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; Kristakis et al., 1990; Collawn et al., 1990; Bansal and Giersasch, 1991; Lobel et al., 1989; Gironés 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 right 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

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Materials and Methods

Insulin Receptor Autophosphorylation

Insulin receptor phosphorylation was assessed by Western blotting with \( \alpha \)-anti-phosphotyrosine (\( \alpha \)-PY) 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 \( \alpha \)-PY 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., 1982). 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 overlaying 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 \( \times 19000\). 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 (Tektronix, type 4953) connected with a microprocessor (IBM AT), allowing the different parameters to be recorded and statistically analyzed.

 Quick Freezing

To visualize the cytoplasmic 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 7.0) 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) rotary 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 \( \times \) 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) and their characteristics are summarized in Fig. 1. All cell lines expressed \(>10^6\) receptors/cell. Autophosphorylation of the insulin receptor \(\beta\)-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-\( \Delta \)960), a point mutation of Tyr960 in the NPXY to Phe (CHO-F960), and a COOH-terminal deletion mutant lacking two autophosphorylation sites (CHO-ACT) was similar (Fig. 2). By contrast, insulin-induced autophosphorylation of the ATP-binding site mutant (CHO-A1018) cells was undetectable, 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,
transfected cells were exposed to tracer concentration (3 × 10⁻¹¹ M) of ¹²⁵I-insulin at 37°C, processed for EM autoradiography, and ¹²⁵I-insulin internalization was quantitated as previously described (Carpentier et al., 1978). CHO-HIRC 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. 3 A). In CHO A1018 cells, in which the insulin receptor tyrosine kinase was inactive, ¹²⁵I-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 ¹²⁵I-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 ¹²⁵I-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 ¹²⁵I-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 ¹²⁵I-insulin internalization in spite of the fact that the insulin

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.

| MUTATION         | HIR    | A 1018  | ΔCT             | F 3TYR | F 1146  | Δ960  | F 960  |
|------------------|--------|---------|-----------------|--------|---------|-------|--------|
| REFERENCE        | Ullrich & al. (1985) | K1018 to A1018 | 43 aa truncation (Δ1301-1343) | Y1146 to F1146 | Y1150 to F1150 | Y1151 to F1151 | Δ954-Δ965 deleted | Y980 to F960 | S962 to T962 |
|                  |        |         |                 |        |         |       |        |        |        |

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 ¹²⁵I-protein A and autoradiography. Insulin-induced tyrosine phosphorylation is observed as an increase in radioactivity of the β-subunit (95-kD band).
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 125I-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). 125I-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).

### 125I-Insulin Internalization in CHO Cells Transfected with Normal (HIRC) or Mutated Insulin Receptors

![Graphs showing internalization of 125I-insulin in CHO cells transfected with normal or mutated insulin receptors.](image)

**Figure 3.** 125I-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.

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 125I-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 125I-insulin to CHO cells preferentially occurs on microvilli (Figs. 4 and 5, and Table I). As a function of incubation time at 37°C, 125I-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 125I-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 position 1146 or positions 1146, 1150, and 1151, 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, 125I-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) $\times$8,000; (B) $\times$56,000; (C) $\times$15,000; (D) $\times$46,000; (E) $\times$22,000; (F) $\times$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 $^{125}\text{I}$-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 $\approx 2,000$ autoradiographic grains were quantitated. Results are expressed as percent of the total number of grains associated with the cell surface ($\pm 250$ nm from the plasma membrane) which center were within a distance of 250 nm from a microvilli.

Figure 6. Association of $^{125}\text{I}$-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 ($\pm 250$ nm from the plasma membrane) which center were within a distance of 250 nm from a clathrin-coated pit.

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

| CHO cell lines | Percent $^{125}\text{I}$-insulin associated with microvilli* | Percent total cell surface occupied by microvilli† | Ratio |
|----------------|-------------------------------------------------------------|--------------------------------------------------|-------|
| HIRC 2         | 75.9 ± 2.0                                                  | 58.3 ± 2.9                                       | 1.30  |
| A1018          | 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  |
| F1146          | 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
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., 1989b; 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 II. 125I-Insulin Association with Clathrin-coated Pits

| CHO cell lines | Percent cell surface occupied by clathrin-coated pits* | Clathrin-coated pits diameter (2 h 4°C) (60 min 37°C) (nm)² |
|---------------|---------------------------------|---------------------------------|
| 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                     | 0.92 ± 0.21                     | 108.4 ± 18.3                   |
| F3TYR         | 0.92 ± 0.21                     | 1.16 ± 0.21                     | 108.4 ± 18.3                   |
| F 1146        | 0.98 ± 0.26                     | 0.98 ± 0.26                     | 104.1 ± 9.3                    |
| Δ960          | 0.98 ± 0.26                     | 1.08 ± 0.29                     | 104.1 ± 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.
² Quantification carried out on quick-freeze replicas. Values are mean ± SEM; n = number of cell analyzed = 20, 17, and 21, respectively.

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

| CHO cell lines | Nonvillous surface* | Villous 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)
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., 1992a). Present data showing that upstream of the NPVY sequence of the insulin receptor could have an additive effect to that of the NPEY sequence (Rajagopalan et al., 1991; Backer et al., 1992a).

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; Kistakis 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. (1999) 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 nonvillus 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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