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JOSEFIN BOLIK

Development of a miniaturised and simplified procedure for evaluation of signal transduction pathways

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Author

Josefin Bolik

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Abstract

A simple model system was developed for analysing phosphorylation status of a membrane bound protein, namely the PDGF β-receptor, in cell lysate. The method was based on a miniaturised sandwich immunoassay integrated into a CD format with a capture antibody directed against the target protein and a fluorescence labelled detecting antibody raised against the phosphorylated amino acid. Response levels reflecting different degree of phosphorylation of target protein could be demonstrated in cell lysates originating from cell cultures stimulated with PDGF-BB and cell cultures treated as controls. Analysis of cell lysate samples, containing significant amounts of detergent, affected antigen-antibody interactions negatively and generated high background. Further developments are needed to generate a quantitative assay for phosphorylation of the PDGF β-receptor.

Keywords

PDGF β-receptor, sandwich immunoassay, PDGF-BB, detergent

Supervisors

Mats Inganäs

Gyros AB

Scientific reviewer

Arne Östman

Ludwig Institute for Cancer Research

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Biology Education Centre Biomedical Center Husargatan 3 Uppsala

Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 555217

Development of a miniaturised and simplified procedure for evaluation of signal transduction pathways

Josefin Bolik

Sammanfattning

Förståelse för cellulär signalering från receptorer på cellernas yta till transkriptions-faktorer som aktiverar transkription av gener är viktigt inom molekylärbiologisk forskning. Aktivering/Inaktivering i signalvägarna bestäms av modifieringar av proteiner såsom fosforylering av aminosyror. Dessa modifieringar är numera möjliga att detektera med specifika antikroppar som utvecklats för dessa ändamål. Många av dagens metoder för att mäta aktiveringsgrad av proteiner är tidskrävande och utveckling av förenklade metoder är önskvärda. PDGF (platelet-derived growth factor) receptorer tillhör familjen receptorer med kinasaktivitet och är involverad i flera signalvägar.

Syftet med projektet var att försöka utveckla en enkel metod för att analysera fosforylerade proteiner (PDGF β-receptorer) i cellysat samt att försöka integrera och miniatyrisera metoden i Gyros CD-plattform. Den assay som utvecklats bygger på immunologisk metodik där en immobiliserad antikropp riktad mot målproteinet, fångar upp proteinet ur provet varefter andelen fosfylerat protein bestäms med en fluorescensmärkt antikropp specifik för fosfotyrosin. Vid stimulering av celler med ligand (PDGF-BB) aktiveras PDGF β-receptorn genom fosforylering av tyrosingrupper på receptorn. Lysat från celler som aktiverats respektive kontroller som inte aktiverats eller saknar PDGF β-receptor har testats med metoden. Resultatet tyder på att det är möjligt att bestämma fosforyleringsgraden av PDGF β-receptorn i metoden.

Examensarbete 20 p i Molekylär bioteknikprogrammet

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1 Introduction
1.1 Methods for analysing signal transduction pathways

The understanding of cellular signal transduction from cell surface receptors to transcription factors is important in research and clinical applications. Activation/Inactivation of the signalling pathways is determined by posttranslational modifications of the proteins, for example phosphorylation, acetylation or ubiquitination of amino acids. These modifications make the proteins in their activation status amenable to immunodetection.

Immunoprecipitation/Western blot, immunohistochemistry and methods similar to ELISA with activation specific antibodies are novel technologies to study phosphorylation cascades intracellulary. In immunoprecipitation, antibodies are used to precipitate an antigen from solution and the creation of insoluble antibody-antigen complexes is correlated to the antigen concentrations. Immunoprecipitation can be exploited in both agar techniques and in solution. In agar techniques, antibody and/or antigen migrate through the gel by simple diffusion or the mixture of proteins containing the antigen is separated with gel electrophoresis followed by diffusion of an antibody through the gel, resulting in visible precipitated antibody-antigen complexes. Soluble immune complexes can be immobilised to immunoglobulin binding protein A or G or anti-immunoglobulin antibody and if the mixture of proteins is radiolabelled (^{125}I) the antigen can be detected with autoradiography [1].

Western blot is used to establish the specificity of antibodies by examining their ability to recognise antigens in crude protein mixtures, for example cell lysates. The separated proteins are transferred from polyacrylamide gels to porous membranes and the blot is probed with an antibody. The antibody-antigen complex is detected with a labelled anti-immunoglobulin reagent or detected with a secondary antibody labelled with ^{125}I or an enzyme [1].

Alternatively, an unlabelled immunoprecipitate can be analysed with SDS-PAGE and immunoblotting with antibodies of known specificity, which can allow information of immunoprecipitated proteins. Detection of the antibody-antigen complex is performed as described above for western blot. Immunoprecipitation in solution has the advantage that the antibody is allowed to react with native rather than partially denatured antigen, as is the case in immunoblots [1].

Immunohistochemical techniques are based on the specific interaction of an antibody with its antigen to locate or to determine the distribution of the antigen in tissues or cells. The primary antibody or the anti-immunoglobulin antibody must be conjugated with fluorophore or enzyme to allow visualisation and the location of the labelled antibody reveals the location of the antigen. The technique requires positive and negative control antibodies for comparison with the test antibody to ensure that the immunostaining is specific. By comparing the results from activation specific antibodies with target protein specific antibodies it is possible to estimate the degree of activation of the target protein [1].

Several of the methods are time consuming and cumbersome and development of simplified methods to measure the degree of activation of proteins in cell lysates is necessary. Pathways from cell surface receptors to transcription factors could be discerned using a fast and simple method for measuring signal profiling intracellularly with the specificity and sensitivity required. Perhaps such methods will contribute to a more efficient process to discover potential drugs?

1.2 PDGF b-receptor and signal transduction pathways

Platelet-derived growth factors (PDGFs) belong to a family of growth factors that stimulate cell growth, survival and motility by exerting their effects by activating two structurally related protein tyrosine kinases, the PDGF α- and β-receptors (Figure 1). The α- and β-receptors have molecular sizes of ~170 and 180 kDa, respectively. The two PDGF receptor types mediate similar, but not identical, cellular responses. The PDGF isoforms are dimeric molecules and therefore have two epitopes for receptor binding; PDGF-AA induces αα receptor dimers, PDGF-AB αα or αβ receptor dimers and PDGF-BB all three possible combinations of receptor dimers. PDGF-AA and PDGF-BB are homodimeric isoforms and PDGF-AB is a heterodimeric isoform [2].

Binding of PDGF to its receptors results in receptor dimerization and this leads to activation of the tyrosine kinase activity. The activation is followed by phosphorylation of multiple tyrosine residues inside the kinase domain and the intracellular parts of the receptor. The functional role of these autophosphorylation sites is to act as docking sites for downstream signal transduction molecules, including signal transduction proteins, docking proteins and transcription factors. Thus, tyrosine kinase receptors can either undergo autophosphorylation or phosphorylate a docking protein at a suitable tyrosine and this will determine which signalling pathway to be activated. The direct interactions are exerted through specific domains, for example Src homology 2 (SH2) domains and phosphotyrosine binding (PTB) domains, which recognise phosphorylated tyrosine residues in specific environments. Phosphatidylinositol 3' kinase (PI_3-kinase) and Phospholipase C-γ (PLC-γ) are two examples of signal transduction molecules with enzymatic activity that bind to PDGF-receptors [2]. Additionally, activation of Ras occurs in PDGF-stimulated cells and this is important for induction of a signal transduction pathway. Activation of the MAP kinase cascade through Ras activation has been shown to be of major importance for growth stimulation and overactivity of different components along this pathway has been shown to lead to cell transformation [2].

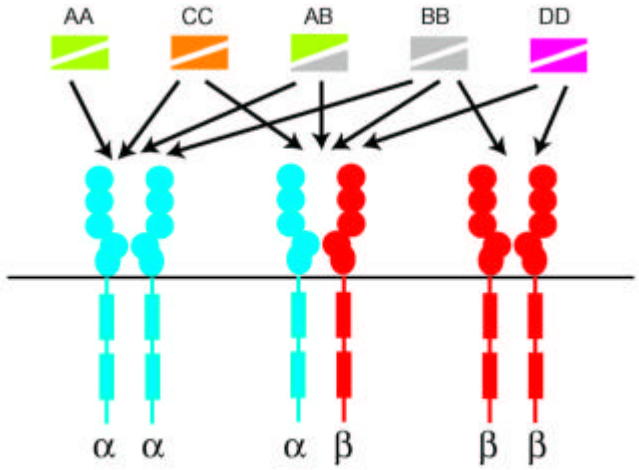


Figure 1. The figure illustrates the different growth factor isoforms that bind to and activate the different PDGF receptor types. The illustration was used with permission from Arne Östman at the Karolinska Institute in Stockholm, Sweden.

Ligand binding induces internalisation of the ligand-receptor complex into endosomes followed by either dissociation of PDGF-receptor complex or degradation and the rate of internalisation of the PDGF β-receptor is dependent on the kinase activity of the receptor. Activated receptors can also undergo ubiquitination followed by cytoplasmic degradation in proteasomes [2].

PDGF receptors have been targets in the study of different pathways since these receptors interact with unusually many SH2 domain proteins. Studies of the components involved in these pathways are becoming more important and can give rise to the discovery of specific and effective antagonists that inhibit signalling. The role of PDGF receptor signalling in malignancies has been an interesting target for cancer therapy. PDGF receptor signalling exerts different effects in different tumours; in certain malignancies mutationally caused changes in expression result in autocrine growth stimulation of tumour cells and in other malignant cancers the PDGF receptor is suggested to regulate tumour development [3].

1.3 Sandwich immunoassay in a CD microlaboratorium

In sandwich-based immunoassays, a capturing reagent is immobilised to a solid phase, which binds or captures the target protein. When the target protein has been captured the detection reagent is added, attaching to the target protein and the protein can be quantified by using a detector system. The diversity of antibodies makes them very useful in many different types of immunoassays. In a sandwich immunoassay the two antibodies recognise different epitopes on the target protein and the method can yield quantitative results with high precision [4].

1.3.1 Gyros AB

Gyros AB was founded in 2000 as a separate company and has developed a technology platform where miniaturisation and integration of laboratory processes into Compact Disc microlaboratories is the main concept. Development of application-specific CD microlaboratories utilises natural forces to control location and flow of liquids, for example centrifugal and capillary force. The CDs are run on an instrument platform, which is called Gyrolab Workstation and Gyrolab software program controls the applications. The system offers flexibility and can be adapted to different applications. Sandwich immunoassay for protein quantification is one example that has been integrated in a CD microlaboratory and the CD contains approximately a hundred of identical, application-specific microstructures, which facilitates parallel sample processing. Miniaturising applications can reduce the sample and reagent consumption, reduce time for analysis due to rapid diffusion of reactants in microsystems and by integrating several steps into a single streamlined process the productivity and automation can be increased [5].

1.3.2 High throughput sandwich immunoassay for signal profiling

The Gyrolab platform has advantages compared to the available technologies mentioned above, for example high throughput and the fact that each analysis consumes a lesser amount of sample compared to conventional methods. Today there are no protocols for automating immunoprecipitation and western blot analyses and the ELISA like assays require larger sampler volumes than Gyros. Furthermore, signal profiling requires very sensitive detection methods where small amounts of proteins can be measured, sometimes at the pM level, so that small differences in levels of activation can be followed. Improved methods for detection of activated signal transduction proteins with activation specific antibodies would be useful in clinical studies [3, 4, 9].

1.4 Antibodies

Antibodies are produced by B-lymphocytes and one antibody molecule consists of two identical heavy chains and two identical light chains. The heavy chains determine the class of the antibody and there are five different classes of antibodies IgG, IgM, IgA, IgE and IgD. Heavy and light chains cooperate to form the antigen-binding site. Germline diversity and recombinatorial diversity are the main reason why antibodies can distinguish small structural differences in molecules. The immunoglobulin loci consist of a large number of segments (V-variability, D-diversity, J-joining and C-constant) that can be combined and a specific combination determines the type of immunoglobulin to be produced and contribute to the wide variability. There are six J_H segments and at least 20 segments of a fourth type of segment, the D_H segment, which lies between the V and J genes. DNA rearrangement begins with V/D/J joining to form the V gene, then linking to one of the C segments. Gene translocation and fusion results in the deletion of intervening DNA. The production of the light chain is similar but the DNA sequence does not include the D-segment (See figure 2 for illustration of antibody). In order to produce antibodies with different specificity the alleles in the heavy and light chains loci can be rearranged. A monoclonal antibody is derived from a single plasma cell and is specific for one epitope on a complex antigen and polyclonal antiserum contains a mixture of antibodies, each specific for one protein or antigen [4]. The antibody class IgG is the most commonly used in sandwich immunoassays.

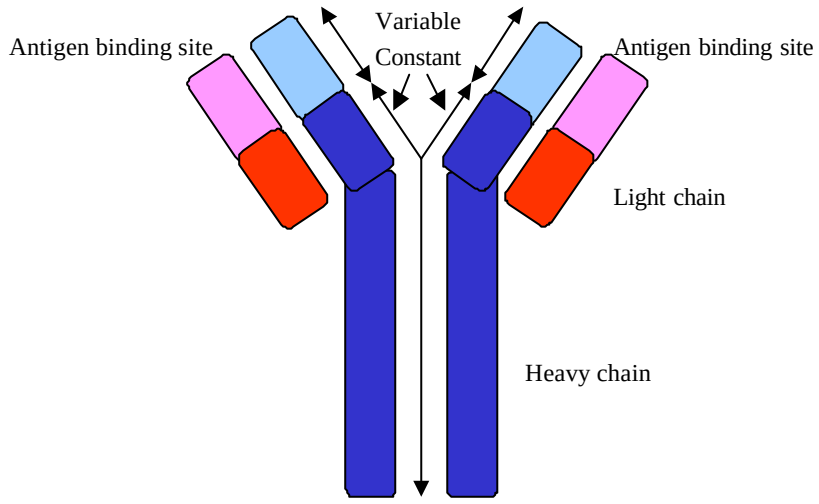


Figure 2. Schematic drawing of an IgG molecule. The illustration was adapted from [6].

1.5 Aim of the project

The aim of the project was to develop a simple method for analysing signalling pathways in cellular responses and try to integrate the process in the Gyrolab Bioaffy platform. As a starting point, porcine aorta endothelial (PAE) cells stably expressing the membrane bound PDGF β-receptor and stimulated with +/- ligand PDGF-BB were analysed and tested in the model system (Cell culture from Ludwig Institute for Cancer Research). The project is based on immunological methods where one antibody is raised against the target protein and another antibody raised against one regulatory phosphorylated aminoacid or site. The antibodies were also tested in immunoprecipitation and western blot performed at Ludwig Institute for Cancer Research in order to control antibody specificity and to compare the results with the results from the sandwich assay performed in the CD microlaboratory.

2 Material and methods
2.1 The Gyrolab

2.1.1 CD microlaboratory

The CDs used in the experiments are called CDBA2 (CD Bioaffy 2), which are developed and produced by Gyros AB and contains 104 identical microstructures arranged in series of eight microstructures per segment. The microstructures are connected to a common distribution channel and contain an individual inlet, a volume definition chamber, an overflow channel, a small affinity column and hydrophobic breaks (See figure 3). The affinity columns incorporated into each microstructure, contain polystyrene (Dynal Biotech, Oslo, N.) particles that are coated with phenyldextran (Amersham Biosciences, Uppsala, S.) to which streptavidin is coupled. In order to control location of liquids in the microstructure, the CD has hydrophobic breaks at distinct places. Plastic surfaces are very hydrophobic and therefore biomolecules adsorb easily to these surfaces and to avoid loss of sample the CD surfaces have been pretreated to become hydrophilic to prevent non-specific binding of proteins. The microstructures are arranged in a manner that allows great flexibility to the distribution of liquids. Wash buffers, reagents and samples can either be applied individually through the specific inlets or simultaneously through the common inlet in the CD (Gyros AB, Uppsala, Sweden).

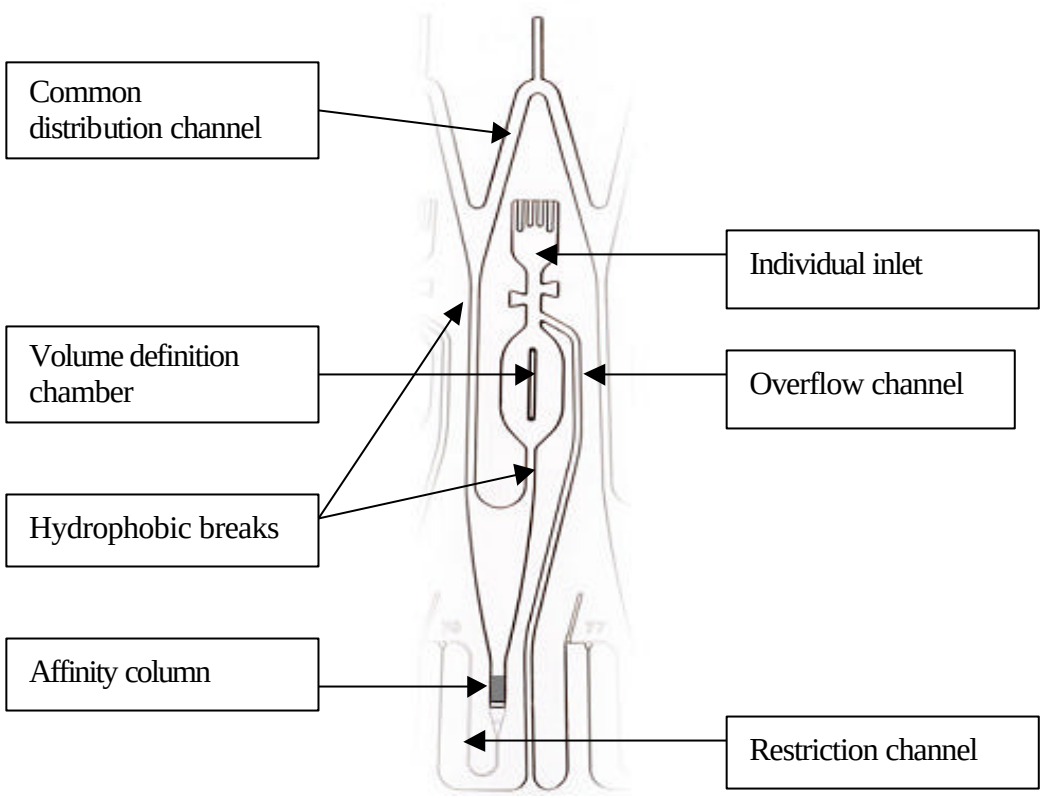


Figure 3. An overview of the microstructure and how the different areas are connected in the CD microlaboratory. The illustration was used with permission from Mats Inganäs at Gyros AB.

Capillary force drives liquid through the nanoliter scale microstructures and the hydrophobic breaks prevent liquid from moving further into the microstructure. Spinning the CD creates

the centrifugal force that moves samples and reagents through each step of the application and thereby over the hydrophobic breaks. When the distribution channel empties, overflow liquid goes through the overflow channel and an exact volume of liquid is passing into the reaction chamber. Volume definition of the liquids eliminates pipetting errors and increases the accuracy of the application. The flow rates over the column can be controlled in every step of the assay by adjusting the rotational speed of the CD together with the design of the microstructures (Gyros AB, Uppsala, Sweden).

2.1.2 Streptavidin-biotin

Streptavidin (Mw 60,000) comes from the bacterium *Streptomyces avidinii* and forms a very strong noncovalent interaction with biotin (vitamin H; Mw 244) with affinity constant > 10^{15} M^{-1}. Streptavidin is a tetrameric protein and each subunit binds one biotin molecule and the strong interaction is almost unaffected by environmental changes, for example extreme pH and denaturing agents. The highly specific and strong interaction can be very useful in biological assays where a biotinylated target protein or ligand is captured through the streptavidin-biotin reaction. In the CD, the solid phase is coated with streptavidin and the biotinylated capture antibodies are fixed onto the solid phase through the rapid interaction between streptavidin and biotin. Before the addition of the capture antibody the streptavidin particles must be reconditioned with wash buffer in order to get an even layer of bound capturing antibodies to the solid phase [7].

2.1.3 The Workstation

The CD microlaboratory is run in the Gyrolab Workstation and is controlled by the Gyrolab Workstation software. Every step in the application runs automatically, CDs are transferred to the spinning station and the detection station and solutions are transferred from microplates to the different inlets in the CD by a robotic arm. The workstation includes a wash station where needles are washed between every application of liquid in the assay (Gyros AB, Uppsala, Sweden).

The light source in the workstation is a HeNe laser with an output wavelength of 632.8 nm as the excitation light source and a Laser Induced Fluorescence (LIF) detector is also incorporated in the workstation, which detects and records fluorescence while the CD is rotating. The laser light is directed to hit the columns in the CD and fluorescent light passes back to the detector through the PickUp unit followed by passage through a filter. The fluorescent light is then directed through the Photo Multiplier Tube where photons are converted to electrons and the electrical signal is relative to the amount of bound fluorescently labelled antibody. Every step in the immunoassay is performed in the CD microlaboratory from volume definition to the fluorescence detection (Gyros AB, Uppsala, Sweden).

2.1.4 Detection using the Fluorescence technique

Fluorescence is produced when certain molecules like fluorophores or fluorescent dyes absorb a photon of energy h?$_{EX}$ that is created by an external light source, for example a laser. This creates an excited electronic singlet state (S_1') and exists for a few nanoseconds. During this time the fluorophore is subject to changes that lead to energy loss yielding a relaxed singlet excited state (S_1). Fluorescence emission is produced when a photon of energy h?$_{EM}$ from stage S_1 is emitted and the fluorophore returns to its ground state S_0. The energy of the emitted photon is lower than the excited photon due to energy loss during the excited state and the difference in energy or wavelength is called the Stokes shift, h?$_{EX}$ - h?$_{EM}$. A fluorophore can be excited and detected many thousands of times and this makes the fluorescence technique sensitive [8].

2.1.5 Result files

The fluorescence distribution is presented as a TIFF image of the columns in the CD and an advanced algorithm calculates the volume of the detected intensity for every column. The intensity or the integrated volume is proportional to the amount of bound protein in the column. A background image is created by a fluorescence detection step before the addition of the detection antibody and this image can be subtracted from the final signal image. A result file with the corresponding integrated signals is always created for every batch run and statistical quantities are automatically calculated. The detection limit is calculated as the average of blank responses times three standard deviations, useful when running a standard curve. The images were illustrated and analysed with the software called LIF Protein Array Analyser also developed by Gyros (Gyros AB, Uppsala, Sweden).

2.1.6 Interferences in immunoassays

Different kinds of immunoassay problems can affect the specificity of the immunoassay and cause inaccurate results, for example unspecific interactions with the capturing antibody, matrix effects or disturbances due to the detection system. The cell lysate is a mixture of proteins and everything except the protein of interest constitute the matrix. In the cell lysate assay there are many components able to contribute to unwanted disturbances that affect the immunochemistry. However, when the true concentration of the analyte is unknown, as in the cell lysate assay, the accuracy of a result is difficult to judge and the best opportunity to detect and characterise interferences is during the evaluation of an assay. Cross-reactivity arises when other molecules with structurally similar or identical epitopes bind to the antibody and one way to decrease this problem is to use more specific antibodies. In addition, sandwich assays with two different antibodies with specificity for different epitopes can increase specificity. Cross-reactivity with polyclonal antibodies can be due to the fact that the antisera consist of a heterogeneous population of antibodies with varying specificity. Addition of excess cross-reactant or anticross-reactant antibody can reduce cross-reactivity by the assumption that the most unspecific antibodies bind the cross-reactant, leaving the specific antibodies to bind the analyte [9].

2.2 Biological material

2.2.1 Cell culture

The cell lysate preparations were performed at Ludwig Institute for Cancer Research in Uppsala. Porcine aorta endothelial (PAE) cells stably expressing the PDGF β-receptor have been used in the sandwich assay. The PAE cells were transfected with PDGF β-receptor and one of the cell cultures were also G-418 selected [10]. PAE cells were grown in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 µg/ml streptomycin and the amino acid glutamine. A nearly confluent monolayer of cells were starved overnight in Ham's F-12 supplemented with 0.1 mg/ml bovine serum albumin (BSA) and +/- stimulated with 100 ng/ml PDGF-BB for 60 minutes on a plate shaker. Unstimulated (- stimulated) cells are used as a control since they have no activated PDGF β-receptors, only unphosphorylated PDGF β-receptors. The receptors are activated and saturated with high concentration of ligand inhibiting internalisation followed by degradation during the stimulation. After the stimulation, cells were washed two times in ice-cold PBS buffer (Phosphate buffer saline) and scraped off with a "rubber police men" in 1 ml PBS. The cell suspension was saved and lysed in 200 µl ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.5% Deoxycholate, 0.5 mM Na_3VO_4 and 1% Trasylol from Bayer) for 15 min on ice. The lysates were centrifuged at 13000 rpm for 15 min at 4°C and the supernatant was

saved in aliquots and stored at –20°C. The cell lysate preparation was done twice and the first time the cells were grown in 75 cm^2 culture dishes and the second time they were grown in 175 cm^2 culture flasks in order to make more concentrated lysates. The quantitation of total protein was done according to BCA Protein Assay Kit Microplate procedure from PIERCE Biotechnology (Boule Nordic AB, Stockholm, S.) and this Protein assay is based on bicinchoninic acid (BCA).

2.2.2 PDGF b-receptor antibodies

The rabbit polyclonal antibody 958 directed against a recombinant protein corresponding to amino acids 958-1106 of the carboxy terminus of human PDGF β-receptor, the goat polyclonal antibody P-20, raised against a peptide of the carboxy terminal of human PDGF β-receptor, and the mouse monoclonal PY99 which detects phosphotyrosine-containing proteins were all from Santa Cruz Biotechnologies (Santa Cruz, CA). The PDGF β-receptor antibodies P-20 and 958 are recommended for the detection of PDGF receptor type β of human and, to a lesser extent, mouse and rat origin by western blotting, immunoprecipitation and immunohistochemistry and should not be cross reactive with PDGF receptor type α. The antibodies have been used extensively in IP and WB experiments with the same cell culture, one example described in reference [11].

Biotinylation is a chemical modification where the biotin reagent is coupled to a molecule or to another material and today the commercially available procedures are very simple to perform [7]. Both of the polyclonal antibodies were biotinylated and fluorescently labelled (P-20 and 958) and the monoclonal antibody was fluorescently labelled (PY99). In the biotinylation procedure a 24 molar excess of biotin reagent (EZ-Link™ Sulfo-NHS-LC-Biotin from PIERCE Biotechnology; Boule Nordic AB, Huddinge, S.) was used for both antibodies. The antibodies were supposed to be in an appropriate buffer PBS pH 7.2 and the concentration 1 mg/ml, which is the recommended concentration for biotinylation. A 1 mg/ml biotin reagent solution was prepared by diluting 1 mg of biotin reagent in 1 ml ice-cold Milli-Q and 9 µl of the biotin reagent solution was added followed by incubation for two hours at room temperature with gentle agitation with a pipette every 20 minutes. Biotinylated antibody was separated from excess (unreacted) biotin reagent with a Nanosep® device (PALL® Life Sciences; VWR International, Stockholm, S.) and biotinylated antibody was recovered with PBS buffer (0.015 M $NaPO_4$, 0.15 M NaCl, pH 7.4). By measuring the absorbance at 280 nm the protein concentrations were calculated according to the following formula:
Concentration (mg/ml) = (A_{280} * dilution factor)/κ * L, where κ is the extinction coefficient in $cm^{-1}M^{-1}Mw^{-1}$ (κ = 1.38 and Mw = 150 000 g/mol) and L is the cyvette length in cm (1 cm).

The antibodies P-20, 958 and PY99 were fluorescently labelled according to the Alexa Flour® 647 Monoclonal Antibody Labelling Kit from Molecular Probes (Termometer fabriken, Gothenburg, S.). The protein concentrations and the degree of labelling were determined by measuring the absorbance according to the manufacturer's instructions.

2.3 Methods

2.3.1 Sandwich-based immunoassay methods

In the IFNγ assay and the cell lysate assay, wash buffers and antibodies were distributed through the common distribution channel and recombinant IFNγ and cell lysate preparation were distributed through the individual inlet. Every batch run included standards in triplicates

and blank samples; IFNγ included six known standard concentrations with several PBS-BSA and lysis buffer blanks and the cell lysate assay included a small dilution serie (+/- PDGF-BB) and several blanks with lysis buffer. All steps in the assays were automatically run in the Gyrolab Workstation.

2.3.1.1 Study of matrix effects: Interferon-g assay (IFNg)
When analysing membrane proteins in cell lysate, high detergent concentrations are used to keep the proteins in solution and attention must be drawn to whether this can interfere with the antibody-antigen interaction.

The sandwich immunoassay with IFNγ was already tested in the Gyrolab system and known to generate specific results at the pM-concentration level. Capturing antibody (R4-6A2) was a monoclonal antibody, labelled with biotin by the supplier and the detecting antibody (AN-18) was a rat monoclonal antibody. Both antibodies were from BD Biosciences Pharmingen (Stockholm, S.) and used in ELISA assays. Recombinant IFNγ (Mw 15.6 kDa) was derived from E.coli and reconstituted and diluted in a suitable buffer, PBS with 1%BSA (R & D Systems (Novakemi, Stockholm, S.)).

First of all, the CDBA2 columns were washed twice with wash buffer PBS-T (0.015 M Na-PO_4 pH 7.4, 0.15 M NaCl, 0.01% NaN_3, 0.01% Tween-20) in order to recondition the streptavidin-coated particles followed by a short spin. The second step was addition of biotinylated capturing antibodies at a concentration of 667 nM followed by a spin and through the streptavidin-biotin interaction the capturing antibodies were bound to the solid phase. The capturing antibodies were diluted in wash buffer described above. After the immobilisation of capturing antibodies to the particles, two wash steps were performed with wash buffer and same short spin as previous wash steps. Standard concentrations for recombinant IFNγ were generated in triplicate (six standards 1.95 pM – 2 nM) and two dilution buffers were used, PBS (same as described but without Tween-20) including 1% BSA and lysis buffer (20 mM Trizma-Base pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.5% Deoxycholate, 0.5 mM Na_3VO_4 and 1% Trasylol). The standard samples were added to the individual inlet in the microstructures. The subsequent spin made volume definition possible and 200 nl of standards were run into the columns, allowing capture antibodies to interact with IFNγ molecules. Two wash steps were performed and the latter followed by a very short spin to ensure that the columns were filled with liquid during the fluorescence detection. The background fluorescence detection included three detection steps with different sensitivity set on the LIF detector, 1%, 5% and 25%. Excess buffer was washed away by a short spin before the addition of detection antibody conjugated with Alexa 647 detection reagent. The detection antibody was diluted in PBS buffer containing 1% BSA and two concentrations were used in the assay, 50 nM and 100 nM. The detection antibody was allowed to bind to IFNγ during the following spin step and the sandwich immunoassay was formed with capture antibody on solid phase, analyte attached to capture antibody and detection antibody bound to analyte. Six wash steps were included, two times with normal wash buffer and four times with wash buffer including isopropanol 20%, to remove all unreacted detection antibody.

2.3.1.2 Cell lysate assay

The purpose was to determine the degree of phosphorylation on the PDGF β-receptor and to compare the results from stimulated with unstimulated cells. By using two PDGF β-receptor specific antibodies and one activation-specific antibody, a phosphotyrosine specific antibody, the degree of phosphorylation can be estimated.

In the cell lysate assay several different combinations of the three antibodies were tested in order to find out what antibody pair that gives the highest degree of specific binding, 958/PY99, 958/P-20, P-20/P-20, P-20/958 and P-20/PY99 (capturing/detecting). The capturing antibody was diluted to 667 nM and titration of the detecting antibody was performed and 400 nM seemed to be a good concentration. The β-PAE cell lysate with +/- PDGF-BB stimulation was diluted in lysis buffer (2 x, 4 x, 8 x) and undiluted cell lysate was also included as a standard point.

In the beginning, cell lysate runs were performed with CDs containing one single affinity column and the same method flow used as the IFNγ assay. The cell lysate method was performed in the same manner as described above but modifications were tested because of difficulties to establish a specific interaction between the PDGF β-receptor and the antibodies. First of all, the analyte and the detection antibody spin program were extended and the linear flow over the column reduced to enable the PDGF β-receptor in the cell lysate to attach to the capturing antibody. After analyte addition, three extra wash steps were included with PBS buffer without Tween-20 and the second and the fourth wash step were followed by a pulsed spin program, allowing the solution to move through the columns by simple diffusion. The final wash steps after detecting antibody were the same with the exception that IPA was excluded in the wash buffer.

2.3.1.3 Double column

Due to a lot of uncontrolled non-specific interactions in the cell lysate assay a double column was loaded in the microstructures. The gel filtration particle Superdex^{TM} peptide (Amersham Biosciences, Uppsala, S.) was loaded in a 5 x dilution slurry onto the existing polystyrene phenyldextran particle bed in all microstructures in CDBA2. The Superdex particle is used for high-resolution gel filtration of peptides and other small biomolecules of 100-7000 molecular weight.

2.3.1.3.1 The effects of normal non-labelled IgG

Efforts have been made to reduce high background effects and one attempt was cross-reactant IgG (non-labelled) added to the detecting antibody in ten molar excess relative the detecting antibody concentration. Two antibody set-ups were tested, capturing 958 together with detecting PY99 (400 nM) including normal rabbit IgG (4 μM) and capturing P-20 in combination with detecting PY99 (400 nM) supplemented with normal goat IgG (4 μM). Both the rabbit IgG and the goat IgG were from ICN Biomedicals, Inc (Aurora, Ohio).

2.3.1.3.2 α-PAE control experiment

α-PAE cells stably expressing the PDGF α-receptor has also been grown and used in a control experiment. The α-PAE cells were grown in a 175 cm^2 culture flask and α-PAE cell lysate prepared as described before for β-PAE cell lysate with the exception that neither starvation nor stimulation was performed. The cell lysate was run in a CD containing Superdex double column with the PDGF β-receptor antibodies in order to verify the specificity.

2.3.2 Immunoprecipitation and Western blot

Nine antibodies were analysed in immunoprecipitation and in western blot, non-labelled P-20 Gyros, P-20 fluorescently labelled, non-labelled P-20 LICR, non-labelled 958 Gyros, 958 fluorescently labelled, non-labelled 958 LICR, non-labelled PY99 Gyros, PY99 fluorescently labelled and non-labelled PY99 LICR. All antibodies were tested with β-PAE cells transfected with PDGF β-receptor and α-PAE cells transfected with PDGF α-receptor as control and cells were +/- stimulated with PDGF-BB.

2.3.2.1 Cell lysate preparation

β-PAE cells and α-PAE cells were grown on 75 cm^2 culture plates and a nearly confluent monoloyer of cells were starved overnight in Ham's F-12 medium including 0.1 mg/ml BSA and the aminoacid glutamine. The cells were stimulated with 100 ng/ml PDGF-BB for 60 minutes on ice followed by two washes in ice-cold PBS. 1 ml lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.5% Deoxycholate, 0.5 mM Na_3VO_4 and 1% Trasylol) was added to each plate and the cells were scraped off by a "rubber policemen" scrape and lysed for 15 minutes on ice. The lysates were clearified by centrifugation at 13,000 rpm for 15 minutes at 4°C and the supernatant saved for precipitation.

2.3.2.2 WGA Sepharose precipitation

PDGF β-receptors and PDGF α-receptors were adsorbed to wheat germ agglutinin (WGA) Sepharose (Amersham Biosciences, Uppsala, S.). Fifty µl WGA Sepharose solution were added to each +/- stimulated β-PAE and α-PAE cell lysate preparation followed by 60 minutes incubation end-over-end at 4°C. The precipitated proteins were washed three times in lysis buffer (0.5 M NaCl) and 50 µl 2 x Sample buffer (0.08 M Tris-HCl pH 8.8, 0.008% bromophenol blue, 28% glycerol, 4% SDS and 2% β-mercaptoethanol) was added to each reaction. In the end, the proteins were denatured for five minutes in 96°C and stored in –20°C.

2.3.2.3 Immunoprecipitation

The antibodies mentioned above were added (1 µg/ml) to the β- and α-cell lysate preparations and the precipitation was performed overnight end-over-end at 4°C. The immunoprecipitates were incubated with 50 µl Protein A Sepharose slurry (Amersham Biosciences, Uppsala, S.) for 60 minutes end-over-end at 4°C. Adsorbed proteins were washed three times in lysis buffer (0.5 M NaCl) and finally 50 µl 2 x Sample buffer was added followed by protein denaturation.

2.3.2.4 Immunoblotting

The precipitated proteins, WGA Sepharose precipitation and immunoprecipitation, were separated by SDS-PAGE (7% polyacrylamide gel, 150 V for 90 minutes) and transferred to nitrocellulose membranes by semidry transfer (15 V for 45 minutes). The membranes were stained with 0.5% Ponceau-S in 10% HAc until the size markers could be visualised and destained with TBS buffer (0.5 M Tris-HCl pH 8.0, 0.138 M NaCl) and subsequently the filters were blocked with 5% BSA in TBS for 30 minutes. After the blocking procedure the membranes were cut in order to separate WGA and IP fractions followed by blotting overnight with primary antibodies in sealed plastic bags on a plate shaker at 4°C. The WGA-precipitates were incubated with the mentioned antibodies (P-20 and PY99 1 µg/ml and 958 2 µg/ml) and the IP-fractions were incubated with P-20 (goat; 1 µg/ml) from Ludwig and all primary antibodies were diluted in 3 ml TBS supplemented with 1% BSA and 1:500 NaN_3. Bound antibodies were visualised by ECL reagent after incubation with horseradish

peroxidase (HRP) conjugated secondary antibodies (Amersham Biosciences, Uppsala, S.;1:10000 antirabbit and antigoat and 1:5000 antimouse), using a Fuji LAS-1000plus camera. Several washes TBS-T (TBS buffer described above with 0.1% Tween-20) were included after the primary and secondary antibody incubations. The bands were analysed using AIDA advanced image data analyser software.

3 Results

3.1 Matrix effects with IFNg

A sandwich immunoassay with IFNγ, known to generate specific and sensitive results in the CD, was performed to investigate the matrix effects of lysis buffer by diluting recombinant IFNγ in lysis buffer and in PBS-BSA as control. Due to the high detergent concentration in the lysis buffer, the hydrophobic breaks were contaminated with detergent and therefore analyte standards in lysis buffer passed the break with no volume definition in some structures as a consequence. When analysing the micro fluidics in the CD during the run, one important notice was that the lysis buffer moves faster than PBS-BSA while spinning the CD. The resulting standard curves were plotted with the integrated volumes of intensity in the columns against the concentrations of IFNγ on a logarithmic scale. The generated standard curves are shown in figure 4.

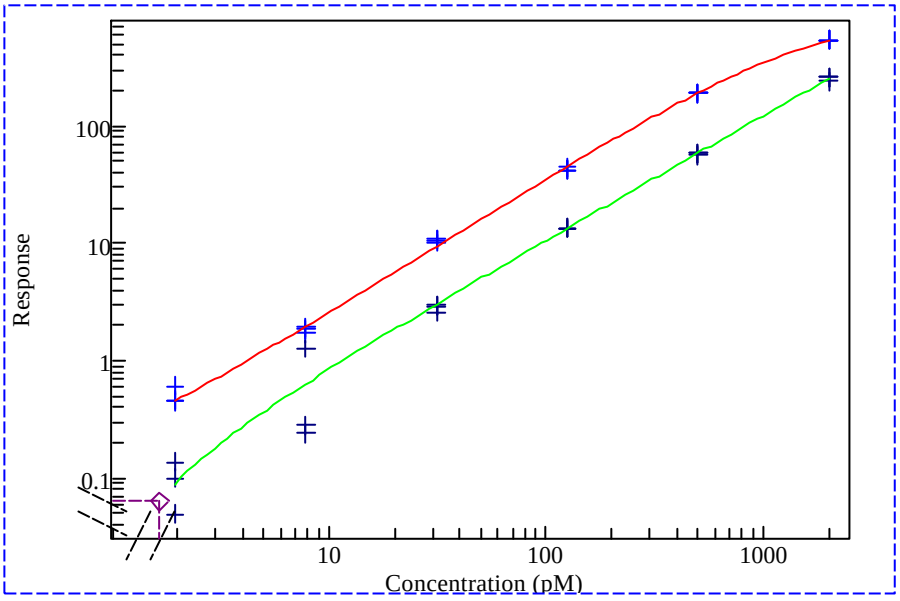


Figure 4. Standard curves for IFNγ in different dilution buffer: the upper curve represents triplicate standards diluted in PBS-1%BSA and the lower curve is triplicate standards diluted in lysis buffer. Detecting antibody has a concentration of 100 nM. In this case volume definition succeeded.

The detection limit concentration was 1.7 pM for the lysis buffer curve and undefined for the normal buffer curve (below the lowest concentration, < 1.95 pM) and the resulting CV % values (Coefficient of Variation) for the triplicate standards showed a satisfactory level. The CV % values varied between 0.5-5% in PBS-BSA samples and somewhat higher values in lysis buffer samples for the higher concentrations. The lower concentrations showed an increase in CV % values for both analyses. The corresponding column images are illustrated in figure 5. Taken together, the results revealed a three times difference in integrated signals for the two matrix experiments.

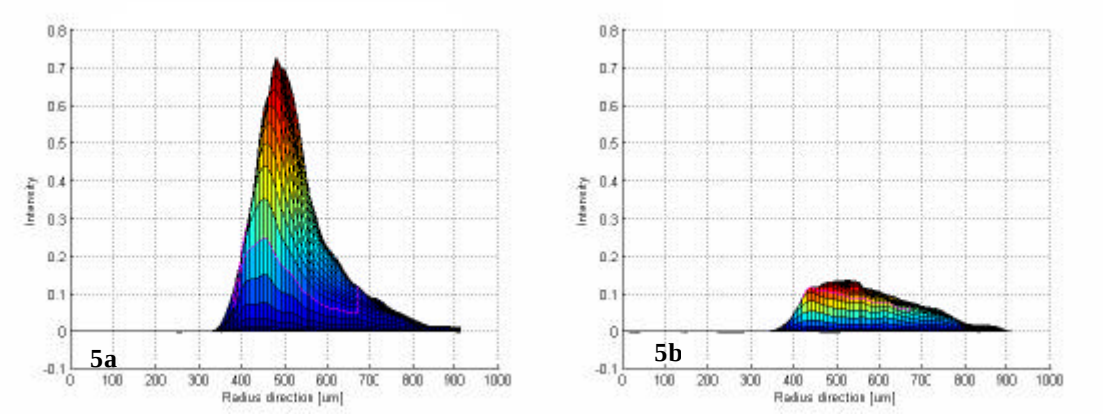


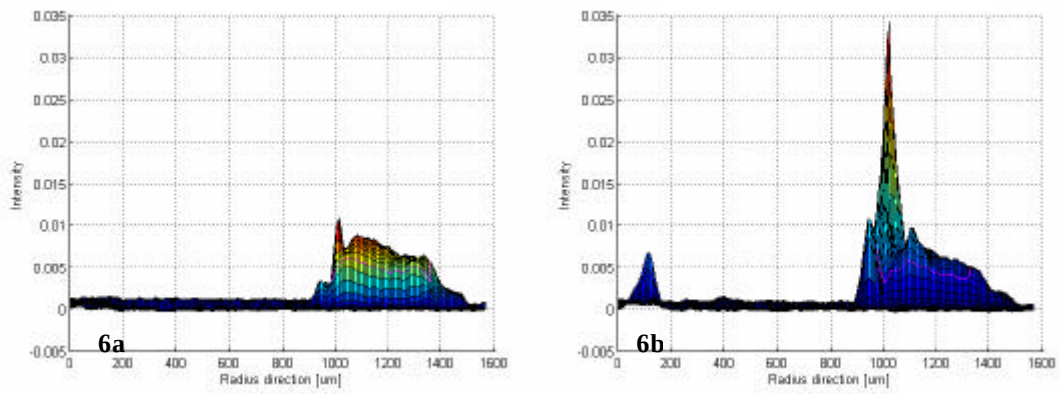
Figure 5. The column profiles illustrates the IFNγ concentration 500 pM for the two matrices, picture **a** shows the PBS-BSA matrix and picture **b** shows the same concentration diluted in lysis buffer.

3.2 Cell lysate assay

To characterise the activation status of a membrane bound protein in cell lysate, namely the PDGF β-receptor, a specific sandwich immunoassay integrated in a miniaturised CD laboratory was tested. In this specific arrangement one antibody was raised against the target protein (PDGF β-receptor) and another antibody was raised against the regulatory phosphorylated tyrosines on activated receptor.

3.2.1 Single column

The titration of detecting antibody was difficult because the assay required a high concentration of detecting antibody to distinguish significant signals at all and a too high concentration gave high background intensity. The detecting antibody concentration finally used was 400 nM and the capturing antibody concentration 667 nM. The hydrophobic breaks managed the high detergent concentration (0.5% Triton) in many cell lysate runs. Several antibody pairs were tested (958/PY99, P-20/PY99 and P-20/958) and two of the combinations, 958/PY99 and P-20/958, showed no significant difference between stimulated and unstimulated cell lysate. The cell lysate signals could not be separated from blank responses, for example see figures 6a-c. Some difference was seen between the cell lysates with the antibody pair P-20/PY99 but the column profiles reveal disturbances, especially in the top of the column (profiles not shown).



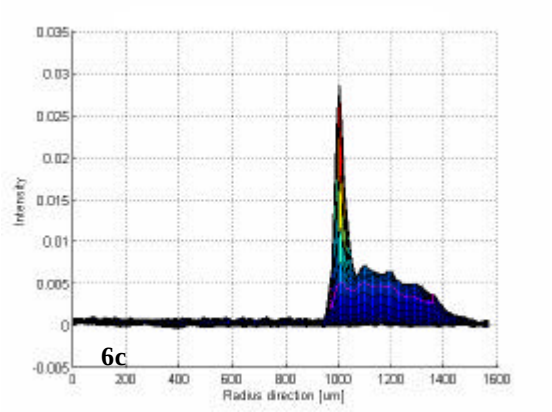


Figure 6. The figure shows the enrichment on column; **a** and **b** showing stimulated and unstimulated cell lysate, respectively and figure **c** is a profile of a blank. 958 is used as capturing antibody (concentration 667 nM) together with PY99 as detecting antibody (concentration 400 nM). The presented images are one of three images; cell lysate samples are performed in triplicates. The blank column profiles reveal a high background.

The following antibody pairs were also tested, 958/P-20, P-20/P-20 and 958/PY99 (normal rabbit IgG was added to PY99) and a high background intensity was detected when fluorophore labelled P-20 was used as detecting antibody (See figure 7a-b).

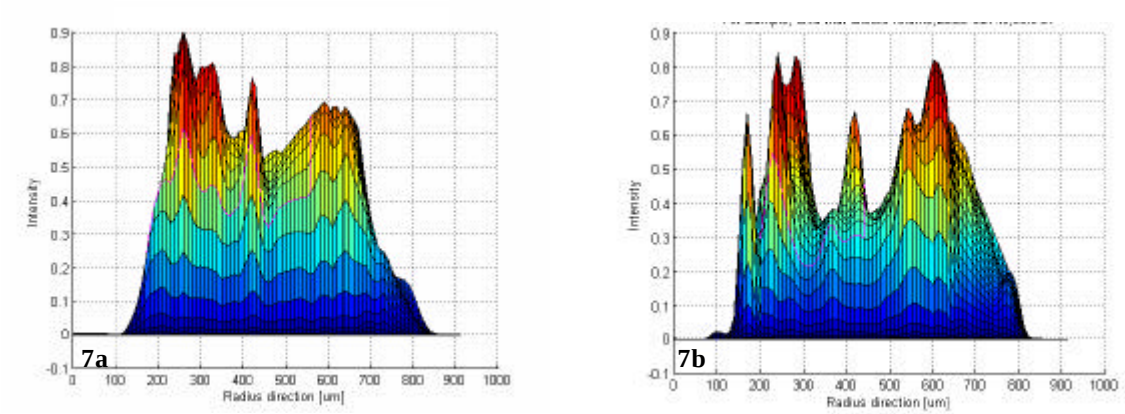


Figure 7. To the left is stimulated cell lysate and to the right is a blank sample. The antibody pair used is P-20/P-20.

In the single column experiments, the antibody PY99 also showed an enhanced background together with 958 as capturing antibody. The effect with high background was getting worse when detecting antibody concentration was raised and true signals were difficult to discriminate from background intensity. The result from 958 as detecting antibody did not show high backgrounds and as a result three antibody pairs could be used in further experiments, P-20/958, P-20/PY99 and 958/PY99.

In general, the cell lysate runs revealed high CV values due to the signal distribution where a lot of variation between the triplicates was seen. In some cases a small difference in signal could be seen between undiluted cell lysate and blank signals but mostly significant signals were hard to distinguish from background signals. The enrichment in the columns was mostly irregular and a general column pattern could not be seen, in some cases the signal had a tendency to progress down the column as seen for the IFNγ columns.

3.2.2 Double column

The idea was that a particle bed loaded on top of the existing streptavidin-coated particles could function as a filter, decreasing the amount of small interfering molecules present in the cell lysate. However, large molecules like the receptors and the antibodies would flow through the gel filtration particle bed. Two types of double columns were tested, a phenyldextran bed on top of the existing column and superdex peptide particles packed on top

of the existing streptavidin-coated phenyldextran particles. The Superdex peptide double columns showed an improvement in signals for three antibody pairs and differences between cell lysate and blank responses could also be discerned, thus the following runs were performed with this double column alternative. Comparison of the different double columns and the simple column is illustrated in figure 8.

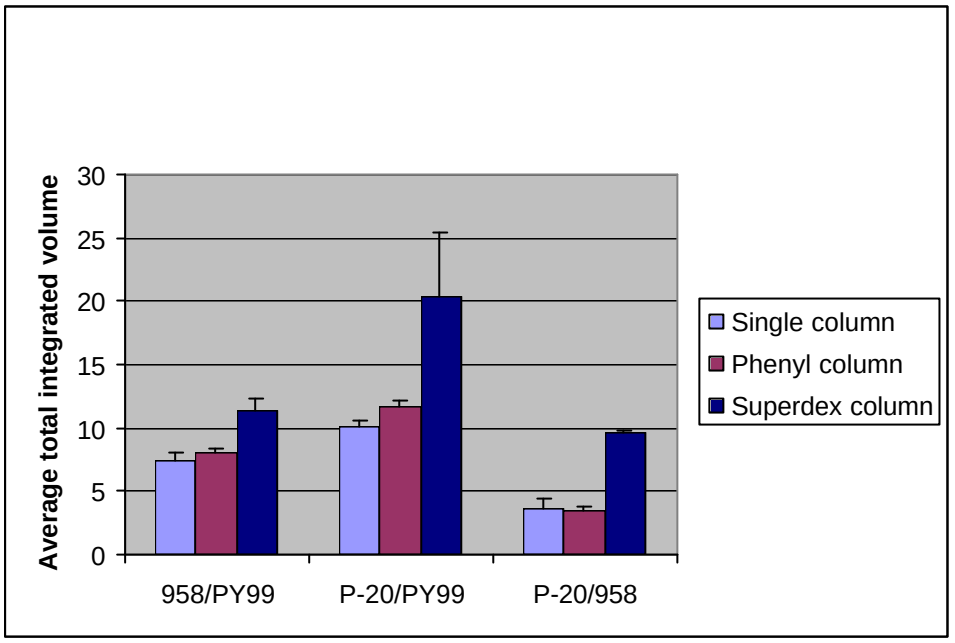
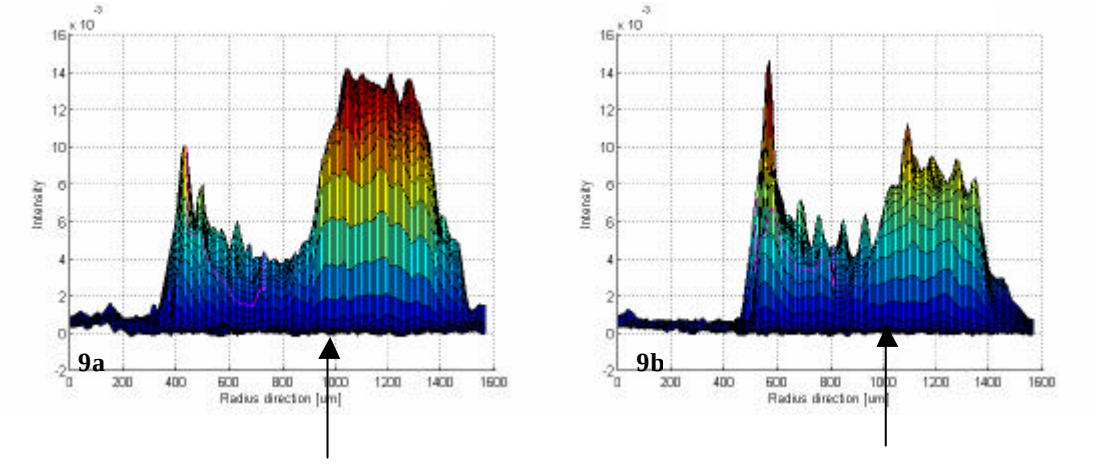


Figure 8. Comparison between single and two different double columns with three antibody pair tested. The bars represent the average total integrated signals from undiluted stimulated cell lysate prepared in triplicates and the error bars indicate the standard deviations. The double column with the Superdex particle bed revealed increased signals for all three antibody combinations.

The antibody pair P-20/PY99 gave the strongest responses and the triplicates were rather homogeneous which yielded relatively low CV % values, mostly between 5 and 15%. Stimulated cell lysate showed higher response values than unstimulated cell lysate and dilution of the cell lysate could also be followed and still have distinguishable signals from blank signals (See column figures 9a-d). The same pattern was seen with the antibody pair 958/PY99 although the integrated signals were lower compared to P-20/PY99 signals (data not shown).



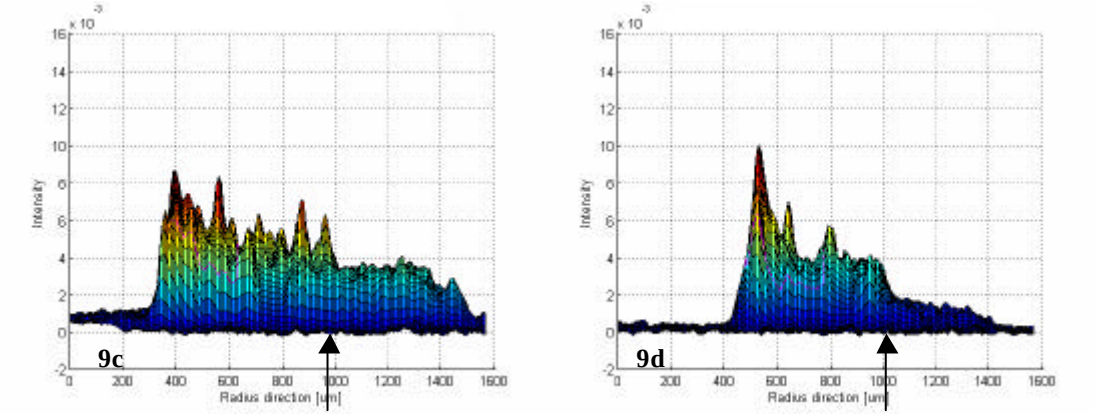


Figure 9. The antibody pair P-20/PY99 in a Superdex double column experiment is illustrated in the column profiles, the concentration of capturing P-20 was 667 nM and the concentration of detecting PY99 was 400 nM. The Superdex column starts around 300 µm and the Streptavidin-coated particle bed starts around 900-1000 µm. Figure **a** and **b** shows stimulated cell lysate, undiluted and an eight fold dilution, respectively. Figure **c** shows unstimulated cell lysate and figure **d** is a blank profile (lysis buffer). The arrows indicate the approximate beginning of the Streptavidin-coated particle bed.

When considering the antibody pair P-20/958, the results from stimulated and unstimulated cell lysate had the same column pattern with equivalent signals (See column figures 10a-c).

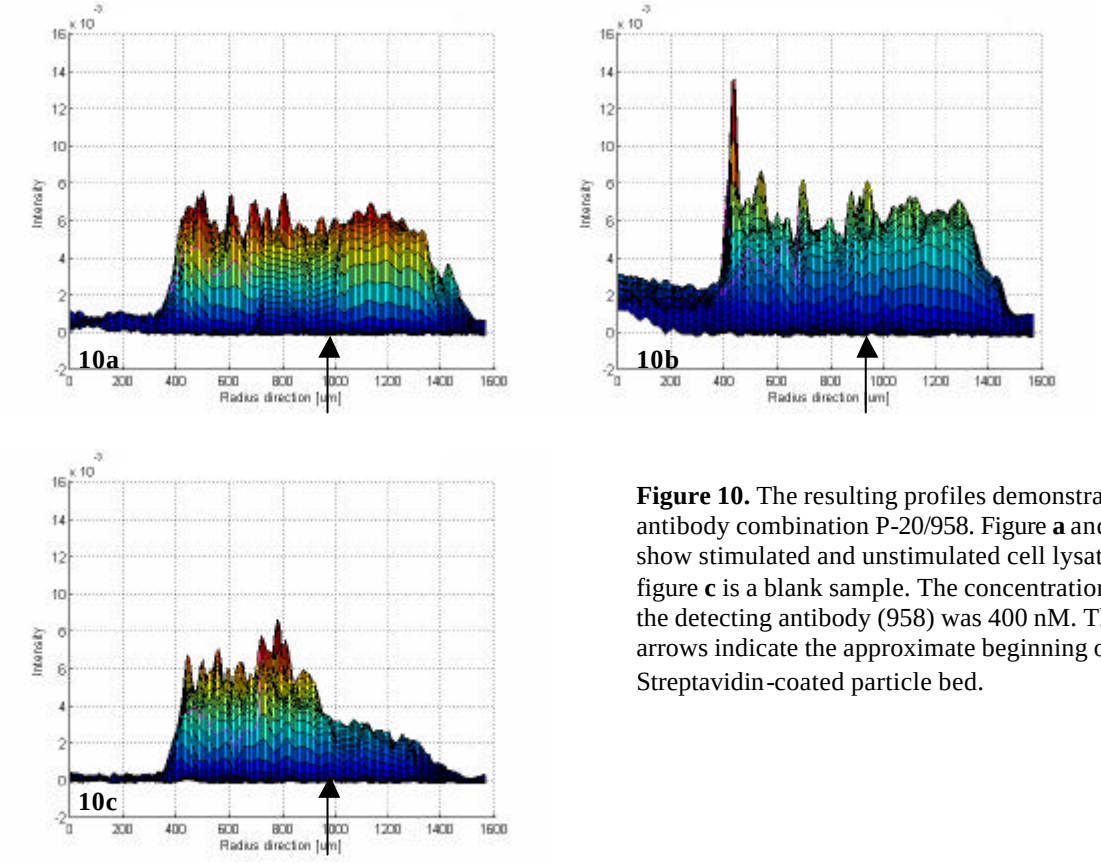


Figure 10. The resulting profiles demonstrate the antibody combination P-20/958. Figure **a** and **b** show stimulated and unstimulated cell lysate and figure **c** is a blank sample. The concentration of the detecting antibody (958) was 400 nM. The arrows indicate the approximate beginning of the Streptavidin-coated particle bed.

The amount of PDGF β-receptor in the two cell lysates is assumed to be the same with some differences due to variations in number of cells. Thus the results from the PDGF β-receptor antibodies follow the expected pattern. The signals are weaker compared to the combination P-20/PY99 and the CV % values between triplicates are generally low. However, the

integrated volumes are lower compared to the runs with PY99 as detecting antibody (See figure 11).

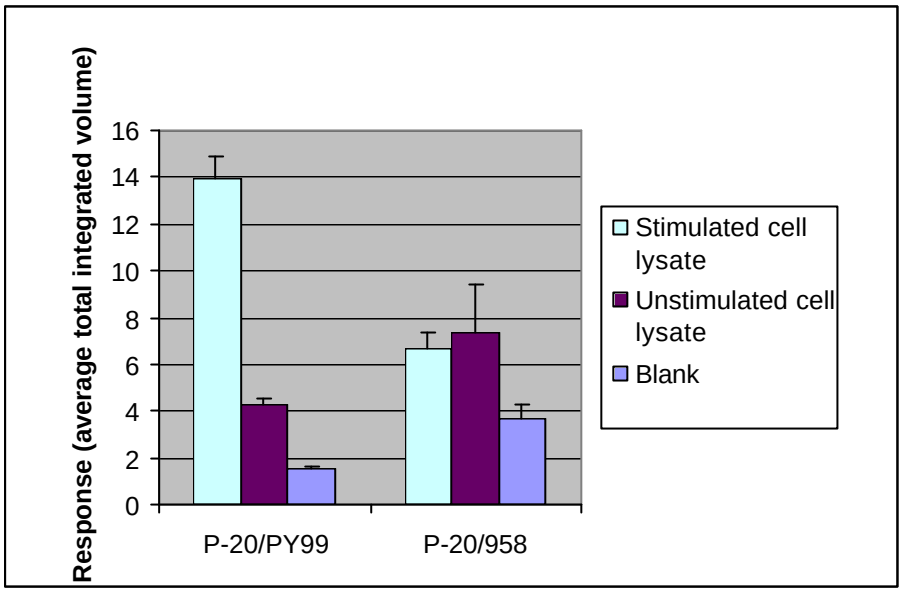


Figure 11. The bars show the average total integrated volumes for the two antibody combinations P-20/PY99 and P-20/958 and the assay is performed with the Superdex double column. The cell lysate samples are performed in triplicates and the error bars indicate the standard deviations.

3.2.2.1 The effects of normal non-labelled IgG

The combination capturing 958 and detecting PY99 (normal rabbit IgG added) revealed interesting results; in the top of the Superdex column a very strong signal peak was observed (See column figures 12a-b) and the integrated signals somewhat decreased over the affinity column compared to result from antibody without normal IgG (data not shown). No noteworthy difference was observed between the combinations P-20/PY99 and P-20/PY99+normal goat IgG (column profiles not shown).

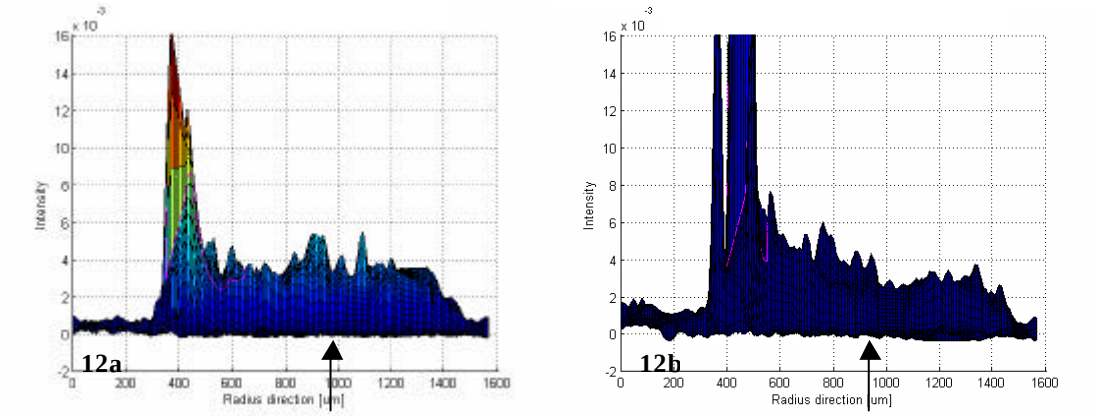


Figure 12. Column profiles illustrate the typical pattern that arises when normal rabbit IgG is added to the detecting antibody PY99 in combination with capturing 958. **a** and **b** shows stimulated and unstimulated cell lysate. The arrows indicate the approximate beginning of the Streptavidin-coated particle bed.

3.2.2.2 a-PAE control experiment

The run yielded higher response signals than previous experiments because the hydrophobic breaks let the cell lysate through and the data indicated variations among triplicates and

showed relatively high integrated signals in the α-PAE samples with the antibody combination P-20/958 (See figure 13).

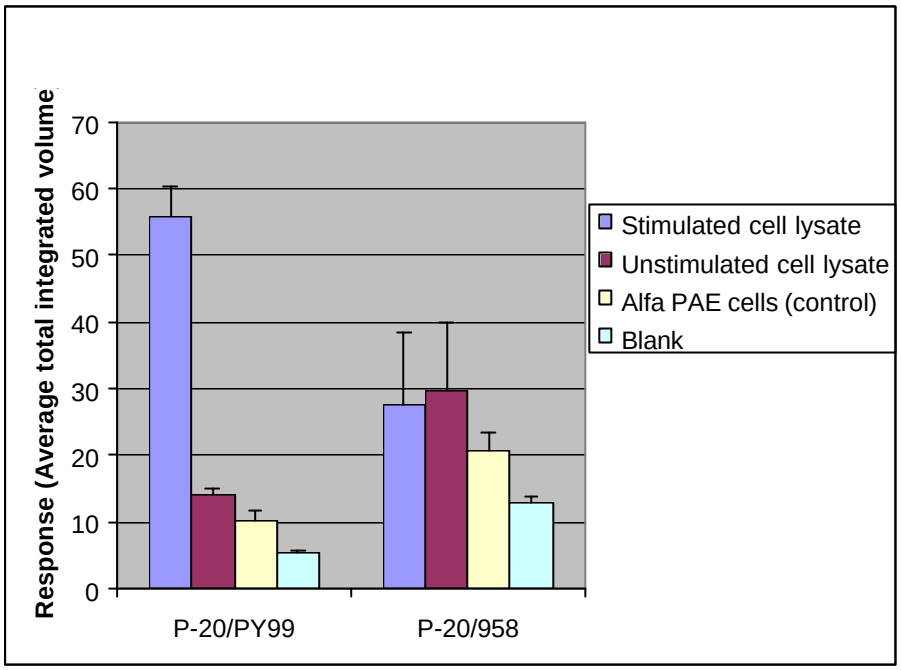


Figure 13. Total integrated volumes over the affinity column are shown for the different cell types performed in triplicates together with the corresponding antibody combinations. The figure indicates a high background with the antibody pair P-20/958. Volume definition did not succeed, explaining the higher integrated signals compared to previous experiments.

3.3 Immunoprecipitation and Western blot

To further evaluate the CD results regarding antibody specificity, immunoprecipitation and western blot was performed with the antibodies tested in the CD model system. The antibodies used in the sandwich immunoassay are frequently used in IP/WB at Ludwig, yielding specific results. β-PAE cells were analysed and α-PAE cells were used as control, both stimulated and unstimulated cell lysate.

3.3.1 WGA-precipitations

First of all, P-20 Gyros bound to the β+ and β- WGA-precipitates in the blotting procedure as expected, fluorescently labelled P-20 and P-20 from LICR showed no bands at all. Furthermore, the blotting with all of the 958 antibodies were negative and 958 in the sandwich immunoassay has revealed weak responses if any. Both unlabelled and fluorescently labelled PY99 from Gyros bound stimulated β-PAE WGA-precipitates as expected but none of them bound corresponding stimulated α-PAE WGA-precipitates. Finally, PY99 from LICR bound stimulated β-PAE fractions and bound to a lesser extent stimulated α-PAE fractions in the blotting. The band with antibody PY99 were more intense than the P-20 bands indicating PY99 as a specific antibody and PY99 as detecting antibody in the CD assay has given strong signals as well.

3.3.2 Immunoprecipitation fractions

The immunoprecipitation experiment failed for all antibody pairs, and for that reason it was not obvious whether the immunoprecipitation failed or if the blotting procedure was unsuccessful, since the positive control with P-20 LICR in the WGA experiment did not

work. A very weak band appeared at the stimulated β-PAE lane for antibody PY99 LICR in the immunoprecipitation experiment. The developed membrane images are not shown but the results are presented in Table 1.

Table 1. Results from the WB/IP-experiment with the mentioned antibodies.

Antibody:	**PAE-cell line**	**PDGF-BB**	**Result WGA (blotting)**	**Result IP (precipitation)**
P-20 Gyros (non-labelled)	α	+	-	-
	α	-	-	-
	β	+	++	-
	β	-	++	-
P-20 Fluorophore labelled	α	+	-	-
	α	-	-	-
	β	+	-	-
	β	-	-	-
P-20 LICR (non-labelled)	α	+	-	-
	α	-	-	-
	β	+	-	-
	β	-	-	-
958 Gyros (non-labelled)	α	+	-	-
	α	-	-	-
	β	+	-	-
	β	-	-	-
958 Fluorophore labelled	α	+	-	-
	α	-	-	-
	β	+	-	-
	β	-	-	-
958 LICR (non-labelled)	α	+	-	-
	α	-	-	-
	β	+	-	-
	β	-	-	-
PY99 Gyros (non-labelled)	α	+	-	-
	α	-	-	-
	β	+	++++	-
	β	-	-	-
PY99 Fluorophore labelled	α	+	-	-
	α	-	-	-
	β	+	++++	-
	β	-	-	-
PY99 LICR (non-labelled)	α	+	+++	-
	α	-	-	-
	β	+	+++++	+
	β	-	-	-

4 Discussion

4.1 Single column

4.1.1 Matrix effects

The matrix effect experiment showed a significant difference (three times) in signals between the matrices, suggesting that the lysis buffer interferes negatively with the antibody-antigen interaction. Antigen loss is abundant in the top of the column where most of the signal is decreased for lysis buffer matrix. Probably, the most critical step is the ability of capturing

antibody to bind antigen due to reduced possibilities getting excess to antigen. One possible explanation is that the antigen might be buried in micelle complexes and that such micelles do not diffuse rapidly enough while still in the capture column. Despite the complications with lysis buffer, the resulting standard curves reveal a similar slope compared to normal matrix PBS-1%BSA.

The observation that the lysis buffer flowed faster through the structures compared to PBS-BSA buffer is expected because of the high detergent concentrations. Mostly the breaks persisted the high detergent concentration and the pattern seemed to be that in almost every liquid distribution, the break in the first structure did not persist to hold the liquid.

4.1.2 Cell lysate assays

The overall picture is that a lot of unspecific interactions due to low antibody specificity made the analysis complicated, significant signals were difficult to distinguish from background and blank samples. The only antibody pair showing signals distinguishable from blank signals was P-20/PY99, however no significant difference could be seen between stimulated and unstimulated cell lysate, which is expected with the phosphotyrosine-specific antibody. Due to the disturbances, method modifications tested were difficult to interpret to improve signals. Isopropanol (IPA) was excluded in the wash buffer because the thought was that it could interfere with the immunochemistry.

4.2 Double column

Specific interaction with the antibody pair P-20/PY99 was observed, signals from unstimulated cell lysate can be regarded as background since no activated receptors (phosphorylated) are supposed to be included. When considering the combination P-20/958 the results point to an unstable assay with unspecific interactions due to high background signals in blank samples. The wide enrichment in the column can be due to diffusion properties, the larger the size of the molecule/complex, the slower diffusion in the column and the molecule follow the liquid flow to a greater extent. The double column effect can have a number of reasons; small molecules and peptides diffuse in the gel filtration particles, decreasing factors that possibly affect the antibody-antigen reaction as well as different types of unspecific interactions involving assay reagents. Presumably, the Superdex column functions as a filter for aggregated antibodies. Large molecules are supposed to pass the Superdex particle bed but very large protein complexes perhaps have difficulties entering the column.

4.2.1 The effects of normal IgG

The high peaks observed in the very beginning of the Superdex column when normal IgG as cross-reactant was added to detecting antibody, especially with the combination 958/PY99 including normal rabbit IgG, were possibly caused by antibodies aggregated to large complexes unable to enter the gel filtration bed. For some reason the PY99 batch maybe contained Ig-fractions with reactivity for rabbit IgG (958) and one proposal to the observation is that the addition of normal rabbit IgG sort out the antibodies with low specificity. The remaining samples in the pool of antibodies are supposed to be specific. Although a monoclonal antibody is by definition, a specific reagent with respect to the binding to epitopes there are several features that can affect assay performance, for example the isotype of a monoclonal is important [12]. The antibody PY99 is of subclass IgG_{2b}, a subclass known to be sticky in immunoassay situations [Personal communications, Mats Inganäs]. Furthermore,

the monoclonal antibody is derived by fusion of mouse myeloma cells with spleen cells from a mouse immunised with phosphotyrosine and purified by affinity chromatography (described by the supplier). Expansion of cells for making the required antibodies can be performed in two ways, expansion of cells in tissue culture medium or expanding clones *in vivo*. In the latter, hybridoma cells are injected in mice able to grow without rejection and the ascites fluid obtained is contaminated with irrelevant mouse immunoglobulins, which is difficult to remove without antigen-based affinity purification. The advantage with this method is that it yields large amounts of specific antibody compared to the tissue culture systems [4]. Thus, if the PY99 clone is expanded *in vivo*, the proposal strengthens that it includes unspecific IgGs.

4.2.2 α-PAE control experiment

The control experiment with α-PAE cells pointed to the fact that the antibody combination P-20/958 gave rise to high background probably as a result of unspecific interactions and consequently the specific interactions are difficult to evaluate. Although the supplier confirms that no cross-reactivity with α-receptor will occur, the observation contradicts the statement in this experiment. The P-20/958 combination also shows high background in blanks, revealing the possibility of unspecific interaction between the two antibodies.

4.3 Immunoprecipitation and western blot

The antibodies were used in immunoprecipitation with both α-PAE and β-PAE cell lysate and in blotting of WGA-precipitates with corresponding cell lysate (positive control). In general, the WGA-precipitation experiment indicated similar results compared to sandwich immunoassay in the CD. Non-labelled P-20 from Gyros used in blotting showed expected result, by binding to β+ and β- WGA-precipitates and this indicates that the P-20 batch ordered by Gyros has a tendency to specific behaviour. When considering the same WGA experiment with the fluorophore labelled P-20 from Gyros, the result pointed to an inactive antibody or to the fact that the secondary antibody was not able to recognise the labelled antibody. The results from fluorophore labelled PY99 together with the outcome from the experiments with P-20 as detecting antibody in the CD, the overall picture supports the hypothesis that the labelled P-20 was inactive for an unknown reason. Maybe the labelling chemistry affected the antibody specificity or induced changes that made the antibody inactive.

Blotting with the different 958 antibodies was unsuccessful in the WGA experiment and this outcome can have a number of possible causes. The fact that 958 was affinity purified (maybe Protein A) may perhaps be a reason because the Ig-fraction might contain a lot of unspecific antibodies unable to bind PDGF β-receptor. As mentioned, the sandwich immunoassay experiments with 958 have experienced unspecific interactions, for example by binding to α-PAE cell lysate and a similar pattern was observed in the western blot results. In contrast, P-20 was peptide affinity purified (information from the supplier), giving more specific results as observed.

The two PY99 antibodies from Gyros were able to bind WGA-precipitates including stimulated β-PAE but PY99 is expected to bind the analogous WGA-precipitates counting stimulated α-PAE as well. One possibility is that the WGA-precipitates came from α-PAE cells mistakenly unstimulated and consequently the precipitate did not contain any phosphorylated receptors. One way to avoid this problem is to pool all cell lysates of the same

type (α+, α-, β+, β-) from the different plates and after that distribute to the different reactions. PY99 from LICR managed to bind both β+ and to a smaller degree α+ WGA-precipitates in the blot.

The immunoprecipitation with the corresponding antibodies did not work at all and the cause was difficult to probe since the positive control also failed, namely P-20 LICR in the WGA-blot. Conclusions were hard to elucidate whether the immunoprecipitation or the blotting procedure failed. The fact that a very weak band was noticed in the immunoprecipitation experiment with PY99 LICR supports the hypothesis that the immunoprecipitation failed with the different antibodies and that the blotting procedure succeeded. One possible explanation to the unsuccessful immunoprecipitation with P-20 (goat) is that Protein A has reduced binding strength for goat IgG relative Protein G [13]. If the experiment was repeated, the immunoprecipitates with goat P-20 perhaps should have been immobilised to Protein G Sepharose as an alternative. This speculation does not explain the outcome from the immunoprecipitation with the other antibodies, rabbit and mouse, which are assumed to bind Protein A [13]. The failure with antibody 958 in the immunoprecipitation is in accordance with the suggestion that the antibody behaves in an unspecific mode. The reasoning with the antibodies P-20 and 958 also supports the theory that the immunoprecipitation was unsuccessful and the blotting was going off well.

The conclusion from the IP/WB experiment is that one experiment is not enough and further investigations need to be done in order to get a more detailed picture of the different antibody actions. An interesting test would be to explore the biotinylated capturing antibody P-20 in the same IP/WB set up.

4.4 Comparison IP/WB and sandwich immunoassay in the CD

Altogether, the results from the different experiments disclose the difficulties to establish a specific interaction with poorly soluble protein in cell lysate. There are many components giving rise to background and proteins or other molecules cannot be excluded from the cell lysate by a simple procedure. Primarily, the model system in the CD demands very specific antibodies because there are many molecules in the cell lysate that can affect the immunochemistry and make interactions uncontrollable. Due to the complications in the CD the IP/WB experiments seem more robust.

Theoretically, the total cell lysate prepared and used in the CD includes a low concentration of PDGF β-receptors (approximately 4000 pM), giving the lysis buffer volume used per culture flask and the following assumptions; every cell contains around one hundred thousand receptors and the fact that one culture flask can hold five million cells. The very sensitive instrumentation in the Gyrolab enables detection of concentrations at this level. When comparing the amount of receptor loaded for each reaction in the WB experiment and the amount loaded onto every column, the difference is approximately a factor of two hundred, given the size of the culture plates/flasks, the amount lysis buffer used and the volumes loaded (200 nl in every microstructure and 500 µl cell lysate is consumed per lane on the gel).

The high detergent concentrations are used to keep the receptor in solution. The cell lysate was not pretreated or enriched before loaded onto the columns compared to the IP/WB experiment where WGA-precipitations (glycosylated proteins) and IP with the specific antibodies were performed to enrich the cell lysate. The cell lysate prepared for CD assays

was concentrated by lysis in 200 µl lysis buffer and the normal amount is 1 ml. In addition, the receptor is totally exposed by the protein separation and the transfer to membranes, which facilitates antibody binding and specific binding is easier to establish. There are substantial differences in time for antibody-receptor binding; in the CD eight minutes for receptor-capture and for detection antibody to bind receptor compared to WB where membranes were incubated overnight with primary antibody.

The antibody consumption for WB is 5-10 µg compared to the CD assay where capture antibody consumption is 4 µg and detection antibody consumption is approximately 1 µg (cell lysate assay). When comparing the data points for the two methods, the CD yields 104 data points compared to the WB yielding 10 data points, for the indicated antibody amounts. Thus, the reagent consumption using the CD-based assay is reduced by a factor of approximately ten per data point.

5 Future perspectives

An interesting approach would be to examine the immunochemistry with the cell lysate in more detail, for example what happens if the receptors are located within large micelle complexes? After the receptor is captured, is it possible to get rid of the detergents by specific washes without affecting the immunochemistry in a negative way? Additionally, detergent relationships can be further explored by testing different lysis buffer compositions and evaluate which constituents that are critical for specific antigen-antibody interaction.

Several improvements need to be performed in the sandwich immunoassay to get more specific and reliable results; different control experiments of the antibodies and explore the possibilities of using another particle bed also streptavidin-coated with the purpose of reducing interferences.

The idea was that the degree of phosphorylation could be estimated with two detecting antibodies labelled with two different fluorophores followed by detection with a red and a green laser system. The two-colour detection system can also be performed in two separate reactions, i.e. two different microstructures, in the same CD with comparable results. For example, the use of detection antibodies specific for different states of an antigen makes it possible to determine the abundance and modification states of a protein simultaneously by estimating a ratio between the red-green intensity.

The flexibility in the Gyrolab offers possibilities to further investigate signal transduction pathways in cell lysate, signal molecules downstream the PDGF β-receptor could be analysed regarding their degree of activation in response to different stimuli. Given a specific cell lysate with corresponding labelled antibodies, an assay can easily be performed in the CD and yields specific results fast. The model system is very straightforward and modifications in the methods are not an impediment.

Improved methods for analysing cell signalling are highly warranted in drug discovery and for investigating the differences between normal and diseased tissues. In a clinical situation, the two-colour system will be valuable for investigating differences between normal and diseased tissues. For example, simplified methods for detection of activated PDGF receptors would be useful in the screening of tumour subsets and development of activation-specific antibodies is highly motivated in such approaches [3].

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