Phosphatidylinositol 3-Kinase (PI3K) Activity Bound to Insulin-like Growth Factor-I (IGF-I) Receptor, which Is Continuously Sustained by IGF-I Stimulation, Is Required for IGF-I-induced Cell Proliferation

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Continuous stimulation of cells with insulin-like growth factors (IGFs) in G1 phase is a well established requirement for IGF-induced cell proliferation; however, the molecular components of this prolonged signaling pathway that is essential for cell cycle progression from G1 to S phase are unclear. IGF-I activates IGF-I receptor (IGF-IR) tyrosine kinase, followed by phosphorylation of substrates such as insulin receptor substrates (IRS) leading to binding of signaling molecules containing SH2 domains, including phosphatidylinositol 3-kinase (PI3K) to IRS and activation of the downstream signaling pathways. In this study, we found prolonged (>9 h) association of PI3K with IGF-IR induced by IGF-I stimulation. PI3K activity was present in this complex in thyrocytes and fibroblasts, although tyrosine phosphorylation of IRS was not yet evident after 9 h of IGF-I stimulation. IGF-I withdrawal in mid-G1 phase impaired the association of PI3K with IGF-IR and suppressed DNA synthesis the same as when PI3K inhibitor was added. Furthermore, we demonstrated that Tyr1316-X-X-Met of IGF-IR functioned as a PI3K binding sequence when this tyrosine is phosphorylated. We then analyzed IGF signaling and proliferation of IGF-IR−/− fibroblasts expressing exogenous mutant IGF-IR in which Tyr1316 was substituted with Phe (Y1316F). In these cells, IGF-I stimulation induced tyrosine phosphorylation of IGF-IR and IRS-1/2, but mutated IGF-IR failed to bind PI3K and to induce maximal phosphorylation of GSK3β and cell proliferation in response to IGF-I. Based on these results, we concluded that PI3K activity bound to IGF-IR, which is continuously sustained by IGF-I stimulation, is required for IGF-I-induced cell proliferation.

Insulin-like growth factors (IGFs) play important roles in embryonic and somatic growth (1). By collaborating with other hormones or growth factors, IGFs promote cell proliferation and differentiation and suppress apoptosis in various cell types (1, 2). It is widely accepted that IGF binding to IGF-I receptor (IGF-IR) on the plasma membranes of target cells activates the receptor intrinsic tyrosine kinase, and it phosphorylates intracellular substrates such as insulin receptor substrate (IRS)-1 and IRS-2 (3). Phosphotyrosyl substrates are then recognized by proteins possessing a Src homology 2 (SH2) domain, including PI3K and growth factor receptor-bound protein 2 (Grb2). In turn, these events cause activation of the PI3K pathway and the MAPK pathway, leading to the induction of IGF-I activities.

Previous studies revealed that IGF-I and other growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) synergistically induce cell proliferation of fibroblasts and epithelial cells derived from various tissues (4–9). Under these conditions, continuous stimulation with IGF-I at least from G0 phase to the mid-G1 phase is required for the induction of DNA synthesis, although other growth factors are required only during the short period from G0 phase to early G1 phase (4–9). These findings pointed out a unique role of...
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IGF-I as a cell cycle progression factor and suggested that continuous stimulation with IGF-I might continue to activate some signal pathways, leading to cell cycle control.

What are the molecular mechanisms that mediate prolonged IGF-I signals? Under experimental conditions where serum-starved cells are stimulated with IGF-I, signals through IRS are often turned off by their dephosphorylation within a short time (10–12), suggesting that other signal molecules may mediate prolonged IGF-I signals. Early studies revealed that IGF-IR could interact with some signal molecules, including PI3K, in response to IGF-I (13, 14). Because activation of the PI3K pathway can play multiple roles to promote cell cycle progression from G1 phase to S phase (15, 16), this putative IGF-IR-PI3K complex may mediate prolonged IGF-I signals, leading to cell cycle control. However, this complex had been observed only in cells overexpressing IGF-IR (13, 17), and its function has not been thoroughly analyzed.

In this study, we elucidate mechanisms that mediate prolonged IGF-I signals leading to cell proliferation. Our results show that continuous stimulation with IGF-I causes prolonged tyrosine phosphorylation of IGF-IR and prolonged association of PI3K with IGF-IR, even in cells expressing endogenous IGF-IR, and PI3K activity bound to IGF-IR is essential for IGF-I-dependent cell proliferation.

EXPERIMENTAL PROCEDURES

Materials—Cells of a line of rat thyroid follicular FRTL-5 (18) (ATCC No. CRL8305) were kindly provided by the late Dr. Leonard Kohn (Ohio University and Edison Biotechnology Institute) and the Interthyr Research Foundation (Baltimore, MD). NIH-3T3 cells overexpressing IGF-1 receptor (NWT10 cells) (19) were kindly donated by Derek LeRoith (The Mount Sinai School of Medicine) and Dr. Hisanori Kato (University of Tokyo). IGF-IR−/− mice embryonic fibroblasts (R−/−) cells were kindly provided by Dr. Renato L. Baserga (Thomas Jefferson University). Recombinant human IGF-1 was kindly donated by Dr. Toshiaki Ohkuma (Fujisawa Pharmaceutical Co., Osaka, Japan). LY294002 was obtained from Sigma Aldrich. bpV(pic) was purchased from Merck (Darmstadt, Germany). Anti-IGF-IR antibody (C-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IRS-1 antibody and anti-IRS-2 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IRS-1 antibody and anti-IRS-2 antibody were produced by immunizing rabbits with carboxyl-terminal peptide of mouse IRS-1 or IRS-2 (amino acids 1211–1233 or amino acids 1302–1321, respectively, synthesized by Qiagen, Tokyo, Japan) and purified with the peptide affinity column (20). Another anti-IRS-2 antibody (06-506, used only for immunoblotting), anti-Shc antibody (06-203), and anti-p85 PI3K antibody (06-195) were obtained from Millipore (Billerica, MA). Anti-Akt antibody (9272), anti-phospho-Akt (Ser473) antibody (9271), anti-glycogen synthase kinase 3 (GSK3) antibody (5676), anti-phospho-GSK3β (Ser9) antibody (9336), anti-Erk antibody (9102), and anti-phospho-Erk (Thr202/Tyr204) antibody (9101) were obtained from Cell Signaling Technology (Danvers, MA). Anti-phosphotyrosine antibody (P4110), anti-FLAG antibody, and anti-FLAG antibody-conjugated agarose beads were obtained from Sigma Aldrich. Anti-glutathione S-transferase (GST) antibody were produced by immunizing rabbits with recombinant Schistosoma japonicum GST produced by Escherichia coli transformed with pGEX vector (GE Healthcare) and purified with GST-conjugated affinity column. Other chemicals were of the reagent grade available commercially.

Construction of Plasmids Encoding IGF-IR—Human IGF-IR cDNA was kindly provided by Dr. Furlanetto (National Institutes of Health, Bethesda, MD) and inserted into the EcoRI-XbaI site in pcDNA6/myc-His A mammalian expression vector (Invitrogen). From this pcDNA6-IGF-IR, plasmids expressing mutant IGF-IR (in which Tyr1316 was substituted to Phe) were generated using a PCR-based method as follows. The first PCR was performed using pcDNA6-IGF-IR as a template, and primer 1 (5′-TCGAAATTAATACGACTCATAAG-3′) and primer 2 (5′-TTCAATGCGGGCGAAAGGCTGTCTCTC-3′), or primer 3 (5′-AGAGCAACCTTTCCTGCCCCACTGAA-3′) and primer 4 (5′-TAAAGCCACAGTCGAGGCTG-3′). The second PCR was performed using two PCR products from the first PCR, as well as primer 1 and primer 4. The second PCR products were cut with EcoRI and XbaI, and inserted into an EcoRI-XbaI site in pcDNA6/myc-His A. The pCMV-IGF-IR-FLAG plasmid for the expression of IGF-IR tagged with FLAG at its carboxyl terminus was kindly provided by Dr. Dr. Baserga (Thomas Jefferson University). Recombinant human IGF-IR was kindly donated by Dr. Toshiaki Ohkuma (Fujisawa Pharmaceutical Co., Osaka, Japan). IGF-IR mutant IGF-IR (in which Tyr1316 was substituted to Phe) were cut with EcoRI and XbaI, and inserted into an EcoRI-XbaI site in pcDNA6/myc-His A. The pCMV-IGF-IR-FLAG, or pcDNA6-IGF-IR (Y1316F), or pcDNA6-IGF-IR (Y1316F) was kindly provided by Dr. Dr. Baserga (Thomas Jefferson University). Recombinant human IGF-IR was kindly donated by Dr. Toshiaki Ohkuma (Fujisawa Pharmaceutical Co., Osaka, Japan). IGF-IR mutant IGF-IR (in which Tyr1316 was substituted to Phe) were cut with EcoRI and XbaI, and inserted into an EcoRI-XbaI site in pcDNA6/myc-His A. The pCMV-IGF-IR-FLAG plasmid, a DNA fragment spanning from Smal site to carboxyl terminus of IGF-IR Y1316 was amplified by PCR, and the corresponding region of pCMV-IGF-IR-FLAG was replaced by it.

Culture, Transfection, and IGF-I Stimulation of Cell—FRTL-5 cells were cultured as described previously (21). NWT10 cells and R− cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, Nissui) and antibiotics. R− cells were transfected with pEGF-N1 (Clontech, Mountain View, CA), pcDNA6-IGF-IR, or pcDNA6-IGF-IR (Y1316F), or pCMV-IGF-IR-FLAG, or pCMV-IGF-IR-FLAG (Y1316F) using Lipofectamine 2000 (Invitrogen). For cells to become quiescent, subconfluent cells were washed twice with Hanks’ balanced salt solution (Nissui) and cultures were continued for an additional 24–48 h in serum-free medium supplemented with 0.1% bovine serum albumin (BSA, Nacalai tesque, Kyoto, Japan). In particular, FRTL-5 cells were then cultured for an additional 24 h in serum-free medium supplemented with 0.1% BSA and 1 μM dibutyryl cAMP (Nacalai tesque) and subsequently washed three times with Hanks’ balanced salt solution and incubated for ~15 min in serum-free medium supplemented with 0.1% BSA. This “cAMP pretreatment” was carried out because it potentiates DNA synthesis in these cells induced by subsequent IGF-I treatment (9). Thereafter, these quiescent cells were stimulated with IGF-I. In some experiments, cells were washed twice with Hanks’ balanced salt solution at the indicated times after the commencement of IGF-I and then cultured in serum-free medium supplemented with 0.1% BSA to examine the effect of IGF-I withdrawal from the culture medium.

DNA Synthesis Assay—Cells were cultured in 48- or 24-well plates, and [methyl-3H]thymidine incorporation into DNA was measured as described previously (21).
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**Immunoprecipitation, Immunoblotting, and PI3K Assay**—Cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 500 μM Na3VO4, 100 kilo international units/ml aprotinin, 20 μg/ml PMSF, 10 μg/ml leupeptin, and 5 μg/ml pepstatin. The lysates were centrifuged at 15,000 × g for 10 min at 4 °C, and the supernatants were used for immunoprecipitation, immunoblotting, and PI3K assay. These procedures were carried out according to standard protocols, as described previously (21). For the immunoprecipitation of FLAG-tagged IGF-IR, anti-FLAG antibody-conjugated agarose beads were used.

**Far Western Blotting**—This procedure was carried out as described previously (22).

**Cell Count Assay**—Cells were treated with PBS supplemented with 0.25% trypsin and 0.02% EDTA. Cell suspension was mixed with equal volume of DMEM supplemented with 10% FBS, and cell numbers were counted using a Thoma-type cell count plate.

**Flow Cytometry Analysis**—Cells were treated with PBS supplemented with 0.25% trypsin and 0.02% EDTA for 5 min and diluted with an equal volume of DMEM supplemented with 10% FBS, and cell numbers were counted using a Thoma-type cell count plate.

**Statistical Analysis**—Statistical analyses of data were performed by Student’s t test and one-way factorial analysis of variance using StatView software (Abacus Concepts, Inc., Berkeley, CA). Fisher’s protected least significant difference (PLSD) and Tukey-Kramer test were performed to study the significance between multiple different conditions. The results shown are the mean ± S.E. p < 0.05 was considered statistically significant.

**RESULTS**

**Long Term Stimulation with IGF-I Is Required for Cell Cycle Progression from G1 to S Phase**—In thyroid FRTL-5 cells, IGF-I effectively induces DNA synthesis when cells are pretreated with TSH or cAMP analog (9), and the cells enter into S phase approximately during 20–32 h after IGF-I stimulation (21). Using this model, we tested effects of IGF-I withdrawal at the indicated time points after commencement of IGF-I stimulation on DNA synthesis (Fig. 1A). As a result, IGF-I withdrawal at 0 (that is, no stimulation with IGF-I), 3, 6, or 12 h after IGF-I stimulation dramatically decreased the DNA synthesis during 20–24 h, indicating that continuous stimulation with IGF-I for more than 12 h is required for maximal DNA synthesis.

To examine whether this phenomenon is observed in other cell types, similar experiments were performed using NWT10 cells, a cell line derived from NIH-3T3 cells stably overexpressing human IGF-IR (Fig. 1B) (19). The result was that IGF-I withdrawal at the time points from 0 to 15 h decreased DNA synthesis during 15–18 h, indicating that the continuous stimulation with IGF-I for >15 h was required for maximal DNA synthesis. We also investigated time course of DNA synthesis induced by IGF-I with or without IGF-I withdrawal at 6 h (Fig. 1C) and observed that IGF-I withdrawal decreased DNA synthesis during 15–18 h to about one-fifth, whereas it had little effect on the timing of maximal DNA synthesis. These results suggested that IGF-I withdrawal reduces numbers of cells that enter into S phase and does not delay cell cycle progression.

**Prolonged Activation of IGF-IR Tyrosine Kinase Is Induced in Response to IGF-I Stimulation, Followed by Activation of PI3K Pathway**—To reveal signal transduction pathway(s) that respond to continuous IGF-I stimulation, we then investigated the time course of protein phosphorylation induced by IGF-I. In FRTL-5 cells (Fig. 2A, left panel), IGF-I-dependent tyrosine phosphorylation of a 110-kDa protein (corresponding to IGF-IR) was sustained at least for 9 h after commencement of IGF-I stimulation and then gradually decreased, but its levels were higher than the basal levels until 24 h. Immunoprecipitation with anti-IGF-IR antibody followed by immunoblotting analysis confirmed that tyrosine phosphorylation of IGF-IR was sustained at least for 9 h (supplemental Fig. S1). Phosphorylation of...
IGF-IR Tyr1131, which is required for kinase activation (23), was also detected at 9 h (supplemental Fig. S1), suggesting that its kinase activity is maintained during this time. In contrast, tyrosine phosphorylation of a 185-kDa protein (corresponding to IRS-1 and IRS-2) was dramatically increased at 1 min and immediately decreased reaching basal levels at least by 9 h (Fig. 2). Consistent with this, we found that protein levels of IRS-1 and IRS-2 were dramatically decreased by 6 h, following the changes of their electrophoretic mobility that may reflect their serine/threonine phosphorylation (24, 25). We also investigated phosphorylation of Akt (at Ser971) and GSK3β (at Ser9), which is known to occur downstream of PI3K (3), using phospho-specific antibodies. As a result, IGF-I-dependent phosphorylation of these proteins was continued at least for 24 h. In contrast, phosphorylation of extracellular signal-regulated kinases (Erks, p42/44 MAPK) was observed only until 3 h. Similar results were obtained using NWT10 cells (Fig. 2B), confirming that IGF-I stimulation induces prolonged tyrosine phosphorylation of IGF-IR and prolonged activation of the PI3K pathway.

Next, we examined the effect of IGF-I withdrawal in mid-G1 phase on protein phosphorylation (Fig. 2A, right panel). We stimulated FRTL-5 cells with IGF-I and then changed the medium to that without IGF-I at 9 h after the commencement of the stimulation, followed by harvesting cells at indicated time. IGF-I withdrawal immediately abolished tyrosine phosphorylation of 110-kDa protein and gradually decreased phosphorylation of Akt and GSK3β. These results indicated that continuous IGF-I stimulation is required for prolonged tyrosine phosphorylation of IGF-IR and prolonged activation of PI3K pathway. We also observed that IGF-I withdrawal partially restored protein levels of IRS-1 and IRS-2, indicating that continuous stimulation with IGF-I lowers their protein levels. These results are consistent with other reports that prolonged insulin/IGF-I stimulation induces proteasomal degradation of IRS depending on the activation of PI3K pathway (26, 27).

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We then tested the roles of PI3K activity in mid-G1 phase for IGF-I-induced cell proliferation. PI3K inhibitor LY294002 was added into the medium at various time points during IGF-I treatment of FRTL-5 cells, and DNA synthesis during 20–24 h was measured (Fig. 3A). The addition of the PI3K inhibitor at 0, 3, 6, 12, or 18 h dramatically decreased DNA synthesis. Similar results were obtained using NWT10 cells (Fig. 3B) in which addition of PI3K inhibitor at 6 h after IGF-I stimulation decreased DNA synthesis during 15–18 h. These results, similar to reported data (28), indicated that prolonged activation of PI3K during G1 phase is required for maximal DNA synthesis induced by IGF-I.

**PI3K Activity Bound to IGF-I Receptor Is Sustained by Long Term IGF-I Stimulation**

Although IRS-1 and IRS-2 are well known to associate with PI3K and lead to activation of PI3K pathway in response to IGF-I, IRS dramatically decreased several hours after IGF-I stimulation in our experiments (Fig. 2). As a result, IGF-I-dependent phosphorylation of these proteins was continued for at least 24 h. In contrast, phosphorylation of extracellular signal-regulated kinases (Erks, p42/44 MAPK) was observed only until 3 h. Similar results were obtained using NWT10 cells (Fig. 2B), confirming that IGF-I stimulation induces prolonged tyrosine phosphorylation of IGF-IR and prolonged activation of the PI3K pathway.
levels, at 1 h after IGF-I stimulation (supplemental Fig. S2B). These results suggest that IRS-1/2 mediate primarily acute activation of PI3K.

It had been reported that, in cells overexpressing IGF-IR, IGF-I stimulation induces the association of IGF-IR with PI3K and increases PI3K activity bound to IGF-IR (13, 17). Thus, we hypothesized that continuous IGF-I stimulation may cause the prolonged association of IGF-IR with PI3K, which leads to prolonged activation of PI3K. To test this, we investigated the association of IGF-IR with PI3K in FRTL-5 in response to prolonged IGF-I stimulation, by co-immunoprecipitation analysis (Fig. 4A). The results were that tyrosine phosphorylation of IGF-IR, the association of IGF-IR with p85 PI3K regulatory subunit, and PI3K activity in IGF-IR-P13K complex were increased, at 1 min and 9.5 h after IGF-I stimulation. IGF-I withdrawal at 9 h abolished tyrosine phosphorylation of IGF-IR, the association of IGF-IR with PI3K, and P13K activity in IGF-IR-P13K complex at 9.5 h. Similar results were obtained using NWT10 cells (Fig. 4B). The time course experiment confirmed that the association of IGF-IR with PI3K was continuously maintained at least for 6 h after IGF-I stimulation in NWT10 cells (Fig. 4C). These results demonstrated that continuous IGF-I stimulation causes prolonged association of IGF-IR with PI3K and prolonged increase in PI3K activity bound to IGF-IR.

**PI3K Recognizes Tyr1316-X-X-Met Motif of IGF-IR when Tyrosine Residue Is Phosphorylated**—IGF-IR possesses a putative PI3K binding sequence around Tyr1316 that may function when the tyrosine residue is phosphorylated. Indeed, this site can be phosphorylated in response to IGF-I at least in vitro (29). Therefore, we examined whether Tyr1316 is necessary for IGF-I-induced association of IGF-IR with PI3K. Far Western blotting analysis using GST-tagged SH2 domain of p85 PI3K subunit as a probe showed that the SH2 domain can directly bind to IGF-IR that was prepared from IGF-I-stimulated cells (Fig. 5A). On the other hand, the binding of the SH2 domain to IGF-IR mutant in which Tyr1316 was substituted with Phe was hardly observed, clearly demonstrating that Tyr1316 is PI3K-binding site.

To examine whether this residue functions as a PI3K-binding site in cells, we used IR− cells (embryonic fibroblasts derived from IGF-IR−/− mice) transfected with plasmids encoding wild-type IGF-IR (WT cells), or with IGF-IR Y1316F mutant (Y1316F cells). Protein levels of IGF-IR were almost equal in these cells (Fig. 5B). In WT cells, IGF-I stimulation induced prolonged phosphorylation of IGF-IR tyrosine residues (Fig. 5C).
and supplemental Fig. S3A), including Tyr1131/1135/1136 (that is necessary for kinase activation (23, 30), and Tyr1316 (supplemental Fig. S3B)). IGF-I stimulation also induced the prolonged association of IGF-IR with p85PI3K regulatory subunit and an increase in PI3K activity bound to IGF-IR (Fig. 5C and supplemental Fig. S3A). On the other hand, in Y1316F cells, IGF-I stimulation induced tyrosine phosphorylation of IGF-IR, but we detected neither the association of IGF-IR with p85PI3K nor increases in PI3K activity bound to IGF-IR (Fig. 5C and supplemental Fig. S3A). These results demonstrated that Tyr1316 functions as a PI3K-binding site in cells and also suggested that the mutation of this site may little affect phosphorylation levels of other tyrosine residues of IGF-IR. Actually, this mutation had no apparent effect on phosphorylation of Tyr1131/1135/1136 (supplemental Fig. S3C).

As described above, IRS-1/2 is well known to associate with IGF-IR and with PI3K in response to IGF-I. Thus, we examined how much PI3K associated with IGF-IR is mediated by IRS. Co-immunoprecipitation analysis using FRTL-5 cells showed that IGF-IR is not co-immunoprecipitated with IRS-1/2 even when cells were stimulated with IGF-I (supplemental Fig. S4), suggesting that IGF-IR and IRS do not form stable protein-complexes but interact with each other transiently as a kinase and a substrate. Therefore, we concluded that PI3K co-immunoprecipitated with IGF-IR is not mediated by IRS.

**PI3K Activity Bound to IGF-IR Is Required for Cell Cycle Progression from G1 to S Phase in Response to IGF-I Stimulation—**

We next investigated effects of the Y1316F mutation of IGF-IR on downstream signaling. IGF-I stimulation induced tyrosine phosphorylation of IRS-1 and IRS-2 both in WT cells and Y1316F cells to a similar extent (Fig. 6, A and C), whereas we observed little tyrosine phosphorylation of IRS in R- fibroblasts transfected with mock plasmids (supplemental Fig. S5A), indicating that Y1316F IGF-IR can phosphorylate IRS and stimulate IRS-mediated signals. Shc, which is another IGF-IR substrate and is known to contribute to the activation of MAPK pathway (3), is also phosphorylated at tyrosine residues both in WT cells and Y1316F cells to a similar extent (Fig. 6, A and C).

We then studied phosphorylation of Akt and GSK3β in WT cells and Y1316F cells. In WT cells, IGF-I stimulation immediately induced phosphorylation of Akt and GSK3β, and their phosphorylation was gradually decreased, but its levels were higher than the basal levels until 9 h (supplemental Fig. S5B). At 9 h, their phosphorylation levels were significantly lower in Y1316F cells than those in WT cells (Fig. 6, B and C), suggesting that increases in PI3K activity bound to IGF-IR enhance the phosphorylation of Akt and GSK3β in mid-G1 phase in WT cells. The Y1316F mutation did not affect phosphorylation of Erk (Fig. 6, A and C).

Lastly, we analyzed cell proliferation induced by IGF-I (Fig. 7). In WT cells, IGF-I induced DNA synthesis during 15–18 h after IGF-I stimulation (Fig. 7A). IGF-I withdrawal at 6 h dramatically decreased DNA synthesis (Fig. 7A), confirming that continuous stimulation with IGF-I is required for DNA synthesis in this model. Continuous stimulation with IGF-I for 24 h induced an ~2-fold increase in WT cell numbers (Fig. 7B). In contrast, we observed no significant increase in Y1316F cell numbers, similar to R- cells transfected with mock plasmids (Fig. 7B). Analyzing the cell cycle at 18 h (Fig. 7C) as well as DNA synthesis at 15–18 h after IGF-I stimulation (Fig. 7D), we found that cell cycle progression from G1 phase to S phase was impaired...
in cells expressing Y1316F IGF-IR. The impairment of DNA synthesis was abrogated by the treatment of cells with bpV(pic), an inhibitor of phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (Fig. 7D) (31), suggesting that the dysfunction observed with the IGF-IR Y1316F mutant is caused by the insufficient activation of PI3K pathway. Taken together, these results indicated that PI3K activity bound to IGF-IR is necessary for IGF-1-dependent cell cycle progression from G1 phase to S phase.

**DISCUSSION**

It has been shown that continuous stimulation of cells with IGFs is required for IGF-induced cell proliferation (4–9), and this role of IGFs as a cell cycle progression factor is distinctive from those of many other growth factors. The molecular components of the prolonged signaling pathway were still unclear. This study is the first report revealing the IGF-IR-PI3K complex as one of components mediating prolonged IGF-I signals. In addition, this is the clearest evidence so far of a function associated with IGF-IR Tyr1316 as a PI3K binding site and of its importance in IGF-1-dependent cell proliferation.

In this study, we found that, after thyroid epithelial FRTL-5 cells and fibroblasts were stimulated with IGF-I, protein levels and tyrosine phosphorylation of IGF-IR were maintained throughout G1 phase (Figs. 2 and 4). When IGF-I was removed from the culture medium during G1 phase, tyrosine phosphorylation of IGF-IR was dramatically decreased within 30 min (Fig. 4, A and B). Similar results were obtained in glial progenitor cells (32). We suppose that enough IGF-IR protein molecules are continuously exposed on the cell surface throughout G1 phase to monitor extracellular IGF-I levels. It is consistent with other reports showing that a substantial fraction of IGF-IR is maintained on the cell surface after IGF-I stimulation (32) and that a large part of internalized IGF-IR is recycled back to the cell surface within the short time after IGF-I stimulation (32, 33). In contrast, ligand-dependent auto-phosphorylation of EGF and PDGF receptors often decrease to basal levels within a much shorter time (34), and the insulin receptor internalizes more efficiently than IGF-IR (33), highlighting a unique property of IGF-IR.

The present study showed IGF-I-induced prolonged association of endogenous IGF-IR with PI3K. Early studies had suggested that phosphotyrosyl IGF-IR and p85 PI3K can interact directly (13), and the carboxyl-terminal region in IGF-IR is important for the association (13, 14, 29). Here, we showed that Tyr1316 of IGF-IR is required for the association (Fig. 5), a strong evidence that this residue is the PI3K binding site. Functional analyses of several tyrosine phosphorylation sites in IGF-IR have been performed, including Tyr1316 (35–37). However, the specific function of this site in the induction of cell proliferation had not been well understood because of the specific experimental conditions (35) and compensatory effects of endogenous wild-type IGF-IR (36, 37). Thus, the present study is the first report demonstrating its importance in IGF-1-dependent cell proliferation. Interestingly, a report showed that Tyr1316 is required for cellular transformation induced by over-expression of IGF-IR (36), indicating that the IGF-IR-PI3K complex may also play roles in cellular transformation.

Insulin receptor is structurally related to IGF-IR, and several reports showed that it also binds to PI3K through its carboxyl-terminal region in a ligand-dependent manner (38–40). It was proposed that PI3K bound to IR plays a negative role in insulin-dependent glucose uptake, possibly through suppressing tyrosine phosphorylation of IRS-1 (40). In contrast, our experiments indicated that PI3K bound to IGF-IR plays a positive role in IGF-I-dependent cell proliferation (Fig. 7), without affecting tyrosine phosphorylation of IRS (Fig. 6A), suggesting distinct characteristics in the IGF-IR-PI3K and IR-PI3K complex.

In FRTL-5 and NWT10 cells, the association of IGF-IR with PI3K was well correlated with phosphorylation of Akt and GSK3β (Figs. 2 and 4). We also observed that phosphorylation of Akt and GSK3β in R− cells expressing Y1316F IGF-IR was lower than control (Fig. 6, B and C), suggesting that these kinases are involved in the signal transduction downstream of the IGF-IR-PI3K complex. However, we have to point out that Akt and GSK3β are phosphorylated both dependently and independently of the IGF-IR-PI3K complex in R− cells expressing IGF-I because the inhibitory effects of the Y1316F mutation on these phosphorylation levels were partial (Fig. 6C). Even so, our results demonstrated that PI3K bound to IGF-IR sig-
nificantly contributes the prolonged activation of the PI3K pathway and is required for the induction of IGF-I mitogenic activity.

It was reported that phosphorylation of GSK3β leads to increases in cyclin D1 protein stability (41). In FRTL-5 cells, we found that prolonged IGF-I signals and PI3K activity are required for the protection of cyclin D1 from proteolysis in mid-G1 phase (supplemental Fig. S6). Thus, we deduced that prolonged increases in PI3K activity bound to IGF-IR lead to the sustained phosphorylation of GSK3β, causing the accumulation of cyclin D1 in FRTL-5 cells. On the other hand, our previous study suggested that IGF-I-induced increases in PI3K activity bound to IRS-2 were transient and lead to increases in cyclin D1 mRNA in early G1 phase and p27Kip1 degradation in this cell line (21). Taking it together with present data, we believed that two different signals through the IGF-IR/PI3K and IRS-2-PI3K complexes are converged at the step of cyclin D1 accumulation, resulting in G1 CDK activation followed by cell cycle progression from G1 to S phase. Unexpectedly, in R– cells expressing IGF-IR, basal levels of cyclin D1 were high, and IGF-I stimulation did not increase cyclin D1 (supplemental Fig. S5B), suggesting that mechanisms downstream of the IGF-IR/PI3K complex to promote cell cycle machinery may be varied among different cell types. In addition, overexpression of IGF-IR in R– cells may exaggerate the contribution of the IGF-IR/PI3K versus the IRS-PI3K complex.

IGF-I also plays important roles in survival and differentiation of various cell types (1), and continuous IGF-I signals are thought to be required for these processes. Actually, prolonged Akt activation in the presence of IGF-I is required for survival of pro-oligodendroblast cells (42). Although molecular mechanisms mediating prolonged IGF-I signals to induce cell survival and differentiation are poorly understood, our study raises the possibility that PI3K bound to IGF-IR may be involved in these events.

In conclusion, we proposed that IGF-IR monitors extracellular IGF-I levels throughout G1 phase, and continuous stimulation with IGF-I causes prolonged increases in PI3K activity bound to IGF-IR and activation of its downstream signal pathways, leading to promotion of cell cycle progression into S phase. Because the concentration/availability of IGF-I in vivo gradually changes depending on nutritional condition and humoral factors (e.g. growth hormone, insulin, and IGF-binding proteins) (43), this novel mechanism may enable fine-tuning of cell proliferation that adequately reflects physiological dynamics of IGF-I levels.

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