Platelet-derived Growth Factor Receptor-mediated Signal Transduction from Endosomes*

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Although accumulated evidence supports the concept of endosomal signaling of receptor tyrosine kinases, most results are generated from studies of epidermal growth factor receptor (EGFR). It is not clear whether the concept of endosomal signaling could be generally applied to the other receptor tyrosine kinases. For example, platelet-derived growth factor receptor (PDGFR) is very similar to EGFR in terms of both signaling and trafficking; however, little is known about the endosomal signaling of PDGFR. In this research, we applied the same approaches from our recent studies regarding EGFR endosomal signaling to investigate the endosomal signaling of PDGFR. We showed in this communication that we are able to establish a system that allows the specific activation of endosome-associated PDGFR without the activation of the plasma membrane-associated PDGFR and without disrupting the overall endocytosis pathway. By using this system, we showed that endosomal activation of PDGFR recruits various signaling proteins including Grb2, SHC, phospholipase C-γ1, and the p85α subunit of phosphatidylinositol 3-kinase into endosomes and forms signaling complexes with PDGFR. We also showed that endosomal PDGFR signaling is sufficient to activate the major signaling pathways implicated in cell proliferation and survival. Moreover, we demonstrate that endosomal PDGFR signaling is sufficient to generate physiological output including cell proliferation and cell survival.

Platelet-derived growth factor (PDGF)-β1 is a connective tissue cell mitogen that binds the PDGFR receptor (PDGFR) with high affinity (1, 2). Like other receptor tyrosine kinases (RTKs), the PDGFR possesses an extracellular region (containing five immunoglobulin-like domains), a single transmembrane segment, a juxtamembrane segment, a protein-tyrosine kinase domain, and a carboxyl-terminal tail (3, 4). Binding of PDGF to PDGFR induces the dimerization of PDGFR (2, 5), which results in the activation of its intrinsic tyrosine kinase activity followed by trans-autophosphorylation (6, 7). The phosphotyrosine recruits signaling proteins containing Tyr(P)-binding domains such as Src homologue 2 or Tyr(P)-binding domains. Several of these signaling proteins have been identified and include Src kinase family members (8), phospholipase C-γ1 (9, 10), the p85α subunit of phosphatidylinositol 3-kinase (PI3K) (11), GTPase-activating protein (12, 13), the phosphotyrosine phosphatase SHP-2 (14, 15), and adaptor proteins such as Grb2 (16), Shc (17), Grb7 (18), and Nck (19). The formation of receptor-signaling protein complexes then initiates the activation of various signaling pathways including the Ras-ERK pathway, the PI3K-Akt pathway, the PLC-γ1 pathway, and the Src pathway. The activation of these pathways eventually leads to cell proliferation and survival. Concomitantly, these ligand-receptor complexes cluster into clathrin-coated pits, internalize into early endosomes, and eventually traffic to lysosomes for degradation (20–24).

Although endocytosis has traditionally been considered a deactivation mechanism for RTKs, accumulated evidence is unveiling a positive signaling role for endosome-localized RTKs (25). It is known that certain RTKs remain autophosphorylated and catalytically active following ligand-induced endocytosis (26–28). The best studied RTK in this regard is epidermal growth factor (EGF) receptor (EGFR). At the endosomal location, EGF-EGFR complexes remain associated with signaling effectors such as Grb2, Shc, p85, and PLC-γ1 (25, 29–34), are also capable of nucleating new complexes (25), and continue to signal downstream through their respective pathways (25, 29, 31). More evidence supporting endosomal signaling comes from endocytosis blocking experiments. Inhibition of EGFR endocytosis modulates EGF-stimulated activation of signaling proteins, especially inhibition of ERK activation (35–38). Moreover, ligand-activated EGFR spends more of its lifetime internally than on the cell surface, which further suggests the importance of endosomal signaling of EGFR. Recently, we established a system to specifically activate endosome-associated EGFR in the absence of any plasma membrane activation (25). By using this system, we examined the effects of endosomal EGFR signaling on the two major physiological outcomes of EGFR activation, cell survival, and proliferation. We showed that endosomal EGFR signaling is sufficient to elicit cell survival through generation of anti-apoptotic signals in response to serum withdrawal (25). We further showed that endosomal EGFR signaling is sufficient to stimulate cell proliferation (54). This demonstrated that endosomal EGFR signaling is sufficient to generate physiological outcome.

However, nothing is known about the endosomal signaling of PDGFR. It is not known whether PDGFR can signal from endosomes and whether the endosomal signaling of PDGFR, if
it exists, can produce any physiological outcomes. Based on the similarity between PDGFR and EGFR in their trafficking and signaling, it is possible that, like EGFR, PDGFR also signals from endosomes. To test this possibility, we applied the same approaches from our EGFR study (25, 39, 54) to investigate the endosomal signaling of PDGFR. We showed in this communication that we are able to establish a system that allows the specific activation of endosome-associated PDGFR without the activation of the plasma membrane-associated PDGFR and without disrupting the overall endocytosis pathway. We treated cells with EGF in the presence of AG1296, a specific PDGFR tyrosine kinase inhibitor (40), and monensin, which blocks recycling of many receptors. This treatment led to the internalization of nonactivated PDGFR-PDGFR complex into endosomes. The endosome-associated PDGFR was then activated by removing AG1296 and monensin. During this procedure we did not observe any detectable surface PDGFR phosphorylation. By using this system, we provided original evidence demonstrating that 1) endosomal activation of PDGFR recruits various signaling proteins including Grb2, SHC, PI3K, and PLC-γ1 into endosomes and forms signaling complexes with PDGFR; 2) endosomal PDGFR signaling is sufficient to activate the major signaling pathways implicated in cell proliferation and survival; and 3) endosomal PDGFR signaling is sufficient to generate physiological output including cell proliferation and cell survival.

MATERIALS AND METHODS

Antibodies and Chemicals—Mouse anti-phosphotyrosine (pY99), anti-phospho-PLC-γ1 (Tyr783), rabbit anti-PDGFR, anti-phospho-PDGFR, anti-ERK, anti-Grb2, anti-Raf, and anti-SHC antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phospho-Akt (Ser473) antibody was from Cell Signaling Technology (Beverly, MA). Glutathione cross-linked to 4% agarose was from Sigma. Protein A-Sepharose 6MB was from Calbiochem (La Jolla, CA). PDGF-B was from Upstate Biotechnology, Inc. Unless otherwise specified, all of the chemicals were purchased from Sigma.

Cell Culture and Treatment—HepG cells (human hepatocellular carcinoma cells) transfected with PDGFR-β receptor and F442 cells (mouse adipocytes) were grown at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and were maintained in a 5% CO₂ atmosphere. For HepG cells, 100 μM nonessential amino acids (Invitrogen) were added. To specifically activate PDGFR after its endocytosis into endosomes, HepG and F442 cells were serum-starved for 24 h. After a 1-h pretreatment with 1 μM AG1296 for 15 min, monensin and PDGFR were added to final concentrations of 100 μM and 10 ng/ml, respectively. After a 30-min treatment, the cells were washed with PBS for three times and then maintained in the serum-free medium for the indicated time.

U0126, an inhibitor of MEK activation, was added to the medium to a final concentration of 10 μM for 1 h. For the depletion of Raf, 10 μM Radicicol was added to the medium, and the cells were cultured for a further period of 40 h. To inhibit PI3K, 100 μM wortmannin was added to the medium for 30 min.

Subcellular Fractionation and Total Cell Lysates—Isolation of plasma membrane, endosomal, and cytosolic fractions was carried out by our previously described method (51). Briefly, following treatment the cells were scraped into homogenization buffer (0.25 M sucrose, 20 mM Tris-HCl, pH 7.5, 1 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 100 mM NaF, 0.5 mM Na₂VO₃, 0.02% NaN₃, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml aprotinin, 1 μM pepstatin A) and homogenized. The homogenates were centrifuged at 200,000 × g for 5 min to remove cell debris and nuclei (p1). The post nuclear supernatant (S1) was then centrifuged at 15000 × g for 10 min to yield a supernatant (S2) and a pellet (P2). Next, P2 was resuspended in homogenization buffer (0.25 M sucrose), over an equal volume of 1.42 M sucrose buffer, and centrifuged at 82,000 × g for 1 h. The pellet of the 0.25–1.42 M interface was collected as the plasma membrane fraction. The S2 fraction was centrifuged at 100,000 × g for 30 min to yield the soluble cytosolic fraction and a microsomal pellet. This pellet was resuspended in 0.25 M sucrose buffer and overlaid upon a discontinuous sucrose gradient containing equal volumes of homogenization buffer at 1.00 and 1.15 M sucrose. The resuspension was centrifuged at 200,000 × g for 1.5 h to obtain the purified endosomal fraction at the 0.25–1.00 M interface.

Indirect Immunofluorescence—Indirect immunofluorescence was performed as described previously (51). Briefly, the cells were grown on glass cover slips and serum-starved for 24 h. After treatment, the cells were fixed by ice-cold methanol and permeabilized with Triton X-100. Next, the cells were incubated with indicated primary antibodies at room temperature for 1 h followed by fluorescence-labeled secondary antibodies for 1 h.

Immunoprecipitation and Immunoblotting—Immunoprecipitation experiments were carried out as described previously (51). The cells were lysed with immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 100 mM NaF, 0.5 mM Na₂VO₃, 0.02% NaN₃, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml aprotinin, 1 μM pepstatin A) overnight at 4°C. The cell lysates were then centrifuged at 21,000 × g for 30 min to remove debris. The supernatants, containing 1 mg of total protein, were used to incubate with 1 μg of rabbit anti-PDGFR antibody to immunoprecipitate PDGFR from HepG and F442 cells. Rabbit anti-SHC antibody was used to immunoprecipitate Shc because the size of Shc is similar to that of IgG.

Ras Activation Assay—Ras activation was assayed by the method described by Herrmann et al. (52). Briefly, HepG or F442 cells that had been treated as required were lysed and subjected to 0.5 ml of a 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 mM NaF, 2.5 mM MgCl₂, 1 mM EDTA) and then centrifuged at 21,000 × g for 30 min. GST fusion Raf-RBD domain (GST-RBD), precoupled to glutathione-agarose beads in BOS buffer, was added, and the lysates were incubated at 4°C for 1 h. The beads were collected by centrifugation and washed three times with BOS buffer, and then loading buffer was added. Ras was detected using the monoclonal anti-Ras antibody, followed by horseradish peroxidase-coupled anti-mouse antibody. Anti-GST blot was used as a loading control.

Immunoblotting—Immunoblotting was performed as described previously (53). For the detection of PDGFR, phospho-PDGFR, phospho-ERK, ERK, phospho-Akt, Akt, phospho-PLC-γ1, and PLC-γ1 in total lysates of HepG and F442 cells, aliquots containing 20 μg of protein from each cell lysate were used. For the detection of PDGFR, Grb2, PLC-γ1 and the p85α subunit of PI3K in the anti-PDGFR immunoprecipitates, PDGFR, and Shc in the anti-SHC immunoprecipitates, one-tenth of the immunoprecipitate from each lysate was used. To examine PDGFR in each subcellular fraction of HepG and F442 cells, aliquots containing 10% of the protein from each fraction were used. The protein samples were separated by electrophoresis through 10% polyacrylamide SDS-containing gels and electrophoretically transferred onto nitrocellulose filter paper. The filters were then probed with the respective primary antibody. The secondary antibody was a polyclonal goat anti-rabbit IgG coupled to horseradish peroxidase or a polyclonal goat anti-mouse IgG coupled to horseradish peroxidase followed by enhanced chemiluminescence development (Pierce) and light detection with Fuji Super RX Film (Tokyo, Japan). Quantification of the results was achieved by using a FluorChem digital imaging system (Alpha Innotech Corporation).

DNA Synthesis Assay—DNA synthesis was assayed by bromodeoxyuridine (BrDUrd) incorporation. The cells (HepG or F442) were plated at 10,000 cells/glass coverslip and serum-starved by incubation in serum-free medium for 24 h. The cells were then treated as necessary in the presence of 25 μM BrdUrd. For discontinuous treatment, BrdUrd was added back after each subsequent pulse or after the whole treatment, the cells were washed and fixed. Following the denaturation of DNA with 2 N HCl for 30 min at room temperature, the cells were incubated with mouse anti-BrdUrd antibody for 1 h before addition of fluorescein isothiocyanate-conjugated anti-mouse IgG for (for detection of BrdUrd) and 50 μg/ml propidium iodide (to stain for total DNA). The nuclei were visualized in the red and green channels, and digital images were quantitated for BrdUrd incorporation. The percentage of DNA synthesis was calculated as the number of BrdUrd positive cells/total number of cells analyzed × 100. For each experimental treatment, a minimum of 500 cells was counted.

TUNEL Assay—HepG and F442 cells (10,000/coverslip) were serum-starved for 24 h to initiate a significant level of apoptosis. Some of the serum-starved cells were treated with AG1296 for 15 min and then with addition of PDGF (20 ng/ml) and monensin for 30 min followed by washing with PBS and incubation with serum-free medium for 12 h. For controls, some of the serum-starved cells were stimulated with...
PDGF for 30 min followed by incubation with serum-free medium for 12 h. Apoptosis was assayed by TUNEL assay using an apoptosis detection system kit (Promega) according to the manufacturer's instructions. The percentage of apoptotic cells was calculated as the number of apoptotic nuclei/total nuclei analyzed × 100. For each experimental treatment, a minimum of 250 cells was counted.
**RESULTS**

Selective Activation of PDGFRs Following Their Endocytosis into Endosomes—We recently established a system to selectively activate endosomal EGFR without the activation of the plasma membrane-associated EGFR (25, 39). Here we apply the same approach to PDGFR. F442 cells and HepG cells that stably express human PDGFR were treated with AG1296 for 15 min and then stimulated with PDGF in the presence of monensin. After 30 min of incubation with PDGF and monensin, the majority of PDGFR was internalized into endosomes (Fig. 1). There is no overlap between PDGFR and Tyr(P) immunostains, indicating that the endosome-associated PDGFRs were not phosphorylated upon internalization (Fig. 1). For control cells treated only with PDGF, PDGFRs were phosphorylated and internalized into endosomes as expected. To more specifically determine the phosphorylation status of endosome-associated PDGFR, F442 and HepG cells were double stained with anti-PDGFR and anti-phospho-PDGFR (p-PDGFR) antibodies following the treatment described above. Our results confirmed that treatment of F442 and HepG cells with AG1296, PDGF, and monensin induced internalization of nonphosphorylated PDGFR into endosomes (Fig. 1).

To determine whether endosome-associated nonphosphorylated PDGFR could be activated, F442 and HepG cells were washed with medium. The endosome-associated PDGFR was significantly activated 30 min following wash (Fig. 1). We did not observe any phosphorylation of plasma membrane-associated PDGFR (Fig. 1). These results suggest that we can specifically activate endosome-associated PDGFR.

These observations were further confirmed by subcellular fractionation experiments (Fig. 2). It is well established that in the absence of ligand, PDGFR is localized on the plasma membrane where it is not phosphorylated; after induction by PDGF for 30 min, the vast majority of PDGFRs is phosphorylated and enriched in endosome fractions. PDGFR itself can therefore be used as a marker for plasma membrane and endosomes. Indeed, our results showed that, for both F442 and HepG cells, in the absence of PDGF PDGFR is primarily localized at the plasma membrane fraction and not phosphorylated, whereas PDGFR was primarily present in the endosome fraction and phosphorylated following standard PDGF stimulation (Fig. 2). Additionally, early endosome autoantigen 1, a marker for endosomes, is highly enriched in our endosome fractions. Treatment with AG1296, monensin, and PDGF resulted in the internalization of nonphosphorylated PDGFR into endosomes, and the endosome-associated PDGFR was phosphorylated following washing and incubation in medium (Fig. 2). Washing to remove AG1296 and monensin did not result in the increase of plasma membrane-associated PDGFR (Fig. 2), which suggests there is no detectable recycling of PDGFR to the plasma membrane following the removal of monensin. More importantly, only very little phosphorylated PDGFR was detected at the plasma membrane (Fig. 2). Together, these results suggest that we have established a system to specifically activate PDGFR in endosomes without meaningful PDGFR activation at the plasma membrane.

The Level and Duration of PDGFR Activation in Our Established System—We next determined the level and duration of PDGFR activity in our system. HepG and F442 cells were treated with AG1296, PDGF, and monensin as described above. Immunoblotting with anti-PDGFR and anti-p-PDGFR antibodies showed that before washing, no PDGFR was phosphorylated (Fig. 3). Washing and incubation gradually induced the phosphorylation of PDGFR. After washing for 30 min, phosphorylation of PDGFR reached the maximum that is 50% of the level following standard PDGF stimulation for 30 min. After washing for 2 h, the level of phosphorylated PDGFR was undetectable (Fig. 3). This pattern of receptor dephosphorylation is similar to that following standard PDGF stimulation (data not shown).

Recruitment of Signaling Proteins into Endosomes by Activation of Endosome-associated PDGFR—We next used this system to specifically study PDGFR-mediated signal transduction from endosomes. First we examined whether activation of endosome-associated PDGFR is able to interact with various signaling proteins and form the signaling complex in endosomes. HepG and F442 cells were treated with AG1296 and then PDGF and monensin as described above. The cell lysates were immunoprecipitated with anti-PDGFR antibody, and the immunoprecipitates were immunoblotted with antibodies to various signaling proteins. As shown in Fig. 4, specific activation of endosome-associated PDGFR resulted in the association of PDGFR with Grb2, PLC-γ1, and the p85α subunit of PI3K. However, we cannot clearly show the SHC bands in the immunoblot. This may be partially due to the similar molecular weight between IgG and SHC and partially due to the poor co-immunoprecipitation or SHC by anti-PDGFR antibody. To further test the interaction between SHC and PDGFR, we immunoprecipitated the total lysates with anti-SHC antibody. Immunoblotting the immunoprecipitates with anti-PDGFR antibody showed the association between PDGFR and SHC following the specific activation of endosome-associated PDGFR (Fig. 4B). The above results indicate that direct activation of endosome-associated PDGFR is sufficient to recruit the major signaling proteins to endosomes to form the signaling complexes with PDGFR.

Stimulation of Various Signaling Pathways by Endosomal Signaling of PDGFR—We showed above that the specific acti-
vation of endosome-associated PDGFR is able to recruit major signaling proteins to endosomes. We further determined whether endosomal signaling of PDGFR is sufficient to stimulate various signaling pathways. It is known that following standard stimulation with PDGF, multiple signal transduction pathways are activated. These pathways include the Ras-ERK pathway, the PI3K-Akt pathway, and the PLC-γ1 pathway. We showed that activation of endosome-associated PDGFR also leads to the phosphorylation of SHC (Fig. 5A), ERK1/2 (Fig. 5C), Akt (Fig. 5D), and PLC-γ1 (Fig. 5E), as well as the activation of Ras as determined by association with the RBD of Raf (Fig. 5B). The maximum phosphorylation level for the activation of endosome-associated PDGFR is ~50% of that following standard PDGF stimulation (Fig. 3), and the activation levels of ERK1/2, Akt, and PLC-γ1 were also ~50% of that following standard PDGF stimulation (Fig. 5), suggesting that activation of endosome-associated PDGFR and standard activation of PDGFR are similarly effective on the induction of these signaling pathways.

To determine whether the mechanism of downstream activation is the same as from standard PDGFR activation, we next examined the effect of specific inhibitors of ERK and Akt activation following stimulation of endosomal PDGFR. Our results showed that the MEK inhibitor U0126 blocked mitogen-activated protein kinase phosphorylation following stimulation of endosomal PDGFR to the same extent as when stimulated by PDGFR initially at the cell surface (Fig. 5C). Moreover, depleting Raf with Radicicol also inhibited the activation of ERK stimulated by endosome-associated PDGFR to the same extent as that stimulated by the standard activated PDGFR (Fig. 5C). Akt phosphorylation induced following stimulation of endosomal PDGFR is blocked by the PI3K inhibitor wortmannin, indicating that Akt activation is mediated by PI3K (Fig. 5D).

Together, these results indicate that endosomal signaling of PDGFR is sufficient to stimulate several signal transduction pathways implicated in cell mitogenesis and survival.

**Support of Cell Survival by Endosomal PDGFR Signaling**—Having positively established a signaling role for PDGFR activated endosomally, we then determined whether these signals are of sufficient potency to produce a biological outcome. We first examined the effects of endosomal PDGFR signaling on serum withdrawal-induced apoptosis by using the TUNEL assay (Fig. 6). HepG and F442 cells cultured in 10% fetal bovine serum have a 5% basal rate of apoptosis; if the cells are then starved of serum for 36 h, this rate increases to 50%. As seen in Fig. 6, a brief 1-h pulse of PDGF, administered after 24 h of starvation, provides a sufficient signal to save approximately half of the cells from apoptotic death. To determine whether an anti-apoptotic effect can be similarly elicited from activating endosome-associated PDGFR, following 24 h of starvation HepG and F442 cells were stimulated with PDGF (10 ng/ml) for 30 min in the presence of AG1296 with or without monensin to allow for the internalization of inactive PDGFR into endosomes. After the subsequent activation of endosome-associated PDGFR by washing and incubating with serum-free medium for the remaining 12 h, the percentage of apoptotic cells was
reduced to levels similar to that following the standard PDGF treatment, in this case a pulse of PDGF for 1 h (Fig. 6). It is important to note that in both cases, the pulse of PDGFR activation is limited to those receptors stimulated over the treatment period, and because the growth factor is thoroughly washed following treatment, no more receptors will contribute to the anti-apoptotic "signal." We show, therefore, that endosomal PDGFR can transduce survival signals of physiological significance.

If the cells were treated with wortmannin, standard PDGF stimulation of 1 h did not promote cell survival (Fig. 6). This is expected, because wortmannin inhibits PI3K and thus prevents transduction downstream to inhibit apoptotic factors. To investigate whether the survival signals elicited from endosome-associated PDGFR originate from the same PI3K-Akt pathway, the cells were pretreated with wortmannin (100 nM) and then treated for 30 min with AG1296 and PDGF with or without monensin before washout. As can be seen in Fig. 6,
activation of endosome-associated PDGFR is unable to suppress apoptosis induced by serum withdrawal. This indicates that similar to standard activation of PDGFR, activation of endosome-associated PDGFR supports cell survival by stimulating the PI3K-Akt pathway.

**The Effect of Endosome-associated PDGFR on Cell Proliferation**—We finally determined whether endosomal PDGFR signaling is sufficient to stimulate cell proliferation. It was shown recently that the sustained requirement (more than 8 h) for PDGF to induce cell proliferation can be replaced with two shorter pulses of PDGF (41). In quiescent cells, the two pulses must coincide first with re-entry into the G1 cell cycle and then during late G1, a time approximate to the restriction point. In many cell types, including our own, the times in which mitogen are required are spaced ~7–9 h apart. These findings allowed us to test whether two pulses of endosomal PDGFR signaling are sufficient to stimulate cell proliferation.

Cell proliferation (DNA synthesis) was assayed by BrdUrd incorporation into cells. As shown in Fig. 7, one pulse of endosomal PDGFR signaling is insufficient to stimulate S phase entry. However, two pulses of endosomal PDGFR signaling can drive cells into the S phase with equal efficacy as two pulses of standard activation from PDGF, in both HepG and F442 cells. Moreover, the percentage of proliferating cells following two-pulse treatment, whether from standard activated or endosome-activated PDGFR, is similar to treating cells continually over 8 h with either PDGF or 10% fetal bovine serum. This demonstrates that PDGFR signals, derived exclusively from the endosome, can lead to cell proliferation; furthermore, the potency of the endosomal signal is kinetically similar to that derived from the standard PDGFR activation.

**DISCUSSION**

It is well established that endocytosis of RTKs from the cell surface to lysosomes results in degradation of the receptor, which can attenuate receptor signaling and may even be conceived of as a tumor suppressor pathway (42, 43). On the other hand, accumulated evidence suggests that the internalized ligand-RTK complex may maintain its ability to generate cell signaling from endosomes. Most studies regarding the endosomal signaling of RTKs have been focused on EGFR. Internalized EGFR is autophosphorylated and catalytically active (26–28). Various signaling molecules that regulate Ras activity, including Grb2, SHC, GTPase-activating protein, and Cbl, are co-internalized with EGFR into endosomes and remain associated with the receptor in endosomes (30, 43–46). Inhibition of EGFR endocytosis significantly modulates the cell signaling mediated by EGFR (47–50). Very recently we established a system to specifically activate endosome-associated EGFR without activation of the plasma membrane-associated EGFR (25, 39, 54). We showed that endosomal signaling of EGFR is sufficient to activate the major signaling pathways and generate physiological outcomes. However, it is not clear whether the concept of endosomal signaling could be generally applied to other RTK. So far only very limited studies have been conducted with other RTKs. PDGFR is very similar to EGFR in terms of both signaling and trafficking; however, little is known about the endosomal signaling of PDGFR. In this research, we applied the same approaches from our EGFR study to investigate the endosomal signaling of PDGFR.

As demonstrated in this communication, we have successfully generated specific endosomal signaling of PDGFR. We first inhibited PDGFR kinase activity by treating the cells with AG1296, a specific PDGFR kinase inhibitor, and then stimulated cells with PDGF in the presence of monensin (which inhibits PDGFR recycling). By these treatments, we induced the internalization and accumulation of inactive PDGFR into endosomes (Figs. 1 and 2). After removing AG1296 by washing with serum-free medium, endosome-associated PDGFR was activated (Figs. 1 and 2). We did not observe any activation of PDGFR associated with the plasma membrane (Figs. 1 and 2). The activation (phosphorylation) level of endosome-associated PDGFR is ~50% of the control (standard PDGFR activation) (Fig. 3). The reduced phosphorylation level could be due to some endosome-associated PDGFR already dissociated from PDGFR and thus will not be
activated following the removal of AG1296. Our results demonstrate that we have established a system in which only endosome-associated PDGFR is specifically activated. This system allows us to study PDGFR-mediated signaling from endosomes without interference from cell surface PDGFRs.

It has been shown that for standard activation by PDGF, various signaling proteins are recruited to PDGFR at the plasma membrane, including Shc, Grb2, PLC-γ1, and the p85α subunit of PI3K (9–11;16;17). Whether endosomally activated PDGFR could directly recruit these signaling proteins into the endosomes was uncertain. Using co-immunoprecipitation of endosomally activated PDGFR, we showed the receptor, activated at the endosome, recruits and remains associated with these same signaling proteins. This indicates that the plasma membrane is not a privileged site for protein recruitment and receptor-signal complex formation (Fig. 4). We then determined whether the activation of endosome-associated PDGFR is able to activate major signaling pathways. We showed that activation of endosome-associated PDGFR stimulated both Ras-ERK pathway and PI3K-Akt pathways (Fig. 5). We further showed that endosomal signaling of PDGFR stimulates ERK activation by a Ras-Raf-MEK-ERK pathway, which is the same as the activation of ERK by standard activation of PDGFR. When wortmannin was used to inhibit the function of PI3K, Akt phosphorylation was totally blocked, indicating that Akt activation is mediated by PI3K when initiated from an endosomally activated receptor (Fig. 5D). We also detected the phosphorylation of PLC-γ1 from total lysates of cells following activation of endosomal PDGFR (Fig. 5), suggesting that PLC-γ1 behaves similarly regardless of the localization of PDGFR signal origin.

Finally, the physiological relevance of PDGFR-mediated signaling from endosomes was investigated. We first looked at the ability of serum-starved cells to withstand the onset of apoptosis by the readidion of mitogenic stimulus. As shown in Fig. 6, a short pulse of either standard PDGFR activation or endosome-associated PDGFR activation was able to rescue ~50% of cells from apoptotic death. We also showed that two discontinuous pulses derived from endosomal PDGFR was sufficient to drive the majority of quiescent cells into the S phase, with kinetics comparable to either standard two-pulse induction of PDGF or continual exposure with mitogen for 8 h (Fig. 7).

We have shown previously that EGFR endosomal signaling is sufficient to activate the major signaling pathways leading to cell proliferation and survival (25). Although PDGFR endosomal signaling, in general, functions similarly to EGFR endosomal signaling, we observe the differences between these two receptors in terms of endosomal signaling. The association between PDGFR and p85 or PLC-γ1 is much stronger than the association between EGFR and p85 or PLC-γ1 following the endosomal activation of these two receptors. Similarly, the activation of PLC-γ1 and Akt is much stronger by endosomal PDGFR signaling than that by endosomal EGFR signaling.

In conclusion, our results indicate that to a large extent, PDGFR-mediated signaling from endosomes is very similar to the standard PDGFR-mediated signaling that includes the signaling from both the plasma membrane and endosomes. The similar results obtained for both PDGFR and EGFR (25) suggest that our recently established system to specifically generate endosomal signaling of EGFR may be suitable for many other RTKs. More importantly, our results suggest that the concept of endosomal signaling may apply to all RTKs.

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