Biologically active colloid-gold complexes were used to compare ligand-induced microaggregation, redistribution, and internalization of insulin receptors on Rat 1 fibroblasts expressing wild type (HIRc) or tyrosine kinase-defective (HIR A/K1018) human insulin receptors. Insulin-like growth factor I (IGF I) and α2-macroglobulin receptors also were compared. On both cell types, all four unoccupied receptor types occurred predominantly as single receptors. Ligand binding caused receptor microaggregation. Microaggregation of wild type or kinase-defective insulin receptors or IGF I receptors was not different. α2-Macroglobulin receptors formed larger microaggregates. Compared to wild type insulin or IGF I receptors, accumulation of kinase-defective insulin receptor microaggregates in endocytic structures was decreased, and the size of microaggregates in coated pits was significantly smaller. As a result, receptor-mediated internalization of gold-insulin by HIR A/K1018 cells was less than 6% of the cell-associated particles compared to ~60% of the particles in HIRc cells. On HIR A/K1018 cells, α2-macroglobulin and IGF I were internalized via coated pits demonstrating that those structures were functional. These results suggest that: 1) ATP binding, receptor autophosphorylation, and activation of receptor kinase activity are not required for receptor microaggregation; 2) receptor microaggregation per se is not sufficient to cause ligand-induced receptor-mediated internalization or the biological effects of insulin; and 3) autophosphorylation of the β-subunit or activation of the receptor kinase activity is required for the insulin-induced concentration of occupied receptors in coated pits.

Insulin binding to its receptor, a complex multifunctional protein, results in a variety of biological responses. Specific structural characteristics of the insulin receptor are likely to play essential roles in the binding of insulin and in transmitting its signal(s) to the cell interior (reviewed in Ref. 1). The structure of the insulin receptor, a transmembrane heterotetrameric glycoprotein with two extracellular α- and two transmembrane β-subunits, has been well characterized (2, 3). Receptors for insulin, a variety of growth factors (IGF I, epidermal growth factor, platelet-derived growth factor, and colony-stimulating factor I), and several transforming proteins and oncogene products share some common structural features (2, 4–7). Among these features are a ligand binding site on the α-subunit and the cytoplasmic kinase domain on the β-subunit. Kasuga et al. (8) were the first to demonstrate that the earliest potential signaling event following insulin binding to the receptor is autophosphorylation of the β-subunit and subsequent activation of the intrinsic receptor kinase. Several intracellular protein substrates exhibiting insulin-sensitive tyrosine phosphorylation have been identified (9–12), but their roles in mediating the pleiotropic effects of insulin have not been demonstrated. Nevertheless, insulin’s effects frequently are manifested by changes in protein phosphorylation (13 and references therein), and substantial evidence suggests that ligand-induced receptor autophosphorylation is an essential prerequisite for signal transduction by insulin (14–18) and other growth factors (19, 20).

Previous ultrastructural and biochemical studies from our laboratory have demonstrated cell-specific differences among a variety of insulin-responsive cell types in the initial organization of unoccupied insulin receptors, ligand-induced microaggregation, and mechanisms of insulin receptor internalization (21 and references therein). Those studies and others have suggested that ligand-induced receptor microaggregation (22–24), and perhaps receptor internalization (25–27), may play important roles in initiating or terminating the transmission of intracellular signals leading to some of the biological effects of growth factors. Biochemical analysis of the structure and function of insulin receptors has resulted from the cloning of receptor cDNAs (2, 3). This advance permitted the hypereexpression of wild type and in vitro mutated receptors in a variety of cell lines (reviewed in Ref. 1). Biochemical studies have demonstrated a variety of mutations in the β-subunit affect insulin’s mitogenic (14–16) or metabolic (14–16, 28, 29) actions and, in some cases, insulin binding (30) and the internalization and cellular processing (14–16) of the ligand-receptor complex. These studies have suggested that ligand-induced receptor microaggregation and receptor autophosphorylation may be causally linked (24, 31) and, in the case of receptors with kinase activity, autophosphorylation may be involved in receptor internalization (14–16, 32).

The present study used ultrastructural techniques to compare insulin-induced microaggregation of wild type human

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‡The abbreviations used are: IGF I, insulin-like growth factor I; PBS, phosphate-buffered saline; MOPS, 3-(N-morpholino)propanesulfonic acid; HIR, human insulin receptor; BSA, bovine serum albumin; Au-Ins, gold-labeled insulin; Au-IGF I, gold-labeled IGF I; Au-α2-MG, gold-labeled α2-macroglobulin.
insulin receptors (HIRc) and kinase-defective human insulin receptors (HIR A/K1018) expressed in Rat 1 fibroblast clones. The HIR A/K1018 cell line expresses insulin receptors in which lysine residue 1018 in the ATP binding site has been replaced by in vitro mutagenesis with alanine (14). These receptors are kinase-defective and biologically inactive and do not undergo insulin-induced endocytosis (14). In this study, no significant differences were found between the insulin-induced microaggregation of wild type or kinase-defective insulin receptors. These results suggest that ATP binding to the β-subunit of the insulin receptor, receptor autophosphorylation, and activation of the receptor tyrosine kinase are not required to form receptor microaggregates. Furthermore, based on the previously demonstrated lack of insulin action or internalization in HIR A/K1018 cells (14), we conclude that receptor microaggregation per se is not sufficient to cause either the biological effects of insulin or the internalization of the insulin-receptor complex. The lack of ligand-induced internalization of the kinase-defective receptors was primarily due to a decrease in the accumulation of kinase-defective insulin receptor microaggregates in coated pits of the cell surface. This observation suggests that autophosphorylation of the β-subunit or activation of the β-subunit kinase activity may be required for the ligand-induced concentration of insulin receptors in coated pits.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Isolation**—The Rat 1 fibroblasts expressing wild type (HIRc) or kinase-defective human insulin receptors (HIR A/K1018) were maintained in culture as previously described (14). Both cell types express ~3 × 10^6 receptors/cell. Cells were washed with phosphate-buffered saline (PBS), pH 7.4, and removed from the culture flask using 0.05% trypsin, 1 mM EDTA in PBS. Trypsin was neutralized after 60 s with trypsin soybean inhibitor, and the cells were collected by centrifugation. The cells were then washed three times and resuspended in Krebs-Ringer MOPS buffer, pH 7.4 (128 mM NaCl, 5 mM KCl, 1.5 mM MgSO4, 5 mM NaH2PO4, 1.5 mM CaCl2, 25 mM MOPS) with 5% insulin-free bovine serum albumin and 2 mM glucose (KRM buffer). Control experiments revealed no significant difference between suspended or attached cells in the amount of specific insulin, IGF I, or α2-macroglobulin binding or internalization (data not shown). Some cells in this study were “prefixed” prior to incubations with ligand to prevent ligand-induced receptor microaggregation and redistribution. Prefixation was accomplished using 0.2-1.0% glutaraldehyde in PBS for 90 s at 37 °C. The cells were then diluted 20-fold in 128 mM NaCl, 50 mM lysine HC1, and 50 mM Tris-HCl, pH 7.4, washed twice with, and then resuspended in, KRM buffer. Prefixation was sufficient to prevent multivalent ligand-induced microaggregation of insulin receptor caused by gold-labeled anti-receptor antibodies (data not shown). Prefixation did not affect the amount of gold-labeled insulin or IGF I binding compared to unincubated cells incubated at 4 °C (see Table I).

**Ligand Preparation and Cell Incubations**—Gold-labeled insulin (Au-Ins) (33-35), IGF I (Au-IGF I) (35), and α2-macroglobulin (Au-α2MG) (36) were prepared and characterized as previously described. In brief, gold colloids were mixed with concentrations of ligands resulting in the absorption of 5-7 ligand molecules per gold particle. Saturating concentrations of bovine serum albumin (BSA) were then added to stabilize the complexes (34). Gold-labeled BSA (Au-BSA) was prepared and used as a control. Au-Ins and Au-IGF I are biologically active and display binding kinetics virtually identical with the unlabeled (125I)-labeled ligands (33-35). Studies characterizing the biochemical and ultrastructural properties of these ligands demonstrated that Au-Ins with 5-7 insulin molecules per gold particle behaved identically with native monovalent insulin and did not cause multivalent ligand-induced microaggregation of insulin receptors on isolated liver plasma membranes (34). In some experiments, cells were incubated simultaneously with both Au-Ins and Au-IGF I. For those experiments, ligands were prepared with 8- or 15-nm diameter particles to be distinguishable from each other. Au-Ins was incubated with Au*Ins and Au*IGF I or Au*Ins and Au*IGF I. The difference in size of the gold particles did not affect the biological activity or binding kinetics of the ligands (data not shown). 125I-Labeled insulin (37), IGF I (38), and α2-macroglobulin (36) were prepared as previously described. Suspended cells (1–5 × 10^6 cells/ml) were incubated under the conditions described in the legends to the figures and tables. Cell-associated ligand in biochemical assays was determined by counting radioactivity associated with cell pellets centrifuged through a layer of 0.25 M sucrose in PBS, pH 7.4, as previously described (39). In biochemical assays, nonspecific binding was determined in the presence of 4.2 μM unlabeled insulin or IGF I or 10 μM α2-macroglobulin. Nonspecific cell-associated ligand was subtracted from total cell associated, and the results were reported as specific binding. In biochemical assays, internalized ligand was determined for IGF I or EDTA wash for α2-macroglobulin as previously described (40).

**Quantitation of Ultrastructural Studies**—In ultrastructural studies, nondisplaceable binding of gold-labeled ligands to viable cells was less than 10% of total cell associated (see Table I) and, except for cell pellets of cellular debris, was not discernible from total binding. No corrections were made for nondisplaceable binding in the ultrastructural studies. Gold particles associated with various domains of the cell membrane or with membranes of intracellular structures were assumed to represent receptors occupied by a substantially intact ligand-gold complex. Analysis of receptor organization on the cell surface was performed by determining the total number of ligand-bound particles and the number of single particles or particles in microaggregates of 2 to >5 molecules based on the distance between nearest neighboring gold particles as previously described (41). The distribution of ligand-receptor complexes and the extent of ligand-induced redistribution was assessed by determining the number of gold particles associated with the microvilli, undifferentiated plasma membrane, noncoated invaginations, and coated pits associated with the cell surface. Intracellular accumulation of ligand in the ultrastructural studies was determined by the apparent location of the gold particles in the thin-sectioned cells. This method may result in overestimation of ligand internalization, since when particles are in structures that are near the plasma membrane and that are open to the extracellular space at a plane above or below the observed section. However, determinations of ligand internalization by ultrastructural methods were in good agreement with the biochemical analysis (see Table II). Gold particles that were not associated with gold-labeled free gold particles at least 500 gold particles were analyzed for each experimental condition. Results presented are the mean values ± S.D. determined in at least three experiments. Significant values were determined with the Student’s t test.

**RESULTS**

**Demonstration of Ligand-Receptor Specificity**—Several different types of controls were used to demonstrate the specificity of the gold-labeled ligands. The data shown in Table I are the results obtained using HIR A/K1018 cells. Similar results were obtained with HIRc cells (data not shown). Gold-labeled BSA (Au-BSA) was used as one control for nonspecific binding because the other ligands used in this study were stabilized with BSA after the addition of the insulin, IGF I, or α2-macroglobulin (33–36) (see “Experimental Procedures”). Au-BSA particles were virtually undetectable under any incubation condition. The addition of 17 nM unlabeled insulin or IGF I or 15 nM α2-macroglobulin did not affect the number of Au-BSA particles associated with the cells (data not shown). Excess homologous ligand (4.2 μM insulin or IGF I; 10 μM α2-macroglobulin) blocked over 90% of the binding of the gold-labeled ligands (17 nM Au-Ins or Au-IGF I; 15 nM Au-α2MG) to viable cells. As we (42) and others (43) have
Insulin-induced Microaggregation of Kinase-defective Receptors

Table I

Receptor specificity of gold-labeled ligands

<table>
<thead>
<tr>
<th>Additions</th>
<th>Gold particles/100 sectioned cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unfixed cells</td>
</tr>
<tr>
<td>Au-BSA</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>Au-Ins</td>
<td>1496 ± 123</td>
</tr>
<tr>
<td>Au-Ins + insulin</td>
<td>119 ± 24</td>
</tr>
<tr>
<td>Au-Ins + IGF I</td>
<td>543 ± 65</td>
</tr>
<tr>
<td>Au-IGF I</td>
<td>876 ± 47</td>
</tr>
<tr>
<td>Au-IGF I + IGF I</td>
<td>79 ± 23</td>
</tr>
<tr>
<td>Au-IGF I + insulin</td>
<td>321 ± 34</td>
</tr>
<tr>
<td>Au-α2MG</td>
<td>914 ± 86</td>
</tr>
<tr>
<td>Au-α2MG + α2MG</td>
<td>86 ± 14</td>
</tr>
<tr>
<td>Au-Ins + Au-IGF I</td>
<td>1386 ± 206 (Ins)</td>
</tr>
<tr>
<td></td>
<td>818 ± 51 (IGF I)</td>
</tr>
</tbody>
</table>

Reported, the relatively large amount of labeled ligand associated with the 1–3% nonviable cells present in the sample is not displaced by excess unlabeled ligand in either biochemical or ultrastructural studies. Nonviable cells are not analyzed in comparable biochemical analyses.

Excess (4.2 µM) heterologous unlabeled IGF I or insulin reduced Au-Ins or Au-IGF I binding, respectively, by about 65%. This observation is consistent with high concentrations of unlabeled insulin or IGF I occupying the heterologous receptor and does not suggest that a high percentage of the gold-labeled ligands occupied the "wrong" receptor. We observed virtually no cross-over binding. Two results suggest greater than 90% of the observed Au-Ins or Au-IGF I binding was to their respective receptors. When 17 nM Au-Ins and Au-IGF I (on different diameter gold particles) were co-incubated with cells at 4 °C, there was no significant reduction in the number of cell-associated particles of either ligand compared to cells incubated with only one ligand (Table I). Secondly, on the HIR A/K1018 cells incubated at 37 °C with Au-Ins and Au-IGF I, either separately or in combination, less than 6% of the cell-associated Au-Ins was found in intracellular structures, whereas 50% of the cell-associated Au-IGF I was internalized (see Table II, below). These data are consistent with biochemical determinations of internalization of the ligand-receptor complexes (see Table II, below). Based on section thickness, average cell diameter, and surface area observed in the cells, the number of Au-Ins and Au-IGF I particles observed is consistent with the number of receptors per cell (44). The data in Table I also demonstrate that with using, excess unlabeled insulin, and IGF I migration, did not significantly affect the amount of binding of these ligands compared to unfixed cells incubated at 4 °C.

It is probable some Au-IGF I was bound to IGF I binding proteins. Biochemical determinations suggest approximately 15% of the binding of tracer concentrations of 125I-IGF I is to binding proteins in both cell types (45). At the concentrations of Au-IGF I used in these studies, the contribution of IGF I binding proteins is probably less than 25% of the total IGF I binding. This estimate was based on the difference between the Au-IGF I particles bound in the presence of 4.2 µM IGF I, representing displacement from receptors and binding proteins, and the particles bound in the presence of 4.2 µM insulin, representing displacement from IGF I receptors that insulin occupied. The difference between these values, ~25% of the total bound, is the maximal amount of binding to IGF I binding proteins.

Quantitative Analysis of Ligand-induced Receptor Microaggregation—The extent of ligand-induced receptor microaggregation was determined by comparing the initial organization of receptors observed on prefixed cells to that found on cells incubated at 4 or 37 °C for 30 min. Incubations at 4 °C decreased insulin receptor internalization (see Table II), but permitted movement of the receptors in the plasma membrane. There were no significant differences in initial insulin receptor organization or the extent of insulin-induced receptor microaggregation on the HIR or HIR A/K1018 cells. On prefixed HIRc cells (Fig. 1a), 89% of the Au-Ins particles bound to single dispersed receptors. Only ~25% of the total binding on unfixed HIRc cells incubated at 4 or 37 °C was in the form of single receptors. The decrease in the percentage of single receptors and the concomitant increase in receptors in groups of two or more implies that insulin-induced receptor microaggregation occurred on HIRc cells expressing the wild type receptor. Similarly, 95% of the tyrosine kinase-defective insulin receptors on prefixed HIR A/K1018 cells (Fig. 1b) were single molecules, but only ~35% were single receptors on the unfixed HIR A/K1018 cells. These data indicate that the tyrosine kinase-defective insulin receptor microaggregated after binding insulin. At 4 °C, the mean size of insulin-induced receptor microaggregates was 1.9 ± 0.3 and 1.7 ± 0.3 particles/group on HIRc and HIR A/K1018 cells, respectively. No significant difference was found in the ability of wild type or kinase-defective insulin receptors to microaggregate after binding insulin.

Ligand-induced microaggregation of IGF I and α2-macroglobulin receptors on the HIRc and HIR A/K1018 cells was examined to evaluate endogenous receptors structurally similar to and different from insulin receptors. The percentage of single IGF I receptors (Fig. 2a) on prefixed HIR A/K1018 cells was slightly lower than that found with insulin (Fig. 1b), and the percentage of receptors in groups of 2 or more molecules was greater. However, there were no significant differences observed between the insulin or IGF I-induced receptor microaggregation at 4 or 37 °C. By contrast, Au-α2MG (Fig. 2b) binding resulted in significantly (p < 0.005) larger receptor microaggregates than either Au-Ins or Au-IGF I. There were only small differences in the microaggregation of IGF I or α2-macroglobulin receptors on HIR A/K1018 and HIRc cells; all receptors on HIRc cells tended to form slightly larger microaggregates (data not shown).

Quantitative Analysis of Ligand-induced Receptor Redistribution—The ligand-induced redistribution of occupied receptors on the cell surface was demonstrated by comparing the initial distribution of receptors on prefixed cells to the distribution found on unfixed cells incubated at 4 or 37 °C. Apparent routes of receptor-mediated endocytosis were accentuated in cells incubated at 4 °C because ligand-receptor complexes accumulated in endocytic structures and receptor internalization was prevented (see Table II, below). Cells incubated at 37 °C were used to demonstrate receptor-mediated ligand internalization.

As shown in Fig. 3, a and b, there were no significant
TABLE II
Internalization of insulin, IGF I, and α2-macroglobulin

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cell type</th>
<th>Biochemical 4°C</th>
<th>Biochemical 37°C</th>
<th>Ultrastructural 4°C</th>
<th>Ultrastructural 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>HIR A/K1018</td>
<td>0.9 ± 0.7</td>
<td>7.1 ± 1.3</td>
<td>1.3 ± 0.9</td>
<td>5.5 ± 1.0</td>
</tr>
<tr>
<td>IGF I</td>
<td>HIR A/K1018</td>
<td>1.2 ± 0.7</td>
<td>48.9 ± 3.4</td>
<td>1.4 ± 0.7</td>
<td>56.7 ± 4.3</td>
</tr>
<tr>
<td>α2-MG</td>
<td>HIR A/K1018</td>
<td>0.8 ± 0.4</td>
<td>31.7 ± 2.4</td>
<td>1.3 ± 0.5</td>
<td>50.6 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>HIRc</td>
<td>0.9 ± 0.6</td>
<td>59.2 ± 2.5</td>
<td>2.0 ± 0.0</td>
<td>73.3 ± 1.2</td>
</tr>
</tbody>
</table>

Fig. 1. Organization of occupied insulin receptors on HIRc (a) and HIR A/K1018 (b) cells. Some cells were prefixed (open bars) as described under “Experimental Procedures.” Those cells were then incubated for 30 min at 4 °C with 17 nM Au-Ins. Unfixed cells were incubated with the same concentration of ligand at 4 °C (solid bars) or 37 °C (cross-hatched bars) for 30 min. The cells were washed and prepared for electron microscopy. The microaggregation of gold particles on the cell surface, representing occupied insulin receptors, was analyzed. Over 500 particles were examined for each incubation condition. Results are the mean ± S.D. of the values obtained in three experiments.

Fig. 2. Organization of occupied IGF I receptors (a) and α2-macroglobulin receptors (b) on HIR A/K1018 cells. Some cells were prefixed (open bars) as described under “Experimental Procedures.” Those cells were then incubated for 30 min at 4 °C with 17 nM Au-IGF I or 15 nM Au-α2-MG. Unfixed cells were incubated with the same concentration of ligand at 4 °C (solid bars) or 37 °C (cross-hatched bars) for 30 min. The cells were washed and prepared for electron microscopy. The microaggregation of gold particles on the cell surface, representing occupied IGF I or α2-macroglobulin receptors, was analyzed. Over 500 particles were examined for each incubation condition. Results are the mean ± S.D. of the values obtained in three experiments.

Significant differences were found between the cells expressing the wild type and kinase-defective receptors in the amount of Au-Ins in putative endocytic structures in unfixed cells. Fig. 3a demonstrates that in HIRc cells incubated at 4 °C, ~12% of the membrane-associated Au-Ins was found in noncoated invaginations (Fig. 4C) and another 12% was associated with coated pits (Fig. 4D). The noncoated invaginations make up 13% of the cell surface, while the coated pits represent only 3% of the cell surface (data not shown). These observations suggest that insulin occupancy of the wild type human insulin receptors resulted in the concentration of differences in the distribution of Au-Ins receptors on prefixed HIRc or HIR A/K1018 cells. On prefixed cells, 60–70% of the receptors were found on microvilli as illustrated in Fig. 4A. On unfixed cells, the Au-Ins-receptor complex on both cell types migrated from its initial binding site on microvilli to the plasma membrane as shown in Fig. 4B. After 30 min at either 4 or 37 °C, only ~20% of the Au-Ins particles were associated with microvilli of either cell type. This finding suggested that the ability of the kinase-defective receptor to migrate from the microvilli was not diminished.
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Fig. 3. Cell surface distribution of occupied insulin receptors on HIRc (a) and HIR A/K1018 (b) cells. Some cells were prefixed (open bars) as described under “Experimental Procedures.” Those cells were then incubated for 30 min at 4 °C with 17 nM Au-Ins. Unfixed cells were incubated with the same concentration of ligand at 4 °C (solid bars) or 37 °C (cross-hatched bars) for 30 min. The cells were washed and prepared for electron microscopy. The distribution of gold particles on the cell surface microvilli, undifferentiated plasma membrane, noncoated invaginations, and coated pits was analyzed. Over 500 particles were examined for each incubation condition. Results are the mean ± S.D. of the values obtained in three experiments.

Insulin-receptor complexes in coated pits, but receptors may be internalized randomly by noncoated invaginations. Since the amount of Au-Ins in noncoated invaginations on the HIRc cells increased at 37 °C compared to 4 °C, but there was no increase in the percentage of Au-Ins particles in noncoated invaginations on the HIR A/K1018 cells, internalized insulin-receptor complexes in HIRc cells may be recycled to the cell surface in noncoated vesicles and invaginations. At 37 °C, the percentage of Au-Ins particles in the coated pits of the HIRc cells was lower than that found at 4 °C (Fig. 3a), presumably due to the rapid internalization of the ligand-receptor complex expected at the higher temperature.

On the HIR A/K1018 cells, the percentage of membrane-associated Au-Ins particles in noncoated invaginations and coated pits was reduced by up to 75% (Fig. 3b) compared to that found in the HIRc cells (Fig. 3a). There were no significant differences in the number or apparent size of noncoated invaginations or coated pits between the two cell types incubated at the same temperature (data not shown). The decrease in the amount of Au-Ins in noncoated invaginations may be caused by the lack of significant internalization and recycling of insulin-receptor complexes. The decrease in Au-Ins particles in coated pits on the HIR A/K1018 cells resulted from a decrease in the size of the receptor microaggregates associated with those structures. As shown above (Fig. 1), insulin receptor microaggregation on the two cell types was comparable when the surface was analyzed as a single entity and there was no difference in the mean size of the receptor microaggregates (1.9 ± 0.3 and 1.7 ± 0.3 particles/group on HIRc and HIR A/K1018 cells, respectively). However, the mean size of microaggregates in coated pits on the HIR A/K1018 cells was 1.4 ± 0.3 particles/pit compared to 3.3 ± 0.4 particles/pit on the HIRc cells. When compared to the mean size of receptor microaggregates on the entire cell surface, these data indicate coated pits on the HIRc cell contained microaggregates significantly (p < 0.01) larger than those on the rest of the cell surface. By contrast, the size of the groups of kinase-defective receptors in coated pits was not different from those on the rest of the cell membrane. These findings suggest the insulin receptor microaggregates on the HIRc cells were collected in coated pits, whereas microaggregates of kinase-defective receptors did not concentrate in coated pits on the HIR A/K1018 cells.

There were no significant differences between the ligand-induced redistribution of Au-IGF I receptors on the HIR A/K1018 cells (Fig. 5a) or Au-Ins receptors on the HIRc cells (Fig. 3a). Like the wild type insulin receptors, IGF I receptors appeared to concentrate in coated pits and be internalized randomly by noncoated invaginations. However, two significant differences were found in the distribution of Au-α2-MG receptors (Fig. 5b) compared to wild type insulin and IGF I receptors. On prefixed cells, unoccupied α2-Macroglobulin receptors were on the plasma membrane, and internalization of α2-macroglobulin occurred only via coated pits. The differences observed in this study between α2-Macroglobulin and insulin or IGF I receptors were consistent with findings in previous reports (21, 36) and indicate that the normal processing pathways for α2-macroglobulin are functional on HIR A/K1018 cells. There were only small differences in the redistribution of IGF I receptors between HIRc and HIR A/K1018 cells. There was a 15–30% reduction in the percentage of Au-IGF I particles in endocytic structures in the HIRc cells compared to HIR A/K1018 cells (not shown).

Quantitative Analysis of Ligand Internalization—Internalization of each of the ligands was determined biochemically and ultrastructurally as shown in Table II. As expected, insulin internalization in the HIR A/K1018 cells was significantly less than that observed in HIRc cells. The reduction in insulin internalization by the kinase-defective receptor was not due to a general cellular defect since both IGF I and α2-Macroglobulin were internalized in both cell types. In fact, IGF I internalization as a percentage of total cell associated receptors was higher in the HIR A/K1018 cells than in the HIRc cells. As shown above, ultrastructural observations revealed Au-Ins and Au-IGF I on HIRc cells were concentrated and were internalized in coated structures. When HIRc cells were incubated with Au-Ins and Au-IGF I, both receptors sometimes internalized in the same coated pit (Fig. 6A). Gold particles from both ligands were observed in various sized vesicular structures and lysosome-like organelles in HIRc cells (Fig. 6B). These data suggest that the internalization process, and perhaps a substantial portion of the intracellular itinerary, are shared by insulin and IGF I in HIRc cells. However, when both Au-Ins and Au-IGF I were incubated with HIR A/K1018 cells, virtually no Au-Ins was observed in intracellular structures (Fig. 6C). Taken together, these observations suggest the mutation in the HIR A/K1018 insulin receptor inhibits internalization by interfering with the concentration of the insulin-receptor complex in coated pits. However, the coated pits are clearly functional since both IGF I and α2-Macroglobulin were by coated pits in HIR A/K1018 cells.

DISCUSSION

This ultrastructural study investigated the effects of a mutation in the ATP binding region of the β-subunit of the
Insulin-induced Microaggregation of Kinase-defective Receptors

FIG. 4. Electron micrographs demonstrating binding of Au-Ins to prefixed HIR A/K1018 cells (A), Au-Ins to HIR A/K1018 cells at 4 °C (B), and Au-Ins to HIRc cells at 4 °C (C and D). Cells were incubated with 17 nM Au\(^{15}\)nm-Ins and prepared for electron microscopy as described under "Experimental Procedures." On prefixed HIR A/K1018 cells (A), Au-Ins (arrowheads) were primarily single molecules preferentially located on microvilli. On unfixed HIR A/K1018 cells (B), Au-Ins particles were found in small groups on the plasma membrane. Au-Ins particles were internalized on HIRc cells via both noncoated (C) and coated (D) endocytic structures. 58,500X, A-C; 70,500X, D.

FIG. 5. Cell surface distribution of occupied IGF I receptors (a) and \(\alpha_2\)-macroglobulin receptors (b) on HIR A/K1018 cells. Some cells were prefixed (open bars) as described under "Experimental Procedures." Those cells were then incubated for 30 min at 4 °C with 17 nM Au-IGF I or 15 nM Au-\(\alpha_2\)-MG. Unfixed cells were incubated with the same concentration of ligand at 4 (solid bars) or 37 °C (cross-hatched bars) for 30 min. The cells were washed and prepared for electron microscopy. The distribution of gold particles on the cell surface microvilli, undifferentiated plasma membrane, noncoated invaginations, and coated pits was analyzed. Over 500 particles were examined for each incubation condition. Results are the mean ± S.D. of the values obtained in three experiments.

FIG. 6. Electron micrographs demonstrating Au\(^{15}\)nm-Ins and Au\(^{8}\)nm-IGF I on HIRc cells incubated at 4 °C (A), Au\(^{15}\)nm-Ins and Au\(^{8}\)nm-IGF I on HIRc cells incubated at 37 °C (B), and Au\(^{8}\)nm-Ins and Au\(^{15}\)nm-IGF I on HIR A/K1018 cells incubated at 37 °C (C). Cells were incubated with 15-nm diameter Au-Ins and 8-nm diameter Au-IGF I (A and B) or 8-nm diameter Au-Ins and 15-nm diameter Au-IGF I (C) for 30 min; all ligands were added at a final concentration of 17 nM. Cells were fixed and prepared for electron microscopy as described under "Experimental Procedures." On HIRc cells, both Au-Ins and Au-IGF I (arrow) were internalized by coated pits, sometimes in the same pit (A). Au-Ins and Au-IGF I (arrow) were frequently found in the same intracellular organelle (B) in HIRc cells. In contrast, on HIR A/K1018 cells, Au-Ins (arrowhead) were found on the surface of HIR A/K1018 cells (C), but failed to internalize. Au-IGF I particles (arrows) were found on the membrane and in intracellular organelles. 70,500X (A-C).

insulin receptor on insulin-induced receptor microaggregation, redistribution, and internalization. Insulin binding results in \(\beta\)-subunit autophosphorylation and activation of an
Physiologic concentrations of insulin affect the internalization of a number of nutritional ligands which utilize coated pits exclusively, both coated and noncoated invaginations, or only noncoated invaginations (21 and references therein). Noncoated invaginations may serve as a nonconcentrative or constitutive receptor-mediated endocytotic pathway for insulin and other ligands (21 and references therein) and frequently do so even in the presence of a concentric coated pit mediated pathway (59). Noncoated invaginations also participate in the recycling of receptors or ligand-receptor complexes to the cell surface (60). The potential of a dual role for noncoated invaginations can obfuscate the morphological assessment of the endocytotic pathway. Despite the possible involvement of the noncoated invaginations in the constitutive endocytosis of insulin and IGF I, a significantly greater amount of insulin and IGF I receptor-mediated internalization in the HIRc cells occurred via a concentrative process in the coated pits, a process lacking in the cells with tyrosine kinase-defective insulin receptors. These results suggest that receptor autophosphorylation or tyrosine kinase activity is required for the concentration of these receptors in coated pits.
Insulin-induced Microaggregation of Kinase-Defective Receptors

on an almost continual basis in vivo. The mechanism by which insulin regulates the uptake of these ligands is not known. It was demonstrated recently that insulin increased the serine phosphorylation of a 125-kDa polypeptide associated with the coated pits of rat adipocytes (65). Neither insulin receptors nor the kinase responsible for the reaction were associated with the coated pits when this effect was seen (65). These findings suggest insulin's effects on the uptake of nutrient ligands through coated pits may be caused by a conformational change in polypeptides within coated pits that interact with cytoplasmic domains of those receptors. Based on the observations in the present study, constitutive (non-concentrating) insulin internalization probably proceeded via noncoated invaginations, whereas ligand-induced internalization used coated pits. In the same manner that insulin regulates uptake of nutrient ligands via coated pits, insulin binding to kinase-active receptors outside coated pits may also regulate ligand-induced internalization of insulin receptors by coated pits. Consequently, impaired internalization of HIR A/K1018 insulin receptors may be related to a lack of the normal effects of insulin on coated pit function. If this hypothesis is true, insulin-regulated uptake of nutrient ligands may be impaired in cells expressing kinase-defective insulin receptors. Mutations in the insulin receptor, such as deletion of the region spanning NPXY990, that impair insulin receptor internalization but not kinase activity (66, 67) may affect the ability of the receptor to associate with coated pit proteins. This mutation would not be expected to affect insulin's regulation of the uptake of other ligands internalized by coated pits.

In summary, the present studies demonstrate the tyrosine kinase activity of the insulin receptor is not required for insulin-induced receptor microaggregation. We conclude insulin receptor microaggregation is not sufficient to cause internalization of the receptor or the biological effects of insulin. Microaggregation of the wild type receptor may, however, be part of a sequence leading to the generation of biological responses and receptor internalization. Receptor-mediated internalization of growth-inhibitory cells expressing the kinase-defective receptor was significantly decreased. This decrease was primarily due to a reduction in the accumulation of occupied kinase-defective insulin receptor microaggregates in coated pit structures. This observation suggests autophosphorylation of the β-subunit and activation of the receptor kinase activity may be required for the ligand-induced interaction of insulin receptors with endocytic structures.

REFERENCES