In Situ Detection of Phosphorylated Platelet-derived Growth Factor Receptor β Using a Generalized Proximity Ligation Method*

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Improved methods are needed for *in situ* characterization of post-translational modifications in cell lines and tissues. For example, it is desirable to monitor the phosphorylation status of individual receptor tyrosine kinases in samples from human tumors treated with inhibitors to evaluate therapeutic responses. Unfortunately the leading methods for observing the dynamics of tissue post-translational modifications *in situ*, immunohistochemistry and immunofluorescence, exhibit limited sensitivity and selectivity. Proximity ligation assay is a novel method that offers improved selectivity through the requirement of dual recognition and increased sensitivity by including DNA amplification as a component of detection of the target molecule. Here we therefore established a generalized *in situ* proximity ligation assay to investigate phosphorylation of platelet-derived growth factor receptor β (PDGFRβ) in cells stimulated with platelet-derived growth factor BB. Antibodies specific for immunoglobulins from different species, modified by attachment of DNA strands, were used as secondary proximity probes together with a pair of primary antibodies from the corresponding species. Dual recognition of receptors and phosphorylated sites by the primary antibodies in combination with the secondary proximity probes was used to generate circular DNA strands; this was followed by signal amplification by replicating the DNA circles via rolling circle amplification. We detected tyrosine phosphorylated PDGFRβ in human embryonic kidney cells stably overexpressing human influenza hemagglutinin-tagged human PDGFRβ in porcine aortic endothelial cells transfected with the β-receptor, but not in cells transfected with the α-receptor, and also in immortalized human foreskin fibroblasts, BJ hTert, endogenously expressing the PDGFRβ. We furthermore visualized tyrosine phosphorylated PDGFRβ in tissue sections from fresh frozen human scar tissue undergoing wound healing. The method should be of great value to study signal transduction, screen for effects of pharmacological agents, and enhance the diagnostic potential in histopathology. ***Molecular & Cellular Proteomics 6:1500–1509, 2007.***

Studies of cell signaling have led to improved understanding of mechanisms governing cellular processes such as proliferation, migration, and apoptosis. They have also firmly established that common diseases such as cancer and cardiovascular diseases are driven by dysregulated signaling (1, 2). The activity of signaling proteins is to a large extent controlled by rapid PTMs,[1] including phosphorylation, ubiquitination, acetylation, and glycosylation (3). Progress in understanding cell signaling will require development of methods for monitoring the spatial and temporal dynamics of specific PTMs. Similarly rational development of inhibitors of signal transduction will depend on improved methods for *in situ* characterization of PTMs in tissues to reveal *e.g.* the tyrosine phosphorylation status of individual receptor tyrosine kinases in human tumor samples (1) and how this is influenced by therapy.

PDGFRβ is a transmembrane protein-tyrosine kinase that becomes dimerized and autophosphorylated at numerous sites upon binding of the cytokines PDGF-BB (4) or -DD (5), leading to activation of several signaling pathways that promote cell proliferation, motility, and survival (6). Studies of these cellular responses have demonstrated the importance of temporal and spatial control of PDGFR phosphorylation (7, 8). Immunofluorescence (IF) analyses with antibodies directed

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[1] The abbreviations used are: PTM, post-translational modification; ATC, anhydrotetracycline hydrochloride; BJ hTert, immortalized human foreskin fibroblasts; HEK, human embryonic kidney; HA, human influenza hemagglutinin; IF, immunofluorescence; PAE, porcine aortic endothelial; PAEα, PAE cells expressing PDGFRα; PAEβ, PAE cells expressing PDGFRβ; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PLA, proximity ligation assay; RCA, rolling circle amplification; RCP, rolling circle product.

against phosphorylated PDGFR remains the predominant method to monitor the distribution of PDGFR phosphorylation *in situ*. Unfortunately this method is limited in sensitivity and selectivity, precluding detailed analysis.

We have recently established a technique referred to as the *in situ* proximity ligation assay (*in situ* PLA, previously called P-LISA) and used it to demonstrate *in situ* protein-protein interactions in cells and tissues (9). *In situ* PLA is a further development of the proximity ligation strategy (10, 11), a protein detection method combining dual recognition of target proteins by pairs of affinity probes generating an amplifiable DNA reporter molecule that acts as a surrogate marker for the detected protein molecule or interacting molecules. *In situ* PLA uses rolling circle amplification (RCA) for localized detection of proteins or protein interactions in fixed cells or tissues. Antibodies covalently linked to oligonucleotides are used as proximity probes, forming templates for circularization of two additional oligonucleotides by enzymatic ligation. This ligation requires coincident binding by two affinity reagents and thereby increases the selectivity compared with single recognition assays. One of the oligonucleotides then serves as a primer for the RCA reaction, amplifying the circular DNA molecule ~1000-fold in 1 h using ϕ29 DNA polymerase (12). The product represents a bundle of single-stranded DNA composed of tandem repeats of complements of the DNA circle. Individual bundles are easily visualized by hybridization of complementary fluorescence-labeled oligonucleotides. Because *in situ* PLA gives rise to a signal at the location where the primary antibodies have bound, it is possible to use it to study the location of proteins and protein complexes in tissues and subcellular compartments.

In the present study we generalized the *in situ* PLA method by using secondary antibodies with attached DNA strands as proximity probes. This approach preserves the sensitivity and selectivity of the *in situ* PLA method while permitting the use of general, species-specific antibodies as proximity probes with any suitable primary antibody pair. We utilized the *in situ* PLA for detection of tyrosine phosphorylation of transfected and endogenous PDGFRβ in fixed cultured cells. The phosphorylated receptors were clearly visible as discrete signals permitting quantitative analyses. We furthermore visualized tyrosine phosphorylated PDGFRβ in tissue sections from fresh frozen human scar tissue.

EXPERIMENTAL PROCEDURES

Cell Culture and Tissue Sections—Human embryonic kidney 293 (HEK293) cells and HEK293 cells stably overexpressing the HA-tagged human PDGFRβ in an expression system that could be inhibited by tetracycline (13, 14) were grown in Dulbecco's modified Eagle's medium-F-12 Ham's nutrient mixture (1:1) (Invitrogen) containing 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin, and 1 mM L-glutamine. The expression of PDGFRβ in HEK293-PDGFRβ-HA cells was down-regulated by the addition of 200 ng/ml anhydrotetracycline hydrochloride (ATC) (Acros Organics, Geel, Belgium) to the medium 1 week prior to stimulation.

Immortalized human foreskin fibroblasts (BJ hTert) were grown in minimum essential medium (Invitrogen) containing 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 50 μg/ml gentamicin. Porcine aortic endothelial (PAE) cells, either untransfected or transfected with the PDGFRα or -β (PAEα and PAEβ), were grown in F-12 Ham's nutrient medium (Sigma) containing 10% FCS, 1% L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Two days before stimulation 23,000 HEK293 cells/well were seeded on Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) coated with growth factor-reduced Matrigel (BD Biosciences). For BJ hTert cells 15,000 cells/well were seeded. After 24 h the cells were starved for 8 h or overnight in medium containing 0.5% FCS. Subsequently the cells were stimulated with 1–100 ng/ml human PDGF-BB (Peprotech, Rocky Hill, NJ; powder dissolved and stored in 20 mM Hepes, pH 7.4, 0.5 M NaCl, 5 mg/ml BSA (New England Biolabs, Beverly, MA)) for 1 h on ice. Cells on glass slides were washed once in ice-cold PBS and fixed for 1 h in ice-cold 70% ethanol.

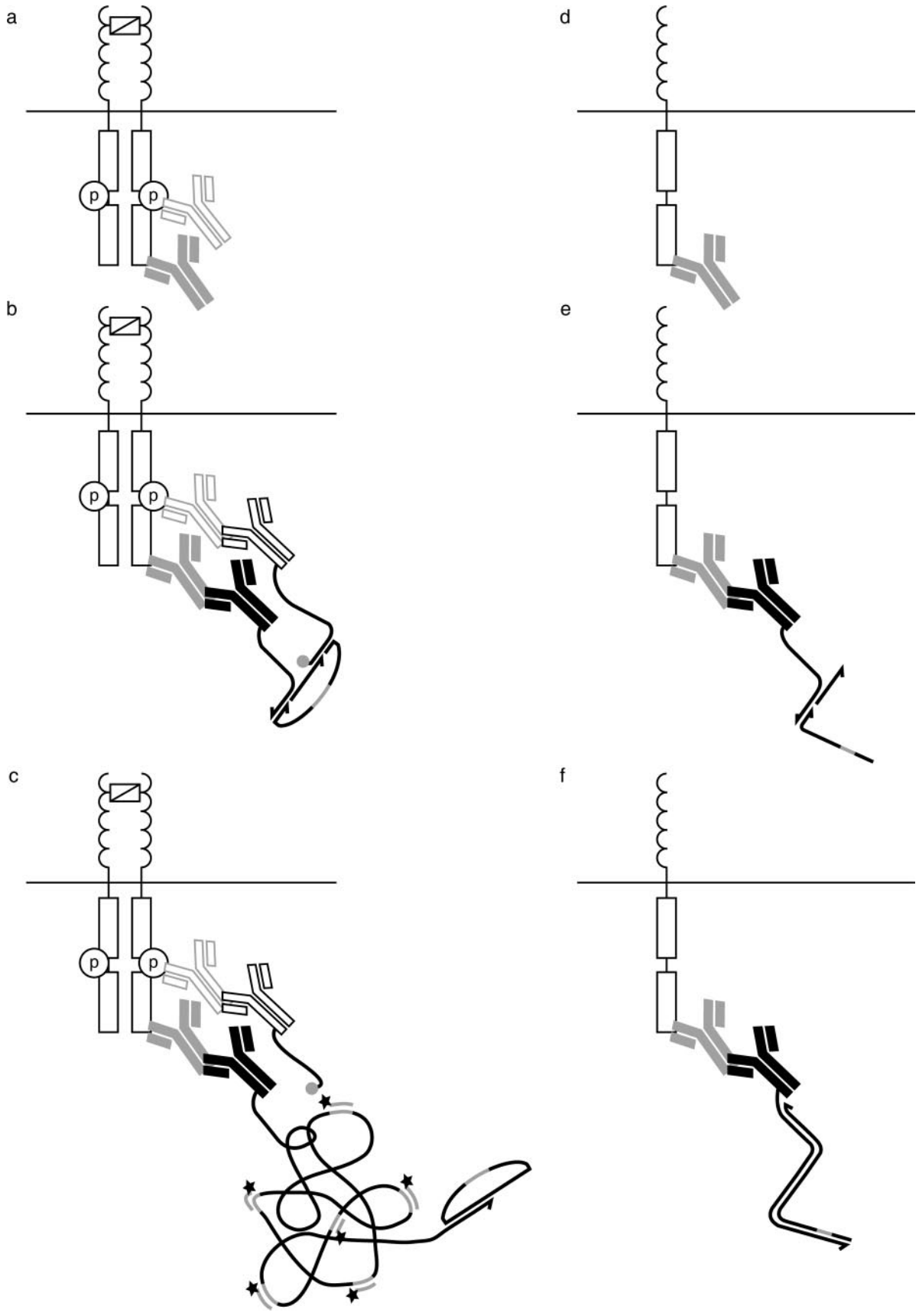
5,000 PAEα or PAEβ cells/well were seeded and 24 h later starved overnight in medium containing 1% FCS following stimulation with or without 100 ng/ml PDGF-BB (Chiron Corporation, Emeryville, CA; powder dissolved and stored in sodium acetate buffer, pH 4.5) on ice for 1 h. Additionally untransfected PAE cells were, after starvation overnight in 1% FCS, incubated with or without 100 μM pervanadate on ice for 1 h. Subsequently the cells were washed once in ice-cold PBS and fixed for 1 h in ice-cold 70% ethanol.

Preparation of Cell Lysates—10^6 BJ hTert cells were seeded on a 10-cm dish and after 2 days starved overnight in medium containing 0.5% FCS following stimulation with or without PDGF-BB (1–100 ng/ml) on ice for 1 h. Cells were then lysed in ice-cold lysis buffer (0.5% Triton X-100, 0.5% deoxycholic acid, 150 mM NaCl, 20 mM Tris, 10 mM EDTA, 30 mM sodium pyrophosphate, pH 7.5) supplemented with 200 μM vanadate, 1% aprotinin, and 2 mM PMSF.

SDS-PAGE/Immunoblotting—Proteins were separated on an 8% SDS-polyacrylamide gel and transferred to a PVDF membrane (Millipore, Billerica, MA) by semidry transfer. The membrane was blocked in TBS containing 5% BSA and 0.05% Tween 20 before overnight incubation with anti-PDGFRβ antibody (0.12 μg/ml; Cell Signaling Technology, Danvers, MA). Bound antibodies were visualized using ECL (GE Healthcare) after incubation with secondary antibodies conjugated with horseradish peroxidase, and signals were captured with a charge-coupled device camera (FUJIFILM Manufacturing U.S.A., Inc., Greenwood, SC). The membranes were then stripped and reprobed with anti-Tyr(P)-751 PDGFRβ antibody (0.8 μg/ml; Cell Signaling Technology) as described above.

Proximity Probe Design—The proximity probes consist of affinity-purified polyclonal antibodies with oligonucleotides covalently linked via their 5′-ends to each antibody. The non-priming proximity probes were composed of donkey anti-mouse antibody (catalog number 715-005-150, Jackson ImmunoResearch Laboratories, West Grove, PA) with the covalently linked amine-modified oligonucleotide (NH_2-AAA AAA AAA AGA CGC TAA TAG TTA AGA CGC TT[U UU] (the sequence within the brackets is 2′*O*-methyl-RNA); Trilink BioTechnologies, San Diego, CA), and for the RCA priming proximity probes donkey anti-rabbit antibodies (catalog number 711-005-152, Jackson ImmunoResearch Laboratories) were covalently linked to the amine-modified oligonucleotide (NH_2-AAA AAA AAA ATA TGA CAG AAC TAG ACA CTC TT; Trilink BioTechnologies). The proximity probes were conjugated by Solulink, San Diego, CA, using hydrazone linkage.

In Situ PLA Analysis of PDGFRβ in Cultured Cells—Glass slides with ethanol-fixed cells were blocked in 20% goat serum (Invitrogen), 2.5 ng/μl sonicated salmon sperm DNA (GE Healthcare), 2.5 mM L-cysteine (Sigma), 50 μg/ml RNase A (Promega, Madison, WI), 0.1% Tween 20, 5 mM EDTA in PBS for 2 h at 37 °C. Afterward slides were rinsed once with PBS, 0.1% Tween 20 before primary antibodies



mouse anti-phospho-PDGFRβ (Tyr(P)-751) (0.16 ng/μl; catalog number 3166, Cell Signaling Technology) and rabbit anti-PDGFRβ (2 ng/μl; catalog number 3169, Cell Signaling Technology) in 20% goat serum, 2.5 ng/μl sonicated salmon sperm DNA, 2.5 mM L-cysteine, 0.1% Tween 20 in PBS containing 5 mM EDTA were applied and incubated overnight at 4 °C.

Unbound primary antibodies were removed by washing the slides three times for 2 min each in PBS with 0.1% Tween 20. After the washing steps the proximity probes were prepared by separately incubating 0.75 ng/μl non-priming proximity probe and 0.15 ng/μl RCA priming proximity probe (final concentrations) per reaction in PBS, 5 mM EDTA, 20% goat serum, 2.5 ng/μl sonicated salmon sperm DNA, 2.5 mM L-cysteine, 1.5 mM sulfobenzoaldehyde (Solulink), 0.05% Tween 20 for 30 min at room temperature. After incubation the proximity probes were mixed, applied to the glass slide, and incubated for 1 h at 37 °C. The slides were washed in 10 mM Tris-HCl, pH 7.5, 0.1% Tween 20 for 5 min, twice in TBS, 0.05% Tween 20 for 2 min, and rinsed once with ligation buffer (10 mM Tris acetate, pH 7.5, 10 mM magnesium acetate, 50 mM potassium acetate). All subsequent washing steps were done in TBS, 0.05% Tween 20 for 2 min if not stated otherwise. Hybridization with 125 nM circularization oligonucleotide probes (5′phosphate-CTA TTA GCG TCC AGT GAA TGC GAG TCC GTC TAA GAG AGT AGT ACA GCA GCC GTC AAG AGT GTC TA and 5′-phosphate-GTT CTG TCA TAT TTA AGC GTC TTA A (Eurogentec, Seraing, Belgium)) in ligation buffer with 250 mM NaCl, 0.25 μg/μl BSA, and 0.05% Tween 20 was done for 30 min at 37 °C. After three washes the slides were rinsed once with ligation buffer. The circularization probes were ligated in ligation buffer containing 250 mM NaCl, 0.05 unit/μl T4 DNA ligase (Fermentas, Vilnius, Lithuania), 1 mM ATP (Fermentas), 0.25 μg/μl BSA, and 0.05% Tween 20 for 30 min at 37 °C. Prior to RCA the slides were washed three times and rinsed once in ϕ29 polymerase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, pH 7.5). RCA was allowed to proceed for 90 min at 37 °C in ϕ29 polymerase buffer containing 0.125 unit/μl ϕ29 DNA polymerase (Fermentas), 1 mM dNTPs (Fermentas), 5% glycerol (USB Corp., Cleveland, OH), 0.25 μg/μl BSA, and 0.05% Tween 20. After three additional washing steps 6.25 nM fluorescence-labeled probe Alexa 555-CAG TGA ATG CGA GTC CGT CT (MWG-BIOTECH, Ebersberg, Germany) was hybridized to the single-stranded RCA product in 2× SSC, 0.25 μg/μl BSA, 7.5 ng/μl poly(A) (Sigma), 0.05% Tween 20 for 30 min at 37 °C. The slides were then stained with a mouse anti-actin antibody (Cederlane, Hornby, Ontario, Canada), subsequently incubated with FITC-labeled rabbit anti-mouse antibody (Jackson ImmunoResearch Laboratories), and in the end counterstained with Hoechst 33342 (Sigma).

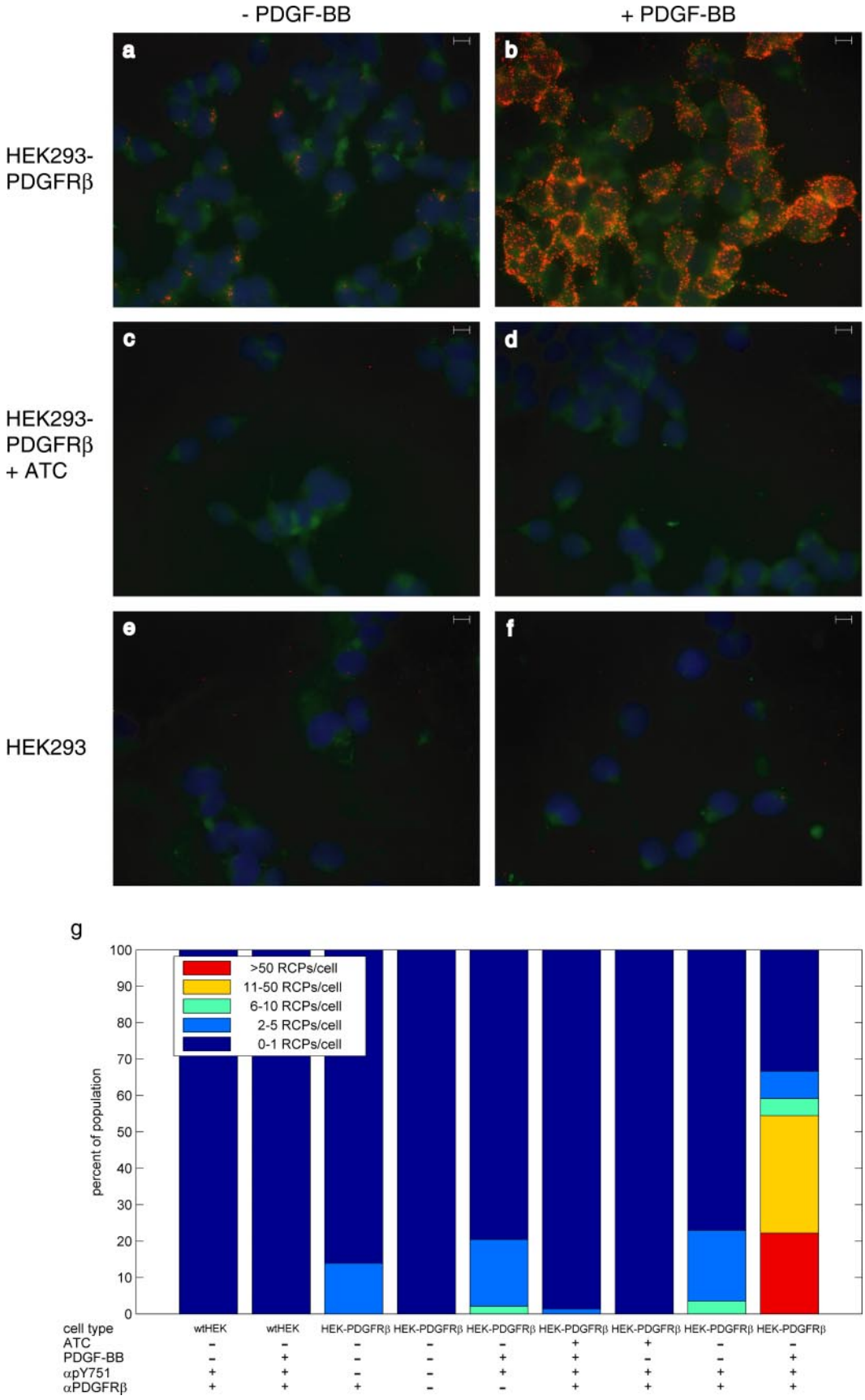
In Situ PLA Analysis of PDGFRβ in Fresh Frozen Tissue—Fully anonymized human tissue samples were obtained from the Fresh Tissue Biobank at the Department of Pathology, Uppsala University Hospital, in accordance with the Swedish Biobank Legislation. Tape-transfer frozen sections (4 μm thick; CryoJane, Instrumedics Inc., Hackensack, NJ) from fresh frozen human scar tissue were fixed in 70% ethanol on ice for 1 h. *In situ* PLA reactions were done as described above for the cultured cell experiments besides the following modifications of antibody and enzyme concentrations: 0.6 ng/μl mouse anti-phospho-PDGFRβ (Tyr(P)-751) antibody and 2 ng/μl rabbit anti-PDGFRβ were applied as primary antibodies. Non-priming proximity probe (1.9 ng/μl) and RCA priming proximity probe (0.43 ng/μl) were used. RCA was performed using 1 unit/μl ϕ29 DNA polymerase. Counterstaining with mouse anti-actin antibody was omitted.

Image Analysis—The number of *in situ* PLA signals per cell was counted by semiautomated image analysis. Fully automated cell delineation was initiated by identifying cell nuclei using the signal from the nuclear Hoechst staining (blue). After intensity thresholding, touching nuclei were separated using a combination of distance transformation and watershed segmentation (15). The image channel showing actin staining (green) was thereafter filtered to enhance regions of high intensity variance, *i.e.* the cytoplasms. Each nuclear delineation was thereafter allowed to expand within these regions to detect the edges of its surrounding cytoplasm. A distance threshold limited the expansion in cases of poor actin staining. Cells whose nuclei were cut by the image border were excluded from the analysis. *In situ* PLA signals were counted by enhancing pointlike signals and defining a true signal as a local intensity maximum above a background threshold (16). The same input parameters were used throughout all experiments. The methods for image analysis were implemented in Matlab (MathWorks Inc., Natick, MA). Fully automated delineation of cytoplasms did not always give a satisfactory result, and some delineations were corrected manually using the interactive tool in Visiopharm Integrator System (Visiopharm, Horsholm, Denmark).

RESULTS

In Situ PLA Using Secondary Proximity Probes—We modified the *in situ* PLA to use secondary antibodies with attached oligonucleotides as proximity probes for a more generally applicable method. These reagents can be used with any pairs of primary antibodies from appropriate species with no need for conjugation. The secondary proximity probes consisted of donkey anti-mouse IgG and donkey anti-rabbit IgG, each covalently conjugated to the 5′-end of different oligonucleotides. Only when both primary antibodies are bound to the target and in turn bound by the proximity probes can the added circularization oligonucleotides be ligated to form a template for RCA. The oligonucleotide of one of the proximity probes acts as a primer for RCA, whereas the oligonucleotide of the other proximity probe is inhibited from priming an RCA by three mismatched, exonuclease-resistant 2′-*O*-methyl RNA nucleotides at the 3′-end. The RCA gives rise to a randomly coiled, single-stranded RCP. Hybridization of complementary, fluorescence-labeled oligonucleotide probes allow individual RCPs to be visualized by fluorescence microscopy as submicrometer-sized objects (Fig. 1).

FIG. 1. **Schematic illustration of *in situ* PLA.** The mechanism of *in situ* PLA with secondary proximity probes is shown. *a*, PDGFRβ molecules stimulated with PDGF-BB are dimerized and phosphorylated. Thereby primary mouse antibodies directed against the phosphorylated Tyr-751 residue and rabbit anti-PDGFRβ antibodies can bind in proximity. *b*, upon addition of proximity probes directed against mouse and rabbit immunoglobulin, their attached DNA strands form templates for ligation of two additional oligonucleotides. *c*, the circular DNA strand that is formed can be replicated in a RCA reaction, giving rise to concatameric molecules that can be visualized by microscopy as an intensely fluorescent spot using fluorophore-labeled oligonucleotides. *d*, in contrast, unstimulated receptors remain monomeric and unphosphorylated, unable to be bound by the anti-Tyr(P)-751 antibody. *e*, only one of the proximity probes can bind the antibody-receptor complex, and the added oligonucleotides are ligated into a linear strand but fail to circularize. *f*, upon addition of a DNA polymerase a short duplex molecule is formed that cannot be visualized by the fluorescent oligonucleotides used to detect RCPs. *P*, phosphorylated Tyr-751 residues.



We used *in situ* PLA with secondary proximity probes to determine the phosphorylation status of PDGFRβs in cells and tissues *in situ*. In the absence of ligand stimulation, PDGFRs are present as unphosphorylated, inactive monomers in the cell membrane, but upon ligand stimulation the receptors dimerize and become autophosphorylated at several sites, including residue Tyr(P)-751, leading to further downstream signaling events. We were able to detect individual phosphorylated receptors *in situ* subsequent to ligand stimulation of the receptor by using a pair of primary antibodies from different species, one directed against the C-terminal part of the PDGFRβ and the other directed against Tyr(P)-751, together with the corresponding secondary proximity probes (Fig. 1).

Enumeration of Phosphorylated PDGFRβ in Single Cells—In unstimulated HEK293 cells stably overexpressing PDGFRβ, minimal signs of specific phosphorylation of the PDGFRβ were detected in the absence of PDGF-BB stimulation (Fig. 2*a*). By contrast, in cells treated with PDGF-BB the amount of signals was dramatically increased (Fig. 2*b*). However, a heterogenous pattern of staining was observed because some cells had lost the expression of the receptor (as confirmed by normal IF, data not shown). The striking difference in the amount of *in situ* PLA signals between the two populations attests to the high selectivity of the method (Fig. 2, *b* and *g*). Moreover when expression of PDGFRβ in HEK293 cells was suppressed using ATC in wild-type HEK293 cells or when one of the primary antibodies was omitted, *in situ* PLA signals were found in negligible amounts regardless of whether the cells were stimulated with PDGF-BB or not (Fig. 2, *c*–*g*). A particular advantage of the *in situ* PLA method is that individual molecules are detected and thus can be enumerated by digital analysis. We therefore recorded the relative number of phosphorylated receptors per cell in the different investigated populations, revealing distinct differences in the amount of signals between the PDGF-BB-stimulated HEK293-PDGFRβ cells and the controls (Fig. 2*g*).

Selective Detection of Phosphorylated PDGFRβ—To investigate whether the two closely homologous isoforms of the PDGFR, α and β, could be distinguished we used PAE cells stably expressing either of the two receptors. Both isoforms are stimulated equally well by PDGF-BB (17). Using *in situ* PLA we detected PDGFRβ phosphorylation in PDGF-BB-stimulated PAE cells expressing the β-isoform of the receptor (Fig. 3*b*) but not in the unstimulated cells (Fig. 3*a*). Similarly only negligible amounts of signals were detected in cells expressing the α-isoform of PDGFR regardless of whether the cells had been stimulated with PDGF-BB or not (Fig. 3, *c* and *d*). Enumeration of fluorescent objects revealed the same clear differences in the amount of signals between the PDGF-BB-stimulated PAEβ cells and the controls, confirming the ability of *in situ* PLA to clearly distinguish the receptor isoforms (Fig. 3*e*).

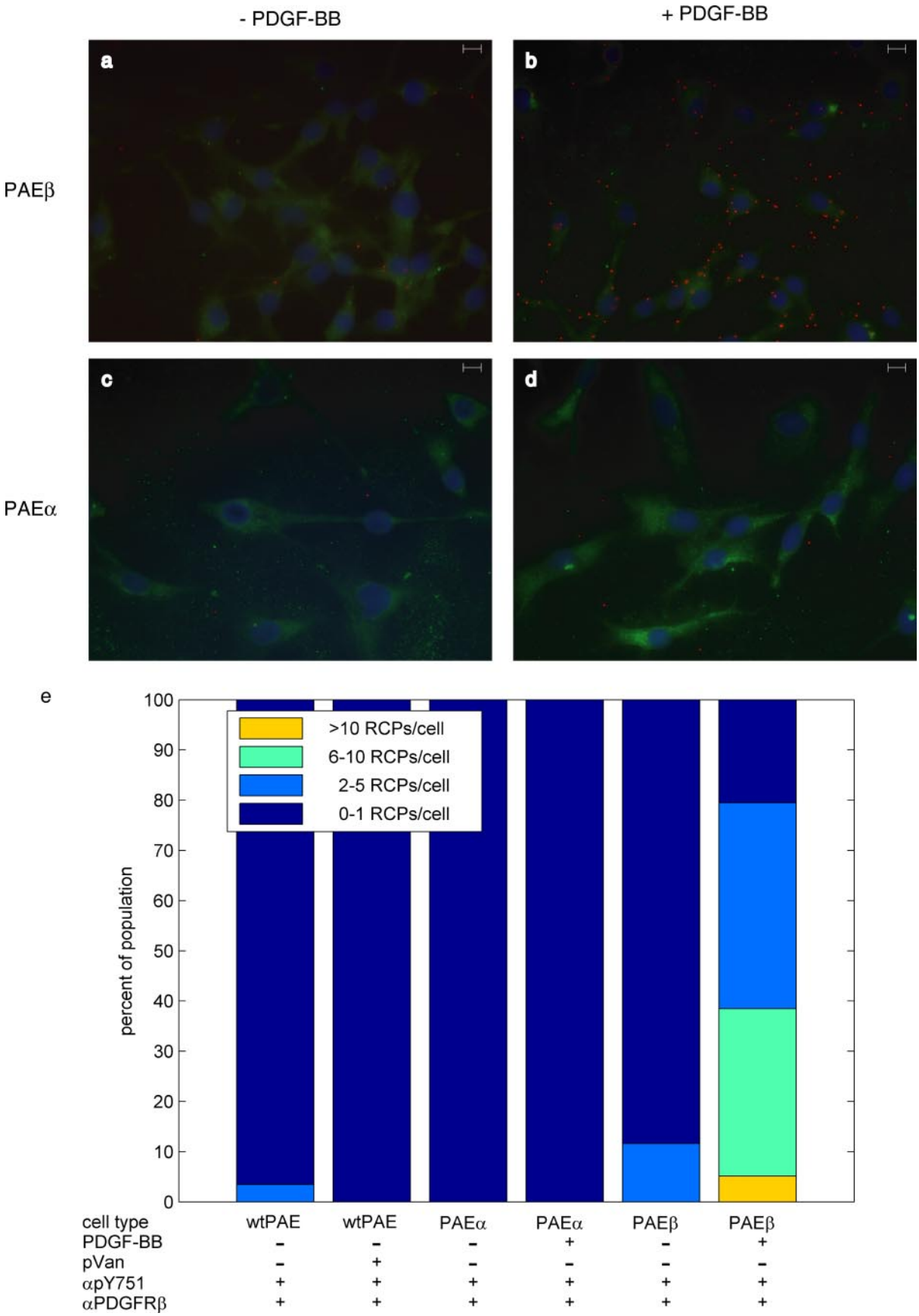
Detection of Phosphorylated Endogenous PDGFRβ—Next we investigated whether phosphorylation of the endogenous PDGFRβ could be detected in BJ hTert cells. As for cells transfected with the receptor, phosphorylated PDGFRβ was detected in the BJ hTert cells upon stimulation with PDGF-BB but not in unstimulated cells (Fig. 4, *a* and *b*). The number of fluorescent spots, reflecting detected phosphorylated receptors, clearly differed between stimulated BJ hTert cells and unstimulated controls (Fig. 4*c*). A comparison of phosphorylation of the receptors in BJ hTert cells exposed to different concentrations of PDGF-BB as measured by *in situ* PLA and immunoblotting revealed similar dose-response curves (Fig. 4*d*).

Detection of Phosphorylated PDGFRβ in Human Tissue—Finally we tested whether the *in situ* PLA technique could also be used to visualize PDGFRβ phosphorylation in histological sections from patient tissue. PDGFRβ is expressed in many stromal fibroblasts and pericytes and can be activated by PDGF-BB secreted during wound healing and scar formation (18). A hematoxylin-eosin-stained frozen section of human scar tissue covered by stratified squamous epithelium is shown (Fig. 5*a*). *In situ* PLA reactivity was clearly demonstrated in the fibrotic dermal stroma beneath the epithelium (Fig. 5*b*) and in the stroma around a venule (Fig. 5*c*).

DISCUSSION

The requirement for dual recognition by pairs of antibodies in combination with a very potent signal amplification makes *in situ* PLA a powerful tool to identify and enumerate interacting proteins (9) and also, as described here, phosphorylation of specific residues in proteins. The analysis allows relative numbers of detected proteins to be identified as well as their subcellular distribution and intercellular variation. However,

FIG. 2. **Detection of phosphorylated PDGFRβ in HEK293 cells stably transfected with HA-tagged human PDGFRβ.** PDGFRβ phosphorylation was detected by *in situ* PLA (*red dots*) in HEK293 cells stably expressing human HA-tagged PDGFRβ in untreated cells (*a*) or in cells stimulated with 100 ng/ml PDGF-BB (*b*). Expression of PDGFRβ in HEK293 cells was suppressed by treatment with ATC, and the cells were then either left unstimulated (*c*) or stimulated with 100 ng/ml PDGF-BB (*d*). Untransfected wild-type HEK293 cells were also left unstimulated (*e*) or stimulated with 100 ng/ml PDGF-BB (*f*). The cells were counterstained with anti-actin (*green*) and Hoechst (*blue*) to visualize the cytoplasm and nucleus, respectively. *Scale bars* represent 10 μm. *g*, quantification of signals per cell in PDGFRβ-expressing HEK293 cells stimulated with PDGF-BB and in a series of negative controls. Treatment of the different controls was done according to the *table under* the *graph*. Additionally RCA priming proximity probe and non-priming proximity probe were added to all tested conditions. The distribution within one group is represented as the percentage of each cell population that shows a certain number of signals. On average 160 cells were investigated for each culture condition in at least three experiments and evaluated using automated image analysis. *αPDGFRβ*, rabbit anti-PDGFRβ antibody; *αpY751*, mouse anti-phospho-PDGFRβ (Tyr(P)-751) antibody; *wtHEK*, non-transfected wild-type HEK293 cells.



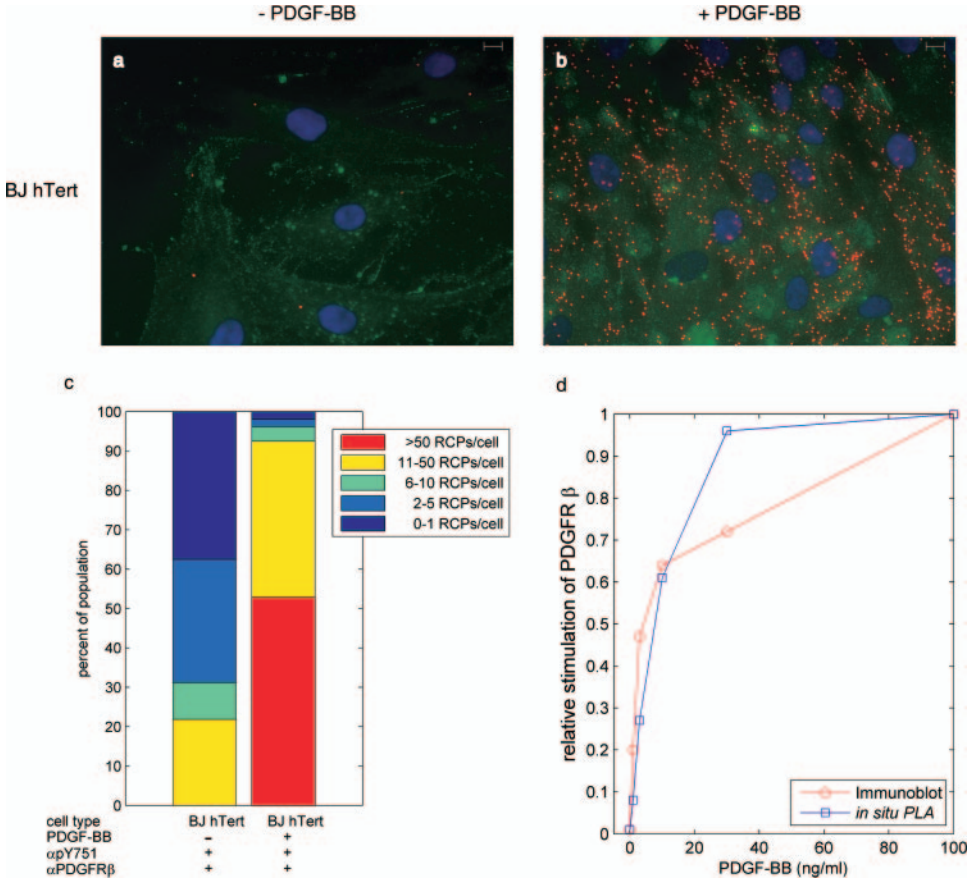


FIG. 4. **Detection of endogenous levels of phosphorylated PDGFRβ.** PDGFRβ phosphorylation was detected by *in situ* PLA (*red dots*) in BJ hTert cells either untreated (*a*) or 100 ng/ml PDGF-BB-stimulated (*b*) before applying *in situ* PLA for detection of phosphorylated PDGFRβ. The cells were counterstained with anti-actin (*green*) and Hoechst (*blue*) to visualize the cytoplasm and nucleus, respectively. *Scale bars* represent 10 μm. *c*, quantification of the numbers of signals per cell in BJ hTert cells in either untreated or PDGF-BB stimulated cells. The distribution within one group is shown as the percentage of each cell population that had a certain number of signals. On average 43 cells per condition were analyzed in three experiments. Treatment of the different conditions was done according to the *table under* the *graph*. Additionally RCA priming proximity probe and non-priming proximity probe were added. *d*, comparison of the effects on PDGFRβ stimulation by variable amounts of PDGF-BB as analyzed by *in situ* PLA and immunoblotting. Cells were treated with 0, 1, 3, 10, 30, and 100 ng/ml PDGF-BB. On average 127 cells per condition were analyzed, and automated image analysis was performed. The Tyr(P)-751 PDGFRβ immunoblot signal was normalized against the anti-PDGFRβ antibody signal for the same blot. Values for immunoblotting and *in situ* PLA were normalized against the value for maximal stimulation to allow a comparison between the different assays. *αPDGFRβ*, rabbit anti-PDGFRβ antibody; *αpY751*, mouse anti-phospho-PDGFRβ (Tyr(P)-751) antibody.

the applicability of the method has so far been limited by difficulties in conjugating oligonucleotides to antibodies. By using secondary proximity probes this obstacle has been overcome, providing a generally applicable reagent set for detection of protein interactions and PTMs using *in situ* PLA. Any pair of primary antibodies can be used to analyze the target proteins together with secondary proximity probes as long as the primary antibodies are derived from a suitable pair of species. Several additional pairs of primary antibodies have been tested with good results for detection of *e.g.* Smad

FIG. 3. **Distinction between the phosphorylated α- and β-isoforms of the PDGFR.** PDGFRβ phosphorylation was detected by *in situ* PLA (*red dots*) in PAEβ cells. PAE cells transfected with PDGFRβ were either left untreated (*a*) or stimulated with 100 ng/ml PDGF-BB (*b*) prior to fixation, and the *in situ* PLA was applied to detect phosphorylated PDGFRβ. As a control for the specific detection of the β-isoform of the receptor PAEα cells used were either untreated (*c*) or stimulated with PDGF-BB (*d*). The cells were counterstained with anti-actin (*green*) and Hoechst (*blue*) to visualize the cytoplasm and nucleus, respectively. *Scale bars* represent 10 μm. *e*, quantification of signals per cell in PAE cells for each treatment condition is illustrated by showing the percentage of cells that had a certain number of signals. Treatment of the different conditions tested was done according to the *table under* the *graph*. RCA priming proximity probe and non-priming proximity probe were added to all tested conditions. On average 30 cells per condition were analyzed in three experiments and subjected to automated image analysis. *αPDGFRβ*, rabbit anti-PDGFRβ antibody; *αpY751*, mouse anti-phospho-PDGFRβ (Tyr(P)-751) antibody; *wtPAE*, wild-type PAE cells; *pVan*, pervanadate.

interactions[2] and HER2 expression (data not shown). Another benefit is that secondary antibodies typically are less expensive and hence can be conjugated in large batches, ensuring reproducible results over time.

We demonstrated herein that *in situ* PLA is suitable to detect stimulation-dependent phosphorylation of PDGFRβ in individual cells with high selectivity and sensitivity. The brightly fluorescent RCPs allowed unbiased enumeration of phosphorylated PDGFRβ molecules in individual cells using dedicated software, revealing intracellular distribution and intercellular variation in the investigated cell populations. All negative control experiments resulted in negligible amounts of signals, whereas abundant signals were observed in positive experiments. Titration of primary antibodies is required to minimize nonspecific signals due to effects such as adsorption of antibodies to surfaces. Because *in situ* PLA signals depend upon having two antibodies bound to a protein or a protein complex, non-saturating levels of antibodies will decrease the amount of obtained signals as many of the targets only will be occupied with one antibody. On the other hand a too high concentration may increase the probability of nonspecific adsorption of antibodies thereby increasing the background of false positive signals. In general, we have found that a concentration of antibodies that will produce good results in immunofluorescence also works well for *in situ* PLA. The method of fixation of cells and tissues as well as the method for antigen retrieval also needs to be determined for compatibility with the primary antibody pair used.

Some of the HEK293 cells transfected with PDGFRβ had lost their expression during cultivation, resulting in a mixture of cells positive and negative for receptor expression. The striking difference in the amount of *in situ* PLA signals between the two populations attests to the high selectivity of the method.

It has proven difficult to distinguish between the phosphorylated isoforms of the receptor by normal IF and immunohistochemistry due to antibody cross-reactivity (19),[3] whereas *in situ* PLA offers greater selectivity due to the requirement for two binding events for detection. Furthermore because in *in situ* PLA RCA is used to amplify the signal it is straightforward to distinguish between RCPs and any autofluorescence or fluorescence from nonspecifically bound detection probes. Using PAE cells that expressed either the α- or the β-isoform of the PDGF receptor, we confirmed that the detected signals were specific for the β-isoform with no cross-reactivity for PDGFRα. Only cells expressing PDGFRβ and stimulated with PDGF-BB yielded any signals even though cells expressing PDGFRα were stimulated to the same extent.

Experiments using the BJ hTert cell line served to demonstrate that the method also can detect phosphorylated forms of the endogenous PDGFRβ upon stimulation with PDGF-BB. There was good agreement between measurements of signals in response to variable amounts of PDGF-BB as determined by *in situ* PLA and by immunoblotting. PAEβ cells expressed about half the amount of receptor compared with

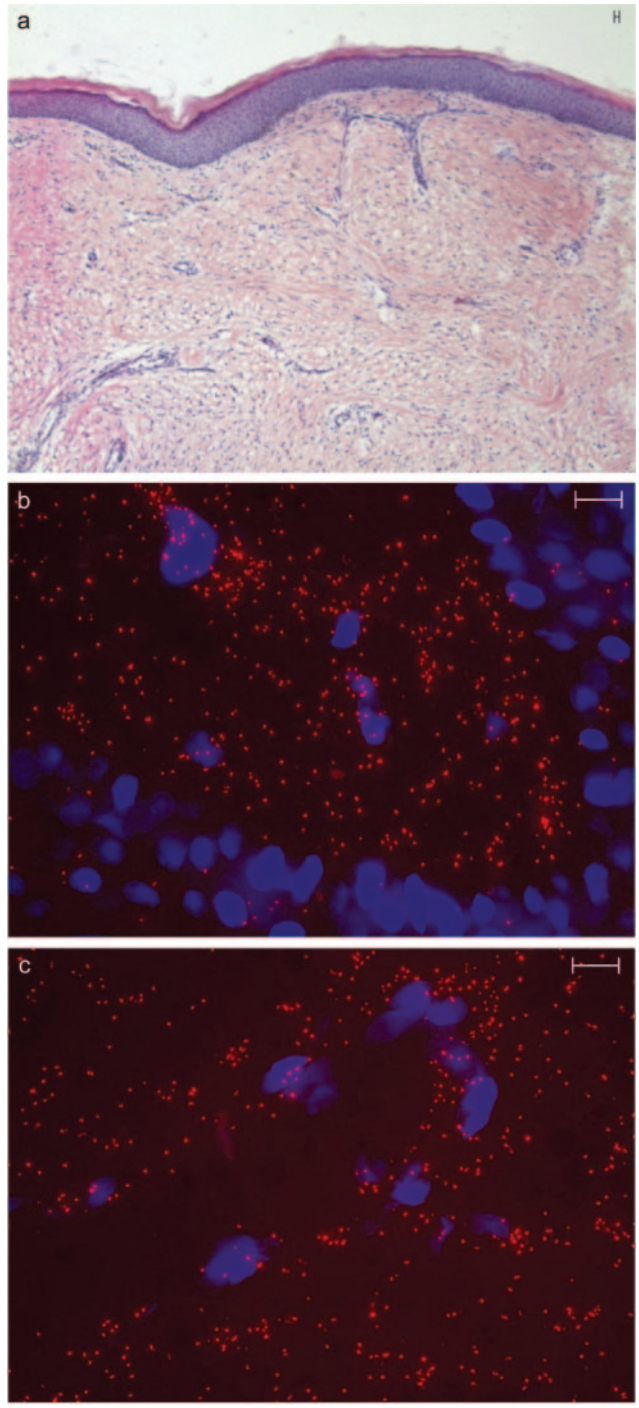


FIG. 5. **Detection of phosphorylated PDGFRβ in human scar tissue.** *a*, hematoxylin-eosin-stained tape-transfer frozen section of scar tissue. PDGFRβ phosphorylation was detected by *in situ* PLA (*red dots*) in the fibrotic scar tissue beneath the surface epithelium (*b*) and in the stroma around a small vessel (*c*). The tissue section was counterstained with Hoechst (*blue*) to visualize the nucleus. *Scale bars* represent 10 μm.

[2] K. Pardali, unpublished data.
[3] J. Paulsson, unpublished data.

the transfected HEK293 cells, but they revealed only one-tenth as many *in situ* PLA signals. This might be due to differences in levels of phosphatases between the cell lines that may affect the extent of ligand-induced receptor phosphorylation. Concerning quantification of the signals it is noted that the *in situ* PLA signal is proportional to the real amount of activated receptors as indicated by the comparison with the results from immunoblotting analyses. However, it is also clear that only a fraction of activated receptors gives a signal. This is most likely reflecting less than 100% efficiency in the multiple steps of the procedure, including binding of antibodies, oligonucleotide ligation, and amplification.

Reliable and sensitive detection of protein-tyrosine kinase receptor phosphorylation by *in situ* PLA in cultured cells and in tissue sections is a new tool of great potential value in basic research and in histopathology. We have previously demonstrated that the proximity ligation mechanism can be extended to using sets of three antibodies (9, 20). This could provide unique opportunities to investigate constellations of several phosphorylations or other PTMs in individual protein molecules, something that would present great difficulties using methods like mass spectrometry.

The *in situ* PLA method should be suitable to investigate pathophysiological processes in *e.g.* inflammatory and neoplastic diseases, and it may be of value in the development of PDGFR inhibitors and for predicting the clinical response to tyrosine kinase inhibitors in patients. Moreover the *in situ* PLA technique could be used to visualize any functional state of proteins in a cell, provided that suitable affinity probes are available, including other PTMs and interactions with other proteins or other macromolecules. On the strength of its selectivity and sensitivity and its potential to investigate inter- and intracellular differences in cells and fresh frozen tissues, *in situ* PLA thus offers unique possibilities in studies of protein functions in basic and clinical research.

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