Two Steps of Insulin Receptor Internalization Depend on Different Domains of the β-Subunit

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Abstract. The internalization of signaling receptors such as the insulin receptor is a complex, multi-step process. The aim of the present work was to determine the various steps in internalization of the insulin receptor and to establish which receptor domains are implicated in each of these by the use of receptors possessing in vitro mutations. We find that kinase activation and autophosphorylation of all three regulatory tyrosines 1146, 1150, and 1151, but not tyrosines 1316 and 1322 in the COOH-terminal domain, are required for the ligand-specific stage of the internalization process; i.e., the surface redistribution of the receptor from microvilli where initial binding occurs to the nonvillous domain of the cell. Early intracellular steps in insulin signal transduction involving the activation of phosphatidylinositol 3'-kinase are not required for this redistribution. The second step of internalization consists in the anchoring of the receptors in clathrin-coated pits. In contrast to the first ligand specific step, this step is common to many receptors including those for transport proteins and occurs in the absence of kinase activation and receptor autophosphorylation, but requires a juxtamembrane cytoplasmic segment of the β-subunit of the receptor including a NPXY sequence. Thus, there are two independent mechanisms controlling insulin receptor internalization which depend on different domains of the β-subunit.

CELL surface receptors taken up by clathrin-coated pits can be divided in two categories. Class I receptors, which include transport protein receptors (i.e., LDL, transferrin, asialoglycoprotein receptors), move spontaneously to clathrin-coated pits and are continuously internalized and recycled even in the absence of ligand. The preferential localization of class I receptors to clathrin-coated pits is presumably dependent on the cytoplasmic domain of these receptors (Brown et al., 1983) and various sequences putatively capable of controlling the internalization of different cell surface receptors have been proposed (Chen et al., 1990; Lazarovits and Roth, 1988; Ktistakis et al., 1990; Collawn et al., 1990; Bansal and Giersasch, 1991; Lobel et al., 1989; Girones et al., 1991). The only common features of these sequences identified thus far are the presence of aromatic residues and the propensity of the amino acid involved to form exposed tight beta turns. Most evidence suggests that these determinants interact specifically with one or more components of clathrin-coated pits such as the 110-kD protein termed β-adaptin which is part of a complex of proteins that also serves to bind clathrin to the plasma membrane (Pearse and Robinson, 1990; Pearse, 1988; Ahles et al., 1988; Beltzer and Spiess, 1991).

In contrast to the class I nutrient receptors, signaling receptors (class II receptors, i.e., insulin and EGF receptors) are not trapped in clathrin-coated pits unless they have bound their ligand (Brown et al., 1983). Their internalization is more complex and involves a higher degree of specificity than class I receptors. The cytoplasmic domain of these receptors contains an intrinsic tyrosine kinase which is activated by ligand binding (Yarden and Ullrich, 1988). In the case of the insulin receptor, endocytosis is triggered by hormone binding (Carpentier et al., 1984; Backer et al., 1989*a*) and, recent evidence from our laboratories has highlighted the central importance of insulin-induced kinase activation in the first step of internalization: the translocation of the receptors from microvilli where they concentrate in their unoccupied form towards the nonvillous area of the cell membrane where clathrin-coated pits are localized (Carpentier et al., 1992). For the EGF receptor, another example of class II receptor, internalization eventually occurs via the same clathrin-coated pits as those used by class I receptors (Carpentier et al., 1982). Autophosphorylation itself is not a sufficient signal to induce internalization. Likewise, deletion of a portion of the intracellular juxtamembrane domain of the insulin receptor which has a minimal effect on autophosphorylation inhibits insulin-stimulated endocytosis

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(Backer et al., 1990; Thies et al., 1990). Since this portion contains a NPXY consensus motif similar to that in the LDL receptor, it is probable that a second regulatory step, common to many receptors including class I receptors, is involved in insulin receptor internalization. Thus, internalization of the insulin receptor appears to involve two potential levels of regulation: a first one controlled by insulin binding and kinase activation, and a second one more general and common to a large number of receptors. The aim of the present work was to characterize these two steps of insulin receptor internalization, to distinguish the exact structural determinants of the β-subunit which are required for each of them and to determine whether internalization is dependent on the early stages of post-receptor insulin signal transduction. To this end, we have characterized morphologically and biochemically, the stages of internalization for insulin receptors possessing a variety of in vitro mutations.

Materials and Methods

Cell Culture and Incubation

The generation and characterization of the stably transformed CHO cell lines [CHO-HIRC, CHO-Δ960, CHO-A1018, CHO-F960, CHO-ΔCT, CHO-F1146, and CHO-F3TYR] expressing the wild-type (CHO-HIRC) and mutated (the others) human insulin receptor (HIR)[1] have been described previously (Backer et al., 1990; Myers et al., 1991; Wilden et al., 1990; White et al., 1988). Each of these cell lines expresses $>10^6$ HIRs per cell, except for CHO-F960 which expresses 5×10^5 HIR per cell. By contrast, untransformed CHO cells express 3×10^2 rodent insulin receptors/cell. Cells were grown in Ham's F-12 medium supplemented with 10% FCS in 35- or 60-mm dishes. All transfected cells were selected for using neomycin resistance and their growth media contained 450 μg/ml of the active neomycin analogue G418.

Before each incubation, cells were washed twice in incubation buffer containing 100 mM Hepes, 120 mM NaCl, 1.2 mM $MgSO_4$, 15 mM CH_3COONa, 10 mM glucose, and 1% BSA (pH 7.4) at 4°C or 37°C. Cells were then incubated either for 2 h at 4°C or for 5, 15, 30, or 60 min at 37°C in the presence of human A14 monoiodo ^{125}I-insulin (3.10^{-11} M) (a generous gift from NOVO, Bagsvaerd, Denmark). At the end of these incubations, the cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 30 min at room temperature.

Insulin Receptor Autophosphorylation

Insulin receptor phosphorylation was assessed by Western blotting with α anti-phosphotyrosine (αPY) antibodies. Cell lysates for Western blot analysis were performed by incubating cells in six-well dishes in the absence or presence of insulin for 2 min, followed by lysis in Laemmli sample buffer (Laemmli, 1974), boiling for 2 min and sonication for 2 s. Proteins were separated on 7.5% resolving SDS-PAGE minigels (Hoeffer Sci. Instrs., San Francisco, CA), transferred to nitrocellulose in Towbin buffer containing 0.02% SDS, and blotted with αPY antibodies as indicated. Proteins were visualized using ^{125}I-protein A (ICN Biomedicals, Costa Mesa, CA) and autoradiography.

Autoradiography

Fixed cells from two different experiences were dehydrated, processed for EM autoradiography, and quantitated as previously described (Carpentier et al., 1978). 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, 18 separate grids were examined out of which ~2,000 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; grains overlying the cytoplasm and >250 nm from the plasma membrane were considered internalized. Grains associated with the plasma membrane were divided into the following classes: (*a*) microvilli, (*b*) clathrin-coated pits, (*c*) nonvillous nonclathrin-coated pits segments, and (*d*) uninterpretable. Grains were considered associated with microvilli or clathrin-coated pits if their center was <250 nm from these surface domains, they were categorized in (*d*) 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 over percent cell surface occupied by microvilli was higher than one indicating that ^{125}I-insulin preferentially associated with microvilli at that early time point.

Morphometry

Evaluation of the percentage of the total cell surface occupied by microvilli and clathrin-coated pits was carried out on randomly photographed pictures of CHO cells. Twelve pictures were taken from four different Epon blocks ($4 \times 12 = 48$) at an initial magnification of ×19,000. The total plasma membrane length, as well as the respective length of microvilli and clathrin-coated pits, were determined on negatives projected onto a graphic tablet (Tektronic, type 4953) connected with a microprocessor (IBM AT), allowing the different parameters to be recorded and statistically analyzed.

Quick Freezing

To visualize the cyroplasmic face of the plasma membrane and the typical clathrin coats, plasma membrane of CHO cells were prepared according to Moore et al. (1987) and Lin et al. (1991) with some modifications and replicas were performed by the quick-freeze rotary shadowed technique of Heuser (1989). Briefly, cells were sedimented on formvar nickel grids coated with poly-L-lysine (1 mg/ml). Adherent cells were disrupted in hypotonic medium (25 mM Hepes, 25 mM KCl, 2.5 mM Mg acetate and 0.2 mM DTT, pH 70) by gentle sonication. Grids were extensively washed to eliminate cell debris and immediately fixed in 0.5% glutaraldehyde for 15 min at 4°C followed by 15 min at room temperature. Grids were next extensively rinsed in water and rapidly frozen in liquid nitrogen. They were then etched in a Balzers 400 (Balzers, Liechtenstein) rotative plate device. Replicas were prepared by platinum and carbon evaporation.

Measurements of the mean size of clathrin-coated pits were carried out on photographic prints at a final magnification of 147,000× onto the same graphic tablet as described above. In each cell line studied, 20 cells were photographed and the diameter of a mean of four clathrin-coated pits were measured per cell.

Results

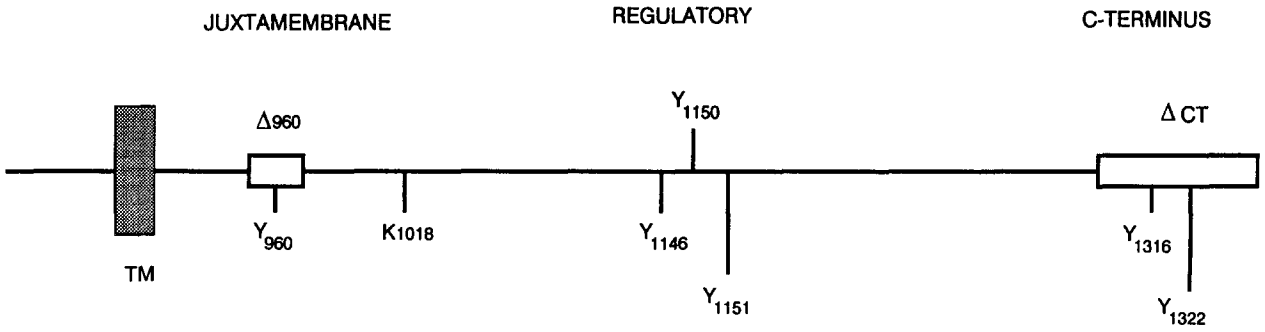
Expression and Autophosphorylation of the Normal and Mutated Insulin Receptors in CHO Cells

The various CHO cell lines employed in this study have been described previously (Chou et al., 1987; Backer et al., 1990; Myers et al., 1991; Wilden et al., 1990; White et al., 1988) and their characteristics are summarized in Fig. 1. All cell lines expressed $>10^6$ receptors/cell. Autophosphorylation of the insulin receptor β-subunit from insulin-stimulated CHO cells expressing the normal receptor (CHO-HIRC), the receptor with a deletion of 12 amino acids in the juxtamembrane domain including the NPXY (CHO-Δ960), a point mutation of Tyr960 in the NPXY to Phe (CHO-F960), and a COOH-terminal deletion mutant lacking two autophosphorylation sites (CHO-ΔCT) was similar (Fig. 2). By contrast, insulin-induced autophosphorylation of the ATP-binding site mutant (CHO-A1018) cells was undetectable, and phosphorylation of CHO-F1146 and CHO-F3TYR (containing Tyr to Phe mutations in one or all three tyrosines in the regulatory domain) was moderately to severely reduced, respectively (Fig. 2), in agreement with previous studies (Wilden et al., 1990).

^{125}I-Insulin Internalization in CHO Cells Expressing Normal or Mutated Insulin Receptors

To investigate their ability to internalize insulin receptors,

1. *Abbreviations used in this paper*: αPY, anti-phosphotyrosine; HIR, human insulin receptor.



	HIR	A 1018	ΔCT	F 3TYR	F 1146	Δ960	F 960
MUTATION	Wild Type	K_{1018} to A_{1018}	43 aa truncation (A_{1301}–S_{1343})	Y_{1146} to F_{1146} Y_{1150} to F_{1150} Y_{1151} to F_{1151}	Y_{1146} to F_{1146}	A_{954}-N_{965} deleted	Y_{960} to F_{960} S_{962} to T_{962}
REFERENCE	Ullrich & al. (1985)	Chou & al. (1987)	Myers & al. (1991)	—	Wilden & al. (1990)	Backer & al. (1990)	White & al. (1988)

Figure 1. Characteristics of mutant insulin receptors. (*Upper part*) A linear model adapted from Myers et al. (1991) of the intracellular domain of the β-subunit indicates the relative position of the transmembrane region (*TM*) and the juxtamembrane, regulatory, and COOH-terminal regions. (*Lower part*) The characteristics and the references of the various mutant insulin receptors used in the present study are listed.

transfected cells were exposed to tracer concentration (3×10^{-11} M) of ^{125}I-insulin at 37°C, processed for EM autoradiography, and ^{125}I-insulin internalization was quantitated as previously described (Carpentier et al., 1978). CHO-HIRC cells progressively internalized ^{125}I-insulin so that by 60 min of incubation, 35–40% of the cell-associated radioactive material was inside the cells (Fig. 3 *A*). In CHO A1018 cells, in which the insulin receptor tyrosine kinase was inactive, ^{125}I-insulin internalization was markedly reduced (Fig. 3 *A*). To determine whether an intact kinase was sufficient or whether, in addition, autophosphorylation of specific tyrosine residues was required, we followed ^{125}I-insulin internalization in cells expressing insulin receptors with deletion or substitution of the major sites of autophosphorylation. Deletion of 43 amino acids of the β-subunit (CHO-ΔCT), which removed two major autophosphorylation sites (1316 and 1322) but maintained a normal kinase activity, was without effect on ^{125}I-insulin internalization (Fig. 3 *B*). By contrast, substitution of the three tyrosines of the regulatory region (1146, 1150, and 1151) for phenylalanine resulted in a decrease in ^{125}I-insulin internalization similar to that of the A1018 mutant (Fig. 3 *B*). Even substitution of tyrosine 1146 alone (CHO-F1146) was sufficient to markedly reduce ^{125}I-insulin internalization in spite of the fact that the insulin

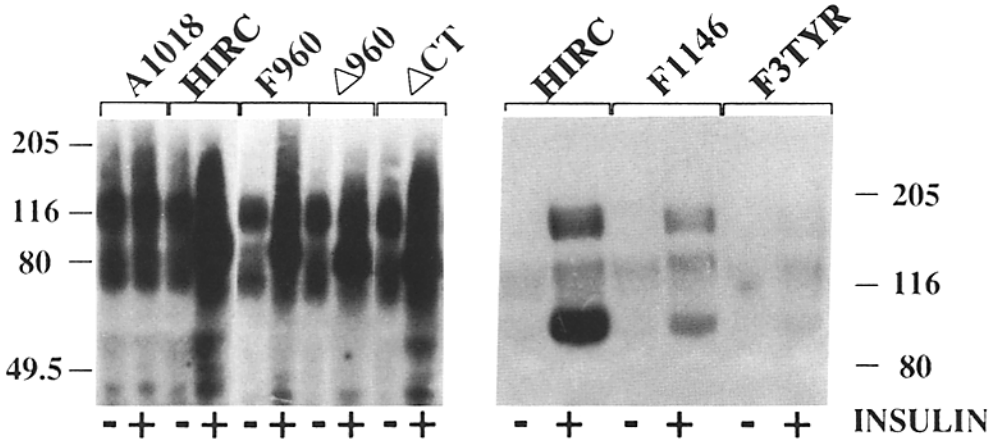


Figure 2. Insulin receptor phosphorylation assessed by Western blotting with αPY antibodies. CHO cells expressing wild-type (HIRC) or mutated insulin receptors (the others) were incubated in absence (−) or presence (+) of 0.1 M of insulin for 2 min, solubilized, electrophoresed on 7.5% SDS-PAGE gel, and transferred to nitrocellulose. Phosphotyrosine residues were visualized on nitrocellulose blots by αPY antibodies, followed by $^{(125)}$protein A and autoradiography. Insulin-induced tyrosine phosphorylation is observed as an increase in radioactivity of the β-subunit (95-kD band).

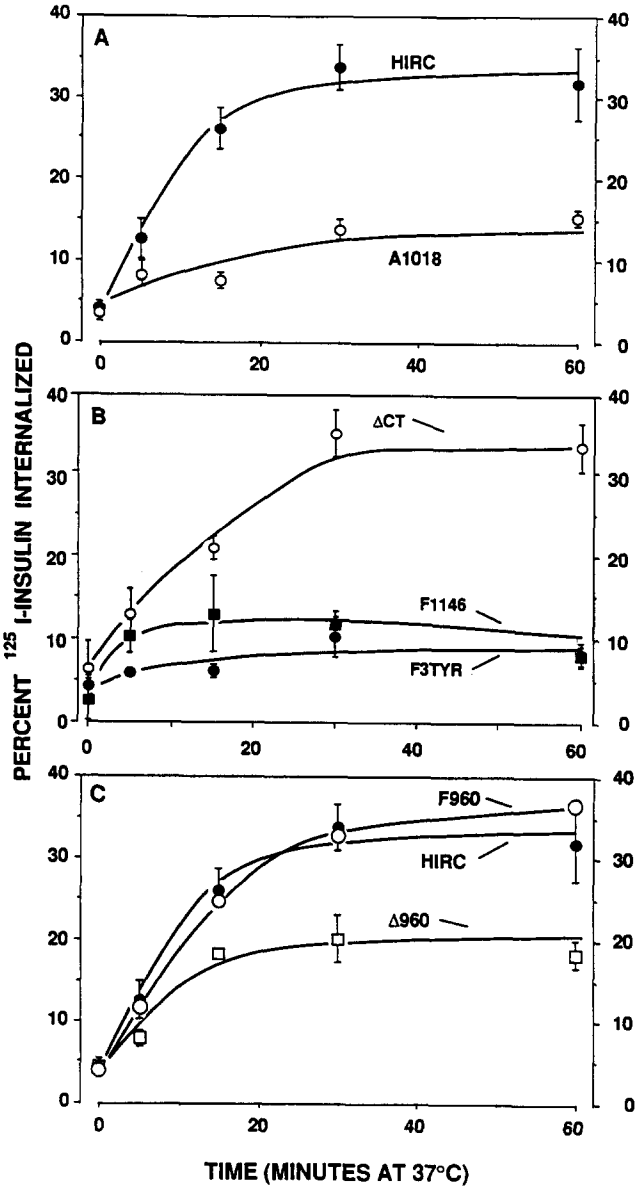


Figure 3. ^{125}I-Insulin internalization in CHO cells transfected with normal (*HIRC*) or mutated (all the others which characteristics are detailed in Fig. 1) insulin receptors. Results presented are the average of the analysis of three different Epon blocks from two different experiments (n = 6). For each time point and each cell line ≅2,000 autoradiographic grains were quantitated. Results are expressed as percent of the total number of grains associated with the cells which center was >250 nm from the plasma membrane.

receptor kinase was activated to ~50% of normal levels (Fig. 3 *B*). Thus insulin receptor internalization requires not only an intact and activable kinase, but also the autophosphorylation of all three tyrosines of the regulatory domain of the receptor.

But, are these necessary events sufficient for the internalization of the insulin receptor in response to insulin binding? Since an amino acid sequence present in the juxtamembrane domain of various receptors could play a role in their endocytosis (Chen et al., 1990; Lazarovits and Roth, 1988; Ktistakis et al., 1990; Collawn et al., 1990; Bansal and Giersasch, 1991; Lobel et al., 1989; Girones et al., 1991), we focused our interest on the juxtamembrane domain of the β-subunit of the insulin receptor. In CHO cells expressing the Δ960 mutant receptor (a deletion of 12 amino acids in the juxtamembrane domain (amino acids 954-965, which include the above-mentioned amino acid sequence), receptor autophosphorylation was normal or near normal (Fig. 2) but internalization of ^{125}I-insulin was markedly reduced (Fig. 3 *C*). It has been suggested that the presence of an aromatic residue in the tyrosine position of NPXY sequence is required for internalization to take place (Davis et al., 1987). This also appears to be true for the insulin receptor (Rajagopalan et al., 1991; Backer et al., 1992). ^{125}I-Insulin internalization in CHO-F960 cells, with a substitution of tyrosine 960 for phenylalanine, is virtually identical to that in CHO-HIRC cells (Fig. 3 *C*).

^{125}I-Insulin Localization on the Surface of CHO Cells Expressing Normal and Mutated Insulin Receptors

Insulin-induced internalization of the insulin receptor requires surface redistribution of the receptor from microvilli to the nonvillous area followed by association of the receptors with clathrin-coated pits which represent the internalization gates (Carpentier et al., 1982, 1985, 1992; Fan et al., 1982). To determine the possible roles of the kinase region, the autophosphorylation sites, and the juxtamembrane domain of the insulin receptor β-subunit in these two steps, we localized ^{125}I-insulin on the surface of the CHO cell lines expressing normal and mutant receptors at different incubation times.

As previously observed in other cell types (Carpentier et al., 1982, 1985, 1992; Fan et al., 1982), initial binding of ^{125}I-insulin to CHO cells preferentially occurs on microvilli (Figs. 4 and 5, and Table I). As a function of incubation time at 37°C, ^{125}I-insulin shifted from microvilli to the nonvillous domains in cells expressing insulin receptors with an active kinase and normal autophosphorylation of the regulatory region (CHO-HIRC, CHO-Δ960, CHO-ΔCT) (Figs. 4 and 5). By contrast, in kinase-inactive cells (CHO A1018), as well as in cells with substitution of tyrosine residues at position 1146 or positions 1146, 1150 and 1151, the redistribution of ^{125}I-insulin was lost (Figs. 4 and 5). These results confirm that insulin activation of the receptor kinase is required for the initial step of the internalization of the receptor, its surface redistribution. Taken together with our previous observation that substitution of the two tyrosines at positions 1150 and 1151 also reduced surface redistribution of the receptor, the present data demonstrate that tyrosines 1146, 1150, and 1151 in the regulatory domain of the β-subunit are required for insulin to induce normal redistribution of its receptor. By contrast, autophosphorylation of tyrosines 1316 and 1322 is not implicated in the redistribution process.

As many other ligand-receptor complexes, in most cell types, the insulin-receptor complexes are internalized via clathrin-coated pits (Carpentier et al., 1982, 1985, 1992; Fan et al., 1982). In CHO-HIRC cells, ^{125}I-insulin association with clathrin-coated pits progressively increased with incubation time at 37°C (Figs. 4 and 6) consistent with the fact that the ligand is internalized via this gating mechanism in these cells. Similar results were obtained in CHO-ΔCT cells (Fig. 6). By contrast, in spite of its shift towards the

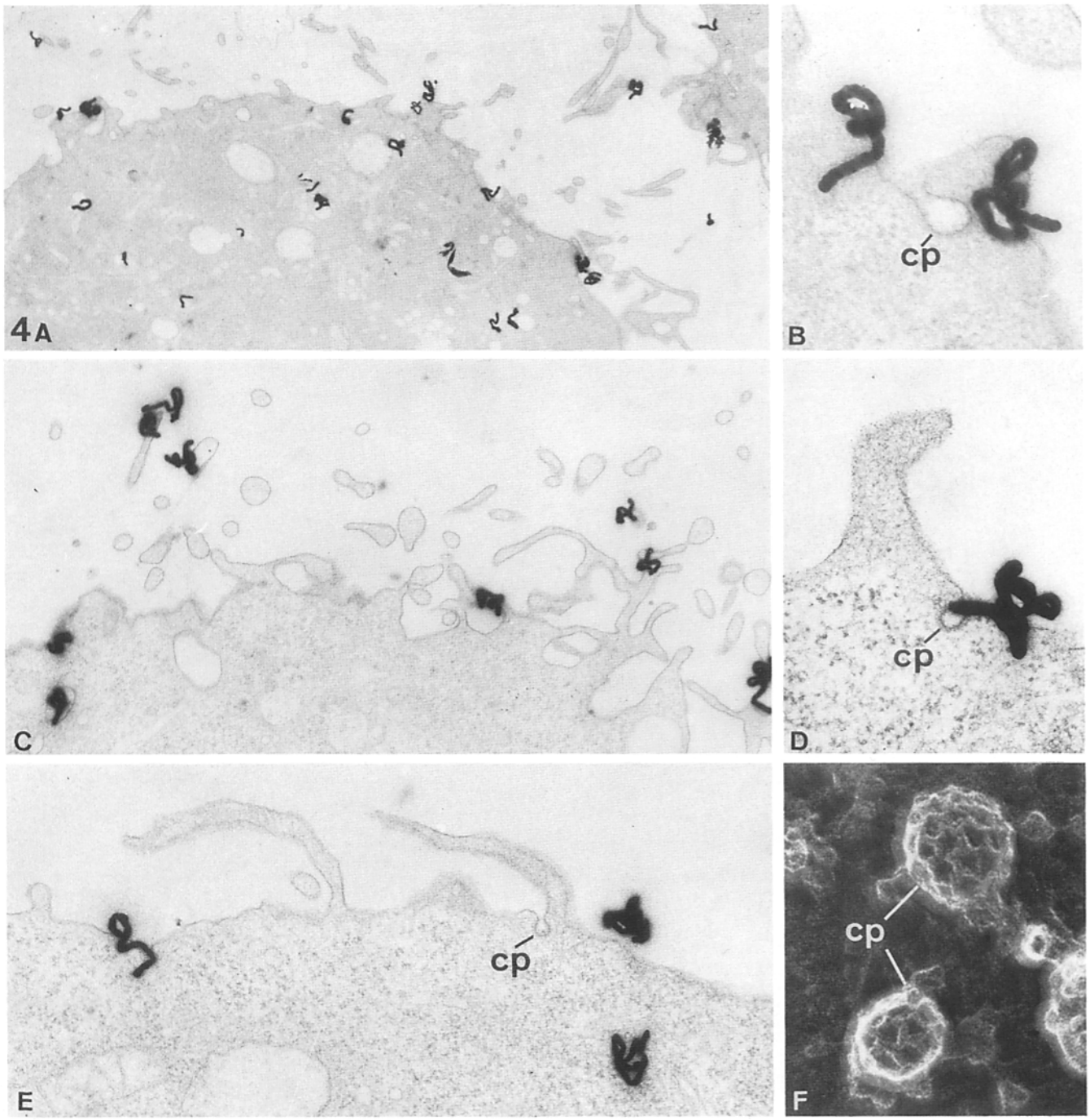


Figure 4. (*A–E*) Representative examples of the localization of autoradiographic grains after 30 min of incubation of the various CHO cells in the presence of ^{125}I-insulin. In CHO-HIR, CHO-ΔCT, and CHO-F960 cells (*A*; *CHO-ΔCT*), ^{125}I-insulin is mostly associated with nonvillous domains of the plasma membrane or internalized. At higher magnification in the same cell lines (*B*; *CHO-HIR*), grains are seen in the vicinity of clathrin-coated pits (*cp*). In CHO-A1018, CHO-F3TYR, and CHO-F1146 autoradiographic grains remain mostly associated with microvilli (*C*; *CHO-F3TYR*). But grains present on the nonvillous domain of the cells are frequently seen close to clathrin-coated pits (*D*; *CHO-A1018*). In CHO.Δ960 cells (*E*), autoradiographic grains are neither seen concentrated on microvilli nor in clathrin-coated pits (*cp*) but are seen on noninvaginated regions of the nonvillous domain of the cell surface. As seen on quick frozen rotatory shadowed replicas, clathrin-coated pits present a general three-dimensional organization which is similar in CHO-HIR and CHO-Δ960 cells (*F*). (*A*) ×8,000; (*B*) ×56,000; (*C*) ×15,000; (*D*) ×46,000; (*E*) ×22,000; (*F*) ×170,000.

nonvillous region of the cell surface, ^{125}I-insulin did not concentrate in clathrin-coated pits in CHO-Δ960 cells (Fig. 6). In the case of CHO-A1018, CHO-F1146, and CHO-F3TYR cells, the percentage of autoradiographic grains associated with clathrin-coated pits remained low at all time points (Fig. 6). This was due to the fact that a large majority (70–75%) of the grains remained on microvilli at all time points (Fig. 5). Indeed, when the association of the radioactivity with clathrin-coated pits was calculated in terms of the autoradiographic grains present on the nonvillous surface, a

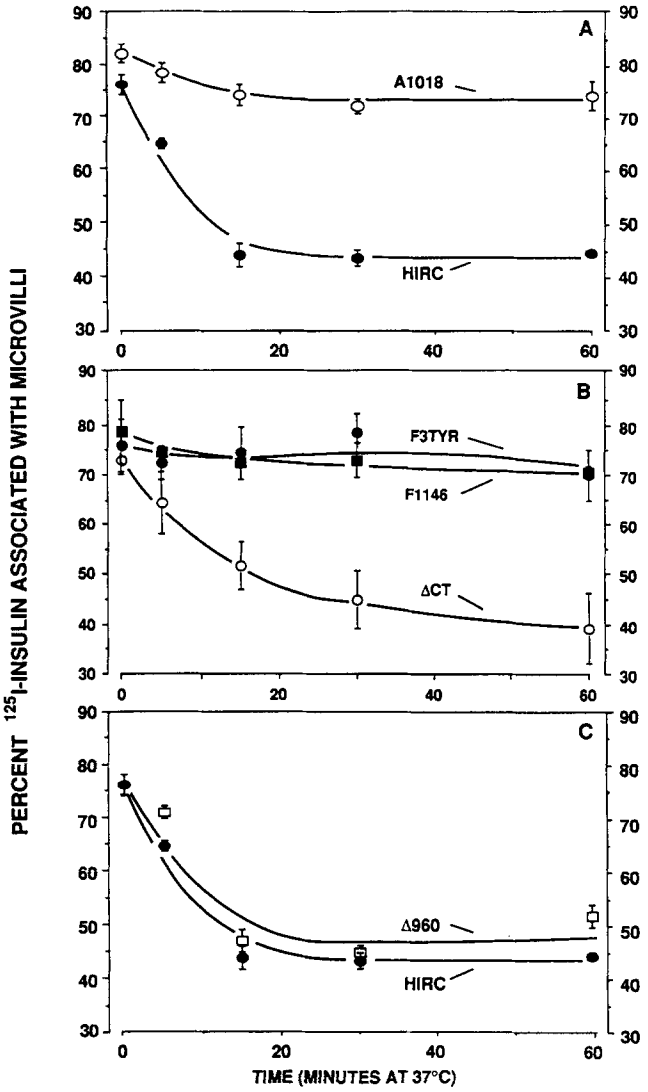


Figure 5. Surface redistribution of ^{125}I-insulin in CHO cells transfected with normal (*HIRC*) or mutated (all the others which characteristics are detailed in Fig. 1) insulin receptors. Results presented are the average of the analysis of three different Epon blocks from two different experiments (n = 6). For each time point and each cell line ≅2,000 autoradiographic grains were quantitated. Results are expressed as percent of the total number of grains associated with the cell surface (±250 nm from the plasma membrane) which center were within a distance of 250 nm from a microvilli.

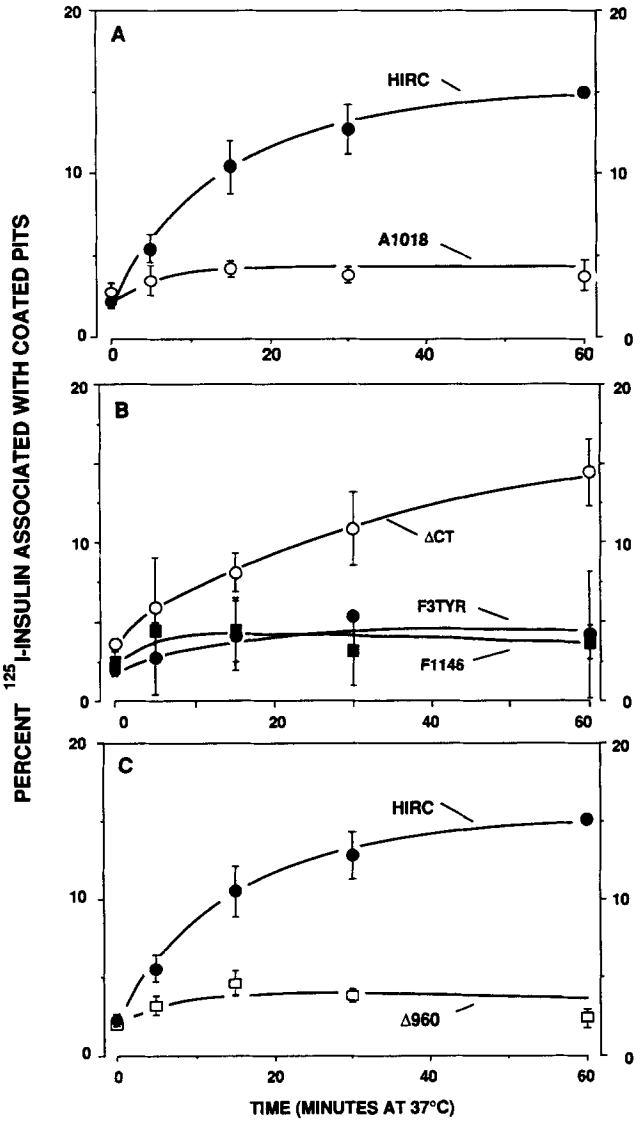


Figure 6. Association of ^{125}I-insulin with clathrin-coated pits on the total surface of CHO cells transfected with normal (*HIRC*) or mutated (all the others which characteristics are detailed in Fig. 1) insulin receptors. Results presented are the average of the analysis of three different Epon blocks from two different experiments (n = 6). Results are expressed as percent of the total number of grains associated with the cell surface (±250 nm from the plasma membrane) which center were within a distance of 250 nm from a clathrin-coated pit.

Table I. Initial Localization of ^{125}I-Insulin on the Surface of CHO Cells

CHO cell lines	Percent ^{125}I-insulin associated with microvilli*	Percent total cell surface occupied by microvilli‡	Ratio
HIRC 2	75.9 ± 2.0	58.3 ± 2.9	1.30
A 1018	81.9 ± 1.8	62.7 ± 2.0	1.31
ΔCT	72.8 ± 1.4	57.7 ± 1.7	1.26
F3TYR	75.9 ± 3.1	56.2 ± 1.9	1.26
F 1146	78.8 ± 3.9	61.1 ± 1.5	1.29
Δ960	75.2 ± 0.9	54.6 ± 3.6	1.38

* Values presented correspond to the 2-h 4°C incubation. In each case n = 6.
‡ n = number of structures analyzed, respectively, from top to bottom: 51, 50, 86, 90, 90, and 43.

similar percentage of grains was associated with clathrin-coated pits in CHO-A1018 cells, CHO-F1146 and CHO-F3TYR cells as in CHO-HIRC and CHO-ΔCT (Table II). By contrast, this value was more than the double of the one obtained in CHO-Δ960 cells (Table II). These results demonstrate that the juxtamembrane domain of the insulin receptor is required for the anchoring of the receptor in clathrin-coated pits and that kinase activation is not needed for rendering this domain of the receptor internalization-competent.

Characteristics of Microvilli and Clathrin-coated Pits on the Surface of the Various CHO Cells

To determine whether the impaired association of insulin

Table II. ^{125}I-Insulin Association with Clathrin-coated Pits

	Total surface*	Nonvillous surface*
HIRC	10.5 ± 1.9	19.1 ± 2.4
A 1018	4.0 ± 0.2	17.5 ± 1.7
ΔCT	9.7 ± 1.8	17.0 ± 2.3
F3TYR	4.6 ± 0.6	18.5 ± 3.5
F 1146	4.1 ± 0.3	17.3 ± 0.7
Δ960	3.5 ± 0.5	7.8 ± 1.2

* Percentage of autoradiographic grains present on, respectively, the total and nonvillous cell surface, that were found associated with clathrin-coated pits. Values are the mean of the values obtained at the 5, 15, 30, and 60 min time points ± SEM.
(n = number of time points = 4)

Table III. Characteristics of Clathrin-coated Pits in CHO Cells

CHO cell lines	Percent cell surface occupied by clathrin-coated pits*		Clathrin-coated pits diameter
	(*2 h 4°C*)	(*60 min 37°C*)	(*nm*)‡
HIRC	1.14 ± 0.32	0.93 ± 0.27	105.9 ± 18.3
A 1018	0.95 ± 0.21	0.90 ± 0.20	108.4 ± 18.3
ΔCT	1.09 ± 0.20	–	–
F3TYR	0.92 ± 0.13	–	–
F 1146	1.16 ± 0.21	–	–
Δ960	0.98 ± 0.26	1.08 ± 0.29	101.7 ± 9.3

* Quantifications carried out on thin sections. Values are mean ± SEM; n = number of cells analyzed from top to bottom: 2 h 4°C = 51, 50, 86, 90, 90, and 43. 60 min 37°C = 45, 50, and 47.
‡ Quantification carried out on quick-freezing replicas. Values are mean ± SEM; n = number of cell analyzed = 20, 17, and 21, respectively.

receptors with clathrin-coated pits in CHO-Δ960 cells was related to an abnormal clathrin-coated pit structure, the organization of these pits and of their clathrin basket was morphologically characterized. A quantitative analysis of the percentage of the total cell surface occupied by microvilli and clathrin-coated pits and of the number of clathrin-coated pits per micron square in the various cell lines, before or at the end of the 37°C incubation, indicate that these parameters were affected by neither the transfections of the various mutated insulin receptors nor the incubation conditions (Table III).

The three dimensional visualization of the cytoplasmic face of the inner leaflet of the plasma membrane by the quick freezing technique allowed a careful analysis of the organization and diameter of clathrin-coated pits. As illustrated in Fig. 4 and as shown by the quantitative analysis in Table III, clathrin-coated pits were not affected by the various transfections studied.

Discussion

In this report, we have studied the role of the cytoplasmic portion of the β-subunit of the insulin receptor in insulin-induced receptor internalization in order to establish the role of various domains of the β-subunit in the process. Based on previous studies, four domains can be defined in the intracellular portion of the receptor β-subunit: a juxtamembrane domain involved in substrate binding, an ATP-binding domain critical for kinase activity, a regulating domain containing three tyrosine sites of autophosphorylation (1146, 1150, and 1151) whose autophosphorylation is important for full kinase activation, and a COOH-terminal domain containing two additional autophosphorylation sites and which have been suggested to be, in some studies, important in insulin's growth effects (Kahn and White, 1988; Thies et al., 1989). The data of the present study demonstrate the existence of two cell surface mechanisms controlling insulin receptor internalization which depend on different domains of the β-subunit. The first step is ligand-specific. It is mediated by insulin-induced receptor kinase activation and autophosphorylation of three tyrosine residues: 1146, 1150, and 1151, but does not depend on the cascade of phosphorylation leading to the activation of phosphatidylinositol 3′-kinase. It consists in the surface redistribution of insulin-receptor complexes from microvilli, where the initial interaction between the receptor and the hormone occurs, to the nonvillous region of the cell surface. The second step is more common to many receptors, including those which are not autophosphorylated and consists in the anchoring of the receptor in clathrin-coated pits. This step requires the presence of a preserved juxtamembrane domain, and especially of an aromatic residue in position 960, but is independent of receptor autophosphorylation and kinase activation.

The requirement of insulin receptor tyrosine kinase activity per se in receptor internalization has been controversial since conflicting evidence for (Hari and Roth, 1987; McClain et al., 1987; Russell et al., 1987; Reynet et al., 1990) and against (Backer et al., 1989*b*; Reddy et al., 1988; Trischitta et al., 1989) its role in this function have been reported. Taken together with previous data from our laboratory (Carpentier et al., 1992), the present results directly demonstrate an important role of kinase activation in mediating insulin-induced receptor internalization in CHO cells. Moreover, they show that the kinase-dependent step is the surface redistribution of the receptor on the cell surface. We also find that insulin-induced internalization requires not only kinase activation but also the phosphorylation of all three of the tyrosine residues phosphorylated in the regulatory domain (1146, 1150, and 1151). By contrast, the autophosphorylation of the tyrosine residues of the COOH-terminal tail of the β-subunit of the receptor is not required, since a deletion of the last 30 amino acids including tyrosines 1316 and 1322, as well as one serine and one threonine site of phosphorylation is without effect.

Whether autophosphorylation frees the receptor from local constraints or whether the receptor is actively transported from the villous region to the nonvillous domain remains an open question. However, we and others have previously described a polar redistribution of insulin on the plasma membrane of cultured human lymphocytes (Barazzone et al., 1980; Majercik and Bourguignon, 1985; Schlessinger et al., 1980). This capping movement is an active process and requires cytoskeleton elements (Bourguignon and Singer, 1977; De Petris, 1975). Thus, one could speculate that insulin binding activates the receptor kinase and allows the phosphorylation of cytoskeleton-associated proteins responsible for the surface displacement of the receptor. Nevertheless, we cannot exclude the possibility that insulin binding and/or autophosphorylation simply releases its receptor from some cytoskeleton elements, as recently

Table IV. Summary of the Functional, Biological and Internalization Characteristics of Mutant Insulin Receptors

	HIRC	A 1018	ΔCT	F 3TYR	F 1146	Δ960	F 960
Kinase activation by insulin	+	−	+	+	+	+	+
Autophosphorylation	+	−	+	±	±	+	+
pp 185 Phosphorylation	+	−	+	−	−	−	−
PtdIns 3′-Kinase	+	−	+	±	±	−	±
Glycogen synthase	+	−	+	−	+	−	−
Thymidine incorporation	+	−	+	−	−	−	−
Internalization	+	−	+	−	−	±	+
Surface Redistribution	+	−	+	−	−	+	ND
Association with Clathrin-coated pits (nonvillous surface)	+	+	+	+	+	−	ND

* This summary was prepared from published data referred to in Fig. 1 together with data from the present study.

shown in the case of IgG binding to Fc receptor (Ohta et al., 1991).

In addition to its implication in receptor internalization, the insulin receptor tyrosine kinase activity is essential for insulin action. Further recent evidence suggest that a phosphoprotein called pp185 or IRS-1 is a substrate of the insulin receptor and plays a significant role in the early stages of post-receptor insulin signal transduction (White et al., 1985, 1987). Recently IRS-1 has been cloned and shown to associate with and activate phosphatidylinositol 3′-kinase (Sun et al., 1991; Backer et al., 1992*a*). Present data showing that two cell lines with a defect of pp185 phosphorylation and PtdIns 3′-kinase activation (CHO-Δ960 and CHO-F960), nevertheless normally redistribute (or internalize) insulin receptors indicate that the substrate phosphorylation and PtdIns 3′-kinase activation are not involved in the triggering of insulin receptor internalization (Table IV). Moreover, the observation that insulin receptor internalization is impaired in two cell lines in which glycogen synthase activation (CHO-F1146) is preserved (Wilden et al., 1990) suggests that insulin receptor internalization is not required for all biological actions of the hormone.

The second step of the internalization process, i.e., anchoring of the receptor in clathrin-coated pits requires an amino acid sequence of the juxtamembrane domain comprised between alanine 954 and asparagine 965. Among these 12 amino acids is a sequence NPEY which is similar to the sequence NPVY required for internalization of the LDL receptor and found also in the cytoplasmic domains of several transmembrane proteins (Chen et al., 1990). In contrast to previously published data (Berhanu et al., 1991), our direct morphological observations support the important role of this sequence in the anchoring of the insulin receptor in clathrin-coated pits. In these plasma membrane invaginations, coat-assembly proteins (AP 2 or HA-II adaptors) appear to mediate the interaction between receptors and clathrin (Pearse and Robinson, 1990). Thus, it is probable that the interaction between the insulin receptor and these adaptors involves a sequence included in the 12 amino acids deleted in the receptor expressed in Δ960 cells. The reduced association of the Δ960 insulin receptor with clathrin-coated pits is not due to a general defect in the clathrin-pathway, since the total number, mean size, and general organization of clathrin-coated pits are similar in Δ960 cells and the various other CHO lines studied. However, even in the Δ960 cells, endocytosis of the insulin receptor is not totally prevented and still proceeds at a significant rate. This can be explained first by the fact that Δ960 cells express insulin receptors whose kinase can be activated by insulin binding and, thus, are rapidly redistributed to the nonvillous region of the cell where endocytotic processes occur, and by the fact that upstream of the NPEY sequence of the insulin receptor is a GPLY sequence which has also recently been implicated in the internalization of the insulin receptor and could have an additive effect to that of the NPEY sequence (Rajagopalan et al., 1991; Backer et al., 1992*a*).

Other sequences capable of controlling the internalization of other surface receptors, i.e., the transferrin receptor, have also been proposed (Chen et al., 1990; Lazarovits and Roth, 1988; Ktistakis et al., 1990; Collawn et al., 1990; Bansal and Giersasch, 1991; Lobel et al., 1989; Girones et al., 1991). At present, the only common features of these sequences are the presence of one aromatic residue and their specification of a particular structure: a tight type 1 β turn. The presence of an aromatic residue at position 960 seems also sufficient for normal internalization of the insulin receptor since the replacement of tyrosine 960 by phenylalanine does not perturb insulin-induced insulin receptor internalization, whereas there is a marked loss of internalization observed when alanine was present at this position. As recently suggested (Lehmann et al., 1992; Jadot et al., 1992), the interchangeability of aromatic residues in this position could be dependent on the length of the cytoplasmic tail of the insulin receptor.

The second step of insulin-induced internalization appears to be independent of kinase activation and of phosphorylation of any tyrosine residue since in CHO-A1018, CHO-F3TYR, and CHO-ΔCT insulin receptors present on the nonvillous region of the cells do concentrate normally in clathrin-coated pits. These direct morphological observations demonstrate that the receptor segment responsible for the anchoring of the insulin receptor in clathrin-coated pits is not unmasked by kinase activation and/or autophosphorylation but is always exposed. These conclusions are in agreement with the previously shown ability of antipeptide antibodies prepared against amino acids 952-962 to immunoprecipitate insulin receptors regardless of their autophosphorylation state (Herrera et al., 1986; Perlman et al., 1989). They also agree with our recent observations that the constitutive internalization of the insulin receptor occurs via clathrin-coated pits (Paccaud et al., 1992). By contrast, Backer et al. (1991) have shown that the internalization of kinase-deficient receptors can be distinguished kinetically and pharmacologically from that of wild-type receptors at low occupancies. Thus,

alternate pathways for the constitutive endocytosis of cell surface receptors cannot be ruled out.

Recently, Smith et al. (1991) followed the surface distribution and internalization of insulin labeled with colloidal gold in cells expressing kinase active and inactive insulin receptors, and concluded that autophosphorylation was not required for the receptor to migrate from microvilli to the nonvillous region of the cell, but was necessary for the concentration of these receptors in clathrin-coated pits. These studies differed from ours in the use of insulin coupled to relatively large electron dense colloidal gold particles which might affect subcellular distribution of the hormone more than simple iodination. These technical differences in the two experimental systems could also explain the long lasting controversy about the internalization gate of insulin (for review see Carpentier, 1989).

In conclusion, we have characterized two initial steps of insulin receptor internalization: the surface redistribution and the anchoring in clathrin-coated pits. These two steps involve structures in different domains (juxtamembrane and kinase regulating domains) of the β-subunit-cytoplasmic portion. Thus, by contrast to what has been described in the course of transport protein receptors, ligand-dependent internalization of signaling receptors (i.e., insulin receptor) internalization is highly specific and involves a complex cellular pathway requiring multiple structural determinants of the receptor.

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