Cross-talk between Insulin Receptor and Integrin α5β1 Signaling Pathways*

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The ligation and clustering of cell surface αβ heterodimeric integrins enhances cell adhesion and initiates signaling pathways that regulate such processes as cell spreading, migration, differentiation, proliferation and apoptosis. Here we show that insulin treatment of Chinese hamster ovary cells expressing insulin receptors (CHO-T) markedly promotes cell adhesion onto a fibronectin matrix, but not onto bovine serum albumin or poly-lysine. Incubation of cells with a GRGDSP peptide that specifically binds integrins (but not the non-specific GRADSP peptide) abolishes this insulin effect, as does the potent phosphoinositide 3-kinase (PI 3-kinase) inhibitor wortmannin. Moreover, a specific blocking monoclonal anti-α5β1 integrin antibody, PB-1, blocks insulin-stimulated cell adhesion onto fibronectin. Conversely, activating α5β1 integrons on CHO-T cells by adherence onto fibronectin markedly potentiates the action of insulin to enhance insulin receptor and insulin receptor substrate (IRS)-1 tyrosine phosphorylation. Activation of α5β1 integrin also markedly potentiates the recruitment of p85-associated PI 3-kinase activity to IRS-1 in response to submaximal levels of insulin in CHO-T cells. These data indicate that insulin potently activates integrin α5β1 mediated CHO-T cell adhesion, while integrin α5β1 signaling in turn enhances insulin receptor kinase activity and formation of complexes containing IRS-1 and PI 3-kinase. These findings raise the hypothesis that insulin receptor and α5β1 integrin signaling act synergistically to enhance cell adhesion.

The tyrosine kinase activity of the cell surface insulin receptor is required to mediate its many biological actions. Insulin receptor activation promotes the rapid autophosphorylation of its β subunit, as well as tyrosine phosphorylation of proteins involved in insulin signaling, such as insulin receptor substrate (IRS1) and Shc proteins (1–4). Insulin-mediated phosphorylation of these proteins is thought to provide tyrosine phosphate docking sites for the recruitment of signaling proteins containing Src homology 2 domains (SH2) (1, 5, 6). One SH2 domain-containing family of proteins which associates with IRS proteins in response to insulin are isoforms of the p85 regulatory subunit of the p110-type PI 3-kinases (1–4, 7). PI 3-kinase activity in such signaling complexes appears to be required for insulin action on many cellular processes, including glucose transport (8, 9), glycogen synthesis (9, 10), stress fiber breakdown (11), and membrane ruffling (12). Thus, inhibition of PI 3-kinase activity by wortmannin or disruption of PI 3-kinase recruitment to IRS proteins by dominant inhibitory constructs of p85 subunits ablate the actions of insulin on these processes (11–13). Our understanding of the downstream elements that mediate the action of the 3'-phosphoinositide products of the PI 3-kinases is incomplete, but appear to include protein kinases such as PDK1 and Akt/protein kinase B (PKB) (14), a family of proteins containing Sec7 homology domains (15), and the zinc finger containing protein EEA1 (16).

Insulin action also promotes dephosphorylation of tyrosine phosphates on such proteins as focal adhesion kinase (FAK) and paxillin (17–19), thought to be involved in cell regulation by integrins. Integrins are αβ heterodimeric transmembrane receptors that mediate interactions between the cell surface and the extracellular matrix and also initiate signaling events (20–22), including tyrosine phosphorylation of FAK and paxillin (23–25), cytoskeletal reorganization (20–22, 25), activation of mitogen-activated protein kinase cascades (26), and regulation of gene expression (22, 27). These biological actions of integrins can overlap with those of growth factors, and there is evidence that signaling pathways initiated by integrins synergize functionally with those triggered by growth factors (22, 28). Thus, cell adhesion has been shown to greatly enhance autophosphorylation of epidermal growth factor and platelet-derived growth factor (PDGF) receptors (25, 29). This in turn potentiates the action of these growth factors in activating mitogen-activated protein kinases, PI 3-kinase, and the downstream protein kinases PKD1 and Akt/PKB (29, 30). The mitogenic effects of insulin have also been shown to be enhanced by interactions of extracellular vitronectin with cell surface αvβ3 integrins, which associate with insulin receptor and IRS-1 in response to insulin (31, 32). However, the mechanism of such synergism in the actions of insulin and integrins is still unclear.

Conversely, it also has been shown that growth factor receptors, such as KIT (33) and PDGF (34) receptors, stimulate integrin-mediated cell adhesion onto fibronectin through a PI 3-kinase-dependent pathway. Thus, in the present studies, we addressed the questions whether insulin may directly activate integrin-mediated cell adhesion and if integrin activation modulates insulin signaling. Here, we show that insulin markedly promotes CHO-T cell adhesion onto a fibronectin matrix by a mechanism that is mediated by α5β1 integrin. Activation of this integrin in turn enhances insulin receptor and IRS-1 tyrosine phosphorylation, as well as recruitment of PI 3-kinase activity to IRS-1 in response to insulin. This cross-talk between
insulin and integrin receptor pathways is likely to play an important role in biological processes regulated by insulin and which depend on integrin engagement.

EXPERIMENTAL PROCEDURES

Materials—Anti-phosphotyrosine (anti-Tyr(p)) mouse monoclonal 4G10, anti-p85 polyclonal, and laminin were purchased from Upstate Biotechnology. Rabbit polyclonal anti-IRS-1 immunoglobulin used for immunoprecipitation was prepared as described previously (35). Anti-insulin receptor monoclonal (CT-1) and polyclonal antibodies were from mouse ascites and from Santa Cruz Biotechnology, respectively. Plasma insulin receptor monoclonal (CT-1) and polyclonal antibodies were from Avanti Polar Lipids.

Cell Culture—CHO-T cells were maintained in Ham’s F-12 medium, 10% fetal bovine serum, and 50 μg/ml streptomycin/penicillin and grown to confluence before use.

Cell Adhesion Assay—To assay cell adhesion on different matrices, cell culture dishes (12-well plate) were coated with fibronectin (0.5 μg/ml), laminin (10 μg/ml), and poly-lysine (0.5 μg/ml) at 4 °C overnight and blocked with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h, prior to plating the cells. Confluent CHO-T cells were serum starved for 12 h in F-12 serum-free medium containing 0.5% BSA and labeled with 35S-labeled protein labeling mix.

RESULTS AND DISCUSSION

Insulin Stimulates Cell Adhesion Mediated by Integrins—To determine whether insulin signaling can activate integrin-mediated cell adhesion, CHO-T cells were detached from culture dishes, treated with 100 nM insulin for 10 min at 37 °C, and plated onto dishes coated with bovine serum albumin, poly-lysine, or fibronectin. As shown in Fig. 1A, adhesion of CHO-T
addition of 0.5 mM GRGDSP peptide to assay media abolished fibronectin binding to integrins (36), on insulin-stimulated CHO-T cell adhesion. Thus, the effect of a specific GRGDSP sequence peptide, which binds to PDGF, and KIT (33, 34). We next investigated whether the action of insulin to increase cell adhesion to fibronectin was mediated by integrins. Thus, the effect of a specific GRGDSP sequence peptide, which binds to integrins (36), on insulin-stimulated CHO-T cell adhesion to fibronectin was examined. As seen in Fig. 1B, the addition of 0.5 mM GRGDSP peptide to assay media abolished basal and insulin-stimulated cell adhesion to fibronectin-coated dishes, whereas an inactive GRADSP peptide at the same concentration did not. Thus, these data suggest that insulin action modulates integrin-mediated CHO-T cell adhesion.

It has previously been established that CHO cells adhere to fibronectin by $\alpha_5 \beta_1$ integrin receptors (37). We thus examined the effect of a monoclonal anti-$\alpha_5 \beta_1$ blocking antibody, PB-1 (37–39), on CHO-T cell adhesion to fibronectin in the presence and absence of insulin. As seen in Fig. 2 (right), addition of PB-1 to assay media markedly inhibited the adhesion of CHO-T cells to a fibronectin matrix under both basal and insulin-stimulated conditions. In contrast, no inhibition of CHO-T cell adhesion onto laminin could be detected by adding PB-1 to the assay media (Fig. 2, left). These data are consistent with a previous report showing PB-1 inhibition of CHO cell adhesion onto fibronectin, but not onto laminin (37). Taken together, the data in Figs. 1 and 2 demonstrate a marked effect of insulin to enhance CHO-T cell adhesion onto fibronectin through $\alpha_5 \beta_1$ integrin.

Insulin-stimulated Cell Adhesion Is Inhibited by Wortmannin—To determine whether insulin-stimulated and integrin-mediated cell adhesion is dependent on activated PI-3 kinase, CHO-T cells were detached from the culture dishes, treated with or without 50 nM of the PI-3 kinase inhibitor wortmannin for 15 min prior to incubation with or without insulin for 10 min, and then replated onto fibronectin or laminin-coated plates. As depicted in Fig. 3, wortmannin treatment of the cells markedly inhibited insulin-stimulated, fibronectin-dependent CHO-T cell adhesion. Similar results were observed when cells were plated onto laminin-coated dishes (Fig. 3). These results suggest that insulin-stimulated cell adhesion in this system is dependent, at least in part, on PI-3 kinase activity. Therefore, we performed experiments in which serum-starved CHO-T cells were detached, held in suspension for 30 min at 37 °C and then stimulated (+) or not (−) with 1 μM insulin for 10 min, replated on fibronectin-coated (2.5 μg/ml) dishes or held in suspension for 20 min. Suspension and adherent cells were lysed, total proteins (25 μg) were resolved by SDS-PAGE on 7.0% gels, electrophoretically transferred to nitrocellulose, blocked, and subsequently incubated with anti-p-Tyr antibody, as described under “Experimental Procedures.” A, anti-p-Tyr immunoblotting of total proteins from cells in suspension or adhered onto fibronectin. Arrowheads indicate bands corresponding to insulin receptor (IR, 95 kDa), IRS-1 (175 kDa), and focal adhesion kinase (FAK, 125 kDa). B, the data shown in panel A for insulin receptor, IRS-1 and FAK tyrosine phosphorylation from suspension cells (−) or adhered on fibronectin (FN, +) were quantified using a scanning densitometer.

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Fig. 3. Wortmannin blocks insulin-stimulated CHO-T cell adhesion onto fibronectin and laminin. $^{35}$S-labeled and serum-free CHO-T cells were detached, treated (+) or not (−) with 50 nM wortmannin (WT) for 15 min at 37 °C and stimulated (+) or not (−) with 100 nM insulin for 10 min at 37 °C. Cells were than replated on fibronectin or laminin-coated dishes and allowed to adhere as described in the legend of Fig. 2. Data presented are average values from five independent experiments ± S.E.

Insulin Action on Integrin-mediated Cell Adhesion

Insulin-stimulated CHO-T Cell Adhesion Increases Insulin Receptor and IRS-1 Tyrosine Phosphorylation in Response to Insulin—Attachment of cells to fibronectin results in increased tyrosine phosphorylation of FAK ($M_p$ = 125,000) and paxillin ($M_p$ = 68,000–75,000) proteins that are associated with focal adhesion complexes (23–25). Also, it has been shown that integrin-mediated cell anchorage promotes increases in tyrosine phosphorylation of epidermal growth factor and PDGF receptors (28, 29). To examine whether the $\alpha_5 \beta_1$ integrin engagement modulates insulin receptor and IRS-1 tyrosine phosphorylation in response to insulin, we performed experiments in which serum-starved CHO-T cells were detached from the culture dish, held in suspension for 30 min at 37 °C, treated with or without 100 nM insulin for 10 min, and then either kept...
in suspension or plated onto fibronectin-coated plates for 20 min. As shown in Fig. 4A and B, CHO-T cell attachment to fibronectin markedly increased tyrosine phosphorylation of the 125-kDa protein that corresponds to FAK as well as 68-kDa proteins. Surprisingly, cell attachment to fibronectin also markedly increased insulin receptor and IRS-1 tyrosine phosphorylation under basal conditions and in response to insulin by 2-3-fold (Fig. 4, A and B). Fig. 4 also shows that insulin treatment of these cells caused dephosphorylation of FAK, consistent with previous reports that insulin induces FAK tyrosine dephosphorylation (17–19).

To examine whether αβ1 integrin engagement potentiates signaling by insulin at submaximal doses, the effect of different concentrations of insulin on insulin receptor and IRS-1 tyrosine phosphorylation was assessed in cells held in suspension or allowed to attach onto fibronectin (Fig. 5). CHO-T cell attachment onto a fibronectin matrix markedly potentiated the ability of insulin to enhance tyrosine phosphorylation of the insulin receptor at all concentrations employed. Similar to this effect observed on insulin receptor phosphorylation, IRS-1 tyrosine phosphorylation in response to all doses of insulin tested was enhanced by CHO-T cell attachment onto fibronectin (Fig. 6A).

Experiments were also conducted to determine the PI 3-kinase activity associated with IRS-1 after treatment of adherent and nonadherent CHO-T cells with or without various concentrations of insulin. Fibronectin engagement of αβ1 integrin receptor was associated with a significant, severalfold increase in basal CHO-T cell PI 3-kinase activity detected in IRS-1.
immunoprecipitates (Fig. 6, B and C). Elevated PI 3-kinase activity associated with IRS-1 was also observed when submaximal or maximal doses of insulin were incubated with adherent cells compared with nonadherent cells. These data are consistent with the hypothesis that activation of αβ$_2$ integrin through cell adhesion onto fibronectin causes enhanced recruitment of p85/p110-type PI 3-kinases to IRS-1, resulting in enhanced catalytic activity of these enzymes.

The findings presented here demonstrating the potentiation of insulin receptor modulation of IRS-1 by αβ$_2$ integrin engagement suggests the concomitant enhancement of signaling events downstream of IRS-1. One function proposed to be regulated by IRS-1/PI 3-kinase signaling complexes is cell proliferation (41), and recent evidence indeed indicates that αβ$_3$ integrin-mediated cell adhesion enhances this insulin action (31). As PI 3-kinase activation appears to also be required for the metabolic actions of insulin (8–10, 13), it will be important in future studies to determine whether integrin ligation may influence such insulin effects. The present work shows for the first time that insulin stimulates cell adhesion, and this effect is dependent upon PI 3-kinase activity (Fig. 3). Cell adhesion is also enhanced upon ligation and clustering of integrins on the cell surface. Thus, our findings are consistent with the hypothesis that potentiation of the insulin signaling pathway through IRS-1/PI 3-kinase by integrins reflects an indirect mechanism to further enhance adhesion over that caused directly by the integrins. Testing this hypothesis will require determining whether IRS-1/PI 3-kinase complexes formed in response to integrin activation actually mediate increased cell adhesion. Such studies will also be important in ultimately probing the full physiological implications of insulin regulation of cell adhesion.

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