Grb7 is a Downstream Signaling Component of Platelet-derived Growth Factor α- and β-Receptors*

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Ligand stimulation of the platelet-derived growth factor (PDGF) α- or β-receptors leads to activation of their intrinsic tyrosine kinases and autophosphorylation of tyrosine residues. Grb7 is an SH2 and PH domain-containing molecule that is known to be overexpressed in some breast cancer tissues and cell lines. Here we show that the SH2 domain of Grb7 can directly bind to the autophosphorylated PDGF β-receptor in vitro. Grb7 association to the PDGF β-receptor was dramatically reduced by replacement of tyrosine residues 716 or 775 with phenylalanine residues. Synthetic phosphorylated peptides containing Tyr-716 or Tyr-775 inhibited binding of the Grb7 SH2 domain to the autophosphorylated PDGF β-receptor in a manner similar to but distinct from the binding of the Grb2 SH2 domain. Grb7 associated with activated PDGF β-receptors in vivo, and the association was dramatically reduced by substitution of Tyr-716 or Tyr-775 with a phenylalanine residue. Furthermore, complex formation between Shc and Grb7 was observed after ligand stimulation of PDGF α- or β-receptors in cells transfected with Grb7 cDNA or in the breast cancer cell line BT-474. Thus, Grb7 is implicated in PDGF signaling pathways in certain cell types by binding to the receptor directly or indirectly via Shc.

Platelet-derived growth factor (PDGF) is a potent mitogen for mesenchymal cells. PDGF plays important roles in the embryonal development and in wound healing as well as in the development of several pathological conditions such as atherosclerosis and tumorigenesis (for review, see Refs. 1 and 2). PDGF is a family of homo- or heterodimeric proteins composed of A- and B-polypeptide chains, which binds to two structurally related protein tyrosine kinase receptors (3–5). The PDGF α-receptor is able to bind either A- or B-chains of PDGF, whereas the β-receptor binds only the B-chain.

PDGF binding activates the intrinsic tyrosine kinase activities of the receptors through receptor dimerization. The receptors then undergo autophosphorylation and phosphorylate intracellular substrates. Autophosphorylation sites in tyrosine kinase receptors provide binding sites for signal transduction molecules containing one or two copies of Src homology 2 (SH2) domains. The SH2 domain is a conserved stretch of about 100 amino acid residues found in several signal transduction molecules, which binds in a specific manner to tyrosine-phosphorylated regions of tyrosine kinase receptors and intracellular signaling molecules (7). Nine and five autophosphorylation sites have so far been identified in the PDGF β- and α-receptors, respectively. Several of these have been shown to interact in a specific manner with certain SH2 domain-containing proteins (for review, see Ref. 8). For the PDGF β-receptor, two autophosphorylation sites in the juxtamembrane region (Tyr-579 and Tyr-581) mediate the binding of Src family tyrosine kinases. There are four autophosphorylation sites in the kinase insert which bind Grb2 (Tyr-716), the regulatory subunit (p85) of phosphatidylinositol 3′-kinase (PI3-kinase; Tyr-740 and Tyr-751), Nck (Tyr-751), and the GTPase-activating protein of Ras (RasGAP; Tyr-716). The two autophosphorylation sites in the carboxyl-terminal tail (Tyr-1009 and Tyr-1021) mediate binding of the SH2 domain-containing phosphatase Syt/PTP1D and phospholipase C-γ, respectively. The adapter proteins Shb and Shc seem to interact with multiple tyrosine residues (9, 10). In the PDGF α-receptor, tyrosine residues 754, 762, 768, 988, and 1018 have been identified as autophosphorylation sites (11–13). Among them, Tyr-1018 has been shown to mediate association of phospholipase C-γ (13). Tyr-731 and Tyr-742 seem to be important for the binding of PI3-kinase (14) and are likely to become phosphorylated, although this has not been shown directly.

Grb7 is a protein identified by expression cloning using autophosphorylated epidermal growth factor receptor carboxy-terminal tail as a probe (15). It has a single SH2 domain, a pleckstrin homology (PH) domain, and a proline-rich region. Thus Grb7 most likely is an adapter protein devoid of intrinsic catalytic activity, similar to Grb2, Shc, Shb, and Nck. Grb7 is coexpressed with HER2/ErbB2 in certain breast cancer tissues and cell lines, and Grb7 can bind to tyrosine-phosphorylated HER2 and Shc via its SH2 domain (16). Furthermore, Mig-10, a protein of high similarity to Grb7, was identified in Caenorhabditis elegans. Mig-10 is involved in long range migration of neuronal cells in embryonic development (32). Thus it is suggested that Grb7 performs an important signaling function. However, whether Grb7 is involved in signaling via receptor tyrosine kinases other than epidermal growth factor receptor family members is not known.

Here we show that Grb7 binds directly to Tyr-716 and Tyr-775 in the PDGF β-receptor. Both PDGF α- and β-receptors induce complex formation between Grb7 and Shc. Thus, Grb7 may have a role in signaling via PDGF receptors in certain cell types.

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¶The abbreviations used are: PDGF, platelet-derived growth factor; PDE cells, porcine aortic endothelial cells; PAGE, polyacrylamide gel electrophoresis; PI3-kinase, phosphatidylinositol 3′-kinase; SH2, Src homology 2; HA, hemagglutinin; GST, glutathione S-transferase.
**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Porcine aortic endothelial (PAE) cells expressing wild-type PDGF α-receptor and wild-type or tyrosine residue-mutated PDGF β-receptors have been described earlier (17, 18, 21). The breast cancer cell line BT-474 was obtained from American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium supplemented with penicillin/streptomycin, 10% fetal calf serum, and 10 μg/ml bovine insulin (Sigma). COS cells and human foreskin fibroblasts AG1518 were obtained from American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium supplemented with penicillin/streptomycin and 10% fetal calf serum.

**Plasmids**—Grb7 cDNA in pmDJ30 expression vector was constructed as described previously (16). For constructing HA-tagged Grb7, the sequence encoding amino acid residues 384–535 of Grb7 cDNA was amplified by polymerase chain reaction, introducing a BglII site before the terminating codon. The polymerase chain reaction product was cleaved with XbaI and BglII and ligated into a pBSKs-HATGAG vector containing hemagglutinin (HA) sequences (a gift from Dr. Y. Xiang, University of North Carolina, Chapel Hill, NC) together with a fragment of Grb7 cDNA cut out from the AElox plasmid (19) with XbaI and SmaI sites. The resulting HA-tagged Grb7 cDNA was cut out with XbaI and EcoRV and then ligated into the expression vector pcDNA3 (Invitrogen). Transient transfection of the plasmids was performed using the calcium phosphate method as described previously (20).

**Antibodies**—The rabbit antisera PDGFR-3 recognizing the PDGF β-receptor (5), PDGFR-7 recognizing the PDGF α-receptor (17), and an antisera against Grb7, denoted #188 (16), have been described earlier. The rabbit antisera YPY, which specifically recognizes HA, was raised against the synthetic peptide YPYDVPDYAGYPYDVPDYA. The anti-glutathione S-transferase (GST) antisera was raised against recombinant GST expressed in bacteria. The monoclonal anti-phosphotyrosine antibody PY20 and affinity-purified polyclonal anti-Shc antisera were from Affiniti Research Products Ltd. The monoclonal antibody against Grb2 was kindly provided by Dr. J. Schlessinger, New York University. Peroxidase-conjugated sheep anti-mouse IgG was from Amersham Corp. Peroxidase-conjugated swine anti-rabbit IgG was from Dakopatts.

**Peptide Synthesis**—The amino acid sequences of the synthetic peptides used in this study, either nonphosphorylated or phosphorylated at tyrosine residues (indicated as Yp), are as follows: peptide Y579, V-S-S-D-G-H-E-Yp-I-Y-V-D-P-M-Q-L-P-Y; peptide Y579, V-S-S-D-G-H-E-Yp-I-Y-V-D-P-M-Q-L-P-Y; peptide Y716, L-Q-H-L-S-D-K-R-R-P-P-S-A-E-L-Yp-S-N-A-L-P-V-G-Y-G; peptide Yp716, L-Q-H-L-S-D-K-R-R-P-P-S-A-E-L-Yp-S-N-A-L-P-V-G-Y-G; peptide Y775, I-E-L-S-N-Y-M-A-P-Y-D-N-Y-V-Y-S-A-P-E-R; peptide Y775, I-E-I-S-N-Y-M-A-P-Y-D-N-Y-V-Y-S-A-P-E-R; peptide Y1021, P-N-E-G-D-N-D-Y-I-I-P-L-P-D-P-K; peptide Y1021, P-N-E-G-D-N-D-Y-I-I-P-L-P-D-P-K. The peptides were synthesized by Fmoc chemistry, as described previously (9).

**Immunoprecipitation and Immunoblotting**—Serum-starved cells were treated with 100 ng/ml PDGF-BB for 5 min at 37 °C, rinsed with ice-cold phosphate-buffered saline (PBS), and then lysed in 1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM EDTA, 500 μM Na3VO4, 1% aprotinin (Trasylol; Bayer) and 1 mM phenylmethylsulfonyl fluoride (Sigma). Immunoprecipitation and immunoblotting were performed as described previously (9). For anti-Shc immunoprecipitation, the anti-Shc antibody was covalently coupled to protein A-Sepharose (11). The blots were reprobed after removal of the first probe by incubation in 5 mM sodium phosphate, pH 7.5, 2% SDS, and 2 mM 2-mercaptoethanol for 30 min at 60 °C.

In Vitro Association of the Autophosphorylated PDGF Receptor with GST Fusion Proteins and Peptide Inhibition Experiment—A fusion protein of GST and the SH2 domain of Grb7 was generated as described previously (16). A plasmid encoding a GST-Grb2 SH2 fusion protein was kindly provided by Dr. Tony Pawson, Mount Sinai Hospital, Toronto. The plasmid encoding GST-Src SH2 fusion protein was kindly provided by Dr. T. Yokote, University of North Carolina, Chapel Hill, NC) together with a fragment of Grb7 cDNA cut out from the AElox plasmid (19) with XbaI and SmaI sites. For the peptide inhibition experiment, the lysate was incubated with immobilized GST expressed in bacteria. The monoclonal anti-phosphotyrosine antibody PY20 and affinity-purified polyclonal anti-Shc antisera were from Affiniti Research Products Ltd. The monoclonal antibody against Grb2 was kindly provided by Dr. J. Schlessinger, New York University. Peroxidase-conjugated sheep anti-mouse IgG was from Amersham Corp. Peroxidase-conjugated swine anti-rabbit IgG was from Dakopatts.

**Results**

Grb7 Associates Directly with the PDGF β-Receptor—It has been shown that the SH2 domain of Grb7 associates with the autophosphorylated epidermal growth factor receptor and ErbB2 (16). We examined whether Grb7 can bind directly also to PDGF receptors via its SH2 domain. Porcine aortic endothelial (PAE) cells expressing wild-type PDGF α- or β-receptors were incubated with wheat germ lectin-Sepharose 6MB (Pharmacia) to enrich the glycoproteins. The samples were subjected to SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was incubated overnight at 4 °C in a blocking solution (20 mM HEPES, pH 7.5, 5% nonfat dry milk, and 0.02% sodium azide) and then probed for 1 h at room temperature in the same buffer using 1 μg/ml Grb7 SH2 domain. The fusion protein was detected by incubation of the membrane with anti-GST antisera (1:200) followed by incubation with peroxidase-conjugated swine anti-rabbit IgG. The membrane was washed four times for 15 min with a buffer containing 10 mM Tris pH 7.5, 150 mM NaCl, and 0.1% Triton X-100 before exposure to film.

**In Vitro Kinase Assay**—The breast cancer cell line BT-474 was incubated with or without 100 ng/ml PDGF-BB, and cells were then lysed and immunoprecipitation was performed with PDGFR-3 or PDGFR-7 antisera. The immunoprecipitated samples were subjected to kinase assays and analyzed by SDS-PAGE and autoradiography as described by Yokote et al. (9).

**Discussion**

Grb7 in PDGF Receptor Signaling

**Fig. 1.** Grb7 SH2 domain associates directly with the autophosphorylated PDGF β-receptor. PAE cells expressing the wild-type α- or β-receptors were stimulated (+) or not (−) with 100 ng/ml PDGF-BB, lysed, and then incubated with wheat germ lectin-Sepharose (WGA). The precipitated samples were separated by electrophoresis and transferred onto nitrocellulose membranes and probed using GST-Grb7 SH2 domain (top). The same blot was reprobed after stripping with anti-phosphotyrosine (anti-PY) antibody (center) and then with anti-PDGF α-receptor antisera R7 or with anti-PDGF β-receptor antisera R3 (bottom). The positions of the PDGF α-receptor (αR) and the β-receptor (βR) are indicated.
Table I

| Tyrosine residues | Surrounding amino acid sequences |
|-------------------|---------------------------------|
| Tyr-716           | A-E-L-Y-S-N-A                   |
| Tyr-771           | S-S-N-Y-M-A-P                   |
| Tyr-775           | M-A-P-Y-D-N-Y                   |
| Tyr-778           | Y-D-N-Y-V-P-S                   |
| Tyr-966           | G-E-G-Y-K-K-K                   |
| Tyr-970           | K-K-K-Y-Q-G-Q-V                 |
| Tyr-1009          | S-V-L-Y-T-A-V                   |

Stripped and immunoblotted with anti-phosphotyrosine antibodies (center) and then reprobed with anti-PDGFB or β-receptor antisera (bottom), which confirmed that ligand-induced autophosphorylation of the receptors occurred. These results suggest that the Grb7 SH2 domain directly binds to the autophosphorylated PDGF β-receptor but not to the PDGF α-receptor.

Grb7 Binds to Tyr-716 and Tyr-775 in the Autophosphorylated PDGF β-Receptor in Vitro—The intracellular domains of the PDGF β- and α-receptors are structurally homologous (8). Since the Grb7 SH2 domain binds to the autophosphorylated PDGF β-receptor but not to the α-receptor, we hypothesized that Grb7 associates with a phosphorylated tyrosine residue in the β-receptor which is not conserved in the α-receptor. The amino acid motifs surrounding phosphorylated tyrosine residues are known to determine the binding specificity for SH2 domains (7). Tyrosine residues 716, 771, 775, 778, 800, 966, 970, and 1009 in the β-receptor have different surrounding motifs compared to the tyrosine residues in the α-receptor (Table I). Therefore, PAE cells expressing various mutant PDGF β-receptors in which either 1 or 2 of these tyrosine residues were replaced with phenylalanine residues were tested for binding of the Grb7 SH2 domain. PAE cells expressing the wild-type or tyrosine residue-mutated PDGF β-receptors were stimulated or not with PDGF-BB and lysed. Ninety percent of the lysates were incubated with GST-Grb7 SH2 domain immobilized on glutathione-Sepharose and 10% with anti-receptor antisera. The precipitated materials were subjected to SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine (anti-PTyr) antibodies (A). In order to estimate the amount of each type of receptors expressed on PAE cells, immunoprecipitation with anti-receptor antisera on 10% of the lysate was performed followed by anti-phosphotyrosine (anti-PTyr) immunoblotting (B). The position of the autophosphorylated PDGF β-receptor is indicated (βR).

Table II

| Receptor                | Fraction of mutant receptor associated with the Grb7 SH2 domain as compared with the wild-type receptor fraction (% of control value) |
|-------------------------|-------------------------------------------------------------------------------------------------|
| Wild type               | 100                                                                                             |
| Y716F                   | 12                                                                                              |
| Y771F                   | 12                                                                                              |
| Y775F                   | 28                                                                                              |
| Y778F                   | 12                                                                                              |
| Y966F/Y970F             | 12                                                                                              |
| Y1009F                  | 28                                                                                              |

In vitro association of GST-Grb7 SH2 fusion protein with the wild-type or tyrosine residue-mutated PDGF β-receptors. PAE cells expressing the wild-type or tyrosine residue-mutated PDGF β-receptors were stimulated (+) or not (−) with 100 ng/ml PDGF-BB and lysed; 90% of the lysate was then incubated with 100 ng of GST-Grb7 SH2 domain immobilized on glutathione-Sepharose. The precipitated materials were subjected to SDS-PAGE and analyzed by immunoblotting using anti-phosphotyrosine (anti-PTyr) antibodies (A). In order to estimate the amount of each type of receptors expressed on PAE cells, immunoprecipitation with anti-receptor antisera on 10% of the lysate was performed followed by anti-phosphotyrosine (anti-PTyr) immunoblotting (B). The position of the autophosphorylated PDGF β-receptor is indicated (βR).
FIG. 3. Inhibition of association between GST-Grb7 SH2 domain and the autophosphorylated PDGF β-receptor by synthetic phosphorylated peptides. PAE cells expressing the wild-type or tyrosine residue-mutated PDGF β-receptors were stimulated (+) or not (−) with 100 ng/ml PDGF-BB, lysed, and then incubated with 100 ng of GST-Grb7 SH2 domain immobilized on glutathione-Sepharose for 1 h. Various concentrations of nonphosphorylated (Y716 and Y775) or tyrosine-phosphorylated (Yp716 and Yp775) synthetic peptides containing Tyr-716 or Tyr-775 of the PDGF β-receptor were added to the mixture, and the incubation was continued for another 2 h. The samples were washed, subjected to SDS-PAGE, and analyzed by immunoblotting with anti-phosphotyrosine (anti-PTyr) antibody (A). The position of the autophosphorylated PDGF β-receptor is indicated (βR). The density of the receptor bands was measured by densitometric scanning. The efficiency of inhibition by the phosphorylated peptides was calculated as the density of the receptor bands in the presence of the peptides compared to that in the presence of the same concentrations of the nonphosphorylated peptides (B). The effect of the phosphorylated peptides on GST-Grb2 SH2 binding to the PDGF β-receptors was examined in the same manner (C). The open circles and solid circles show the effects of the Yp716 and Yp775 peptides, respectively. Effect of the peptides derived from other autophosphorylation sites in the PDGF β-receptor on the binding of the wild-type β-receptor to Grb7 (D) or Grb2 (E) SH2 domains was examined in a similar manner. Effect of Yp716 and Yp775 peptides on complex formation between β-receptor and GST-Src SH2 domain was also examined (F). All of the peptides were added to the lysate/GST-SH2 mixture at a final concentration of 50 μM in the experiments shown in D, E, and F.
in Vivo—

Yp775 peptides (Fig. 3) ed by the Yp579 peptide but not influenced by either Yp716 or Grb7 (antibody). The positions of the PDGF receptors (ad, cR) and analyzed by immunoblotting with anti-phosphotyrosine (anti-PTyr) antibody. The positions of the PDGF receptors (ad, cR) are indicated. A 65-kDa tyrosine-phosphorylated protein is indicated by an arrow.

The cells were stimulated (+) or not (−) with 100 ng/ml PDGF-BB and then lysed. Ten percent of the lysate was immunoprecipitated with anti-receptor antisera (A). Ninety percent of the lysate was immunoprecipitated with anti-Grb2 SH2 domain or the Grb2 SH2 domain (EC50, 14 mM) than to the Grb2 SH2 domain (EC50, >20 μM; Fig. 3C). On the other hand, neither Yp579 peptide nor Yp1021 peptide inhibited binding of the Grb7 SH2 domain or the Grb2 SH2 domain to the autophosphorylated wild-type β-receptor at the concentration of 50 μM (Fig. 3, D and E). Binding of the autophosphorylated wild-type β-receptor to Src SH2 domain was specifically inhibited by the Yp579 peptide but not influenced by either Yp716 or Yp775 peptides (Fig. 3F).

Grb7 Associates with Autophosphorylated PDGF β-Receptor in Vivo—In order to examine in vivo association of Grb7 with PDGF receptors, PAE cells expressing wild-type or tyrosine residue-mutated PDGF α- or β-receptors were transiently transfected either with HA-tagged Grb7 cDNA in an eukaryotic expression vector or with the empty vector. The cells were treated or not with PDGF-BB, lysed, and immunoprecipitated with either anti-receptor antisera (10% of the lysate) or with anti-HA tag antisera (90% of the lysate). The samples were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibodies. Fig. 4A shows that both the wild-type and mutant receptors were expressed in COS cells at similar levels. As shown in Fig. 4B, the autophosphorylated wild-type PDGF β-receptor was coprecipitated with Grb7 after ligand stimulation. Compared to the wild-type receptor, considerably less of Y716F or Y775F PDGF β-receptors were co-precipitated with Grb7, which was visualized after longer exposure (data not shown). Unlike the wild-type PDGF β-receptor, no significant binding of the wild-type PDGF α-receptor to Grb7 was observed. Tyrosine-phosphorylated components of ~65 kDa were precipitated in the Grb7-transfected samples upon stimulation of both the wild-type and tyrosine residue-mutated PDGF α- and β-receptors, but not in the samples transfected with empty vector (Fig. 4B, arrow). The phosphorylated protein could not be detected by anti-Grb7 immunoblotting but comigrated with the 66-kDa form of Shc (data not shown).

Both the PDGF α- and β-Receptors Are Able to Induce Complex Formation between Shc and Grb7—It has been reported that Shc, which contains a tyrosine-phosphorylatable Y-X-N motif, binds Grb7 upon activation of the epidermal growth factor receptor (16). In order to examine the ability of the PDGF α- and β-receptors to induce complex formation between Shc and Grb7, PAE cells expressing either wild-type PDGF α-receptor or β-receptor were transiently transfected with Grb7 cDNA in an eukaryotic expression vector. The cells were serum-starved, stimulated or not with PDGF-BB, lysed, and immunoprecipitated with an anti-Shc antibody. The samples were subjected to SDS-PAGE and then immunoblotted with antibodies against Grb7, Shc, or Grb2. As shown in Fig. 5A, Grb7 (top) and Grb2 (bottom) were detected in the anti-Shc immunoprecipitates upon PDGF stimulation in PDGF α- or β-receptor-expressing cells. Thus, both the PDGF α- and β-receptors are capable of inducing complex formation between Shc and Grb7 in response to PDGF.

PDGF Stimulation of Breast Cancer Cells Induces Complex Formation between Grb7 and Shc—Certain types of breast cancer cell lines, such as BT-474 and SK-BR3, are known to overexpress Grb7 (16). In order to examine PDGF-induced Grb7 responses in these cells, anti-Shc immunoprecipitation was performed on PDGF-BB-stimulated BT-474 cell lysate. Human foreskin fibroblasts AG1518 expressing endogenous PDGF receptors but not Grb7 (data not shown) were also subjected to the same procedure. The samples were separated by SDS-PAGE, and immunoblotting was performed using anti-Grb7 antisera. As shown in Fig. 5B, top, a Grb7-Shc complex was detected in PDGF-BB-stimulated BT-474 cells but not in AG1518 cells or in unstimulated cells. After stripping, the same filter was blotted with anti-Shc antibody, which showed that equal amount of Shc proteins were immunoprecipitated from unstimulated and stimulated lysates (Fig. 5B, center). AG1518 cells showed expression of all three isoforms of Shc proteins (p46, p52, and p66), and BT-474 cells expressed p46 and p52 but not p66 (Fig. 5B, center). For comparison, the low molecular weight region of the same filter was immunblotted with an anti-Grb2 antibody, which revealed that Shc formed complex with Grb2 upon PDGF-BB stimulation in AG1518 cells. A Grb2-Shc complex was also seen in BT-474 cells, but it was constitutively formed and was not affected by ligand stimulation in these cells (Fig. 5B, bottom).

In order to confirm that BT-474 cells express functional PDGF receptors, an in vitro kinase assay on anti-receptor immunoprecipititates was performed (Fig. 6). Bands at the sizes of 170 and 85 kDa were detected in anti-PDGF α-receptor immunoprecipititates upon ligand stimulation but not in anti-PDGF β-receptor immunoprecipitates. It is likely that the 170-kDa band represents the PDGF α-receptor. However, we have failed to confirm this notion by detection of the α-receptor by anti-receptor immunoblotting, which is a less sensitive technique than an in vitro kinase assay. We speculated that the 85-kDa band represents the p85 subunit of PI3-kinase, as this SH2
domain protein is known to be a substrate for the PDGF receptors. Confirming this possibility, tyrosine phosphorylation of a protein of 85 kDa was detected when an antibody against p85 of PI3-kinase was used for immunoprecipitation of a lysate of BT-474 cells treated with PDGF-BB (data not shown). Thus, BT-474 cells express PDGF \( \alpha \)-receptors capable of initiating intracellular signaling; PDGF stimulation of these cells induces phosphorylation of Shc and formation of a complex between Shc and Grb7.

**DISCUSSION**

In this study, we demonstrate that Grb7 binds directly to Tyr-716 and Tyr-775 in the PDGF \( \beta \)-receptor upon ligand stimulation. Activation of PDGF \( \alpha \)- and \( \beta \)-receptors induced complex formation between Shc and Grb7.

Grb7 has been shown to associate with tyrosine-phosphorylated HER2 and with Shc after epidermal growth factor stimulation of the breast cancer cell line SK-BR3 (16). It is known that a major tyrosine phosphorylation site in Shc is Tyr-317, which has a Y-V-N motif, serving as a Grb2 binding site (23). Shc was shown to bind Grb7 with affinity lower than Grb2 (16). In our study, binding of Grb7 SH2 domain to the autophosphorylated PDGF \( \beta \)-receptor was dramatically reduced by replacing either Tyr-716 or Tyr-775 with a phenylalanine residue. Both these tyrosine residues have Y-V-L-N motifs, and Tyr-716 has been implicated as a Grb2 binding site in the PDGF \( \beta \)-receptor (21). We also observed that the Y778F mutant \( \beta \)-receptor showed increased binding of Grb7 SH2 domain. We have previously reported that phosphorylation of a tyrosine or a serine residue in the PDGF receptors can be increased by mutation of an adjacent tyrosine residue to a phenylalanine residue (11). In

**FIG. 5.** Complex formation between Shc and Grb7 upon activation of PDGF receptors. A, PAE cells expressing the wild-type PDGF \( \alpha \)- or \( \beta \)-receptors were transiently transfected with Grb7 cDNA in pMJ30 vector. Cells were stimulated (+) or not (-) with PDGF-BB, lysed, and then immunoprecipitated with anti-Shc antibody covalently coupled to protein A-Sepharose. The samples were analyzed by SDS-PAGE followed by immunoblotting using anti-Grb7 antiserum (top). The same blot was stripped and then reprobed with anti-Shc antibody (center). The lower molecular region of the blot was probed with anti-Grb2 antibody (bottom). The sizes of Grb7, Shc, and Grb2 are indicated.

**FIG. 6.** Ligand-induced kinase activation of the PDGF \( \alpha \)-receptor in BT-474 cells. The breast cancer cell line BT-474 was stimulated (+) or not (-) with PDGF-BB, lysed, and the lysate was immunoprecipitated with an antiserum against the PDGF \( \alpha \)-receptor (anti-\( \alpha \)R) or with an antiserum against the PDGF \( \beta \)-receptor (anti-\( \beta \)R). The samples were subjected to in vitro kinase assay, subjected to SDS-PAGE, and then examined by Bioimager BAS2000 (Fuji Film Ltd.). The positions of the PDGF \( \alpha \)-receptor (\( \alpha \)R) and a 85-kDa band (p85) are indicated.
In our peptide inhibition experiments, synthetic phosphopeptides containing phosphorylated Tyr-775 efficiently and specifically blocked the β-receptor binding to both Grb2 and Grb7 SH2 domains. However, the Tyr-716 phosphopeptide inhibited binding of the receptor to the Grb2 SH2 domain more efficiently than to the Grb7 SH2 domain. Thus, theoretically, phosphorylated Tyr-775 can both serve as an efficient binding site for Grb2 and Grb7 SH2 domains. However, the Tyr-716 phosphopeptide in the src SH2 domain is important for determination of its specificity regarding amino acid residues at positions +1 and +2 carboxyl-terminal of the phosphorylated tyrosine residue (24). As shown in Fig. 7, the amino acid residue predicted to contact the +2 amino acid in the phosphopeptide is identical for Grb2 and Grb7 SH2 domains (βD’1, leucine). However, the residues that serve to stabilize the +1 amino acid in the phosphopeptide are different between the SH2 domains of Grb2 (βD3, glutamine and βD5, phenylalanine) and Grb7 (βD3, lysine and βD5, tyrosine). Interestingly, the combination of lysine and tyrosine at βD3 and βD5 in the Grb7 SH2 domain is identical to that of the src SH2 domain, which is known to select an acidic residue at position +1 relative to the phosphorylated residue (25). This is consistent with our finding that the Grb7 SH2 domain appears to prefer Tyr-775 (Y-D-N) to Tyr-716 (Y-S-N). In addition, an amino acid residue at the EF1 position (the loop in between βE and βF) has been shown to contribute to the specificity of the SH2 domain by generating a pocket in which the amino acid residue in position +3 can bind (24, 26). The amino acid residues in the EF region are not conserved between the Grb2 and Grb7 SH2 domains (Fig. 7), which may also contribute to their different specificities. There is also the possibility that amino acid residues further downstream in the phosphopeptides or motifs amino-terminal of the phosphorylase may contribute to the affinity of the SH2 binding (27–29). Further investigation is required to understand why the mutation of either Tyr-716 or Tyr-775 dramatically decreased binding of the PDGF β-receptor to Grb7.

Grb7 coprecipitated a phosphorylated protein of about 65 kDa upon activation of the β-receptor as well as the β-receptor (Fig. 4). The protein comigrated with the 66-kDa Shc protein, which suggested that Grb7 forms complex with another adapter molecule, Shc, upon activation of both PDGF α-receptor and β-receptor. In experiments using PAE cells expressing the wild-type α- or β-receptors transiently transfected with Grb7 cDNA, both Grb2 and Grb7 formed complex with Shc after stimulation with ligand. However, in the case of the breast cancer cell line BT-474, the Grb2-Shc complex was constitutively formed, whereas the formation of the Grb7-Shc complex was dependent on PDGF stimulation (Fig. 5B). The Grb2-Shc complex seen in the absence of ligand stimulation is probably due to constitutive activation of HER2, which is overexpressed in this cell line (16). Grb2 has been shown to be able to compete with the binding of Grb7 to Shc, which suggests that Grb2 and Grb7 bind to the same site on Shc (16). Tyr-317 is known as a phosphorylation site in Shc that binds Grb2 (23). The reason for the PDGF dependence in Shc binding of Grb7 but not Grb2 is unknown. It is possible that Grb2 binds with higher affinity than Grb7 to Shc phosphorylated on Tyr-317 by HER2 and that Grb7 can bind to Shc only after the pool of phosphorylated Shc has been increased after PDGF stimulation. Alternatively, a tyrosine phosphorylation site other than the major phosphorylation site Tyr-317 may be present in Shc, which has a higher affinity for the Grb7 SH2 domain in vivo than for the Grb2 SH2 domain. It has been reported that Shc can be phosphorylated by activated c-Met even if Tyr-317 is replaced by a phenylalanine residue (23). We are currently investigating whether tyrosine residues other than Tyr-317 become phosphorylated in Shc upon activation of PDGF receptors.

BT-474 cells express a low amount of functional PDGF α-receptors. It has been reported that certain other epithelial cell lines such as HC-11 (30) or HepG2 cells express PDGF receptors (31). It would therefore be of interest to investigate further the role of Grb7 in PDGF receptor signaling pathways in these and other nonmesenchymal cells and tissues. Recently, the mammalian protein Grb10 and the C. elegans protein Mig-10, which are similar in structure to Shc, were reported (32, 33). Mig-10 is known to be important in mediating a signal for cell migration in C. elegans. Grb7, Grb10, and Mig-10 share a central domain referred to as GM (Grbs and Mig) domain. GM domain is implicated to be involved in protein/protein or protein/lipid interactions because it contains a PH domain (32). Characterization of interactions of the proline-rich region and the GM domain in Grb7 may clarify the role of Grb7 in different signaling pathways.

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