Previously we demonstrated that insulin-like growth factor-I mediates the sustained phosphorylation of Akt, which is essential for long term survival and protection of glial progenitors from glutamate toxicity. These prosurvival effects correlated with prolonged activation and stability of the insulin-like growth factor type-I receptor. In the present study, we investigated the mechanisms whereby insulin-like growth factor-I signaling, through the insulin-like growth factor type-I receptor, mediates the sustained phosphorylation of Akt. We showed that insulin-like growth factor-I stimulation induced loss of receptors from the cell surface but that surface receptors recovered over time. Blocking receptor internalization inhibited Akt phosphorylation, whereas inhibition of receptor trafficking blocked receptor recovery at the cell surface and the sustained phosphorylation of Akt. Moreover the insulin-like growth factor type-I receptor localized with the transferrin receptor and Rab11-positive endosomes in a ligand-dependent manner, further supporting the conclusion that this receptor follows a recycling pathway. Our results provide evidence that ligand stimulation leads to internalization of the insulin-like growth factor type-I receptor, which mediates Akt phosphorylation, and that receptor recycling sustains Akt phosphorylation in glial progenitors. Mathematical modeling of receptor trafficking further supports these results and predicts an additional kinetic state of the receptor consistent with sustained Akt phosphorylation.

Receptor tyrosine kinases mediate the activity of various signal transduction pathways, which regulate cell survival, proliferation, migration, or differentiation. Classically the activity of these signaling pathways is transient because receptor tyrosine kinases often are internalized followed by ligand/receptor dissociation and degradation. This paradigm of transient receptor tyrosine kinase signaling leading to ligand and receptor degradation has been well studied, particularly for the epidermal growth factor receptor, and is thought to be a major mechanism for the termination of signal transduction (1).

Few studies have addressed the mechanisms that mediate long term activation of signal transduction. We previously reported that stimulation of the insulin-like growth factor (IGF) type-I receptor (IGF-IR) mediates sustained activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in the absence of receptor down-regulation in glial progenitor cells (2–4). We further demonstrated that sustained Akt phosphorylation is essential for long term survival and IGF-1-mediated protection of these cells from trophic factor deprivation or excitotoxicity (2, 4). In contrast, neurotrophin-3 promotes only short term survival of the progenitor cells; this is correlated with transient Akt phosphorylation followed by down-regulation of the TrkC receptor (2, 5).

IGF-1/IGF-IR signaling is important for the survival and maintenance of multiple cell types within the central nervous system (6). Loss of IGF-1 results in a decrease in the number of neurons and oligodendrocytes (7–9), whereas overexpression of IGF-1 results in brain overgrowth and hypermyelination (10–15). Neurological deficits also have been reported in embryonic brains of IGF-IR null mice, which invariably die at birth (16). In more recent studies, Zeger et al. (17) demonstrated an essential role for the IGF-IR in oligodendrogial development using cell-specific deletion of the IGF-IR.

Although the downstream properties of IGF-IR signaling have been well studied, less is known about the subcellular trafficking of the IGF-IR and regulation of signaling pathway activation in neural cells. Moreover few studies have reported long term receptor stability and downstream activity for the IGF-IR or other receptor tyrosine kinases (18). Thus, it is of considerable interest to understand the mechanisms by which the IGF-IR sustains the phosphorylation of Akt and promotes long term survival of progenitor cells in the central nervous system.

The temporal and spatial regulation of signal transduction has been attributed to the subcellular localization of receptors and their downstream effector molecules (19). That signal transduction occurs rapidly and with high fidelity lends

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3 The abbreviations used are: IGF, insulin-like growth factor; IGF-I, insulin-like growth factor type-I; IGF-IR, insulin-like growth factor type-I receptor; ERK, extracellular signal-regulated kinase; Trk, transfrerin receptor; NHS, N-hydroxysuccinimide; CG, central glial; PBS, phosphate-buffered saline; OPC, oligodendrocyte progenitor cell; E3, ubiquitin-protein isopeptide ligase; PI3K, phosphatidylinositol 3-kinase.

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support to the hypothesis that receptors and signaling molecules are organized into specific subcellular compartments. Surface-localized receptor tyrosine kinases activate distinct signaling pathways compared with internalized, endosome-associated receptor tyrosine kinases leading to specific biological responses (20). Chow et al. (21) demonstrated that blocking internalization of the IGF-IR in Chinese hamster ovary cells inhibits ERK activation without affecting IGF-I-mediated Akt activation. Likewise blocking epidermal growth factor receptor internalization results in a decrease in ERK activation but an increase in protein kinase C activation (22). Furthermore nerve growth factor-mediated survival of PC12 cells is associated with surface-localized TrkA via the activation of the PI3K/Akt pathway, whereas internalized, endosome-associated TrkA induces nerve growth factor-mediated differentiation of these cells via the ERK pathway (23). Taken together, these studies suggest that the activation of specific signal transduction pathways and consequent biological responses are mediated through the subcellular compartmentalization of receptor tyrosine kinases.

In the present study we combined empirical studies and mathematical modeling to investigate how IGF-IR trafficking promotes and sustains phosphorylation of Akt in glial progenitor cells. We tested the hypothesis that internalization and recycling of the IGF-IR mediates sustained Akt phosphorylation. Our results show that internalization of the IGF-IR was associated with initial Akt phosphorylation; however, recycling factor-mediated differentiation of these cells via the ERK pathway (23). Taken together, these studies suggest that the activation of specific signal transduction pathways and consequent biological responses are mediated through the subcellular compartmentalization of receptor tyrosine kinases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture medium (minimum essential medium and Dulbecco’s modified Eagle’s medium/F-12) and fetal bovine serum were purchased from Invitrogen. Additional cell culture medium supplements and chemical inhibitors were purchased from Sigma. Recombinant human IGF-I was purchased from Upstate Biochemicals (Lake Placid, NY). Antibodies to Akt, phospho(Ser-473)-Akt, and phospho(Ser-9)-GSK-3β were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to cyclin D1, early endosomal antigen-1, and the IGF-IRα and β subunits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The transferrin receptor antibody and sheep anti-mouse Dynal magnetic beads were purchased from Invitrogen. The β-actin antibody and goat anti-rabbit and -mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The antibodies to Rab11 and GSK-3β were purchased from BD Transduction Laboratories. The glucose transporter 1 antibody was a generous gift from Ian Simpson (24). Sulfo-NHS-biotin and ImmunoPure streptavidin were purchased from Pierce.

**Cell Culture and Treatment Conditions**—The central glial (CG)-4 cell line, an immortalized glial progenitor that gives rise to astrocytes and oligodendrocytes, was a generous gift from Lynn Hudson (National Institutes of Health) (25). Cells were maintained in N2S medium composed of 1) 66% N2B2 medium (Dulbecco’s modified Eagle’s medium/F-12 supplemented with 0.6 mg/ml bovine serum albumin, 10 ng/ml β-biotin, 5 μg/ml insulin, 20 nM progesterone, 100 μM putrescine, 5 ng/ml selenium, 50 μg/ml apotransferrin, 100 units/ml penicillin, and 100 μg/ml streptomycin), 2) 34% B104 conditioning medium, 3) 5 ng/ml FGF-2, and 4) 0.5% fetal bovine serum. CG-4 cells were plated on poly-D-lysine-coated dishes at a density of 2.0 × 10^4 cells/cm^2 in N2S medium. Prior to all experiments cells were serum-starved in N1A (N2B2 medium without insulin) for 2–4 h and subsequently treated with 1 nM IGF-I with and without pharmacological inhibitors at the following concentrations: 100 μM dansylcadaverine, 10 μM monensin, and 10 μg/ml cycloheximide. For all inhibitor groups, cells were pretreated for 15 min.

**Western Blot Analysis**—Following treatments, cells were washed in ice-cold PBS, and total cell lysates were isolated in SDS sample buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol) containing 1:100 protease inhibitor mixture, 1 mM sodium vanadate, and 1 mM sodium fluoride (Sigma). Lysates were briefly sonicated on ice and quantified by a detergent-compatible protein assay (Bio-Rad). Approximately 20 μg of protein from each sample was boiled for 5 min, separated by SDS-PAGE on 4–12% minigels (Invitrogen), and subsequently electrophoretically transferred to nitrocellulose membranes. Membranes were blocked in 5% milk in Tris-buffered saline, 0.05 or 0.1% Tween for 1 h and incubated with primary antibodies overnight (1:1000; except cyclin D1, which was used at 1:250) at 4 °C in blocking buffer with gentle rocking. Membranes were incubated with the appropriate goat anti-rabbit or -mouse antibody at a dilution of 1:5000 in blocking buffer for 1 h at room temperature. The detection of horseradish peroxidase-conjugated secondary antibodies was performed with enhanced chemiluminescence (PerkinElmer Life Sciences). Membranes were exposed on film (Eastman Kodak Co.) within a linear range and digitally captured and quantified by NIH Image 1.62.

**Cell Surface Biotinylation Assay**—After IGF-I/inhibitor treatments, cells were rinsed with PBS (pH 7.4) and incubated with 0.3 mg/ml sulfo-NHS-biotin for 1 h at 4 °C. Reactions were quenched with 100 mM glycine for 10 min to remove unreacted biotin. Cells were then scraped in ice-cold PBS (plus protease inhibitors), and the volume of each sample was adjusted to 500 μl. Triton X-100 was added to a final concentration of 1% (v/v). Cells were passed through a 23-gauge needle and incubated on ice for 30 min with constant rocking. Lysates were briefly sonicated, and an aliquot was taken for protein normalization. Samples were incubated with ImmunoPure streptavidin for 1 h at 4 °C with end-over-end rocking. Streptavidin–precipitated complexes were rinsed three times in PBS containing 1% Triton X-100 and once in PBS. Biotinylated proteins were eluted with 2× SDS buffer and boiling. Protein samples were separated by SDS-PAGE and processed for Western blot analysis (as described above) for surface and total IGF-IR subunit immunoreactivity.
**IGF-IR Trafficking and Sustained Akt Phosphorylation**

*Endosome Separation*—Procedures were performed as described previously (26) with slight modifications. Briefly progenitor cells were homogenized in 0.5 ml of buffer (150 mM NaCl, 10 mM HEPES, pH 7.5, 1 mM EDTA, 0.1 mM MgCl2, 250 mM sucrose). Homogenates were centrifuged at 800 × g for 10 min at 4 °C to separate nuclei. Postnuclear supernatants were further clarified by centrifugation at 10,000 × g for 10 min. The clarified supernatants were loaded onto a 10–50% linear sucrose gradient (4.5 ml) and centrifuged for 16 h in an SW 55Ti rotor at 48,000 rpm at 4 °C. Eight (0.6-ml) fractions were collected from the top of each gradient. Protein was precipitated overnight with 2 volumes of ice-cold ethanol at −20 °C and resuspended in SDS buffer.

*Organelle Immunoisolation*—Cells were incubated with sulfo-NHS-biotin, as described above for the biotinylation assay, to eliminate surface proteins. Subsequently TfR-positive endosomes were isolated with Dynabead M-500 (Invitrogen) according to the manufacturer’s protocol. Briefly a mouse anti-human transferrin receptor antibody was incubated with surface protein-cleared homogenates overnight and next incubated with sheep anti-mouse Dynal magnetic beads for 2 h at 4 °C with end-over-end rocking. Immunoisolated endosomes were eluted from beads with SDS buffer and boiling.

Mathematical Analyses—Mathematical models of the experimental data were constructed as systems of ordinary differential equations in which each equation of the system corresponds to an identifiable biological state of the IGF-IR or Akt. The IGF-IR states were represented as the fractional occupancy of each state (i.e. the states sum to 1.0). Simple rate parameters, fitted by hand, govern the transitions between states of the IGF-IR or activation of Akt. The transitions do not directly correspond to biological transition/activation kinetics but instead represent several distinct steps collapsed together. The models used here are “minimal models” representing the simplest description that is consistent with the experimental data. The models were coded in MATLAB® Version 7.0.4 and solved using the ode23s routine.

The four-state model (see “Results” for a description of states and Table 1 for rate constants) is as follows.

\[
\frac{dRM1}{dt} = -k_{M1RM2}RM1 + k_{M2RM1}RM2 + k_{RM1 RI2} \quad (Eq. 1)
\]

\[
\frac{dRM2}{dt} = k_{M1RM2}RM1 - k_{M2RM1}RM2 - k_{RM2 RI1} \quad (Eq. 2)
\]

\[
\frac{dRI1}{dt} = k_{M2RI2}RM2 - k_{RI1RI1} \quad (Eq. 3)
\]

\[
\frac{dRI2}{dt} = k_{RI1RI1} - k_{RI2RI2} \quad (Eq. 4)
\]

\[
\frac{dAkt}{dt} = k_{AktRI1} - k_{AktAkt} \quad (Eq. 5)
\]

The five-state model (see “Results” for a description of states and Table 2 for rate constants) is as follows.

\[
\frac{dRM1}{dt} = -k_{M1RM2}RM1 + k_{M2RM1}RM2 + k_{M3RM3} \quad (Eq. 6)
\]

\[
\frac{dRM2}{dt} = k_{M1RM2}RM1 - k_{M2RM1}RM2 - k_{M3RM2} \quad (Eq. 7)
\]

\[
\frac{dRI1}{dt} = k_{M2RI2}RM2 - k_{RI1RI1} \quad (Eq. 8)
\]

\[
\frac{dRI2}{dt} = k_{RI1RI1} - k_{M2RI2} \quad (Eq. 9)
\]

\[
\frac{dRM3}{dt} = k_{M3RI2}RM2 - k_{M3RM1}RM3 \quad (Eq. 10)
\]

\[
\frac{dAkt}{dt} = k_{AktRI1} - k_{AktAkt} \quad (Eq. 11)
\]

Statistical Analyses—Statistical analyses were performed using the Statview statistical software program. For all experiments, one way analysis of variance followed by a Fisher’s posthoc test was used to assess the statistical significance between treatment groups. All experiments were repeated at least three times.

RESULTS

**IGF-I Is Required for Sustained IGF-IR and Akt Phosphorylation**—Previously we demonstrated that IGF-I promotes the survival and long term protection of primary rat oligodendrocyte progenitor cells (OPCs) from trophic factor deprivation and glutamate-mediated toxicity through sustained phosphorylation of Akt (2–4). In the present study, we utilized the CG-4 cell line of rat OPCs to better understand the mechanisms by which the IGF-IR mediates sustained Akt phosphorylation (27). To verify that IGF-I activates its receptor similarly in primary and CG-4 progenitors, we treated CG-4 cells with IGF-I or basal medium for 24 h (Fig. 1). Similar to our previous results in primary OPCs, phosphorylation of the IGF-IR subunit in IGF-I-treated cells though 24 h was significant over t0 and control groups (p = 0.01; Fig. 1, A and B). No changes in total IGF-IR protein levels were observed (Fig. 1, A and C).

To determine whether IGF-I sustains Akt phosphorylation in CG-4 progenitors and whether the presence of IGF-I is required for the sustained phosphorylation of Akt, CG-4 progenitors were stimulated with IGF-I for 24 h or for 30 min and then incubated in basal medium without IGF-I for 24 h. In the continual presence of IGF-I, Akt phosphorylation was sustained through 24 h (p < 0.05; Fig. 2, A and B), consistent with our previous results in primary OPCs (3). However, removal of IGF-I after 30 min of stimulation resulted in decreased phosphorylation of Akt to control (t0) levels within 30 min (Fig. 2, A and B). Similarly the phosphorylation of GSK-3β, a direct downstream substrate of Akt and indicator of Akt activity, was also sustained through 24 h in the presence of IGF-I (p < 0.05) and was markedly decreased after IGF-I removal to control (t0) levels (Fig. 2, A and C). The results demonstrating that the continual presence of ligand is required to sustain Akt phosphorylation indicate that the localization of the IGF-IR at the cell
surface also is required. In addition, these experiments demonstrate that the kinetics of IGF-I stimulation of its receptor and downstream targets are similar in CG-4 and primary OPCs. Therefore, we utilized the CG-4 progenitor cell line in the subsequent experiments to elucidate the mechanisms of IGF-IR-mediated sustained Akt phosphorylation.

**IGF-I Stimulation Alters the Surface Availability of the IGF-IR**—Whereas total levels of IGF-IR protein remained unchanged during IGF-I treatment in these and our previous studies (2, 3), we were interested in determining whether surface localization of the IGF-IR was altered by ligand stimulation. Using a biotinylation assay, we measured IGF-IR availability at the cell surface following IGF-I stimulation. Progenitor cells were serum-starved for 120 min and then treated with IGF-I or basal medium (N1A) from 0 to 120 min. Surface IGF-IRs were quantified following cell surface biotinylation and streptavidin precipitation. The IGF-IR was constitutively present at the cell surface in control cells in basal medium through 60 min. However, prolonged absence of IGF-I (4 h) induced an approximate 80% increase in surface IGF-IRs \( p < 0.005 \); Fig. 3, A and B). In contrast, IGF-I stimulation decreased IGF-IR surface availability by 32% after 15 min \( p < 0.04 \); Fig. 3, A and B) and 86% after 30 min \( p < 0.04 \); Fig. 3, A and B) compared with \( t_0 \). The decrease in surface IGF-IR was followed by recovery to 36% after 60 min \( p < 0.002 \); Fig. 3, A and B) and 90% after 120 min (Fig. 3, A and B). Cells treated with IGF-I, without prior sulfo-NHS-biotin incubation, were negative for surface IGF-IRβ subunit immunoreactivity (data not shown). These data...
suggest that the IGF-IR is internalized and subsequently recovers at the cell surface following stimulation with IGF-I. Moreover, the data suggest that growth factor-deprived cells are sensitized to IGF-I through the up-regulation of surface IGF-IRs. Dansylcadaverine Blocks IGF-IR Internalization and Akt Phosphorylation—To confirm that the loss of surface IGF-IR after IGF-I stimulation is due to receptor internalization and to examine the relationship between Akt phosphorylation and surface availability of the IGF-IR, we treated cells with dansylcadaverine, a pharmacological inhibitor of transglutaminase activity and receptor internalization (28). Dansylcadaverine was used previously to block IGF-IR internalization in Chinese hamster ovary cells (21) and in mouse embryo fibroblasts (29). CG-4 progenitors were treated with IGF-I with or without dansylcadaverine for 30 min, the time when we observed maximal loss of surface IGF-IR following IGF-I exposure (see Fig. 3). As in previous experiments, IGF-IR surface availability was markedly decreased after 30 min of IGF-I stimulation (p < 0.05; Fig. 4, A and B). However, in the presence of dansylcadaverine, receptor surface availability remained at the level of the untreated control (t0) cells (Fig. 4, A and B). IGF-IR phosphorylation was equivalent in cells treated with IGF-I plus dansylcadaverine compared with cells treated with only IGF-I (p < 0.005 versus t0; Fig. 4, A and C). To determine whether dansylcadaverine altered IGF-I-mediated Akt phosphorylation, progenitors were treated with IGF-I plus or minus dansylcadaverine for 5, 20, and 30 min. The presence of dansylcadaverine blocked IGF-I-mediated Akt phosphorylation at all time points versus cells treated with IGF-I alone (p < 0.0001 versus t0; Fig. 4, D and E). The results of these experiments demonstrate that dansylcadaverine blocks internalization of the IGF-IR as well as IGF-I-mediated Akt phosphorylation without affecting IGF-IR phosphorylation.

Recycling Is Required for IGF-IR Surface Recovery and Sustained Akt Phosphorylation—Our biotinylation data showed that IGF-IRs recover at the cell surface after 120 min of IGF-I stimulation (see Fig. 3). These receptors could originate either from recycling or from de novo synthesis of IGF-IRs. To investigate the role of recycling in surface recovery of the IGF-IR and sustained phosphorylation of Akt, we utilized the pharmacological inhibitor monensin, which blocks acidification of endosomal vesicles and consequently prevents trafficking to the lysosome and recycling compartment (30). Monensin was used in previous studies to block IGF-IR and insulin receptor endosomal trafficking in rat hippocampal neurons (31). Using the surface biotinylation assay, we again observed loss and recovery of surface IGF-IR at 30 and 120 min of IGF-I treatment, respectively (p < 0.03 versus t0; Fig. 5, A and B). However, the presence of monensin prevented receptor recovery at 120 min (p < 0.03; Fig. 5, A and B). To determine the effects of monensin on Akt phosphorylation, progenitors were treated with IGF-I plus or minus monensin for 15 or 120 min. The phosphorylation of Akt in IGF-I-treated cells at 15 min (p < 0.03) and 120 min (p < 0.0001) was significant over control groups. However, the presence of monensin significantly blocked Akt phosphorylation at 120 min compared with cells treated with IGF-I alone at 120 min (p < 0.0003; Fig. 5, C and D). Monensin had no effect on Akt phosphorylation at 15 min.

The previous data suggest that IGF-I recycling contributes to recovery of the receptor at the cell surface after exposure to IGF-I; however, it does not preclude an additional contribution from synthesis of new receptors. Although total IGF-I levels appear stable through 24 h (see Fig. 1), this could be the result of steady-state kinetics of receptor synthesis and degradation. To determine whether de novo synthesis of the IGF-IR contributes to the IGF-I-mediated phosphorylation of Akt, cells were treated with IGF-I plus or minus cycloheximide pre-treatment to block protein synthesis. Phosphorylation of Akt in IGF-I-treated cells was significantly above that in control cells in the presence and absence of cycloheximide (p < 0.0001 versus t0; Fig. 6, A and B). Cycloheximide had no effect on total IGF-I protein levels (Fig. 6, A and C). Analysis of cyclin D1 as a positive control for cycloheximide activity revealed decreased cyclin D1 protein levels in the same cells treated with cycloheximide (p < 0.0001; Fig. 6, A and D). These data suggest that recycling, but not de novo synthesis, of the IGF-IR is required for sustained Akt phosphorylation.

The IGF-IR Co-fractionates and Co-localizes with Markers for the Endosomal Recycling Compartment—To further confirm that IGF-IRs follow a recycling pathway, we analyzed whether the IGF-IR co-fractionates with markers of the endosomal recycling compartment. Progenitor cell homogenates were fractionated on a 10–50% linear sucrose gradient and subsequently were analyzed for markers of distinct endosomal vesicles as described under “Experimental Procedures.” Early endosome antigen 1, a marker for the early endosome, and glucose transporter 1, a marker for the plasma membrane (32),

FIGURE 3. IGF-I alters IGF-IR surface availability. Cells were serum-starved and treated with 1 nM IGF-I or basal medium (N1A) for the indicated times. Cells were labeled with 0.3 mg/ml sulfo-NHS-biotin. Total and surface precipitated lysates were processed for SDS-PAGE and Western blot analysis for IGF-IR subunit immunoreactivity. A, representative Western blot. B, surface availability of IGF-IRβ represented as a ratio of surface to total IGF-IRβ and as a percentage of t0. *, p < 0.05 versus t0; **, p < 0.004 versus t0. Data shown represent the mean ± S.E. (n = 3).
localized predominantly in fraction 3 (Fig. 7, A and B). The TfR, a marker for the recycling endosome (33), was distributed in fractions 2–6 with maximal expression in fraction 4 (Fig. 7, A and C). The distribution of the IGF-IR was similar to that of the TfR, localizing in fractions 3–5 (Fig. 7, A, C, and D).

To determine whether the IGF-IR and the TfR also co-localize, we isolated TfR-positive endosomes from control or IGF-I-treated cells that were first stripped of surface proteins (see “Experimental Procedures”). Surface protein-cleared homogenates were immunoprecipitated with a TfR antibody and subsequently conjugated to magnetic beads. Whereas the small GTPase Rab11 co-precipitated with the TfR antibody and subsequently conjugated to magnetic beads. Whereas the small GTPase Rab11 co-precipitated with the TfR in both IGF-I-treated (Fig. 8, lanes 3 and 4) and untreated lysates (Fig. 8, lanes 1 and 2), the IGF-IR co-precipitated with TfR only in the presence of IGF-I. In contrast, immunoprecipitates were negative for Akt immunoreactivity, suggesting that they are specific to TfR-positive endosomes. Together these data show that IGF-IRs are associated with TfR- and Rab11-positive endosomes in a ligand-dependent manner, and they support the conclusion that IGF-IRs are targeted for recycling.

A Four-state Mathematical Model for IGF-IR Trafficking Is Inconsistent with Empirical Data—Based on our data from the present study, we propose that the surface loss and recovery of the IGF-IR in OPCs is due to receptor internalization followed by receptor recycling. Our data support the hypothesis that receptor internalization and recycling are essential for the ability of IGF-I to sustain Akt phosphorylation and promote long term survival of glial progenitors.

To assist in interpretation of the experimental results, we performed computational analyses of the experimental data. The goal of these analyses was not to build a comprehensive model of the signaling system underlying the response of CG-4 cells to IGF-I. Rather the goal was to reproduce the primary kinetic profiles of both the time course of IGF-IR surface avai-
ability, provided by our biotinylation assay, and the sustained activation of Akt. The aim was to validate the interpretation of these results based on the understanding of the underlying cellular processes. The model was built to be consistent with the minimal set of processes that could be considered to be involved in the agonist-induced response, i.e. that

1. receptors are unbound and in a “resting” state prior to being exposed to the agonist, IGF-I;
2. introduction of IGF-I induces reversible binding of the agonist with surface receptors;
3. activated IGF-I receptors are internalized, effectively coupling them to the signaling machinery; and
4. IGF-I receptors are recycled to the surface membrane where they can continue to participate in signaling in the sustained presence of IGF-I.

The simplest model that is consistent with the data and these processes contains four receptor states (for rate constants see Table 1): RM1, unbound receptors at the plasma membrane (representing the resting state of the receptor); RM2, receptor-ligand complexes at the plasma membrane (the “bound” receptors); RI1, internalized receptors competent to activate Akt; and RI2, internalized receptors in the recycling compartment (representing all forms of the internalized recycling receptors). These states are shown in Fig. 9A, and we term this model the “four-state model.” In this model, Akt is activated by receptors in the RI1 state because we observed that dansylcadaverine inhibited IGF-IR-mediated Akt phosphorylation and internalization (see Fig. 3). The fractional occupancy of the IGF-IR in each state was coded as a set of ordinary differential equations (see “Experimental Procedures”) and solved for the predicted Akt phosphorylation and IGF-IR surface occupancy (Fig. 9, B–D). The states of the two membrane-bound forms of the receptor, RM1 and RM2, were summed to provide the total surface receptors (Fig. 9C).

The four-state model reproduced the essential kinetics of the experimentally observed sustained phosphorylation of Akt (Fig. 9D). However, this model was able to produce only moderate loss of receptors at the cell surface, and more importantly, the kinetics of surface receptors showed no recovery over the 120-min time period (Fig. 9C). Over a wide range of tested values, no set of parameters was able to display loss and recovery of surface receptors. This model therefore appears insufficient to reproduce the essential kinetics of surface receptors. Thus, although the components of this four-state receptor trafficking model are consistent with our interpretation of the experimental data, once the components are forced to adhere to quantitative “rules” imposed by formulating the interpretation as a mathematical model, we found inconsistencies with the observed experimental results. This suggests that the interpretation of the results is incomplete and additional processes must be included to fully explain the kinetics of the experimental observations.

FIGURE 5. Recycling is required for IGF-IR surface recovery and sustained Akt phosphorylation. Cells were serum-starved and treated with 1 nM IGF-I for 15, 30, or 120 min ± 10 μM monensin (M). After biotinylation, total and surface precipitated lysates were subjected to PAGE and Western blot analysis for IGF-IR immunoreactivity. A and C, representative Western blots. B, surface availability was represented as a ratio of surface to total IGF-IR and as a percentage of t0. D, phosphorylated Akt protein (pAkt) normalized to total Akt. *, p < 0.03 versus control; **, p < 0.003 versus IGF-I 120 min; ***, p < 0.0001 versus control. Data shown represent the mean ± S.E. (n = 3). mon, monensin.
A Five-state Mathematical Model of IGF-IR Trafficking Recapitulates the Empirical Data—The analysis of the four-state model revealed a discrepancy between the experimental data and our initial interpretation of the data. In other words, there is information in the data that is not easily understood and utilized from a qualitative interpretation. Having revealed this “knowledge gap,” the model can further be used to hypothesize what essential additional process(es) may be operating in these cells. For example, in one approach we added an additional internalized receptor state, hypothesizing that a delay in receptor recycling, representing intracellular organelle trafficking, might explain the observed kinetics of receptor recycling. However, this did not improve the ability of the model to recapitulate the data, with no observed recovery of surface receptors over 120 min, irrespective of the parameter values used (data not shown). It should also be noted that placing the activated receptor state that is responsible for PI3K/Akt pathway stimulation on the cell surface also did not improve the predicted data. However, in a further approach, when a state that represents receptors recycled to the cell surface but not yet competent to bind the agonist IGF-I was added to the model, we were able to reproduce the essential kinetics of the experimental results (Fig. 9) (for rate constants see Table 2). This new state, RM3, is counted as part of the surface receptors. In this five-state model, the IGF-IR surface occupancy curve recapitulated the essential kinetics observed in our surface receptor biotinylation studies with significant surface loss of the receptor followed by receptor recovery at 120 min (Fig. 10, C and D). The fit for the Akt response for the four- and five-state model is slightly different. However, qualitatively the essential dynamics of the Akt response were reproduced in both cases, whereas only the five-state model reproduced the essential dynamics of the surface receptors. Changing the parameters of the four-state model to loosen the fit to the Akt data did not cause the recovery of the surface receptors to occur. That aspect seems critically dependent on the structure of the model (i.e. the additional surface receptor state) rather than the choice of parameters for the respective models.

We were also able to show that the five-state model recapitulated the data using dansylcadaverine (no activation of Akt and no receptor internalization) and monensin (transient Akt activation and receptor internalization but not recovery). Taken together, computational analyses predict a model of IGF-IR trafficking in which the IGF-IR is internalized and recovers at the plasma membrane for further activation. The five-state model also predicts an additional kinetic state of the receptor at the cell surface that was not elucidated by empirical analyses and is represented by the transition from RM3 to RM1.

DISCUSSION

Previously we demonstrated that IGF-I sustains phosphorylation of both the IGF-1R and Akt, leading to long term survival

FIGURE 6. De novo synthesis is not required for IGF-I-mediated Akt phosphorylation. Cells were serum-starved and treated with 1 nM IGF-I ± 20 ng/ml cycloheximide (Cy) for 120 min. Cell lysates were isolated, and protein was processed for SDS-PAGE and Western blot analysis. A, representative Western blot. B, phosphorylated Akt protein (pAkt) normalized to total Akt. C, total IGF-IR protein normalized to β-actin. D, cyclin D1 protein normalized to β-actin. *, p < 0.0001 versus control (con) (t0). Data shown represent the mean ± S.E. (n = 3).
of primary OPCs (2). In the present study, we used a combination of biochemical approaches, including the use of trafficking inhibitors and the direct measure of surface receptors, to investigate the role of IGF-IR trafficking in Akt phosphorylation. The results of these experiments collectively provide evidence that IGF-IR internalization and recycling are required for sustained Akt phosphorylation in glial progenitor cells. Moreover, computational analyses support a model of IGF-IR trafficking that is consistent with and extends our empirical data.

**Sustained Phosphorylation of Akt Requires IGF-IR Internalization**

We report that the continual presence of IGF-I was requisite to sustain the phosphorylation of Akt and its downstream substrate, GSK-3β. Whereas total IGF-IR protein levels remained unchanged, the localization of the IGF-IR was altered during IGF-I stimulation such that the levels of receptor on the cell surface initially decreased and then recovered. We observed maximal loss of the IGF-IR from the cell surface within 30 min after IGF-I exposure; this is consistent with other studies that have shown maximal internalization of Akt phosphorylation in glial progenitor cells. Moreover, computational analyses support a model of IGF-IR trafficking that is consistent with and extends our empirical data.

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**IGF-IR Trafficking and Sustained Akt Phosphorylation**

**TABLE 1**

Four-state model rate constants

Receptor states are in arbitrary units, representing fractional occupancy of the total receptor states (which sum to 1.0); time is in minutes. Akt is in arbitrary units corresponding to normalized values of optical density measurements from the experiments. M, membrane; I, internal (or intracellular); A, Akt; a, activation; d, deactivation.

| Constant | Value |
|----------|-------|
| $k_{M1M2}$ | 0.05 |
| $k_{M2M1}$ | 0.002 |
| $k_{M1I1}$ | 0.04 |
| $k_{I1I2}$ | 0.06 |
| $k_{I2M1}$ | 0.04 |
| $k_a$ | 1.8 |
| $k_d$ | 0.6 |

**FIGURE 7. IGF-IRs co-fractionate with the transferrin receptor.** Progenitor cells were cultured for 24 h in N2S medium containing 5 μg/ml insulin (sufficient to stimulate the IGF-IR). Cell homogenates were fractionated on a 10–50% linear sucrose gradient as described under "Experimental Procedures." Fractions were taken from the top of the gradients and were processed for SDS-PAGE and Western blot analysis. Band densities are in arbitrary units and represented as percentage of total signal. A, representative Western blot; Lane T, total lysates; lanes 1–8, fractions 1–8. B, percent localization of early endosome antigen 1 (EEA1) and glucose transporter 1 (GLUT1). C, percent localization of the TfR and IGF-IR. D, merged data from B and C.

**FIGURE 8. IGF-IRs co-precipitate with the transferrin receptor and Rab11-positive endosomes.** Cells were serum-starved in basal medium and treated with 1 nM IGF-I for 16 h. Cells were incubated with sulfo-NHS-biotin, and surface proteins were precipitated with ImmunoPure streptavidin as described under "Experimental Procedures." Surface protein-cleared homogenates from control (Ctl) (lanes 1 and 2) and IGF-I-treated groups (lanes 3 and 4) were incubated plus (+) or minus (−) a mouse anti-human transferrin receptor antibody and subsequently incubated with sheep anti-mouse magnetic beads. Total lysates, surface protein-cleared lysates, and immunoprecipitates (IP) were processed for SDS-PAGE and Western blot analysis. A representative Western blot is shown for duplicate samples.
receptor tyrosine kinases, including the IGF-IR, between 15 and 30 min after ligand stimulation in various cell types (19, 31, 34).

Results from previous studies suggest that Akt phosphorylation occurs at the plasma membrane. For example, Chow et al. (21) showed that IGF-IR internalization in Chinese hamster ovary cells is blocked by dansylcadaverine without affecting IGF-I-mediated Akt phosphorylation. In contrast, we found that this inhibitor blocked IGF-IR internalization and completely abrogated IGF-I-mediated Akt phosphorylation in glial progenitors. The difference between these results and those of Chow et al. (21) may be due to differences in cell type and/or the

FIGURE 9. Four-state mathematical model for IGF-IR trafficking is inconsistent with empirical data. The time course of IGF-IR surface availability during IGF-I stimulation was used as the basis for constructing a minimal mathematical model. The simplest model based on the empirical data includes four receptor states. A, RM1, unbound receptor at the cell surface; RM2, ligand-receptor complex at the cell surface; RI1, internalized activated receptors competent to activate Akt; RI2, internalized receptor in the recycling compartment. B–D, the fractional occupancy of the IGF-IR in each receptor state was coded as a set of ordinary differential equations (B) and solved for the theoretical IGF-IR surface occupancy (RM1 + RM2) (C) and Akt activation (D) over time. Expt, experimental.

TABLE 2

| Constant | Value |
|----------|-------|
| $k_{M1}$ | 0.08  |
| $k_{M2}$ | 0.002 |
| $k_{I1}$ | 0.09  |
| $k_{I2}$ | 0.1   |
| $k_{M3}$ | 0.015 |
| $k_{A}$  | 0.16  |
| $k_{Ad}$ | 0.015 |

FIGURE 10. Five-state IGF-IR trafficking model recapitulates the empirical data. A, an additional receptor state (RM3) was added to the original four-state model shown in Fig. 9. B–D, the fractional occupancy of the IGF-IR in each state was coded as a set of ordinary differential equations (B) and solved for the theoretical IGF-IR surface occupancy (RM1 + RM2 + RM3) (C) and Akt activation (D) over time. Expt, experimental.
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site for PI3K/Akt activation. Consistent with our results, Wang et al. (35) demonstrated recently that platelet-derived growth factor receptors activate signal transduction pathways within endosomes, including PI3K/Akt, in a human hepatocellular carcinoma cell line. The notion that Akt phosphorylation and its subsequent activation can occur within endosomes is further supported by evidence that the regulatory subunit of PI3K, p85, is associated with early endosomes in sensory neurons (36). Taken together, these data suggest that endosomes are competent for signaling in various cell types and that, in some cases, internalization may be required for ligand-bound receptors to interact with the appropriate signaling complexes.

The IGF-IR Follows a Recycling Pathway—Although our data support the hypothesis that IGF-IR internalization is required to initiate Akt phosphorylation, receptor recycling appears to be important for the IGF-mediated sustained phosphorylation of Akt. The internalization of ligand–receptor complexes can function to desensitize and target a receptor for degradation (37, 38). However, receptors also can recycle to the cell surface for further ligand binding. The recycling of cellular proteins to the plasma membrane is a common phenomenon that is more energy-efficient compared with de novo synthesis (39). We observed surface recovery of the IGF-IR after 120 min of IGF-I stimulation and determined that these receptors originate from a recycling receptor pool. We further demonstrated that IGF-IRs fractionated with the TfR and co-precipitated with the TfR and Rab11, classical makers of the recycling pathway, in a ligand-dependent manner.

Previous studies in rat fibroblasts demonstrated that ~60% of IGF-1/IGF-IR complexes recycle to the cell surface after 120 min of IGF-1 stimulation, suggesting that this receptor may consistently follow a recycling pathway in multiple cell types (40). Furthermore IGF-IR recovery at the cell surface at 120 min is consistent with a slow recycling compartment, which is known to associate with Rab11 and with the constitutive trafficking of the TfR (41). In the glial progenitors, the recovery of IGF-IRs at the cell surface is correlated with sustained Akt phosphorylation. Similarly recycling of the neurotensin-1 receptor leads to prolonged activation of mitogen-activated protein kinases (p42/44) in neuroblastoma cells (42). Taken together, these data support the idea that the recovery of receptors at the cell surface leads to long term activation of signaling pathways.

The decision of whether a receptor will be targeted for degradation or recycling is critical to short term as well as long term signaling and downstream biological effects. Receptors are internalized within distinct regions of the plasma membrane, forming endosomes in which proteins are targeted for transport to various subcellular compartments, including the lysosome, trans-Golgi network, or plasma membrane (43, 44). Previously we reported that TrkC, a member of the receptor tyrosine kinase family, is degraded in response to neurotrophin-3 in primary OPCs (2). In contrast, IGF-IR protein levels do not change in response to continual ligand stimulation in the primary OPCs (2) and in the CG-4 progenitors used for these studies. Thus, although stimulation of either receptor leads to phosphorylation of Akt initially, only stimulation of the IGF-IR sustains Akt phosphorylation and promotes long term survival of OPCs.

The mechanisms that control the differential subcellular fates of TrkC and the IGF-IR in OPCs are currently unknown. In general, the regulation of receptor sorting is attributed to several proteins, including cbl, an E3 ubiquitin ligase, which is involved in targeting proteins for degradation, as well as members of the Rab protein family of small GTPases, which are involved in budding and fusion of various endosomal compartments (44). IGF-IR stability is regulated in mouse embryo fibroblasts by association with Grb10, which promotes the recruitment of an E3 ubiquitin ligase and culminates in receptor ubiquitylation and a 4-fold decrease in total receptor protein levels (29).

Mathematical Modeling Predicts a Cell Surface Receptor State—To further elucidate IGF-IR trafficking in the glial progenitors we used mathematical modeling, which was used previously to delineate the mechanisms of receptor dimerization and signaling pathway activation (45). We found that a five-state receptor model was necessary and sufficient to recapitulate the essential kinetics of our empirical data. This model contains a receptor state (RM3) at the cell surface that represents putatively recycled receptors that are not yet able to engage in signaling. This receptor state was required to fully explain the empirical data but was not readily apparent from our qualitative interpretation of the data. These results highlight the value of mathematical modeling for the purpose of extracting the maximum amount of information from the experimental data.

We hypothesize that the transition from RM3 to RM1 may represent a state of the receptors in which additional processing (e.g. (de)phosphorylation that occurs as part of the recycling process) is required prior to reactivation by binding to IGF-I. Alternatively the receptors may be recycled to an area of the plasma membrane where they are incompetent to re-engage in signaling and must relocate before this can occur. These hypotheses, however, are not mutually exclusive and may both contribute to the kinetics of sustained Akt phosphorylation. Whereas receptor dephosphorylation is traditionally regarded as an endosomal event (46, 47), there is precedence for dephosphorylation of the IGF-IR at the cell surface. Specifically Maile and Clemmons (48) demonstrated that IGF-IR dephosphorylation is promoted by recruitment of a tyrosine phosphatase, SHP-2, to the plasma membrane in a ligand-dependent manner in smooth muscle cells. Thus, these studies provide support for proposing that the transition of RM3 to RM1 is a putative dephosphorylation step at the plasma membrane.

The results of these studies provide the basis for understanding how IGF-I initiates and sustains activation of the PI3K/Akt signaling pathway in glial progenitor cells and promotes their long term survival. IGF-I is an important survival factor for multiple cell types through this pathway; however, few studies have addressed sustained activation of PI3K/Akt by IGF-I or other trophic factors. The ability of receptors to sustain signaling pathway activation through receptor trafficking may be an important mechanism for achieving specific biological outcomes in cells. However, this process is likely highly regulated and restricted to certain receptors and/or cell types due to the
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potentially negative consequences of globally sustained signal pathway activation.

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