The Induction of Serine/Threonine Protein Phosphorylations by a PDGFR/TrkA Chimera in Stably Transfected PC12 Cells*

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Stably transfected PC12 cells expressing a chimeric receptor composed of the extracellular domain of the platelet-derived growth factor receptor BB and the transmembrane and intracellular domains of TrkA, the nerve growth factor receptor, were stimulated for 20 min with platelet-derived growth factor and the resulting phosphoproteome was determined from affinity purified tryptic peptides identified by tandem MS (MS/MS) analyses. The changes in the levels of individual phosphorylation sites in stimulated cells versus control were ascertained by the stable isotope labeling of amino acids in cell culture technique. A total of 2035 peptides (806 proteins) were identified and quantified in both data sets. Of these, 424 phosphopeptides on 259 proteins were found to be up-regulated and 392 sites on 206 proteins were down-regulated (1.8-fold or more). Protein kinases and phosphatases, as well as sites in many proteins involved in G-protein signaling, were prominently represented in the up-regulated group and more than half of the kinase up-regulated phosphosites could be clustered into three sequence motifs; a similar distribution was also found for the down-regulated sites. A comparison of the up-regulated motif profile observed to that calculated from a previous study of the EGFR-induced phosphoproteome in human HeLa cells at the same time point showed a considerable amount of similarity, supporting the view that RTK signal transduction pathways and downstream modifications are likely to be extensively overlapping. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.013375, 15–30, 2012.

Extracellular signals constitute a fundamental biological activity by which cells communicate with their environment by responding to changes in their external milieu. In higher eukaryotes, these signals are essential for the coordination of organ/organism function and are generally regulated through electrical and chemical networks that constitute the nervous and endocrine systems, respectively (1). In the latter case, with the exception of lipid soluble messengers, e.g. steroids, the mechanism of transmission is through the activation of plasma membrane-bound receptors following specific binding of the signaling entities. These ligand-receptor complexes trigger a response by activating the intracellular domain of the receptor that is then propagated and amplified via signaling cascades of varying complexity (2). The ultimate targets are usually transcription factors that are activated/deactivated, leading to modulations in gene expression. However, many intracellular proteins are affected by these transmission processes (positively or negatively) and contribute to other changes in cellular activity independent of the terminal nuclear events. The principal mechanism for the perpetration of these signaling events is via protein post-translational modification, i.e. the immediate signaling responses, as opposed to the long term changes, depend on the regulation of existing proteins (3, 4).

The extracellular ligands are commonly, although not exclusively, soluble proteins and, in large part, consist of hormones and growth factors, that are exocytosed and act on the cells of origin (autocrine), neighboring, but different, cells (paracrine) and distant cells (endocrine); the means of transport for this last group being blood (5). The different classes of receptors that recognize these entities use a variety of signaling mechanisms; chief among these is the induction of tyrosine phosphorylation. However, there are a far greater number of protein kinases with specificity for serine/threonine modifications in eukaryotic cells (6) and many of these are activated downstream by the various amplified signaling stimuli. Thus the overwhelming amount of the total protein phosphorylation events that result from external stimulation ultimately occur on serine and threonine residues, as reflected in the observed distribution of serine/threonine/tyrosine phosphorylations on cellular proteins (7).

The receptor tyrosine kinase (RTK) family is one of the main groups of transmembrane receptors and consists of 19 different subfamilies collectively containing 58 members (6).

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1 The abbreviations used are: EGF, epidermal growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PTR, PDGFR-TrkA chimeric receptor; PC12c, control PC12 cells, unstimulated; PC12-PTRs, transfected PC12 cells, unstimulated; PC12-PTRs, transfected PC12 cells, stimulated; PTM, post-translational modification; RTK, receptor tyrosine kinase; SILAC, stable isotope labeling of amino acids in culture; SLIP score, site localization in peptide score.
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Several have been extensively studied, such as those containing the receptors for insulin, EGF, the FGFs, PDGF, and the neurotrophins and many have been directly connected to human disease. However, to date, there have only been a limited number of phosphoproteomic analyses of receptors of this type, and many of these have been focused on the early steps, i.e. tyrosine modifications (see, e.g. (8)). These are known to occur very rapidly, generally peaking after only a couple of minutes following stimulation, and then rapidly falling off, whereas serine/threonine phosphorylations can persist for several hours, although these tend to peak at about 20 min following stimulation. Olsen et al. (9) have reported the only extensive analysis of RTK-initiated downstream modifications using the EGF receptor in HeLa cells; this study provided a list of 6600 phosphorylation sites (2244 proteins) in a kinetic study that covered the first 20 min after the addition of growth factor. Other studies have dissected aspects of the phosphorylation responses to insulin (10, 11), PDGF (12) and the ephrin B1/ephrinB2 receptor interaction (13). Similarly, analyses of oncogenic signaling in nonsmall cell lung cancer (14) and with a modified FMS-like tyrosine kinase 3 (FLT3-ITD), a member of the PDGF receptor family (15), have revealed aberrant modifications that presumably underlie abnormal signaling pathways and mechanisms.

Nerve growth factor (NGF) utilizes two types of receptors, P75 and TrkA, for its various functions, both in neural and nonneural tissues (16). The former is a member of the TNF-receptor family and is activated not only by NGF but also the three other homologs that with NGF make up the neurotrophin family (BDNF and neurotrophins 3 and 4). TrkA, along with the two other Trk receptors (B and C) that mediate the functions of the other NGF-related proteins, is a member of the RTK superfamily and activation by NGF binding induces a phosphorylation cascade that is associated with neurite proliferation and neural differentiation (17, 18). However, no detailed analyses of the downstream TrkA stimulated phosphoproteome in an appropriate cell line has been reported.

In this study, a chimeric receptor composed of the ectodomain of the human PDGF receptor and the transmembrane and endodomain of the TrkA receptor from rat (the chimera is denoted PTR; Fig. 1A) was stably transfected into PC12 cells (19). Using this receptor it was possible to selectively stimulate TrkA signaling without activation of the p75 receptor, and thus allowed determination of the serine/threonine phosphoproteome specific to the TrkA receptor following 20 min of stimulation. Cells were labeled with heavy and light lysine/arginine residues (stable isotope labeling of amino acids in culture (SILAC) technique (20)) to allow quantification of the changes observed and the results were also compared with the activation of another RTK.

EXPERIMENTAL PROCEDURES

Construction and Stable Transfection of PTR in PC12 Cells—The construction of PTR from the extracellular domain of human PDGFR

and the transmembrane and intracellular domains of rat TrkA has been described (19). Briefly, an EcoRI/MseI restriction fragment containing the cDNA sequence for the human βPDGF-R extracellular domain fused to a cDNA sequence coding for the transmembrane and intracellular domains of rat Trk created by PCR was cloned into the retroviral vector pLEN. All PCR derived sequences were completely resequenced to exclude any PCR errors. Ecotrophic retrovirus were generated with the help of PA317 and GP+E-B6 producer cell lines. The level of expression of PTR of the clone used in these studies was not directly measured but indirect measures of activity following stimulation indicated that the levels were comparable to native expression, based on comparison to clones that had been analyzed.

Cell Culture and SILAC Labeling—PC12 cells, with or without the stably transfected chimeric receptor PTR, were grown in 15-cm Petri dishes in light medium containing Dulbecco’s modified Eagle’s medium, 4.5 g/L glucose, 10% horse serum, 5% calf serum, 50 units/ml penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively) and sodium pyruvate (0.11 mg/ml). After starving the cells for 24 h, the cells were stimulated for 20 min with human PDGF (Austral Biologicals, San Ramon, CA) (50 μg/ml) and lysed in 10 ml of Trizol® reagent. Proteins were purified according to the manufacturer’s protocol and resuspended in 6 M guanidine.HCl. After centrifugation (5 min, 4 °C at 14,000 × g), an aliquot of the supernatant was analyzed by the Bradford test to measure the concentration of protein in each sample. For SILAC, PC12 cells stably transfected with the chimeric receptor PTR were grown in Dulbecco’s modified Eagle’s medium deficient in normal arginine and lysine and supplemented with 10% dialyzed fetal bovine serum (Hyclone®, thermo Fisher Scientific, San Jose, CA), penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively), sodium pyruvate (0.11 mg/ml), 200 mg/L l-proline (Thermo Fisher Scientific, San Jose, CA) and L-arginine-13C₆-15N₄.HCl and L-lysine-13C₆-15N₂.HCl (Thermo Fisher Scientific, San Jose, CA and Sigma, respectively). Cells were grown in a humidified incubator with 5% atmospheric CO₂ at 37 °C for at least six passages to obtain 100% incorporation of the isotopically labeled amino acids. The incorporation level was determined after each cell passage by checking for the presence of the light version of each peptide identified when running only the heavy sample. There was no evidence for any unlabeled peptides in the sample used as reference for these studies. After starvation for 24 h, seven Petri dishes of cells were stimulated 20 min with PDGF (50 mg/ml) in the same way as the light samples and lysed in Trizol reagent. Proteins were extracted and resuspended in 700 μl 6 M guanidine.HCl. This sample constituted the “heavy standard” used for quantification. PDGF was not added to the untransfected control PC12 cells, as they do not express PDGF receptors and independent analyses of these cells has indicated that there is no measurable response to this treatment. To measure the neurite outgrowth, PC12 cells and PC12 cells stably transfected with PTR were cultivated on 5 μg/cm² rat tail collagen I (BD Bioscience, San Jose, CA) and stimulated for 3 days with NGF (50 μg/ml) or PDGF-BB (50 μg/ml).

Protein Digestion—Three milligrams of each sample, untransfected/unstimulated PC12 cells (PC12c) or truncated (with the chimeric receptor PTR) unstimulated PC12 cells (PTRu), or transfected/stimulated (with hPDGF) PC12 cells (PTRs) were mixed with 3 mg of the heavy standard (Fig 1B). Each sample was reduced for 1 h at 57 °C with 2.1 mM tris (2-carboxyethyl)phosphine.HCl to reduce cysteine/cysteine side chains and alkylated with 4.2 mM iodoacetamide in the dark for 45 min at 21 °C. Proteins were digested using 2% (w/w) modified trypsin (Promega, Madison, WI) in 1 mM guanidine.HCl, 25 mM ammonium bicarbonate, pH 8, for 16 h at 37 °C. Peptides were desalted on a C18 Sep-Pak cartridge® (Waters, Milford, MA) and eluted in 70% acetonitrile/0.1% formic acid. The peptides were vac-
Peptides were enriched using 5 μm titanium dioxide beads (GL Sciences, Tokyo, Japan) (21, 22) packed into an analytical guard column with a 62 μl packing volume (Upchurch Scientific, Oak Harbor, WA). Peptides were passed over the titanium dioxide column with 4.4 ml of loading buffer B1 (35% acetonitrile/200 mM NaCl/0.4% trifluoroacetic acid) followed by 6.5 ml of wash buffer A1 (5% ACN/0.1% TFA). The nonphosphorylated peptides were collected during the first step. Phosphorylated peptides were then eluted from the titanium dioxide column directly onto a C18 macrotrap peptide column (Michrom Bioresources, Auburn, CA) using 15 ml of elution buffer A2 (1 mM KH₂PO₄). The C18 column was washed with 17.1 ml of wash buffer A1 and the peptides were eluted using 500 μl of organic elution buffer B2 (50% acetonitrile/0.1% trifluoroacetic acid). This collected fraction was lyophilized to dryness. Three separations were performed for each sample (2 mg/run).

**Separation of Phosphopeptides by Strong Cation Exchange (SCX) Chromatography**—SCX chromatography was performed using an ÄKTA Purifier (GE Healthcare, Piscataway, NJ, USA) equipped with a Tricorn S/200 column (GE Healthcare, Piscatawy, NJ, USA) packed in house with 5-μm 300-Å polysulfoethyl A resin (Western Analytical, Lake Elsinore, CA). Phosphopeptides enriched from each sample were loaded onto the column in 30% acetonitrile/5 mM KH₂PO₄, pH 2.7 (buffer A). Buffer B consisted of buffer A with 350 mM KCl. The gradient went from 1% B to 72% B over 16.5 ml, from 72% B to 100% B over 1.5 ml, and from 100% B to 1% B over 2 ml at a flow rate of 0.3 ml/min. Fractions (53 and 30) were collected for the phosphopeptides and the nonphosphopeptides, respectively, and were desalted using C18 ZipTips (Millipore), dried down and resuspended in 25 μl of water/0.1% formic acid. A quarter of each fraction was analyzed by liquid chromatography-tandem MS (LC-MS/MS).

**LC-MS/MS**—Mass spectrometry was performed using an LTQ-Orbitrap XL (Thermo Fisher Scientific, San Jose, CA). Chromatography was performed with a NanoAcuity ultra performance liquid chromatography (UPLC) system (Waters, Milford, MA) at a flow rate of 300 nL/min on a column of BEH130 C18 75 μm ID X 150 mm (Waters, Milford, MA) with a 90-min gradient. Solvent A was water/0.1% formic acid, and solvent B was acetonitrile/0.1% formic acid; peptides were eluted by a gradient from 2% to 28% solvent B over 70 min followed by a short wash at 50% solvent B, before returning to the starting conditions. Peptide components eluted over a period of ∼60 min during these runs. After a precursor scan of intact peptides was measured in the Orbitrap by scanning from m/z 350–1800 (with a resolution of 60,000), the seven most intense multiply-charged precursors were selected for collision-induced dissociation (CID) analysis in the linear ion trap. Activation times were 30 msec for CID fragmentation with normalized collision energy of 35.0. Automatic gain control targets were 100,000 ions for Orbitrap scans and 10,000 for MS/MS scans. Dynamic exclusion for 60 s was used to prevent repeated analysis of the same components.

**Peptide and Protein Identification**—Fragmentation data were converted to peaklists using in-house software based on the Raw_Extract script from Xcalibur v2.4 (Thermo Fisher Scientific, San Jose, CA) and the CID data for each sample were searched using Protein Prospector version 5.8 (23) in two separate searches against the UniProt Rodent database (downloaded June 6, 2010 with a total of 44,512 entries), to which a randomized version of all entries had been concatenated. Both searches used the following parameters: mass tolerances in MS and MS/MS modes were 30 ppm and 0.6 Daltons, respectively. Trypsin was designated as the enzyme and up to two missed cleavages were allowed. S-carbamidomethylation of cysteine residues was designated as a fixed modification. Variable modifications allowed were N-terminal acetylation, N-terminal glutamine conversion to pyroglutamate, methionine oxidation, and phosphorylation of serine, threonine, or tyrosine residues. In the second search, the same parameters were employed but with heavy arginine and lysine residues considered as fixed modifications. The two searches were then merged into a single result file. The maximum expectation value allowed was set as 0.01 (protein) and 0.05 (peptide). At these thresholds the peptide false positive rate was estimated to be 0.5% for each SILAC experiment according to the concatenated database search results (24); the protein false positive rate was estimated to be 3.5%. As the proteins reported in this study are only those identified in both SILAC experiments, and the incorrect identifications are probably mainly identified in a single experiment, the actual false discovery rate is likely to be lower than this estimate.

**Quantification**—SILAC quantification measurements were extracted from the raw data by Search Compare in Protein Prospector (http://prospector.ucsf.edu). Search Compare averaged together MS scans from −10 s to +30 s from the time at which the MS/MS spectrum was acquired in order to produce measurements averaged over the elution of the peptide. Search Compare calculates a noise level in the averaged spectrum. Only peaks with a signal to noise of greater than 10 are used in quantification measurements. If one of the SILAC pair is above this threshold and the other is below, then the ratio is reported with a > or < (see supplementary tables), indicating one value was below the noise level, so the ratio reported is a minimum estimate. If a phosphopeptide was identified from multiple MS/MS spectra, the median of the calculated SILAC ratios from all the replicate identifications of the same peptide was reported. These ratios were then corrected for differences in protein level using data from the nonphosphorylated peptides (where SILAC ratios should be 1:1). The protein ratio for practically all proteins was the same between samples, as twenty minutes of stimulation is not long enough to instigate measurable changes in protein expression levels. The average of the standard deviation of the ratios for each peptide was 0.08. Three times this standard deviation should include 99.7% of the data according to a Gaussian distribution, so a 1.8-fold difference should correspond to a significant change.

**Phosphorylation Site Assignment**—The reliability of phosphorylation site assignments was measured using the site localization in peptide score in Protein Prospector version 5.8 (25). Briefly, the site localization in peptide score is derived from determining the difference in expectation value for a peptide identification with a particular modification site assignment in comparison to the next highest scoring assignment to the same peptide with a different localization of the modification. Previous analyses of standard data sets acquired by ion trap CID fragmentation showed that the results reported with a site localization in peptide score of 6 had a local false localization rate of 5% and that results with this score or higher were close to 99% correct in their site assignments.

**Western Blots**—Twelve micrograms of PC12c, PTRu and PTRs (treated as described above) were separated on a 4–20% tris-HCl ready gel (Bio-Rad, Hercules, CA) and transferred onto a polyvinylidene difluoride membrane (Polyscreen®). The blocking step was performed using 5% bovine serum albumin in Tris buffered saline/0.5% tween 20 buffer for 1 h. The first antibody was used at 1/1000 dilution for 1 h followed by 3 washes of 5 min in TBS/0.5% tween 20 buffer. The secondary antibody coupled to HRP (Bio-Rad, Hercules, CA) was incubated for 30 min. After 3 washes of 5 min with Tris buffered saline/0.5% tween 20, the substrate ECL-plus (GE Healthcare, Piscataway, NJ) was added to the membrane and the detection performed.

**Bioinformatics**—Phosphorylation motif analysis: In order to determine candidate kinases that may be activated by TrkA stimulation, the frequency of occurrence of different kinase motifs within the phos-
Phosphorylation sites identified as a whole and those that appeared to be regulated by receptor stimulation were characterized. The queried kinase motifs were from those reported in the HPRD phosphorylation database (26) and those previously queried in an EGF signaling proteomics study (9).

For each motif, an enrichment factor was calculated, which corresponded to the frequency of detection of a particular motif in comparison to how often one would expect to find it at random in the database queried. The frequency of occurrence of different motifs in the database was measured using MS-Pattern (27). This software is designed to search protein databases for occurrence of amino acid sequences (that can include unknown residues). Hence, it is able to report the number of occurrences of a given motif in the queried database. For the phosphorylation data in this study, rodent entries in Uniprot from June 2010 were queried; for the EGF data (9), occurrences of each motif in the human entries from the same Uniprot database were counted. The expected number of random identifications of a given motif was calculated by determining the fraction of all serines and/or threonines (depending on the motif) in the database that occurred within the given motif. This value was multiplied by the number of phosphopeptides identified. For example, the motif PX[S/T]P occurred 45,172 times and there were 5,183,918 serines and threonines in these rodent protein entries. So, if 1633 phosphorylation sites were reported then the expected number to randomly contain this motif would be 14.2 (1633 x (45172/5183918)). In actual fact, this motif was observed 134 times in the 1633 phosphorylation sites, so the enrichment factor for this motif was 9.4 (134/14.2).

To determine whether differences between two enrichment factors were statistically significant a measure of accuracy/error for the enrichment factors was determined by splitting a motif into submotifs, then producing enrichment factors for each of these submotifs. For example, the motif PX[S/T]P was split into six sub-motifs where in each case the X could only correspond to one of six or seven amino acids. This allowed six measurements for the PX[S/T]P motif enrichment factor, from which a mean and variance could be reported. Significance of different enrichment factors was then calculated by a two-sample t test (allowing for unequal variances).

Motif-x — motif-x  (http://motif-x.med.harvard.edu) parameters were set up as: significance of 0.000001 with an occurrence of 30.

The IPI rat database was used as background data. The sequences of all phosphopeptides were submitted that had at least 6 amino acids before and after the phosphorylation site.

GO Annotation — The proteins were submitted to DAVID Bioinformatics Resources 6.7 (28) with their Uniprot accession numbers. The different categories of biological processes, cellular components and molecular functions were manually associated to each protein. The terms “metabolic,” “signal,” “transport,” “morphogenesis,” “cell cycle,” “apoptosis,” “mitosis,” “melosis,” and “chrom” (for chromatin and chromosome) were queried for the biological processes associated to each protein; the terms “cytoplasm” and “cytosol” (joined in the same section), “nuclear,” “plasma membrane,” “mitochondria,” and “mitochondrion” (joined in the section “mitochondria”), “endoplasmic,” “golgi,” “cytoskeleton,” “endoplasmic,” and “ribo” (for ribosome and ribonucleoprotein) were queried for the cellular component; the terms “kinase activity,” “phosphatase activity,” “transcription activity,” “acetyl transferase activity,” “deacetylase activity,” “translation factor activity,” “GTPase activator activity,” “guanyl-nucleotide exchange factor activity” were queried for the molecular function associated to each protein.

STRING — The list of identified proteins were submitted using their Uniprot accession numbers from MOUSE to the STRING database (29) considering the combined score of experimental and database interactions. The confidence was set to medium (0.400). The results were visualized with Cytoscape v.2.7.0 attributing the ratio strength to the nodes.

RESULTS

Strategy: Definition of Samples — In this study, the phosphorylation changes present after 20 min of stimulation of the stably transfected PTR receptor, corresponding to the activation responses of TrkA, were measured. Three different samples were analyzed: PC12c are nontransfected PC12 cells, PTRu are PC12 cells stably transfected with the PTR receptor but unstimulated by added ligand and PTRs, consisting of PC12 cells stably transfected with the PTR receptor and stimulated for 20 min with hPDGF-BB (human platelet-derived growth factor-BB) (Fig. 1B). The first two samples represent the cells in a nonstimulated state, but still growing and dividing; the third corresponds to cells in a stimulated state. As shown in Fig. 2, both transfected and untransfected cells respond morphologically to NGF (because of the expression of endogenous TrkA receptors) while only the chimera expressing cells show neurite proliferation in response to PDGF.

The activation of the PTR receptor with hPDGF-BB for 20 min induces a wide range of serine/threonine phosphorylations that can be monitored in part by using commercially available antibodies against specific phosphorylations. By Western blotting (Fig. 3), the phosphorylation of PLCγ (Y783), TrkA (Y490), AKT (T308 and S473), GSK3 α/β (S21/S9), ERK 1/2 (T202/Y204; T183/Y185) were shown to be up-regulated upon stimulation. The first measurements confirm the activation (autophosphorylation) of PTR and the downstream effectors PLCγ, AKT, and ERK1/2, indicating that the adaptors dock correctly and lead to the activation of signaling pathways previously described for TrkA (18). The PTRu sample shows a similar profile to PC12c but the overexpression of the PTR receptor, even before the activation by ligand addition, causes a higher background, as has been seen previously with other stably transfected chimeras in this cell type (30).

Thus, the untransfected PC12 cells (PC12c) were used as the principal control and the data from the PTRu samples was only used to validate the reliability of the data. Analysis of the PTRu data showed that the log(ratios) presented a comparable profile to PC12c, with quantification measures generally somewhere between PC12c and PTRs results (supplemental Fig. S1), suggesting a higher level of basal level of activation. Comparing the ratios for phosphorylation sites identified as regulated according to PC12c to PTRs differences with those observed in the PTRu sample there was a Pearson correlation coefficient of 0.56, indicating the results did generally agree, although there were minor differences. The PC12c sample was chosen as a control to allow the measurement of a maximum of phosphorylation changes.

After enrichment, separation, and analysis of the samples PC12c and PTRs, 3452 and 4315 unique phosphopeptides, respectively, at a false discovery rate of ~0.5% for peptides and ~3.5% for proteins, were identified. After merging these
data sets, 2035 unique phosphopeptides from 806 proteins (listed in supplemental Table S1) were identified and quantified in both of these conditions (Fig. 4A). The relative abundance of each phosphopeptide was quantified as the median of the area of its MS peak for each replicate compared with the ones identified in the heavy standard. The calculation and correction of the ratios were performed as indicated in the Experimental Procedures section. Fig. 4B plots the distribution of the ratios observed normalized against the ratio of the peptides from the PTRs sample. The dashed lines correspond to a ± 1.8 fold difference (± 0.25 on a logarithmic scale); the threshold used to identify peptides that are up or down-regulated after stimulation of the chimeric receptor PTR. There were, respectively, 424 up-regulated phosphopeptides on 259 proteins and 392 down-regulated phosphopeptides on 206 proteins. The threshold of 1.8 fold was considered to be a significant change as explained under “Experimental Procedures.”

Validation of SILAC Quantification—In order to validate the reliability of the SILAC methodology, the phosphorylations of GSK3α, ERK1, and ERK2 measured on Western blots were compared with the relative changes found by mass spectro-
Fig. 2. Neurite outgrowth induced by TrkA and PTR. Comparison of the neurite outgrowth induced by a stimulation with NGF (50 μg/ml) or PDGF-BB (50 μg/ml) of PC12 cells and PC12 cells stably transfectcd with PTR. The cells were cultivated on rat tail collagen I (5 μg/cm²) and either stimulated or not for 3 days.

Fig. 3. Western blot analyses of selected downstream signaling entities activated by PTR. Following stimulation of PTR with human PDGF-BB (50 μg/ml) for 20 min, selected proteins were monitored by Western blot analysis using antibodies against specific phosphorylation sites. The phosphorylation of PLCγ (Y783), TrkA (Y490), Akt (T308 and S473), GSK3α/β (S21/S9), ERK 1/2 (T202/Y204; T183/Y185) were detected in 3 different samples: PC12 cells (PC12c), PC12-PTR unstimulated (PTRu), and PC12-PTR stimulated by human PDGF-BB (PTRs). Glyceralddehyde-3-phosphat dehydrogenase (GAPDH) was the loading control for each sample.

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Fig. 5 shows the MS peaks of three different phosphorylated peptides from GSK3α (S21), ERK1 (T202/Y204) and ERK2 (T183/Y185). For each protein, the peaks of the three light (L) samples from PC12c, PTRu, PTRs, mixed with the same heavy standards (H), are depicted. The ratios below each panel, representing the relative quantity in each sample, are comparable with what was observed on the Western blots (Fig. 3).

GO Annotation: Biological Processes and Cellular Components—The biological processes (BP) and the cellular components (CC) of the phosphoproteins identified were characterized using DAVID Bioinformatics Resources 6.7 (28) and are given in Fig. 6. Each protein was manually assigned using the gene ontology terms found in the DAVID database. Common phosphoproteins shared by both conditions (PC12c and PTRs) were mainly associated with metabolism (31%), signaling (14%) and transport (14%). Fig. 6B shows that the up-regulated phosphorylations targeted preferentially proteins that are part of the plasma membrane and cytoskeleton, whereas the down-regulated phosphorylations were more frequently found associated with ribosomes and RNP (ribonucleoproteins).

The phosphoproteome of PTR after 20 min ligand-induced Stimulation—Fig. 7 represents a list of regulatory proteins normally associated with signal transduction pathways for which phosphorylation was modified wholly or in part by stimulation of PTR. They are classified according to their molecular functions, their regulation (up and down) and the strength of this regulation (ratio). Three classes of ratios have been indicated: the first two (more than twofold and between 1.8 and twofold) correspond to significant changes at the threshold selected (±0.25 on a logarithmic scale). The changes between 1.5- and 1.8-fold likely include proteins involved in signal transmission that show faster or slower kinetics of modification.

Twenty-six phosphorylations on 21 kinases were up-regulated as compared with the seven down-regulated sites emphasizing the important role of kinases in RTK signal transduction. Many of the phosphorylation sites are in as yet undefined domains of these kinases, although about a third are in the catalytic domains. The up-regulated sites in ERK1/2 are known to be the main sites responsible for enzyme activation, whereas removal of phosphorylation on serine 14 of CDK1 (phosphorylation that inactivates the enzyme) is a key checkpoint event required for transition from G2 to M. The phosphorylation on Serine 279 of Mnat1 is located in the
C-terminal part of the protein that interacts with the CDK7/cyclin H complex. Four of these were tyrosine kinases and four were phosphoinositide kinases. In addition, nine phosphorylations on eight phosphatases were up-regulated (four were tyrosine-phosphatases, and one was a phosphatidylinositol phosphatase) showing the retro-control of the signal. Further analyses showed the up-regulation of phosphorylation of GAPs (GTPase-Activating Proteins, 14 sites on 14 proteins) and GEFs (guanine nucleotide exchange factors, 15 sites on 12 proteins) and the down-regulation of GAPs (2 sites on 2 proteins) and GEFs (1 site on 1 protein). These large differences suggest the importance of the recruitment of these proteins following the stimulation of the receptor. Additionally many transcription factors and translation factors were found to be modified.

Other PTMs seem to be involved in the signaling pathways activated by TrkA as up-regulated phosphorylations were observed on two acetyltransferases (MED24 and mgea5) and down-regulated phosphorylation on two deacetylases. The protein mgea5 is also the O-GlcNAcase that catalyzes the cleavage of β-O-linked GlcNAc (O-GlcNAc) from modified nuclear and cytoplasmic proteins, suggesting the involvement of this PTM in TrkA responses as well.

**Phosphorylation Motifs**—An initial analysis of the kinase motifs contained in the phosphopeptides identified was performed using motif-x (http://motif-x.med.harvard.edu/) that allows the extraction of enriched phosphorylation motifs (supplemental Fig. S2). Of the 426 up-regulated phosphopeptides, 259 could be clustered into three different motifs: a pSP motif targeted by the proline-directed kinases, such as the MAPKs or the CDKs; an acidic motif containing 1, 2, or 3 aspartic acid (D) or glutamic acid (E) residues after the phosphorylation site, characteristic of the casein kinase II (CK2) or CK2-like substrates; and a basophilic motif RXXpS, with an arginine (R) at the -3 position, that can be phosphorylated by automatic gain control kinases (cAMP dependent, cGMP (guanosine 3',5'-monophosphate)–dependent, and protein kinase C) such as AKT, CAMK2, AKT-like, and PKD. These were also enriched in the down-regulated phosphopeptide set.

A more detailed analysis of a larger number (16) of kinase motifs is represented in Fig. 8, which shows the enrichment in different populations, according to the human protein reference database (http://www.hprd.org/). The upper panel represents the motifs of the phosphopeptides identified in this study. The enrichment factor, indicated above each point of the chart, was calculated as described in the Experimental Procedures, taking into account the statistical representation of each motif in the Uniprot database. Automatic gain control kinases, CK2 and CK2-like phosphorylation motifs are enriched in the up and down-regulated phosphopeptides. The ERK1/2 motif (PX[pS/T]p or VX[pS/pT]p) is clearly up-regulated after stimulation of PTR and the difference with the down-regulated phosphopeptides is statistically significant (p < 0.0001).
FIG. 5. Example of SILAC quantifications. MS peaks of three different phosphorylated peptides are shown in A (GSK3α (S21)), B (ERK1 (T202/Y204)), and C (ERK2 (T183/Y185)). For each protein, the MS peaks of the three unlabeled (light, L) samples PC12c, PTRu, PTRs, mixed with the same heavy standards (H), are depicted. The ratios represent the relative quantity in each sample normalized against PTRs.
Protein Interaction Network—In order to identify protein-protein networks regulated by a 20 min stimulation of PTR, the STRING database, which reports a combined score for several types of interaction between proteins, was utilized (29). By visualizing the results with Cytoscape v_2.7.0, showing the interaction score and the relative change of the phosphorylation identified on these proteins, the data revealed two highly regulated subnetworks (Fig. 9). If more than one phosphorylation was identified for a protein, it was represented by only the highest change, and if the phosphorylations identified were up and down-regulated, both were reported. The first subnetwork represents proteins from the spliceosome linked to several nuclear pore proteins. The second sub-network shows proteins related to cell morphogenesis, cell migration, axon guidance, cytoskeletal changes, and neurite outgrowth, all processes associated with neurite proliferation that is the phenotypic response of PC12 cells stimulated by NGF (through TrkA activation) (See Fig. 2). The detection of several of these phosphorylations in the PTRu sample confirms the specificity of the response to the stimulation of the receptor and not to its overexpression.

DISCUSSION

Although the importance of protein phosphorylation as a regulatory modification has been known since the pioneering work of Krebs and Fischer (31), it has taken the unbiased approaches of mass spectrometric analyses to appreciate the extent to which this modification occurs in eukaryotic cells, even at rest (9). By using a variety of affinity capture techniques to enrich for phosphopeptides, typically hundreds to thousands of sites have been identified in single samples and in many cases these have been quantified to allow investigators to determine changes with time following a germane stimulus (32). There are a number of technical difficulties in identifying the phosphoesters of serine and threonine and there are even greater problems in ascertaining the correct site of modification in peptides where more than one possibility exists (33). Nonetheless, there are growing catalogs (atlases) that clearly indicate that this modification is so extensive that it seems likely that the vast majority of intracellular proteins are probably modified either during a stimulus by a growth factor or other appropriate moiety or at the basal level. A great number of these occur at quite a low level (partial site occupancy) but modification of multiple sites on a single protein seems to be the norm rather than the exception. It remains a substantially unanswered questions in most paradigms studied to date as to how many of these phosphorylations are physiologically relevant (to either the resting cell or the stimulus response). Thus, it is clear that to understand signaling cascades, quantitative phosphoproteomic analyses are essential.

Phosphoproteomics on RTKs have been usually performed with the aim of identifying the phosphotyrosine proteome.
In general, the tyrosine-phosphorylated proteins are immunoprecipitated and analyzed by high-resolution mass spectrometry in combination with the SILAC (or other quantifiable) method. Here is presented a quantitative analysis of mainly the serine/threonine phosphoproteome of the receptor TrkA (as manifested in the chimera PTR) after 20 min of stimulation, in order to characterize the downstream response subsequent to the early tyrosine phosphorylations triggered by TrkA.

Regulated phosphoproteins associated with signal transduction defined by selected molecular function. The closed and open symbols indicate up-regulated and down-regulated phosphorylations, respectively. The changes are classified according to the magnitude of change: more than twofold (square), between 1.8- and twofold (triangle), and between 1.5- and 1.8-fold (circle). Lipid kinases are indicated in gray.
by activation of the receptor. 2035 unique phosphopeptides from 806 proteins were identified and quantified in a non-stimulated and stimulated state from which 40% were regulated. Direct measurements of protein levels for those found to be modified in this study were not performed, as it was considered unlikely that stimulation would alter protein levels to any significant extent over a 20 min time frame.

Comparison with EGFR—The comparison to other RTK phosphoproteomes should allow a better understanding of the similarities and differences between the signaling pathways that they activate, leading to a specific effect on responsive cells. Olsen and coworkers (9) studied "the global in vivo phosphoproteome and its temporal dynamics upon growth factor stimulation" of EGFR, another RTK. The serine/threonine phosphoproteome determined for TrkA has been compared with the EGFR data in terms of phosphorylation motifs identified (Fig. 8, lower panel). At 0 and 20 min of stimulation, time points, which correspond to the study reported herein, 2479 unique phosphopeptides could be extracted from the Olsen et al. data set (this includes both the ones with confident site assignments and the ones with a level of ambiguity) with quantitative data, from which 343 were up-regulated after stimulation with a fold increase of ≥1.8 times. Comparative analysis shows a similar pattern in the motifs of the up-regulated phosphopeptides by the two growth factor receptors, TrkA and EGFR, suggesting that the pathways stimulated are quite similar. A significant difference of (p < 0.011) shows that the cdc2 (CDK1) phosphorylation motif [pS/pT]PX[R/K] is more enriched in the EGF up-regulated phosphopeptides. This is consistent with the observations from previous studies showing that NGF stops cell cycle progression whereas EGF tends to increase proliferation (37). The phosphorylation of T14 of CDK1, which has been described as inhibiting the kinase activity (38, 39), was down-regulated upon stimulation, suggesting that this kinase is regulated by TrkA, but the motif KRXX[pS/pT], recognized by CDK1, seems to be slightly down-regulated, showing the importance of cell cycle regulation.

In comparing the TrkA phosphorylations to those reported for EGFR (9), it is important to note that two different cell lines (PC12 and HeLa) and two different species (rat and human) were used. Furthermore, both are transformed cell lines and may have alterations in their expressed proteins that would not be manifested in "normal " or primary cell lines from these species. Indeed, the levels of phosphorylation may be elevated in both studies as well (because of their transformed state) although phosphoproteome analyses of native cells indicate that even basal levels of protein phosphorylation are extensive (40–42). Nonetheless, there is clearly, even at this level of comparison, a considerable degree of similarity that is not inconsistent with the view that RTK signaling is generally overlapping. It is well known, for example, that when activated, the Trks and FGFRs cause the differentiation of PC12 cells, and that the EGFR receptor, if

**Fig. 8. Phosphorylation motifs.** Sixteen phosphorylation motifs modified by different kinases that are represented at the top of the figure were analyzed for the regulated phosphopeptides by determining their enrichment in each population. The upper panel represents the enrichment factor (see text for definition) of each motif in the up (dark gray bars) and down- (light gray bars) regulated phosphopeptides upon stimulation of PTR. The lower panel shows the same analysis on up-regulated phosphopeptides upon stimulation of PTR (dark gray bars) and EGFR in Hela cells (9) (white bars).
over expressed, will also have the same effect (43). Thus all three of these RTKs induce the pathways necessary for this biological response.

TrkA Signaling Pathways—The main signaling pathways activated by NGF are well known, but there are clearly additional potential entities involved that can be identified by large-scale phosphoproteomic studies. The use of a chimeric receptor also provides information about the contributions of specific NGF receptors (TrkA and/or p75) that control the regulation/modification of these proteins. Fig. 10 shows a representative scheme of the main signaling pathways activated by the TrkA receptor as previously described (18, 44, 45). Each protein identified in the present study is depicted in gray with the phosphorylation site associated (the sites represented are from rat protein sequences). If the peptide is multiply phosphorylated, it is represented by residue numbers separated by a forward slash (e.g. 337/340); if the site could not be confirmed, it is represented by residue numbers separated by hyphens (e.g. 34-35-36). The data reported herein confirm the involvement of several proteins, such as Gab-1/2, caspase 9, GSk3α/β, RAPTOR, elF4G, Bcl2-antagonist of cell death (BAD) in the PI3K/AKT pathway; MAP3K3, ERK1/2, P90RSK in the MAPK pathways, and Rap-1 and B-raf. Some phosphorylations were observed to be down-regulated upon the stimulation of the receptor, e.g. BAD, 4EBP1, and B-raf. Interestingly, the 2 sites on BAD contain the kinase motif RXRXXS/T that corresponds to sites described as substrates for AKT (46). The major phosphorylation sites on PI3K, PDK1, and AKT that would confirm their activation were not observed but the up-regulation of phosphorylations on several proteins downstream of these kinases, such as caspase 9, GSk3α, and elF4G suggest that they were activated. Moreover, the Western blotting (Fig. 3) showed that the T308 and S473 of AKT were up-regulated.

**Fig. 9. Proteins sub-networks.** Two representative subnetworks containing proteins (A) related to nuclear pore and spliceosome or proteins (B) related to cell morphogenesis, cell migration, axon guidance, cytoskeleton, and neurite outgrowth are highly regulated upon stimulation. The orange and blue circles represent proteins with an up and down-regulated phosphor site after stimulation, respectively. If more than one phosphorylation change were identified, only the biggest one is depicted.
The phosphorylation on Y279/216 on GSK3/ is described as necessary for the activation of the kinase (47, 48), does not change upon stimulation, contrary to the site on S9/S21 (inhibitory site). This confirms that GSK3/ is inactivated by NGF (after 20 min) as has been previously shown (49). However, in contrast to those findings, in which they show that this effect is due to p75, the data reported herein suggests that at least part of this signal is because of TrkA activation as well.

The doubly phosphorylated peptides on ERK1 (T202/Y204) and ERK2 (T183/Y185) are up-regulated after stimulation. These sites have been described as necessary for the activation of these two kinases and they have already been shown to be up-regulated upon stimulation of the TrkA receptor by NGF (50). The diphosphopeptides (T179/Y185 on ERK2), or the singly phosphorylated peptides (Y204 and Y185 for ERK1 and ERK2, respectively) were up-regulated as well, which shows the importance of these two kinases in the signaling pathway.

**Fig. 10. TrkA signaling pathway.** Composite scheme of the main signaling pathways activated by the TrkA receptor as previously described (44, 45). Each protein identified in this study is depicted in gray with the associated phosphorylation site (the sites represented are from rat proteins). A site represented by multiple residue numbers indicates a multiply phosphorylated peptide (e.g. 337/340) or a not confirmed site (e.g. 34–35-36). The open circle shows a down-regulated phosphorylation of 1.8 fold or greater upon stimulation; the crossed circles represent a phosphorylation that is not affected by the stimulation at 20 min; the black circles and black squares symbolize a up-regulated phosphorylation of more than 1.5- and 1.8-fold upon stimulation, respectively. The asterisks indicate RXRSS/T motif phosphorylation site.
pathway of the TrkA receptor and the variable kinetics for the different phosphorylations.

Regulation of Cytoskeleton and Spliceosome—The GO annotation analysis revealed some differences between the up-regulated and the down-regulated phosphoproteins. Proteins associated with the plasma membrane and the cytoskeleton were more represented in the first category. This is consistent with a receptor stimulation that involves transmission of signal from the plasma membrane to the cell interior. For example, phosphorylations on GAPs and GEFs, which are highly regulated, finely modulate the signal by switching on and off the various signaling entities. The involvement of proteins related to cytoskeleton is also consistent with the TrkA-induced differentiation. The formation of neurites requires some morphogenetic changes and protein transport into the new extensions is a function of the cytoskeleton. Some of these proteins, gathered into a subnetwork, have already been associated with the regulation of the actin cytoskeleton and cell shape (Fgd1), cell migration (Arhgef7), cell motility and polarization, dendritic spine morphology (Arhgef2), axonal branching, synapse formation and dendritic morphogenesis (Rgnef) or lamelipodia formation (ARHGAP22), organization of cell structure (Cortactin), remodeling of the actin cytoskeleton (Myo9b), or developmental neurite growth regulatory factor (reticulon 4).

Another interesting observation was the high level of down-regulation of phosphorylation on ribonucleoproteins combined with up-regulation of phosphorylation of nuclear proteins, suggesting an important role of the spliceosome in this response. The spliceosome is a complex containing up to 300 distinct proteins, although half of these are factors only temporarily associated with the complex (51). It is formed from several ribonucleoprotein subunits, termed uridine-rich small nuclear ribonucleoproteins that are assembled in the cytoplasm before being imported into the nucleus, where they are active. As such, the transport through nuclear pores is a prerequisite for the assembly and the function of the spliceosome. Both phosphorylation and dephosphorylation regulate the different steps of the splicing process (52–59). For example, the phosphorylation of the spliceosomal RNA helicase PRP28 by SRPK2 is required for the spliceosome assembly (52). Moreover, Shi et al. (58) showed that dephosphorylation of spliceosome components by phosphatases PP1 and PP2A facilitates essential structural rearrangements in the complex during the splicing process. Inhibitors of these two phosphatases block catalytic steps of splicing without affecting the assembly (53). In this study, SRPK2 kinase and protein phosphatase 1 regulatory subunit 12A present a phosphorylation up-regulation of fivefold and ~30-fold, respectively, after stimulation, supporting the involvement of the spliceosome in the signaling pathways. In eukaryotic cells, regulation of protein expression in response to intracellular and extracellular signals can be performed at the transcriptional level but also at the post-transcriptional stage, notably during the splicing of pre-mRNA. This step is essential for the splicing, the expression, the turnover, the silencing and the localization of RNA (reviewed in (60)).

Many of the regulated spliceosome components identified in our study such as SRSF5 are involved in alternative splicing (61); i.e. determining which splice sites are chosen, and are known to be regulated by phosphorylation, although the mechanisms are not well understood. The phosphorylation of the spliceosome components identified in this study could play a role in the cell cycle. Indeed, it has been demonstrated that the phosphorylation of SRp38, a splicing regulator, changes at the beginning of mitosis (62). Both up-regulated and/or down-regulated phosphorylations were observed in each of these sub-networks suggesting the equal importance of the appearance/disappearance of phosphorylation modifications.

Involvement of Other Post-translational Modifications—Other post-translational modifications seem to interface in the signaling of the PRT receptor. Two acetyltransferases (mgea5 and MED24) displayed increased phosphorylation upon stimulation and three deacetylases show up-regulated phosphorylation (Mta2) and down-regulated phosphorylations (HDAC1 and 2). Lysine acetylation is a reversible modification that plays a crucial role in regulating gene expression through the acetylation of histones but has been more recently associated with a large variety of biological processes, cellular component and molecular function (63, 64). More particularly, acetylation has been identified on proteins related to RNA splicing, DNA damage repair, cell cycle, nuclear transport, actin cytoskeleton, chaperones, and ribosomes (63). The protein mgea5 catalyzes the removal of O-GlcNAc modification from nuclear and cytoplasmic proteins. This modification, another PTM that regulates protein function and sometimes competes with phosphorylation at the same sites, can regulate RTK signaling. For example, the addition of O-GlcNAc modification after stimulation with insulin increases and has been shown to attenuate the insulin signaling by competing with phosphorylation (65, 66), thus the involvement of this enzyme in the signaling by TrkA is not surprising.

Although it is clear that the experiments described in this report do not give complete coverage of all the phosphosites present after 20 min of stimulation, those that were identified offer a representative group. Many of the known effectors/adaptors of TrkA signaling were found and new sites (proteins) were also identified. The comparison to the responses to EGFR stimulation at the same time point also indicates a similar profile with respect to kinase motifs, further establishing the overall redundancies of RTK signaling. TrkA is also attractive as a model for further dissecting its signaling potential because it putatively uses only two main docking sites on its intracellular domain (Y490 and Y785) to perpetrate its downstream signals (18). Phosphoproteomic analyses of mutant receptors in which these sites have been altered will therefore allow a more exact delineation of RTK signaling in terms of various pathways affected.
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This article contains supplemental Figs. S1 and S2 and Tables S1 and S2.

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REFERENCES

1. Bradshaw, R. A., and Dennis, E. A., eds. Handbook of Cell Signaling. 2nd ed. 2009. Elsevier Academic Press: San Diego, CA 3047

2. Kholodenko, B. N. (2006) Cell-signalling dynamics in time and space. Nat. Rev. Mol. Cell. Biol. 7, 165–176

3. Hunter, T. (2000) Signaling - 2000 and beyond. Cell 100, 113–127

4. Schlessinger, J. (2000) Cell signaling by receptor tyrosine kinases. Cell 103, 211–225

5. Bradshaw, R. A., and Sporn, M. B. (1983) Polypeptide growth factors and the regulation of cell growth and differentiation: Introduction. Fed. Proc. 42, 2590–2591

6. Blume-Jensen, P., and Hunter, T. (2001) Oncogenic kinase signaling. Nature 411, 355–365

7. Hunter, T., and Setton, B. M. (1980) Transforming gene product of the Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. 77, 1311–1315

8. Choudhary, C., and Mann, M. (2010) Decoding signalling networks by mass spectrometry-based proteomics. Nat. Rev. Mol. Cell. Biol. 11, 427–439

9. Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006) Global, In Vivo, and Site-Specific Phosphorylation Dynamics in Signaling Networks. Cell 127, 635–648

10. Schmelzle, K., Kane, S., Gridley, S., Lienhard, G. E., and White, F. M. (2006) Temporal dynamics of tyrosine phosphorylation in insulin signaling. Diabet. 55, 2171–2179

11. Krüger, M., Kratckmarova, I., Blagoev, B., Tseng, Y. H., Kahn, C. R., and Mann, M. (2008) Dissection of the insulin signaling pathway via quantitative phosphoproteomics. Proc. Natl. Acad. Sci. U.S.A. 105, 2451–2456

12. Kratckmarova, I., Blagoev, B., Haack-Sorensen, M., Kasseem, M., and Mann, M. (2005) Mechanism of divergent growth factor effects in mRNA localization scoring integrated into a search engine. Mol. Cell. Proteomics M111.008078

13. Amanchy, R., Periaswamy, B., Mathivanan, S., Reddy, R., Tattikota, S. G., and Pandey, A. (2007) A curated compendium of phosphorylation motifs. Nat. Biotechnol. 25, 285–286

14. Chalkley, R. J., Baker, P. R., Medzhiradszky, K. F., Lynn, A. J., and Burlingame, A. L. (2008) In-depth analysis of tandem mass spectrometry data from disparate instrument types. Mol. Cell. Proteom. 7, 2386–2398

15. Elias, J. E., and Gygi, S. P. (2007) Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat. Methods 4, 207–214

16. Effer, P., Trinidad, J. C., and Chalkley, R. J. (2011) Modification site localization scoring integrated into a search engine. Mol. Cell. Proteomics M111.008078

17. Chao, M. V. (2003) Neurotrophins and their receptors: A convergence point for the regulation of cell growth and differentiation: Introduction. Fed. Proc. 42, 2590–2591

18. Jörgenen, C., Sherman, A., Chen, G. I., Pasculescu, A., Poliakov, A., Mann, M., and Serve, H. (2009) Mislocalized activation of oncogenic kinases through specific inhibition of the Cdk kinases and induction of cyclin D1. J. Neurosci. 10, 6000–6021

19. Chow, J. P., Siu, W. Y., Ho, H. T., Ma, K. C., Ho, C. C., and Poon, R. Y. (2003) Differential contribution of inhibitory phosphorylation of CDC2 and CDK2 for unperturbed cell cycle control and DNA integrity checkpoints. J. Biol. Chem. 278, 40815–40828

20. Mueller, P. R., Coleman, T. M., Kurnagai, A., and Dunphy, W. G. (1995) Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. Science 270, 86–90

21. Swaney, D. L., Wenger, C. D., Thomson, J. A., and Coon, J. J. (2009) Human embryonic stem cell phosphorylome revealed by electron transfer dissociation tandem mass spectrometry. Proc. Natl. Acad. Sci.
41. Monetti, M., Nagaraj, N., Sharma, K., and Mann, M. (2011) Large-scale phosphosite quantification in tissues by a spike-in SILAC method. Nat. Methods 8, 655–658
42. Trinidad, J. C., Thalhammer, A., Specht, C. G., Lynn, A. J., Baker, P. R., Schoepfer, R., and Burlingame, A. L. (2008) Quantitative analysis of synaptic phosphoproteome and protein expression. Mol. Cell. Proteomics 7, 684–696
43. Raffioni, S., and Bradshaw, R. A. (1995) Staurosporine Causes Epidermal Growth Factor to Induce Differentiation in PC12 Cells via Receptor Up-regulation. J. Biol. Chem. 270, 7568–7572
44. Huang, E. J., and Reichardt, L. F. (2003) Trk Receptors: Roles in Neuronal Signaling. Annu. Rev. Biochem. 72, 609–642
45. Reichardt, L. F. (2006) Neurotrophin-regulated signalling pathways. Phil. Trans. R. Soc. B 361, 1545–1564
46. Obata, T., Yaffe, M. B., Leparc, G. G., Piro, E. T., Maegawa, H., Kashiwagi, A., Kikkawa, R., and Cantley, L. C. (2000) Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. J. Biol. Chem. 275, 36108–36115
47. Hughes, K., Nikolakaki, E., Plyte, S. E., Totty, N. F., and Woodgett, J. R. (1993) Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. EMBO J. 12, 803–808
48. Wang, Q. M., Fiol, C. J., DePaoli-Roach, A. A., and Roach, P. J. (1994) Glycogen synthase kinase-3 beta is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. J. Biol. Chem. 269, 14566–14574
49. Arevalo, M. A., and Rodríguez-Tébar, A. (2006) Activation of cascin kinase II and inhibition of phosphatase and tensin homologue deleted on chromosome 10 phosphatase by nerve growth factor/p75NTR inhibit glycogen synthase kinase-3beta and stimulate axonal growth. Mol. Biol. Cell. 17, 3369–3377
50. Stephens, R. M., Loeb, D. M., Copeland, T. D., Pawson, T., Greene, L. A., and Kaplan, D. R. (1994) Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. Neuron 12, 691–705
51. Jurica, M. S., and Moore, M. J. (2003) Pre-mRNA splicing: awash in a sea of proteins. Mol. Cell. 12, 5–14
52. Mathew, R., Hartmuth, K., Möhmann, S., Urlaub, H., Ficner, R., and Lührmann, R. (2008) Phosphorylation of human PRP28 by SRPK2 is required for integration of the U4/U6-U5 tri-snRNP into the spliceosome. Nat. Struct. Mol. Biol. 15, 436–443
53. Mermod, J. E., Cohen, P., and Lamond, A. I. (1992) Ser/Thr-specific protein phosphatases are required for both catalytic steps of pre-mRNA splicing. Nucleic Acids Res. 20, 5263–5269
54. Tazi, J., Kornstädt, U., Rossi, F., Jeanneau, P., Cathala, G., Brunel, C., and Lührmann, R. (1993) Thiophosphorylation of U1–70K protein inhibits pre-mRNA splicing. Nature 363, 283–286
55. Cao, W., Jamison, S. F., and Garcia-Blanco, M. A. (1997) Both phosphorylation and dephosphorylation of ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing. Genes Dev. 11, 334–344
56. Xiao, S. H., and Manley, J. L. (1997) Phosphorylation of the ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing. Genes Dev. 11, 334–344