Functional Proteomics Analysis of Signal Transduction Pathways of the Platelet-Derived Growth Factor β Receptor†

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Received August 28, 1998; Revised Manuscript Received November 16, 1998

ABSTRACT: We report efficient methods for using functional proteomics to study signal transduction pathways in mouse fibroblasts following stimulation with PDGF. After stimulation, complete cellular proteins were separated using two-dimensional electrophoresis and phosphorylated proteins were detected with anti-phosphotyrosine and anti-phosphoserine antibodies. About 260 and 300 phosphorylated proteins were detected with the anti-phosphotyrosine and anti-phosphoserine antibodies, respectively, at least 100 of which showed prominent changes in phosphorylation as a function of time after stimulation. Proteins showing major time-dependent changes in phosphorylation were subjected to in-gel digestion with trypsin and identified by mass spectroscopy using MALDI-TOF mass fingerprinting and ESI peptide sequencing. We have observed phosphorylated proteins known to be part of the PDGF signal transduction pathway such as ERK 1, serine/threonine protein kinase akt and protein tyrosine phosphatase syp, proteins such as proto-oncogene tyrosine kinase fgr previously known to participate in other signal transduction pathways, and some proteins such as plexin-like protein with no previously known function in signal transduction. Information about the phosphorylation site was obtained for proto-oncogene tyrosine kinase fgr and for cardiac α-actin. The methods used here have proven to be suitable for the identification of time-dependent changes in large numbers of proteins involved in signal transduction pathways.

Platelet-derived growth factor (PDGF)[1] and epidermal growth factor (EGF) are known for their growth-promoting activity in various cell types (*1*, *2*). They have been shown to play important roles in cell growth regulation, differentiation, chemotaxis, tumor progression, and wound healing (*3*). PDGF is reported to be involved in the development of malignancies, atherosclerosis, inflammatory joint disease, and fibrotic conditions (*4*). Molecular and genetic approaches have demonstrated an absolute requirement for PDGF signaling in the vertebrate embryo (*5*). There are three PDGF isoforms, PDGF-AA, -BB, and -AB, with different affinities for two PDGF receptors, α and β (*6*). The PDGF-B chain is functionally and structurally similar to the transforming protein v-Sis of simian sarcoma virus (*7*, *8*). Binding of PDGF to its cell surface receptor induces receptor dimerization and subsequent activation of its intrinsic tyrosine kinase activities followed by receptor autophosphorylation and phosphorylation of numerous cellular proteins. Subsequently, diverse signal transduction cascades are initiated, leading to cell division and DNA synthesis (*2*). Activation and signaling of receptor tyrosine kinases involve the recruitment of cellular proteins that contain SH2 domains (*9*) such as phospholipase Cγ, phosphatidylinositol 3-kinase (PI3K), the GTPase-activating protein of Ras, Src kinases, and some adapter proteins such as Shc, Grb2, and Nck (*10*, *11*). PDGF receptor signaling is known for its cross-talk with other signal transduction pathways (*12*−*15*). Although investigations of the signaling mechanisms triggered by the binding of PDGF to its receptor have rapidly advanced in the past several years, the number of proteins involved in the signal transduction cascades, the complexity of the signal transduction pathways, and the presence of cross-talk give us reason to believe that current knowledge about this system is still far from complete.

Since much of the cellular response to PDGF involves post-translational modifications of proteins, these processes are not easily amenable to analysis by currently used genomic methods. There is a need for efficient methods for analyzing in parallel large numbers of proteins in the cell at as close to physiological conditions as possible, i.e., a proteome-based analysis of signal transduction pathways. High-resolution two-dimensional (2D) electrophoresis is an excellent tool for separating thousands of protein components in a cell (*16*−*18*), and the development by several groups of mass

† This work was supported by Deutsche Forschungsgemeinschaft Grant Go-639/1-2 to J.G.-Z.

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[1] Abbreviations: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; ESI, electrospray ionization; MS/MS, tandem mass spectrometry.

10.1021/bi982093r CCC: $18.00
Published on Web 01/16/1999

spectrometric fingerprinting (*19*−*23*) for very high-sensitivity, rapid identification of proteins provides powerful methods for investigation of complex processes in cells. On a large scale, mass fingerprinting has been successfully applied to the process of identification of proteins from the budding yeast *Saccharomyces cerevisiae* whose genome has been completed and for which construction of a 2D electrophoresis map of cellular proteins is well advanced (*24*−*26*). In this paper, we report efficient methods for studying signal transduction pathways in mouse fibroblasts upon stimulation with PDGF by means of functional proteomics. These methods have proven to be suitable for identification of large numbers of proteins involved in the signal transduction pathway, for study of signal transduction cross-talk, and for following kinetic changes in phosphorylation of tyrosine and serine at various times after cell stimulation.

EXPERIMENTAL PROCEDURES

Materials. NIH 3T3 cells were from American Type Culture Collection. Platelet-derived growth factor (PDGF-BB), monoclonal anti-phosphoserine antibodies, sodium orthovanadate, sodium fluoride, sodium pyrophosphate, and Chaps were obtained from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). Monoclonal anti-phosphotyrosine antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany); nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany). Immobiline DryStrips were from Pharmacia Biotech (Freiburg, Germany); NBT/BCIP solution and Complete mini protease inhibitor cocktail were from Boehringer (Mannheim, Germany). DMEM media and reagents for tissue culture were obtained from GibcoBRL (Eggenstain, Germany), and Duracryl (30% acrylamide, 0.65% BIS) was from ESA. Trypsin was from Promega (Madison, WI). Other chemicals (Merck and Roth) were of the best grade available.

Cell Culture. NIH 3T3 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a water-saturated, 10% CO_2 atmosphere at 37 °C in 75 cm^2 polystyrene Petri dishes. Confluent cultures were made quiescent by extensive washing with PBS and subsequently by switching to DMEM medium containing 0.5% fetal calf serum for 24 h.

PDGF Induction. Quiescent NIH 3T3 cells were incubated with or without PDGF-BB (50 ng/mL) for various periods of time at 37 °C. After the induction time, the cells were washed twice with ice-cold PBS containing 30 mM sodium pyrophosphate, 50 mM NaF, and 100 μM Na_3VO_4. The cells were lysed with 240 μL of buffer containing 0.3% SDS, 200 mM DTT, 28 mM Tris-HCl, 22 mM Tris, 1 mM Na_3VO_4, 1 mM NaF, 2 mM EDTA, 1 mM sodium pyrophosphate, Complete mini protease inhibitor cocktail, and DNAse I/RNAse A cocktail (25 μg/mL DNAse I and 7 μg/mL RNAse A). After centrifugation at 14 000 rpm for 20 min at 4 °C, the proteins in the supernatant were precipitated by adding 80% (v/v) cold acetone. The reaction was carried out on ice for 40 min. The precipitated proteins were collected by centrifugation at 14 000 rpm for 20 min at 4 °C and subsequently were dissolved in 200 μL of IEF sample buffer containing 7 M urea, 2 M thiourea, 4% Chaps, 1% Triton X-100, 0.8% Pharmalyte 3-10, 1% DTT, 20 mM Tris, and 5 mM Pefabloc. Dissolved proteins were stored at −80 °C.

2D Electrophoresis. Ready-to-use Immobiline DryStrips (pH 4−7) were reswollen overnight in 250 μL of protein solution (750 μg of proteins for Coomassie Brilliant Blue staining and 300 μg of proteins for immunoblotting). The IEF was carried out up to a total of 70 kVh. Prior to SDS gel electrophoresis, the gels were incubated in a solution of 10 mg/mL DTT in equilibration buffer for 20 min and subsequently in a solution of 45 mg/mL iodoacetamide in the same buffer for 20 min. SDS−PAGE was performed in 11.5% polyacrylamide gels (160 mm × 140 mm × 1 mm) at 11 °C at a constant current of 50 mA for 3 h. Gels were stained with Coomassie Brilliant Blue or the Sigma Rapid Silver Staining Kit.

Immunoblot Analysis. Proteins from SDS−PAGE were electroblotted onto a nitrocellulose membrane (Schleicher & Schuell). The blot was incubated with anti-phosphoserine antibody at a 1:250 dilution or anti-phosphotyrosine antibody at a 1:200 dilution in 3% BSA in TBS at room temperature for 2 h. The protein−antibody complexes were visualized with an alkaline phosphatase−goat anti-mouse immunoglobulin G conjugate at a 1:5000 dilution and stained with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (ready-made solution from Boehringer, Mannheim). Gels and blots were scanned using a XRS OmniMedia Scanner 12cx (XRS, Torrance, CA) and a UMAX UC1260 (UMAX Data System, Hsinchu, Taiwan) ROC scanner. Image analysis and spot matching were performed using the BioImage software package (BioImage, Ann Arbor, MI) on a Sun workstation or the NIH Image program available at the web site of the NIH (http://rsb.info.nih.gov/nih-image). For determination of the p*I*, we used the values specified by the manufacturer of the p*I* strips (Pharmacia).

In-Gel Tryptic Protein Digestion. After visualization, the gel was destained with a solution of 25 mM ammonium bicarbonate/50% acetonitrile. The proteins were digested in the gel according to the modified procedure of ref *27*.

Mass Spectrometric Analysis. For MALDI-TOF mass spectrometry, samples were dissolved in 5 μL of 50% acetonitrile/0.1% TFA and sonicated for a few minutes. Aliquots of 0.5 μL were applied onto a target disk and allowed to air-dry. Subsequently, 0.3 μL of matrix solution [1% (w/v) α-cyano-4-hydroxycynnamic acid in 50% acetonitrile/0.1% (v/v) TFA] was applied to the dried sample and the sample again allowed to dry. Spectra were obtained using a Bruker Biflex MALDI time-of-flight mass spectrometer. MS/MS analysis was carried out using a Finnigan Mat (San Jose, CA) LCQ ion trap mass spectrometer. For interpretation of the MS and MS/MS spectra of protein digests, we used the Sherpa software (*28*), the MS-Fit program available at the World Wide Web site at the University of California at San Francisco (http://rafael.ucsf.edu/cgi-bin/msfit), the ProFound program at the World Wide Web site of Rockefeller University (http://prowl.rockefeller.edu/cgi-bin/ProFound), the PepSearch program at the World Wide Web site of EMBL in Heidelberg (http://.www.mann.embl-heidelberg.de/Services/PeptideSearch/FR_peptideSearchForm.html), and TagIdent available on the ExPASy World Wide Web server.

RESULTS

PDGF Stimulation and 2D Electrophoresis. NIH 3T3 fibroblast cells were stimulated with PDGF; the reaction was

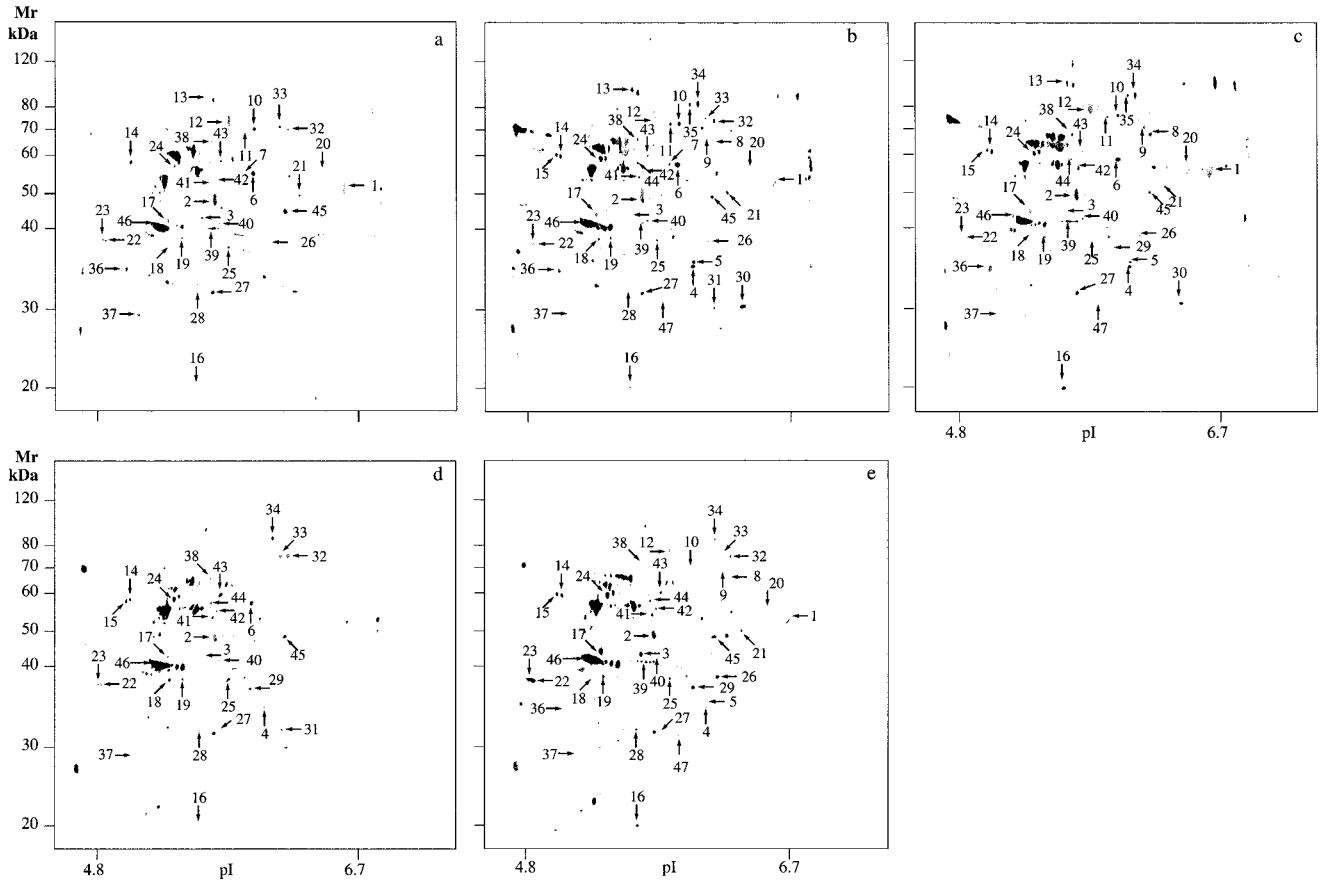

FIGURE 1: Immunoblots of proteins detected with anti-phosphoserine antibodies following stimulation of NIH 3T3 cells with PDGF. Western blotting was carried out after 2D electrophoresis of the total protein from unstimulated cells (a) and from cells after stimulation for 3 (b), 5 (c), 10 (d), and 20 min (e) using anti-phosphoserine antibodies. The spots marked with arrows and numbers denote proteins for which major changes in intensity of the spots were observed as a function of stimulation time.

terminated at various times, and the cellular proteins were separated by 2D electrophoresis. To observe as many proteins as possible, we did not apply protein prefractionation or immunoprecipitation prior to electrophoresis. However, enrichment of the total protein amount by total protein precipitation was necessary to detect proteins present in low amounts. Although we have presently limited our 2D electrophoretic analysis to a p*I* range of 4−7 and a molecular mass range of 15−120 kDa, about 3000 protein spots could be detected in the silver-stained gels (gels not shown). Proteins containing phosphoserine and phosphotyrosine were identified by detection with anti-phosphotyrosine and anti-phosphoserine antibodies, respectively (Figure 1), on immunoblots obtained for gels from unstimulated cells and from gels with termination of stimulation at 3, 5, 10, and 20 min. We observed at least 300 proteins containing phosphoserine and 260 proteins containing phosphotyrosine and about 47 and 41 proteins in the Ser and Tyr series, respectively, which showed strong intensity changes as a function of time after stimulation with PDGF. Although it cannot be guaranteed that the presently used antibodies detect all proteins containing phosphoserine and phosphotyrosine, it is clear that large numbers of phosphorylated proteins can be detected and that a surprisingly large number of these proteins show substantial changes in the intensities observed on the antibody blots. Individual protein phosphorylations are readily followed as a function of time after stimulation, as indicated by the expanded plots in Figure 2 showing blots covering a molecular mass range of 30−40 kDa and a p*I* range of 5.6−6.2 (phosphoserine series) and ranges of 50−70 kDa and p*I* 5.1−5.7 (phosphotyrosine series). These two expanded regions have been chosen to illustrate two types of behavior in the observed intensities. In the phosphoserine series (Figure 2a), some proteins show virtually constant intensities, e.g., s27, while other proteins vary between being undetectable and giving very intense spots, e.g., s4 and s5. In the phosphotyrosine series (Figure 2b), a number of proteins show more gradual intensity changes. Although such intensity changes might in some cases reflect multiple phosphorylation sites, and hence changes in the number of antibodies reacting with a single protein, it seems probable that in most cases the intensities in the blots will instead reflect the proportion of a protein which is present in the phosphorylated form. For example, in the Tyr series, there were at least 13 proteins which were not observed in unstimulated cells, but which became observable 3 min after stimulation. For these proteins, it seems likely that in the basal (unstimulated) state, virtually all of the protein is unphosphorylated, but is rapidly converted fully or partially to a phosphorylated form following stimulation with PDGF. Observation of the intensities on the immunoblots for individual proteins following stimulation revealed a wide diversity of kinetic behavior (see below).

Protein Analysis by MALDI-TOF and Ion Trap ESI Mass Spectrometry. We have begun the process of identifying the several hundred phosphorylated proteins by concentrating

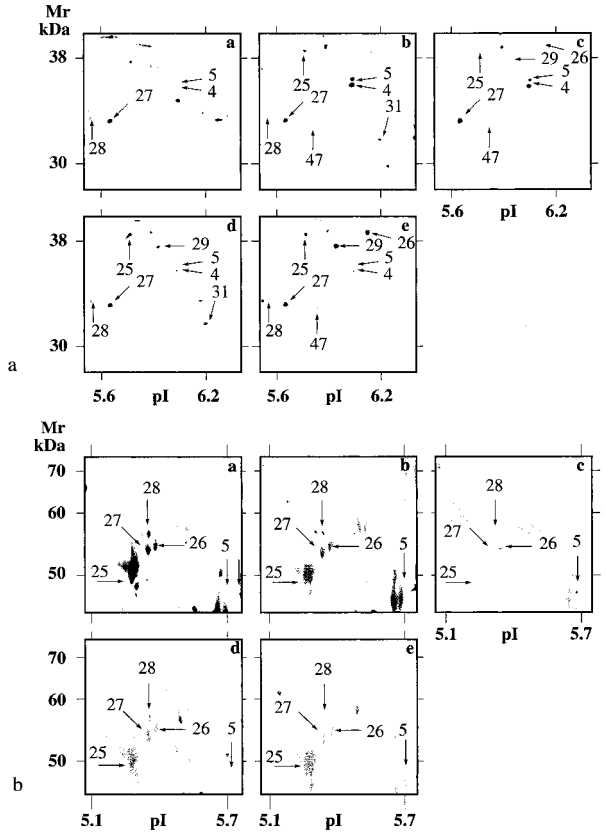

FIGURE 2: Expanded plots of immunoblots of proteins detected with anti-phosphoserine and anti-phosphotyrosine antibodies following stimulation of NIH 3T3 cells with PDGF. (a) Proteins phosphorylated on serine in the ranges of 30−40 kDa and p*I* 5.6−6.4. (b) Proteins phosphorylated on tyrosine in the ranges of 50−70 kDa and p*I* 5.1−5.7. Stimulation times are the same as in Figure 1.

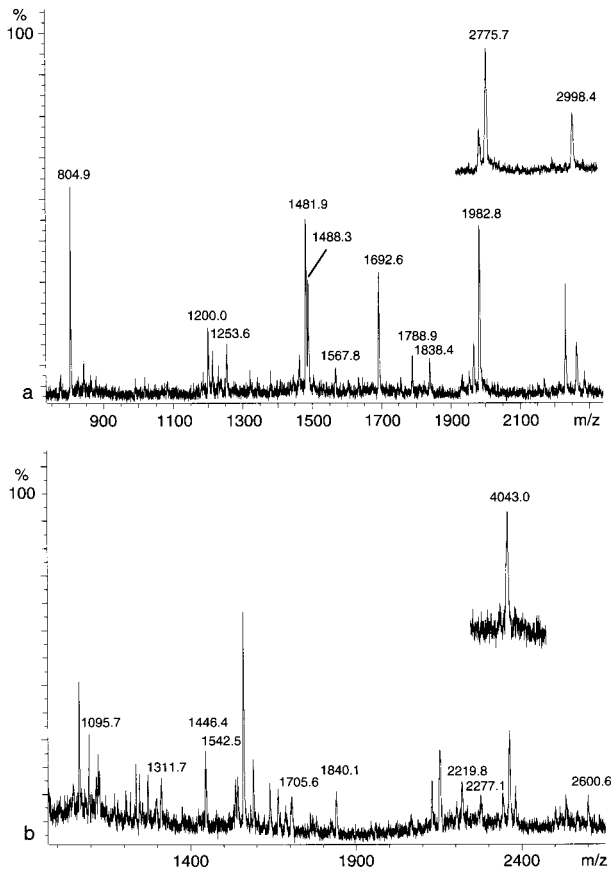

FIGURE 3: MALDI-TOF mass spectrometry of peptides obtained by in-gel trypsin digestion of phosphorylated proteins. Spectra of (a) s12, heat shock 73 kDa protein, and (b) s24, proto-oncogene tyrosine kinase FGR.

on some of the proteins that showed large intensity changes as a function of time. Protein spots detected on the immunoblots were cut out from the corresponding gel and subjected to in-gel digestion with trypsin (*27*). A mass fingerprint of the unfractionated peptide mixture was obtained by MALDI mass spectrometry following procedures previously described (*29*). Selected MALDI mass spectra for the tryptic digests of spots s12 and s24 from the phosphoserine series are presented in Figure 3. Even for those spots which were quite weak on the immunoblots, e.g., spot s47 at 3 min in Figure 2a, adequate material was available to obtain accurate masses (ca. ≤1 ppm) of peptides and peptide fragments by MALDI mass spectrometry as well as for subsequent sequence analysis by ion trap ESI mass spectrometry. Although we have not attempted to strictly quantitate the amounts of the various proteins on the gels, we estimate that the weakest spots used for the mass spectrometric analysis contained less than 1 pmol, which corresponds to about 10^4 copies/cell.

A variety of available software packages have been used to search for proteins consistent with the observed peptide masses obtained by MALDI-TOF mass spectrometry. The different search programs use several widely available sequence databases. We report only the NCBI protein accession numbers to avoid confusion. Initial searches were performed using a maximum ±0.3 Da mass tolerance, a single trypsin missed cleavage, and no phosphorylation modification. For initial identification of a protein, we required that the peptides identified covered a minimum of 20% of the protein sequence. On average, about 10 peptide masses were matched. Once the protein was identified, manual corrections based on the theoretical protein digest were used to identify peptides with up to three missed tryptic site cleavages and phosphorylated peptides. In all cases, as described previously (*29*), the identity of the protein was confirmed by subjecting selected peptides to MS/MS analysis of the peptide sequence using ion trap electrospray mass spectrometry of the unseparated peptide mixture previously analyzed by MALDI-TOF. Once the protein or peptide was confirmed by MS/MS (collision-induced dissociation) fragmentation, use of a slightly larger mass tolerance allowed further peptides to be identified from the MALDI-TOF mass fingerprinting. Table 1 shows examples of proteins identified to be participants in PDGF-induced signal transduction. It should be noted that for the proteins shown in Table 1, no evidence for comigration with other proteins on the 2D gels was detected in the MS analyses. In a few cases, the peptides that were selected for further analysis by ion trap ESI mass spectrometry showed the characteristic mass increase of 80 Da which is indicative of a single phosphorylation site. As described in some detail elsewhere (*29*), MS/MS sequencing of these peptides allowed the site of phosphorylation to be determined; i.e., we have found phospho-^{60}Ser in cardiac α-actin and phospho-^{13}Ser in proto-oncogene tyrosine kinase fgr. In some cases, the peptide masses observed in the MALDI-TOF mass fingerprint did not identify a protein in the available sequence data for the mouse genome. For

Table 1: Phosphorylated Proteins Identified by Mass Spectrometry

spot	identified protein	NCBI entry	mass (Da) expected	mass (Da) measured	p*I* expected	p*I* measured	no. of peptides[a]	Δ mass[b]	% sequence[c]
				Phosphotyrosine					
s8	calmodulin kinase II	125284	54 325	53 000	6.8	6.6	12	0.7 (−1.8 to 0.8)	24.5
s23	guanine nucleotide binding protein G(Q), α subunit	585189	41 478	40 000	5.7	5.7	7	0.3 (−0.4 to 0.7)	22.7
s27	proto-oncogene tyrosine kinase FGR	280989	58 883	58 000	5.4	5.3	10	0.5 (−0.7 to 0.7)	32.1
s25	vimentin	138536	53 556	54 000	4.9	5.2	11	0.4 (−0.3 to 0.5)	20.6
s13	guanine nucleotide binding protein, α11 subunit	120982	42 024	42 000	5.7	5.3	7	0.4 (−0.5 to 0.5)	20.6
s4	γ-actin	113278	41 793	43 000	5.2	5.6	11	0.3 (−0.8 to 0.6)	41.6
s5	γ-actin	113278	41 793	43 000	5.2	5.7	8	0.4 (−0.9 to 0.8)	24.8
s26	plexin-like protein (human)	1508821	53 069	56 000	6.5	5.2	15	0.3 (−0.4 to 0.7)	35.6
s28	Src substrate cortactin	2498955	61 620	60 000	5.1	5.2	11	0.5 (−0.8 to 0.8)	27.6
s42	protein tyrosine phosphatase SYP	464495	66 816	65 000	6.5	6.2	8	0.3 (−0.3 to 0.6)	22.5
s43	phospholipase Cα	130232	56 621	60 000	6.1	6.0	12	0.4 (−0.5 to 0.4)	32.5
s44	Rac β-serine/threonine kinase, AKT2 kinase (human)	1170703	56 000	55 769	6.0	6.3	6	0.3 (−0.3 to 0.4)	23.8
				Phosphoserine					
s12	heat shock 73 kDa protein	123651	70 871	70 000	5.1	5.7	15	0.4 (−0.9 to 1.0)	28.9
s1	calmodulin kinase II	125284	54 325	53 000	6.8	6.6	12	0.7 (−1.8 to 0.8)	
s2	cardiac α-actin	309090	41 826	46 000	5.2	5.7	10	0.3 (−0.8 to 1.1)	28.8
s20	serine/threonine protein kinase akt	479298	55 707	56 000	5.8	6.5	12	0.6 (−0.8 to 1.1)	32.6
s46	phosphotyrosine phosphatase, PTP-2	464491	44 572	43 000	8.4	5.3	6	0.4 (0.0 to −0.8)	28.0
s47	extracellular signal-regulated kinase 1 (ERK 1)	2499593	36 198	34 000	5.8	5.8	7	0.5 (−0.1 to 0.9)	35.6
s24	proto-oncogene tyrosine kinase FGR	280989	58 883	58 000	5.4	5.3	10	0.5 (−0.7 to 0.7)	32.1

example, the protein from spot s26 of the phosphotyrosine series showed strong homology to human plexin-like protein in both the MALDI-TOF mass fingerprint and in further analysis by ion trap ESI mass spectrometry. This protein seems to represent the C-terminal segment of the much larger protein plexin, whose function is presently unclear.

Kinetic Changes in Phosphorylation. The intensities observed on the immunoblots as a function of time after stimulation reveal very interesting kinetic behaviors. We have divided the intensities observed in the 2D immunoblots into five approximate classes (++++, +++, ++, +, and 0), corresponding qualitatively to very high, high, moderate, low, and very low or undetectable intensities. Figure 4 shows the intensities of the peaks detected on blots with the phosphotyrosine antibody as a function of time for some of the proteins which initially showed high intensity levels (upper panel) or initially showed very weak or undetectable levels with this antibody (lower panel). It should be noted that apart from essentially identical apparent p*I* and M_r values on the 2D gels, the identity of the proteins at different times can be rapidly verified by mass fingerprints using MALDI-TOF mass spectrometry. From the plots in Figure 4, it is clear that stimulation with PDGF initiates a very complex series of kinetic changes in phosphorylation levels. Among the proteins with high initial intensities, some such as s4 (γ-actin), s5 (γ-actin), s6, s7, and s27 (proto-oncogene tyrosine kinase fgr) see their intensities decline to low or very low or undetectable levels over the first 3−5 min and then show no further change. It is interesting to note that s27 (proto-oncogene tyrosine kinase fgr) was also detected by the phosphoserine antibody and that in this case the intensity

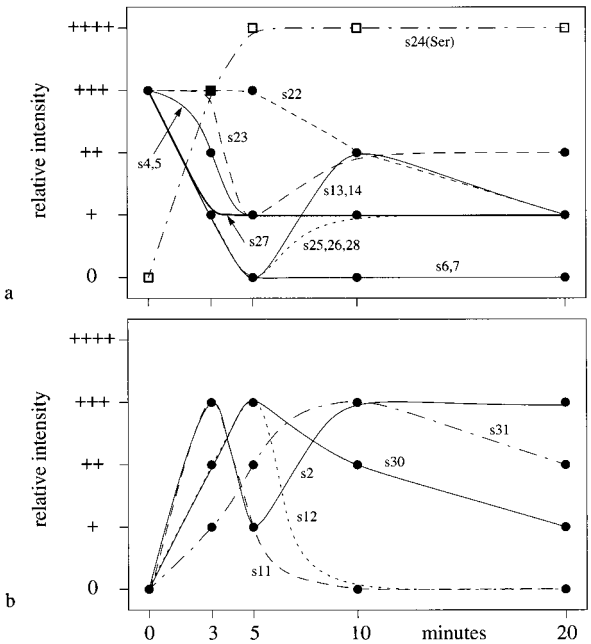

FIGURE 4: Time dependence of the intensities (*I*) observed with the phosphotyrosine antibody as a function of time after stimulation with PDGF. (a) Proteins for which high intensity was observed in unstimulated cells. The intensity scale (++++, +++, ++, +, and 0) corresponds qualitatively to very high, high, moderate, low, and very weak or undetectable intensities on the immunoblots. (b) Proteins for which very weak or no intensity was observed in unstimulated cells.

goes from very low to very high over the first 5 min and then remains at a very high intensity [□, s24 (Ser) in the upper panel]. This indicates that in response to PDGF

stimulation this protein shows a switch in the dominant type of phosphorylation from phosphotyrosine to phosphoserine. Other proteins show more complex kinetic behavior. The intensities of s26, s25 (vimentin), and s28 (Src substrate cortactin) as well as s14 and s13 (guanine nucleotide binding protein, α11 subunit) decline from high levels prior to stimulation to very low or undetectable levels at 5 min followed by recovery to low or moderate intensities at 10 and 20 min. Other proteins such as s23 and s22 show no initial response to PDGF stimulation over the course of 3 or 5 min, respectively, but do show substantial intensity changes after longer times. Similar diversity in kinetic behavior is observed among the proteins which were initially very weak or undetectable by the phosphotyrosine antibody (Figure 4, lower panel). The intensities of s11 and s12 initially increase to high levels at 3 or 5 min and then decline to undetectable levels at 10 min. Spot s2 also shows a rapid increase and decline in intensity over the first 5 min, but then increases to a high intensity after long periods of time. s30 and s31 show increases to high intensity at 5 or 10 min and then slow decreases in intensity. As noted already for proto-oncogene tyrosine kinase fgr, similarly complex kinetic behavior has also been observed as a function of time for proteins detected with the phosphoserine antibody (see also Figures 1 and 2a). Although the evaluations of intensity changes on the blots are presently at best semiquantitative, it appears that approximate measures of the time scale for the kinetics of phosphorylation and dephosphorylation of different proteins can be obtained with the present methods.

DISCUSSION

In an attempt to establish proteomic methods for analysis of signal transduction systems, it did not seem sensible to identify several thousand proteins on silver-stained 2D gels prior to investigating the interesting cellular phenomena, and we were therefore interested in establishing methods which would allow the analyses to concentrate on proteins which are more or less directly involved in the signal transduction system of interest. Although the number of phosphorylated proteins showing substantial intensity changes is surprisingly large, the use of antibodies against phosphoserine and phosphotyrosine together with specific stimulation with PDGF has reduced the number of proteins that need to be analyzed, at least initially, by perhaps 100-fold. On the other hand, in comparison with more conventional methods such as immunoprecipitation, the present method allows the parallel observation of large numbers of proteins without preselection of the proteins to be observed and with at least qualitative characterization of kinetic phenomena. In the future, more quantitative analyses may well be established. We believe that the use of similar selective stimulation and detection strategies will in many cases be more efficient than trying to map all cellular proteins and expect that much ingenuity will be forthcoming in future stimulation and detection strategies. Since the term proteome seems in the present vernacular to be synonymous with attempts to map all cellular proteins, the term "functional proteomics" is appropriate for characterizing strategies based on selective excitation and observation of specific cellular protein networks.

We have chosen to start with a system, PDGF, for which a very substantial body of research already exists and which therefore can be used to validate the present approach. Almost 100 proteins which show strong changes in phosphorylation following stimulation with PDGF have been observed. Simple visual inspection of the immunoblots revealed rapid phosphorylation and the appearance of new phosphorylated proteins at very early stages of the stimulation. This observation agrees with literature data on PDGF signaling **(***12*, and references therein). We have chosen to also monitor changes after longer periods of time to detect not only direct, but also distant and delayed, signaling processes triggered by stimulation of NIH 3T3 cells with PDGF. Although there is still much work to be done in identifying all of the protein components which have been observed, the present results are already very promising both in terms of the observation of known components of this system and in terms of new information.

Many of the proteins whose gel spots we have identified by mass spectrometry have previously been shown to be part of the PDGF signaling system. These proteins include ERK 1 kinase (s47 of the phosphoserine series, Figure 2a). This protein was absent from the immunoblot before stimulation and showed a rapid increase in intensity at 3 and 5 min, a complete absence at 10 min, and reappearance at 20 min. This behavior of ERK kinase is consistent with previous data for this protein. It is known that ERKs are activated extremely rapidly by phosphorylation and that their activation after mitogen stimulation occurs within 5 min, after which time most of the activity is lost. In some cell types, another peak of activity may follow (*30*, *31*). In the phosphoserine series, the serine/threonine protein kinase akt (s20), which is well-known to be stimulated by the PDGF receptor (*32*, *33*), was also observed. Calmodulin kinase II, α chain, was observed in both the phosphotyrosine (s8) and phosphoserine (s2) blots. Calmodulin is a ubiquitous Ca^{2+}-binding protein, and the Ca^{2+}−calmodulin complex interacts with various target proteins, resulting in functional changes (*34*). It is well-known that Ca^{2+} activates diverse intracellular signaling pathways via protein phosphorylation, and these kinases are thought to play a central role in Ca^{2+}-dependent signal transduction in cells.

Two types of G-proteins, G-protein α11 subunit (s13, phosphotyrosine series) and G-protein (Q) α subunit (s23, phosphotyrosine series), were identified as being modulated in phosphorylation by PDGF stimulation. Guanidine nucleotide binding proteins are known to be involved as modulators or transducers in various transmembrane signaling systems. Our observation of phosphorylation of these G-proteins on tyrosine, but not on serine, is consistent with data showing that the G-protein α subunit is phosphorylated at tyrosine by EGFR kinase (*35*, *36*) and by pp60c-src (*37*) in the case of the α s subunit. Thus, our finding may uncover a novel signaling link from the activated PDGF receptor to G-proteins.

It has previously been reported that G-proteins and actin associate on 2D gels (*38*). In some of our 2D gel separations, spot s13 (phosphotyrosine series) showed peptides characteristic of both *β*-actin and the G-protein α11 subunit. This spot corresponds to spot s17 (phosphoserine series) which was identified as *β*-actin. *β*-Actin and this G-protein differ in their theoretical p*I* values by ca. 0.6 unit. Their apparent comigration might be due to a p*I* shift of the G-protein due to post-translational modification or to association on the

gels. Gq- and G11-proteins are known to associate with the actin cytoskeleton in a variety of cell types (*39*), suggesting that actin-associated G α q or G α11 proteins play a role in signal transduction. Although further development would certainly be needed, the present results suggest that in the future it may be possible to detect strong intermolecular protein−protein interactions with methods similar to those presented here.

Changes in tyrosine phosphorylation have also been observed for some proteins known to be prominent phosphorylated proteins in various cell types, including vimentin (s25), actins (s4 and s5), and Src cortactin substrate (s28). These proteins are involved in PDGF and a variety of other signal transduction pathways as exemplified by phosphorylation of Src cortactin substrate on tyrosine by fibroblast growth factor and kinases of the Src family (*40*, *41*) and by phosphorylation through phospholipase C kinase and calmodulin kinase II induced by vimentin (*42*).

We have also observed changes in phosphorylation of three proteins, heat shock protein, proto-oncogene tyrosine kinase fgr, and phosphotyrosine phosphatase PTP-2 which have been implicated in signal transduction, although we are not aware of literature in which direct involvement of these proteins in PDGF signaling has been reported. The proto-oncogene tyrosine fgr, which belongs to the Src family, was identified in both the phosphotyrosine blots (s27) and the phosphoserine blots (s24). This kinase is known to undergo tyrosine autophosphorylation, and it contains a copy of both SH2 and SH3 domains (*43*). MS/MS analysis revealed that ^{13}Ser is phosphorylated, which is in accordance with reports that Src kinases are phosophorylated at serine in their N-terminal sequence (*44*). Involvement of Src kinases in PDGF signal transduction is well-documented (*10*, *11*), but there seems to be no direct evidence for the role or involvment of proto-oncogene kinase fgr. Another protein with no direct evidence for involvment in PDGF signal transduction is tyrosine protein phosphatase PTP-2 (s46 of the phosphoserine series), although other phosphotyrosine phosphatases such as PTP-1B and CD45 are known to be serine phosphorylated (*45*, *46*). One protein, plexin-like protein, whose involvement in signal transduction seems not to have previously been reported was identified.

The present results have shown that it is possible to follow time-dependent changes in phosphorylation patterns using 2D electrophoresis in combination with anti-phosphotyrosine and anti-phosphoserine antibodies. The proteins which have been identified by mass spectrometry include many that are known to be involved in either PDGF or other signaling pathways, thereby giving us confidence that the observed phosphorylation patterns reflect signaling pathways induced by PDGF stimulation of quiescent cells and providing motivation for further investigating the roles of proteins not previously known to be involved in PDGF pathways or even signal transduction. Further elucidation of the signaling pathways will require identification of more proteins by mass spectrometry, but it also appears that careful attention to the kinetics of phosphorylation will be important. In this respect, we note that although there is relatively little data available on the kinetics of phosphorylation for the proteins so far identified, for ERK kinase we have observed behavior which is consistent with previous data reported for this protein (*30*, *31*). Similarly, pulsed behavior of signaling pathways is known to exist in cellular systems, in particular in connection with calcium signaling which shows very complex temporal and spatial characteristics that cause pulsatile behavior of intracellular Ca^{2+} (*47*). Although we presently have only a limited number of time points, it is interesting to note that the phosphorylation of calmodulin kinase II seems also to show oscillatory behavior (s2 in Figure 1). Finally, we note that completion of the mapping of the various phosphorylated proteins promises to provide a very powerful means for investigating a wide variety of signaling phenomena, including cross-talk between different signal pathways, activation or inactivation of particular pathways by endogenous or exogenous cofactors, and even the nature of genetic defects in signaling cascades (*48*, *49*).

ACKNOWLEDGMENT

We thank Ms. H. Keilhack for valuable discussions and E. Nyakatura for excellent technical help and support.

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BI982093R