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RESEARCH ARTICLE

PDGF-induced internalisation promotes proteolytic cleavage of PDGFRβ in mesenchymal cells

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ABSTRACT

Platelet-derived growth factor (PDGF)-induced signalling via PDGF receptor β (PDGFRβ) leads to activation of downstream signalling pathways which regulate multiple cellular responses. It is unclear how PDGFRβ is degraded; both lysosomal and proteasomal degradation have been suggested. In this study, we have characterised the proteolytic cleavage of ligand-activated PDGFRβ, which results in two fragments: a larger fragment containing the extracellular domain, the transmembrane segment, and a part of the intracellular juxtamembrane region with a molecular mass of ~130 kDa, and an intracellular ~70 kDa fragment released into the cytoplasm. The proteolytic processing did not take place without internalisation of PDGFRβ. In addition, chelation of intracellular Ca^{2+} inhibited proteolytic processing. Inhibition of the proteasome affected signal transduction by increasing the phosphorylation of PDGFRβ, PLCγ, and STAT3 while reducing it on Erk1/2 and not affecting Akt. The proteolytic cleavage was observed in fibroblasts or cells that had undergone epithelial-mesenchymal transition.

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PDGFR; bortezomib; proteasome; proteolysis; cleavage; RTK

Introduction

Platelet-derived growth factor receptors (PDGFRs) belong to the receptor tyrosine kinase (RTK) family and exist as two isoforms, PDGFRα and PDGFRβ, with different preferences for binding of the five dimeric ligands, i.e. PDGF-AA, -BB, -CC, -DD and -AB (Heldin, Lennartsson, and Westermark 2018). Upon ligand binding, PDGFRβ dimerises into homo- and heterodimers, depending on the ligand isoform, and autophosphorylation occurs at conserved tyrosine residues throughout the intracellular domain. Thereby, docking sites for SH2-domain-containing signalling proteins are created that lead to activation of, among others, phospholipase Cγ1 (PLCγ), signal transducer and activator of transcription (STAT), phosphatidylinositol 3′-kinase (PI3K)/Akt, Src and Erk1/2 mitogen-activated protein kinase (MAPK) signalling pathways. Activated PDGFR often promotes cell migration, proliferation, survival and in some cases differentiation (Heldin and Lennartsson 2013). PDGFRβ is expressed primarily in cells of mesenchymal origin, such as fibroblasts, pericytes and vascular smooth muscle cells, and dysregulation of PDGFRβ is linked to diseases associated with over-proliferation, such as cancers, fibrotic conditions and vascular disorders (Heldin, Lennartsson, and Westermark 2018; Heldin and Lennartsson 2013).

Following ligand-induced activation, PDGFRβ initially translocates from the cell surface to early endosomes, mainly via clathrin-mediated endocytosis (Wåhlén et al. 2023), and then further to multivesicular bodies and late endosomes, followed by lysosomal degradation (Goh and Sorkin 2013). As PDGFRβ is transported through the different subcellular compartments, it engages with signalling proteins at different locations (Von Zastrow and Sorkin 2021). Internalisation and the sorting process are promoted by PDGFRβ ubiquitination

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primarily by members of the Cbl family of E3 ubiquitin ligases (Miyake et al. 1999; Rorsman et al. 2016). At the same time, basal levels of PDGFRβ are controlled by the TRIM21 ubiquitin ligase (Sarri et al. 2023). In addition, proteasomes have been suggested to be involved in the degradation of PDGFRβ (Rorsman et al. 2016).

It has been observed that some receptor tyrosine kinases (RTKs) undergo proteolytic cleavage in response to ligand stimulation. In some cases, the extracellular domain is shed, which may sequester ligands, thus interfering with ligand-induced activation of full-length receptors. Such a process has been shown for the RTKs c-Kit, where the cleavage is performed by the protease TACE (ADAM17) (Cruz et al. 2004), epidermal growth factor receptor (EGFR) cleaved by the serine protease prostasin (Chen et al. 2008), vascular endothelial growth factor receptor 2 (VEGFR2) by matrix metalloproteases (Basagiannis and Christoforidis 2016), and PDGFRβ by ADAM10 (Mendelson et al. 2010). In some cases, ectodomain shedding is further coupled to regulated intramembrane proteolysis where integral membrane proteins are subject to intramembrane cleavage, often by γ-secretase, as in the case of Notch (Selkoe and Kopan 2003) and several RTKs, including ErbB4 and the CSF1R, where the resulting intracellular fragments then translocate to the nucleus (Ni et al. 2001; Wilhelmsen and van der Geer 2004). VEGFR2 has also been reported to be cleaved in its intracellular domain to form an intracellular fragment (Bruns et al. 2010; Ewan et al. 2006; Smith et al. 2016), the significance of which is not yet clarified.

In the present study, we have characterised the proteolytic cleavage of ligand-stimulated PDGFRβ. We found that a cleavage occurs between amino acid residue numbers 581 and 857, that the larger transmembrane fragment is phosphorylated and may participate in signal transduction, and that the cleavage requires the PDGFRβ to be internalised. Furthermore, this proteolytic processing was observed in mesenchymal cells.

Material & methods

Reagents and antibodies

Table S1 lists the reagents and antibodies, as well as the providers and concentrations at which they were used in this study.

Cell culture

Immortalised human foreskin fibroblasts Bj-hTERT were cultured in Dulbecco's Modified Eagle's Medium (DMEM) + Glutamax, supplemented with 10% foetal bovine serum (FBS) at 37 °C in a 5% CO_2 humidified atmosphere. For serum starvation, Bj-hTERT cells were incubated overnight in the same medium containing 0.2% FBS. Human primary foreskin fibroblasts AG01523 were cultured in Minimum Essential Medium Eagle (Sigma-Aldrich, M5650), supplemented with 2 mM of L-glutamine and 10% FBS, at 37 °C in a 5% CO_2 humidified atmosphere. For serum starvation, AG01523 cells were starved overnight in MEM medium, supplemented with 0.1% FBS and 2 mM L-glutamine.

Generation of cell lines

The pPB[Exp]-EGFP/Neo-CMV > {PDGFRB-BIOID} plasmid (Vectorbuilder) was designed to express PDGFRβ with the C-terminal fused to N-terminal of the sequence from miniTurbo from Addgene plasmid #107172 (Branon et al. 2018) from the Mammalian Gene Expression PiggyBac Vector backbone with EGFP/Neomycin marker and the CMV promoter. To create a HaCaT cell line stably expressing the PDGFRB-BIOID construct, HaCaT cells seeded at a density of 35,000 cells/cm^2 in 24-well plates, grown to 80% confluency, and co-transfected with 1500 ng/well pPB[Exp]-EGFP/Neo-CMV > {PDGFRB-BIOID} (Vectorbuilder) and pRP[Exp]-mCherry-CAG > hyPBase (Vectorbuilder) in 3:1, 1:1 and 3:1 molar ratios using Lipofectamine 3000 (Thermo Fischer), as indicated by the manufacturer. After 48 hours, the medium was changed to a selection medium consisting of DMEM, 10% FBS, and 600 µg/ml G418 (Thermo Fischer), and cells were cultured in the selection medium for at least 10 days until all nontransfected cells had died. Polyclonal HaCaT-PDGFRβ-BioID were subjected to cell sorting based on EGFP expression using FACSAriaIII (BD) and EGFP-positive individual cells were cultured until confluency and tested for expression of the fusion construct PDGFRβ-BioID with immunoblotting, as described below.

Treatment and stimulation of cells

Bj-hTERT cells were seeded in 6-well plates at a density of 50,000 cells/cm^2 and left to adhere overnight. AG01523 cells were seeded in 6-well plates, grown to 70% confluence, and serum-starved overnight. The cells were incubated at 37 °C with inhibitors in starvation medium with the concentrations listed in Table S1 or equal volume DMSO or EtOH as control for the following time periods: bortezomib and chloroquine for 4 hours; cycloheximide, Dyngo-4a, E-64, CA 074, L 006236, SID 026681509, β-Secretase Inhibitor IV and γ-Secretase Inhibitor IX, 1 hour; and EDTA

and BAPTA-AM, 30 minutes. Cells were stimulated with 20 ng/ml PDGF-BB (Peprotech) at 37 °C for the indicated time periods. Experiments with stimulation on ice were performed as previously described (Heldin et al. 2019). HaCaT-PDGFRβ-BioID were seeded in 6-well plates at 35,000 cells/cm^2 and left to adhere overnight. The following day the cells were starved overnight in DMEM without FBS. The medium was changed to fresh starvation medium containing TGFβ-1 to a final concentration of 5 ng/ml or starvation medium alone for 72 hours, with medium replacement once daily. In the last 60 minutes of TGFβ1 treatment, the cells were additionally stimulated with 20 ng/ml PDGF-BB.

To prepare cell lysates, cells were briefly washed in ice-cold PBS. For Bj-hTERT and HaCaT-PDGFRβ-BioID, cells were lysed in 150 µl lysis buffer (0.1% sodium dodecyl sulphate (SDS), 1% Nonidet, 0.5% sodium deoxycholate, 20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA), supplemented with PhosSTOP and complete mini EDTA free protease inhibitor tablets (both Roche) for 20 minutes on ice. The cell lysates were cleared by centrifugation at 13,000 x g at 4 °C for 15 minutes and the supernatant combined with NuPAGE 4x lithium dodecyl sulphate sample buffer (ThermoFisher scientific) containing 20 mM dithiothreitol (DTT). For AG01523, the cells were lysed in 2x Laemmli Sample Buffer (5% SDS, 25% glycerol, 150 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue), supplemented with 1 mM DTT. Cell lysates were then collected and heated for 10 min at 95 °C. Next, samples were centrifuged at 12,000 rpm for 15 minutes and the supernatants were collected.

Immunoblotting

For experiments using Bj-hTERT and HaCaT-PDGFRβ-BioID cells, samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with Chameleon Duo Pre-stained Protein Ladder (LI-COR Biosciences) as a size marker. Proteins were transferred to Immobilon-FL polyvinylidene difluoride (PVDF) membranes (Merck). Membranes were blocked in Odyssey or Intercept Tris-buffered saline (TBS) Blocking Buffer (LI-COR Biosciences; diluted 1:3 in TBS) for 2 hours at room temperature. Membranes were incubated overnight with primary antibodies diluted in blocking buffer with 0.1% Tween-20 (see Table S1) and washed 3 times for 10 minutes each in TBS with 0.05% Tween-20 (TBST). Membranes were then incubated with fluorescently labelled secondary antibodies (see Table S1) diluted in blocking buffer for 1 hour at room temperature and washed again 3 times 10 minutes in TBST. Membranes were scanned using an Odyssey Scanner, and the bands were quantified using ImageStudio v5.2.5 software (both LI-COR Biosciences).

For experiments using AG01523 cells, samples were separated by SDS-PAGE and transferred to Immobilon-P PVDF membranes (Merck), then blocked for one hour in 5% bovine serum albumin (BSA) in PBS containing 0.06% Tween-20, followed by incubation with the primary antibodies (see Table S1) overnight at 4 °C. After three washes in PBS, 0.06% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (see Table S1) for one hour at room temperature. Proteins were visualised with the enhanced chemiluminescence (ECL) detection system using SuperSignal West Dura Extended Duration Substrate (ThermoFisher scientific) and a charge-coupled device (CCD) camera (Bio-Rad).

Proximity ligation assay (PLA)

The cells were seeded and treated as described above in the "Treatment and stimulation of cells" section. After removal of the culture medium, cells were then fixed in 4% formaldehyde for 15 minutes on ice, washed 3 times 5 minutes with PBS and then permeabilized with 0.2% Triton X-100 for 10 minutes. After this proximity ligation assay (PLA; NaveniFlex MR, Navinci Diagnostics) was performed according to the manufacturer's instructions. The primary antibodies used are described in Supplementary Table 1.

Cell surface biotinylation and internalisation assay

AG01523 cells were cultured in 60-mm petri dishes to 70% confluence and then starved for 16 hours. To analyse cell surface PDGFRβ, cells stimulated or not with PDGF-BB, were washed with PBS, incubated in 0.3 mg/ml of EZ-Link Sulfo-NHS-SS-Biotin (ThermoFisher scientific, #21331), dissolved in PBS on ice for 1 hour, quenched with 50 mM Tris-HCl, pH 7.4, for 15 min, rinsed with ice-cold PBS, and lysed in RIPA buffer (0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 20 mM Tris-HCl, pH 7.5, 10% glycerol, 150 mM NaCl) on ice for 15 minutes. To analyse PDGFRβ internalisation, cells were incubated in 0.3 mg/ml of EZ-Link Sulfo-NHS-SS-Biotin (ThermoFisher scientific, #21331) for 30 minutes at 4 °C to label cell surface proteins, followed by stimulation with PDGF-BB for different time periods at 37 °C. Labelled PDGFRβ remaining at the cell surface was stripped of biotin by

three subsequent incubations for 15 min with ice-cold 100 mM 2-mercaptoethane sulphonic acid (Sigma-Aldrich) dissolved in 50 mM Tris (pH 8.8), 100 mM NaCl, 1 mM EDTA, and 0.2% BSA. Stripped cells were solubilised in RIPA lysis buffer on ice. Cell extracts were cleared from cell debris by 15 minutes of centrifugation at 13,000 rpm. Pierce Streptavidin Agarose (ThermoFisher Scientific, 20359) was pre-washed and added to the cleared cell extracts, followed by rotation at 4 °C for 1 hour, washing three times with RIPA buffer, and heating at 95 °C in reducing SDS-sample buffer. The desorbed protein samples were then subjected to SDS-PAGE and membrane transfer, as described above. The membranes were blocked in TBST containing 5% BSA for 1 hour, incubated in primary antibodies diluted in TBST containing 1% BSA overnight at 4 °C, washed three times in TBST for 5 minutes each time, incubated in peroxidase-conjugated secondary antibodies for 1 hour, and then washed three times in TBST. Proteins were visualised as described above.

Statistical analysis

Statistical differences were analysed using a two-sided Mann-Whitney test.

Results

PDGFRβ is cleaved into two fragments upon stimulation with PDGF-BB

To investigate the kinetics of PDGFRβ degradation, Bj-hTERT cells were stimulated with 20 ng/ml PDGF-BB for different time periods up to 90 minutes. Immunoblotting was performed using antibodies targeting the extracellular domain (ECD) and intracellular domain (ICD) of PDGFRβ, i.e. AF385 and CTβ, respectively (Figure 1A–C). The antibody against the ECD recognised a 130 kDa fragment and the antibody against the ICD a 70 kDa fragment: in addition, both antibodies also recognised the uncleaved full-length receptor which migrated as a 190 kDa protein. The amount of full-length PDGFRβ started to decline after 30 minutes, while at the same time, an increase in the ~130 kDa fragment was detected, peaking at 60 minutes after which it began to decline (Figure 1A, B). Immunoblotting with the ICD antibody showed that a fragment of ~70 kDa appeared after 60 minutes of stimulation and then declined (Figure 1A, C). siRNA knockdown of PDGFRβ confirmed the specificity of the ECD antibody in detecting PDGFRβ bands (Figure S1). Cells were treated with cycloheximide to prevent protein synthesis to exclude the possibility that the 130 kDa fragment represented a newly synthesised receptor precursor. Under these conditions, the full-length PDGFRβ was still degraded and a 130 kDa fragment appeared after 30 minutes (Figure 1D). The intracellular fragment band was faint and not always possible to capture in our assays, most likely due to the limited sensitivity of our antibody; therefore, for further experiments, we focused on the larger 130 kDa fragment.

Since many proteases are Ca^{2+}-dependent, we investigated whether the cleavage involved Ca^{2+}-dependent proteases. We used EDTA to chelate Ca^{2+} outside the cells and BAPTA-AM, which is cell permeable, to chelate Ca^{2+} inside and outside the cells. Bj-hTERT cells were pre-treated for 30 minutes with 2 mM EDTA or 100 µM BAPTA-AM, then stimulated with 20 ng/ml PDGF-BB for 60 min, followed by immunoblotting against PDGFRβ. EDTA did not prevent the cleavage of PDGFRβ, while BAPTA-AM blocked the formation of the 130 kDa fragment (Figure 1E). To narrow down the region in which the cleavage occurred and elucidate if the 130 kDa fragment contained phosphorylated tyrosine residues capable of initiating signal transduction, we performed streptavidin pulldown of biotinylated internalised proteins, followed by immunoblotting with site-specific phosphoantibodies recognising PDGFRβ phosphorylated at Y579/581 or Y857 (Figure 1F). A 130 kDa band was observed when blotting for phosphorylated (p)Y579/581 but not for pY857.

These observations suggest that PDGFRβ is cleaved in the region between Y579 and Y857 following ligand-induced activation to form two distinct fragments. Based on the size of the fragments, the cleavage is likely to occur in the juxtamembrane region close to the Y579/581 phosphorylation site (Figure 1G). The larger ECD fragment of 130 kDa contains the extracellular-, transmembrane- and juxtamembrane domains, and the intracellular ICD fragment of 70 kDa contains most or all of the tyrosine kinase domain with its non-kinase insert, and the C-terminal tail. The 70 kDa fragment contains most of the autophosphorylation sites, but the 130 kDa fragment contains phosphorylated Y579/581 and hence both fragments may interact with signalling proteins.

PDGFRβ cleavage is prevented by bortezomib

The generally accepted model of the fate of PDGFRβ after ligand stimulation is that active PDGFRβ is ubiquitinated and internalised via clathrin-mediated endocytosis and sorted through the endosomal/lysosomal pathway, where it is eventually degraded (Goh and Sorkin 2013). Previous experiments using a

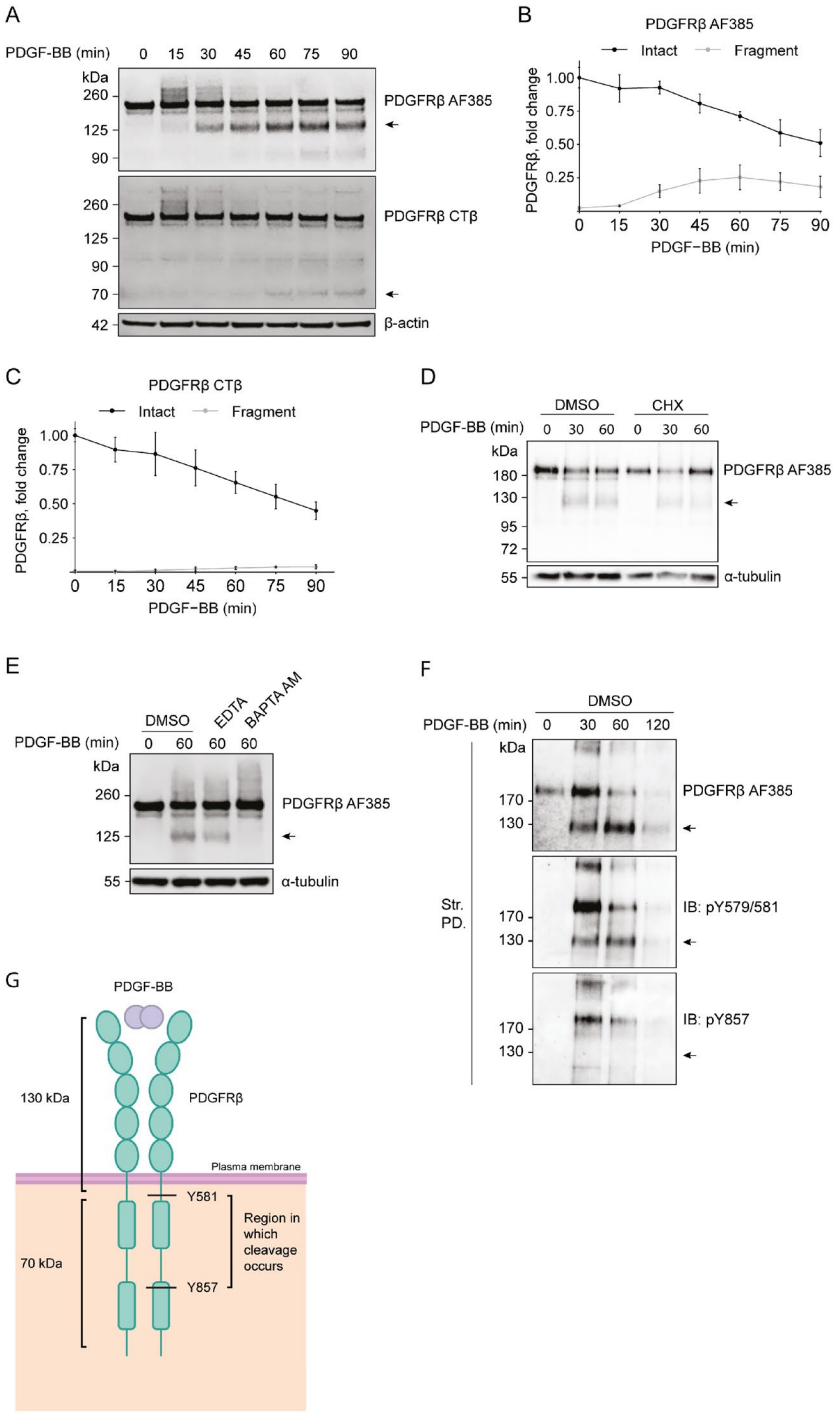

Figure 1. PDGFRβ is cleaved into two distinct fragments upon stimulation with PDGF-BB. (A) Bj-hTERT cells were serum-starved overnight and stimulated with 20 ng/ml PDGF-BB for the indicated time periods. After cell lysis, proteins were separated by SDS-PAGE and subjected to immunoblotting (IB) with antibodies against the ICD (CTβ) and ECD (AF385) of PDGFRβ. (B, C) Quantification of intact PDGFRβ and 130 kDa (B) and 70 kDa fragments (C) of PDGFRβ. Error bars represent the standard deviation (n = 3). (D) AG01523 cells were serum-starved and treated with 50 µM cycloheximide (CHX) for 1 hour and stimulated with 20 ng/ml PDGF-BB for the indicated time periods, followed by cell lysis, SDS-PAGE and immunoblotting for PDGFRβ (AF385). (E) Bj-hTERT cells were serum-starved overnight and treated with 2 mM EDTA and 100 µM BAPTA-AM for 30 minutes before stimulation with PDGF-BB for the indicated time periods, followed by cell lysis, SDS-PAGE and immunoblotting for PDGFRβ (AF385). (F) AG01523 cells were serum-starved overnight and then biotinylated, followed by stimulation with PDGF-BB for the indicated time periods. Next, biotin was removed from cell surface proteins and cells lysed, and biotinylated intracellular proteins were adsorbed to streptavidin beads (Str. PD.). Samples were analysed by SDS-PAGE and immunoblotting for the 130 kDa fragment of PDGFRβ (AF385) and PDGFRβ phosphorylated at Y579/Y581 and Y857. (G) Based on the results in panel (F), the cartoon depicts PDGFRβ with the 130 and 70 kDa fragments indicated and the region containing the cleavage site. In panels A, D, E and F, the 130 and 70 kDa fragments are indicated with arrows.

PDGFRβ antibody against the intracellular domain showed that lysosomal inhibition did not prevent degradation, but proteasomal inhibition did, thus suggesting that proteasomes are involved in the degradation of PDGFRβ (Rorsman et al. 2016). To investigate the involvement of proteasomes in PDGFRβ cleavage and degradation, we used the proteasome inhibitor bortezomib. Cells were treated with 500 nM bortezomib or an equal volume of DMSO for 4 hours before stimulation with 20 ng/ml PDGF-BB for 0, 30 and 60 minutes, followed by immunoblotting with antibodies against the ECD of PDGFRβ. Bortezomib prevented fragmentation of PDGFRβ, as detected by the reduced formation of the extracellular 130 kDa fragment in both Bj-hTERT (Figure 2A–C) and AG01523 cells (Figure 2D–E). Similar results were obtained using the proteasome inhibitors MG132 or lactacystin (data not shown).

Internalisation is required for cleavage of PDGFRβ

The 130 kDa fragment appeared after 30 minutes of ligand stimulation (Figure 1A, B), while PDGFRβ internalisation starts within 5 minutes of PDGF-BB stimulation (Heldin et al. 2019). Based on the kinetics of receptor internalisation and the emergence of the cleavage product, it is likely that PDGFRβ cleavage took place after ligand-induced internalisation and not at the plasma membrane. We investigated this possibility by blocking internalisation from the plasma membrane by employing low temperature, which prevents internalisation while allowing for receptor activation. Stimulation of Bj-hTERT with PDGF-BB on ice inhibited the degradation of PDGFRβ and the formation of the 130 kDa fragment (Figure 3A). Blotting with an antibody specific for PDGFRβ phosphorylated at Y751 showed an increase in phosphorylated full-length receptor after 60 minutes of stimulation consistent with the lack of internalisation and degradation. Our observations thus suggest that the cleavage of PDGFRβ depends on internalisation and does not occur at the plasma membrane.

To further substantiate this finding, we used an alternative and unrelated method to block internalisation using the dynamin inhibitor Dyngo-4a (McCluskey et al. 2013). AG01523 cells were treated with 30 μM Dyngo-4a for 1 hour before stimulation with 20 ng/ml PDGF-BB for 0, 30 and 60 minutes, followed by immunoblotting with antibodies against the ECD of PDGFRβ (Figure 3B). Inhibition of endocytosis by Dyngo-4a treatment prevented the formation of the 130 kDa fragment and the degradation of full-length PDGFRβ. Taken together, these observations support the notion that receptor cleavage is dependent on PDGFRβ internalisation from the plasma membrane.

Since bortezomib and BAPTA-AM both efficiently blocked receptor cleavage (Figures 1E and 2A), and PDGFRβ internalisation was needed for cleavage (Figure 3A, B), we investigated whether bortezomib or BAPTA-AM could affect receptor internalisation. To this end, we performed streptavidin pulldowns of biotinylated cell surface receptors, followed by immunoblotting using an antibody specific for the ECD of PDGFRβ; we found that the full-length receptor was stabilised at the plasma membrane after treatment with the proteasome inhibitor bortezomib, while treating the cells with the lysosomal inhibitor chloroquine did reduce, but not completely block, the cleavage (Figure 3C). The ability of bortezomib to impair PDGFRβ internalisation was confirmed using the proximity ligation assay to measure the proximity between PDGFRβ and the early endosome marker EEA1 after PDGF-BB stimulation (Figure 3D). In addition, BAPTA-AM treatment also inhibited cell surface clearance of PDGFRβ (Figure 3E). Thus, upon treatment with bortezomib or BAPTA-AM, most PDGFRβ remained in an uncleaved form at the plasma membrane for a longer period of time.

Bortezomib increases ligand-induced autophosphorylation of PDGFRβ and phosphorylation of PLCγ and STAT3

PDGFRβ signalling is compartmentalised and regulated by the subcellular location of both receptor and signalling protein (Wang et al. 2004; Villaseñor, Kalaidzidis, and Zerial 2016). Since bortezomib treatment affected internalisation, we determined whether it also affects the activation of signalling downstream from PDGFRβ. Bj-hTERT cells were treated with bortezomib or DMSO for 4 hours and then stimulated with PDGF-BB for 0, 30 and 60 minutes, followed by immunoblotting with total and phospho-specific antibodies for PDGFRβ, PLCγ, STAT3, Akt and Erk1/2. Ligand-induced phosphorylation of PDGFRβ at Y751 was increased upon treatment with bortezomib at 30 and 60 minutes of PDGF-BB stimulation (Figure 4A, B), as expected due to the decreased receptor internalisation. Activation of PLCγ, measured as phosphorylation of Y783, and activation of STAT3, as determined by phosphorylation of Y705, were also increased (Figure 4A, C and D). In contrast, phosphorylation of Akt, downstream of PI3K, was unaffected by treatment with bortezomib (Figure 4A, E). For Erk1/2, the basal activation level was higher in bortezomib-treated cells and was only slightly induced

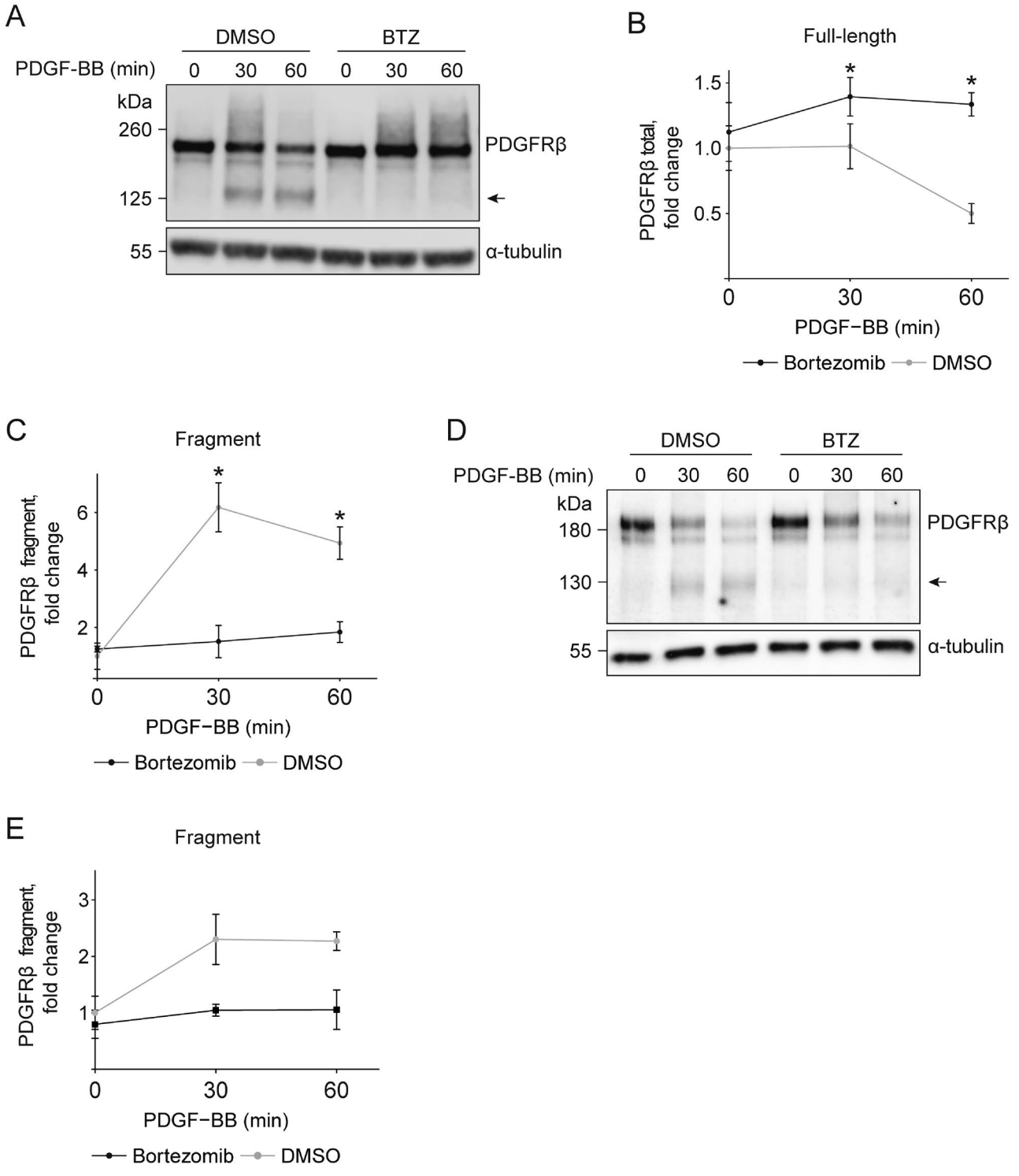

Figure 2. PDGFRβ internalization and cleavage is prevented by bortezomib. (A) Bj-hTERT cells were serum-starved overnight and treated with 500 nM bortezomib (BTZ) before stimulation with 20 ng/ml PDGF-BB for the indicated time periods, followed by cell lysis, SDS-PAGE and immunoblotting using the PDGFRβ ECD antibody (AF385). (B, C) Quantification of full-length PDGFRβ (B) and PDGFRβ 130 kDa fragment (C). Error bars represent the standard deviation (n = 4). *$p < 0.05$. (D, E) AG01523 cells were serum-starved overnight and treated with 500 nM bortezomib for 4 hours before stimulation with PDGF-BB for the indicated time periods, followed by lysis, SDS-PAGE and immunoblotting using the PDGFRβ ECD antibody (AF385). In panels a and D, arrows indicate the 130 kDa fragment.

by PDGF-BB, resulting in a decrease in ligand-induced Erk1/2 MAPK activation in bortezomib-treated cells, compared to DMSO-treated control cells (Figure 4A, F). In summary, bortezomib treatment affected PDGF-BB-induced signalling pathways in a distinct manner: phosphorylation of PLCγ and STAT3 was increased, Akt

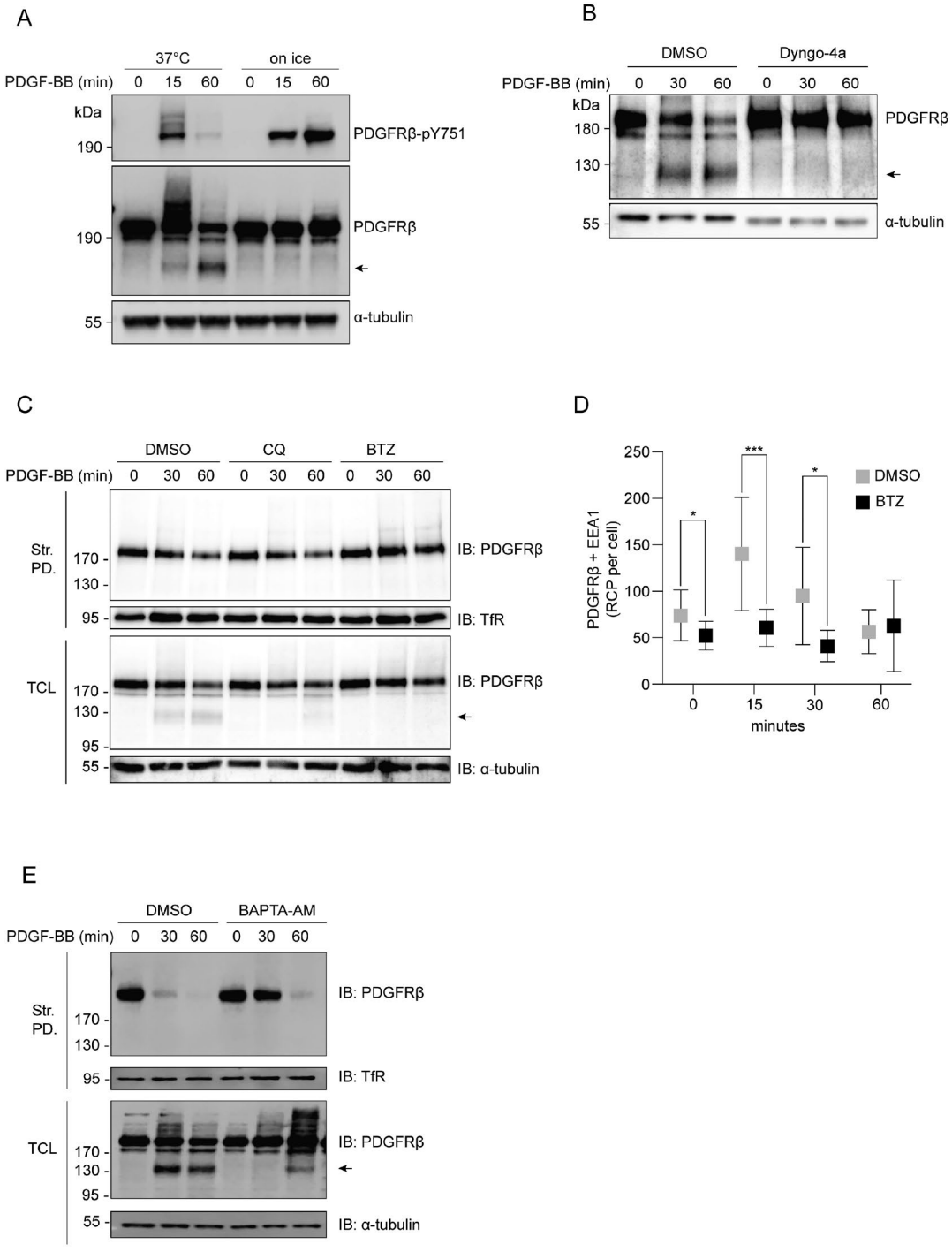

Figure 3. Internalisation is necessary for PDGFRβ cleavage. (A) Bj-hTERT cells were serum-starved overnight before stimulation with 20 ng/ml PDGF-BB for the indicated time periods, either at 37 °C or on ice, followed by cell lysis, SDS-PAGE and immunoblotting using the PDGFRβ ECD antibody (AF385). (B) AG01523 cells were serum-starved overnight and treated with 30 µM Dyngo-4a for 1 hour before stimulation with PDGF-BB for the indicated time periods, followed by lysis, SDS-PAGE and immunoblotting using the PDGFRβ ECD antibody (AF385). (C) Cell surface biotinylation of PDGFRβ. AG01523 cells were serum-starved overnight and treated with 50 µM chloroquine (CQ) or 500 nM bortezomib (BTZ) for 4 hours before stimulation with 20 ng/ml PDGF-BB for the indicated time periods, followed by cell surface biotinylation, cell lysis, pull-down with streptavidin beads (PD), SDS-PAGE and immunoblotting using the PDGFRβ ECD antibody (AF385). (D) Bj-hTERT cells were serum-starved overnight before stimulation with 20 ng/ml PDGF-BB in the presence or absence of bortezomib for the indicated time periods. Co-localisation between PDGFRβ to the early endosomal marker EEA1 was analysed using the proximity ligation assay (PLA). Panel D shows the quantification of PLA rolling circle products (RCP). (E) Bj-hTERT cells were serum-starved overnight and treated with 100 µM BAPTA-AM for 30 minutes before stimulation with 20 ng/ml PDGF-BB for the indicated time periods at 37 °C, followed by cell surface biotinylation, cell lysis, pull-down with streptavidin beads (PD), SDS-PAGE and immunoblotting using the PDGFRβ ECD antibody (AF385). Total cell lysate (TCL) was also subject to SDS-PAGE and immunoblotting (IB) for 130 kDa fragment of PDGFRβ. Arrows indicate the 130 kDa fragment.

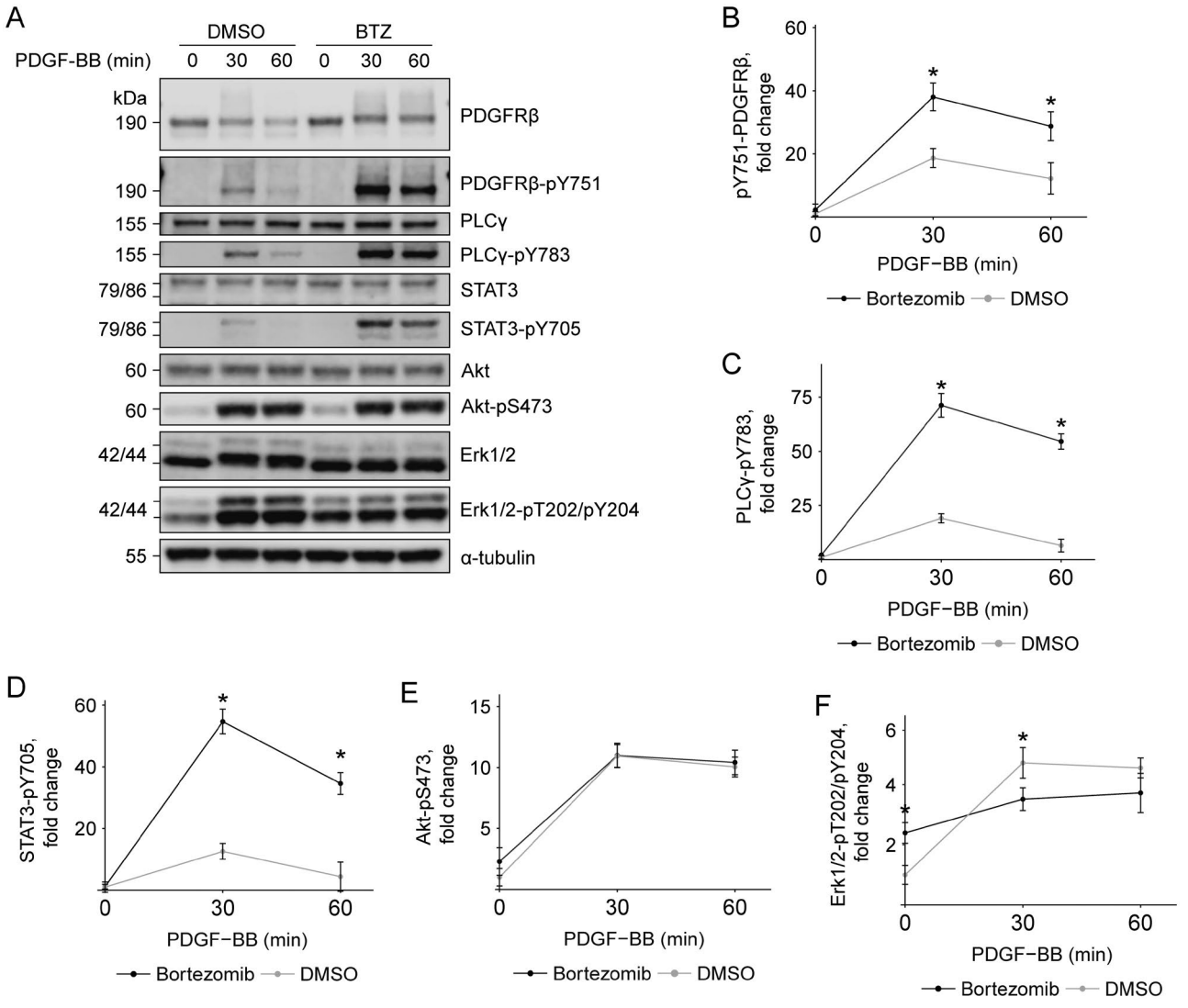

Figure 4. Bortezomib increases activation of PDGFRβ and phosphorylation of PLCγ and STAT3. (A) Bj-hTERT cells were serum-starved overnight and treated with 500 nM bortezomib (BTZ) for 4 hours before stimulation with 20 ng/ml PDGF-BB for the indicated time periods, followed by lysis, SDS-PAGE and immunoblotting for total protein or specific phosphorylation sites of PDGFRβ, PLCγ, STAT3, Akt and Erk1/2. (B–F) Quantification of PDGFRβ-pY751 (B), PLCγ-pY783 (C), STAT3-pY705 (D), Akt-pS473 (E) and ERK1/2-pT202/pY204 (F). Error bars represent the standard deviation (n = 4). *$p < 0.05$.

phosphorylation was not affected, whereas Erk1/2 phosphorylation was reduced.

Calpain, cathepsins and γ-secretase are not involved in ligand-induced PDGFRβ cleavage

A previous report (Ek and Heldin 1986) and results presented in this study have indicated that PDGFRβ can be cleaved in a Ca^{2+}-dependent manner. Thus, in an attempt to identify the protease responsible for the PDGFRβ cleavage, we used a panel of different protease inhibitors, including inhibitors targeting Ca^{2+}-dependent cysteine proteases, i.e. calpain and cathepsin (E-64, 10 μM; CA 074, 25 μM; L 006235, 10 μM; and SID 26681509, 50 μM). Bj-hTERT cells were treated with inhibitors for 1 hour before stimulation with 20 ng/ml PDGF-BB, followed by immunoblotting using an antibody against the extracellular domain of PDGFRβ (Figure 5A). The results showed no inhibitory effect on PDGFRβ fragmentation, leading us to conclude that calpains and cathepsins are either not involved in the proteolysis or that there may be redundancy among different proteases.

γ-Secretase is an intramembrane protease residing in the lipid bilayer, known for its cleavage of APP in Alzheimer's disease. β-Secretase is another transmembrane protease involved in the cleavage of APP (Hemming et al. 2009). Treatment of cells with inhibitors of the aspartic acid proteases γ-Secretase Inhibitor IX (also known as DAPT; used at 100 μM), or β-Secretase Inhibitor IV (used at 1 μM) did not affect the formation of an extracellular fragment of PDGFRβ

(Figure 5A). Thus, β- and γ-secretases are not involved in the cleavage of PDGFRβ.

PDGFRβ is only cleaved into the 130 kDa fragment in mesenchymal cells

The proteolytic processing of PDGFRβ into 130 kDa and 70 kDa fragments could only be observed in the fibroblast cell lines Bj-hTERT and AG01523, but not in other PDGFRβ-expressing cell types tested, such as the osteosarcoma U2OS (Figure S2) and the glioblastoma T98G cells (data not shown). This suggests that the cleavage may be specific to cells of mesenchymal origin. To explore this possibility further, epithelial HaCaT cells transfected with PDGFRβ were stimulated or not with PDGF-BB; as expected, the receptor was not cleaved in these cells (Figure 5B). However, after treatment of these cells with TGFβ to induce epithelial-to-mesenchymal transition (EMT), we observed cleavage of the PDGFRβ. In the mesenchymal state, the cleavage occurred with or without exogenously added PDGF-BB, which may be related to the production of endogenous PDGF during the EMT process.

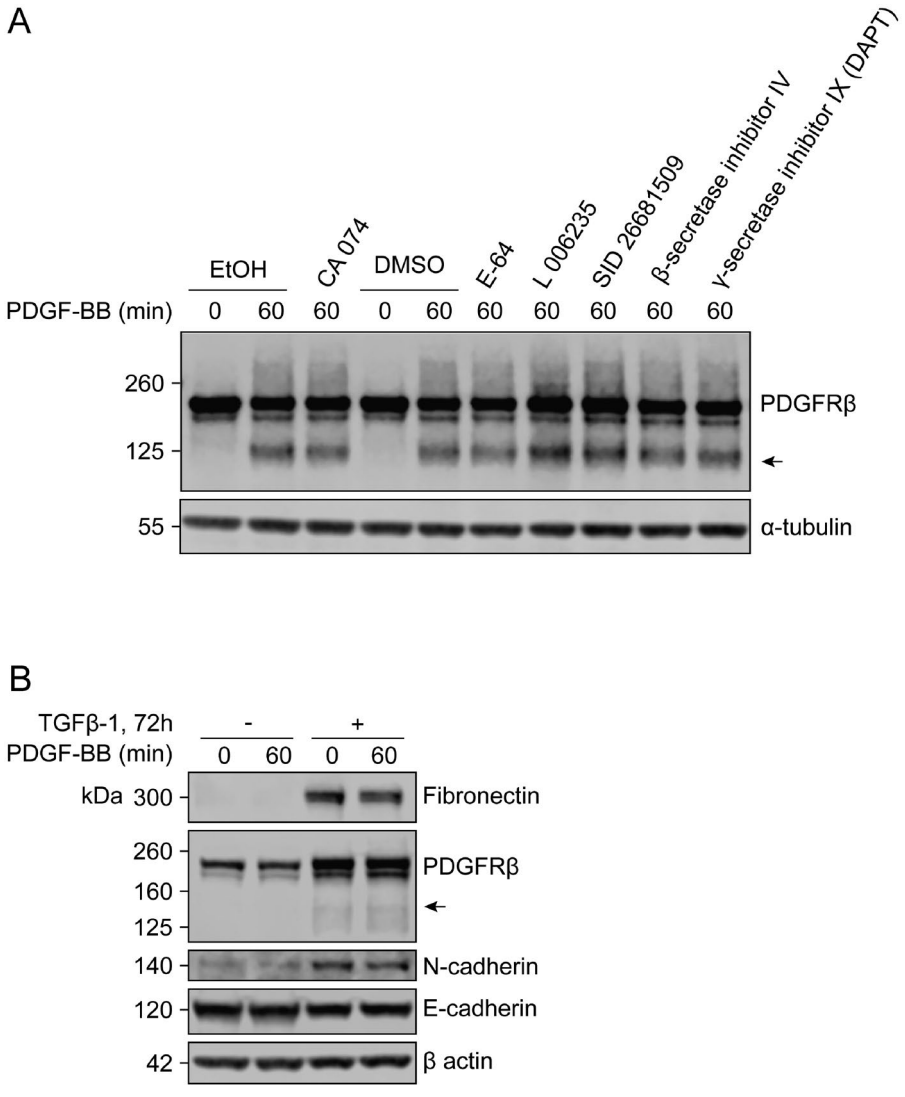

Figure 5. (A) Inhibitors of calpain, cathepsins, and β- and γ-secretase do not affect PDGFRβ cleavage. Bj-hTERT cells were serum-starved overnight and treated with the calpain and cathepsin inhibitors CA 074 (25 μM), E-64 (10 μM), L 006235 (10 μM), SID 026681509 (50 μM), the β-secretase inhibitor IV (1 μM) and the γ-secretase inhibitor IX (100 μM) for 1 hour before stimulation with PDGF-BB for the indicated time periods, followed by cell lysis, SDS-PAGE and immunoblotting using the PDGFRβ ECD antibody (AF385). The arrow indicates the extracellular 130 kDa fragment. (B) PDGFRβ is cleaved in cells that have undergone EMT. HaCaT cells stably expressing a PDGFRβ-BIOID construct were treated or not with 5 ng/ml TGFβ-1 under starvation for 72 hours and then stimulated with 20 ng/ml PDGF-BB for indicated time periods, followed by cell lysis, SDS-PAGE and immunoblotting for the 130 kDa fragment of PDGFRβ and markers of EMT. The arrow indicates the 130 kDa fragment.

Discussion

PDGFRβ degradation has been proposed to take place in the endosomal/lysosomal pathway (Heldin, Wasteson, and Westermark 1982; Nilsson et al. 1983; Sorkin et al. 1991). Moreover, a role for the proteasome in PDGFRβ degradation has also been proposed (Mori et al. 1995; Rorsman et al. 2016). Thus, both pathways may contribute, as suggested in a model by Mori et al. (Mori et al. 1995). Previous studies of PDGFRβ degradation focused only on the full-length PDGFRβ, so cleaved receptor fragments may not have been noticed. In addition, most studies have used PDGFRβ antibodies that recognise the C-terminal tail of the receptor, and thus, extracellular receptor fragments may not have been noticed.

Proteolytic cleavage of various receptor tyrosine kinases, including AXL, MET, Trk, and ERBB4, has been documented to release intracellular or extracellular fragments (Huang 2021). These fragments may play novel roles in signalling, influence cellular processes, such as apoptosis and migration, and contribute to the progression of diseases like cancer and neurodegeneration.

This study aimed to investigate ligand-induced cleavage and degradation of PDGFRβ. We found that ligand binding leads to cleavage of PDGFRβ into two fragments. This ligand-induced fragmentation may be specific for mesenchymal cells since we have not been able to detect PDGFRβ cleavage in other cell types (Figure S2).

By following ligand-induced degradation of PDGRβ over time, a decrease in the intensity of the full-length band was observed, while the two fragments appeared after 30 minutes of PDGF-BB stimulation; the smaller 70 kDa fragment was challenging to detect, which may be due to the low affinity of the antibody available, or that the small fragment is rapidly degraded. Using phospho-specific antibodies against PDGFRβ, we could show that PDGFRβ is cleaved in the region between Y581 and Y857. Based on the fragment sizes, the cleavage likely occurs closer to Y581 in the juxtamembrane region of the receptor. Phosphorylated Y579/581, which is situated in the intracellular juxtamembrane region, was detected in the 130 kDa fragment, suggesting that the ECD fragment can engage in signal transduction emanating from phosphorylated Y579/581, e.g. activating the tyrosine kinase Src which binds to these residues We could not detect any phosphorylated tyrosine residues in the 70 kDa fragment, possibly due to the low levels of this fragment; whether the ECD or ICD fragments affect signal transduction events is an interesting possibility that remains to be elucidated.

PDGFRβ internalisation occurs almost immediately after ligand binding; after 5 minutes of PDGF-BB stimulation, PDGFRβ can be detected in the early endosomes, with a peak after 30 minutes of stimulation (Heldin et al. 2019). The fact that cleavage occurred after 30 minutes or longer of PDGF-BB stimulation suggests an endosomal localisation for the cleavage process. Inhibiting internalisation by maintaining cells on ice or treating cells with Dyngo-4a, a dynamin 2 inhibitor, prevented receptor cleavage. These findings support the notion that cleavage occurred after internalisation and not at the plasma membrane.

We found that treating cells with a proteasomal inhibitor also prevented PDGFRβ cleavage, suggesting that the cleavage occurred in proteasomes. Proteasomes normally degrade proteins into small fragments, making it challenging to envision how they can cleave the receptor at a single site in the middle of the protein. Alternatively, active proteasomes may be required for the PDGFRβ to be internalised. Indeed, proteasomal inhibition decreased ligand-induced internalisation of PDGFRβ, as well as its cleavage and degradation. Furthermore, using the cell-permeable Ca^{2+} chelator BAPTA-AM, we also observed a reduced internalisation correlated with reduced PDGFRβ cleavage. Use of inhibitors of Ca^{2+}-dependent proteases and γ-secretase, known to cleave membrane proteins, did not enable us to identify the protease responsible for this process. Since PDGFRβ cleavage predominantly occurs in mesenchymal cells, a protease specific to such cell types is likely to be involved.

Activation of signalling pathways by PDGFRβ has been proposed to be compartmentalised and regulated by the subcellular localisation of the receptor (Villaseñor, Kalaidzidis, and Zerial 2016; Wang et al. 2004). For instance, Akt and Erk1/2 are activated at endosomes (Palamidessi et al. 2008; Schenck et al. 2008; Teis, Wunderlich, and Huber 2002), while other signalling proteins, like PI3K and PLCγ, are activated at the plasma membrane (Haugh and Meyer 2002). Our results showed increased phosphorylation of PLCγ upon bortezomib treatment, which is in line with the observation that PDGFRβ is more phosphorylated and remains at the plasma membrane for an extended period of time after treatment with bortezomib. We also found an increased phosphorylation of STAT3, which shuttles between the cytoplasm and nucleus (Levy and Darnell 2002; Liu, McBride, and Reich 2005) and can potentially be phosphorylated at several cellular locations. STAT3 activation downstream of PDGFRβ has been shown to involve Src kinases that reside at the plasma membrane through a lipid modification (Mori et al. 1993; Wang et al. 2000; Yeatman 2004).

The observed increase in PLCγ and STAT3 phosphorylation and unaffected levels of Akt phosphorylation are in line with previous reports that have shown increases in PLCγ and STAT3 activation in cells depleted of the E3 ubiquitin ligases c-Cbl and Cbl-b, which also reduced receptor internalisation, while no effect on phosphorylation of Akt was observed (Rorsman et al. 2016). However, other studies have shown a reduced level of STAT3 phosphorylation under conditions where PDGFRβ endocytosis was inhibited, suggesting that PDGFRβ may activate STAT3 differentially in different cell types (Jastrzębski et al. 2017; Sadowski et al. 2013).

It has been proposed that the Erk1/2 is primarily activated on the surface of endosomes (Teis, Wunderlich, and Huber 2002). The fact that fewer receptors arrive at the endosome under conditions where the proteasome is inhibited may explain why we observed a decrease in Erk1/2 phosphorylation after bortezomib treatment despite increased receptor phosphorylation and activation of other pathways.

Interestingly, ligand-induced proteolytic cleavage of VEGFR2 in its intracellular part, similar to what we have found for PDGFRβ, has been reported (Bruns et al. 2010; Ewan et al. 2006). The cleavage of VEGFR2 was also sensitive to proteasomal inhibition. Furthermore, the VEGFR2 fragmentation could be induced by PKC activation. Since several PKC isoforms require Ca^{2+} this is consistent with our observation that Ca^{2+} chelators inhibited the fragmentation. Functionally, it was shown that preventing VEGFR2 cleavage promoted cell migration. To clarify the functional importance of the cleavage on PDGFRβ function, it would be helpful to identify the exact cleavage site to make it possible to construct a mutated receptor that cannot be cleaved.

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Additional information

This work has been performed in compliance with the Declaration of Helsinki. Reagents used in the study are available upon reasonable request.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data in this investigation are available from the corresponding author upon reasonable request.

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