Journal of Cell Science 111, 111-120 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS1471

Platelet-derived growth factor (PDGF)-induced actin rearrangement is deregulated in cells expressing a mutant Y778F PDGF β-receptor

Aino Ruusala[1], Christian Sundberg[2], Ann-Kristin Arvidsson[1,*], Eva Rupp-Thuresson[1,†], Carl-Henrik Heldin[1] and Lena Claesson-Welsh[2,‡]

[1]The Ludwig Institute for Cancer Research, Biomedical Center, Box 595, S-751 24 Uppsala, Sweden
[2]Department of Medical and Physiological Chemistry, Biomedical Center, Box 575, S-751 23, Uppsala, Sweden
*Present address: Department of Medical Pharmacology, Biomedical Center, Box 593, S-751 24 Uppsala, Sweden
†Present address: Pharmacia and Upjohn AB, Strandbergsgatan 49, S-112 87 Stockholm, Sweden
‡Author for correspondence (e-mail: Lena.Claesson-Welsh@bmc.uu.se)

Accepted 27 October 1997; published on WWW 11 December 1997

SUMMARY

Platelet-derived growth factor-stimulated actin rearrangement and edge ruffle formation have previously been shown to be dependent on activation of phosphatidylinositol 3′-kinase, the activity of which also is important for directed migration of cells. This lipid kinase binds to phosphorylated tyrosine residues Y740 and Y751 in the kinase insert of the human platelet-derived growth factor β-receptor. We examined the role of two other tyrosine residues in the kinase insert of this receptor, Y775 and Y778, for ligand-induced actin rearrangement. Both were shown to be phosphorylation sites; Y775 was only marginally phosphorylated in cells expressing the wild-type β-receptor, whereas Y778 was phosphorylated at higher stoichiometry. Mutant receptors Y775F, Y778F and Y775/778F were active kinases and mediated proliferative responses when expressed in porcine aortic endothelial cells. Fluorescence staining of actin in platelet-derived growth factor-stimulated PAE cells revealed that Y778 is involved in regulation of the actin cytoskeleton since the cells contained, apart from edge ruffles and circular ruffles, a novel type of giant ruffle on the dorsal side of the cell, which consisted of irregular multilayered actin structures. Mutation at Y778 had no effect on activation of phosphatidylinositol 3′-kinase, nor on the GTPase activating protein of Ras and phospholipase Cγ, and the extent of directed migration towards platelet-derived growth factor of these cells was not changed. We conclude that actin rearrangement is regulated in part by Y778 in the platelet-derived growth factor β-receptor, potentially through binding of a novel signaling molecule to this site.

Key words: PDGF, Actin, Ruffle, Tyrosine phosphorylation

INTRODUCTION

The platelet-derived growth factor (PDGF) is a dimeric protein composed of A- and B-chains, which combine to form three isoforms, PDGF-AA, -AB and -BB (Heldin and Westermark, 1996). The α-granules of platelets contain PDGF, which becomes released upon platelet aggregation. In addition, the different PDGF isoforms are synthesized by a variety of cell types, such as mesenchymal cells and by macrophages and monocytes. The isoforms bind to different extents to two receptor types, denoted α and β. All three isoforms bind with high affinity to the PDGF α-receptor, whereas only PDGF-BB binds with high affinity to the PDGF β-receptor. The receptors are independently expressed on mesenchymal cells, such as fibroblasts, glia cells and capillary endothelial cells, but also on epithelial and neuronal cells (Heldin and Westermark, 1996). The structural organization of the receptors is similar, with an extracellular part containing five immunoglobulin-like domains, a single transmembrane stretch, and an intracellular part which contains the ligand-stimulatable tyrosine kinase domain (Claesson-Welsh, 1994). Binding of PDGF leads to dimerization of the receptors and activation of the kinase domains. Dependent on the PDGF isoform, receptor homo- or heterodimers are formed. Activation of the kinase domain leads to phosphorylation of tyrosine residues in the receptors, through a trans-phosphorylation reaction, whereby docking sites for downstream signal transduction molecules containing Src homology 2 (SH2) domains are formed (Pawson, 1995). The signal transduction molecules are often equipped with enzymatic activity; alternatively, they are adaptors, which may associate with an enzymatic activity. Binding to the receptors induces activation of the intrinsic or indirectly associated enzymatic activities. Thereby, signaling cascades are initiated, which ultimately result in initiation of cell proliferation, chemotaxis, actin reorganization and other cellular responses.

A number of phosphorylated tyrosine residues have been identified in the PDGF α- and β-receptors and in many cases, the associating signal transduction molecule(s) has been determined. Thus, the human PDGF β-receptor binds the cytoplasmic tyrosine kinase Src (at Tyr579 and Tyr581), phosphatidylinositol 3′-kinase (PI3-kinase; at Tyr740 and Tyr751), the GTPase activating protein of Ras (RasGAP; at

Tyr771), phospholipase Cγ (PLC-γ; at Tyr1009 and Tyr1021) and the tyrosine phosphatase PTP 1D (SHP-2; at Tyr1009). In addition, the PDGF β-receptor binds the adaptor molecules Grb2 (at Tyr716), Shc (Tyr579, Tyr581 and Tyr740) and Nck (Tyr751) (see Claesson-Welsh, 1994, for a review).

Several of these signal transduction molecules, such as Grb2, Shc and SHP-2, connect the β-receptor with signal transduction pathways leading to mitogenic responses. The PI3-kinase and Src family members have also been assigned roles in mitogenic signal transduction. In addition, PLC-γ has been implicated in mitogenic signal transduction in certain experimental models. PLC-γ catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, eventually leading to activation of protein kinase C and increased cytoplasmic Ca^{2+} concentration (reviewed by Rhee et al., 1989; Wahl and Carpenter, 1991). Motogenic signal transduction on the other hand, appears primarily to be controlled by PI3-kinase and PLC-γ activities (Hansen et al., 1996; Kundra et al., 1994; Wennström et al., 1994b). Thus, cells expressing a PDGF β-receptor mutant lacking the PI3-kinase binding site, i.e. a Y740/751F mutant, are unable to migrate directionally towards PDGF. PLC-γ appears to play a modulatory role in signal transduction leading to cellular migration. PI3-kinase is composed of a regulatory subunit, p85, which exists in constitutive complex with the catalytic subunit, p110 (Panayotou and Waterfield, 1992). The molecular mechanism underlying the regulation of cellular migration by PI3-kinase is not clear at present, but a coupling between PI3-kinase and the small GTP-binding molecule Rac has been demonstrated (Hawkins et al., 1995). Rac has been shown to regulate actin reorganization in the form of edge ruffles, i.e. membrane ruffles formed around the margins of the cells (Ridley et al., 1992). In accordance, PDGF-stimulated cells expressing the mutant Y740/751F PDGF β-receptor lack edge ruffles (Wennström et al., 1994a). Thus, it is possible that actin reorganization and edge ruffle formation is a prerequisite for cellular migration.

In this report, we demonstrate phosphorylation of two novel sites in the PDGF β-receptor kinase insert, Tyr775 and Tyr778. The Y775F as well as the Y778F mutant receptors are active kinases and able to mediate mitogenic as well as motogenic responses. Actin reorganization in cells expressing the Y778F mutant receptor was, however, different from that of wild-type receptor expressing cells. Novel ruffle-formations were visualized by phalloidin-staining, revealing large disorganized structures of actin at the margins of the cells or on the cell body. Activation of PI3-kinase and PLC-γ were not affected by the mutation, suggesting the possibility that novel signal transduction molecule(s) affecting actin reorganization associate with the PDGF β-receptor at Tyr778.

MATERIALS AND METHODS

Cell culture and mutagenesis

Porcine aortic endothelial (PAE) cells were cultured in Ham's F12 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin.

Site-directed mutagenesis was performed on the full-length PDGF β-receptor cDNA cloned into the Altered sites II mutagenesis system (Promega Corp.) vector. The following oligonucleotides were used for mutagenesis; 5′-TACATGGCCCCTTTCGATAACTACGTT-3′ (Y775F) and 5′-TACGATAACTTCGTTCCCTCT-3′ (Y778F). Mutations were confirmed by nucleotide sequencing and the cDNA's were inserted into the eukaryotic expression vector pcDNA3 (Invitrogen). Transfection of PAE cells and selection of expressing cell clones were performed using electroporation followed by neomycin selection, as described previously (Claesson-Welsh et al., 1988).

In vitro kinase assay

PAE cells expressing wild-type or mutant PDGF β-receptors were incubated in the presence or absence of 100 ng/ml PDGF-BB in Ham's F12 medium on ice for 1 hour, followed by 5 minutes incubation at 37°C. The cells were lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol (DTT), 100 μM Na_3VO_4, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cell lysates were immunoprecipitated using a PDGF β-receptor antiserum (PDGFR-3). Kinase assays were performed in 20 mM Hepes, pH 7.5, 10 mM $MnCl_2$, 1 mM DTT, and 5 μCi [γ-^{32}P]ATP. The assays were performed at 30°C for 10 minutes, whereafter ATP was added to a final concentration of 150 μM and incubations were continued for another 10 minutes at 30°C. The reactions were stopped by adding SDS-sample buffer (4% SDS, 0.2 M Tris-HCl, pH 8.8, 0.5 M sucrose, 5 mM EDTA, 0.01% Bromophenol Blue, 2% 2-mercaptoethanol) and boiled for 3 minutes. The samples were analyzed by SDS-polyacrylamide gel electrophoresis. After completion of the electrophoresis, the gel was fixed in 10% glutaraldehyde, soaked in 1 M KOH at 55°C for 1 hour, to hydrolyze serine-phosphorylation, dried and analyzed by autoradiography.

[^{3}H]Thymidine incorporation assay

PAE cells expressing wild-type or mutant PDGF β-receptors were seeded on 24-well dishes at 3×10^4 cells/well. After 24 hours of culture, the medium was changed to Ham's F12 medium, supplemented with 0.3% FBS, and incubation was continued for another 48 hours. Cells were then incubated with PDGF-BB at different concentrations (0-10 ng/ml) or 10% FBS and 0.2 μCi [^{3}H]thymidine in Ham's F12 medium, 0.3% FBS for another 24 hours. The medium was removed and the cell monolayers were incubated in 10% trichloroacetic acid for 5 minutes on ice, rinsed with water and ^{3}H-radioactivity was solubilized using 1 M NaOH. Samples were neutralized and subjected to liquid scintillation counting.

Actin reorganization

The different PAE cell lines were seeded sparsely on coverslips. The following day, the cells were stimulated with 100 ng/ml PDGF-BB in serum-free Ham's F12 for 5 minutes at 37°C. The medium was removed and the cells were fixed for 10 minutes with 2% paraformaldehyde, washed with phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100 in PBS. After blocking in 10 mM glycine in PBS for 1 hour at room temperature, the cells were stained with 50 ng/ml TRITC-labeled phalloidin (Sigma) for 30 minutes. The coverslips were washed with PBS and mounted with Fluoromont and the cells were viewed and photographed using an Olympus microscope.

The effect of wortmannin (Sigma Chemical Co., St Louis, MO) was examined by pretreating the cells with 30 nM wortmannin for 10 minutes at 37°C prior to stimulation with 100 ng/ml PDGF-BB for 10 minutes in the presence of wortmannin at the same temperature. Wortmannin was dissolved in dimethyl sulfoxide (Merck, Germany) and the final concentration of dimethyl sulfoxide in the assay medium was 0.01%. For the phalloidin staining the cells were treated and analyzed as above.

[^{32}P]Orthophosphate labeling, Edman degradation and phosphoamino acid analysis

PAE cells expressing the different mutant PDGF β-receptors were washed with phosphate-free Ham's F12 medium supplemented with 0.1% dialyzed FBS, 10 mM Hepes, pH 7.4, 50 μM Na_3VO_4 and incubated for 3 hours in the same medium supplemented with 3 mCi/ml

[^{32}P]orthophosphate (Amersham). The cells were stimulated with 100 ng/ml PDGF-BB for 1 hour on ice and then washed with ice-cold PBS, followed by lysis in 20 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 0.5% deoxycholate, 0.15 M NaCl, 5 mM EDTA, 30 mM $Na_7P_2O_7$, 100 μM Na_3VO_4, 1% aprotinin and 1 mM PMSF. The cleared cell lysates were immunoprecipitated with anti-phosphotyrosine antibody (PY20; Transduction Laboratories) and the proteins were separated by SDS-PAGE. After completed electrophoresis, the proteins were electrotransferred onto Hybond C-extra nitrocellulose membrane and the receptor band was visualized by autoradiography. The filter piece containing the receptor band was washed with water and incubated in the presence of 100 mg/ml CNBr in 70% formic acid for 2 hours. The digest was lyophilized and washed with 0.1 M ammonium bicarbonate containing 0.1% Triton X-100. The pellet was dissolved in 50 mM ammonium bicarbonate and immunoprecipitated with a peptide antiserum raised against amino acid residues 771-789 in the human PDGF β-receptor, kindly provided by T. O. Daniel, Vanderbilt University, Nashville, TN (Kumjian et al., 1989). After washing the immunoprecipitate with 0.005% Triton X-100, 50 mM ammonium bicarbonate, the CNBr-cleaved fragments were eluted with 1% diethylamine, pH 11.5, and lyophilized. For peptide sequencing the lyophilized peptides were coupled to a Sequelon-AA membrane (Milligen/Bioresearch, Milford, MA) according to the manufacturer's instructions and Edman degradation was performed in an Applied Biosystem gas-phase sequencer modified for solid-phase sequencing. The fractions were applied on a thin-layer chromatography (TLC) cellulose plate and radioactivity in the fractions was estimated by exposure and analysis of the plate in a Fuji BioImager.

Confocal microscopy

PAE cells were cultured and stained with phalloidin, as described above. Cells were examined using a Multiprobe 2001 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA) equipped with a Nikon inverted microscope and argon/krypton laser. The FITC filter set 488/510DRLP/510EFLP (laser wavelength/primary beamsplitter/barrier filter) was purchased from Molecular Dynamics. Series of sequential optical sections through entire PAE cells were recorded, digitalized in 1,024×1,024 pixels (p) and stored for further analysis. A Nikon PlanApo ×60 NA objective was used. Pixel size and step size during scanning were optimized in order to avoid distortions in three-dimensional (3-D) structure. Furthermore, interpolation programs were used to enable computation, which corrects for unavoidable loss of information between adjacent sections in a section series. In order to emphasize edge definition in the image, section series were subjected to a 3-D-gradient filter using a kernel size of 3p×3p×3p. This filter measures rate of change in grey-scale intensities between adjacent pixels within the kernel.

In order to examine the 3-D distribution of phalloidin-stained structures, the slice viewer program (Molecular Dynamics) was employed on the filtered section series (see Fig. 5A,B,E,F). Using this program, it is possible to navigate within section series (depicted by the white solid lines) enabling simultaneous viewing of the phalloidin staining in all three planes (horizontal, *x-y*; planar, *x-z*; and sagittal, *y-z*).

In order to further characterize actin organization within PAE cells, surface projections of phalloidin stained structures were acquired as follows: Phalloidin-stained PAE cells were scanned, stored and gradient filtered, as described above. The section series were then subjected to a 3-D gaussian filter using a kernel size of 3p×3p×3p in order to reduce background noise. Thereafter, the information within the section series was depth coded, thereby converting the 3-D section series into a 2-D depth map. Brighter pixels represent surface pixels higher up in the section series stack, i.e. closest to the objective, whereas darker pixels represent surface pixels lower down in the section series, i.e. furthest away from the objective. Black represents the surface of the coverslip. In order to obtain a 3-D image of the surface of the phalloidin-stained structures, the 2-D depth map was subjected to the plotting program, fish net, using the surface setting and a line density of 1/2. This program converts the depth map into a 2-D column diagram (in the *x-y* plane) where each column is 2 pixels wide (line density). This diagram was then tilted 60° in the *y*-axis in order to give depth perception to the image (see Fig. 5C,D,G,H).

Sub-region programs together with ray-modelling was employed to study certain regions of the unfiltered section series in more detail. The ray-model allows the transparency of the image to be regulated enabling a more detailed view of very dense structures (see Fig. 5I).

Affinity chromatography on immobilized Y778 peptide

A peptide comprising Y778 (D-Y771-M-A-P-Y775-D-N-Y778-V-P-S-A-P-Q) either unphosphorylated or phosphorylated at Y778, was coupled to Affigel-10 in 20 mM Hepes, pH 7.5. PAE cells were cultured in MCDB medium lacking methionine and cysteine, supplemented with [^{35}S]methionine/cysteine (Promix; Amersham), for 3 hours at 37°C. The cells were lysed in 20 mM Hepes, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 1% SDS, 100 μM Na_3VO_4, 1% aprotinin, and 1 mM PMSF. Unsolubilized material was pelleted and the supernatant was precleared by incubation with rabbit serum coupled to Affigel-10. Precleared cell lysates were incubated with Affigel-coupled peptides for 1 hour at +4°C. The peptide-beads were washed three times with 20 mM Hepes, pH 7.5, 500 mM NaCl, 1% Nonidet P-40, 100 μM Na_3VO_4, 1% aprotinin, and 1 mM PMSF, and once with lysis buffer. The samples were boiled in SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

RESULTS

Y775 and Y778 are phosphorylation sites in vivo

The kinase insert domain of the PDGF β-receptor contains seven tyrosine residues, of which Y716, Y740, Y751 and Y771 have previously been shown to be autophosphorylation sites (Claesson-Welsh, 1994). We examined whether phosphorylation also occur on residues Y775 and Y778, since their surrounding amino acid residues indicated the possibility that this region of the molecule could participate in interactions with SH2 domain-containing signal transduction molecules.

PAE cell lines expressing the wild-type PDGF β-receptor, or mutant receptors in which either one or both of Y775 or Y778 had been replaced with phenylalanine residues, were established by transfection of cells. The binding of iodinated PDGF was used to select several cell lines expressing each of the different receptors, at similar levels. The different cell lines were then labeled in vivo with [^{32}P]orthophosphate. The cultures were stimulated with PDGF, followed by lysis and immunoprecipitation with a PDGF β-receptor antiserum. The samples were separated by SDS-PAGE and transferred to nitrocellulose membrane, which was exposed on film. Areas of the membrane corresponding to ^{32}P-labeled wild-type and mutant receptors were cut out and incubated in the presence of CNBr. The digests were neutralized and subjected to immunoprecipitation, using an antibody raised against amino acid residues 771-798 in the PDGF β-receptor (Kumjian et al., 1989). The immunoprecipitated material was analyzed by Edman degradation. Fig. 1A shows the content of ^{32}P-radioactivity in the series of cycles resulting from Edman degradation of the CNBr fragment of the wild-type receptor. A small peak of radioactivity at the expected position of Y775 (fraction 3) was seen, as well as a considerable peak at the position of Y778 (fraction 6). In addition, there was trailing of radioactivity in the fractions following fractions 3 and 6, typically arising as a result of the less than 100% repetitive yield for each cycle. Edman degradation of

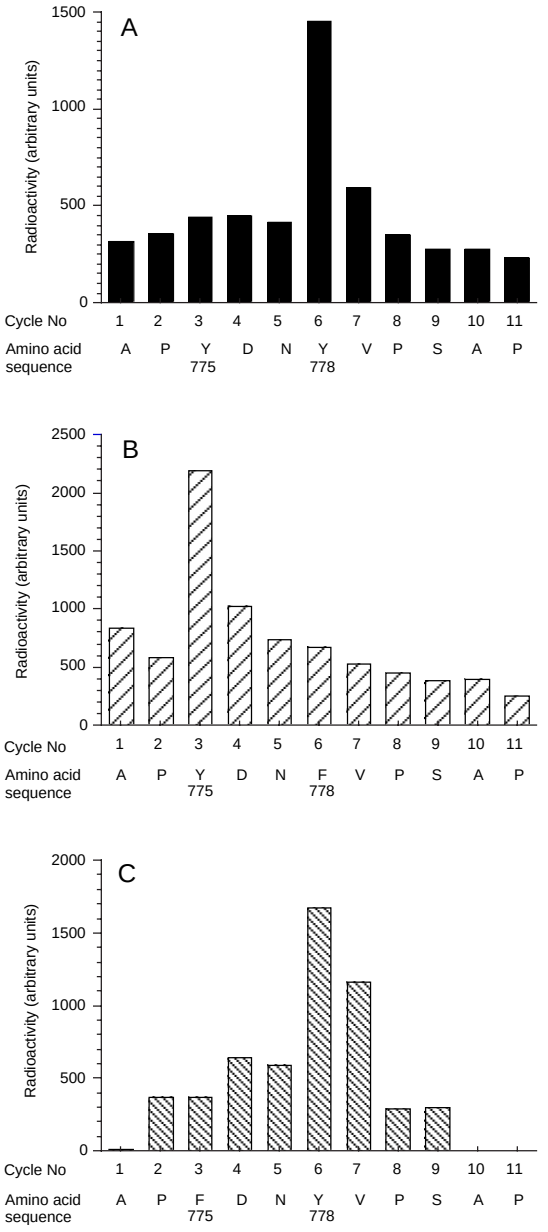

Fig. 1. Edman degradation of immunoprecipitated material from CNBr-digested wild-type and mutated PDGF β-receptors. Cells expressing the wild-type or mutant PDGF β-receptors were labeled with [^{32}P]orthophosphate, lysed and immunoprecipitated with anti-phosphotyrosine antibody. After SDS-PAGE and electroblotting, the piece of membrane containing the receptor was cut out and incubated with CNBr. Digested fragments were subjected to immunoprecipitation using an antibody against the human PDGF β-receptor, amino acid residues 771-789. The immunoprecipitated material was subjected to Edman degradation and the ^{32}P-radioactivity in different cycles was determined. Analyses of the wild-type PDGF β-receptor (A), as well as of the receptor mutant Y778F (B) and the receptor mutant Y775F (C) are shown. The sequence shown below the histograms represents the CNBr fragment containing Y775 and Y778.

immunoprecipitated CNBr-digested Y778F mutant receptor (Fig. 1B) lacked radioactivity in fraction 6, corresponding to Y778F, confirming the identity of the immunoprecipitated CNBr-peptide. On the other hand, the fraction corresponding to Y775 contained a threefold increased level of radioactivity, as compared with the background, i.e. considerably more than in the wild-type receptor. Thus, phosphorylation of Y775 occurred with an increased stoichiometry in the mutant receptor lacking Y778, compared with the wild-type receptor. Fig. 1C shows the result of Edman degradation of CNBr-digested immunoprecipitated material from the Y775F mutant receptor, which as expected, shows loss of radioactivity in fraction 3, corresponding to Y775F. The amount of radioactivity in fraction 6, corresponding to Y778, was similar to that of the wild-type PDGF β-receptor. These results show that both Y775 and Y778 are phosphorylation sites in vivo, with a higher stoichiometry of phosphorylation on Y778 than Y775, and, moreover that a mutant receptor lacking Y778 shows an increased phosphorylation at Y775.

The mutant receptors possess intact PDGF-stimulatable kinase activity and ability to mediate increase in DNA synthesis

To ensure that the mutant receptors were functional tyrosine kinases, we used PAE cells expressing wild-type PDGF β-receptors, or Y775F, Y778F and Y775/778F mutant receptors in an in vitro immunocomplex kinase assay. The different cell lines were serum-starved over-night, stimulated with PDGF for 5 minutes at 37°C, lysed and immunoprecipitated with a PDGF β-receptor antiserum. The immunoprecipitates were immobilized on Protein A-Sepharose beads and incubated in the presence of [γ-^{32}P]ATP. The samples were analyzed by SDS-PAGE (Fig. 2). The different mutant receptors showed increased incorporation of ^{32}P-radioactivity in response to PDGF, on the receptor itself and on associated molecules, indicating that replacement of Y775 or Y778, or both, with phenylalanine residues is compatible with intact kinase activity. The pattern of phosphorylated, receptor-associated components was, however, different between cells expressing the wild-type and the various mutant receptors, since a 130 kDa molecule was lost in cells expressing the Y778F and Y775/778F mutant receptors. In cells expressing the Y775F mutant, the association of the 130 kDa was considerably reduced.

The PAE cell lines expressing wild-type and mutant PDGF β-receptors were analyzed for their capacity to mediate increase in DNA synthesis in response to PDGF-treatment. Serum-starved cells were incubated for 20 hours in the presence of [^{3}H]thymidine and 1 or 10 ng/ml PDGF-BB or 10% FBS. Fig. 3 shows that the extent of incorporation of [^{3}H]thymidine was similar for the wild-type and the different mutant PDGF β-receptor expressing cells, although the basal levels of incorporation were slightly increased in cells expressing the Y775F and Y775/778F mutant receptors. These results were reproduced with several independent cell lines for each mutant receptor type.

These data show that point mutations of Y775, Y778 or both residues simultaneously, do not impair ligand-induced receptor kinase activity or signal transduction mediating increased DNA synthesis.

Actin rearrangement is changed in the PDGF β-receptor Y778F mutant

PDGF is known to induce proliferation and migration of receptor expressing cells (Claesson-Welsh, 1994). In conjunction with these responses, the actin cytoskeleton is

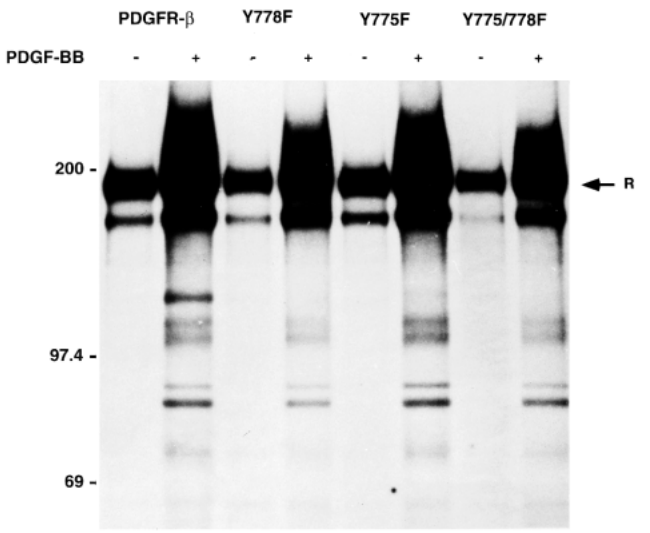

Fig. 2. In vitro kinase assay on PDGF β-receptor immunoprecipitates from cells expressing wild-type and mutant receptors. PAE cells expressing wild-type, or Y775F, Y778F or Y775/778F mutant receptors were stimulated or not with PDGF-BB (100 ng/ml), lysed and subjected to immunoprecipitation with a PDGF β-receptor antiserum. After washing, the immobilized immunoprecipitates were incubated in the presence of kinase buffer and [γ-^{32}P]ATP. The samples were separated by SDS-PAGE, whereafter the gel was dried and exposed on film. To the left is shown migration rates of marker proteins run in parallel; to the right is indicated the migration rate of the PDGF β-receptor.

rearranged. We examined actin rearrangement in PAE cells expressing the wild-type and mutant PDGF β-receptors (Fig. 4). The cells were stained with phalloidin, after PDGF-stimulation, to visualize the actin fibers. As seen in Fig. 4, the wild-type PDGF β-receptor expressing PAE cells exhibited edge ruffles as well as more centrally located circular ruffles. In PAE cells expressing the Y778F mutant, a new type of ruffle appeared, which was exaggerated and showed no apparent organization (Fig. 4). About 35% of the PDGF-stimulated Y778F cells exhibited these 'giant' ruffles (see below). In the Y775F mutant expressing cells, staining with phalloidin after PDGF-stimulation showed a pattern of actin distribution very similar to that of the wild-type receptor expressing cells. The Y775/778F double mutant on the other hand, showed the same phenotype as the Y778F mutant, with intensely stained patches of actin at the rim of the cells. However, both Y778F and Y775/778F expressing PAE cells contained circular ruffles and occasional normal edge ruffles.

Analysis of cytoskeletal rearrangement by confocal microscopy

In order to further delineate the structure of the novel type of PDGF-induced actin ruffles in the Y778F mutant receptor cells, confocal microscopy combined with special imaging techniques was employed. PAE cells expressing wild-type receptors or Y775F or Y778F mutant receptors were stimulated with PDGF-BB for 5 minutes and stained for actin using FITC-phalloidin, as described above. Series of optical sections through entire PAE cells were recorded and processed using the slice viewer program (Fig. 5A,B,E,F), enabling analysis of the phalloidin staining pattern in the planar, sagittal and horizontal axes. The patterns of actin organization were similar in PDGF-BB-stimulated wild-type and Y775F mutant receptor expressing cells (data not shown). Unstimulated wild-type and Y778F mutant receptor cells also did not differ in their actin organization (Fig. 5A,B). When cells where stimulated with PDGF-BB, actin organization was markedly different in the Y778F mutant receptor cells as compared with the wild-type receptor cells. In the wild-type receptor cells, edge ruffles were discerned in the planar and sagittal sections as uniform in appearance, consisting of rounded unilayer actin bundles of homogenous size running in parallel to each other (Fig. 5E). Similar edge ruffle structures were seen in the Y778F mutant receptor expressing PAE cells (Fig. 5F). In these cells, the novel ruffle structures seen by the conventional fluorescence microscopy described above, were strikingly large and multilayered and appeared in the planar and sagittal sections as irregular in shape and size (Fig. 5F).

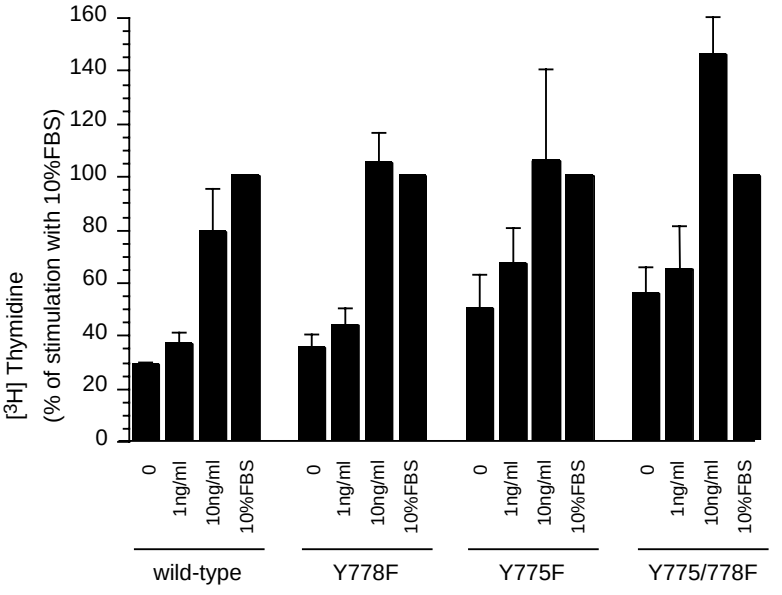

Fig. 3. Increased DNA synthesis in PDGF-stimulated wild-type and mutant PDGF β-receptor expressing cells. PAE cells expressing the wild-type PDGF β-receptor or Y775F, Y778F or Y775/778F mutant receptors were serum-starved and then either left unstimulated or stimulated with different concentrations of PDGF-BB or with 10% FBS, in the presence of [^{3}H]thymidine. The incorporation of [^{3}H]thymidine into DNA is shown.

In order to further elucidate the organization of these structures, the same series of sections were processed differently, so that the surface of the phalloidin staining could be appreciated (Fig. 5C,D,G,H). In unstimulated cells, no difference could be discerned in the PAE cells expressing wild-type receptors, as compared with cells expressing the Y778F mutant receptor (Fig. 5C and D). Using this imaging technique, edge ruffles in the PDGF-BB-stimulated wild-type receptor cells (Fig. 5G) consisted of upward protrusions of uniform structure confined to the edge of the cell. In contrast, the novel ruffle type in the PDGF-BB-stimulated Y778F mutant receptor cells exhibited a bulky appearance with a broad base that was not confined to the cell edges (Fig. 5H). By use of ray-modelling, an imaging technique which allows for regulation of the transparency of the section series, the internal structures of the different ruffle types were further examined (Fig. 5I). In this analysis, conventional edge ruffles in wild-type and Y778F mutant receptor cells appeared as a dense, uniform actin front, followed by perpendicularly oriented fine actin bundles (Fig. 5I, and data not shown). In the aberrant ruffles, actin organization was chaotic in appearance and no distinct pattern could be discerned within a single aberrant actin ruffle, or when comparing different aberrant actin ruffles with each other (Fig. 5I).

Time and dose-dependent ruffle formation in the different cell lines were examined; no differences in formation of the different ruffle types were seen when stimulating with 5, 20 or 100 ng/ml PDGF-BB (data not shown). Stimulation with PDGF-BB was performed for 0, 2, 5, 10, 20 and 60 minutes.

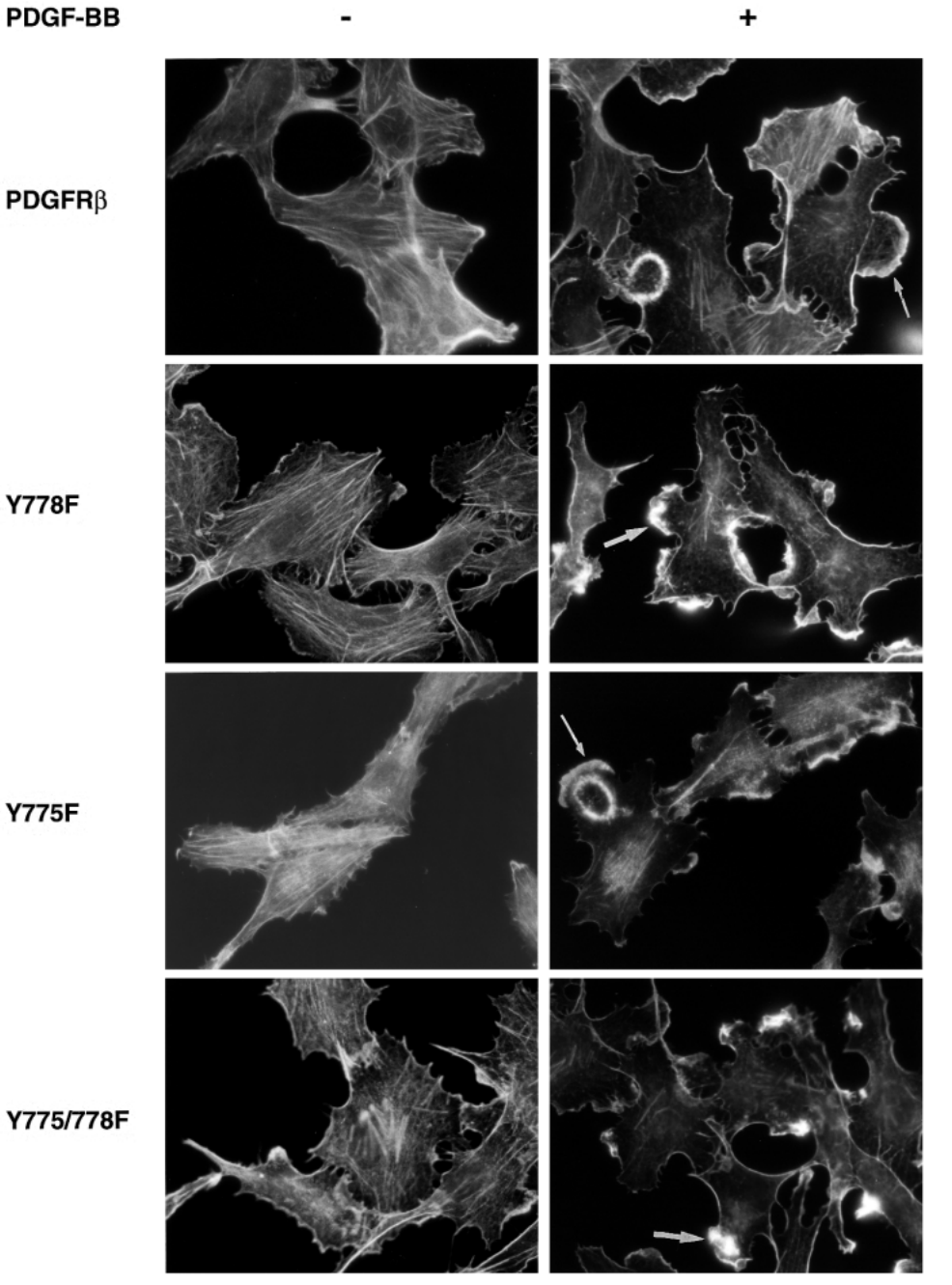

Fig. 4. Actin rearrangement in PDGF-BB stimulated PAE cells expressing wild-type and mutant PDGF β-receptors. Unstimulated (–) or PDGF-BB-stimulated (+; 100 ng/ml for 5 minutes at 37°C). PAE cells expressing wild-type or Y775F, Y778F, or Y775/778F mutant receptors were fixed, permeabilized and stained with TRITC-phalloidin and analyzed by fluorescence microscopy. Thin arrow in the Y775F sample indicates an edge ruffle adjacent to a circular ruffle. Thick arrow in the Y778F sample indicates an aberrant ruffle.

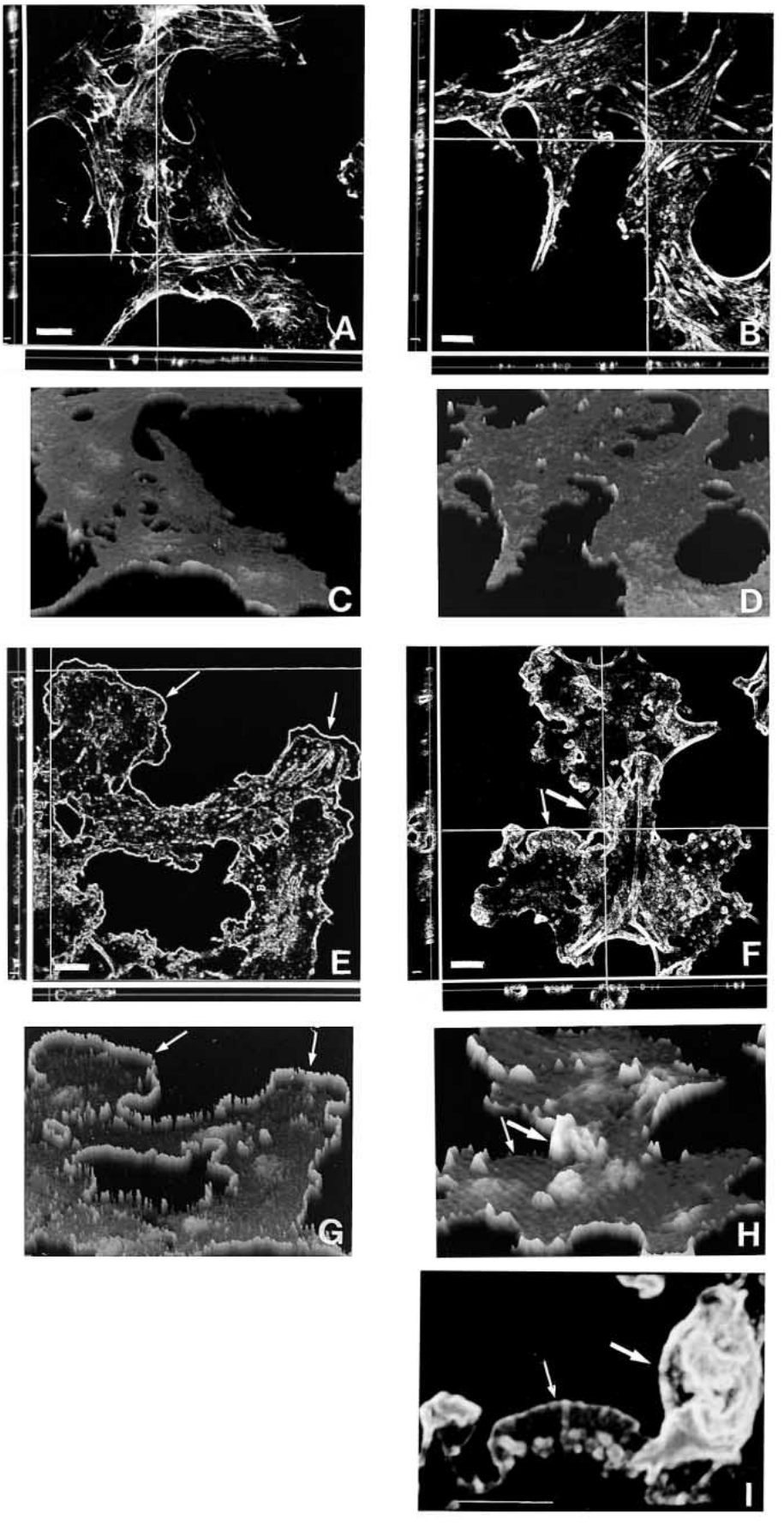

Fig. 5. Characterization of effects of PDGF-BB on actin reorganization using confocal microscopy. PAE cells expressing the wild-type receptor (A,C,E,G) or the Y778F mutant receptor (B,D,F,H,I) were left unstimulated (A-D) or were stimulated with PDGF-BB (E-I) and stained using FITC-phalloidin. Section series were analyzed using the slice viewer program (A,B,E,F), by depth coding combined with fish plotting (C,D,G,H) and by ray-modelling (I). Thin arrows indicate edge ruffles and thick arrows indicate aberrant ruffles. (I) A subregion of the same PDGF-BB stimulated mutant Y778F cell as in F and H, but depicted using the ray-model mode. In the slice viewer projections, the main frame depicts a horizontal (*x*-*y* view) optical section through the cell. The subwindow to the left of the main frame depicts the section series in the sagittal angle (*y*-*z* view). The subwindow below the frame depicts the section series in the planar angle (*x*-*z* view). The white solid lines in the main frame depict where the sagittal and planar angles have been chosen. Thick bars, 5 μM; thin bars, 5 μM; subwindow bars, 2 μM.

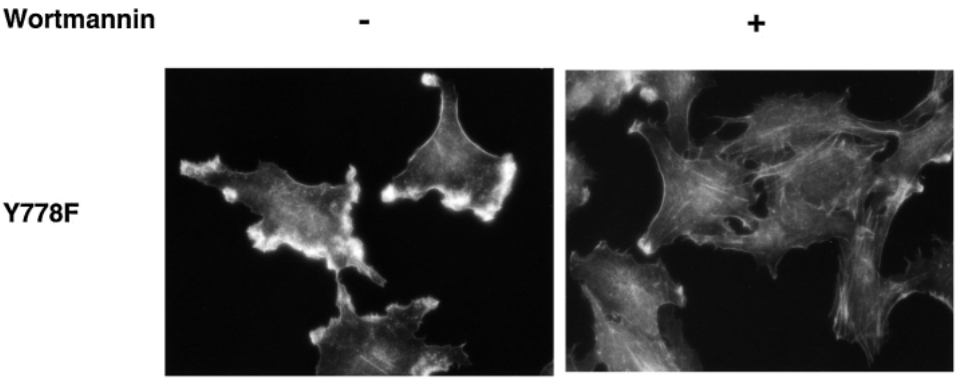

Fig. 6. PAE cells expressing the Y778F mutant were treated (+) or not (–) with 30 nM wortmannin for 10 minutes at 37°C, before stimulation with 100 ng/ml PDGF-BB for 10 minutes at 37°C in the presence of 30 nM wortmannin in the (+) sample. Cells were processed for staining with TRITC-phalloidin, as described for Fig. 4.

In the wild-type cells, circular ruffles and edge ruffles appeared after 2 minutes of stimulation and were to a large extent gone after 20 minutes. In the Y778F mutant receptor cells, circular and edge ruffles, as well as aberrant ruffles were apparent after 2 minutes of stimulation. After 20 minutes, circular and edge ruffles were gone, similar as for the wild-type receptor cells. The aberrant ruffles, however, prevailed at the 60 minute time point, although at decreased numbers as compared with the earlier time points.

Signal transduction involving PI3-kinase, PLC-γ and RasGAP in the wild-type and mutant receptor expressing cells

We have previously shown that edge ruffling and chemotaxis require receptor-binding and activation of PI3-kinase (Wennström et al., 1994a,b). Evidence for an involvement of PLC-γ and RasGAP in PDGF-induced chemotaxis has also been presented (Kundra et al., 1994). Furthermore, cells expressing a constitutively active PI3-kinase (Reif et al., 1996) exhibit ruffles very similar to the giant ruffles in PDGF-stimulated Y778F cells. We examined whether the novel type of ruffle-formation in the Y778F expressing cells could be due to disturbances in the binding or activation of these signal transduction molecules. The levels of PI3-kinase activity, and tyrosine phosphorylation of PLC-γ and RasGAP were, however, similar in cells expressing the wild-type receptor as compared with cells expressing the Y778F mutant receptor (data not shown). Treatment of cells expressing the Y778F mutant receptor with the fungal derivative wortmannin (Arcaro and Wymann, 1993), to inhibit PI3-kinase activity, attenuated formation of the giant ruffles (Fig. 6). These data indicate that PI3-kinase activity is necessary for formation of the giant ruffles, but that the phenotype of the Y778F mutation cannot be explained on the basis of deregulation of PI3-kinase activity.

71 and 78 kDa components bind to immobilized phosphorylated peptide containing Y778

In order to test whether phosphorylated Y778 would be capable of interacting with potential signal transduction molecules, affinity chromatography using immobilized synthetic phosphorylated peptides, was performed. Incubation of beads with immobilized phosphorylated peptide corresponding to the region around Y778 with cell lysates derived from [^{35}S]methionine/cysteine-labeled cells resulted in retention of several components including molecules with the apparent molecular masses of 71 and 78 kDa; the binding was competed by addition of 200 μM free phosphorylated Y778(P) peptide during the affinity chromatography (Fig. 7, lane 6), but not by addition of 200 μM free unphosphorylated Y778 peptide (Fig. 7, lane 5). Moreover, no binding of these components to immobilized unphosphorylated peptide was observed.

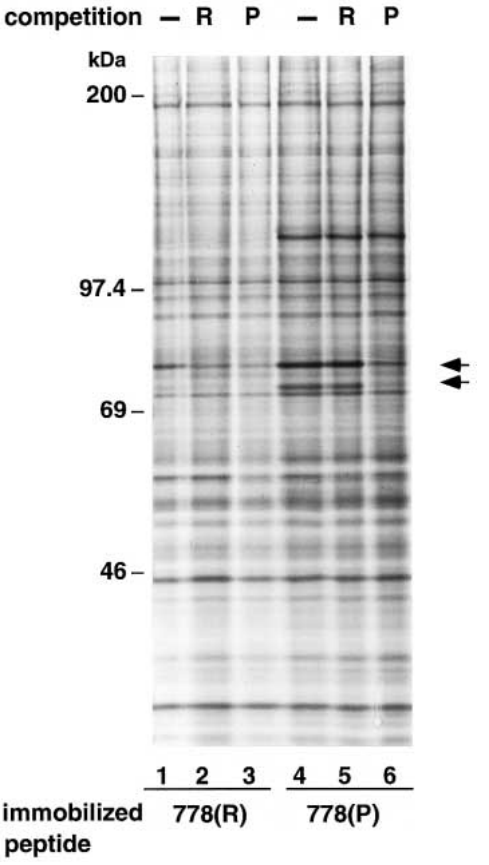

Fig. 7. Binding of [^{35}S]methionine/cysteine labeled proteins to immobilized phosphorylated or unphosphorylated peptides. PDGF β-receptor expressing PAE cells were labeled with [^{35}S]methionine/cysteine mix for 3 hours and then lysed. The cleared cell lysate was incubated with synthetic phosphorylated or unphosphorylated Y778 peptides immobilized on Affigel. Soluble phosphorylated (P) or unphosphorylated (R) peptides were included in the reaction to demonstrate phosphotyrosine-dependent binding of the 71 and 78 kDa components, indicated by arrows to the right. To the left is shown migration rates of marker proteins run in parallel.

DISCUSSION

In this work, we describe two novel phosphorylation sites in the PDGF β-receptor kinase insert, at Y775 and Y778. Mutation of these tyrosine residues to phenylalanine residues did not affect kinase activity or mitogenic signal transduction. Cells expressing the Y778F point mutant exhibited in response to PDGF-BB a novel type of disorganized actin-containing ruffle structure, indicating that Y778 is involved in regulation of the actin cytoskeleton.

The small GTP-binding proteins Rac, Rho and cdc42 have been shown to be critical for regulation of the actin cytoskeleton (Lim et al., 1996). Microinjection of active, GTP-bound Rac induces formation of edge ruffles in Swiss 3T3 cells (Ridley et al., 1992), whereas Rho is involved in formation of actin stress fibers (Ridley and Hall, 1992). Cdc42 on the other hand appears to regulate formation of microspikes (Nobes and Hall, 1995). These different actin-containing structures appear on most adherent cell types in tissue culture immediately upon stimulation, e.g. with growth factors, but the consequences of the actin structures for cellular responses in vivo are in most cases not clear. The level of GTP-bound Rac has been shown to be affected by activation of PI3-kinase (Hawkins et al., 1995). In agreement, the appearance of edge ruffles on PDGF-stimulated cells is lost when the target cells express a mutant PDGF β-receptor, unable to bind and mediate activation of PI3-kinase. It has also been shown that PI3-kinase activity is important for PDGF-directed migration of cells. This indicates that edge ruffles may represent lamellopodia. Lamellopodia are structures on migrating cells, which extend the cells towards the stimulus and through which the leading front of the cell makes new connections with the substratum. It is possible that the giant ruffles in cells expressing the Y778F mutant reflect an increased propensity for lamellopodia formation. On the other hand, conventional edge ruffles were also present on PDGF-stimulated PAE cells expressing the mutant Y778F receptor, and these cells migrated towards PDGF in a manner similar to that of cells expressing the wild-type PDGF β-receptor (data not shown).

The novel aberrant ruffles described in this work as large disorganized structures appearing in the dorsal side of PDGF-stimulated cells expressing a mutant Y778F PDGF β-receptor, could possibly arise as a result of deregulation of any of Rac, Rho or cdc42. However, PI3-kinase activity, measured as phosphorylation in vitro of phosphatidylinositol, was similar in PDGF-stimulated PAE cells expressing wild-type or mutant Y778F PDGF β-receptors. The signal transduction molecules PLC-γ and RasGAP, which have shown to be involved in regulation of cellular migration, but not in actin reorganization, were also not affected by the Y778F mutation. Treatment of cells with wortmannin to inhibit PI3-kinase activity, inhibited formation of giant ruffles, indicating that PI3-kinase activity is a prerequisite for this deregulated form of cytoskeletal arrangement.

Phosphorylation of Y775 has previously been shown to create a binding site for Grb7, an SH2 domain-containing protein which is amplified in breast cancer (Yokote et al., 1996). In addition, the transcription factor Stat5 is able to bind to phosphorylated Y775 (Valgeirsdóttir et al., 1997). It is, however, unlikely that the phenotype of the Y778F receptor mutant is due to an increased interaction with these signaling molecules, as a result of the observed increased phosphorylation of Y775 (Fig. 1), since the Y775/778F mutant receptor also induced aberrant ruffles. We therefore analyzed whether phosphorylated Y778 in the PDGF β-receptor is capable of interacting with signal transduction molecule(s) that potentially could mediate the effect on actin organization, e.g. via regulation of the activity of small GTP-binding proteins. Thus, phosphopeptides corresponding to the region around Y778 in the PDGF β-receptor were shown to bind cytoplasmic components in a phosphorylation-dependent manner. The migration rate of two of these components (71 and 78 kDa) are not compatible with those of already characterized signal transduction molecules. Another candidate molecule with potential signal transduction properties downstream of phosphorylated Y778, is the 130 kDa component, which is present in the in vitro kinase assay on sample from PDGF-stimulated cells expressing the wild-type PDGF β-receptor, but which is lost in the PDGF-stimulated Y778F sample (Fig. 2). Large scale purification and cDNA cloning will be undertaken to determine the structure and function of these molecules.

We thank T. O. Daniel, Vanderbilt University, Nashville, TN, USA, for the PDGF β-receptor antiserum, Mathilda Johnell and Agneta Siegbahn, Department of Clinical Chemistry, Uppsala Akademiska Hospital, Uppsala, Sweden, for performing chemotaxis assays and Ingegärd Schiller for preparation of the manuscript. These studies were partially supported by a grant from the Axel and Margaret Ax:son Johnson Foundation. Lena Claesson-Welsh was supported by the Swedish Cancer Society.

REFERENCES

Arcaro, A. and Wymann, M. P. (1993). Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem. J.* **296**, 297-301.

Claesson-Welsh, L. (1994). Platelet-derived growth factor receptor signals. *J. Biol. Chem.* **269**, 32023-32026.

Claesson-Welsh, L., Eriksson, A., Morén, A., Severinsson, L., Ek, B., Östman, A., Betsholtz, C. and Heldin, C.-H. (1988). cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules. *Mol. Cell. Biol.* **8**, 3476-3486.

Hansen, K., Johnell, M., Siegbahn, A., Rorsman, C., Engström, U., Wernstedt, C., Heldin, C.-H. and Rönnstrand, L. (1996). Mutation of a Src phosphorylation site in the PDGF β-receptor leads to increased PDGF-stimulated chemotaxis but decreased mitogenesis. *EMBO J.* **15**, 5299-5313.

Hawkins, P. T., Eguinoa, A., Qiu, R.-G., Stokoe, D., Cooke, F. T., Walters, R., Wennström, S., Claesson-Welsh, L., Evans, T., Symons, M. and Stephens, L. (1995). PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. *Curr. Biol.* **5**, 393-403.

Heldin, C.-H. and Westermark, B. (1996). Role of platelet-derived growth factor *in vivo*. In *The Molecular and Cellular Biology of Wound Repair* (ed. R. A. F. Clark), pp. 249-273. Plenum Press, New York.

Kumjian, D. A., Wahl, M. I., Rhee, S. G. and Daniel, T. O. (1989). Platelet-derived growth factor (PDGF) binding promotes physical association of PDGF receptor with phospholipase C. *Proc. Nat. Acad. Sci. USA* **86**, 8232-8236.

Kundra, V., Escobedo, J. A., Kazlauskas, A., Kim, H. K., Rhee, S. G., Williams, L. T. and Zetter, B. R. (1994). Regulation of chemotaxis by the platelet-derived growth factor receptor-β. *Nature* **367**, 474-476.

Lim, L., Manser, E., Leung, T. and Hall, C. (1996). Regulation of phosphorylation pathways by p21 GTPases. The p21 Ras-related Rho subfamily and its role in phosphorylation signalling pathways. *Eur. J. Biochem.* **242**, 171-185.

Nobes, C. D. and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia and filopodia. *Cell* **81**, 53-62.

Panayotou, G. and Waterfield, M. D. (1992). Phosphatidyl-inositol 3-kinase: a key enzyme in diverse signalling processes. *Trends Cell Biol.* **2**, 358-360.

Pawson, T. (1995). Protein modules and signalling networks. *Nature* **373**, 573-580.

Reif, K., Nobes, C. D., Thomas, G., Hall, A. and Cantrell, D. A. (1996). Phosphatidylinositol 3-kinase signals activate a selective subset of Rac/Rho-dependent effector pathways. *Curr. Biol.* **6**, 1445-1455.

Rhee, S. G., Suh, P.-G., Ryu, S.-H. and Lee, S. Y. (1989). Studies of inositol phospholipid-specific phospholipase C. *Science* **244**, 546-550.

Ridley, A. J. and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389-399.

Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401-410.

Valgeirsdóttir, S., Paukku, K., Silvennoinen, O., Heldin, C.-H. and Claesson-Welsh, L. (1997). Activation of Stat5 by platelet-derived growth factor (PDGF) is dependent on phosphorylation sites in PDGF β-receptor juxtamembrane and kinase insert domains. *Oncogene* (in press).

Wahl, M. and Carpenter, G. (1991). Selective phospholipase C activation. *BioEssays* **13**, 107-113.

Wennström, S., Hawkins, P., Cooke, F., Hara, K., Yonezawa, K., Kasuga, M., Jackson, T., Claesson-Welsh, L. and Stephens, L. (1994a). Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. *Curr. Biol.* **4**, 385-393.

Wennström, S., Siegbahn, A., Yokote, K., Arvidsson, A.-K., Heldin, C.-H., Mori, S. and Claesson-Welsh, L. (1994b). Membrane ruffling and chemotaxis transduced by the PDGF β-receptor require the binding site for phosphatidylinositol 3′ kinase. *Oncogene* **9**, 651-660.

Yokote, K., Margolis, B., Heldin, C.-H. and Claesson-Welsh, L. (1996). Grb7 is a downstream signaling component of platelet-derived growth factor α- and β-receptors. *J. Biol. Chem.* **271**, 30942-30949.