Insulin Receptors Internalize by a Rapid, Saturable Pathway Requiring Receptor Autophosphorylation and an Intact Juxtamembrane Region

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Abstract. The effect of receptor occupancy on insulin receptor endocytosis was examined in CHO cells expressing normal human insulin receptors (CHO/IR), autophosphorylation- and internalization-deficient receptors (CHO/IR_{A1018}), and receptors which undergo autophosphorylation but lack a sequence required for internalization ($CHO/IR_{\Delta 960}$). The rate of [^{125}I]insulin internalization in CHO/IR cells at 37°C was rapid at physiological concentrations, but decreased markedly in the presence of increasing unlabeled insulin (ED_{50} = 1–3 nM insulin, or 75,000 occupied receptors/cell). In contrast, [^{125}I]insulin internalization by CHO/IR_{A1018} and $CHO/IR_{\Delta 960}$ cells was slow and was not inhibited by unlabeled insulin. At saturating insulin concentrations, the rate of internalization by wild-type and mutant receptors was similar. Moreover, depletion of intracellular potassium, which has been shown to disrupt coated pit formation, inhibited the rapid internalization of [^{125}I]insulin at physiological insulin concentrations by CHO/IR cells, but had little or no effect on [^{125}I]insulin uptake by $CHO/IR_{\Delta 960}$ and CHO/IR_{A1018} cells or wild-type cells at high insulin concentrations. These data suggest that the insulin-stimulated entry of the insulin receptor into a rapid, coated pit-mediated internalization pathway is saturable and requires receptor autophosphorylation and an intact juxtamembrane region. Furthermore, CHO cells also contain a constitutive nonsaturable pathway which does not require receptor autophosphorylation or an intact juxtamembrane region; this second pathway is unaffected by depletion of intracellular potassium, and therefore may be independent of coated pits. Our data suggest that the ligand-stimulated internalization of the insulin receptor may require specific saturable interactions between the receptor and components of the endocytic system.

THE uptake of a variety of hormones and nutrients by eukaryotic cells occurs by receptor-mediated endocytosis (Goldstein et al., 1985). Occupied cell surface receptors internalize via clathrin-coated vesicles and are transported to endosomes, where ligands dissociate during the gradual acidification of the endosomal lumen (Tycko et al., 1983). Recently, a number of these events have been reconstituted in vitro: the formation of coated pits onto isolated membranes, the endocytosis of receptors from coated pits to coated vesicles, and the fusion of endosomal vesicles have all been demonstrated in cell-free or broken-cell systems (Mahaffey et al., 1989; Smythe et al., 1989; Podbilewicz and Mellman, 1990; Gruenberg and Howell, 1989; Braell, 1987; Diaz et al., 1989; Davey et al., 1985; Mullock et al., 1989; Ward et al., 1990). However, the mechanisms which govern the entry of receptors from the plasma membrane into the coated pit/coated vesicle/endosome continuum remain poorly defined.

Cell surface receptors have been broadly divided into class I receptors, which are constitutively located in coated pits under basal conditions, and class II receptors, which move from non-coated to coated regions of the plasma membrane when stimulated by ligand (Goldstein et al., 1985). The designation of class I and II receptors in this manuscript refers only to the distinction between constitutive and ligand-stimulated internalization, and should not be confused with the division of tyrosyl kinase receptors into EGF-related (class I) or insulin receptor-related (class II) kinases as proposed by Yarden and Ullrich (1988). The preferential localization of class I receptors to coated pits is presumably dependent on the cytoplasmic domains of these receptors, as mutant receptors lacking this domain are randomly dispersed in the plasma membrane and internalize poorly (Anderson et al., 1977; Goldstein et al., 1985; Lehrman et al., 1985; Iacopetta et al., 1988; Mostov et al., 1986). Several endocytosis-competent receptors contain aromatic residues near the cytoplasmic face of the membrane which are necessary for internalization (Davis et al., 1987; Lazrovits and Roth, 1988; Lobel et al., 1989; Jing et al., 1990; Breitfeld et al., 1990; Johnson et al., 1990; Valiquette et al., 1990). Moreover, Chen et al. (1990) have shown that a tetrapeptide sequence in the juxtamembrane region of the LDL receptor, $NPXY_{807}$, is required for efficient internalization. While only a few cell surface receptors contain this putative consensus sequence, it has recently been proposed that the internalization signal for class I receptors is an aromatic

residue in a tight turn (Collawn et al., 1990) or a short surface loop stabilized by hydrogen bonds between residues on either side of the aromatic amino acid (Ktistakis et al., 1990). The $NPVY_{807}$ sequence in the LDL receptor, as well as the sequence $Y_{20}XRF$ required for transferrin receptor internalization, are predicted to form turns (Collawn et al., 1990). Furthermore, the introduction of a tyrosine residue immediately distal to residues favoring turn formation in the cytoplasmic tail of glycophorin caused this molecule, normally excluded from coated pits, to be efficiently internalized (Ktistakis et al., 1990).

The internalization of class II receptors, which include the insulin and EGF receptors, is more complex. The insulin receptor contains an intrinsic tyrosyl kinase activity, and ligand binding stimulates the autophosphorylation and the internalization of the receptor (Kasuga et al., 1982; Bergeron et al., 1985). In several cell types, insulin-stimulated autophosphorylation or kinase activity of the insulin receptor appears to be essential for internalization (Russell et al., 1987; McClain et al., 1987; Hari and Roth, 1987). However, autophosphorylation may not be a sufficient signal for internalization. Deletion of a portion of the juxtamembrane region of the insulin receptor containing the $NPXY_{960}$ motif has minimal effects on autophosphorylation but significantly inhibits insulin-stimulated endocytosis (Backer et al., 1990*a*; Thies et al., 1990). Thus, internalization of the insulin receptor may require both autophosphorylation and amino acid sequences similar to those required for the constitutive internalization of the LDL receptor.

This study explores the possibility that insulin receptor endocytosis requires specific and saturable interactions with cellular components. The internalization of [^{125}I]insulin by CHO cells expressing 10^6 wild-type human insulin receptors/cell (CHO/IR) is inhibited by increasing receptor occupancy; half-maximal inhibition is observed when 75,000 receptors are occupied by insulin. In contrast, the internalization of [^{125}I]insulin by cells expressing kinase-deficient receptors (CHO/IR_{A1018}) or receptors lacking a portion of the juxtamembrane region containing the $NPXY_{960}$ motif ($CHO/IR_{\Delta 960}$) is significantly slower and is nearly unaffected by increasing insulin concentrations. Moreover, the rapid internalization of [^{125}I]insulin observed in CHO/IR cells at low receptor occupancy is inhibited by 80% when coated pit formation is disrupted by depletion of cellular potassium; [^{125}I]insulin internalization in the $CHO/IR_{\Delta 960}$ and CHO/IR_{A0108} cells is inhibited little or not at all. These data suggest that CHO cells possess two internalization pathways: a rapid saturable coated pit-dependent pathway that requires receptor autophosphorylation and an intact juxtamembrane region, as well as a constitutive nonsaturable pathway which is unaffected by mutations in the juxtamembrane region or ATP binding site and which may not utilize coated pits.

Materials and Methods

Cell Culture, Expression Plasmids, and Transfection of CHO Cells

CHO/IR were maintained as previously described and were used on the first day of confluence (Backer et al., 1990*a*). The normal human insulin receptor expression plasmid pCVSVHIRc and expression plasmids encoding a deletion of amino acids A954-D965 ($IR_{\Delta 960}$) and the substitution of Tyr_{960} with phenylalanine and Ser_{962} with threonine (IR_{F960}) have been previously described (Backer et al., 1990*a*). The expression plasmid encoding the substitution of Lys_{1018} with alanine (IR_{A1018}) was a gift from Dr. O. M. Rosen (deceased) (Chou et al., 1987). CHO cells were transfected and selected as previously described (Backer et al., 1990*a*; Maron et al., 1984). Clonal cell lines, obtained by plating at limiting dilution, expressed approximately 1.2×10^6 receptors/cell. Scatchard analysis indicated that insulin binding affinity was normal in all lines (Backer et al., 1990*a*; data not shown).

Uptake of [^{125}I]insulin by CHO Cells Expressing Wild-type or Mutant Insulin

Confluent monolayers of CHO/IR, $CHO/IR_{\Delta 960}$, CHO/IR_{F960}, or CHO/IR_{A1018} cells were grown in 6- or 24-well dishes. The cells were washed twice in PBS, pH 7.4, and incubated at 37°C in "binding buffer" (F-12 medium containing 50 mM Hepes, pH 7.4, 0.1% BSA, and 5×10^6 cpm/ml [^{125}I]insulin [[^{125}I]iodotyrosyl A14-insulin, 2,000 Ci/mmol]). Where indicated, incubations also included various concentrations of unlabeled insulin. At various times between 0 and 10 min, the medium was removed and the cells were rapidly chilled by immersion in ice-cold PBS. Cells were washed twice in PBS, to determine total cell-associated radioactivity, or acidic PBS (pH 3.5), to determine intracellular radioactivity. The washed cells were solubilized in 0.1% SDS/0.1 N NaOH and counted in a Tracor GammaTrac gamma counter. Nonspecific binding was determined in the presence of 10^{-6} M insulin, and was found to be approximately 0.25% of total at 37°C. Data are representative of two to five separate experiments.

Analysis of Internalization Data

The internalization rate constant (k_e) for each receptor mutant was determined during a 0–10-min incubation with [^{125}I]insulin as described by Lund et al. (1990) for internalization of the EGF receptor. Ligand recycling and degradation in CHO cells is minimal within this time interval (Backer et al., 1990*b*; data not shown). The instantaneous velocity of receptor-mediated internalization of a ligand (dL_{int}/dt) is expressed as

$$dL_{int}/dt = k_e(LR_s), \quad (1)$$

where L_{int} is the amount of internalized ligand, LR_s is the concentration of ligand-receptor complexes at the cell surface, and k_e is the rate constant for receptor internalization (11,105). Integration of both sides yields:

$$L_{int} = k_e \int_0^t (LR_s)dt. \quad (2)$$

As shown by Lund et al. (1990), a plot of internalized ligand (L_{int}) versus the integral with respect to time of surface-bound ligand ($\int_0^t (LR_s)dt$) yields a straight line whose slope is equal to the internalization rate constant k_e. Integrals were approximated by the trapezoidal rule, using an interval of $\Delta t = 2$ min. Slopes were determined by linear regression; values for r^2 were 0.99 for CHO/IR and $CHO/IR_{\Delta 960}$ cells, and 0.91 for CHO/IR_{A1018} cells. The data reflect duplicate determinations and are representative of five separate experiments.

At steady state, the relationship between receptor occupancy and internalization velocity can be described by the Eadie-Scatchard equation as modified by Lund et al. (1990) to study the internalization of receptor-ligand complexes:

$$v/LR_s = -k_{IC}v + k_{IC}V_{max}, \quad (3)$$

where v is the velocity of ligand uptake, LR_s is the concentration of receptor-ligand complexes at the cell surface, and V_{max} is the maximum uptake velocity of the system. k_{IC} is a combination of individual rate constants for formation and dissociation of receptor/coated pit complexes, the dissociation of ligand from receptor/pit complexes, and the actual rate of coated pit internalization (Lund et al., 1990).

Since the velocity of internalization v is equivalent to dL_{int}/dt, then from equation (1) above $v = k_e(LR_s)$ and

$$k_e = -k_{IC}v + k_{IC}V_{max}. \quad (4)$$

A plot of k_e against v, as both parameters change with increasing ligand concentrations, is analogous to an Eadie-Scatchard plot. The internalization rate constant (k_e), determined as described above at each insulin concentration, was plotted against the average velocity of insulin uptake between 2 and 10 min at each insulin concentration. The slope of the apparently saturable component in each cell line was determined by linear regression; the x-intercept of the calculated line yields the apparent V_{max}. The half-saturating surface occupancy was calculated, using linear regression, by determining the insulin uptake velocity at which k_e was 50% of maximum;

the corresponding number of surface-bound insulin molecules was then determined directly from the binding measurements used to construct the plots of equation 2 at varying insulin concentrations. The data reflect duplicate determinations, and are representative of five separate experiments.

Affinity Labeling of CHO Cells with [^{125}I]-BPA Insulin and Internalization of Labeled Receptors

Benzoylphenylalanine (Bpa) was synthesized as described by DeGrado and coworkers (Kauer et al., 1986). B-25-BPA insulin was synthesized as described (Shoelson, S. E., C. S. Lynch, S. Chatterjee, Y.-M. Feng, K. R. Williams, and J. M. Backer, manuscript in preparation). B-25-BPA-insulin was iodinated at the A-14 position by the method of Lioubin et al. (1984) as modified by Shoelson, S. E. (manuscript in preparation) and purified by reverse phase HPLC (Waters Instruments, Inc.; Rochester, NY). Confluent monolayers of CHO/IR, CHO/IR$_{A1018}$, or CHO/IR$_{\Delta 960}$ cells in 6-well dishes (Nunc, Roskilde, Denmark) were incubated for 3 h at 4°C with 0.5 cc "binding buffer" containing 1–2 × 10^5 CPM/ml [^{125}I]-BPA insulin (0.02–0.04 pM). The cells were irradiated for 30 min at 4°C in a Rayonet photochemical reactor (350 nm) at 5 cm from the light source, washed with PBS at 4°C, and internalization was initiated by rapid warming to 37°C in the absence or presence of unlabeled insulin (concentrations as indicated). At various times, the cells were chilled in ice-cold PBS and incubated for 30 min on ice in 0.5 mg/ml trypsin (Worthington Biochemical Corp., Freehold, NJ). Proteolysis was terminated by the addition of 0.5 ml ice cold PBS containing 5 mg/ml soy bean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO). The cells were scraped, washed in cold PBS containing 0.5 mg/ml soy bean trypsin inhibitor, and solubilized in 50 mM Hepes, pH 7.4, containing 1% Triton X-100, 350 μg/ml PMSF, 100 μg/ml aprotinin (Sigma Chemical Co.), and 1 μg/ml leupeptin. Insulin receptors were immunoprecipitated with antiinsulin receptor antibody (provided by K. Siddle, University of Cambridge), eluted with Laemmli sample buffer (Laemmli, 1970) containing 10 mM oxidized glutathione. Proteins were separated by nonreducing SDS-PAGE (5% resolving) and intact insulin receptors were visualized by autoradiography. Receptor bands were quantitated by scanning densitometry or counting of receptor bands in a γ-counter. The data is representative of two to three separate experiments.

Surface Iodination and Internalization of Insulin Receptors in CHO Cells

CHO/IR, CHO/IR$_{A1018}$ or CHO/IR$_{\Delta 960}$ cells were surface iodinated by incubation in the presence of lactoperoxidase/glucose oxidase, glucose, and [^{125}I]iodine (1 mCi/10-cm dish) as previously described (Backer et al., 1989). The cells were washed five times in cold PBS and then incubated in the absence or presence of 100 nM insulin at 37°C. At various times, the cells were chilled with cold PBS, trypsinized, washed, and solubilized and insulin receptors were immunoprecipitated as described above. Samples were eluted and reduced in Laemmli sample buffer (Laemmli, 1970) containing 100 mM DTT. Proteins were separated by reducing SDS-PAGE (7.5% resolving) and intact insulin receptors were visualized and quantified as described above. The data reflects two to four separate experiments, with the exception of Fig. 5 *a*.

Potassium Depletion Experiments

Cells were depleted of potassium as described by Larkin et al. (1983, 1986). CHO cells expressing wild-type or mutant receptors, grown in 24-well dishes, were washed twice at 37°C in either 50 mM Hepes (pH 7.4)/100 mM NaCl (buffer A) or 50 mM Hepes (pH 7.4)/80 mM NaCl/20 mM KCl (buffer B). The cells were incubated for 5 min at 37°C in F-12 media diluted 1:1 with water, followed by an additional 30-min incubation in buffer A or B. The internalization of [^{125}I]insulin was measured as described above, with the substitution of buffer A or B for "binding buffer," and the k_e for each mutant under each condition was calculated as described above. The data is derived from triplicate determinations, and reflects two separate experiments. Control experiments, in which receptors from insulin-stimulated CHO/IR cells were solubilized in Laemmli sample buffer, separated by SDS-PAGE, and Western blotted with antiphosphotyrosine antibody, showed that potassium depletion did not inhibit insulin-stimulated receptor autophosphorylation or endogenous substrate phosphorylation.

1. *Abbreviation used in this paper*: Bpa, benzoylphenylalanine.

Inhibition of [^{125}I]IGF-I and [^{125}I]IGF-II Internalization by Insulin

CHO/IR or CHO/neo cells were incubated with "binding buffer" containing 100,000 CPM [^{125}I]IGF-I or [^{125}I]IGF-II for 3 h. The cells were washed with PBS at 4°C and incubated at 37°C in binding buffer in the absence or presence of 100 nM insulin. At various times, the cells were chilled by immersion in ice-cold PBS and surface-bound ligand was removed by washing twice in acid PBS (pH 3.0 for IGF-I; pH 2.5 for IGF-II). The cells were solubilized and counted as described above to measure internalized ligand. The data reflect triplicate determinations and are representative of four separate experiments.

Results

Internalization of [^{125}I]insulin Is Diminished in CHO Cells Expressing Mutant Insulin Receptors

The endocytosis of [^{125}I]insulin at 37°C was compared in CHO/IR, kinase-deficient mutant receptors (CHO/IR$_{A1018}$), and mutant receptors from which 12 amino acids in the cytoplasmic juxtamembrane, including the NPXY$_{960}$ motif, were deleted (CHO/IR$_{\Delta 960}$). Insulin binding at 37°C was similar in the three cell lines, with surface-bound insulin approaching steady state after 10 min (Fig. 1). Internalization of surface-bound insulin by CHO/IR cells was rapid (Fig. 1*A*); however, internalization was markedly slower in the CHO/IR$_{A1018}$ cells, which do not undergo insulin-stimulated autophosphorylation, and in the CHO/IR$_{\Delta 960}$ cells (Fig. 1, *B* and *C*). Although the IR$_{\Delta 960}$ receptors undergo autophosphorylation in vivo (Backer et al., 1990*a*), they lack the putative internalization signal sequence NPXY$_{960}$ (Chen et al., 1990). Therefore, endocytosis of insulin at physiological concentrations by CHO cells requires receptor autophosphorylation, and is diminished by deletions in the cytoplasmic juxtamembrane region of the receptor.

Internalization of [^{125}I]insulin in CHO Cells at Varying Insulin Concentrations

The effect of receptor occupancy on the internalization of [^{125}I]insulin was examined in CHO cells expressing 10^6 normal or mutant receptors (Fig. 2). CHO cells were incubated for 10 min at 37°C with [^{125}I]insulin in the presence of increasing concentrations of unlabeled insulin. At 0.1 nM insulin, approximately 50% of cell associated radioactivity was

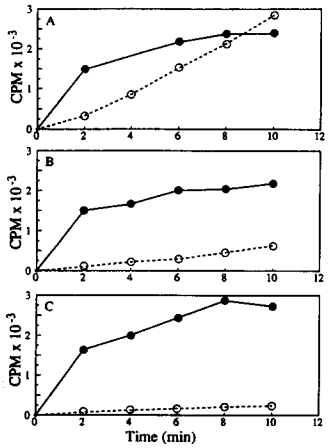


Figure 1. Internalization of [^{125}I]insulin by CHO cells expressing normal and mutant insulin receptors. CHO cells were incubated with [^{125}I]insulin (10^5 CPM/ml) at 37°C for various times. The cells were chilled and washed in PBS or acidic (pH 3.0) PBS to determine cell-associated and intracellular radioactivity, respectively. (●) Surface bound radioactivity; (○) intracellular radioactivity. (*A*) CHO/IR cells. (*B*) CHO/IR$_{\Delta 960}$ cells. (*C*) CHO/IR$_{A1018}$ cells. All points reflect the means of duplicate determinations.

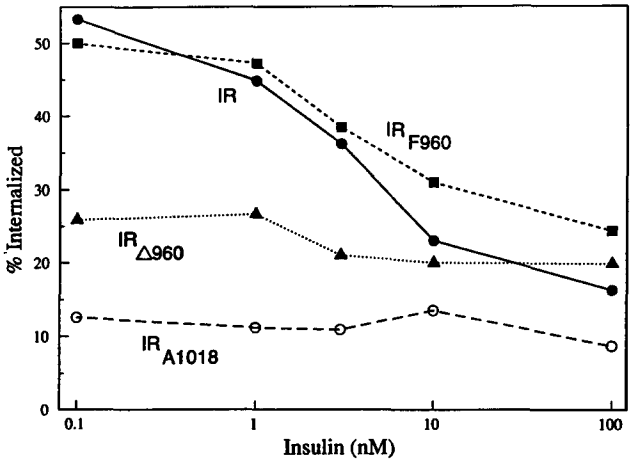


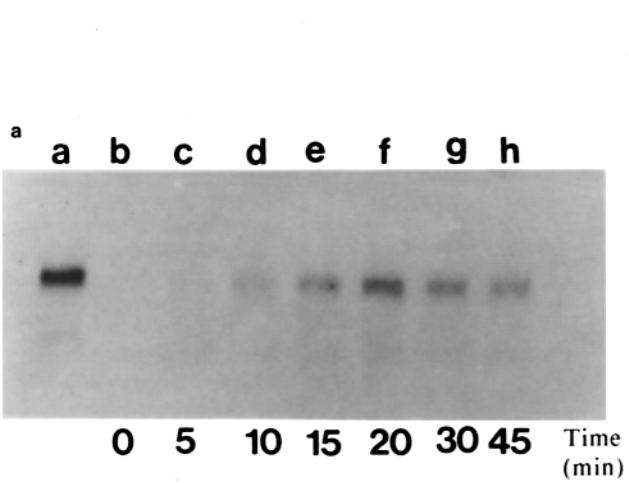
Figure 2. The effect of increasing concentrations of unlabeled insulin on the internalization of [^{125}I]insulin. CHO cells expressing normal or mutant insulin receptors were incubated with [^{125}I]insulin (10^5 CPM/ml) plus varying concentrations of unlabeled insulin for 10 min, chilled and washed as described in Fig. 1 to determine cell-associated and intracellular radioactivity. Internalized insulin was calculated as a percentage of total cell-associated insulin at each concentration. (●) CHO/IR cells; (■) CHO/IR$_{F960}$ cells; (▲) CHO/IR$_{\Delta 960}$ cells; (○) CHO/IR$_{A1018}$ cells.

internalized by CHO/IR cells. However, the internalization of [^{125}I]insulin was inhibited at higher concentrations of insulin, with a half-maximal decrease at ~3 nM insulin. Similar results were seen in cells expressing a mutant receptor containing phenylalanine in place of Tyr$_{960}$ and threonine in place of Ser$_{962}$ (IR$_{F960}$); this mutant receptor internalizes normally, but is defective in mediating insulin bioeffects or the phosphorylation of endogenous substrates (Backer et al., 1990*a*; White et al., 1988). The decreased uptake in these two cell lines was not due to the displacement of [^{125}I]insulin with unlabeled insulin, as internalization was calculated as a percentage of total cell binding. In contrast, the internalization of [^{125}I]insulin by the CHO/IR$_{\Delta 960}$ and CHO/IR$_{A1018}$ cells was reduced by 60–80% relative to the CHO/IR cells at 0.1 nM insulin, and showed no further decrease at higher insulin concentrations. Thus, increasing occupancy had differential effects on insulin internalization by wild-type and mutant receptors: the internalization of wild-type receptors appeared to saturate at high insulin concentrations corresponding to high receptor occupancy, whereas the internalization of internalization-defective mutant receptors was unaffected.

Internalization of Covalently Linked Insulin-receptor Complexes in CHO Cells

The internalization of the insulin receptor was directly measured using a novel insulin derivative containing the photoactivatable amino acid BPA at the B-25 position (BPA-insulin). This derivative binds specifically to the human insulin receptor, can be iodinated without altering binding or compromising the photoactive residue, and forms a covalent bond to the insulin receptor α-subunit after exposure to UV light (350 nm) (Shoelson, S. E., C. S. Lynch, S. Chatterjee, Y.-M. Feng, K. P. Williams, and J. M. Backer, manuscript in preparation).

CHO cells expressing the human insulin receptor were covalently labeled with [^{125}I]-BPA-insulin at 4°C, warmed to 37°C for various times, and then trypsinized at 4°C. The time-dependent internalization of labeled receptors into a trypsin-resistant compartment was determined by immunoprecipitation and nonreducing SDS-PAGE (Fig. 3 *a*). Affinity-labeled insulin receptors from CHO/IR cells appeared as a major band at ~360 kD (Fig. 3, lane *a*). Trypsinization at 4°C completely removed the 360-kD band (Fig. 3, lane *b*). When cells were incubated at 37°C before trypsinization, the accumulation of trypsin-resistant receptors was apparent



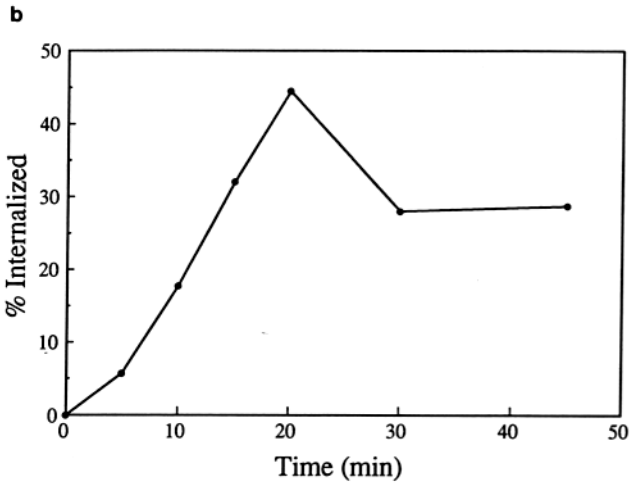


Figure 3. Internalization of insulin receptors covalently labeled with [^{125}I]-BPA-insulin. (*A*) CHO/IR cells were photolabeled with [^{125}I]-BPA-insulin at 4°C as described, solubilized, immunoprecipitated with anti-insulin receptor antibody, and analyzed by nonreducing SDS-PAGE. The cells were either solubilized without additional treatment (lane *a*), or were first incubated at 37°C for varying times and then trypsinized for 30 min at 4°C to destroy cell surface receptors (lanes *b–h*). Photolabeled receptors migrated at ~360 kD. (*B*) The internalization of receptors labeled with [^{125}I]-BPA-insulin. Photoaffinity labeling was performed at 4°C, followed by incubation at 37°C for various times. Internalized photolabeled receptors were measured by trypsinization, immunoprecipitation and SDS-PAGE as described above, and quantitated by scanning densitometry. Internalization is expressed as percent of total labeled receptors.

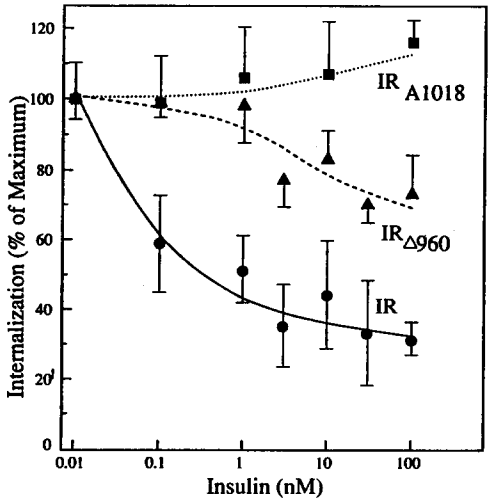


Figure 4. Internalization of photolabeled insulin receptors in CHO cells expressing normal and mutant receptors. CHO cells expressing normal or mutant insulin receptors were photolabeled with [^{125}I]BPA-insulin at 4°C as described above and then incubated at 37°C for 10 min in the presence of varying concentrations of unlabeled insulin. The cells were trypsinized at 4°C and solubilized, and receptors were immunoprecipitated and analyzed by nonreducing SDS-PAGE, and quantified by counting of receptor bands in a γ-counter. Internalization is expressed as percent of maximum. All points represent the mean ± SD of triplicates. (●) CHO/IR cells; (▲) CHO/IR$_{\Delta 960}$ cells; (■) CHO/IR$_{A1018}$ cells.

(Fig. 3, lanes *c–h*). The internalization of [^{125}I]-labeled receptors reached maximal levels after 15–20 min, with a plateau in the percentage of internalized receptors due to the recycling of receptors to the cell surface (Backer et al., 1989*a*; Hueckstaedt et al., 1986) (Fig. 3 *b*). The initial rate of uptake was similar to that seen with a single cohort of non-covalently surface-bound [^{125}I]insulin (data not shown), suggesting that the covalently labeled receptors internalized normally.

The internalization of wild-type and mutant receptors covalently labeled with [^{125}I]BPA-insulin was measured during a 10-min incubation at 37°C in the presence of varying concentrations of unlabeled insulin (Fig. 4). In CHO/IR cells, the internalization of [^{125}I]-labeled receptors was half-maximally inhibited by the addition of 0.1 nM unlabeled insulin. This dose was 10-fold less than the half-maximal dose observed in Fig. 2; although the reason for this difference is not clear, the overall effect of increasing receptor occupancy was similar. In contrast, internalization of [^{125}I]-labeled receptors in CHO/IR$_{A1018}$ cells was unaffected by the addition of unlabeled insulin. Interestingly, internalization of [^{125}I]-labeled receptors in CHO/IR$_{\Delta 960}$ cells was partially inhibited by insulin, although with a 100-fold decrease in sensitivity relative to the normal receptor. These data confirm that the internalization wild-type and mutant receptors are differentially effected by increases in receptor occupancy, as internalization of the wild-type receptor appears to saturate at high insulin concentrations. These data furthermore eliminate the possibility that the decreases in insulin uptake observed in Fig. 2 are caused by changes in insulin binding at high ligand concentrations due to negative cooperativity (De Meyts and Roth, 1975), since only covalently linked receptor-ligand complexes were measured in Fig. 4.

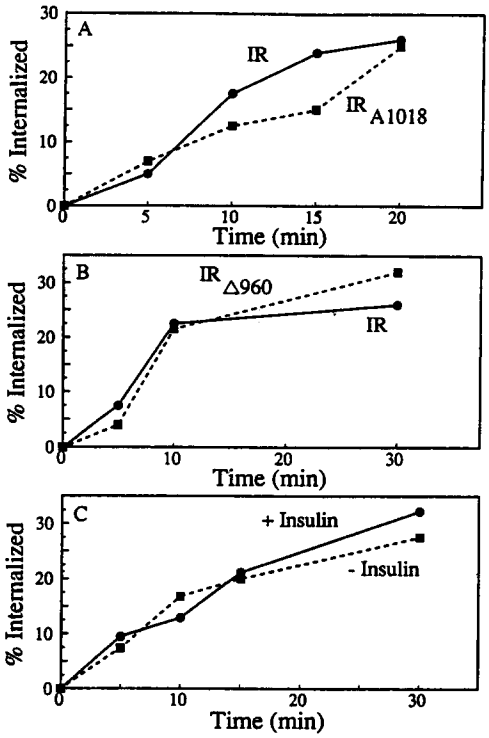


Figure 5. Internalization of surface [^{125}I]iodinated insulin receptors in CHO cells expressing normal or mutant receptors. CHO cells were labeled with [^{125}I]iodine at 4°C as described, and incubated at 37°C in the absence or presence of 100 nM insulin for varying times. The cells were trypsinized at 4°C, solubilized, and immunoprecipitated with anti-receptor antibody, and the percentage of intact insulin receptors was analyzed by reducing SDS-PAGE (*A*) (●) CHO/IR cells or (■) CHO/IR$_{A1018}$ cells in the presence of 100 nM insulin. (*B*) (●) CHO/IR cells or (■) CHO/IR$_{\Delta 960}$ cells in the presence of 100 nM insulin. (*C*) CHO/IR cells in the absence (■) or presence (●) of 100 nM insulin.

The Internalization of Wild-type and Mutant Receptors at High Insulin Concentrations

The previous experiments suggest that at high receptor occupancies, the endocytosis of [^{125}I]insulin occurs at similar velocities in CHO cells expressing wild-type or mutant receptors. Insulin receptor endocytosis was directly measured using surface [^{125}I]iodinated CHO/IR, CHO/IR$_{A1018}$ and CHO/IR$_{\Delta 960}$ cells, which were incubated at 37°C in the presence of 100 nM insulin for varying times; previous studies have shown that surface iodination does not affect insulin receptor internalization or autophosphorylation (Backer et al., 1989*a*). Internalization of surface-iodinated receptors was assessed by measuring the time-dependent accumulation of receptors resistant to trypsinization at 4°C. These experimental conditions measure the total number of cell surface receptors that internalize per unit time, and thus reflect the contributions of both ligand stimulated and constitutive internalization. Interestingly, no differences in the initial rates of insulin receptor internalization were seen when CHO/IR cells were compared to CHO/IR$_{A1018}$ (Fig. 5 *A*) or CHO/IR$_{\Delta 960}$ (Fig. 5 *B*) cells. Furthermore, the initial rate of internalization of surface-iodinated wild-type receptors was unaffected by 100 nM insulin in CHO/IR cells (Fig. 5 *C*). Thus, the net rate of receptor internalization was similar for

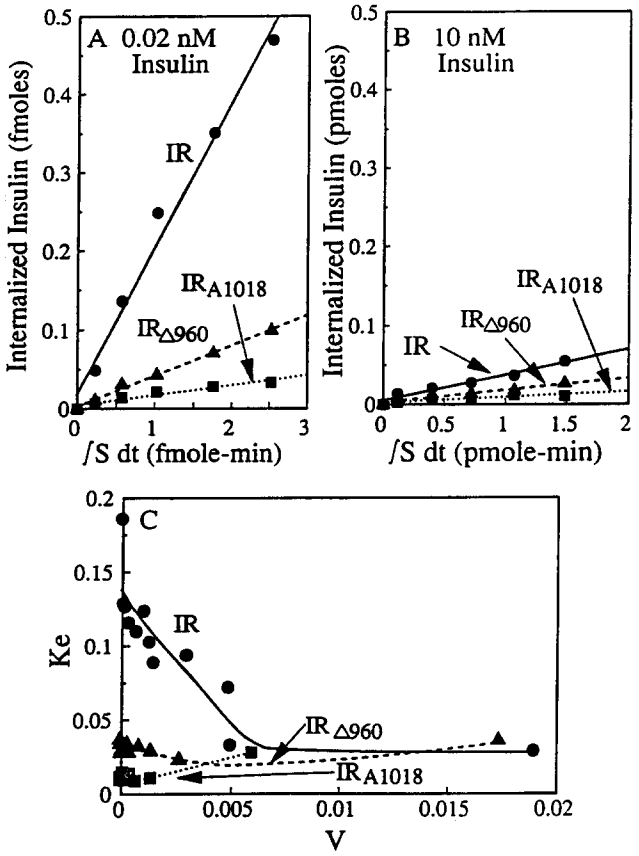


Figure 6. Quantitation of [^{125}I]insulin internalization in CHO cells at varying insulin concentrations. CHO cells expressing normal or mutant insulin receptors were incubated at 37°C with [^{125}I]insulin for varying times, and cell-associated and internalized radioactivity was determined as described in Fig. 1. Plots of internalized insulin versus the integral with respect to time of surface bound integral were constructed as described by Lund et al. (1990). (*A*) Internalization of [^{125}I]insulin by CHO/IR (●), CHO/IR$_{\Delta 960}$ (▲), or CHO/IR$_{A1018}$ (■) cells in the presence of 0.02 nM insulin. (*B*) Internalization of [^{125}I]insulin by CHO/IR (●), CHO/IR$_{\Delta 960}$ (▲) or CHO/IR$_{A1018}$ (■) cells in the presence of 10 nM insulin. (*C*) The internalization rate constant, k_e, was determined for CHO cells expressing normal or mutant receptors at each insulin concentration. The plot of k_e versus velocity was constructed as described by Lund et al. (1990) for the CHO/IR (●), CHO/IR$_{\Delta 960}$ (▲) or CHO/IR$_{A1018}$ (■) cells.

normal and mutant insulin receptors at high receptor occupancies, and this rate was not stimulated by insulin. These data are in marked contrast to the internalization of [^{125}I]insulin at physiological ligand concentrations, which clearly distinguished between wild-type and mutant cell lines (Fig. 1). These data suggest that capacity of the internalization pathway which discriminates between wild-type and mutant receptors is small relative to the total flux of receptors from the plasma membrane into the cell. However, it is also possible that this pathway is not operable at high insulin receptor occupancy levels, perhaps due to an insulin-dependent inhibition.

Quantitation of Insulin Uptake by Wild-type and Internalization-deficient Receptors

To quantitate changes in the rate of internalization of the normal and mutant receptors at varying insulin concentrations, the uptake of [^{125}I]insulin by CHO cells was analyzed as described by Lund et al. (1990). The plots of internalized [^{125}I]insulin versus the integral with respect to time of surface-bound [^{125}I]insulin were linear in all cases; at 0.02 nM insulin (Fig. 6 *A*), the resultant slopes gave a rate constant for internalization (k_e) of 0.186 ± 0.01 min^{-1} for the normal IR, 0.037 ± 0.002 min^{-1} for IR$_{\Delta 960}$ and 0.010 ± 0.001 min^{-1} for IR$_{A1018}$ (Fig. 6 *A*). Thus, the internalization rates for the IR$_{\Delta 960}$ and IR$_{A1018}$ receptors were reduced by 80 or 95%, respectively, as compared to the normal receptor. In contrast, the internalization rate constants for all three receptors were similar at 10 nM insulin (0.033 ± 0.003 min^{-1}, 0.023 ± 0.003 min^{-1}, and 0.012 ± 0.002 min^{-1} for IR, IR$_{\Delta 960}$, and IR$_{A1018}$ receptors, respectively) (Fig. 6 *B*).

The relationship between surface occupancy and internalization rate for the wild-type and mutant receptors was analyzed as described by Lund et al. (1990) for the EGF receptor. The rate constant for internalization (k_e) was determined at varying insulin concentrations and plotted against the velocity of insulin uptake at each concentration. This plot is analogous to an Eadie-Scatchard plot (Lund et al., 1990) in which the slope is the negative value of Michaelis constant k_{IC} and the X-intercept is V_{max}. In cells expressing normal insulin receptors, the Eadie-Scatchard plot was biphasic, with a rapid component that appeared to saturate at increasing insulin concentrations as well as a slow component which showed no saturable behavior (Fig. 6 *C*). Analysis of the rapid component by linear regression yielded a value for apparent half-maximal saturation of ~75,000 occupied receptors per cell, or 6% of total cell surface receptors; this level of occupancy occurred at an insulin concentration of ~2 nM (data not shown). The V_{max} value for the rapid component was 14,000 molecules/min/cell, similar to the calculated V_{max} for internalization of the EGF receptor in A431 cells (Lund et al., 1990).

In contrast to the CHO/IR cells, a flat line was obtained for the internalization-deficient CHO/IR$_{A1018}$, with no insulin-induced decrease in internalization rate constant. The plot for the IR$_{\Delta 960}$ mutant was slightly biphasic, although the slope of the rapid component could not be accurately determined. The calculated internalization rate constants of 0.01 ± 0.002 min^{-1} and 0.035 ± 0.002 min^{-1} for the CHO/IR$_{A1018}$ and CHO/IR$_{\Delta 960}$ cells, respectively, were similar to that of the slow component in the CHO/IR cells (0.03 ± 0.003 min^{-1}). Thus, both surface iodination experiments and kinetic analysis of insulin uptake show that wild-type and mutant receptors internalize at similar rates in the presence of high concentrations of insulin.

Disruption of Coated Pits Distinguishes between Wild-type and Mutant Receptor Internalization

Our data suggest that CHO cells possess two internalization pathways: a rapid pathway requiring an intact ATP binding site and juxtamembrane region, and a slow pathway which does not require autophosphorylation and which predominates at high insulin concentrations. To test this hypothesis, CHO cells expressing normal or mutant receptors were depleted of intracellular potassium, which has been shown to inhibit the formation of coated pits (Larkin et al., 1983,

1986) and is thus a pharmacological marker for coated pit-mediated internalization. The rate constant for internalization of [^{125}I]insulin was then determined in untreated and treated cells; control experiments demonstrated that the potassium-depletion protocol did not inhibit insulin receptor autophosphorylation or endogenous substrate phosphorylation (data not shown). [^{125}I]insulin internalization was inhibited by 80% in potassium-depleted CHO/IR cells, suggesting that at physiological insulin concentrations most [^{125}I]insulin uptake occurs via coated pits (Fig. 7). In contrast, [^{125}I]insulin internalization in CHO/IR$_{A1018}$ cells was not inhibited by potassium depletion. Insulin internalization by IR$_{\Delta 960}$ cells was inhibited by ~65% in potassium-depleted cells, intermediate between the CHO/IR and CHO/IR$_{A1018}$ cells. Strikingly, the rate constant for internalization of [^{125}I] insulin by wild-type cells in the presence of 100 nM cold insulin was similar to that of the IR$_{A1018}$ cells, and was unaffected by potassium depletion. These data show that insulin internalization in the IR$_{A1018}$ cells proceeds primarily via a pathway which is uneffected by potassium depletion; this pathway thus appears to be mechanistically distinct from the pathway utilized by the wild-type receptor, and may not involve coated pits. This second pathway appears to predominate even for the wild-type receptor at 100 nM insulin, consistent with the similar rates of internalization of wild-type and mutant receptors at high occupancy levels. The IR$_{\Delta 960}$ mutant appears to internalize by both pathways; the finding that these receptors retain some ability to enter the rapid, potassium-dependent pathway is consistent with data in Figs. 4 and 6 showing some saturable behavior.

Internalization of IGF-I and IGF-II in Cells Over-expressing the Wild-type Insulin Receptor

The apparent saturability of insulin internalization in the CHO/IR cells suggests that ligand-stimulated receptors may compete for entry into a rapid, coated pit–dependent pathway whose capacity is limited. Alternatively, high insulin levels may cause a general disruption of coated pit–mediated internalization. To examine this possibility, we determined the effect of insulin on the internalization of a receptor which constitutively resides in coated pits, the IGF-II/Mannose 6-phosphate receptor (Oka and Czech, 1986). Uptake of surface-bound [^{125}I]IGF-II by CHO/IR cells was only slightly affected by the presence of 100 nM insulin (Fig. 8 *C*). Similarly, calculation of the endocytic rate constant for IGF-II uptake as described (Wiley and Cunningham, 1981, 1982; Lund et al., 1990) revealed no significant differences in the absence or presence of insulin (data not shown). These data show that the effects of insulin on the internalization of the insulin receptor in CHO/IR cells did not result from a general modulation of the endocytic system or a nonspecific derangement of coated pit function.

The effect of insulin receptor occupancy on the internalization of another ligand-stimulated receptor, the IGF-I receptor, was also determined. The internalization of surface-bound [^{125}I]IGF-I in CHO/IR and control CHO/neo cells was measured in the absence or presence of 100 nM unlabeled insulin (Fig. 8, *A* and *B*). Surface-bound IGF-I was taken up rapidly in both cell lines, with maximal and identical levels of internalization reached after 10–15 min. The addition of 100 nM insulin caused a 90% decrease in [^{125}I]-IGF-I internalization by the CHO/IR cells (Fig. 8 *a*). However, insulin caused only a partial decrease in [^{125}I]IGF-I internalization by CHO/neo cells, which express 20-fold fewer insulin receptors (Fig. 8 *B*). The significantly greater inhibition of [^{125}I]IGF-I internalization by insulin in the CHO/IR cells reflects the greater level of insulin receptor expression in these cells. The decrease is unlikely to be caused by displacement of surface-bound [^{125}I]IGF-I by insulin, since little displacement of [^{125}I]IGF-I by 100 nM insulin was seen in equilibrium binding assays (data not shown). The inhibition could reflect competition between insulin receptors and chimeric insulin/IGF-I receptors. However, in control experiments cells were incubated at 4°C with [^{125}I]IGF-I in the presence 0–10^{-6} M concentrations of a monoclonal anti-insulin receptor antibody (provided by K. Siddle, University of Cambridge) which has been previously shown to inhibit [^{125}I]IGF-I binding to insulin/IGF-I chimeric receptors (Soos et al., 1990); no inhibition of [^{125}I]IGF-I binding to the CHO/IR cells by antibody was detected. The observation that the inhibition of [^{125}I]IGF-I uptake is directly related to the large number of occupied insulin receptors in CHO/IR cells suggests that insulin receptors can compete with other ligand-stimulated receptors for entry into the endocytic system.

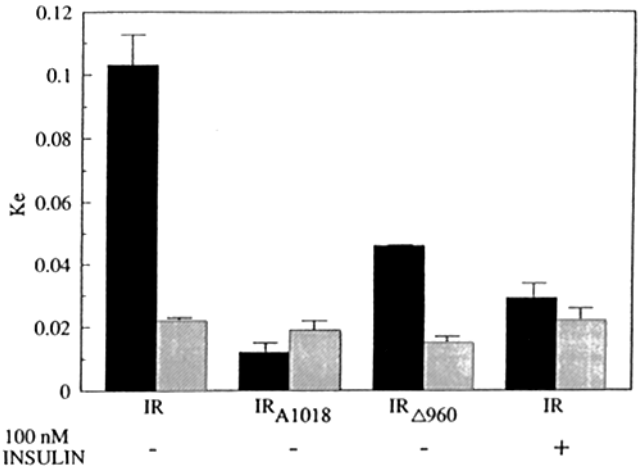


Figure 7. Internalization of normal and mutant insulin receptors in potassium-depleted CHO cells. CHO/IR, CHO/IR$_{A1018}$, or CHO/IR$_{\Delta 960}$ cells were subjected to hypotonic shock and incubated in potassium-containing or potassium-free buffer at 37°C for 30 min. The cells were then incubated for an additional 0–10 min in the same buffer containing 100,000 CPM/ml [^{125}I]insulin. The internalization of [^{125}I]insulin in each cell line was measured and analyzed in triplicate, and the internalization rate constants ± SD were calculated as described in Fig. 6. (*solid bars*) Potassium-containing buffer. (*hatched bars*) Potassium-free buffer.

Discussion

CHO cells appear to possess two distinct pathways for insulin receptor internalization that can be distinguished by their internalization rates, inhibition by potassium depletion, and response to high receptor occupancy levels. At physiological levels of insulin, wild type receptors internalize predominantly by a coated-pit dependent pathway that is 80% inhibited by depletion of cytoplasmic potassium, which disrupts coated pits (Larkin et al., 1983, 1986). Although

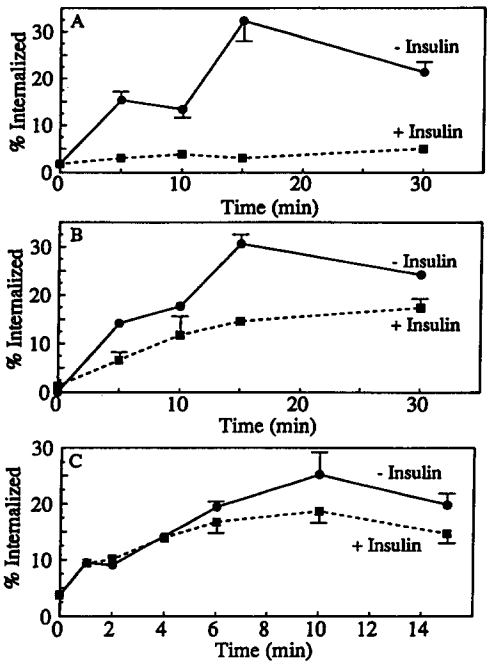


Figure 8. Internalization of surface-bound IGF-I and IGF-II in the absence or presence of insulin. CHO/IR cells (*A* and *C*) or control CHO/neo cells (*B*) were incubated with 10^5 CPM/ml [^{125}I]IGF-I or [^{125}I]IGF-II for 3 h at 4°C. The cells were washed to remove unbound ligand and incubated at 37°C for varying times in the absence or presence of 100 nM insulin. The cells were washed in acidic PBS at 4°C to remove surface-bound radioactivity, and the internalized radioactivity was calculated as percent of initial bound; all points represent the mean ± SD of triplicates. (*A* and *B*) [^{125}I]IGF-I; (*C*) [^{125}I]IGF-II. (●) no insulin; (■) 100 nM insulin.

[^{125}I]insulin internalization is rapid at physiological insulin concentrations, access of receptor/ligand complexes to this pathway decreases at increasing levels of receptor occupancy, with apparent half-maximal saturation observed at 75,000 occupied receptors/cell. In contrast, [^{125}I]insulin internalization in cells expressing a kinase deficient insulin receptor mutant is 10–20-fold slower than wild-type at physiological insulin concentrations, is not inhibited by depletion of cellular potassium, and is unaffected by increasing receptor occupancy. Thus, the kinase-deficient receptors appear to internalize primarily by a slow pathway which may not depend on coated pits. Internalization of [^{125}I]insulin in the CHO/IR$_{\Delta 960}$ cells is four- to fivefold slower than in CHO/IR cells at physiological insulin concentrations, is inhibited by potassium depletion to a lesser extent, and decreases only slightly at high insulin levels. The IR$_{\Delta 960}$ receptor thus retains some characteristics of the wild-type receptor, but has an impaired ability to enter the rapid pathway.

The rate of [^{125}I]insulin internalization by wild type receptors at high insulin concentrations is similar to that of mutant receptors and is unaffected by depletion of intracellular potassium. Thus, at high insulin concentrations, internalization of [^{125}I]insulin by the wild-type receptor is excluded from the rapid pathway and occurs predominantly by the slower alternate pathway. The shunting of wild-type receptors to the slower pathway at high ligand levels strongly suggests that the rapid uptake pathway is saturable, with a limited capacity for insulin-receptor complexes. These data could result from a specific modulation of insulin receptor endocytosis by insulin. However, the occupancy-dependent decrease in internalization rate is observed with either [^{125}I]insulin or covalently radiolabeled receptors, and is thus not due to nonspecific binding effects or negative cooperativity. It is also not due to a general insulin-stimulated perturbation of the endocytic apparatus, because IGF-II uptake is unaffected by insulin. Furthermore, the same saturable behavior is seen in cells expressing the mutant receptor IR$_{F960}$, which is defective in cellular signaling and endogenous substrate phosphorylation (White et al., 1988); therefore, the phenomenon does not depend on an intact signaling cascade. The data is best explained by a competition between ligand-stimulated insulin or IGF-I receptors for a saturable pathway, and suggests that ligand-stimulated internalization requires specific interactions between receptors and some component(s) of the endocytic system.

CHO/IR cells express ~1.2×10^6 receptors per cell, and the rapid pathway is half-saturated at an occupancy of 75,000 receptors, or ~6% of total. The contribution of this pathway is difficult to detect in a surface iodination experiment, which measures the total number of internalizing receptors and thus reflects both the saturable and non-saturable pathways; surface iodination experiments in fact could not detect a significant insulin-stimulated increase in the number of internalizing receptors in CHO/IR cells. This quantitative analysis explains why differences between the wild type and mutant receptors which are clearly seen at low concentrations of ligand are obscured at high ligand concentrations in CHO cells.

Internalization of the EGF receptor also displays saturable characteristics (Wiley, 1988; Benveniste et al., 1988). Entry of the EGF receptor into the saturable pathway appears to require receptor autophosphorylation, although the internalization of kinase deficient EGF receptor mutants remains controversial (Lund et al., 1990; Honegger et al., 1987; Chen et al., 1987, 1989; Felder et al., 1990). Thus, despite differences in intracellular itinerary (Hueksteadt, 1986; Beguinot et al., 1984), internalization of the insulin and EGF receptors appear to utilize similar mechanisms. Our data is also consistent with a previous report that the rate constant for insulin internalization decreases at high insulin levels in adipocytes (Jochen et al., 1989). Similarly, the accumulation of intracellular insulin receptors was found to be saturable in Hep G2 hepatoma cells (McClain and Olefsky, 1988). However, in this case the saturable behavior reflected an increase in receptor recycling and not a decrease in the internalization rate. The finding that the rate of insulin receptor internalization was not saturable in human hepatoma cells might be due to an unusually large endocytic capacity in these cells, or an insufficient receptor number to saturate the pathway even at high levels of receptor occupancy. Cell type–specific differences in endocytic capacity have been reported for EGF receptor internalization (Lund et al., 1990).

Our data suggest that ligand-stimulated internalization of the insulin receptor is mediated through a specific interaction between the receptor and an unidentified intracellular component, which limits the capacity of the endocytic system. At receptor occupancy levels that exceed this capacity, the excess occupied receptors spill over into the slower constitutive pathway. The ability of insulin receptors to inhibit the internalization of IGF-I receptors suggests that multiple

ligand-stimulated receptors may utilize the same presumably coated pit-dependent pathway. However, insulin and IGF-I receptors are highly homologous in their intracellular β-subunits (Ullrich et al., 1985, 1986), and it is not clear whether insulin receptors would compete with an unrelated receptor such as the EGF receptor. Insulin receptors do not interfere with the internalization of the IGF-II/mannose 6-phosphate receptor, which constitutively associates with coated pits (Oka and Czech, 1986). This data is in agreement with those of Wiley (1988), who showed that EGF does not inhibit transferrin internalization in A-431 cells. The internalization pathway which accommodates ligand-stimulated tyrosyl-kinase receptors may therefore be distinct at least in part from that which handles the internalization of the transferrin and mannose 6-phosphate/IGF-II receptors. The saturable component might in fact serve as a gating mechanism for the entry of ligand-stimulated receptors into coated pits; receptors like the mannose 6-phosphate/IGF-II receptor, which constitutively reside in coated pits, may be able to bypass this step.

Rapid insulin receptor internalization in CHO cells appears to require receptor autophosphorylation, since kinase-deficient mutants receptors are excluded. It is not clear whether receptor autophosphorylation provides an independent signal favoring rapid internalization, or causes a conformational change which exposes other regions of the receptor that are needed for rapid endocytosis. Insulin-stimulated conformational changes in the insulin receptor kinase domain have been detected with anti-peptide antibodies (Perlman et al., 1989; Herrera et al., 1985, 1986), and might play a role in endocytosis. A similar suggestion has been made for the EGF receptor, since COOH-terminal truncations partially restored rapid internalization to a kinase- and internalization-deficient mutant (Chen et al., 1989). Alternatively, it is possible that insulin-stimulated internalization is an insulin bioeffect which requires activation of the receptor kinase and transmission of an insulin signal. However, a biologically inactive mutant receptor in which Tyr_{960} is replaced by phenylalanine internalizes normally and can enter the saturable pathway (Backer et al., 1990*a*, White et al., 1988), suggesting that insulin signal transduction is not required for internalization.

The juxtamembrane deletion mutant $IR_{\Delta 960}$ also internalizes slowly. The 12 amino acids deleted from this mutant contain the sequence $NPEY_{960}$, which is similar to the sequence $NPVY_{807}$ required for rapid internalization of the LDL receptor. The Chou-Fasman algorithms for analysis of secondary structure (Chou and Fasman, 1978) predict that the insulin receptor sequence $NPEY_{960}$ specifies a tyrosine-containing β-turn (data not shown). Two recent studies have proposed that an aromatic residue in a tight turn, or a short loop stabilized by hydrogen bonding between residues on either side of the aromatic amino acid, may serve as a general internalization signal for Class I receptors (Ktistakis et al., 1990; Collawn et al., 1990). These observations suggest that the mechanisms which direct ligand-stimulated and constitutive receptors to coated pits may share some common features.

The inability of the IR_{A1018} and $IR_{\Delta 960}$ receptors to enter the saturable pathway suggests that these mutations limit interactions between the mutant receptor and a saturable component of the endocytic system. However, it must be noted that the location of the saturable step in the internalization pathway cannot be determined from biochemical studies alone, and will require a morphological characterization of the internalization defect in the $IR_{\Delta 960}$ and IR_{A1018} receptors. Thus, the decreased entry of either mutant into the saturable pathway may also result from an inability to traverse a downstream step in the endocytic pathway, resulting in an apparent inability to interact with the saturable component.

While the role of insulin receptor autophosphorylation and juxtamembrane sequences in the saturable pathway are uncertain, they do not appear to be required for entry into the nonsaturable pathway. Insulin internalization by the $IR_{\Delta 960}$ and IR_{A1018} mutants is similar to that of the wild-type receptor at high insulin concentrations, conditions under which the nonsaturable pathway predominates. Similarly, the surface iodination experiments, which measure the total number of internalized receptors and thus reflect primarily the nonsaturable pathway, suggest that the nonsaturable pathway is independent of mutations in the juxtamembrane region and receptor autophosphorylation. The latter result is consistent with the finding that the internalization of surface iodinated wild-type receptors is not stimulated by incubation with 100 nM insulin. Thus, the nonsaturable pathway is constitutive. Although the mechanism by which receptors enter the cell through the nonsaturable pathway is not clear, this pathway is not inhibited by depletion of intracellular potassium and thus may not involve coated pits. Morphological studies have in fact shown that the kinase deficient IR_{A1018} receptor does not enter coated pits in Rat-1 fibroblasts (Smith et al., 1991), although conflicting data has been reported for kinase-deficient EGF receptors in NIH 3T3 cells (Felder et al., 1990). Morphological studies on the internalization of the IR_{A1018} and $IR_{\Delta 960}$ receptors, as well as the wild-type insulin receptor at saturating insulin concentrations, will be required to determine the role of coated pits in the nonsaturable pathway in CHO cells. In this regard, the folate receptor internalizes by a pathway which does not utilize coated pits (Rothberg et al., 1990), and insulin receptor internalization has been demonstrated in rat adipocytes, which possess few coated pits (Smith and Jarett, 1983). Studies in Hep G-2 hepatoma cells have also suggested the existence of both coated pit dependent and independent pathways for insulin receptor endocytosis (McClain and Olefsky, 1988).

The identity of the saturable intracellular component that limits the capacity of the endocytic system is unknown. Pearse and co-workers have suggested that clathrin accessory proteins (adaptins) may specifically bind to the cytoplasmic tails of receptors which internalize via coated pits (Pearse, 1988). There is not yet evidence, however, for the association of adaptins with Class II receptors which lie outside coated pits, or for associations with Class I receptors in vivo. It is also not clear whether the saturable components that limit the number of class I receptors entering the endocytic system are the same as those interacting with the putative β-turn-aromatic internalization signal. The identification of the protein(s) which mediate the entry of surface receptors into the endocytic system will greatly increase our understanding of receptor-mediated endocytosis.

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