000, pH 7.4). Cells were centrifuged at 12,000 g for 15 min at 4 °C, and the supernatants were collected. Aliquots (0.5–1.0 mg) were incubated for 2 h with polyclonal IRS-1 antibodies coupled to 20 μl of protein A-Sepharose beads. Immunocomplexes were washed twice with buffer A and twice with PBS. Immunocomplexes were resolved by means of SDS-PAGE and immunoblotted with the indicated antibodies.

Adhesion of CHO-T Cells to Different ECM Proteins—Six-centimeter dishes were precoated with fibronectin (10 μg/ml), gelatin (25 μg/ml), or collagen (10 μg/ml) or, 2 h at 25 °C. CHO-T cells, grown on tissue culture plates, were detached from the plates with 5 mM EDTA and seeded in serum-free medium on the plates coated with the different ECM proteins. Cells were allowed to adhere for 6 h at 37 °C and then subjected to further treatments as indicated.

Assays of Insulin Internalization—Cells were incubated with 125I-insulin (10−10 M) for 16 h at 4 °C in buffer B (serum-free F-12 medium, 50 mM Hepes, 1 mg/ml bovine serum albumin (radioimmunoassay grade), pH 7.5). The cells were washed 3× with PBS, buffer B was re-added, and the cells were transferred to 37 °C for the indicated times. Thereafter, cells were washed 2× with buffer B (titrated to pH 4.0). Following wash, the cells were solubilized in PBS containing 1% Triton X-100 and 1% SDS, and the amount of intracellular 125I was counted in a gamma counter.

Assays of Receptor Internalization—CHO-T cells were allowed to adhere to plates coated with different ECM proteins as described above. Thereafter, the cells were cooled to 4 °C and labeled for 45 min with sulfo-NHS-LC-biotin (0.5 mg/ml) in buffer D (0.1 mM CaCl2, 1 mM MgCl2, pH 7.4 in PBS). The labeled cells were washed 3× with buffer E (0.1 mM CaCl2, 1 mM MgCl2, 15 mM glycerol in PBS, pH 7.4) and further incubated with the indicated concentrations of insulin (in buffer B) at 15 °C for 3 h to allow for insulin binding. To initiate receptor internalization, the cells were transferred to 37 °C for 20 min and back to 4 °C to stop the internalization process. To remove any remaining cell surface receptor, the cells were subjected to trypsinization (30 min at 4 °C) with 1 mg/ml trypsin in buffer F (serum-free F12 medium, 20 mM Hepes, pH 7.5) followed by washing with buffer G (serum-free F12 medium containing 10 mg/ml BSA, pH 7.5). Cells extracts were prepared in buffer C (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40 and protease inhibitor, 1:1000, pH 7.4) and biotin labeled-proteins were precipitated with immobilized NeutrAvidin for 2 h at 4 °C while shaking. The beads were washed 2× with buffer C and 2× with PBS and then boiled in 50 μl of Laemmli sample buffer. Samples were resolved by means of 10% SDS-PAGE and immunoblotted with IR antibody (α subunit).

Immunofluorescence Analysis—CHO-T cells were allowed to adhere for 6 h at 37 °C to cover glasses precoated with the indicated ECM proteins. Cells were washed and fixed for 30 min with paraformaldehyde-Triton X-100 (2:0.5%). Following fixation, the cells were stained with TRITC-phalloidin, and actin organization was assessed under a fluorescence microscope.

RESULTS

Deletion of the PH Domain of IRS-1 Impairs Its Tyr Phosphorylation without Affecting IR Internalization—Internalization of the insulin receptor is a multi-step process requiring an active insulin receptor kinase. However, the involvement of IR substrates in this process remains unclear. To assess the role of IRS-1 in mediating IR internalization, we made use of IRS-1, whose PH domain has been deleted (IRS-1ΔPH). We have shown previously that such deletion impairs the ability of IRS-1 to properly juxtapose to the insulin receptor, making it a poor
IRS-1
higher than that of wild-type IRS-1. Tyr phosphorylation of IRS-1 mediated by the PH-PTB domain of IRS-1 (CHO-T PH-PTB) were incubated for 16 h in serum-free F-12 prior to stimulation with or without 100 ng/ml insulin for the indicated times at 37 °C. Cell extracts were prepared, and samples (100 μg) were resolved by means of 10% SDS-PAGE and immunoblotted (IB) with anti-Tyr(P) (pTyr) (section I) or anti-Myc (section II) antibodies. CHO-T and CHO-T PH-PTB were incubated with 125I-insulin (10−10 μM) for 16 h at 4 °C. 125I-insulin internalization was then assayed following transfer of the cells to 37 °C as described under “Experimental Procedures.” Results are mean ± S.D. of duplicates measurements.

![Figure 2](image)

**Fig. 2.** Effect of overexpression of the PH-PTB domain of IRS-1 on insulin-induced Tyr phosphorylation of IRS-1 and IR internalization. A, naive CHO-T cells or cells overexpressing a Myc-tagged PH-PTB domain of IRS-1 (CHO-T PH-PTB) were incubated for 16 h in serum-free F-12 prior to stimulation with or without 100 ng/ml insulin for the indicated times at 37 °C. Cell extracts were prepared, and samples (100 μg) were resolved by means of 10% SDS-PAGE and immunoblotted (IB) with anti-Tyr(P) (pTyr) (section I) or anti-Myc (section II) antibodies. CHO-T and CHO-T PH-PTB were incubated with 125I-insulin (10−10 μM) for 16 h at 4 °C. 125I-insulin internalization was then assayed following transfer of the cells to 37 °C as described under “Experimental Procedures.” Results are mean ± S.D. of duplicates measurements.

B, CHO-T and CHO-T PH(CHO-TD) when compared with CHO cells expressing the insulin receptor and wild-type IRS-1 (CHO-TS). This occurred despite the fact that the cellular content of IRS-1 was higher than that of wild-type IRS-1. Tyr phosphorylation of IRS-1 was lower by about 30% even when compared with the phosphorylation of the endogenous IRS-1 protein in CHO-T cells stably overexpressing just the insulin receptor. Still, as shown in Fig. 1B, insulin internalization, which represents IR internalization, preceded at comparable rates in CHO-TS and CHO-TD cells, indicating that insulin receptor internalization occurs independent of IRS-1 phosphorylation.

**Overexpression of the Isolated PH-PTB Domain of IRS-1 Inhibits Insulin-induced Tyr Phosphorylation of IRS-1 but Not IR Internalization**—IRS proteins contain at their N terminus a PH domain flanked by a PTB domain. To generate dominant negative inhibitors to IRS-1, a construct encoding the (Myc tagged) PH-PTB region was introduced into CHO-T cells. As shown in Fig. 2, insulin-induced Tyr phosphorylation of IRS was decreased by ~45% in cells stably overexpressing the PH-PTB domains, but IR internalization (assayed by insulin internalization) was not altered upon expression of this construct. Hence, the results presented in Figs. 1 and 2 support the notion that insulin receptor internalization occurs, by and large, independent of IRS-1 phosphorylation, although we cannot rule out the possibility that the residual Tyr-phosphorylated IRS-1 could affect IR internalization.

**Ligand-induced Insulin Receptor Internalization Is Modulated by Extracellular Matrix Proteins (ECMs)—**Agents and conditions that modulate cell adhesion were shown to affect insulin receptor signaling. For example, activation of α5β1 integrins upon adherence of CHO-T cells onto fibronectin markedly potentiates the Tyr phosphorylation of IR and IRS-1 (21). Conversely, when adherent cells are maintained in suspension, the extent of insulin-stimulated IR autophosphorylation is largely diminished (21). To determine whether ECM proteins could regulate insulin receptor internalization, we made use of ECM proteins having different characteristics. In general, two kinds of ECM proteins were utilized, i.e. those that interact with integrins through protein-protein interactions, represented by fibronectin, and those that interact with integrins through protein-sugar interactions, represented by the mammalian lectin galectin-8, a β-galactoside-binding protein (22, 23). As shown in Fig. 3, cells adherent onto fibronectin, laminin, and collagen showed a similar extent of IR internalization. However, IR internalization was inhibited ~65% in cells adherent onto galectin-8. These results indicate that extracellular matrix proteins modulate insulin receptor internalization. They further suggest that ligation of integrins via protein-protein interactions better supports IR endocytosis.

**Insulin Receptor Signaling and Basal Internalization Rates Are Regulated by ECM Proteins**—To determine whether the reduced internalization of cells adherent onto galectin-8 reflects impairments in IRK activity, insulin-stimulated Tyr phosphorylation of IR and IRS proteins was assayed using CHO-T cells grown on different matrices. As shown in Fig. 4 (top), maximal insulin-induced Tyr phosphorylation of IR, assayed at 100 nM insulin, was decreased in cells adherent to galectin-8 when compared with cells adherent to fibronectin, laminin or poly-L-lysine. The decrease in Tyr phosphorylation of IRS-1 was much less severe. To determine whether the reduced autophosphorylation of IR could account for its impaired internalization, we established conditions in which insulin-induced Tyr phosphorylation of IR and IRS-1 were comparable in cells grown on either matrix. As shown in Fig. 4
(bottom), this could be achieved when cells grown on fibronectin or galectin-8 were stimulated for 3 min with 3 and 100 nM insulin, respectively. Pretreatment of cells grown on galectin-8 but not on fibronectin with vanadate, an inhibitor of protein tyrosine phosphatases, also resulted in comparable levels of Tyr phosphorylation of IR and IRS-1 (not shown).

IR internalization was then re-examined under conditions in which insulin-induced Tyr phosphorylation of IR and IRS were comparable. As shown Fig. 5, IR internalization, assayed following its biotinylation, was still severely impaired in cells adherent to galectin-8 even though the insulin-induced Tyr phosphorylations of IR and IRS-1 were comparable. Defects in IR internalization were evident whether the internalization of IR itself (Fig. 5) or 125I-insulin (Fig. 6) was monitored. These results indicate that inhibition of IR internalization, which occurs upon cell adhesion to galectin-8, cannot be attributed solely to differences in insulin-induced Tyr phosphorylation of IR and IRS-1. Cell adhesion to galectin-8 did not inhibit only the ligand-stimulated internalization of IR but also impaired its basal internalization rate. As shown in Fig. 7, the basal (ligand-independent) rate of IR internalization was inhibited ~30% in cells adherent to galectin-8, indicating that cell adhesion to galectin-8 also affects the steady-state surface expression of insulin receptors even in the absence of a ligand.

**Fig. 4.** Effects of cell adhesion molecules on insulin-stimulated Tyr phosphorylation of IR and IRS-1. *A*, six-centimeter plates were precoated with fibronectin (10 μg/ml), galectin-8 (25 μg/ml), laminin (10 μg/ml), or poly-L-lysine (100 μg/ml). CHO-T cells, in serum-free medium, were seeded on the coated plates for 6 h at 37 °C. After additional incubation at 4 °C for 16 h, cells were stimulated with 100 nM insulin for 5 min. Cytosolic extracts were prepared and boiled in Laemmli sample buffer. Samples (100 μg) were resolved by means of 10% SDS-PAGE and immunoblotted (IB) with anti Tyr(P) (pTyr) or IR antibodies. *B*, six-centimeter plates were precoated with fibronectin or galectin-8, and CHO-T cells in serum-free medium were seeded on the precoated plates as described above. After incubation at 37 °C for 10 h, the cells were stimulated with the indicated insulin concentrations for 3 min. Extracts were prepared, and samples (100 μg) were resolved by means of 10% SDS-PAGE and immunoblotted (IB) with Tyr(P) (pTyr) antibodies.

**Fig. 5.** Effect of galectin-8 and fibronectin on IR internalization under conditions where insulin induces comparable level of Tyr phosphorylation of IR and IRS-1. Six-centimeter plates were precoated with fibronectin (10 μg/ml) or galectin-8 (25 μg/ml), and CHO-T cells in serum-free medium were seeded on the precoated plates. After incubation at 37 °C for 6 h, the cells were further incubated at 4 °C for 16 h and then surface labeled with sulfo-NHS-LC-biotin and IR internalization was assayed as described under “Experimental Procedures.” Results are mean ± S.D. of duplicates measurements.

**Fig. 6.** Effect of vanadate on insulin internalization in cells adherent to galectin-8. Six-centimeter plates were precoated with fibronectin (10 μg/ml) or galectin-8 (25 μg/ml), and CHO-T cells in serum-free medium were seeded on the precoated plates. The cells were incubated with 125I-insulin (10−12 M) for 16 h at 4 °C in the absence or presence of 50 μM sodium orthovanadate, as indicated. 125I-insulin internalization was assayed as described under “Experimental Procedures.” Results are mean ± S.D. of duplicates measurements.
Cytoskeletal Organization Affects IR Internalization—A key feature that distinguishes cells adherent to galectin-8 is their unique cytoskeletal organization. Prominent stress fibers that traverse the cell body are readily detected in cells adherent onto fibronectin but are less abundant in cells adherent to galectin-8 (Fig. 8). Instead, the formation of cortical actin (23) and the generation of an F-actin microspike (24) characterize adhesion to galectin-8. Second, although vinculin and paxillin are associated with large focal contacts in cells adherent to fibronectin, the number and size of vinculin-and paxillin-containing focal contacts is reduced in cells attached to galectin-8 (23). The differences in actin-microfilament organization are not accompanied by differences in microtubules organization, and a similar microtubular network develops when cell adhere to galectin-8 or fibronectin (24).

To establish a role for actin filaments in IR endocytosis, insulin-induced IR internalization was assayed in CHO-T cells (adherent onto fibronectin) whose actin filaments were disrupted upon treatment with latrunculin B, a drug that sequesters actin monomers. Latrunculin B did not affect insulin-induced Tyr-phosphorylation of IR or the ability of IRK to phosphorylate its substrates IRS-1 and annexin II (Fig. 9A). Accordingly, Latrunculin B did not impair activation of downstream effectors of IR such as mitogen-activated protein kinase (MAPK) (not shown). Still a 30–50% reduction in the rate of IR internalization was observed in cells treated with latrunculin B (Fig. 9B). These results indicate that proper actin organization is a critical requirement for IR internalization.

Next, we wished to determine whether disruption of microtubule filaments, which inhibit insulin-induced GLUT-4 translocation (25), affects IR internalization. CHO-T cells adherent onto fibronectin were treated with nocodazole, and IR internalization was measured. As shown in Fig. 9C, there was no prominent change in IR internalization in cells treated with nocodazole when compared with control. However, we could demonstrate a ~25% reduction in insulin-induced Tyr phosphorylation of IRS-1 in cells treated with nocodazole (Fig. 9D). These results indicate that microtubule integrity is not required for insulin-stimulated IR internalization despite its importance for insulin-induced GLUT-4 translocation.

**DISCUSSION**

The present study provides evidence that the cellular environment into which the cells adhere directly affects insulin signaling and insulin responsiveness. In particular, we show that the interaction of different ECM proteins with cell surface integrins results in a different cytoskeletal organization that affects the rate of endocytosis of the insulin receptor. The effects of the ECM proteins on receptor internalization are independent of their effects on IRK activity and its ability to phosphorylate downstream effectors such as the IRS proteins. Hence, insulin signaling and insulin responsiveness are dually regulated by the adhesive properties of the cells. On one hand, ligation of ECM proteins by cell surface receptors such as integrins generates signaling cascades that modulate the activity of the insulin receptor kinase, whereas, on the other hand, the alterations in cytoskeletal organization that take place upon cell adhesion dictate the rate and extent of internalization of the insulin receptor.

Several lines of evidence support this conclusion. First, we could show that insulin receptor internalization occurs independent of its ability to phosphorylate the IRS proteins that play a central role in insulin signaling (15, 26). Impairment of
IRS-1 signaling either by the introduction of IRS-1 whose PH domain has been deleted, making it a poor substrate for IRK, or overexpression of the isolated PH/PTB domain of IRS-1, which acts as a partial dominant negative inhibitor of endogenous IRS-1, did not affect the rate and extent of IR endocytosis. Although we cannot rule out the possibility that the residual Tyr-phosphorylated IRS-1 is necessary to promote IR internalization, our findings strongly suggest that downstream effectors of IRS proteins such as P13K have little role in promoting this process.

Although modulators of IRS-1 signaling do not affect IR endocytosis, the nature of the ECM proteins on which the cells adhere seems to have a profound effect on this process. ECM proteins exert a dual effect on the internalization rate of IR. First, ECM proteins such as galectin-8, which inhibit IR internalization, fail to support IRK activity at the same intensity as other ECM proteins, represented by fibronectin. Because a functional IRK is a prerequisite for proper IR internalization (8–13), partial inhibition of its activity when cells adhere to galectin-8 could account for the reduction in IR internalization. Second, and more important, the differences in cytoskeletal organization, which take place when cells adhere to different matrices, seem to play a critical role. Hence, whereas cells adherent to fibronectin develop an elaborated network of actin bundles associated with well developed focal contacts, cells attached to galectin-8 are characterized by the formation of cortical actin, elaborate F-actin microspikes, and a poorly organized network of actin microfilaments, which is associated with small focal contacts distributed mainly at the cell periphery (23, 24). Many of these adhesion sites contain lower amounts of vinculin or paxillin (23).

The differences in cytoskeletal organization observed when cells adhere either to galectin-8 or to fibronectin stem from fundamental differences in their structure, function, and mode of interaction with integrins. Whereas fibronectin is considered to be a conventional ECM protein that forms protein-protein interactions with integrins, galectin-8 (18, 22–24, 30), a member of the galectin family (31), is a mammalian lectin that interacts with sugar moieties of fibronectin in promoting cell adhesion and spreading, effects that involve interactions of galectin-8 with sugar-moieties of integrins (23). Accordingly, cell adhesion to galectin-8 is potentiated in the presence of Mn$^{2+}$, whereas adhesion is interrupted in the presence of soluble galectin-8, integrin $\beta_i$ inhibitory antibodies, EDTA, or thiodigalactoside, but not by RGD peptides (23). Hence, formation of protein-protein complexes upon binding of integrins to fibronectin versus the formation of protein-sugar complexes between galectin-8 and integrins offers a molecular aspect for the differences in cytoskeletal organization and signaling induced by these two matrices. Indeed, the less developed pattern of actin filaments and focal contacts associated with small focal contacts distributed mainly at the cell periphery (23, 24). Many of these adhesion sites contain lower amounts of vinculin or paxillin (23).
observed in cells seeded on galectin-8 resembles the appearance of cells whose integrins were aggregated in the absence of a ligand (e.g. RGD peptide) (32), suggesting that galectin-8 presumably fails to occupy the protein-ligand binding site of integrins, although it effectively induces aggregation of these receptors (23).

The differences in cytoskeletal organization of cells adherent to galectin-8 or fibronectin involve numerous proteins, each having the potential to modulate the internalization of the insulin receptor. We can rule out the effects of cytoskeletal organization on protein Tyr phosphatases (PTPs), because reduced internalization of IR in cells adherent to galectin-8 could not be potentiated in the presence of vanadate, a potent inhibitor of PTP activity. Our results do implicite actin as one of the key components involved. Actin has been proposed to play both a positive and negative role in the regulation of various vesicle trafficking events. In some systems, polymerized actin has been implicated as a barrier that undergoes depolymerization during vesicle trafficking. On the other hand, actin is also thought to play a positive role by forming scaffolds for transport vesicles to move along during vesicle sorting decisions (33, 34).

Actin has already been implicated as playing a role in insulin action and as a key component of insulin-induced GLUT4 translocation (35). Actin disassembly abolishes insulin-induced phosphorylation and activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) but does not prevent activation of PI3K and its downstream effectors (36). It has also been shown that the actin cytoskeleton is required for transferrin endocytosis in A431 cells (37). However, the importance of actin filaments for IR endocytosis was not studied previously. The present findings indicate that disruption of actin filaments by latrunculin B inhibits GLUT4 translocation (38, 39). Certain aspects of insulin-induced cytoskeletal remodeling related to the inhibition of IR endocytosis could be potentiated in the presence of vanadate, a potent inhibitor of PTP activity. Our results do implicate actin as one of the key components involved. Actin has been proposed to play both a positive and negative role in the regulation of various vesicle trafficking events. In some systems, polymerized actin has been implicated as a barrier that undergoes depolymerization during vesicle trafficking. On the other hand, actin is also thought to play a positive role by forming scaffolds for transport vesicles to move along during vesicle sorting decisions (33, 34).

The impact of insulin itself on cytoskeletal organization is well documented. Several studies have shown that insulin induces cell membrane ruffling, stress fiber breakdown, and microspike formation as well as dynamical cortical actin remodeling (38–40). Certain aspects of insulin-induced cytoskeletal remodeling, such as the induction of membrane ruffling, are impaired by inhibitors of PI3K, indicating that such elements might not be directly involved in insulin-induced IR endocytosis. In contrast, other features associated with actin organization that are insensitive to PI3K inhibitors, such as the formation of cortical actin, which are induced either upon ligation of integrins by galectin-8 or upon insulin stimulation, could be related to the inhibition of IR endocytosis. The organization of microtubules seems to be less critical for proper IR endocytosis, because impairment of microtubular organization upon the addition of nocodazole fails to affect IR endocytosis. In that respect, insulin-induced endocytosis of the insulin receptor differs from insulin-induced endocytosis of the glucose transporter GLUT4 that depends upon intact microtubular network and the presence of microtubule motors (25).

Regardless of the nature of the cytoskeletal elements involved in modulating IR endocytosis, our results clearly indicate that the constituents of the extracellular matrix onto which the cells adhere modulate both IRK activity and the signaling pathways emitted thereof, as well as the itinerary, surface levels, and cellular content of IR. This conclusion has somewhat broader implications, because it suggests that the agents and conditions that modulate the structural components constituting the extracellular matrix might induce insulin resistance and diabetes by altering the surface availability of the insulin receptor. Indeed, carcioembryonic antigen-related cell adhesion molecule 1 serves as an IR substrate and up-regulates receptor-mediated insulin endocytosis and degradation to enhance insulin clearance in liver (41). Recent studies have also reported the presence of high serum concentrations of soluble adhesion molecules (sICAM-1, sVCAM-1, and the mammalian lectin sE-selectin) in patients with type 2 diabetes (42, 43). Moreover, the levels of sE-selectin correlate positively with the degree of hyperglycemia (44). The elevated levels of sE-selectin are of particular relevance, because they suggest that mammalian lectins such as selectins or galectins might affect insulin action under physiological or pathological conditions by modulation of IR endocytosis. Further studies are required to put this intriguing hypothesis to the test.

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