THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 1, Issue of January 7, pp. 100–104, 1994
Printed in U.S.A.

Functional Domains of the Insulin Receptor Responsible for Chemotactic Signaling*

(Received for publication, July 16, 1993, and in revised form, September 3, 1993)

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The insulin receptor mediates a variety of cellular responses to insulin, including glucose transport, endocytosis, and cell proliferation. The role of the insulin receptor in mediating cellular motility has not, however, been extensively investigated. In this report, we demonstrate that chinese hamster ovary (CHO) cells that normally have low concentrations of insulin receptor display chemotaxis toward insulin after overexpression of the wild type human insulin receptor. Chemotaxis toward insulin proceeded through a pertussis toxin-sensitive pathway and required both tyrosine kinase activity and tyrosine autophosphorylation of the regulatory region of the β-subunit. In contrast, the autophosphorylation sites in the carboxyl terminus of the receptor were not required for chemotactic activity. A mutation in the juxtamembrane region, which disabled tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1), also prevented the chemotactic response, suggesting a possible role for IRS-1 in chemotactic signaling. In the absence of insulin receptor, however, the presence of excess transfected IRS-1 was not sufficient to mediate chemotaxis toward insulin. These results demonstrate that the intact insulin receptor can stimulate a chemotactic signaling pathway and that this initial pathway more closely correlates with that for insulin-stimulated cell proliferation than for insulin-stimulated receptor endocytosis.

Receptors that mediate chemotaxis fall into two classes: the G-protein-linked seven membrane-spanning domain receptors, such as the anaphylatoxin C5a and interleukin-8 receptors (1–3), and the tyrosine kinase receptors that bind factors such as the platelet-derived growth factor, the hepatocyte growth factor (also known as scatter factor), and the insulin-like growth factor-1 (4–6). Although initially studied for their ability to stimulate cell proliferation, these "growth factor" tyrosine kinase receptors also stimulate directional cell motility (chemotaxis) in response to a gradient of the appropriate ligand. Although the precise mechanisms underlying chemotactic signal transduction by the tyrosine kinase receptors are not completely understood, they are generally thought to involve receptor autophosphorylation followed by subsequent phosphorylation of SH2 and SH3 domain-containing signaling molecules whose activity results in a complex series of phosphorylation events that lead to directional cell translocation by the stimulated cell.

Although certain insulin-like molecules are known to stimulate chemotaxis, the role of the insulin receptor in stimulating cell motility has not been extensively explored. This receptor has been more commonly studied with regard to its role in stimulating glucose transport, endocytosis, and cell proliferation in responsive cells. In general, insulin activity is considered to be initiated by a series of autophosphorylation reactions which take place on tyrosine residues in distinct regions of the receptor molecule; a regulatory region consisting of 3 tyrosines at positions 1146, 1150, and 1151 and a carboxyl-terminal region with 2 additional tyrosines at positions 1316 and 1322 (7). The insulin receptor differs, however, from other well characterized tyrosine kinases such as the epidermal growth factor receptor and platelet-derived growth factor receptor. These other receptors bind directly and phosphorylate a number of SH2 domain-containing signaling molecules such as phospholipase C-γ, phosphatidylinositol-3′ kinase, the ras-GTP-associated protein (ras-GAP),[1] the growth factor receptor-binding protein (GRB-2), and a variety of intracellular protein phosphatases. In contrast, the insulin receptor acts, in part, through one or more intermediate signaling molecules which are bound and activated by the insulin receptor and subsequently phosphorylate and activate downstream signaling molecules (8). One such molecule, termed the insulin receptor substrate-1 (IRS-1), is now known to bind and activate both phosphatidylinositol-3′ kinase (9) and GRB-2 (10) after stimulation by the insulin receptor and may mediate activation of additional downstream signaling molecules. Mutation of a single tyrosine at position 960 in the juxtamembrane region of the insulin receptor prevents activation of IRS-1 and activation of subsequent signaling pathways (11).

Although insulin receptor activation can result in the stimulation of a variety of cellular responses, the particular receptor domains necessary for each of these responses are not known in detail. We have now employed a series of insulin receptor and insulin receptor substrate mutations to investigate the role of the insulin receptor in mediating insulin-directed chemotaxis. We employed mutations that affect the receptor's ability to undergo phosphorylation and to initiate distinct parts of the signaling cascade. Our results clearly show that the insulin receptor is capable of initiating directional cell motility in response to a gradient of insulin and that this chemotactic response depends on phosphorylation of tyrosines in the receptor regulatory region and on the integrity of sites that allow binding of the receptor to IRS-1. Insulin-stimulated chemotaxis is, in addition, blocked by cellular pretreatment with pertussis toxin, implying the role of a G-protein in insulin-stimulated chemotaxis.

* This work was supported by United States National Institutes of Health Grants DK 38712 and DK 43808. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ras-GAP, ras-GTP-associated protein; GRB-2, growth factor receptor binding protein-2; IRS-1, insulin receptor substrate-1; CHO, Chinese hamster ovary; HIRC, CHO cells expressing human insulin receptor.

TABLE I
Summary of CHO cell lines

Cell type	Description
CHO	Approximately 30,000 endogenous insulin receptors/cell; express low levels of IRS-1
HIRC	CHO cells expressing 10_6 insulin receptors/cell
IR_{A1018}	HIRC cells expressing ATP binding-deficient insulin receptor
$IR_{\delta CT}$	HIRC cells expressing insulin receptor in which the C-terminal 43 amino acids are deleted, including phosphorylation sites Y1316 and Y1322
IR_{F1146}	HIRC cells expressing single regulatory region tyrosine autophosphorylation mutant insulin receptor
$IR_{F1146,1151}$	HIRC cells expressing double regulatory region tyrosine autophosphorylation mutant insulin receptor
$IR_{F1146,1150,1151}$	HIRC cells expressing triple regulatory region tyrosine autophosphorylation mutant insulin receptors
IR_{A960}	HIRC cells expressing juxtamembrane IRS-1 binding tyrosine autophosphorylation mutant insulin receptors
IRS-1	CHO cells expressing a 20-fold increase of IRS-1
IR/IRS-1	HIRC cells expressing a 20-fold increase of IRS-1

MATERIALS AND METHODS

Insulin Receptor Mutants—The preparation and transfection into CHO cells of the insulin receptor mutants used in this study have been described previously, and a summary of cell lines used is provided in Table I (12–14). Briefly, vectors containing mutant insulin receptors were generated using oligonucleotide-directed mutagenesis. These expression vectors were then transfected into CHO cells by calcium phosphate precipitation and cell lines were selected using fluorescence-activated cell sorting. Expression levels were determined by insulin binding and/or metabolic labeling, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and autoradiography.

Chemotactic Boyden Chamber Assay—25 × 80-mm 8-μm polyvinylpyrrolidine-free filters (Nucleopore Corp., Pleasanton, CA) were coated for 15 min with fibronectin (Sigma) diluted in phosphate-buffered saline (without calcium or magnesium) to a final concentration of 13.3 μg/ml. A dry coated filter was placed on a 48-blind well chamber (Neuroprobe, Cabin John, MD) containing the indicated amounts of insulin diluted in Ham's F-12 medium (Life Technologies, Inc.). After trypsinization and dilution in Ham's F-12 serum-free media, 15,000 cells in 50 μl of F-12 were added to the top wells. The chamber was then incubated at 37 °C, 5% CO_2 for 4 h. The side of the filter onto which the cells were loaded was then scraped free of cells. The migrating cells were then fixed in formalin for 45 min. The filter was washed in phosphate-buffered saline and stained overnight in Gill's triple strength hematoxylin (Polysciences, Warrington, PA), washed again in phosphate-buffered saline, and mounted in glycerol. All cells within an area representing a well were counted visually. In some experiments, the cells were pretreated for 2 h with the indicated amounts of pertussis toxin and then assayed in the Boyden chamber as described above.

RESULTS

The Chemotactic Response to Insulin Is Mediated by the Insulin Receptor—The ability to migrate toward an insulin gradient was assayed in a modified Boyden multiwell chemotaxis chamber. Fig. 1 shows that control cells (CHO) did not move in response to insulin, whereas cells transfected with the wild type insulin receptor (HIRC) migrated in a dose-dependent manner with maximum stimulation at 5 nM. Receptor expression had no effect on unstimulated cell motility as both the control and insulin receptor transfected cell lines showed a similar low level of background motility. Fig. 2 shows that insulin-mediated chemotaxis is pertussis toxin-sensitive in a dose-dependent manner, with maximum inhibition at 1 μg/ml.

Chemokinesis (stimulated random cell motility) was not seen when equal concentrations of insulin were placed on both sides of the filter (Fig. 3, *diagonal*). In contrast, an insulin gradient created by increasing the concentration of insulin in the bottom chamber caused a much greater stimulation of chemotaxis (directed cell movement) (Fig. 3, *first column*). The inability of insulin to stimulate random motility was confirmed using a quantitative phagokinetic track assay (15), which measures the ability of cells to move across a particle-coated surface in response to a nondirectional stimulus (data not shown). Thus, the principal motile response to insulin is chemotactic and results in directed rather than random motility.

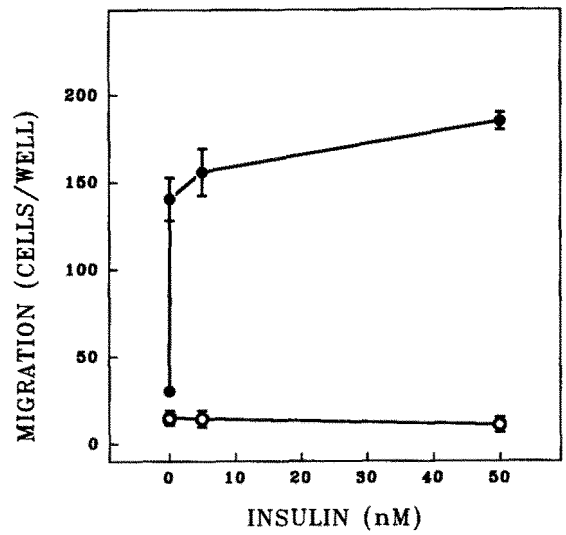

FIG. 1. **Migration of CHO cells toward insulin.** Migration of CHO cells (○; transfected with drug resistance gene only) and HIRC cells (●; transfected with wild type human insulin receptor gene). Data points are the average value of three or four replicate wells, and *graphs* depict results of a single representative experiment. *Error bars* represent standard error of the mean.

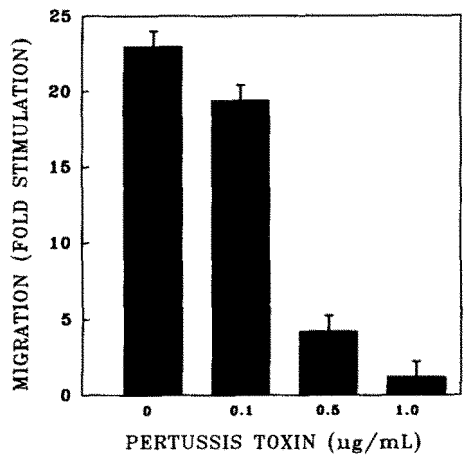

FIG. 2. **Insulin-stimulated chemotaxis of HIRC cells is pertussis toxin-sensitive.** Inhibition of insulin-mediated chemotaxis by pertussis toxin. Cells were pretreated for 2 h with the indicated amount of pertussis toxin. *Bars* indicate S.E. (n = 4).

Insulin Receptor Kinase Activity, Juxtamembrane Autophosphorylation, and Normal Kinase Activation Are Required for Insulin Receptor-mediated Chemotaxis—A kinase-deficient insulin receptor (IR_{A1018}), which displays normal insulin binding, did not mediate insulin-stimulated chemotaxis (Fig. 4*A*). In contrast, deletion of 43 amino acids from the COOH terminus of the β-subunit, including two autophosphorylation sites (Tyr-1316 and Tyr-1322), had no effect on insulin-stimulated chemotaxis (Fig. 4*B*). Thus, although kinase activity is required for chemotaxis, autophosphorylation sites in the COOH-termi-

nal region of the insulin receptor are not involved in mediating chemotaxis toward insulin.

The intracellular juxtamembrane region of the β-subunit is highly phosphorylated on serine residues before and after insulin stimulation and contains at least one site of tyrosine phosphorylation (14). This region is especially important for insulin receptor endocytosis and mitogenesis signaling (12, 13). Moreover, the integrity of the juxtamembrane region, especially Tyr-960, is required for phosphorylation of IRS-1 (11). CHO cell lines in which Tyr-960 has been substituted to phenylalanine (IR_{A960}) are deficient in insulin-stimulated IRS-1 tyrosine phosphorylation (16). In our experiments, the IR_{A960} cell line failed to migrate toward insulin in the Boyden chamber assay (Fig. 4*C*). This result demonstrates a role for the juxtamembrane region and suggests that phosphorylation of IRS-1 or other substrates may be necessary for insulin-stimulated chemotaxis.

[INSULIN] LOWER WELL (nM) \ [INSULIN] UPPER WELL (nM)	0	0.5	1.0	5.0
0	9	12	13	13
0.5	19	68	17	30
1.0	77	87	35	22
5.0	130	123	97	55

FIG. 3. **Checkerboard analysis of HIRC cell migration toward insulin.** For this assay, the first column has an increasing gradient of chemoattractant, and the *diagonal* has equal amounts in both the top and the bottom wells and therefore no gradient of chemoattractant. Therefore the gradient-dependent chemotactic response of cells is demonstrated in the *first column*, whereas the gradient-independent chemokinetic response is shown in the wells along the *diagonal* of the checkerboard diagram. S.E. less than 10% of the given values (n = 2).

There are three tyrosine autophosphorylation sites in the regulatory region of the insulin receptor β-subunit. Autophosphorylation of these sites following insulin stimulation increases the tyrosine kinase activity of the insulin receptor (17, 20). The triple-Phe mutant ($IR_{F1146,1150,1151}$) did not mediate chemotaxis (Fig. 5*A*). The double-Phe mutant ($IR_{F1146,1151}$) partially mediated chemotaxis, whereas the single-Phe mutant (IR_{F1146}) was only slightly impaired (Fig. 5, *B* and *C*, and Fig. 6). These experiments demonstrate that the chemotactic response is graded depending on the number of regulatory tyrosine residues available. This result is similar to that previously observed for insulin-stimulated DNA, glycogen synthesis, and IRS-1 phosphorylation; however, none of these mutant receptors affect insulin-stimulated endocytosis, suggesting that receptor endocytosis and chemotaxis are regulated by distinct mechanisms (19, 20).

The Role of IRS-1 in the Insulin Receptor-mediated Chemotactic Response—IRS-1 is a cytosolic phosphoprotein which is tyrosine phosphorylated on multiple sites upon insulin stimulation (8, 16). IRS-1 appears to act as a docking protein for signaling molecules with src-homology 2 (SH2) domains such as PI-3 kinase (16) and GRB-2 (10). Overexpression of IRS-1 alone is insufficient to induce the chemotactic response, since these cells (IRS-1) fail to respond chemotactically to insulin (Fig. 7*A*). These results demonstrate that the addition of excess IRS-1 to CHO cells, which have endogenous IRS-1, is not sufficient to induce a chemotactic response. A cell line that expresses both the insulin receptor and excess IRS-1 (IR/IRS-1) responds the same as the HIRC cells that are transfected with wild type insulin receptor only, up to a concentration of 10 nM insulin, but then fails to respond at 100 nM insulin (Fig. 7*B*).

DISCUSSION

Our results demonstrate that the insulin receptor can transmit a chemotactic signal to CHO cells and that the signaling pathway contains a pertussis toxin-sensitive element. It is possible that the element is the β-subunit of the insulin receptor itself, since it has been reported that autophosphorylation of the insulin receptor is pertussis toxin-sensitive (18). This result suggests a potential difference between the receptors for insu-

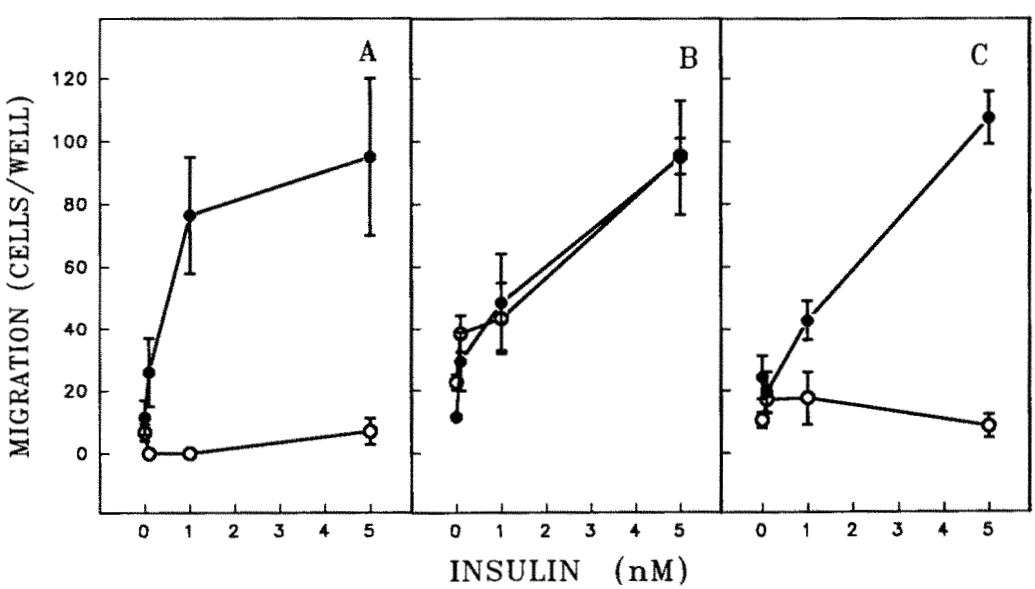

FIG. 4. **Migration of CHO cells transfected with kinase-deficient insulin receptor and COOH-terminal-deleted insulin receptor toward insulin.** *A*, migration of HIRC cells (●) and IR_{A1018} cells (○; expressing insulin receptors with conserved lysine in ATP binding region replaced with alanine, resulting in a kinase deficient receptor). *B*, migration of HIRC cells (●) and IR_{SCT} cells (○; expressing insulin receptors in which the COOH-terminal 43 amino acids have been deleted). *C*, migration of HIRC cells (●) and IR_{A960} cells (○; expressing insulin receptors in which the intracellular juxtamembrane autophosphorylation site Tyr-960 has been replaced with alanine). *Bars* represent S.E. (n = 4).

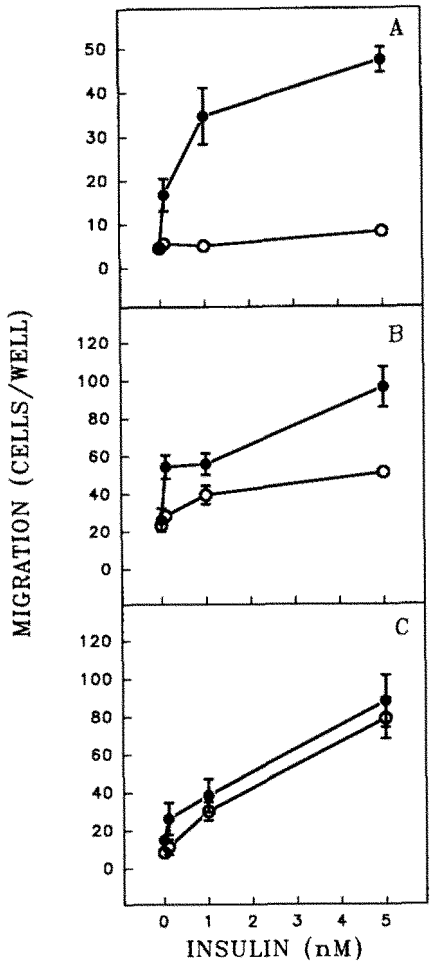

FIG. 5. **Migration of CHO cells transfected with insulin receptors containing regulatory region autophosphorylation site mutations.** *A*, migration of HIRC cells (●) and $IR_{F1146,1150,1151}$ cells (○; expressing insulin receptors in which tyrosines 1146, 1150, and 1151 in the autophosphorylation regulatory domain have been replaced by phenylalanines, resulting in an autophosphorylation-deficient insulin receptor). *B*, migration of HIRC cells (●) and $IR_{F1146,1151}$ cells (○; expressing insulin receptors in which tyrosines 1146 and 1151 have been replaced with phenylalanines). *C*, migration of HIRC cells (●) and IR_{F1146} cells (○; expressing insulin receptors in which tyrosine 1146 has been replaced by phenylalanine). *Bars* represent S.E. (n = 3 or 4).

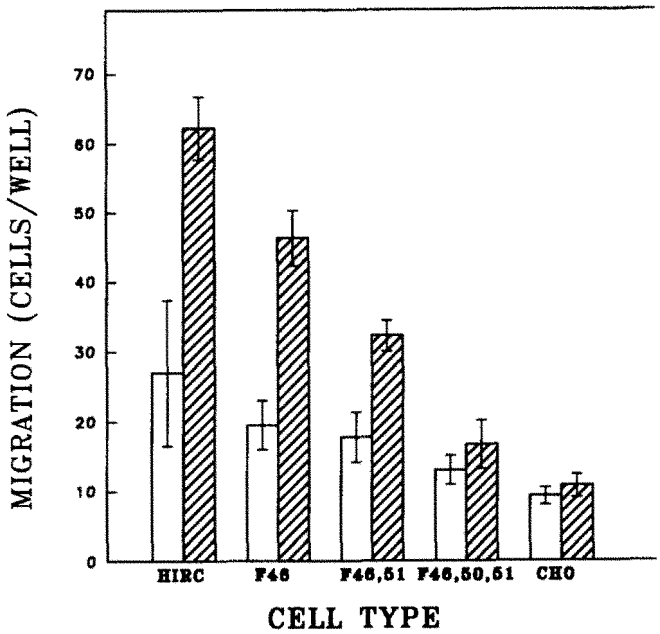

FIG. 6. **Comparison of the chemotactic responses of CHO cells transfected with regulatory autophosphorylation site mutant insulin receptors.** Migration of HIRC, IR_{F1146}, $IR_{F1146,1151}$, $IR_{F1146,1150,1151}$, and CHO cells in response to no chemoattractant (*empty bars*) or 0.1 nM insulin (*hatched bars*). Bars represent S.E. (n = 3 or 4).

lin and the insulin-like growth factor-1, since it was reported previously that insulin-like growth factor-1-stimulated chemotaxis is not blocked by pertussis toxin in A2058 cells (6). However, a direct comparison of the receptors would require similar experiments to be done in one cell type. The kinase activity and normal activation, via autophosphorylation, are necessary for the chemotactic response, whereas the COOH-terminal region is not involved in chemotactic signaling by the insulin receptor. The COOH-terminal region recently has been shown to modulate both the glucose transport and glycogen synthase responses to insulin but has no effect on insulin-stimulated mitogenesis (21). In this regard, the signaling mechanisms for insulin-stimulated chemotaxis more closely resemble those for mitogenesis than for any of the other insulin-induced responses.

We also demonstrate that the intracellular juxtamembrane region is required for insulin receptor-mediated chemotaxis. These results are consistent with previous work showing a similar structure/function relationship between the insulin receptor and mitogenesis (16). Overexpression of IRS-1 alone is not sufficient for chemotaxis, suggesting that it is the insulin receptor-mediated phosphorylation of IRS-1 rather than its expression levels that induces chemotaxis toward insulin. Overexpression of IRS-1 cells in insulin receptor-expressing CHO cells appears to reduce the chemotactic response to high insulin concentrations (100 nM), but not to lower concentrations, a result similar to that reported previously for mitogenesis in these cells (16).

Identification of the insulin receptor as a chemotactic receptor adds a novel family of tyrosine kinase receptor to those that have been shown to mediate both chemotaxis and mitogenesis. Our current data indicates that the early events in insulin-stimulated chemotactic signaling are functionally the same as those through which insulin mediates mitogenesis, but differ, in part, from those that induce endocytosis. The observation that this pathway is pertussis toxin-sensitive suggests that the insulin receptor and the seven transmembrane domain receptors may converge on a common chemotactic pathway downstream of the receptors themselves and provides support for the hypothesis that some insulin-mediated activities involve heterotrimeric G-proteins (22–24). However, the nature of the interaction of the insulin receptor and heterotrimeric G-proteins is not known.

Additional downstream effectors of insulin receptor signaling have recently been identified. Clearly, insulin stimulation of its receptor results in activation of Ras protein by means of an increased guanine nucleotide exchange activity (25) that is likely mediated by the mammalian homolog of the *Drosophila* Son of sevenless (dSos) protein (26). Sos has been shown recently to form a complex with IRS-1 (27) that may function to activate Ras in insulin- stimulated cells. The docking protein GRB-2 likely serves to link IRS-1 to Sos (28) in a multifunctional complex that also includes the activated PI-3 kinase. Thus, activation of the insulin receptor results in activation of both Ras-dependent and PI-3 kinase dependent pathways.

Future studies should define which downstream molecules are involved in the insulin receptor-mediated chemotactic pathway and determine where the pathways for chemotaxis and mitogenesis may diverge. In other tyrosine kinase receptor families, tentative distinctions have been made between the

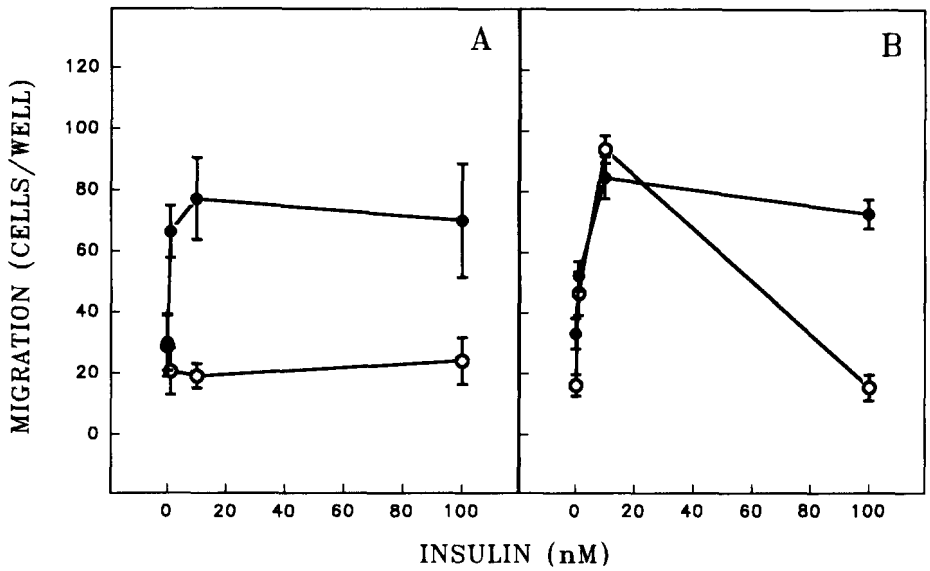

FIG. 7. **Migration of CHO cells transfected with IRS-1 alone, or IRS-1 and wild type insulin receptor.** *A*, migration of HIRC cells (●) and IRS-1 cells (○; overexpressing IRS-1). *B*, migration of HIRC cells (●) and IR/IRS-1 cells (○; overexpressing both IRS-1 and insulin receptor). *Bars* represent S.E. (n = 3 or 4).

two pathways. For example, for the platelet-derived growth factor-β receptor we have shown that GTPase-activating protein (GAP) negatively regulates chemotaxis, but has no effect on mitogenesis.[2] GAP does not appear to bind to IRS-1 (29), and the effects of GAP mutations on insulin function have not yet been investigated. Several types of cells exhibit pleiotropic responses to insulin. The fact that it has now been identified as a chemoattractant may have physiological implications. Insulin-stimulated cell migration may be important in physiologic processes such as development and neoplasia. Chemotactic effects of insulin may also be involved pattern formation and other aspects of development. For example, patients in which the gene for the insulin receptor is nonfunctional have a wide variety of abnormalities, such as an elfin appearance and lack of subcutaneous fat (30–32). This phenotype is known as leprechaunism and may be in part due to aberrant pattern formation during development. Finally, because tumor metastasis depends, in part, on cell motility (33), tumor cells that express increased numbers of insulin receptors may be found to have an increased metastatic potential.

Acknowledgment—We thank Martin G. Myers Jr. for helpful discussions.

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