Identification of an Insulin-responsive, Slow Endocytic Recycling Mechanism in Chinese Hamster Ovary Cells

Amy O. Johnson‡, Agathe Subtil‡, Rebecca Petrush‡, Keith Kobylarz‡, Susanna R. Keller§, and Timothy E. McGraw¶¶

From the ‡Department of Biochemistry, Cornell University Medical College, New York, New York 10021 and the ¶Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755

In adipocytes, the insulin-regulated aminopeptidase (IRAP) is trafficked through the same insulin-regulated recycling pathway as the GLUT4 glucose transporter. We find that a chimera, containing the cytoplasmic domain of IRAP fused to transmembrane and extracellular domains of the transferrin receptor, is slowly recycled and rapidly internalized in Chinese hamster ovary cells. Morphological studies indicate that the chimera is slowly trafficked through the general endosomal recycling compartment rather than being sorted to a specialized recycling pathway. A chimera in which a di-leucine sequence within the cytoplasmic domain of IRAP has been mutated to alanines is rapidly internalized and rapidly recycled, indicating that this di-leucine is required for the slow recycling but not for the rapid internalization. Insulin stimulates a 2–3-fold increase in the recycling of the chimera and only a 1.2-fold increase in the recycling of the transferrin receptor. The effect of insulin on the recycling of the chimera is blocked by wortmannin, a phosphatidylinositol 3'-kinase inhibitor. GTP\(\gamma\)S (guanosine 5'-3-O-(thio)triphosphate) increases the recycling of the chimera by 50% but has no effect on the recycling of the transferrin receptor. In these studies, we have identified in Chinese hamster ovary cells a novel, slow endocytic recycling mechanism that is regulated by insulin.

GLUT4 is a glucose transporter isoform predominantly expressed in fat and muscle cells (reviewed in Ref. 1). In the absence of insulin, ~90% of GLUT4 is localized to intracellular compartments. Exposure of fat and muscle cells to insulin stimulates a translocation of GLUT4-containing vesicles to the plasma membrane, shifting the distribution to a new steady state in which 50% of GLUT4 is on the cell surface. In both the basal and insulin-stimulated states GLUT4 is continually internalized and recycled back to the cell surface (2, 3). Insulin induces the redistribution of GLUT4 to the cell surface predominantly by increasing the recycling rate of GLUT4 by 3–9-fold (2–5). Insulin also affects general membrane trafficking; however, these effects are small compared with the effect on GLUT4 (6). Both the carboxyl- and amino-terminal cytoplasmic domains of GLUT4 contain information that targets GLUT4 to the insulin-responsive pathway (7–9). When GLUT4 is expressed in cell types other than fat or muscle it is concentrated intracellularly in the absence of insulin, yet insulin does not cause a large translocation to the cell surface (see, e.g., Refs. 10–14).

A type II membrane protein with aminopeptidase activity has been isolated from GLUT4-containing vesicles (15, 16). This protein, originally named vp165 or gp160, is now referred to as IRAP, for insulin-regulated aminopeptidase (17). In fat and muscle cells, IRAP has the same trafficking characteristics as GLUT4 (15, 16, 18, 19). The information required for targeting IRAP to the insulin-regulated membrane trafficking pathway is likely to be contained within its single 109-amino acid cytoplasmic domain because, as noted above, the information that regulates GLUT4 trafficking is found within its cytoplasmic domains.

The internalization and recycling of the transferrin receptor (TR) has been extensively characterized. Briefly, TR binds dfferic transferrin (Tf) and is rapidly internalized through clathrin-coated pits into acidic endosomes. At the acidic intralumenal pH of endosomes, Fe is released from Tf and the iron-free Tf remains bound to its receptor (20). The TfTR complex is returned to the plasma membrane, and Tf is released from the TR at the neutral extracellular pH. We and others have used TR chimeras as reporter molecules to study endocytic trafficking (see, e.g., Refs. 21 and 22). Advantages of using the TR for these studies are that substitution of cytoplasmic domains for the cytoplasmic domain of the TR does not affect the binding of Tf to the TR, and therefore Tf can be used in biochemical and microscopy studies to characterize the internalization, intracellular trafficking, and recycling back to the cell surface of the chimeras. The only known endocytic trafficking motif of the TR is a cytoplasmic YTRF internalization motif (23). Unlike internalization, efficient recycling back to the plasma membrane does not require a specific recycling motif (24–26). Consequently, the transmembrane and extracellular domains of the TR do not contribute to the trafficking characteristics of chimeras containing these domains.

In this report, we characterize the behavior of chimeras between IRAP and the human TR. In one chimera, vpTM-TR, the cytoplasmic domain of IRAP was substituted for the single amino-terminal cytoplasmic domain of the TR, and in the other, vpTM-TR, the cytoplasmic and transmembrane domains of IRAP were fused to the extracellular domain of the TR. Both

* This work was supported by National Institutes of Health Grant RO1-DK32552 (to T. E. M.), a research grant from Metabolix, Inc. (to T. E. M.), National Institutes of Health Grant RO1-DR5536 (to S. R. K.), and a fellowship from the Charles H. Revson Foundation (to A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, Cornell University Medical College, 1300 York Ave., New York, NY 10021. Tel.: 212-746-4982; Fax: 212-746-8875; E-mail: temcgraw@mail.med.cornell.edu.

† The abbreviations used are: TR, transferrin receptor; Tf, transferrin; CHO, Chinese hamster ovary; TGN, trans-Golgi network; PCR, polymerase chain reaction; SM, sphingomyelin; TM, transmembrane; GTP\(\gamma\)S, guanosine 5’-3-O-(thio)triphosphate; MES, 4-morpholineethanesulfonic acid.
chimeras are recycled at about one-third the rate of the wild type TR and internalized at the same rate as the TR. These data demonstrate that the cytoplasmic domain of IRAP contains information that promotes rapid internalization and slow recycling. Morphological studies indicate that the vp-TR is slowly trafficked through the general endosomal recycling compartments rather than being sorted to a specialized recycling pathway. The slow recycling of vp-TR is dependent on a di-leucine sequence located at position 76 in the cytoplasmic domain of IRAP because a mutant in which alanines have been substituted for these leucines is recycled at the same rate as wild type TR. Insulin stimulates the recycling of vp-TR by 2–3-fold and causes a much smaller increase, 1.2-fold, in the recycling rate of the wild type TR. The effect of insulin on the recycling of the chimera is blocked by wortmannin, a phosphatidylinositol 3'-kinase inhibitor. GTPyS increases the recycling of the chimera but has no effect on the recycling of the TR. We conclude that CHO cells possess a specialized, slow recycling mechanism that is regulated by insulin.

MATERIALS AND METHODS

Cell Culture—TRb, a CHO cell line that does not express functional endogenous TR, was used for transfection studies (27). The cells were transfected with the vp-TR chimera using Lipofectin (Life Technologies, Inc.) according to the manufacturer's recommendations. The G418 resistance plasmid, pSV3Neo, was co-transfected as a selectable marker. Cells were selected in 1 mg/ml G418, and individual colonies were isolated with cloning cylinders. The isolation and characterization of TRb cells expressing the wild type human TR and the Δ3–59 TR deletion construct have been described (27, 28). Cells were grown in either Ham's F-12 medium or McCoy's 5A medium containing 5% fetal bovine serum, penicillin-streptomycin (Life Technologies, Inc.), and 220 mM sodium bicarbonate (Sigma).

Ligands—Human Tf was obtained from Sigma and further purified by Sephacryl S-500 gel filtration. Differ T9, T11-Tf, and 52FeCl3 were prepared as described previously (21). 125I and 55Fe2 were purchased from NEN Life Science Products. Tf was labeled with the fluorescent dye Cy3 (Biological Detection Systems, Pittsburgh, PA) according to the manufacturer's instructions.

Chimera Construction—Oligonucleotides were purchased from Operon Technologies (Alameda, CA) or Life Technologies, Inc. CDAs of IRAP (16) and the human TR cloned into pUC8 vector (27) were used to prepare cDNAs of dye Cy3 (Biological Detection Systems, Pittsburgh, PA) according to the manufacturer's recommendations. The G418 resistance plasmid, pSV3Neo, was co-transfected as a selectable marker. The cells were selected in 1 mg/ml G418, and individual colonies were isolated with cloning cylinders. The isolation and characterization of TRb cells expressing the wild type human TR and the Δ3–59 TR deletion construct have been described (27, 28). Cells were grown in either Ham's F-12 medium or McCoy's 5A medium containing 5% fetal bovine serum, penicillin-streptomycin (Life Technologies, Inc.), and 220 mM sodium bicarbonate (Sigma).

Ligands—Human Tf was obtained from Sigma and further purified by Sephacryl S-500 gel filtration. Differ T9, T11-Tf, and 52FeCl3 were prepared as described previously (21). 125I and 55Fe2 were purchased from NEN Life Science Products. Tf was labeled with the fluorescent dye Cy3 (Biological Detection Systems, Pittsburgh, PA) according to the manufacturer's instructions.

Chimera Construction—Oligonucleotides were purchased from Operon Technologies (Alameda, CA) or Life Technologies, Inc. CDAs of IRAP (16) and the human TR cloned into pUC8 vector (27) were used to prepare cDNAs of dye Cy3 (Biological Detection Systems, Pittsburgh, PA) according to the manufacturer's recommendations. The G418 resistance plasmid, pSV3Neo, was co-transfected as a selectable marker. The cells were selected in 1 mg/ml G418, and individual colonies were isolated with cloning cylinders. The isolation and characterization of TRb cells expressing the wild type human TR and the Δ3–59 TR deletion construct have been described (27, 28). Cells were grown in either Ham's F-12 medium or McCoy's 5A medium containing 5% fetal bovine serum, penicillin-streptomycin (Life Technologies, Inc.), and 220 mM sodium bicarbonate (Sigma).

Insulin-regulated Endocytic Recycling in CHO Cells

Steady-state Distribution—The amount of chimera on the surface was determined using a previously described assay (30).

Internalization Assay—Cells were incubated for 1 h in medium 1 at 37 °C, followed by an incubation in medium 1 with 5 μg/ml iodinated Tf for 30 min. The cells were washed, and incubated for 5 min at 4 °C in 0.5 mM NaCl, 0.5 mM glacial acetic acid, pH 3.0. The cells were then washed with medium 2 (4 °C), solubilized, and the radioactivity measured. This value represents the TF internalized during the incubation at 37 °C. The steady-state amount of Tf bound to the cell surface is determined in parallel dishes by incubating cells with iodinated Tf for 2 h at 4 °C. The slope of the plot of the ratio of internal Tf to surface Tf versus time is the internalization rate constant.

55Fe Accumulation—Cells were incubated in medium 1 for 1 h at 37 °C, and then incubated in 3 μg/ml 55Fe2 Tf at 37 °C. At the times indicated, the cells were washed three times with medium 2, solubilized, and cell-associated 55Fe determined. To compare Fe accumulation data among cells expressing varying amounts of chimera or TR, the Fe accumulation data were normalized to surface Tf binding of 30 ng/well. Actual surface Tf binding was determined in parallel dishes as described above.

Recycling Assay—Cells were preincubated for 1 h in medium 1 at 37 °C, followed by an incubation in medium 1 with 5 μg/ml iodinated Tf for 30 min at 37 °C. The cells were washed with medium 2 and incubated for 2 min in 0.2 mM NaCl, 50 mM MES, pH 5.0, followed by three washes with medium 2 supplemented with 3 μg/ml unlabeled Tf. Incubation in the acidic medium releases Tf bound to TR on the cell surface. The cells were incubated at 37 °C in medium 1 with 3 μg/ml unlabeled Tf and 100 μg/ml of the iron chelator desferoxamine (eflux medium). At the times indicated, the medium was collected and the cells solubilized. The radioactivity in the medium is the Tf released from the cells during the incubation, and the cell-associated radioactivity is the Tf remaining inside cells. The recycling rate constant is the slope of the natural logarithm of the percent Tf remaining cell-associated versus time. To examine the effect of insulin on recycling, cells were treated as above except that the eflux medium was made 100 nM insulin. For experiments measuring the effect of wortmannin on recycling, 100 nM wortmannin was added to cells for the last 30 min of incubation with the iodinated Tf, and 100 nM wortmannin was added to all subsequent buffers used in the assay. For experiments measuring the effect of nocardazole on the recycling of the vp-TR chimera, 10 μg/ml nocardazole was added for the last 30 min of incubation with iodinated Tf and 10 μg/ml nocardazole was included in all subsequent incubations.

Endocytosis in Permeabilized Cells—The assay to measure recycling in streptolysin O-permeabilized cells has been described in detail (31). Streptolysin O was purchased from Murex, Inc. (Dartford, United Kingdom). Donor cytosol was prepared from mouse liver and used at a protein concentration of 9 mg/ml (31). GTPyS was used at a concentration of 50 μM.

Microscopy—Cells, grown on coverslip bottom dishes, were incubated with 3 μg/ml Cy3-Tf in medium 1 at 37 °C for >2 h. The cells were labeled with bodipy-sphingomyelin (SM) as described previously (26). Briefly, the cells were pre-incubated with fluorescent Tf (Cy3-Tf) for 3 h at 37 °C, chilled to 4 °C, washed once with medium 2, and the plasma membrane labeled with fluorescent SM by incubating with bodipy-SM liposomes for 30 min at 4 °C (26). The cells were then washed three times with medium 2 and incubated at 37 °C for 20 min in medium 2 containing 3 μg/ml Cy3-Tf. Following this incubation, the cells were placed on ice and washed three times, 5 min each wash, with 5 mg/ml fatty acid-free bovine serum albumin. To examine the steady-state distributions of Tf and SM, the cells were fixed in 3% formaldehyde in medium 2. To examine the recycling of Tf and SM, the cells were incubated at 37 °C for 30 min in medium 2 fixed with 5 µg/ml fatty acid-free bovine serum albumin and 3 μg/ml Tf. At the end of the incubation, the cells were washed once with medium 2 and fixed in 3% formaldehyde in medium 2. For the efflux experiments, cells were singly labeled with either Cy3-Tf or bodipy-SM and processed in parallel.

For experiments using nocardazole, 10 μg/ml nocardazole was added to the cells during the first incubation with Cy3-Tf. Nocardazole was also added to the buffers used for incubations at 37 °C. The cells were imaged in a Leitz DM-IRB fluorescence microscope with a cooled CCD camera (Princeton Instruments Inc., West Chester, PA). Images were collected using Metamorph Image processing software (Universal Imaging Corporation, West Chester, PA). Immunofluorescence with a monoclonal antibody specific for the extracellular domain of the human TR (B93/25; Boehringer Mannheim) was performed as described previously (21).
RESULTS

Expression of the vp-TR Chimera in CHO Cells—To determine the role that the cytoplasmic and transmembrane domains play in determining the endocytic trafficking of IRAP, two chimeras were constructed. In one, vp-TR, the 109-amino acid cytoplasmic domain of IRAP was fused to the transmembrane and extracellular domains of the human TR, and in the other, vpTM-TR, the cytoplasmic and transmembrane domains of IRAP were fused to the extracellular domain of the human TR (Fig. 1). TRVb CHO cells were transfected with these constructs and single colonies were isolated. TRVb CHO cells do not express functional endogenous TR and therefore provide a TR-free background for studies of the trafficking of transfected TR constructs (27). Individual clonal lines expressing the chimeras were identified by immunofluorescence using a monoclonal antibody against the extracellular domain of the human TR, B3/25 (27). The wild type human TR and a cytoplasmic deletion mutant of the human TR (Δ3–59 TR) have previously been transfected into TRVb CHO cells (25, 27). Uptake of iodinated Tf was examined for each clone and clonal lines that express less of the chimeras than control cells express of the wild type TR were chosen for study. Several independently isolated clonal lines expressing the chimeras were studied, and in no case were there significant differences among clones expressing the same construct.

vp-TR and vpTM-TR Are More Concentrated Intracellularly than the Wild Type TR—Steady-state conditions were established as follows. Cells were incubated with iodinated Tf at 37 °C, and the amount of cell-associated radioactivity was determined as a function of time. Cell-associated radioactivity asymptotically approaches a plateau level in cells expressing the vp-TR chimera (Fig. 2A). Binding of Tf to TR on the surface saturates within 5 min at 37 °C (20), and the increase in cell-associated radioactive Tf reflects the delivery of internalized chimeras to the surface which at the start of the experiment are unoccupied with iodinated Tf. Thus, the time required to reach the plateau level is the time required for the internal population of the chimera to cycle through the plasma membrane. In cells expressing the vp-TR chimera or the vpTM-TR chimera, the plateau level is reached within 100 min. In subsequent experiments, incubations with Tf of at least 2 h were used to measure the steady-state trafficking kinetics of the chimeras. The steady-state distributions of the vp-TR and vpTM-TR chimeras between the surface and the cell interior were determined. At steady-state, ~10% of the vp-TR and vpTM-TR chimeras are on the surface, whereas ~30% of the wild type TR and ~70% of the Δ3–59 TR are on the surface (Fig. 2B). The distribution of the Δ3–59 TR is shifted to the cell surface, relative to the TR, because deletion of the cytoplasmic domain of the TR removes the internalization motif and thereby slows internalization. The observation that the distribution of vp-TR is different from the distribution of the Δ3–59 TR demonstrates that the cytoplasmic domain of IRAP contains information that regulates endocytic trafficking. The finding that the two chimeras have the same distribution indicates that the transmembrane domain of IRAP does not contain information that affects endocytic trafficking in CHO cells. This conclusion was confirmed upon further characterization of the trafficking parameters (see below). The observation that the distribution of vp-TR is different from that of the TR indicates differences in the trafficking of the vp-TR relative to the TR. The greater intracellular concentration of vp-TR, relative to the TR, could be due to an increased rate of internalization, a decreased rate of recycling, or changes in both parameters.

The vp-TR Chimera Is Rapidly Internalized from the Plasma Membrane—The internalization rate constants of the chimeras were measured (Fig. 3A). The vp-TR and vpTM-TR chimeras are internalized at essentially the same rate as the TR, which is ~7-fold faster than the rate of Δ3–59 TR internalization. These data show that the cytoplasmic domain of IRAP contains a motif that promotes rapid internalization of the vp-TR and vpTM-TR.

Delivery of Fe to cells is the biological function of Tf internalization. Efficient Fe accumulation by cells is dependent on internalization of Tf into acidic endosomal compartments, where Fe is released from Tf. To determine whether Tf internalized by the vp-TR chimera is capable of delivering Fe to
cells, the ability of cells expressing vpTR to accumulate Fe from diferric Tf was measured (Fig. 3B). Cells expressing the vpTR chimera accumulate Fe from diferric Tf as efficiently as do cells expressing the TR, indicating that vpTR delivers Tf to acidic endosomal compartments.

The vpTR and vpTM/TR Chimeras Are More Slowly Recycled than the TR—We next determined the rate at which the chimeras are recycled. The results from a representative experiment are shown in Fig. 4 (A and B). In cells expressing the wild type TR, greater than 80% of the Tf is released from cells within 60 min. In cells expressing the vpTR chimera, the decrease in cell-associated Tf is slow relative to that observed in cells expressing the TR. Although the release of Tf is slower in cells expressing the vpTR chimera, Tf is recycled from these cells to the same degree as it is in cells expressing the TR, with greater than 80% of the Tf released by 120 min. The slow recycling of Tf in cells expressing the vpTR chimera is not due to inefficient Fe release from Tf, inasmuch as Fe is efficiently released from Tf internalized into cells by vpTR, as shown in Fig. 3B. Greater than 90% of the Tf released from cells expressing either vpTR or wild type TR was precipitated with 15% trichloroacetic acid at 4 °C. These data indicate that Tf is recycled intact and it is not being significantly targeted to degradative endosomal compartments.

To quantify the differences in recycling rates between vpTR and the TR, the initial recycling rate constants were calculated by plotting the natural log of the percent cell-associated Tf versus time (Fig. 4B). The mean recycling rate constants for the TR, the Δ3–59 TR, and the vpTR and vpTM/TR chimeras are presented in Fig. 4C. The recycling rate of the vpTR and vpTM/TR chimeras is ~3-fold slower than the recycling rate of the TR. The Δ3–59 TR is recycled at the same rate as the TR because the recycling of membrane proteins back to the cells surface is by a bulk flow process (26, 32). Thus, finding that vpTR and vpTM/TR are more slowly recycled than either the Δ3–59 TR or the TR demonstrates that the cytoplasmic domain of IRAP contains information that slows recycling.

At steady state, the distribution of a membrane protein between the surface and interior of cells is determined by the ratio of the recycling to internalization rate constants (25). The measured rates of internalization and recycling for the vpTR and vpTM/TR chimeras are consistent with 8% of the chimeras being on the cell surface at steady state, which is comparable to the measured values of ~10% (see Fig. 2B). Thus, the slower recycling of the vpTR and vpTM/TR chimeras, relative to the TR, accounts for the more pronounced intracellular localization of the chimera.

Because we find no differences in the kinetics of trafficking of vpTR and vpTM/TR, unless noted otherwise, the following microscopy experiments were performed on clonal lines expressing the vpTR chimera.

The vpTR Chimera Traffics through the General Endosomal System—Two general mechanisms could account for the slow recycling of the vpTR relative to the wild type TR. The vpTR chimera could traffic through different intracellular endosomal compartments or through the transcytotic pathway. Careful analysis of the Tf efflux experiment presented in Fig. 4A, however, suggests that the slower recycling of the vpTR is not due to a different route of endocytosis.

To determine whether vpTR traffics through the transcytotic pathway, we examined the Tf efflux pattern in cells expressing the vpTR chimera. The Tf efflux experiment showed that the vpTR chimera was recycled at a rate of 15% of the Tf associated with the cell surface at steady state. As with the Tf efflux experiment, the vpTR chimera accumulated Fe from diferric Tf with the same rate as the TR (Fig. 3B). These results are consistent with the Tf efflux experiment showing that the faster recycling of Tf in cells expressing the TR is not due to a difference in the route of endocytosis.
compartments than the TR, in which case the cytoplasmic domain of vpTR would contain information that targets the chimera to a slowly recycling pathway. Alternatively, vpTR could traffic more slowly through the same endosomal compartments as the TR. In this case, the slow recycling of vpTR would reflect regulated trafficking of vpTR through the general endosomal recycling pathway, and the cytoplasmic domain of IRAP would contain information that slows traffic of vpTR through the endosomal recycling system. In the former model, the vpTR chimera would be found in different intracellular compartments than the TR, whereas in the latter model, vpTR would be in the same intracellular compartments as the TR.

To determine whether the vpTR chimera traffics through the general endosomal recycling compartments, the intracellular compartments containing Tf internalized by the vpTR chimera were compared with those containing fluorescent sphingomyelin internalized from the plasma membrane. Previous studies have shown that fluorescent sphingomyelin inserted into the plasma membrane is trafficked through the same endosomal compartments as the TR (26, 33). In cells expressing vpTR, the intracellular compartments labeled with the fluorescent Tf internalized from the medium also contain bodipy-SM internalized from the plasma membrane (Fig. 5, A and B). The majority of Tf and bodipy-SM are concentrated as a single fluorescent patch located in the peri-centriolar region of the cell (arrows). In CHO cells, this juxta-nuclear compartment has been previously characterized as a compartment along the general endosomal recycling pathway (see, e.g., Refs. 26, 34, and 35). Tf and other recycling molecules accumulate in this compartment because the rate-limiting step in return to the surface is transport from this compartment back to the cell surface (26). Tf and bodipy-SM are also observed in peripheral, small punctate structures which are early sorting endosomes (36). Finding co-localization of Tf and bodipy-SM indicates that the vpTR chimera trafficks through the general endosomal recycling system. A similar co-localization of the vpTM-TR chimera and bodipy-SM was observed (not shown). The distributions of the Tf and bodipy-SM in cells expressing the wild type TR are similar to the distributions observed in cells expressing the chimera (Fig. 5, C and D), providing further support for the conclusion that the vpTR chimera is slowly recycled through the general recycling system rather than being targeted to a distinct slowly recycling pathway.

A number of membrane compartments are localized to the juxta-nuclear region of cells, including the Golgi, TGN, and lysosomes (37). To more rigorously demonstrate a co-localization between bodipy-SM and the chimera, we examined the co-distribution of vpTR and bodipy-SM in cells in which the microtubule cytoskeleton had been depolymerized with nocodazole. The peri-centriolar, endocytic recycling compartment is vesiculated and dispersed when cells are treated with nocodazole (34). The peri-centriolar localization of vpTR is fragmented and dispersed by treatment of cells with nocodazole, and most significantly, the fluorescent Tf remains co-distributed with the bodipy-SM (Fig. 5, E and F). The wild type TR and the bodipy-SM also co-distribute in nocodazole-treated cells (Fig. 5, G and H). These results indicate that the vpTR chimera and the bodipy-SM are in the same compartment because if they were in distinct, nocodazole-sensitive peri-centriolar compartments, they would not necessarily remain co-localized following nocodazole treatment. Treatment of cells with nocodazole does not promote aberrant mixing of distinct compartments located in the peri-centriolar region, as there is no mixing of the peri-centriolar, endocytic recycling compartment and the TGN in cells treated with nocodazole (not shown). Although nocodazole treatment disrupts the morphology of the recycling compartment, it has no effect on the recycling rate of the wild type TR (34). The recycling rate constant for vpTR is unchanged by nocodazole, demonstrating that an intact microtubule cytoskeleton is not required for slow recycling of the chimera (data not shown).

Endogenous Hamster IRAP Co-localizes with TR in CHO Cells—To examine the distribution of endogenous IRAP, CHO cells expressing the wild type human TR were incubated with Cy3-Tf to label endosomal compartments, and the endogenous
IRAP was localized using polyclonal antibodies against the cytoplasmic domain of IRAP (16). The endogenous IRAP co-localizes with the TR (Fig. 6). This finding indicates that the endogenous IRAP, like the vp/TR chimera, is in the general endosomal system. It is not possible to examine the co-distribution of the vp/TR chimera and endogenous IRAP, inasmuch as the only available antibody to IRAP recognizes the cytoplasmic domain and it therefore reacts with the vp/TR chimera.

**Expression of vp/TR Does Not Slow General Membrane Recycling**—We next sought to determine whether the slow recycling of vp/TR is characteristic of the chimera or if expression of the chimera slows the recycling of other molecules. To address this question, we examined the recycling of bodipy-SM in cells expressing the vp/TR chimera. Endosomal compartments were labeled with bodipy-SM and either fixed (A, C, E, and G) or re-incubated at 37 °C for 30 min (B, D, F, and H), during which time the Tf and SM are recycled back to the cell surface (see “Materials and Methods”). Size bar is 10 μm.

![Fig. 7. Efflux of Cy3-Tf (A–D) and bodipy-SM (E–H) from cells expressing vp(TR (A, B, E, and F) or human TR (C, D, G, and H). Intracellular endosomal compartments were labeled with Cy3-Tf or bodipy-SM and either fixed (A, C, E, and G) or re-incubated at 37 °C for 30 min (B, D, F, and H), during which time the Tf and SM are recycled back to the cell surface (see “Materials and Methods”). Size bar is 10 μm.](image)

![Fig. 8. A, mean internalization rate constants for clonal lines expressing vp/TR, LL53AA vp/TR, LL76AA vp/TR or LL53,76AA vp/TR. The values are the means ± S.E. of at least five independent experiments. The internalization rate constants were calculated as described under “Materials and Methods.” Similar results were obtained using other clonal lines expressing the various constructs. B, mean recycling rate constants for clonal lines expressing wild type TR, vp/TR, LL53AA vp/TR, LL76AA vp/TR, or LL53,76AA vp/TR. The values are the means ± S.E. of at least five independent experiments. The recycling rate constants were calculated as described under “Materials and Methods.” Similar results were obtained using other clonal lines expressing the various constructs.](image)

more slowly through the recycling compartments than is bodipy-SM, and consequently that the vp/TR chimera is specifically retained within the general endosomal recycling compartment.

The Di-leucine Sequence at Positions 76–77 of the Cytoplasmic Domain of IRAP Plays a Role in the Slow Recycling of the vp/TR Chimera—The above results demonstrate that the cytoplasmic domain of IRAP contains information that promotes rapid internalization and slows recycling. One class of endocytic trafficking motifs is based on di-leucine sequences (38). These motifs can function as both internalization and intracellular trafficking motifs. The cytoplasmic domain of IRAP contains two di-leucine motifs, one at positions 53–54 and the other at positions 76–77 (Fig. 1). To investigate the role of these sequences in the trafficking of vp/TR three constructs were created: LL53AA, in which alanines were substituted for the leucines at positions 53–54; LL76AA, in which alanines were substituted for the leucines at positions 76–77; and LL53,76AA, in which alanines were substituted for the leucines at positions 53–54 and 76–77. The mutant cDNAs were transfected into TRVb cells, clonal lines isolated and the endocytic behaviors of the constructs characterized. The mean internalization rate constants derived from a number of independent experiments are shown in Fig. 8A. Neither of the di-leucine sequences are necessary for internalization as all three mutant constructs are internalized at the same rate as vp/TR. These
findings demonstrate that sequences other than the di-leucine sequences are responsible for the rapid internalization of the vpTR chimera.

The mean recycling rate constants derived from a number of independent experiments are presented in Fig. 8B. The LL53AA mutant is recycled at the same rate as vpTR, indicating that the di-leucine sequence at positions 53–54 is not required for the slow recycling of vpTR. Mutation of the di-leucine sequence at positions 76–77, however, increases the recycling rate to approximately the recycling rate of the wild type TR. This finding demonstrates that the di-leucine at positions 76–77 of the cytoplasmic domain of IRAP is involved in regulating the recycling of the vpTR chimera. This conclusion is further supported by the finding that the construct containing mutations of both di-leucine sequences, LL53,76AA, is also rapidly recycled.

In fluorescence microscopy analysis, both the rapidly recycled LL76AA and the slowly recycled LL53AA, like the vp-TR chimera, are localized to the peri-centriolar, endocytic recycling compartment (not shown). Therefore, mutation of the di-leucine at positions 76–77 affects the kinetics of trafficking without detectably affecting the intracellular itinerary of the chimera. These data are consistent with the proposal that the slow recycling of vpTR is achieved by regulating its trafficking through the general endosomal recycling pathway.

Recycling of the vp-TR Chimera Is Regulated by Insulin—In fat and muscle cells, IRAP trafficking is regulated by insulin (15, 16, 18, 19). To examine the effect of insulin on the trafficking of vpTR in CHO cells, the recycling rate constant of the vp-TR chimera and the TR were measured in the presence of 100 nM insulin. Data from representative experiments on the effect of insulin on the recycling of the wild type TR and vp-TR are shown in Fig. 9 (A and B), and a summary of a number of experiments on the effect of insulin on the recycling of the TR, vpTR, vpTM-TR, LL53AA, and LL76AA are presented in Fig. 9C. Insulin had a large effect on the recycling rate constant of the vpTR and vpTM-TR chimeras, increasing the rate constant by 2–3-fold, whereas insulin had a small effect on the recycling of the TR, increasing the recycling rate constant by 1.2-fold. These data demonstrate that the recycling of the vpTR chimera is regulated by insulin. Analysis of the effect of insulin on the recycling of the LL53AA and LL76AA chimeras demonstrates that slow recycling is linked to the insulin response, as insulin has a large effect on the recycling of the slowly recycled LL53AA mutant and a small effect on the rapidly recycled LL76AA mutant (Fig. 9C).

The effect of insulin on expression of the vp-TR chimera and the TR on the cell surface was also examined. Cells were incubated for 10 min in the presence or absence of 100 nM insulin, chilled to 4 °C, and surface iodinated Tf binding measured at 4 °C. Insulin caused a 2.3 ± 0.3-fold (± S.D., n = 4) increase in surface expression of the vpTR chimera, and a 1.3 ± 0.4-fold (± S.D., n = 4) increase in surface expression of the TR. The increased surface expression of vpTR can be accounted for by the insulin-induced increase in the recycling rate of the vpTR chimera, and this indicates that insulin does not have a large effect on the internalization rate of the chimera.

The Insulin-stimulated Recycling of the vpTR Chimera Is Inhibited by Wortmannin—The insulin-induced translocation of GLUT4 to the cell surface is inhibited by wortmannin (see, e.g., Ref. 39). We therefore examined whether the insulin-regulated recycling of the vpTR chimera in CHO cells is also inhibited by wortmannin. Treatment of cells with 100 nM wortmannin, a concentration that inhibits the phosphatidylinositol 3′-kinase in CHO cells (40), blocks the insulin-induced increase in recycling of the vpTR chimera (Fig. 10A).

The Recycling of the vp-TR Chimera Is Stimulated by GTPγS—In permeabilized cells, GTPγS induces the translocation of GLUT4 to the surface (41, 42). We examined the effects of insulin and GTPγS on the recycling of the vpTR chimera reconstituted in permeabilized CHO cells. We have previously shown that GTPγS does not affect TR recycling reconstituted in semi-permeabilized CHO cells (31). Both GTPγS and insulin increased the recycling rate of the vpTR chimera in permeabilized cells by about 50%, and their effects on recycling were not additive (Fig. 10B). Neither insulin, GTPγS, nor insulin and GTPγS together had a large effect on the recycling of the TR in semi-permeabilized CHO cells, providing evidence that the effect of insulin on vpTR trafficking is not a consequence of alterations in general endocytic recycling.

**DISCUSSION**

In this report, we characterized the endocytic trafficking of chimeras that contain either the cytoplasmic domain of IRAP and the transmembrane and extracellular domains of the human TR (vpTR), or the cytoplasmic and transmembrane domains of IRAP and the extracellular domain of the TR (vpTM-TR). When expressed in CHO cells, vpTR and vpTM-TR, like the TR, are rapidly internalized, trafficked to the peri-
centriolar, endocytic recycling compartment, and recycled back to the cell surface. The trafficking of the chimeras differ from that of the TR in that the chimeras are recycled 3-fold more slowly. We do not observe any differences in the trafficking of vp-TR and vpTM-TR, indicating that the transmembrane domain of IRAP does not contain information which regulates endocytic trafficking. Thus, the 109-amino acid cytoplasmic domain of IRAP contains information that promotes rapid internalization from the plasma membrane as well as information that slows transport from the endocytic recycling compartment to the plasma membrane. A chimera in which the di-leucine at positions 76–77 is mutated to di-alanine is rapidly recycled and rapidly internalized, demonstrating that this di-leucine is required for slow recycling but not for rapid internalization. Insulin increases the recycling of vp-TR and vpTM-TR to near the rate observed for the wild type TR. These studies demonstrate that CHO cells possess the machinery that allows for the insulin-regulated dynamic retention of membrane proteins within the endocytic system.

Our data are consistent with vp-TR trafficking through the same endosomal recycling compartment as other more rapidly recycled molecules. This conclusion is based on the observation that vp-TR co-localizes in the peri-centriolar region of the cells with fluorescent-SM internalized from the plasma membrane. Previous work has shown that fluorescent-SM is recycled through the same peri-centriolar, endocytic recycling compart-

ment as is the TR (26). Importantly, the vp-TR chimera remains co-localized with the bodipy-SM following nocodazole-induced vesiculation of the peri-centriolar recycling compartment. This result argues against the chimera residing in a compartment adjacent to the recycling compartment that is not resolved as a distinct compartment in light microscopy, because if that were the case, vp-TR and bodipy-SM would not necessarily co-distribute when the compartments were vesiculated. The endogenous hamster IRAP is also in the peri-centriolar recycling compartment, providing evidence that the vp-TR chimera behaves like endogenous IRAP.

The steady-state localization of vp-TR in the peri-centriolar recycling compartment demonstrates that the rate-limiting step in the recycling of the vp-TR chimera is transport from this compartment, as has been previously shown for other recycled molecules (26, 32). Our results show that different membrane proteins can be transported from the recycling compartment at different rates. A number of previous observations demonstrate that the kinetics of trafficking from the peri-centriolar, endocytic recycling compartment can be slowed. Transport of the TR from the recycling compartment is slowed when endosomes are alkalized by pharmacological treatments or in acidification-defective mutant cells (25, 29, 32). Inhibition of endosome acidification has a greater effect on the recycling of the TR than it does on the recycling of lipid, indicating that transport of the TR from the peri-centriolar, endocytic recycling compartment is slowed relative to lipid (32). Another example of regulated transport from the peri-centriolar, endocytic recycling compartment is the inhibition of TR recycling by binding to multimeric Tf complexes (43). In the case of the vp-TR chimera, a di-leucine-based motif in the cytoplasmic domain of IRAP (a protein normally expressed in CHO cells) slows recycling of the vp-TR chimera, indicating that CHO cells dynamically retain certain proteins within the peri-centriolar, endocytic recycling compartment. These results are the first report of the regulated dynamic retention of a transmembrane protein within the endocytic system. Because the physiological role of IRAP is not known, it is not prudent to speculate on why IRAP is dynamically retained within the recycling compartment.

Di-leucine-based motifs have been shown to function as membrane protein trafficking motifs (see, e.g., Ref. 38). The molecular mechanism by which these motifs regulate membrane protein trafficking is not known, although they are believed to regulate targeting via a specific interaction with proteins that regulate formation of transport vesicles (e.g., coat and/or adaptin proteins). The di-leucine sequence required for the dynamic retention of vp-TR in CHO cells (positions 76–77) is in a region of the cytoplasmic domain of IRAP (residues 55–82), which, based on microinjection studies, interacts with the cellular machinery that regulates IRAP and GLUT4 distribution in fat cells (44). Thus, the results are consistent with the di-leucine at 76–77 being involved in insulin-regulated trafficking in both CHO and fat cells. GLUT4 has a di-leucine motif on its carboxyl cytoplasmic domain, which functions as an internalization motif but does not regulate recycling (7, 8, 9, 30, 45, 46). As discussed below, it is not known whether IRAP and GLUT4 traffic through the same pathway in CHO cells; therefore, a comparison in CHO cells of the function of targeting motifs of GLUT4 to those of IRAP is not warranted at this time.

Two possible models accounting for the slow recycling of vp-TR are depicted in Fig. 11. In one model, insulin-regulated retention, flow of vp-TR into the recycling vesicles that transport TR and other rapidly recycled molecules back to the cell surface is impeded, thereby slowing recycling of the chimera. In this case, an interaction of the cytoplasmic sequences of IRAP (based in part on the di-leucine sequence at positions 76–77)
Insulin-regulated Endocytic Recycling in CHO Cells

**Comparison between the Insulin-regulated Trafficking in CHO and Fat Cells**—Our data demonstrate that CHO cells, like fat and muscle cells, have a slow endocytic recycling mechanism that is regulated by insulin. In CHO cells, insulin increases the recycling of the TR by 1.2-fold and the recycling of vpTR by 2–3-fold. It is this large difference which shows that the chimera is trafficked by a specialized insulin-regulated mechanism, because if the effects of insulin on the trafficking of the vpTR chimera were due to effects on general membrane flow, then insulin would not have a greater effect on the recycling of vpTR than on the recycling of the TR. In 3T3-L1 adipocytes, insulin has a small effect on the trafficking of the TR (−2-fold increase in recycling) compared with its large effect on GLUT4 and IRAP (−9-fold increase) (see, e.g., Refs. 3, 6, and 18). As is the case in CHO cells, this large difference in response to insulin is one feature which establishes that GLUT4 and IRAP are trafficked by a specialized mechanism.

In addition to these kinetic similarities between the pathways in fat and CHO cells, two observations suggest that the molecular mechanisms of these insulin-regulated pathways are similar. 1) Wortmannin inhibits the insulin-stimulated recycling of the vpTR chimera in CHO cells as well as the insulin-stimulated translocation of GLUT4 to the cell surface in fat cells (see, e.g., Ref. 39). 2) GTPγS stimulates recycling of the vpTR chimera in permeabilized CHO cells as well as the translocation of GLUT4 in permeabilized fat cells (41, 42). Although these are consistent with mechanistic similarities in the insulin-regulated pathways in fat and CHO cells, inhibition of phosphatidylinositol 3'-kinase affects a number of trafficking pathways, including recycling of the TR (40, 49, 50), and the molecular mechanism underlying the effect of GTPγS on translocation of GLUT4 is not known.

Although the parallels between insulin-regulated trafficking in CHO and fat cells are provocative, there are a number of important distinctions. 1) As noted above, the magnitude of the effect of insulin on the recycling of the chimera in CHO cells is small compared with the effect of insulin on the recycling of IRAP in fat cells. 2) In fat cells, IRAP appears to be segregated away from the general recycling pathway to an insulin-regulated pathway (53–55), indicating that the IRAP dynamic retention mechanism in fat cells is different from that in CHO cells.

**Are GLUT4 and IRAP Trafficked by the Same Pathway in CHO Cells?**—In fat and muscle cells, IRAP and GLUT4 are trafficked by the same insulin-responsive mechanism (15, 16, 18, 19, 44). Unlike IRAP, GLUT4 is not endogenously expressed by fibroblasts. When expressed in CHO cells, GLUT4, like vpTR, is dynamically retained within cells in the absence of insulin, and slow recycling is the determining step in achieving this dynamic intracellular concentration (see, e.g., Ref. 51). However, a number of observations indicate that GLUT4 and the vpTR may not be trafficked by the same pathway in CHO cells. Epitope-tagged GLUT4 expressed in CHO cells was found enriched in a population of small vesicles that are not enriched in TR (56). This is not in agreement with our findings that both vpTR and hamster IRAP are in the general (TR-containing) recycling compartment. In addition, a number of studies have shown that GLUT4 expressed in CHO cells is not translocated to the surface by insulin (12, 14, 56, 57), in contrast to our findings that insulin increases the recycling of vpTR in CHO cells. Taken together, these findings indicate that GLUT4 and vpTR are not trafficked by the same mechanism in CHO cells.

In this regard, it has recently been shown in atrial cardiomyocytes that IRAP and GLUT4 do not completely co-distribute, indicating that in cell types other than CHO, IRAP and GLUT4

---

**Fig. 11. Models for the dynamic retention of the vpTR chimera in the peri-centriolar, endocytic recycling compartment of CHO cells.** In the top panels, the insulin-regulated retention model is shown. A, in the basal state, traffic of vpTR into recycling vesicles is inhibited by an interaction between its cytoplasmic domain and a cytoplasmic protein. The behavior of the LL76AA mutant vpTR indicates that the di-leucine at positions 76–77 is required for this interaction. B, insulin disrupts this interaction, allowing the vp-TR chimera to be recycled back to the plasma membrane in the same vesicles carrying the TR and other recycled molecules. In the bottom panels, the insulin-regulated budding model is shown. C, in the basal state vpTR is recycled to the plasma membrane by different vesicles than those that carry the TR and other rapidly recycled molecules. The di-leucine at positions 76–77 would be involved in concentrating vpTR into these vesicles. The vesicles that carry vpTR form more slowly, in the basal state, than those that carry the TR. D, insulin increases the formation of the vpTR-containing vesicles, thereby increasing recycling. The structure labeled ERC is the peri-centriolar endocytic recycling compartment; PM denotes the plasma membrane.

---

with proteins in the cytosol would prohibit the vpTR chimera from efficiently entering recycling vesicles formed from the endocytic recycling compartment (Fig. 11A). This model is conceptually similar to the mechanism proposed for the dynamic retention of proteins in the TGN (see, e.g., Ref. 47). Insulin would disrupt the interaction between the cytoplasmic domain of IRAP and the cytosolic proteins, freeing the vpTR chimera to enter the recycling vesicles and to be rapidly recycled to the cell surface (Fig. 11B). An alternative model, insulin-regulated budding, is that vpTR is transported to the surface in vesicles that are slowly formed from the endocytic recycling compartment (Fig. 11C). These vesicles are different from those that transport TR and other rapidly recycled molecules back to the cell surface. In this model, the cytoplasmic sequences of IRAP would interact with proteins that concentrate the vpTR chimera in the more slowly forming vesicles. This model requires the formation of two different types of vesicles from the peri-centriolar, endocytic recycling compartment, and is conceptually similar to the formation of distinct vesicles from the TGN that are destined for the apical or basolateral membrane (49).

In this model, insulin would increase recycling of the vpTR chimera by increasing the rate of formation of the vesicles enriched in vpTR (Fig. 11D).
may be differentially trafficked (54). Insulin regulation of GLUT4 surface expression in CHO cells has been observed when CHO cells when the insulin receptor is co-transfected with the epitope-tagged GLUT4 (57, 58). It is not known why the insulin receptor must be overexpressed to detect an effect of insulin on GLUT4 distribution in CHO cells. The CHO cells we have use not been transfected with the insulin receptor; thus, the effect we see on the trafficking of vpTR is based on the endogenous level of insulin receptor expression.

**CHO Cells Possess Specialized Membrane Trafficking Pathways**—A number of recent reports demonstrate specialized membrane trafficking pathways in CHO cells. In one report, a Ca+-regulated secretion pathway was identified (59), and in another report, apical- and basolateral-like transport vesicles formed from the TGN of CHO cells were identified (60). Like the insulin-regulated trafficking we have observed, Ca+-regulated exocytosis and polarized sorting from the TGN were thought to be characteristic of specialized cell types. Our findings, together with these other reports, indicate that CHO cells have specialized membrane trafficking mechanisms.

**REFERENCES**

1. James, D. E., and Piper, R. C. (1996) J. Cell Biol. 136, 1123–1126
2. Jhun, B., Rampla, A., Lui, H., Lachaal, M., and Jung, C. (1992) J. Biol. Chem. 267, 17710–17715
3. Yang, J., and Holman, G. D. (1993) J. Biol. Chem. 268, 4600–4603
4. Satoh, S., Nishimura, H., Clark, A. E., Kozka, I. J., Vannucci, S. J., Simpson, I. A., Quon, M. J., Cushman, S. W., and Holman, G. H. (1993) J. Biol. Chem. 268, 17820–17829
5. Yeh, J., Verhey, K. J., and Birnbaum, M. J. (1995) Biochem. J. 314, 15523–15531
6. Tanner, L. I., and Lienhard, G. E. (1987) J. Biol. Chem. 262, 8975–8980
7. Haney, P. M., Levy, M., Strube, M. S., and Mueckler, M. (1995) J. Cell Biol. 129, 641–658
8. Mar, B. J., Alm, R. A., McIntosh, S. R., and James, D. E. (1995) J. Cell Biol. 130, 1081–1091
9. Verhey, K. S., Yeh, J., and Birnbaum, M. J. (1995) J. Cell Biol. 130, 1071–1079
10. Haney, P. M., Slot, J. W., Lui, H., James, D. E., and Mueckler, M. (1991) J. Cell Biol. 114, 699–699
11. Hudson, A. W., Ruis, M. L., and Birnbaum, M. J. (1992) J. Cell Biol. 116, 785–791
12. Asano, T., Takata, K., Katagiri, H., Tsukuda, K., Lin, J. L., Ishihara, H., Inukai, K., Hirano, H., Yazaki, Y., and Oka, Y. (1992) J. Biol. Chem. 267, 19836–19841
13. Czech, M. P., Chawla, A., Woon, C.-H., Buxton, J., Armoni, M., Tang, W., Joly, M., and Corvera, S. (1995) J. Cell Biol. 131, 127–135
14. Kanai, F., Nishio, Y., Hayashi, H., Kamohara, S., Todaka, M., and Ebina, Y. (1995) J. Biol. Chem. 269, 14523–14526
15. Kandor, K. V., Yu, L., and Pilch, P. F. (1994) J. Biol. Chem. 269, 30777–30780
16. Keller, S. R., Scott, H. M., Morris, N. A., Leung, W.-Y., Mie, P., Lian, L. G., and Keller, S. R. (1996) J. Cell Biol. 130, 1081–1091
17. Keller, S. R. (1998) in *Handbook of Proteolytic Enzymes*, Eds. Rawlings, N., and Barlett, A. J., ed. Academic Press, Orlando, FL, in press
18. Ross, S. A., Scott, H. M., and Bakke, O. (1994) Trends Cell Biol. 4, 292–297
19. Clarkson, J. P., Young, P. W., Yonezawa, K., Kasuga, M., and Holman, G. D. (1994) Biochem. J. 306, 631–635
20. Keller, S., Wjasow, C., Gangi, D. M., Kielian, M. C., McGraw, T. E., and Backer, J. M. (1996) J. Biol. Chem. 271, 10953–10962
21. Baldini, G., Hohman, R., Charron, M. J., Lodish, H. F. (1991) J. Biol. Chem. 266, 4037–4040
22. Roblin, L., Pang, S., Harris, D., Heuser, J., and James, D. E. (1992) J. Cell Biol. 117, 1118–1123
23. Marsh, E. W., Leopold, P. L., Jones, N. L., and McGraw, F. R. (1995) J. Cell Biol. 129, 1599–1522
24. Waters, S. B., D’Auria, M., Martin, S. S., Nguyen, C., Kuzma, L. M., and Luksey, K. L. (1997) J. Biol. Chem. 272, 23233–23237
25. Verhey, K., and Birnbaum, M. J. (1994) J. Biol. Chem. 269, 2353–2356
26. Luzio, J. P., and Banting, G. (1993) Trends Biochem. Sci. 18, 395–398
27. Rodriguez-Boulan, E., and Powell, S. K. (1992) *Annu. Rev. Cell Biol.* 8, 395–427
28. Joly, M., Kazlauskas, A., and Corvera, S. (1995) J. Biol. Chem. 270, 12325–12330
29. Spiro, D. J., Boll, W., Kirchhausen, T., and Wassling-Resnick, M. (1996) *Mol. Biol. Cell* 7, 335–367
30. Araki, S., Yang, J., Hashimoto, M., Tamori, Y., Kasuga, M., and Holman, G. D. (1996) *Biochem. J.* 315, 153–159
31. Martin, S., Rice, J. E., Gould, G. W., Keller, S. R., Slot, J. W., and James, D. E. (1997) J. Cell Sci. 110, 2281–2291
32. Malide, D., St-Jenis, D.-F., Keller, S. R., and Cushman, S. W. (1997) *FEBS Lett.* 409, 461–468
33. Martin, S., Tellam, J., Lestone, G., Slot, J. W., Gould, G. W., and James, D. E. (1996) J. Cell Biol. 134, 625–635
34. Wei, M. L., Bensoliz, F., Scully, R. M., Kelly, R. B., and Herman, G. A. (1998) *J. Biol. Chem.* 273, 565–576
35. Shibaoka, K., Asano, T., Lu, J.-L., Tsukuda, K., Lin, J. L., Kasugia, H., Ishihara, H., Inukai, K., Yazaki, Y., and Oka, Y. (1992) *Biochem. J.* 281, 829–834
36. Kanai, F., Nishio, Y., Hayashi, H., Kamohara, S., Todaka, M., and Ebina, Y. (1993) *J. Biol. Chem.* 268, 14523–14526
37. Todaka, M., Hayashi, H., Imanaka, T., Mitan, Y., Kamohara, S., Kishi, K., Tamaoka, K., Kanai, F., Shichiri, M., Morii, N., Narumiya, S., and Ebina, Y. (1996) *Biochem. J.* 315, 873–882
38. Chavez, R. A., Miller, S. G., and Moore, H.-P. (1996) *J. Biol. Chem.* 271, 133, 1177–1191
39. Yoshimura, T., Keller, P., Roth, M. G., and Simon, K. E. (1996) *J. Cell Biol.* 133, 247–256