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Expression and purification of functional PDGF receptor beta

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ABSTRACT

Platelet Derived Growth Factor receptors (PDGFRs), members of receptor tyrosine kinase superfamily, play essential roles in early hematopoiesis, angiogenesis and organ development. Dysregulation of PDGF receptor signaling under pathological conditions associates with cancers, vascular diseases, and fibrotic diseases. Therefore, they are attractive targets in drug development. Like any other membrane proteins with a single-pass transmembrane domain, the high-resolution structural information of the full-length PDGF receptors is still not resolved. It is caused, at least in part, by the technical challenges in the expression and purification of the functional, full-length PDGF receptors. Herein, we reported our experimental details in expression and purification of the full-length PDGFRβ from mammalian cells. We found that purified PDGFRβ remained in two different oligomeric states, presumably the monomer and the dimer, with basal kinase activity in detergent micelles. Addition of PDGF-B promoted dimerization and elevated kinase activity of the receptor, suggesting that purified receptors were functional.

1. Introduction

Platelet Derived Growth Factor (PDGF) receptors, including the PDGFRα and PDGFRβ, play a pivotal role under physiology and pathology conditions. They regulate early hematopoiesis, angiogenesis and the development of multiple organs. Dysregulation of PDGF receptor signaling, by genetic alteration or abnormal activation, associates with various human diseases, including cancers, vascular diseases, and fibrotic diseases [1–5]. Hence, they are attractive therapeutic targets.

PDGF receptors, together with Colony Stimulating Factor-1 Receptor (CSF1R), Stem Cell Factor Receptor (c-Kit) and Fms Like Tyrosine Kinase 3 (FLT3), belong to the Class III receptor tyrosine kinase subfamily. They contain an extracellular domain, which is composed of five immunoglobulin-like (Ig) domains, for ligand recognition (Fig. 1A and B). Linked to the extracellular domain, is the receptor single-pass transmembrane domain, followed by its intracellular juxtamembrane domain, kinase domain, and a long, disordered C-terminal tail (Fig. 1A and B). Specialized to the Class III receptor tyrosine kinases, a structurally uncharacterized insert splits the kinase domain into two separate fragments in sequence (Fig. 1B). At the receptor kinase insert, the intracellular juxtamembrane domain, and the C-terminal tail, there are multiple tyrosine phosphorylation sites [6–10]. These sites can be transphosphorylated by the receptor upon stimulation, and then serve as regulatory sites for kinase activation or docking sites for binding downstream signaling molecules (Fig. 1) [6–10].

Four different PDGF growth factors had been identified in human. They are named from PDGF-A to PDGF-D. These growth factors can form homodimers as well as heterodimers. Different PDGF growth factor dimers are recognized by their receptors in a pleiotropic manner [11].

The activation of PDGF receptor kinase requires the dimerization of the receptor and the transphosphorylation of its regulatory tyrosines at the juxtamembrane or the kinase activation loop (Fig. 1A) [12]. In the absence of ligand, the PDGF receptor remains in an inactive state, in which the receptor juxtamembrane domain is folded upon the substrate binding site of its kinase and blocks the access of its substrates [13–15]. The stimulation of PDGF receptor by dimeric PDGF growth factors leads to the proximity of PDGF receptor extracellular domains, which, in turn, causes kinase transphosphorylation at regulatory sites through the lipid

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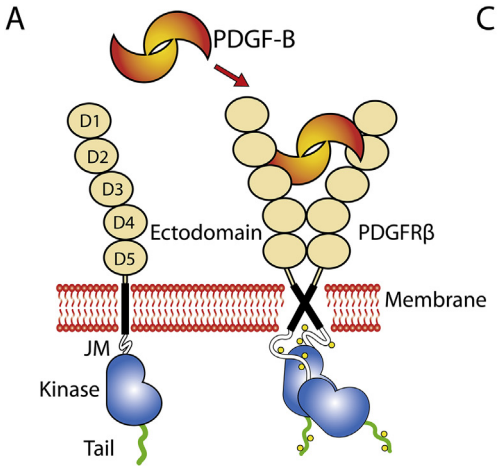

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Glycosylation sites	Autophosphorylation sites
N45	Y562
N89	Y579
N103	Y581
N215	Y716
N230	Y740
N292	Y751
N307	Y763
N354	Y771
N371	Y775
N468	Y778
N479	Y857
	Y1009
	Y1021

Fig. 1. Diagram of PDGFRβ Structure and Activation. A) The Binding of dimeric growth factor, PDGF-B, triggers the dimerization and kinase activation of PDGFRβ. The ectodomain of PDGFRβ, which is required for ligand recognition, is composed of five Ig-like domains, denoted as D1 to D5. Multiple autophosphorylation sites at the intracellular side of the PDGF receptor, marked in yellow circles, serve as regulatory sites in the control of kinase activities or as docking sites in binding downstream signaling molecules. The kinases of liganded PDGFR dimer were believed to be activated by transphosphorylation of intracellular regulatory tyrosines upon dimerization. B) The primary structure of PDGFRβ. TM, transmembrane domain; JM, juxtamembrane domain; KD1, kinase domain 1; KI, kinase insert; KD2, kinase domain 2. Glycosylation sites were red hexagons and autophosphorylation sites were yellow circles. These sites were listed in panel C.

membrane. As such, the autoinhibitory interactions between the receptor juxtamembrane and kinase domain are released, and the catalytic activities of the kinases are enhanced [12,16]. Through a series of catalytic cascades, the signaling is further propagated to the nucleus to regulate cell growth, differentiation, and migration.

Although the functional significance of PDGF receptors had been well established, the structural information of the full-length receptor, especially those regarding the signaling across its single-pass transmembrane, was still not fully resolved. To date, the crystallographic structures of PDGFRβ ectodomain in complex with PDGF-B and PDGFRα kinase domain had been reported [15,17]. These structures provided atomic details for understanding the receptor ligand recognition and kinase autoinhibition. In addition, a low-resolution negative-stain Electron Microscopy (EM) structure of PDGFRβ in complex with PDGF-B had been determined by using Gradient Ultracentrifugation and Fixation (GraFix) technique, which used chemical cross-linkers to stabilize the receptor complex [18]. In order to elucidate the molecular mechanisms for transmembrane signaling of the PDGF receptors, however, it necessitates the purification of the full-length receptors in their functional states.

Herein, we reported our experimental results in expression and purification of the full-length PDGFRβ from mammalian cells. We found that unliganded PDGFRβ remained in two different oligomeric states, presumably the monomer and the dimer, with basal kinase activity in detergent micelles. Addition of PDGF-B promoted dimerization of the receptor and elevation of kinase activity, suggesting that the purified receptors were functional.

2. Materials and methods

2.1. Materials

The cDNA of PDGFRβ was kindly provided by Dr. Jiahuai Han at Xiamen University. The original pEF1-puro plasmid was a gift from Dr. Timothy A. Springer (Addgene plasmid #59973). Restriction enzymes and Gibson assembly kit were purchased from NEB (MA, USA), and DNA Polymerases were from Vazyme (China). Recombinant human PDGF-B from ACRO Biosystems (USA) was reconstituted in water at a final concentration of 4 μM and stored at −20 °C. Strep-tactin resin was purchased from IBA (Germany). Antibodies to protein C and to phosphotyrosine (clone 4G10) were from Genscript (China) and Merck Millipore (USA), respectively.

2.2. Cloning of PDGFRβ into pEF1-puro plasmid

The gene coding for human PDGFRβ$_{33\text{-}1106}$ was amplified by PCR from cDNA of PDGFRβ with the following primers: 5'-CTGGGGTTGCTCTTGCTCTGGTTTCCTGGCTCGA-GATGCCTGGTCGTCACACCCCCGGG-3' (forward); and 5'-TTGATCTT-CAACTGGACCTTGGAAGTA-CAGTTCACGCGTCAGGAAGCTATCCTCTGCTT-3' (reverse). Then, the PDGFRβ gene was subcloned into a bicistronic expression vector modified from the pEF1-puro vector. In the bicistronic vector, an Internal Ribosome Entry Site (IRES) and the EGFP gene were added to the 3' end of the PDGFRβ gene by Gibson cloning. For enhanced expression of PDGFRβ, the signal peptide of human PDGFRβ was replaced with murine immunoglobulin κ chain signal peptide. In addition, tandem tags were fused to the C-terminus of PDGFRβ. This construct was verified by Sanger sequencing.

2.3. Mammalian cell culture and transfection

HEK293T and BHK-21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS in humidified atmosphere with 5% CO_2. These cells seeded in 6-well plates were transiently transfected at 65–75% confluency with 5 μg plasmid of PDGFRβ per well using 25 kDa linear polyethylenimine (PEI). The

DNA to PEI ratios in transfection of HEK293T and BHK-21 cells were 1:10 (w/w) and 1:2, respectively. At 5 h posttransfection, the culture medium for HEK293T was changed to ExCell 293 serum free medium (SAFC Biosciences) supplemented with 6 mM Glutamine, and the medium for BHK-21 was changed to BHK-SFM (Neuronbc). The transfection efficiencies were evaluated at 2 days post-transfection from the emitted fluorescence of co-expressed EGFP.

To compare the expression levels of PDGFRβ from HEK293T and BHK-21 cells, the transfected cells were lysed with 300 μL/well Radioimmunoprecipitation assay(RIPA) buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM Na_3VO_4, 10 μM Sunitinib, 2 mM PMSF, 2× Complete protease inhibitor cocktail) at 3 days posttransfection, and the lysates were cleared by centrifugation at 13,200*g* for 10 min. The supernatants were mixed with 6× SDS loading buffer (0.35 M Tris-HCl, pH 6.8, 10.28% (w/v) SDS, 36% (v/v) glycerol, 5% β-mercaptoethanol, 0.012% (w/v) bromophenol blue), and were subjected to reducing SDS-PAGE electrophoresis. The expressed PDGFRβ was detected by Western blotting, using anti-protein C antibody.

HEK293F cells adapted for suspension culture were used for large-scale transient transfection. HEK 293F cells were cultured in 2L conical flasks filled with 1L SMM-293Ti medium per flask rotating at 120 rpm/min in 5% CO_2 incubator. Cells were transfected at the density of 1.5 × 10^6 cells/mL with every 1 mg plasmids per litter culture medium, which were mixed and incubated with 3 folds of PEI at room temperature for 15 min. Transfected cells were harvested at 3 days posttransfection and stored at −80 °C.

2.4. Cell-based kinase activity assay

HEK293T cells in 6-well plates were transfected at 65%–75% confluency with 1.4 μg DNA per well using 14 μg/well PEI. After 5 h, cells were starved in DMEM for 24 h. Transfected cells were treated with or without 4 nM PDGF-B in 2 mL/well DMEM for 20 min at 37 °C. Then, the medium were removed, and cells were lysed in 300 μL/well RIPA buffer. The lysed cells were cleared by centrifugation at 13,200*g* for 10 min, and the supernatants were analyzed by reducing SDS-PAGE and Western blotting. The phosphorylated and totally-expressed PDGFRβ were detected with 4G10 and protein C antibodies, respectively.

2.5. Purification of PDGFRβ

The frozen pellets of transfected HEK293F cells were resuspended in lysis buffer (50 mL/10 g cell pellets; 20 mM Tris, pH 8.0, 400 mM NaCl, 1 mM EDTA, 10% glycerol, 0.15% Triton X-100, 100 μM Na_3VO_4, 0.5 mM TCEP, 1× Complete protease inhibitor cocktail), and then lysed at 4 °C for 1 h. The lysed cells were cleared by centrifugation at 50,000*g* for 30 min at 4 °C, and then the supernatant was loaded onto a strep-tactin column pre-equilibrated with 5 column volumes (CVs) of wash buffer (20 mM Tris, pH8.0, 400 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 0.5 mM TCEP, 2 mM PMSF). The column was washed with 25 CVs of wash buffer, and bound proteins were eluted from the column with 5 mM desthiobiotin in wash buffer. The eluates were collected at 0.5 CVs each for seven consecutive fractions.

The wash and eluates were analyzed by Coomassie-stain SDS-PAGE. The eluates containing purified PDGFRβ were divided into aliquots, flash-frozen in liquid nitrogen, and stored at −80 °C.

2.6. In vitro kinase assay

Purified PDGFRβ in aliquots were treated with or without 1.6 μM PDGF-B in 40 μL of reaction buffer (20 mM Tris, pH 8.0, 400 mM NaCl, 10% glycerol, 2 mM $MnCl_2$, 0.1% Triton X-100, 0.5 mM TCEP) on ice for 30 min. Kinase phosphorylation was initiated by adding 20 μM ATP on ice. After 5 min, the reactions were terminated by adding 10 μL6× SDS loading buffer into each sample. The total and phosphorylated PDGFRβ were detected by Western blotting with protein C and 4G10 antibodies, respectively.

2.7. Negative-stain electron microscopy

30 μL purified PDGFRβ were treated with or without 2 μM PDGF-B on ice for 30 min. Then, the samples were loaded onto a Superose 6 Increase 3.2/300 (GE Healthcare) pre-equilibrated with 0.2 mM Dodecyl Maltoside (DDM). The running buffer for gel filtration chromatograph contains 20 mM Tris, pH 8.0, 200 mM NaCl, 0.2 mM DDM, and 0.5 mM TCEP. The peak fractions corresponding to the ligand-free and ligand-bound PDGFRβs were collected, and were immediately stained with uranyl acetate on glow-discharged thin-carbon-coated grids. Images of stained samples were collected using a Gatan US4000 CCD camera at a nominal magnification of 78,000× on an FEI F20 TEM operated at 200 kV.

3. Results

3.1. Design of a bicistronic vector for expression of PDGFRβ

As human PDGFRβ has multiple glycosylation and phosphorylation sites (Fig. 1C), we decided to express and purify functional PDGFRβ from mammalian cells. To that end, a bicistronic vector was designed to co-express PDGFRβ with EGFP (Fig. 2A). In this design, the transcription of PDGFRβ and EGFP were driven by the same EF-1α promoter, but these two genes were separated by an Internal Ribosome Entry Site (IRES). This design enabled us to monitor the transient transfection efficiency and the transcription of PDGFRβ from coexpressed EGFP using fluorescent microscopy or flowcytometry. To facilitate the purification and detection process, tandem affinity tags and a protein C antibody recognition site were appended to the C-terminus of PDGFRβ in this construct (Fig. 2A).

3.2. Expression of PDGFRβ in mammalian cells

For comparison, PDGFRβ was transiently transfected and expressed in two different mammalian cell lines: HEK293T and BHK-21. HEK293T was widely used in the expression of exogenous proteins from mammals, while BHK-21, a fibroblast cell line, was chosen for that endogenous PDGFRβ was reported to be selectively expressed in fibroblast [19,20].

The transfection efficiencies of PDGFRβ into these two cell lines were comparable, as detected by the green fluorescence of co-expressed EGFP using fluorescent microscopy (Fig. 2B and C). However, the expression level of PDGFRβ in BHK-21 cells was much lower than that in HEK293T cells (Fig. 2D). In addition, the patterns of post translational modification of PDGFRβ in these two transfected cell lines were different (Fig. 2D). From transfected HEK293T cells, three bands at molecular weights of 210 kDa, 191 kDa and 173 kDa were reproducibly detected by western blotting using a specific anti-protein C antibody. In contrast, only two bands at 210 kDa and 185 kDa, were detected from transfected BHK-21 cells. The difference in the pattern might arise from the difference of expressed PDGFRβ in glycosylation, phosphorylation, or degradation. Nevertheless, HEK293T cell was selected in the following experiments for its high expression level of PDGFRβ.

The functionality of expressed PDGFRβ was verified in cell-based experiments. As shown by western-blotting experiments using *anti*-phosphotyrosine antibody 4G10, the addition of 4 nM PDGF-B to transiently transfected, starved HEK293T cells at 37 °C

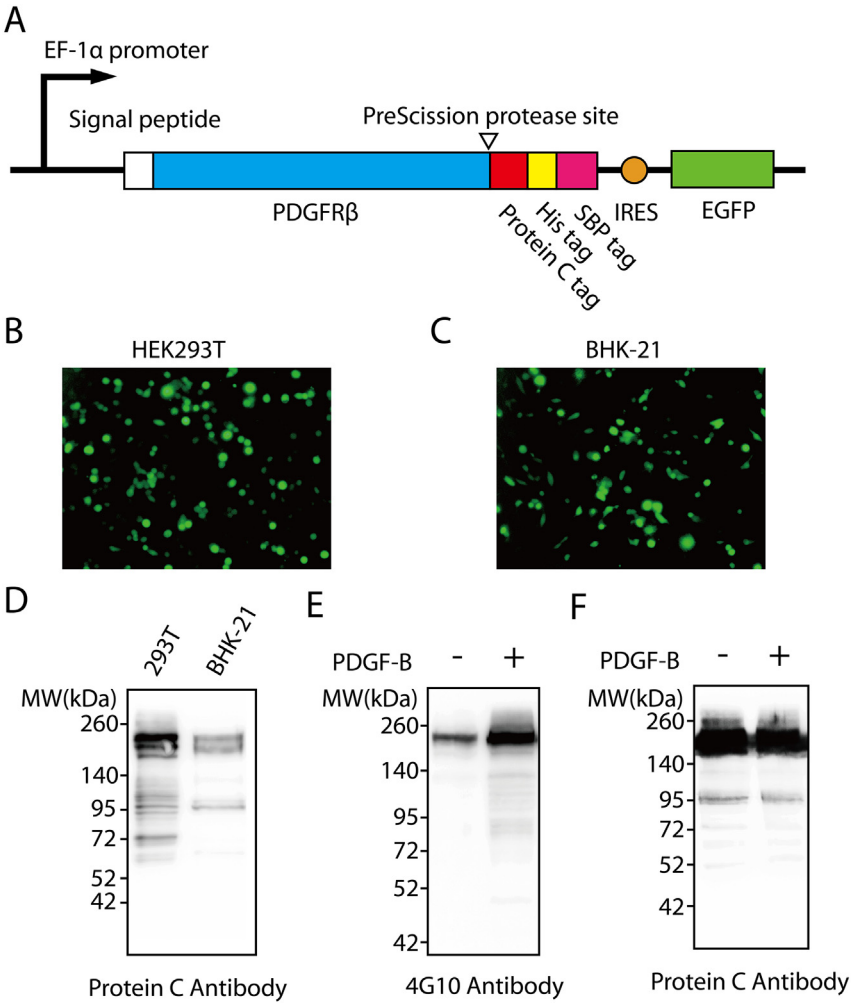

Fig. 2. Expression of PDGFRβ in Mammalian cells. A) Schematic diagram of PDGFRβ expression vector. The transcription of PDGFRβ and EGFP were driven by the EF-1α promoter, as an IRES was inserted between these two genes. A PreScission protease site followed by a protein C tag, a His tag, and a SBP tag was added to the C-terminus of PDGFRβ. The signal peptide of PDGFRβ was replaced with the one from murine immunoglobulin κ chain. B, C) Fluorescence microscopy of transfected HEK293T (B) and BHK-21 cells (C). These cells were transiently transfected with PDGFRβ plasmids, and the green fluorescence from co-expressed EGFP was monitored at 2 days posttransfection for evaluation of transfection efficiency. D) The expression levels of PDGFRβ from transfected HEK293T and BHK-21 cells were compared by Western blotting with a specific protein C antibody. E, F) The activities of expressed PDGFRβ. Transiently transfected HEK 293T cells were starved overnight, treated with or without 4 nM PDGF-B at 37 °C for 20 min. And then the phosphorylated and totally-expressed PDGFRβ were blotted with phosphotyrosine antibody 4G10 (E) or protein C antibody (F), respectively.

for 20 min stimulated the autophosphorylation of PDGFRβ (Fig. 2E and F). This experiment validated the biological activities of expressed PDGFRβ, and suggested that adding multiple tags to the C-terminus of PDGFRβ had little, if any, effect on PDGFRβ functions.

Then, the expression of PDGFRβ was scaled up using HEK293F cells, which were adapted for suspension culture.

3.3. *Purification of functional PDGFRβ from transiently transfected HEK293F cells*

Two technical challenges need to be resolved in the purification of functional PDGFRβ. One was the selection of a suitable detergent in solubilization and purification of PDGFRβ from lipid membrane. The detergent should be able to solubilize the PDGFRβ in a monodispersed state while it should keep the receptor activities for ligand-binding and kinase activation. Triton X-100 was selected and tested in our experiments as it was successfully applied in the purification of Epidermal Growth Factor Receptor (EGFR) and in functional studies of PDGFR [21,22]. The other challenge was the instability of type I membrane proteins in detergent micelles. For example, the half-life of EGFR activity in 0.2 mM DDM is only 3 h [21]. That demands the shortening of the purification process. Benefited from the high affinity and specificity of SBP tag to strep-tactin resin, we were able to finish the affinity purification of PDGFRβ within 5 h starting from the solubilization of transfected cells.

Through a single strep-tactin affinity purification, PDGFRβ was purified to over 80% purity as judged by Coomassie-stain SDS-PAGE (Fig. 3A). In addition, the purified receptor was monodispersed in gel-filtration chromatography (Fig. 3B). Only a single, symmetric peak with apparent molecular weight of 613 kDa was detected in gel-filtration chromatography using a Superose 6 Increase 3.2/300 column equilibrated with 0.2 mM DDM. Treatment of the sample with 2 μM PDGF-B on ice for 30 min shifted the gel-filtration chromatography profile toward to a higher apparent molecular weight of 1493 kDa, indicating the formation of ligand-bound receptor oligomers (Fig. 3B).

The activity of purified PDGFRβ was characterized by western blotting experiments. Trace, but noticeable, amount of purified receptors was phosphorylated in the absence of ATP and Mn^{2+}

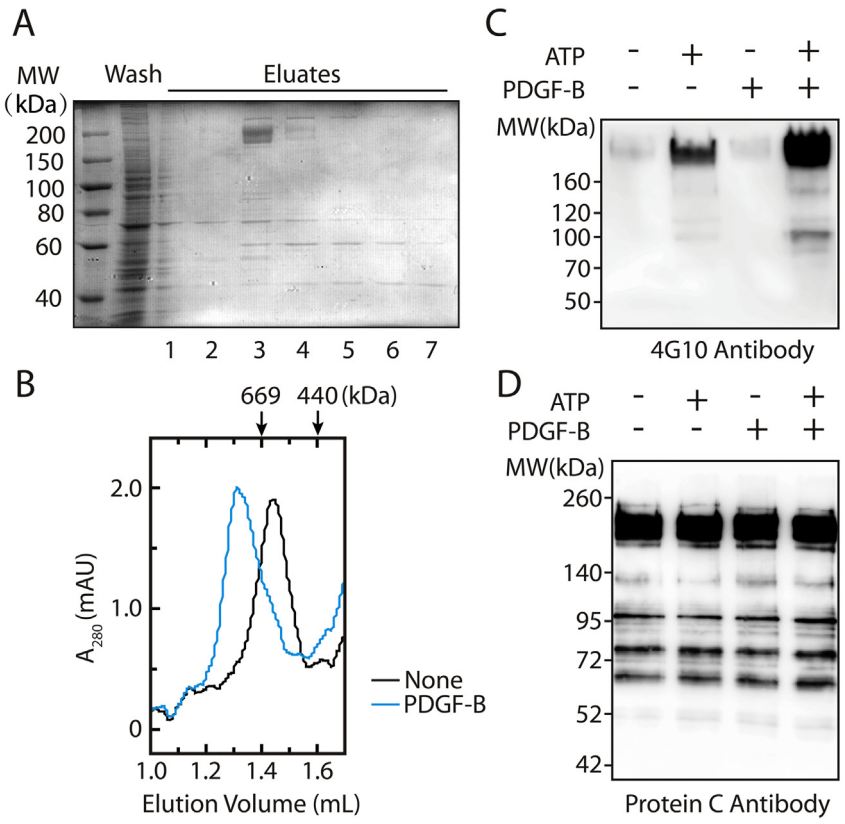

Fig. 3. The purification of functional PDGFRβ. A) Coomassie-stain SDS-PAGE of strep-tactin affinity- purified PDGFRβ. B) The gel-filtration profiles of affinity purified PDGFRβ alone or in complex with 2 μM PDGF-B. C, D) The activities of affinity-purified PDGFRβ. Purified samples were treated with or without 1.6 μM PDGF-B on ice for 30 min. The activities of ligand treated and untreated samples were compared in the presence or absence of 20 μM ATP and 2 mM Mn^{2+}. The phosphorylated and total PDGFRβ were detected with 4G10 (C) and protein C antibodies (D), respectively.

(Fig. 3C and D). However, the basal activity of unliganded PDGFRβ was readily detectable in the presence of ATP and Mn^{2+} using *anti*-phosphotyrosine antibody 4G10 (Fig. 3C and D). Adding 1.6 μM PDGF-B to purified receptors on ice for 30 min could further stimulate the phosphorylation of the receptor over its basal level by over 3 folds (Fig. 3C and D). These results indicated that purified receptors were functional and suitable for structural studies.

3.4. Characterization of purified PDGFRβ by negative-stain EM

The negative-stain EM images of PDGFRβ were visualized in the presence and absence of PDGF-B (Fig. 4). Both samples were heterogeneous. In the absence of the ligand, two different-sized particles coexisted (Fig. 4A). One of them was nearly doubled in size of the other. They were assumed to be the dimeric and monomeric PDGFRβ. The monomeric PDGFRβ exhibited an elongated shape, while the dimeric particles were broadened along the short axis of the monomer. At current resolution, however, it was challenge to point out the extracellular domain from the intracellular kinase of the receptor. The binding of PDGF-B promoted the dimerization of PDGFRβ, as more dimeric particles were identified (Fig. 4B). However, attempts of 2D classification of these particles failed to reveal enough defined features. The details of PDGFR dimeric interactions and the molecular mechanisms for the receptor transmembrane signaling need to be addressed in future studies.

4. Discussion

The purification of functional membrane proteins, especially those with a single-pass transmembrane domain, is not a trivial task. In contrast to thousands of high resolution structures obtained for membrane proteins with multi-pass transmembrane domains, to our knowledge, none of high resolution structure for any membrane proteins with a single-pass transmembrane domain had been determined [23]. This may reflect the instable nature of the single-pass transmembrane helix and its flanking region in the absence of membrane contacts [23,24]. For example, the half-lives of purified EGFR activity in different detergent conditions varied from 0.5 to 4 h [21]. But the EGFR reconstituted in lipid vesicles or nanodiscs was functionally stable for over 24 h [21]. PDGFRβ is another example. Due to the flexibility or instability of the full-length receptor, ligand-bound PDGFRβ had to be fixed by using GraFix technique in early negative-stain EM studies [18]. GraFix technique, however, is incompatible with the requirements for determination of high-resolution structure of the full-length PDGFRβ. In this report, we provided details for purification of functional, full-length PDGFRβ from transiently transfected mammalian cells, and characterized the purified receptors by negative-stain EM. These information provided a starting point for future functional and structural studies of the PDGFRβ transmembrane signaling.

The purified PDGFRβ receptor was functional as evidenced by ligand-induced dimerization in gel-filtration chromatography and kinase activation in phosphorylation assays (Fig. 3). In the expression and purification, we considered several factors that could potentially impact the functional or structural stability of the receptor. These include the mild detergents, high affinity tags, and shortening of purification process. In addition, the activity of the receptor was followed at every step in the expression and purification process. Certainly, it would be better to reconstitute the

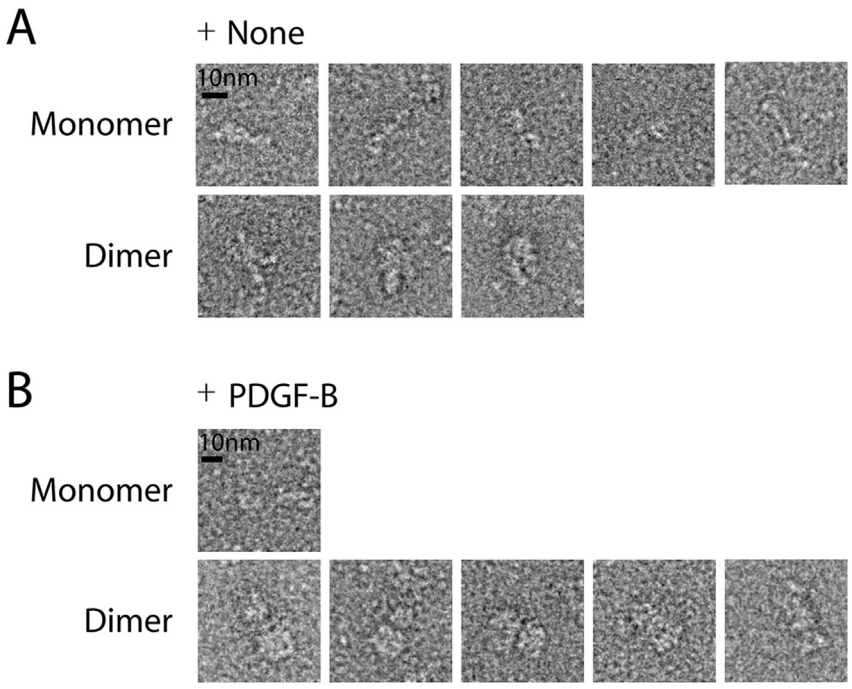

Fig. 4. Negative-stain EM of purified PDGFRβ. A, B) Representative images of PDGFRβ in the absence (A) or presence (B) of PDGF-B treatment. The strep-tactin-affinity purified PDGFRβ was treated with or without 2 μM PDGF-B on ice for 30 min. Then the samples were fractionated using Superose 6 Increase 3.2/300 column equilibrated with 0.2 mM DDM. The peak fractions of ligand-free and ligand-bound PDGFRβ were collected, stained with uranyl acetate, and visualized under FEI F20 microscope.

purified receptors into lipid environment for further stabilization of the receptor functions [21].

Although our results were compatible with ligand induced dimerization in the activation of the PDGFRβ receptor [25], we did visualize some dimers in the absence of PDGF-B treatment by negative-stain EM (Fig. 4A). These dimers were presumably unliganded and inactive, as transfected cells were starved for over 48 h and only basal activity was detected from the purified receptors. Indeed, it had been reported that preexisting PDGFRβ oligomers could be detected on cell surface by image correlation spectroscopy [26]. Therefore, we tempted not to exclude the possibility that those unliganded dimers could be activated, presumably by a different mechanism.

Author contributions

QS, SFS, and LZM designed the experiments. QS and MW carried out the biochemical studies. LZ and XW performed the EM experiments. QS, LZ, SFS, and LZM analyzed the data and wrote the paper.

Conflict of interest

The authors declare no conflict of interest in this study.

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