Specific Factors Are Required for Kinase-dependent Endocytosis of Insulin Receptors

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Mouse B82 cells that support high affinity saturable endocytosis of epidermal growth factor receptors (EGFR) exhibited only low rates of nonsaturable internalization of insulin receptors (InsR). To investigate the defect in endocytosis of InsR in B82 cells, we examined the role of sequence motifs and tyrosine kinase, the two receptor components shown to be required for efficient saturable endocytosis of InsR in Rat 1 cells. Placement of residues encoded by exon 16 of the InsR onto an EGFR truncated to residue 958 restored EGF-induced internalization of this mutant receptor indicating that the sequence codes in exon 16 are recognized by B82 cells. To determine whether the kinase function could be provided in trans, a B82 cell expressing both receptors was established. EGF-activated EGFR kinase was not able to restore insulin-dependent rapid endocytosis to InsR. However, fusion of untransfected Rat1 cells with InsR-expressing B82 cells enabled rapid endocytosis of InsR, indicating that the internalization defect can be complemented. These results indicate that, although internalization codes can function in the context of other receptors, activation of tyrosine kinase receptors requires an additional specific component.

INTRODUCTION

Endocytosis of many cell surface receptors depends on specific cytoplasmic sequence "codes" consisting of four to six amino acids that include an aromatic residue (Davis *et al.*, 1986; Collawn *et al.*, 1990; Johnson *et al.*, 1990; Ktistakis *et al.*, 1990). NMR analysis of peptides corresponding to functionally identified endocytic codes confirmed the prediction of Collawn *et al.* (1990), that these exist in a tight turn structure (Bansal and Gierasch, 1991; Eberle *et al.*, 1991; Backer *et al.*, 1992). Receptors, such as those for low density lipoproteins and transferrin, associate with clathrin-coated pits constitutively, and endocytosis is independent of ligand binding (Anderson *et al.*, 1977; Ajioka and Kaplan, 1986). In contrast, epidermal growth factor and insulin receptors (EGFR and InsR) are diffusely distributed on the cell surface and redistribute to coated pits upon ligand binding (Haigler *et al.*, 1978; Pilch *et al.*, 1983). These receptors exhibit occupancy-induced endocytosis (Lund *et al.*, 1990; Backer *et al.*, 1991; Wiley *et al.*, 1991), a process that is saturable and requires endocytic codes

as well as intrinsic protein tyrosine kinase activity (Honegger *et al.*, 1987; Russell *et al.*, 1987; Chen *et al.*, 1989; Backer *et al.*, 1990; Thies *et al.*, 1990; Chang *et al.*, 1993).

The endocytic codes of InsR are localized to the juxtamembrane region of the cytoplasmic domain encoded by exon 16. Although the stronger code is GPLY, optimal endocytosis requires two codes (Rajagopalan *et al.*, 1991; Backer *et al.*, 1992). Addition of a third code gives even stronger effects (Carpentier *et al.*, 1992). The role of intrinsic tyrosine kinase activity, which is strictly required for ligand-induced internalization of both InsR and EGFR, has remained obscure. The observation that endocytic codes from EGFR can substitute for the endocytic code of transferrin receptors suggested that receptor autophosphorylation may be required to expose the endocytic codes (Chang *et al.*, 1993). However, Backer *et al.* (1989) presented evidence that tyrosine phosphorylation of InsR is not required for internalization. Additionally, mutant EGFR that contain endocytic codes, but lack sites of tyrosine self-phosphorylation, remain dependent on tyrosine kinase activity for in-

duced internalization (Chang *et al.*, 1993). These results suggest that tyrosine phosphorylation of a cellular component is necessary for ligand-induced internalization of both InsR and EGFR.

In the present study we present evidence that mouse B82 cells have a specific defect in occupancy-induced internalization of InsR. Fusion of residues encoded by exon 16 of human InsR to the carboxy terminus of kinase-active mutant c'958 EGFR restored EGF-induced internalization of EGFR, indicating that B82 cells can recognize the endocytic codes of InsR. EGF-activated EGFR were not able to restore insulin-dependent high affinity endocytosis to InsR, indicating the tyrosine kinase function could not be provided in trans. However, when B82 cells that express InsR were fused with endocytosis-competent Rat1 cells, ligand-induced internalization was restored. These results indicate that a specific component is necessary for kinase-dependent internalization of occupied InsR.

MATERIALS AND METHODS

Preparation of Mutant InsR and EGFR

Human InsR cDNA as a 4.6-kilobase insert in pSP64 was a gift of Dr. Graeme Bell, University of Chicago (Seino *et al.*, 1989). Wild-type (WT) InsR used in these studies lacks exon 11 residues found in HUMINSR (Ebina *et al.*, 1985b). The cDNA was excised from the vector and subcloned into the phagemid pBS KS+ for site-directed mutagenesis using the procedure of Kunkel (1985). To generate an internal deletion of exon 16, an oligonucleotide 5' ATTCCTGA-GAAAACGCGTGTTCATGCTCT 3' was used. This oligonucleotide generate a unique *MluI* restriction site for diagnostic and construction uses and deleted 22 residues from Q944 to D965 of InsR. All oligonucleotides were made on an Applied Biosystems 380B DNA synthesizer (Foster City, CA). To generate a kinase-inactive InsR, an oligonucleotide 5' ACCCGCGTGGCGGTGGCCACGGTCAACGAG 3' was used to change K1018 to A and to provide an *MscI* restriction site. WT, Δ exon 16, and A1018 InsR cDNAs were inserted into the px expression plasmid that contains a mutant dihydrofolate reductase gene (Chen *et al.*, 1987) to generate pxHIR, pxHIR Δ 16, and pxHIRK⁻ plasmids.

Exon 16 of human InsR was placed onto the C' terminus of an internalization-defective EGFR truncated after residue 958 using oligonucleotides corresponding to exon 16. The oligonucleotides 5' GATAGTCGACCAACCAGATGGTCCCCCTAGGACCGCTATAC-GCGTCTTCGAATCCG 3' and 5' CTATTAAGCTTAATCGCTAGC-ACTTAAGTACTCCGGATTCTGAAGACGCGTATAGCG 3' were annealed, filled in with the Klenow fragment of DNA polymerase, cut with *Sall* and *HindIII*, and subcloned into pBS KS+ for amplification. The expression plasmid pxEGFR c'958f 993–1022 was constructed such that the fusion segment encoding residues 993–1022 of EGFR could be excised as a *Sall* to *HindIII* fragment (Chang *et al.*, 1991). Reestablishment of the *Sall* site places codons for V and D at the junction; the *HindIII* site is followed by an ochre stop codon. Sequences corresponding to exon 16 from pBS KS+ replaced the 993–1022 coding sequence using a two-step ligation. This yielded pxEGFR c'958f exon 16 corresponding to intact EGFR to residue 958 followed by the InsR-derived sequence VDQPDGPLYASSNPEYLSASDZ. To establish doubly transfected cell lines, holo EGFR was placed in the Rc/CMV vector that contains a CMV promoter and the neo^R gene under control of the SV40 early promoter (Invitrogen, San Diego, CA). The polylinker of Rc/CMV was exchanged for the polylinker of pBS KS+ to allow correct orientation. EGFR was excised from the pxER plasmid as an intact *XbaI* to *HindIII* fragment and cloned into

the modified Rc/CMV plasmid to yield pCMVEGFR. All constructions were verified by DNA sequencing.

Preparation and Propagation of Cells Expressing InsR and EGFR

Mouse B82L cells were transfected with various cDNA expression plasmids using calcium phosphate precipitation. Rat1 cells were transfected using a modification in which calcium phosphate precipitates were formed in the presence of balanced electrolyte solution instead of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Ishiura *et al.*, 1982). Cells were placed under selection in 400 nM methotrexate; clonal lines were isolated and characterized by fluorescence-activated cell sorter (FACS) analysis using 528 anti-EGFR and 5D9 anti-InsR monoclonal antibodies (Kawamoto *et al.*, 1983; Gustafson and Rutter, 1990). For dual expression of InsR and EGFR, B82 cells stably expressing InsR under 1 μ M methotrexate selection were transfected with pCMVEGFR and placed under selection with both 400 μ g/ml G418 and methotrexate. B82 cells expressing holo EGFR, kinase-inactive M721 EGFR, and c'958 EGFR have been described previously (Chen *et al.*, 1987, 1989).

Preparation of B82-Rat1 Cell Hybrid

A hybrid cell line was generated by fusion of untransfected Rat1 cells with B82 cells expressing InsR by the polyethylene glycol technique (Norwood *et al.*, 1976). Confluent dishes of both cell types were split at a 4:1 dilution together into 35-mm dishes. After 24 h, the cells were rinsed with serum-free Dulbecco's modified Eagle's medium (DME) and incubated with 0.5 ml of 45% polyethylene glycol (Sigma cell fusion grade), 10% dimethylsulfoxide for 2 min at 20°C. Cells were rinsed three times in DME, allowed to recover for 24 h, and then replated into 100-mm dishes for selection. Cells were placed under selection with HAT (hypoxanthine, aminopterin, thymine) and 400 nM methotrexate. Hybrid cells were resistant to methotrexate by virtue of the DHFR gene included in the InsR expression plasmid and were resistant to HAT by virtue of the intact thymidine kinase gene of the Rat1 cell line. Colonies were isolated by limiting dilution and screened for the presence of human InsR expression with the 5D9 monoclonal antibody using FACS analysis.

All cell lines were grown in DME supplemented with 5% dialyzed newborn calf serum and antibiotics (penicillin G 100 U/ml, streptomycin sulfate 100 μ g/ml, and fungizone 0.25 μ g/ml). Cells were grown in humidified incubators at 37°C with 5% CO₂.

Binding Studies

Receptor number and affinity were analyzed by the procedure of Scatchard using ¹²⁵I-labeled insulin (3-[¹²⁵I] iodotyrosyl A14 insulin) (Amersham, Arlington Heights, IL) or ¹²⁵I-labeled EGF prepared using iodo-beads (Pierce Chemical, Rockford, IL) following the manufacturer's instructions. Approximately 3.5 $\times$ 10⁵ cells were plated on each well of a 12-well plate, allowed to adhere overnight, and placed in 0.5 ml of cold binding buffer (DME, 1 mg/ml bovine serum albumin, 20 mM HEPES pH 7.4). A constant amount of labeled ligand was added in the presence of increasing amounts of unlabeled ligand, and cells were incubated for 4 h at 4°C on an oscillating platform. Cells were rinsed and lysed in 0.5 ml 1 N NaOH, and radioactivity was determined at ligand concentrations ranging from 1 to 25 ng/ml.

Specific internalization rates for the different receptors were determined as described (Lund *et al.*, 1990). Measurements were made using ligand concentrations ranging from 1 to 100 ng/ml, and each rate constant determination was derived from five data points at 1, 2, 3, 4, and 5 min after addition of ligand. Values for surface bound and internalized ligand were corrected for nonspecific binding and spillover from the interior and surface of the cell, respectively (Wiley and Cunningham, 1982). Specific internalization rates were determined by plotting the integral of surface-associated ligand against the amount internalized, and the slopes were determined by linear

regression. Correlation coefficients of internalization plots were generally >0.98 . The internalization velocity was plotted against k_e , the specific internalization rate constant, yielding a saturation of internalization (SatIn) plot. The data were analyzed using templates and macros written for Microsoft Excel as previously described (Lund *et al.*, 1990), which are available from the authors.

Insulin-stimulated Tyrosine Phosphorylation

Transfected B82 or Rat1 cells were grown to confluence in duplicate 10-cm plates. One hour before the assay, cells were rinsed in phosphate-buffered saline (PBS) and returned to the incubator in serum-free medium. Cells were exposed to 0 or 100 nM insulin for 5 min at 37°C, then rinsed in ice-cold PBS with 100 μ M orthovanadate. Cells were harvested with a rubber policeman into centrifuge tubes, spun for 2 min at $500 \times g$, and resuspended in 50 μ l PBS with 20 mM 3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate and 100 μ M orthovanadate. This mixture was centrifuged for 10 min at $10\,000 \times g$, and the pellets were discarded. Aliquots of the cell extracts were mixed with an equal volume of boiling Laemmli sample buffer (2% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 1% glycerol, 0.01% bromophenol blue) just before running on 7.5% polyacrylamide gels. The proteins were transferred to immobilon membranes and visualized using the antiphosphotyrosine antibody PY-20 (Glenney *et al.*, 1988).

2-Deoxyglucose Uptake

Rat1 cells expressing InsR and B82 cells expressing either WT or kinase-inactive InsR were grown to confluence on 12-well plates. Cells were exposed to 10 mM 2-deoxy-D-glucose in complete medium for 5 h before the experiment (Ebina *et al.*, 1985a) then washed and exposed to 1 or 100 ng/ml insulin and 0.4 μ Ci 2-[3 H] deoxy-D-glucose (Amersham) (7.7 nM brought up to 10 mM with unlabeled 2-deoxy-D-glucose) for the indicated times. Cells were then washed with PBS, solubilized in 1 N NaOH, and assayed for radioactivity.

RESULTS

Expression of Receptors

The receptors used in this study are shown in Figure 1. Kinase-inactive InsR contains a mutation of K1018 to A in the ATP binding site, whereas the Δ exon 16 InsR lacks amino acids 944–965. The EGFR mutant c'958 is C' truncated at amino acid 958, and the c'958f exon 16 EGFR has the 22 amino acids of InsR exon 16 fused to residue 958 of EGFR. Table 1 summarizes results of Scatchard analysis of receptors expressed in the clonal cell lines used in this study. The contribution of endogenous InsR to total insulin binding and internalization in either B82 or Rat1 cells was insignificant ($\sim 7 \times 10^3$ receptors per cell, 1–5%) compared to ligand bound by transfected cells. Because B82 cells lack endogenous EGFR, all binding of that ligand was mediated by the expressed human receptor.

Differential Endocytosis of InsR in Rat1 Compared to B82 Cells

Previous work indicates that the insulin receptor undergoes ligand-induced internalization through a saturable mechanism dependent on receptor sequences encoded by exon 16 (Backer *et al.*, 1990, 1991; Thies *et al.*, 1990). The characteristics of this "high affinity" en-

Figure 1. Schematic diagram of receptors for EGF, insulin, and the mutations used in this study. □, extracellular domains; ▨, transmembrane domains; ▩, intracellular domains. In the InsR diagram, fine stippling represents the alpha subunit, and coarse stippling represents the extracellular portion of the beta subunit. The kinase domains are indicated by a shaded background. →, exon 16 of the InsR. *, the K1018 to A mutation.

docytic system are very similar to those which mediate ligand-induced internalization of EGFR (Lund *et al.*, 1990; Wiley *et al.*, 1991). To confirm these observations, we evaluated the endocytosis of InsR in Rat1 cells, a system that has previously been shown to support efficient internalization of the InsR (Thies *et al.*, 1990; Rajagopalan *et al.*, 1991). Shown in Figure 2, A and B are internalization plots of the WT InsR and the Δ exon 16 InsR, respectively, using insulin concentrations ranging from 1 to 100 ng/ml. The relative amount of surface binding has been normalized to facilitate comparisons between different cells and ligand concentrations. The slope of an internalization plot is directly proportional to the specific internalization rate of the receptor (Lund *et al.*, 1990). The data shows that internalization of the WT InsR is efficient at low ligand concentrations but decreases with progressive receptor occupancy (Figure 2A). In contrast, the Δ exon 16 InsR displays a low specific internalization rate at all ligand concentrations (Figure 2B).

The specific internalization rates of both WT and Δ exon 16 InsR were plotted against their absolute internalization velocity at each ligand concentration. The resultant SatIn plot, shown in Figure 2C, indicates that the WT InsR is internalized by both a specific, high affinity, saturable pathway and a nonspecific pathway

Table 1. Expression of mutant EGF and insulin receptors in transfected cells

Receptor ^a	Parent cell ^b	K _d (nM)	Receptors per cell (×10 ⁻³)	C.C. ^c
WT InsR	B82	1.7	720	0.97
WT InsR	Rat1	1.3	160	0.94
Δ exon 16 InsR	Rat1	2.8	621	0.97
Kinase-inactive InsR	Rat1	1.3	240	0.99
c958f InsR exon 16 EGFR	B82	3.1	266	0.87
c958 EGFR	B82	2.5	390	0.98

^a The designation corresponds to descriptions given in the text.^b The cell line expressing the receptor.^c Correlation coefficients of the Scatchard plots used in estimating receptor number. Values were determined from two to five separate closely agreeing measurements.

(Lund *et al.*, 1990; Wiley *et al.*, 1991). In contrast, the SatIn plot of the Δ exon 16 InsR has a slope near 0, indicating that these receptors only undergo nonspecific internalization. Although high ligand concentrations resulted in a greater net internalization for the Δ exon 16 InsR relative to the WT InsR, this was because of its higher levels of receptor expression (Table 1). At low, but equivalent levels of receptor occupancy, the WT InsR was internalized at a three- to fivefold greater rate (Figure 2C). These data confirm previous studies on the behavior of the InsR in Rat1 cells as well as the necessity of exon 16 in the InsR for efficient internalization (Thies *et al.*, 1990).

To confirm observations initially reported for EGFR that the rate of endocytosis at low concentrations of ligand is independent of the number of receptors per cell (Lund *et al.*, 1990), we also analyzed endocytosis of InsR in HIRc-B cells that express ~10-fold higher concentrations of InsR (1.25×10^6 cell⁻¹) (McClain *et al.*, 1987). The key value at low concentrations of insulin was 0.21 min⁻¹ falling to 0.07 min⁻¹ at higher concentrations of insulin similar to the results shown in Figure 2.

Because internalization of both the InsR and EGFR appear similar with respect to saturable behavior and requirement for intrinsic receptor tyrosine kinase activity, it seemed possible that they use the same endocytic machinery. To explore this possibility, we expressed the InsR in the B82 cell type that supports efficient internalization of the EGF receptor (Chen *et al.*, 1989; Chang *et al.*, 1993). Surprisingly, we found that the specific internalization rate of the WT InsR was extremely low in these cells. Whereas the specific internalization rate of EGFR at a ligand concentration of 1 ng/ml was 0.32 min⁻¹ (Wiley *et al.*, 1991), that of the InsR was 10-fold lower at 0.03 min⁻¹. This low specific internalization rate was independent of insulin concentration and appeared very similar to that observed for kinase-inactive

InsR in Rat1 cells (Figure 3A). Satin plot analysis indicates that both WT and kinase-inactive InsR in B82 cells utilize a nonspecific internalization pathway (Figure 3B). This suggests that B82 cells either lack a specific component required for the ligand-induced endocytosis of the InsR or express an inhibitor of this process.

Biological Activity of Human Insulin Receptors in B82 Cells

It seemed possible that defective endocytosis of human InsR in B82 cells could be because of a secondary, inactivating mutation that suppressed its biological activity. To examine this, we measured several parameters of receptor activity unrelated to endocytosis. As shown in Figure 4A, 2-deoxyglucose uptake by either Rat1 or B82 cells transfected with the WT InsR was stimulated to a similar degree by insulin. B82 cells transfected with kinase inactive InsR did not show increased 2-deoxyglucose uptake in response to insulin, demonstrating that this response was mediated by the transfected WT InsR (Figure 4A). Similarly, both Rat1 and B82 cells transfected with the WT InsR display insulin-induced

Figure 2. Comparison of endocytosis of WT and Δ exon 16 InsR in Rat1 cells. (A) Internalization plots of WT InsR at 1 (○), 3 (■), 10 (□), 30 (▲), and 100 (Δ) ng/ml insulin. (B) Internalization plots of Δ exon 16 InsR at 1 (○), 10 (□), and 100 (Δ) ng/ml insulin. All scales have been normalized to total surface binding during the 5-min internalization time. (C) Satin plot of endocytic rate constants vs. internalization velocities of WT InsR (○) and Δ exon 16 InsR (●).

Figure 3. Low rates of endocytosis of InsR in B82 cells. (A) Internalization plots of WT InsR in B82 cells exposed to 1 (—○—) and 100 (—●—) ng/ml insulin. Endocytic rate constants are 0.03 and 0.02 min⁻¹, respectively. Data from kinase-inactive InsR (—△—, dashed line) and WT InsR expressed in Rat1 cells (—□—, dashed line) are shown for comparison. Endocytic rate constants were 0.02 and 0.17 min⁻¹, respectively. (B) Satin plot of endocytic rate constants vs. internalization velocities for WT (—○—) and kinase-inactive (—△—) InsR expressed in B82 cells. Data from WT InsR expressed in Rat1 cells (—□—, dashed line) is presented for comparison.

receptor autophosphorylation and phosphorylation of endogenous substrates (Figure 4B). The major insulin dependent tyrosine phosphorylated substrate corresponded to the β subunit of InsR in both cell types. The Δ exon 16 InsR mutant expressed in Rat1 cells was also found to respond to insulin stimulation by autophosphorylation. As expected, Rat1 or B82 cells transfected with kinase-inactivated InsR as well as untransfected cells did not show an increase in phosphotyrosine-containing proteins in response to insulin stimulation (Figure 4B). Thus, by both biochemical and biological criteria, the InsR expressed in B82 cells retains ligand-induced biological activities.

Endocytosis of Activated EGFR/InsR Chimeras in B82 Cells

We have previously shown that the endocytic code of EGFR will function constitutively when substituted for

the endogenous code of the transferrin receptor. In contrast, the endocytic code of the transferrin receptor will operate in the context of EGFR only when the hybrid receptor has tyrosine kinase activity (Chang *et al.*, 1993). This suggests that ligand-induced internalization of tyrosine kinase receptors consists of at least two steps: "activation" followed by interaction with coated pits. In view of the apparent defect in InsR endocytosis shown by transfected B82 cells, it was important to determine whether the previously defined endocytic codes of the InsR could interact with the endocytic machinery of B82 cells. A mutant EGFR that was C' truncated at residue 958 to delete endocytic codes (Chen *et al.*, 1989) was modified so as to present the endocytic codes of InsR at its extreme C' terminus. This receptor construct was then stably expressed in B82 cells. As shown in

Figure 4. Responses to insulin in both Rat1 and B82 cells expressing transfected InsR. (A) [³H] 2-deoxyglucose uptake in B82 cells expressing WT or kinase-inactive InsR and in Rat1 cells expressing WT InsR. Cells were treated without (open symbols) or with (closed symbols) 100 nM insulin and internalized. [³H] 2-deoxyglucose was measured at the indicated times. B82 cells expressing WT InsR (—□—, —■—), B82 cells expressing kinase-inactive InsR (—○—, —▲—), and Rat1 cells expressing WT InsR (—○—, —●—). (B) Anti-phosphotyrosine Western blot of B82 (lanes 1 and 2) and Rat1 (lanes 3 and 4) cells expressing the WT InsR and exposed to 0 (lanes 1 and 3) or 100 nM (lanes 2 and 4) insulin for 5 min. Lanes 5 and 6, untransfected Rat1 cells exposed to 0 (lane 5) or 100 nM (lane 6) insulin for 5 min. The arrow indicates the InsR β subunit; molecular weight standard mobilities are indicated on the right.

Figure 5. Endocytosis of a truncated EGFR fused to InsR exon 16-encoded residues in B82 cells. (A) Internalization plots for the mutant receptor at 1 (—○—), 3 (—■—), 10 (—□—), 30 (—▲—), and 100 (—△—) ng/ml EGF. Data from c'958 mutant EGFR exposed to 100 ng/ml EGF (—×—) is shown for comparison. (B) SatIn plots of endocytic rate constants vs. internalization velocities for the c'958f InsR exon 16 EGFR chimera (—○—) and the c'958 EGFR (—×—).

Figure 5, the endocytic codes of InsR were able to mediate rapid receptor internalization when expressed in the context of EGFR. The biphasic SatIn plot (Figure 5B) obtained with the chimeric receptor was very similar to that obtained using WT EGFR, indicating that it can use the saturable, high affinity endocytic pathway normally used by EGFR. The ability of B82 cells to use the InsR endocytic codes only in the context of the EGFR suggests that the defect in WT InsR internalization is at the level of its activation.

Endocytosis of InsR in the Presence of Activated EGFR

Tyrosine kinase activity could act either directly or indirectly in activating InsR. If the effect is indirect, then the EGFR kinase should be able to complement the InsR receptor in trans, perhaps by phosphorylating a diffus-

ible substrate that interacts with the activated InsR intracellular domain. To test this hypothesis, we expressed the WT InsR and either the WT EGFR or a kinase-inactivated EGFR in B82 cells. After treatment with 100 nM EGF, internalization of InsR was evaluated using ¹²⁵I-labeled insulin. As shown in Figure 6, InsR internalization remained at a low rate of internalization in either the presence or absence of activated EGFR. Therefore, activated EGFR cannot act in trans to enable high affinity InsR endocytosis in B82 cells. This suggests that the kinase activity must reside on the receptor being internalized.

Endocytosis of InsR in B82-Rat1 Hybrid Cells

B82 cells could lack a critical factor required for internalization of the InsR. Alternately, they could express an inhibitor of the process. To discriminate between these two possibilities, we constructed a hybrid cell line by fusing B82 cells expressing the human InsR with the internalization-competent (but human InsR negative) Rat1 cell line. Analysis of the resultant hybrid lines showed that the InsR was indeed able to undergo rapid internalization in these cells (Figure 7A). Similar results were obtained from all independently isolated hybrid cell lines. Analysis of specific internalization rates at 1 and 100 ng/ml insulin (Figure 7B) demonstrated saturability of the endocytic apparatus in the hybrid cells, demonstrating that Rat1 cells can complement the defect in InsR internalization in B82 cells. These results suggest that the lack of a specific component in B82 cells is responsible for their internalization-negative phenotype.

DISCUSSION

Previous studies of both the EGF and insulin receptors have shown that ligand-induced endocytosis requires

Figure 6. Rates of InsR internalization in B82 cells cotransfected with EGFR. Cells bearing the WT InsR were cotransfected with kinase-inactive (Kin-) or WT (Kin+) EGFR and assayed for specific internalization rate of InsR in the presence or absence of EGF.

Figure 7. Restoration of efficient InsR endocytosis by fusion of Rat1 cells with InsR-bearing B82 cells. (A) Comparison of the specific internalization rates between the InsR in the parental B82 cells (—●—) and after fusion with Rat1 cells (—○—). Insulin concentration was 1 ng/ml. Specific rates of internalization are 0.03 and 0.13 min⁻¹, respectively. (B) Endocytic rate constants of InsR in the two cell lines at 1 and 100 ng/ml insulin. ■, B82-Rat1 hybrid cells; ▨, B82 cells.

both intrinsic protein-tyrosine kinase activity and sequence information located in regulatory regions of their cytoplasmic domains (Chen *et al.*, 1989; Wiley *et al.*, 1991). Kinetic studies of various EGFR mutants have suggested that there are two distinct processes in internalization of receptors: binding to an internalization component (IC) that is required for rapid endocytosis and binding to coated pits (Chang *et al.*, 1993). Because the characteristics of InsR endocytosis have been reported to be very similar to the EGFR system (Backer *et al.*, 1991), we thought it important to evaluate their similarity by expressing both receptors in a common cell type.

We found that the internalization rates of InsR in B82 cells were close to those of basal membrane turnover (Wiley *et al.*, 1991). This was unexpected as those cells are very efficient in supporting rapid internalization of EGFR. A different line of mouse fibroblasts can efficiently internalize human InsR, ruling out the possibility that the defect was specific to mouse cells (Hofmann *et*

al., 1989). The observation that Rat1 cells transfected with the identical InsR expression vectors efficiently internalized activated InsR confirmed that mutations in the InsR expression vector did not account for the defect in B82 cells. Finding similar *in vivo* insulin-stimulated tyrosine kinase activity and similar insulin-stimulated 2-deoxyglucose uptake in transfected Rat1 and B82 cells confirmed that InsR was active in B82 cells by functional and biochemical criteria.

Internalization sequences from InsR restored internalization of endocytosis-defective EGFR in B82 cells. This observation is consistent with a model in which internalization codes assume functional conformations independently of surrounding residues (Trowbridge, 1991) and argues against the hypothesis that the B82 cells are unable to recognize InsR exon 16 sequences. Doubly transfected B82 cells bearing both InsR and EGFR were used to test the hypothesis that only EGFR can phosphorylate a necessary component of the endocytic apparatus in B82 cells. The inability of activated EGFR to restore insulin-stimulated InsR uptake indicates that activation of the EGFR endocytic pathway does not facilitate uptake of other receptors. If EGFR does phosphorylate a protein carrier necessary for endocytosis, the carrier either fails to reach the activated InsR or fails to interact productively with it.

Fusion of InsR-expressing B82 cells with Rat1 cells resulted in a rat/mouse hybrid cell line in which InsR could be rapidly internalized. Although this could be because of inactivation of an inhibitor of endocytosis, we think a more reasonable hypothesis is that B82 cells lack an InsR-specific internalization component that was furnished by the Rat1 fusion partner. It is known that EGF and transferrin receptors do not compete with each other for internalization (Wiley, 1988). Because both receptors have interchangeable internalization sequences, another level of specificity must exist for recruitment of receptors into the endocytic pathway. Because insulin and insulin-like growth factor-I receptors appear to compete with each other for internalization (Backer *et al.*, 1991), absolute specificity between a receptor and the endocytic machinery appears unlikely. However, the inability of the InsR to utilize the EGFR pathway in B82 cells suggests that multiple classes of internalization components probably exist. Rat1 cells express the InsR-specific component whereas the B82 cells do not.

The cell-specific aspect of InsR internalization appears to be at the kinase-dependent step. This supports our previously proposed model in which the occupied receptor phosphorylates the internalization component (Wiley *et al.*, 1991), leading to the formation of an "active" receptor:IC complex that binds to coated pits. Morphological analysis of InsR trafficking indicated that tyrosine kinase activity was necessary for movement of receptors from microvilli to coated pits (Carpentier *et al.*, 1992). The formation of an active receptor complex

could also be required for other biological actions of insulin. For example, Androlewicz *et al.* (1989) found that PG19 mouse melanoma cells lacked many biological responses to insulin as well as the ability to internalize the InsR. The insulin resistance of PG19 cells and their inability to internalize InsR were reversed by fusion with the normal mouse cells. Because of the high degree of constitutive metabolic activity in B82 cells, it is difficult to directly compare our results with those of Androlewicz *et al.* (1989). It is interesting to note, however, that in separate, earlier studies aimed at localizing the human gene for EGFR, it was found that both PG19 and B82 cells lack EGFR (Davies *et al.*, 1980). The failure to bind EGF was not reversible by fusing the two cell lines, and it was suggested that these cells may bear the same defect: deletion of the mouse EGFR gene. It is of interest that both B82 and PG19 cells are defective in EGFR expression and in high affinity internalization of InsR.

The present studies provide evidence for specificity in the tyrosine kinase function in addition to the requirement for endocytic codes for ligand-induced internalization of receptors. Because the defect in B82 cells can be restored by cell fusion, these cells could provide a convenient system for assessing the function of any putative InsR-specific internalization component.

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REFERENCES

Ajioka, R.S., and Kaplan, J. (1986). Intracellular pools of transferrin receptors result from constitutive internalization of unoccupied receptors. *Proc. Natl. Acad. Sci. USA* 83, 6445-6449.

Anderson, R.G., Brown, M.S., and Goldstein, J.L. (1977). Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell* 10, 351-364.

Androlewicz, M.J., Brandenburg, D.F., and Straus, D.S. (1989). Insulin receptor internalization defect in an insulin-resistant mouse melanoma cell line. *Biochemistry* 28, 9750-9757.

Backer, J.M., Kahn, C.R., Cahill, D.A., Ullrich, A., and White, M.F. (1990). Receptor-mediated internalization of insulin requires a 12-amino acid sequence in the juxtamembrane region of the insulin receptor beta-subunit. *J. Biol. Chem.* 265, 16450-16454.

Backer, J.M., Kahn, C.R., and White, M.F. (1989). Tyrosine phosphorylation of the insulin receptor is not required for receptor internalization: studies in 2,4-dinitrophenol-treated cells. *Proc. Natl. Acad. Sci. USA* 86, 3209-3213.

Backer, J.M., Shoelson, S.E., Haring, E., and White, M.F. (1991). Insulin receptors internalize by a rapid, saturable pathway requiring receptor autophosphorylation and an intact juxtamembrane region. *J. Cell Biol.* 115, 1535-1545.

Backer, J.M., Shoelson, S.E., Weiss, M.A., Hua, Q.X., Cheatham, R.B., Haring, E., Cahill, D.C., and White, M.F. (1992). The insulin receptor

juxtamembrane region contains two independent tyrosine/beta-turn internalization signals. *J. Cell Biol.* 118, 831-839.

Bansal, A., and Gierasch, L.M. (1991). The NPXY internalization signal of the LDL receptor adopts a reverse-turn conformation. *Cell* 67, 1195-1201.

Carpentier, J.L., Paccaud, J.P., Gorden, P., Rutter, W.J., and Orci, L. (1992). Insulin-induced surface redistribution regulates internalization of the insulin receptor and requires its autophosphorylation. *Proc. Natl. Acad. Sci. USA* 89, 162-166.

Chang, C.-P., Lazar, C.S., Kao, J.P., Wells, A., Tsien, R.Y., Wiley, H.S., Gill, G.N., and Rosenfeld, M.G. (1991). Ligand-induced internalization and calcium mobilization are mediated via distinct structural elements in the carboxy terminus of the epidermal growth factor receptor. *J. Biol. Chem.* 266, 23467-23470.

Chang, C.-P., Lazar, C.S., Walsh, B.J., Komuro, M., Collawn, J.F., Kuhn, L.A., Tainer, J.A., Trowbridge, I.S., Farquhar, M.G., Rosenfeld, M.G., Wiley, H.S., and Gill, G.N. (1993). Ligand-induced internalization of the epidermal growth factor receptor is mediated by multiple endocytic codes analogous to the tyrosine motif found in constitutively internalized receptors. *J. Biol. Chem.* 268, 19312-19320.

Chen, W.S., Lazar, C.S., Lund, K.A., Welsh, J.B., Chang, C.P., Walton, G.M., Der, C.J., Wiley, H.S., Gill, G.N., and Rosenfeld, M.G. (1989). Functional independence of the epidermal growth factor receptor from a domain required for ligand-induced internalization and calcium regulation. *Cell* 59, 33-43.

Chen, W.S., Lazar, C.S., Poenie, M., Tsien, R.Y., Gill, G.N., and Rosenfeld, M.G. (1987). Requirement for the intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. *Nature* 328, 820-823.

Collawn, J.F., Stangel, M., Kuhn, L.A., Esekogwu, V., Jing, S.Q., Trowbridge, I.S., and Tainer, J.A. (1990). Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. *Cell* 63, 1061-1072.

Davies, R.L., Grosse, V.A., Kucherlapati, R., and Bothwell, M. (1980). Genetic analysis of epidermal growth factor action: assignment of human epidermal growth factor receptor gene to chromosome 7. *Proc. Natl. Acad. Sci. USA* 77, 4188-4192.

Davis, C.G., Lehrman, M.A., Russell, D.W., Anderson, R.G.W., Brown, M.S., and Goldstein, J.L. (1986). The J.D. mutation in familial hypercholesterolemia: amino acid substitution in cytoplasmic domain impedes internalization of LDL receptors. *Cell* 45, 15-24.

Eberle, W., Sander, C., Klaus, W., Schmidt, B., von Figura, K., and Peters, C. (1991). The essential tyrosine of the internalization signal in lysosomal acid phosphatase is part of a beta turn. *Cell* 67, 1203-1209.

Ebina, Y., Edery, M., Ellis, L., Standing, D., Beaudoin, J., Roth, R.A., and Rutter, W.J. (1985a). Expression of a functional human insulin receptor from a cloned cDNA in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA* 82, 8014-8018.

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.H., Masiarz, F., Kan, Y.W., Goldfine, I.D., Roth, R., and Rutter, W.J. (1985b). The human insulin receptor cDNA: the structural basis for hormone-activated transmembrane signalling. *Cell* 40, 747-758.

Glenney, J.R., Jr., Zokas, L., and Kamps, M.P. (1988). Monoclonal antibodies to phosphotyrosine. *J. Immunol. Methods* 109, 277-285.

Gustafson, T.A., and Rutter, W.J. (1990). The cysteine-rich domains of the insulin and insulin-like growth factor I receptors are primary determinants of hormone binding specificity. Evidence from receptor chimeras. *J. Biol. Chem.* 265, 18663-18667.

Haigler, H., Ash, J.F., Singer, S.J., and Cohen, S. (1978). Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells A-431. *Proc. Natl. Acad. Sci. USA* 75, 3317-3321.

- Hofmann, C., White, M.F., and Whittaker, J. (1989). Human insulin receptors expressed in insulin-insensitive mouse fibroblasts couple with extant cellular effector systems to confer insulin sensitivity and responsiveness. *Endocrinology* 124, 257–264.
- Honegger, A.M., Dull, T.J., Felder, S., Van Obberghen, E., Bellot, F., Szapary, D., Schmidt, A., Ullrich, A., and Schlessinger, J. (1987). Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. *Cell* 51, 199–209.
- Ishiura, M., Hirose, S., Uchida, T., Hamada, Y., Suzuki, Y., and Okada, Y. (1982). Phage particle-mediated gene transfer to cultured mammalian cells. *Mol. Cell. Biol.* 2, 607–616.
- Johnson, K.F., Chan, W., and Kornfeld, S. (1990). Cation-dependent mannose 6-phosphate receptor contains two internalization signals in its cytoplasmic domain. *Proc. Natl. Acad. Sci. USA* 87, 10010–10014.
- Kawamoto, T., Sato, J.D., Le, A., Polikoff, J., Sato, G.H., and Mendelsohn, J. (1983). Growth stimulation of A431 cells by epidermal growth factor: identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc. Natl. Acad. Sci. USA* 80, 1337–1341.
- Ktistakis, N.T., Thomas, D., and Roth, M.G. (1990). Characteristics of the tyrosine recognition signal for internalization of transmembrane surface glycoproteins. *J. Cell Biol.* 111, 1393–1407.
- Kunkel, T. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* 82, 488–492.
- Lund, K.A., Opreko, L.K., Starbuck, C., Walsh, B.J., and Wiley, H.S. (1990). Quantitative analysis of the endocytic system involved in hormone-induced receptor internalization. *J. Biol. Chem.* 265, 15713–15723.
- McClain, D.A., Maegawa, H., Lee, J., Dull, T.J., Ulrich, A., and Olefsky, J.M. (1987). A mutant insulin receptor with defective tyrosine kinase displays no biologic activity and does not undergo endocytosis. *J. Biol. Chem.* 262, 14663–14671.
- Norwood, T.H., Zeigler, C.J., and Martin, G.M. (1976). Dimethyl sulfoxide enhances polyethylene glycol-mediated somatic cell fusion. *Somatic Cell Genet.* 2, 263–270.
- Pilch, P.F., Shia, M.A., Benson, R.J., and Fine, R.E. (1983). Coated vesicles participate in the receptor-mediated endocytosis of insulin. *J. Cell Biol.* 93, 133–138.
- Rajagopalan, M., Neidigh, J.L., and McClain, D.A. (1991). Amino acid sequences Gly-Pro-Leu-Tyr and Asn-Pro-Glu-Tyr in the submembranous domain of the insulin receptor are required for normal endocytosis. *J. Biol. Chem.* 266, 23068–23073.
- Russell, D.S., Gherzi, R., Johnson, E.L., Chou, C.-K., and Rosen, O.M. (1987). The protein tyrosine kinase activity of the insulin receptor is necessary for insulin-mediated receptor down-regulation. *J. Biol. Chem.* 262, 11833–11840.
- Seino, S., Seino, M., Nishi, S., and Bell, G.I. (1989). Structure of the human insulin receptor gene and characterization of its promoter. *Proc. Natl. Acad. Sci. USA* 86, 114–118.
- Thies, R.S., Webster, N.J., and McClain, D.A. (1990). A domain of the insulin receptor required for endocytosis in rat fibroblasts. *J. Biol. Chem.* 265, 10132–10137.
- Trowbridge, I.S. (1991). Endocytosis and signals for internalization. *Curr. Opin. Cell Biol.* 3, 634–641.
- Wiley, H.S., and Cunningham, D.D. (1982). The endocytic rate constant: a cellular parameter for quantitating receptor-mediated endocytosis. *J. Biol. Chem.* 257, 4222–4229.
- Wiley, H.S., Herbst, J.J., Walsh, B.J., Lauffenburger, D.A., Rosenfeld, M.G., and Gill, G.N. (1991). The role of tyrosine kinase activity in endocytosis, compartmentation, and down-regulation of the epidermal growth factor receptor. *J. Biol. Chem.* 266, 11083–11094.