

Insulin-induced surface redistribution regulates internalization of the insulin receptor and requires its autophosphorylation

(phosphorylation/endocytosis)

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ABSTRACT The role of insulin-induced receptor autophosphorylation in its internalization was analyzed by comparing ¹²⁵I-labeled insulin (¹²⁵I-insulin) internalization in Chinese hamster ovary (CHO) cell lines transfected with normal (CHO.T) or mutated insulin receptors. In four cell lines with a defect of insulin-induced autophosphorylation, ¹²⁵I-insulin internalization was impaired. By contrast, in CHO.T cells and in two other CHO cell lines with amino acid deletions or insertions that do not perturb autophosphorylation, ¹²⁵I-insulin internalization was not affected. A morphological analysis showed that the inhibition is linked to the ligand-specific surface redistribution in which the insulin–receptor complexes leave microvilli and concentrate on nonvillous segments of the membrane where endocytosis occurs.

Characterization of the cellular and molecular mechanisms governing insulin receptor internalization will help in understanding the role of this process in insulin receptor regulation and insulin action. We have shown that the initial binding of insulin to its receptor preferentially occurs on thin digitations of the cell surface, the microvilli (1–4). The insulin–receptor complex then redistributes in the plane of the membrane and concentrates on nonvillous segments from which it is internalized by pinching off the coated pits that form coated vesicles (1–4). Through regulating the number of surface insulin receptors and by facilitating the intracellular degradation of insulin, stimulation of the internalization of the insulin receptor plays a key role in the biological action of the hormone.

The binding of insulin to the α subunit of the receptor immediately stimulates the phosphorylation of tyrosine residues of the β subunit; recent studies suggest that this tyrosine kinase activity plays a central role in the transmission of the insulin signal (5, 6). Key issues, however, remain unresolved. (i) Is autophosphorylation required for internalization? (ii) If so, how does autophosphorylation mediate internalization. In the present study, we have addressed this issue directly at the ultrastructural level. We have utilized Chinese hamster ovary (CHO) cells transfected with either normal human insulin receptors (hIRs) or hIRs mutated so as to affect various steps of insulin-induced receptor autophosphorylation.

MATERIALS AND METHODS

Cell Culture and Incubation. The generation and characterization of the stably transformed CHO cell lines CHO.T, CHO.IN7, CHO.IN56, CHO.ARG, CHO.YF3, CHO.D-30, and CHO.D-95 have been described (7–11). CHO.T cells

express the wild-type receptor that undergoes normal autophosphorylation, whereas the other six cell lines contain mutations in the β subunit that either do affect or do not affect autophosphorylation. Each of these cell lines expresses $>10^5$ hIRs per cell, except for CHO.ARG cells, which express 5×10^4 hIRs per cell. By contrast, untransformed CHO cells express 2800 rodent insulin receptors per cell (7). Cells were grown in Ham's F-12 medium supplemented with 10% fetal calf serum in 35- or 60-mm Petri dishes. Since transfected cells were selected for neomycin resistance, their growth medium contained 450 μg of the active neomycin analogue G418 per ml (11).

Before each incubation, cells were washed twice in incubation buffer containing 100 mM Hepes, 120 mM NaCl, 1.2 mM MgSO₄, 15 mM CH₃CO₂Na, 10 mM glucose, and 1% bovine serum albumin (pH 7.4) at 4°C or 37°C. Cells were then incubated either for 2 hr at 4°C or for 5, 15, 30, or 60 min at 37°C in the presence of human insulin labeled with ¹²⁵I at position 14 of the A chain (¹²⁵I-insulin; 0.03 nM) (gift from NOVO, Bagsvaerd, Denmark). At the end of these incubations, the cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min at room temperature (12).

Insulin Receptor Autophosphorylation. Insulin receptor phosphorylation was assessed as described by Kasuga *et al.* (13). Cells grown on 60-mm dishes were incubated for 2 hr in phosphate-free Krebs Ringer buffer containing 0.1 mCi of ³²P_i per ml (1 Ci = 37 GBq; Amersham) at 37°C. Phosphorylation was initiated by adding insulin (0.1 μM), and the incubation was stopped at various time intervals by removing the incubation buffer and adding liquid nitrogen. Monolayers were thawed by adding 500 μl of extraction buffer containing 1% Triton X-100 and phosphatase inhibitors. Extracts were ultracentrifuged (60,000 $\times g$ for 30 min), and solubilized receptors were precipitated with monoclonal anti-insulin receptor antibody 83-14 (14) followed by addition of 3 mg of protein A-Sepharose (Pharmacia). The washed immunoprecipitates were analyzed by SDS/PAGE and autoradiography.

Autoradiography. Fixed cells were dehydrated, processed for electron microscope (EM) autoradiography, and quantitated as described (1–3, 12). Three experiments were performed. For each incubation time analyzed, three Epon blocks were prepared and three sections were cut from each block. Thus, for each time point studied, for each cell line, 27 separate grids were examined, from which about 3000 grains were analyzed from all cells judged to be morphologically intact. Grains within a distance of 250 nm from the plasma membrane were considered associated with the cell surface, whereas grains overlying the cytoplasm and >250 nm from the plasma membrane were considered internalized. Grains

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Abbreviations: CHO, Chinese hamster ovary; hIR, human insulin receptor; EM, electron microscope.

associated with the plasma membrane were divided into the following classes: (i) microvilli, (ii) nonvillous segments, (iii) uninterpretable. Grains were considered associated with microvilli if their center was <250 nm from these surface domains, they were categorized in *iii* when the structures underlying the grain could not be unequivocally identified. In all cell lines, by 5 min of incubation at 37°C, the ratio of percent grains associated with microvilli to percent cell surface occupied by microvilli was >1, indicating that ¹²⁵I-insulin preferentially associated with microvilli at that early time point.

¹²⁵I-Insulin Degradation. Cells grown on 35-mm dishes were incubated in buffer for 2 hr at 4°C in the presence of ¹²⁵I-insulin and further incubated or not incubated for 60 min at 37°C in the same incubation medium. At the end of the 2 hr of incubation at 4°C or the subsequent 60 min of incubation at 37°C, the incubation medium was removed and the amount of degraded ¹²⁵I-insulin was measured by adding ice-cold trichloroacetic acid (final concentration, 10%) to the medium. The percentage of trichloroacetic acid-soluble radioactivity was taken as an estimate of insulin degradation.

RESULTS AND DISCUSSION

To be certain that the seven cell lines under our conditions of growth and maintenance conformed to the expected phosphorylation state, we studied phosphorylation directly. We found that CHO.IN7 and CHO.D-30 cells underwent insulin-stimulated autophosphorylation similar to that of CHO.T cells expressing normal hIR, whereas CHO.IN56, CHO.ARG, and CHO.D-95 cells underwent little or no autophosphorylation (Table 1 and Fig. 1). In several different experiments, CHO.YF3 cells with mutations at Tyr-1162 and Tyr-1163 underwent low to normal phosphorylation (Fig. 1). This is similar to the results of others who have studied this cell line (11, 15, 16).

To investigate their respective ability to internalize insulin receptors, transfected cells were exposed to a tracer concentration (0.03 nM) of ¹²⁵I-insulin at 37°C and processed for EM autoradiography; ¹²⁵I-insulin internalization was quantitated as described (12). CHO.T cells progressively internalized ¹²⁵I-insulin so that by 60 min of incubation, 35–40% of the cell-associated radioactive material was inside the cells (Fig. 2A). Similar results were obtained with CHO.D-30 cells, whereas in the case of CHO.IN7 cells, the internalization was increased (Fig. 2A). By contrast, ¹²⁵I-insulin internalization was inhibited in the four CHO cell lines transfected with mutant insulin receptors, in which the insulin-induced autophosphorylation was compromised (Fig. 2A). Interestingly, this system shows further specificity in the CHO.YF3 cell line, where Tyr-1162 and Tyr-1163 are not phosphorylated and internalization is very low. Since insulin degradation depends upon receptor-mediated endocytosis (4), we have assessed the degradation of ¹²⁵I-insulin in the incubation medium of the various CHO cell lines at the end of a 60-min incubation period at 37°C. The level of ¹²⁵I-insulin degradation was markedly reduced in CHO.ARG, CHO.IN56, CHO.D-95, and CHO.YF3 cells compared to the degradation observed in CHO.T, CHO.IN7, and CHO.D-30 cells (Fig. 3).



FIG. 1. Insulin-induced receptor phosphorylation in the various CHO cell lines under study. Cells were loaded with [³²P]orthophosphate (0.1 mCi/ml) for 2 hr and incubated with 0.1 μM insulin for 15 min. Solubilized receptors were precipitated with monoclonal antibody 83-14 (14). The immunoprecipitates were analyzed by SDS/PAGE. Gels illustrated are representative of three different experiments. In the case of CHO.YF3 cells, insulin-induced autophosphorylation varied from low to normal.

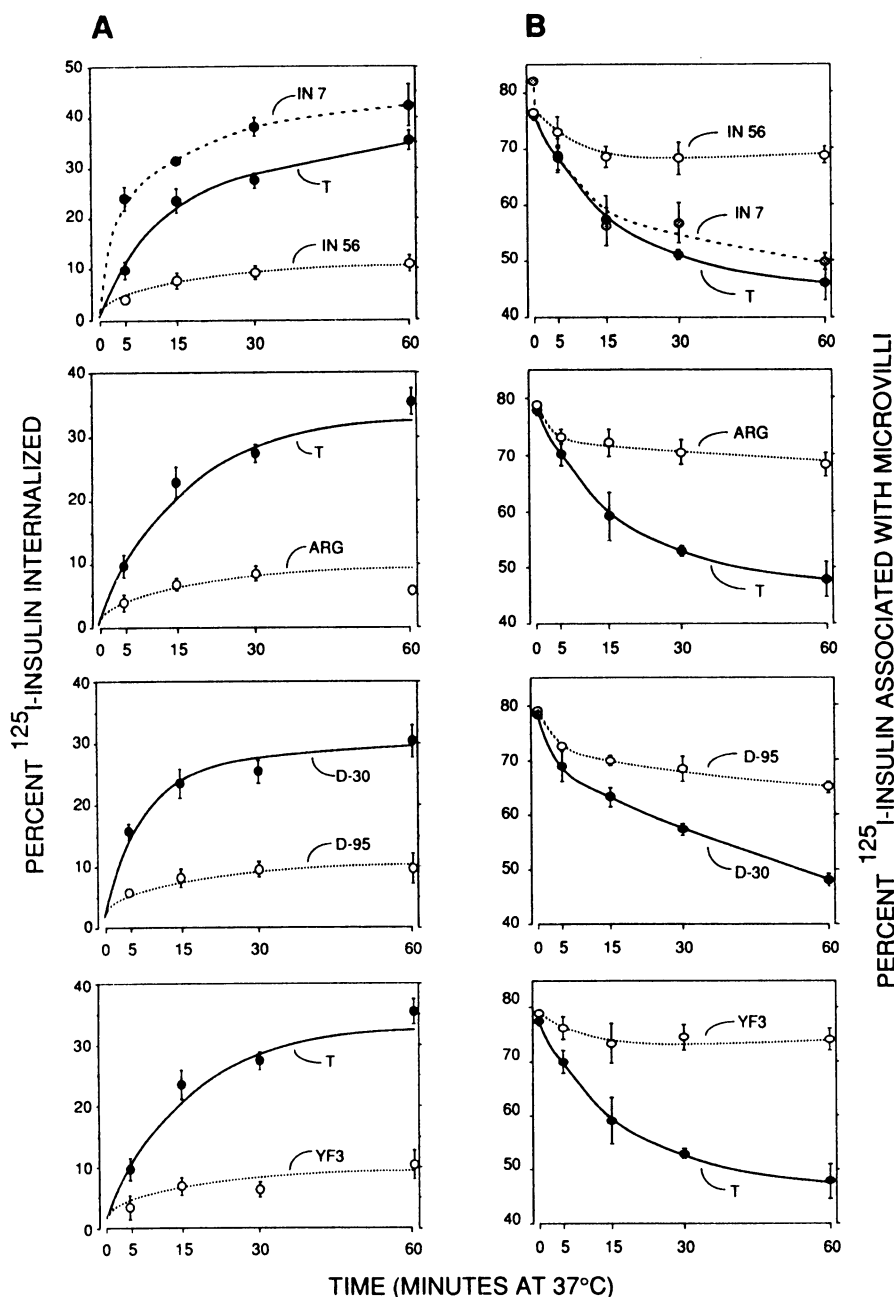
Thus ¹²⁵I-insulin internalization and degradation correlate strongly with receptor autophosphorylation. Moreover, the extent of internalization of polypeptide hormone–receptor complexes is related to their degree of lateral mobility (17). To determine whether autophosphorylation is required for insulin-induced surface redistribution of the receptor, we localized ¹²⁵I-insulin at the surface of the various CHO cells at different incubation times. As previously observed in various cell types (1–3), there is a preferential initial association of ¹²⁵I-insulin with microvilli in CHO cells (Fig. 1B). As a function of incubation time at 37°C, the association of ¹²⁵I-insulin shifted from microvilli to nonvillous domains in all cell lines in which insulin receptors were normally autophosphorylated (Fig. 2B and Fig. 4). By contrast, in autophosphorylation-deficient cells, the redistribution of ¹²⁵I-insulin was inhibited (Fig. 2B and Fig. 4). Thus, insulin-induced receptor autophosphorylation is required for the ligand-specific step of insulin receptor internalization—the insulin-induced surface redistribution of the receptor.

Previous studies have reported that inhibition of receptor autophosphorylation is associated with defective internalization as in the present work (18–20). However, others have reported that insulin internalization persists even when receptor autophosphorylation was reduced or absent. The discrepancies could be related either to the incomplete abolition of insulin-induced kinase activity (21, 22) or to the fact that the insulin receptors were cross-linked with antibodies (23). In other studies using CHO.D-95 and CHO.IN7 cells, internalization was reported to be normal and decreased, respectively (10, 24). These studies differed from ours in that the rate of internalization was lower and a photoreactive cross-linking probe was used to study internalization. Thus, there are important technical differences in the two experimental systems.

CHO cell lines that display a defect in ¹²⁵I-insulin internalization have in common the expression of hIR with compromised receptor autophosphorylation (Fig. 1), but the causes of these defects are quite different from one mutant to another: inhibition of signal transduction in CHO.IN56 cells, deletion of the C-terminal “juxta-kinase” domain in

Table 1. Characteristics of CHO cell lines transfected with normal or mutated hIR

Cell line	Characteristic	Autophosphorylation	Ref.
CHO.T	Normal hIR	+	7
CHO.IN7	Dimerization of residues 958–964	+	8
CHO.IN56	Multimerization (eight times) of residues 958–964	–	8
CHO.ARG	Substitution of lysine 1030 for arginine	–	9
CHO.D-30	Truncation of 30 C-terminal amino acids	+	10
CHO.D-95	Truncation of 95 C-terminal amino acids	–	10
CHO.YF3	Substitution of tyrosines 1162–1163 for phenylalanine	±	11



PERCENT ¹²⁵I-INSULIN ASSOCIATED WITH MICROVILLI

FIG. 2. ¹²⁵I-insulin internalization (A) and surface distribution (B) in CHO cells transfected with normal (T) or mutated hIR. Results presented are the average of three separate experiments. For each time point, and each cell line, ≈2000–3000 grains were quantitated. Dotted lines correspond to cell lines with an altered insulin-induced receptor autophosphorylation (CHO.IN56, CHO.ARG, CHO.D-95, CHO.YF3). Dashed and full curves illustrate corresponding control cell lines transfected with normal hIRs or with receptors with insertion or deletion without effect on the tyrosine kinase activity (CHO.T, CHO.IN7, CHO.D-30).

CHO.D-95 cells, mutation of the ATP binding site in CHO. ARG cells, and substitution of two crucial tyrosine residues in CHO.YF3 cells. Taken together with the observation that internalization is conserved in the case of insulin receptor mutations where a normal kinase activity was preserved (CHO.IN7 and CHO.D-30), these data suggest that inhibition of receptor internalization was not due to conformational changes in the β subunit that might affect its recognition by the endocytic system. The data with CHO.YF3 cells are particularly meaningful. As previously shown, these cells maintain measurable insulin-stimulated phosphorylation *in vivo* (11, 15, 16). Since ¹²⁵I-insulin internalization is ordinarily preserved under conditions where a residual kinase activity is measured (21, 22), we conclude that insulin receptor internalization requires not only an active kinase but also the phosphorylation of specific tyrosine residues such as the ones located at positions 1162 and 1163.

A cytoplasmic juxtamembrane domain (NPXY sequence) has recently been implicated in the coated pit internalization process of various receptors, including the insulin receptor (25–27). This sequence appears necessary for ligand-

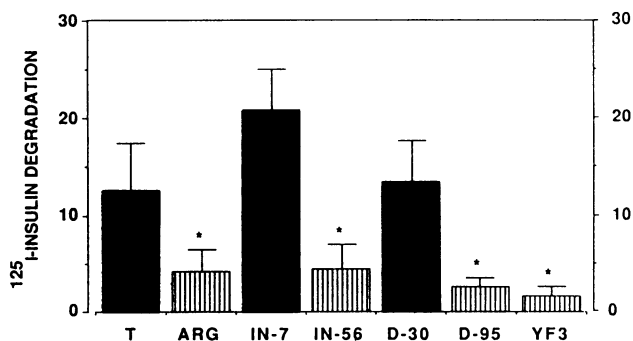


FIG. 3. ¹²⁵I-insulin degradation by the various CHO cell lines under study. Cells were incubated for 2 hr at 4°C in the presence of ¹²⁵I-insulin and further incubated for 60 min at 37°C in the same incubation medium. At the end of the incubations at 4°C or 37°C, the amount of degraded ¹²⁵I-insulin in the incubation medium was estimated by trichloroacetic acid precipitation. Values are the mean ± SEM of four experiments. Asterisks indicate *P* < 0.05 versus respective control.

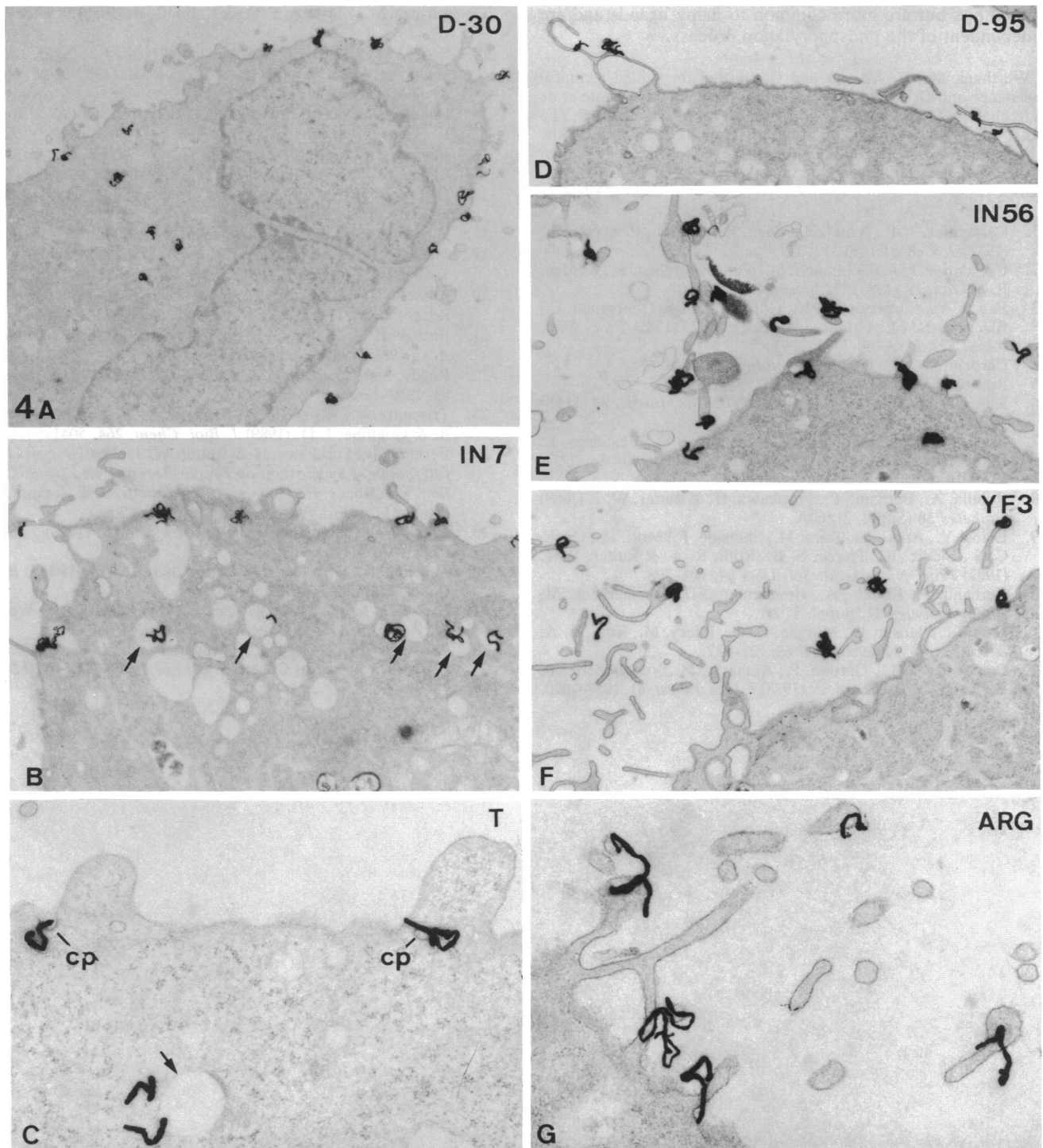


FIG. 4. Representative examples of the localization of autoradiographic grains by 30 min of incubation at 37°C in the presence of ^{125}I -insulin. In CHO.D-30 (A), CHO.IN7 (B), and CHO.T (C) cells, most grains are associated with nonvillous segments of the plasma membrane and with cytoplasmic vesicles (arrows), whereas in CHO.D-95 (D), CHO.IN56 (E), CHO.YF3 (F), and CHO.ARG (G) cells, most grains are associated with microvilli. At higher magnification (C), grains associated with nonvillous segments are frequently seen close to or overlying the coated pits (cp). (A, $\times 5800$; B, $\times 8500$; C, $\times 19,000$; D, $\times 5800$; E, $\times 8500$; F, $\times 8500$; G, $\times 24,000$.)

dependent and ligand-independent internalization; thus, this sequence must be required for a ligand-independent step [such as in the coated pit, where receptors of the two categories meet before being internalized (28)]. In the case of the insulin receptor, the activity of the NPXY sequence may be masked in the absence of insulin but may become uncovered under the influence of some ligand-mediated stimulus (25).

Our data suggest a model in which insulin binds to receptors on the microvillous surface of the cell; autophosphory-

lation of the receptor then results in redistribution of the ligand-receptor complex to the nonvillous surface. One could speculate that insulin-induced receptor autophosphorylation is the ligand-specific mechanism freeing the insulin receptor from constraints (i.e., cytoskeleton) that maintain the unoccupied receptor on microvilli. These events constitute the ligand-specific mechanism involved in the internalization process. The next steps appear more general and may involve specific sequences, such as NPXY, which affect processes in

coated pits but are more common to many ligands and are independent of the phosphorylation process.

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