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Authors

Biarc, Jordane
Chalkley, Robert J
Burlingame, AL
et al.

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Dissecting the Roles of Tyrosines 490 and 785 of TrkA Protein in the Induction of Downstream Protein Phosphorylation Using Chimeric Receptors*[S]

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Jordane Biarc[‡1], **Robert J. Chalkley**[‡2], **A. L. Burlingame**[‡], **and Ralph A. Bradshaw**[‡§]

From the [‡]*Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94158 and the* [§]*Department of Physiology and Biophysics, University of California, Irvine, California 92697*

Background: TrkA propagates downstream signaling mainly through two known phosphotyrosine-docking sites.
Results: Through mutating these tyrosines, it was possible to dissect which phosphorylations are driven through each of these sites.
Conclusion: Some signals are transmitted through only one of these sites and some through both, and some are propagated through new, unknown docking site(s).
Significance: Evidence is uncovered for additional docking site(s) on TrkA.

Receptor tyrosine kinases generally act by forming phosphotyrosine-docking sites on their own endodomains that propagate signals through cascades of post-translational modifications driven by the binding of adaptor/effector proteins. The pathways that are stimulated in any given receptor tyrosine kinase are a function of the initial docking sites that are activated and the availability of downstream participants. In the case of the Trk receptors, which are activated by nerve growth factor, there are only two established phosphotyrosine-docking sites (Tyr-490 and Tyr-785 on TrkA) that are known to be directly involved in signal transduction. Taking advantage of this limited repertoire of docking sites and the availability of PC12 cell lines stably transfected with chimeric receptors composed of the extracellular domain of the PDGF receptor and the transmembrane and intracellular domains of TrkA, the downstream TrkA-induced phosphoproteome was assessed for the "native" receptor and mutants lacking Tyr-490 or both Tyr-490 and Tyr-785. Basal phosphorylation levels were compared with those formed after 20 min of stimulation with PDGF. Several thousand phosphopeptides were identified after TiO_2 enrichment, and many were up- or down-regulated by receptor activation. The modified proteins in the native sample contained many of the well established participants in TrkA signaling. The results from the mutant receptors allowed grouping of these downstream targets by their dependence on the two characterized docking site(s). A clear subset that was not dependent on either Tyr-490 or Tyr-785 emerged, providing direct evidence that there are other sites on TrkA that are involved in downstream signaling.

Responding to signals emanating from the extracellular environment constitutes a fundamental biological activity that allows cells to relay external information, such as changes in environment, to interior compartments and components. In multicellular organisms, this is essential to coordinate the activities of different tissues and organs within the context of the organism as a whole. The mechanism of transmission of these signals is usually through the activation of plasma membrane-bound receptors following specific binding of germane signaling entities, with the view of eventually modulating protein synthesis through the activation/deactivation of transcription factors (1).

Each receptor family has a distinct mechanism for activation and propagation of signals (2), including the instigation of multiple (both number and kind) post-translational modifications. For the most part, these are characterized by transient alterations of existing proteins that regulate their function, location, or turnover, often by affecting macromolecular interactions. Many of these are catalyzed by the over 500 protein kinases (in humans) (3), producing a plethora of phosphorylations on tyrosine, threonine, and serine residues of a vast array of intracellular proteins. Indeed, these modifications have been shown to be very extensive (4–6), and there are now detailed maps and atlases documenting a large number of them. However, it is also clear that the complete description of this type of modification in any cell type under any condition has certainly not been elucidated.

One of the reasons that protein phosphorylation has been studied so extensively is the fact that one receptor class, the so-called receptor tyrosine kinases (RTKs),[3] plays a central role in a very broad range of cellular responses and is heavily implicated in a spectrum of human pathologies, notably cancer (7, 8).

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[S] This article contains supplemental Figs. 1 and 2 and Table 1.
[1] Present address: UMR 5280, CNRS, and Université de Lyon, 69622 Villeurbanne, France.
[2] To whom correspondence should be addressed: University of California San Francisco, 600 16th St., Genentech Hall, Rm. N474A, San Francisco, CA 94158-2517. E-mail: chalkley@cgl.ucsf.edu.

[3] The abbreviations used are: RTK, receptor tyrosine kinase; PTR, PDGF receptor-TrkA chimeric receptor; PC12c, control PC12 cells unstimulated; PTRu, transfected PC12 cells unstimulated; PTRs, transfected PC12 cells stimulated; PTR Y490F(s), transfected PC12 cells with PTR Y490F stimulated; PTM, post-translational modification; SILAC, stable isotope labeling of amino acids in culture; DAG, diacylglycerol; ACN, acetonitrile.

The RTK family consists of 19 different subfamilies containing a total of 58 members (9). The protomers are characterized by the presence of an intracellular tyrosine kinase domain and assemble into higher oligomeric forms that, when activated by ligand binding to the extracellular domain, catalyze the trans-autophosphorylation of a number of tyrosine residues found in the kinase domain itself or in its N- or C-terminal flanking sequences. These can serve as docking sites for a variety of adaptor/effector/scaffold proteins, which are similarly modified in turn, leading to the propagation and amplification of the signal. There is a limited subset of such proteins, and there is considerable overlap in the pathways that are activated by the various RTK families. However, there is quite a bit of variation in the binding profiles of each family and, hence to some degree, on the downstream phosphoproteome thus produced. For example, the EGF receptor has a large (~200 residues) C-terminal extension beyond the kinase, which contributes most of its phosphotyrosine-docking sites, and there is considerable redundancy in the entities bound (10, 11), although the FGF receptor family has its many fewer phosphorylated tyrosines located mostly in the kinase domain itself and does not apparently bind effector/adaptor molecules through these sites (12). Of course, all of the RTK family has tyrosine residues in the activation loop that become phosphorylated, and these are essential for signaling in every case except the EGF receptor (13).

TrkA, the receptor for NGF, has in addition to the activation loop tyrosines (Tyr-670, Tyr-674, and Tyr-675) two other principal sites, Tyr-490 and Tyr-785, both of which lie outside of the kinase domain. These provide docking sites that can lead to the stimulation of several signaling pathways. For example, the Ras/mitogen-activated protein kinase (MAPK or ERK) and the phosphatidylinositol 3-kinase (PI3K)/AKT pathways have been shown to be associated with Tyr-490, and the phospholipase Cγ (PLCγ) pathway is associated with Tyr-785 (14). However, no detailed analysis of the downstream TrkA phosphorylations specifically tied to Tyr-490 or Tyr-785 has been reported.

In this study, chimeric receptors composed of the ectodomain of the human PDGF receptor and the transmembrane and endodomain of the TrkA receptor from rat (denoted PTR), in which Tyr-490 or Tyr-490/785 were changed to phenylalanine by site-directed mutagenesis, were stably transfected into PC12 cells (14) and the phosphoproteomes, determined after 20 min of stimulation, were compared with that of the activated wild-type PTR receptor and unstimulated cells (15). Because of the lack of PDGF receptors in PC12 cells, the chimeric construction avoids any contributions from other receptors, including the exogenous Trk receptors and the pan-neurotrophin receptor p75, which also uses NGF as a ligand. The phosphopeptides that were observed to significantly increase or decrease across a comparison of all four samples indicate those events dependent on Tyr-490 and Tyr-785 or not dependent on either. This last group apparently arises from other, as yet unidentified, binding sites on TrkA.

EXPERIMENTAL PROCEDURES

Construction and Stable Transfection of PTR and PTR Mutants in PC12 Cells—Cell lines stably transfected with the chimeric receptors PTR, PTR Y490F, and PTR Y490F/Y785F were generated as described previously (14). Briefly, an EcoRII/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 TrkA was cloned into the retroviral vector pLEN. Ecotropic retroviruses were generated with the help of PA317, GP+E-86 PTR, and PTR Y490F producer cell lines. In the case of PTR Y490F/Y785F, 15 μg of the construction (cloned into p-Babe-puro plasmid given by E. Gonzalez-Munoz and C. Petritsch) were transfected into Phoenix packaging cells (PTR Y490F/Y785F) using polyethyleneimine. After 48 h, viruses were collected, concentrated, and incubated with PC12 cells. After 8 days, puromycin was added to the culture medium at 0.5 μg/ml. All PCR-derived sequences were completely resequenced to exclude any PCR errors. The level of expression of PTR receptors used in these studies was not directly measured, but indirect measures of activity following stimulation indicated that the levels were comparable with native expression, based on comparison with clones that had been analyzed.

Cell Culture and SILAC Labeling—PC12 cells, with or without the stably transfected chimeric receptor PTR, PTR Y490F, and PTR Y490F/Y785F, were grown in 15-cm Petri dishes in light medium containing Dulbecco's modified Eagle's medium (DMEM), 4.5 g/liter glucose, 10% horse serum, 5% calf serum, 50 units/ml penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively), and 200 mg/liter L-proline (Thermo Fisher Scientific, San Jose, CA). After starving the cells for 24 h, the cells were stimulated for 20 min with PDGF-BB (Austral Biologicals, San Ramon, CA) (50 ng/ml) and lysed in 10 ml of TRIzol® reagent. Proteins were purified according to the TRIzol® reagent protocol and resuspended in 6 M guanidine hydrochloride. 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 DMEM deficient in normal arginine and lysine and supplemented with 15% dialyzed FBS (Hyclone®, Thermo Fisher Scientific), penicillin and streptomycin (100 units/ml and 100 μg/ml, respectively), 200 mg/liter L-proline (Thermo Fisher Scientific), and L-arginine-$^{13}C_6$-$^{15}N_4$·HCl and L-lysine-$^{13}C_6$-$^{15}N_2$·HCl (Thermo Fisher Scientific and Sigma, respectively). Cells were grown in a humidified incubator with 5% atmospheric CO_2 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 μg/ml) in the same way as the light samples and lysed in TRIzol® reagent. Proteins from the seven dishes were each suspended in 700 μl of 6 M guanidine·HCl. This sample constituted the "heavy standard" used for quantification. To measure the neurite outgrowth, PC12 cells and PC12 cells stably transfected with PTR or PTR mutants were cultivated on 5 μg/cm^2 rat tail collagen I (BD Biosciences) and stimulated for 3 days with NGF or PDGF-BB (50 ng/ml).

Protein Digestion—Three mg of sample from each mutant PTR transfected and stimulated cell line were mixed with 3 mg of the heavy standard. Each sample was reduced for 1 h at 57 °C with 2.1 mM tris(2-carboxyethyl)phosphine hydrochloride to reduce 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 M guanidine hydrochloride, 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 vacuum-dried and resuspended in 750 μl of 35% ACN, 200 mM NaCl, 0.4% TFA for phosphopeptide enrichment.

Phosphopeptide Enrichment by Titanium Dioxide Chromatography—Peptides were enriched using 5 μm of titanium dioxide beads (GL Sciences, Tokyo, Japan) (23, 24) 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% ACN, ,200 mM NaCl, 0.4% TFA) 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 M KH_2PO_4). 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% ACN, 0.1% TFA). This collected fraction was lyophilized to dryness. Three separations were performed for each sample (2 mg/run).

Separation of Phosphopeptides by Strong Cation Exchange Chromatography—Strong cation exchange chromatography was performed using an ÄKTA purifier (GE Healthcare) equipped with a Tricorn 5/200 column (GE Healthcare) packed in house with 5 μm of 300 Å of polysulfoethyl A resin (Western Analytical, Lake Elsinore, CA). Phosphopeptides enriched from each sample were loaded onto the column in 30% ACN, 5 mM KH_2PO_4, 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 0.350 ml/min. Fractions (53 and 30) were collected of 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 LC-MS/MS.

LC-MS/MS—Mass spectrometry was performed using an LTQ-Orbitrap XL (Thermo Fisher Scientific). Chromatography was performed using a NanoAcquity ultraperformance liquid chromatography system (Waters) at a flow rate of 300 nl/min on a column of BEH130 C18 75 μm inner diameter × 150 mm (Waters), 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 −1,800 (with a resolution of 60,000), the seven most intense multiply charged precursors were selected for collision-induced dissociation analysis in the linear ion trap. Activation times were 30 ms for collision-induced dissociation fragmentation with a 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 reduce repeated analysis of the same components.

Peptide and Protein Identification—Fragmentation data were converted to peak lists using an in-house script based on the Raw_Extract script in Xcalibur version 2.4 (Thermo Fisher Scientific), and the collision-induced dissociation data for each sample were searched using Protein Prospector version 5.8 (16) 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 considered 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 up at 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 concatenated database search results (17); the protein false-positive rate was estimated to be 3.5%. As the proteins reported in this study are only those identified in all SILAC experiments, and the incorrect identifications are probably only identified in a single experiment, the actual false discovery rate is likely to be lower than this estimate. The reliability of phosphorylation site assignments was measured using the site localization in peptide score in Protein Prospector (18).

Quantification—SILAC quantification measurements were extracted from the raw data by Search Compare in Protein Prospector.[4] Search Compare averaged together MS scans from −10 s to +30 s from the time at which the MS/MS spectrum was acquired 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 were 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 supplemental Tables), indicating one value was below the noise level, so the ratio reported is a minimum estimate. If a phosphopeptide was identified from multiple MSMS spectra, the median of the calculated SILAC ratios from all the replicate identifications of

[4] P. R. Baker, N. J. Agard, A. L. Burlingame, and R. J. Chalkley, poster presented at 58th ASMS Conference of Mass Spectrometry and Allied Topics, Salt Lake City, UT (May 23–27, 2010).

the same peptide was reported. These ratios were then corrected for differences in protein level using data from the non-phosphorylated peptides (where SILAC ratios should be 1:1). The protein ratio for practically all proteins was the same between samples, as 20 min of stimulation is not long enough to instigate measurable changes in protein expression levels. The average of the standard deviation of the log(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 greater than 1.8-fold difference (0.25 = log(1.8)) should correspond to a significant change and a less than a 1.3-fold difference (0.125 = log(1.3)) should not be considered as significant.

Phosphorylation Motif Analysis—The frequency of occurrence of different kinase motifs within the phosphorylation sites identified as a whole, and those that were regulated by stimulation of different receptor versions were characterized. The queried kinase motifs were from those reported in the Human Protein Reference Database phosphorylation database (19). The frequency of occurrence of different motifs in the database were determined using MS-Pattern (20), as described previously (15). The plotted enrichment factor reports how often a given motif was observed compared with how often it would be expected to be observed at random.

GO Annotation—The proteins were submitted to DAVID Bioinformatics Resources 6.7 (21) with their Uniprot accession numbers to analyze their membership of different categories of biological processes, cellular components, and molecular functions, as described previously (15).

Heatmaps—The lists of identified peptides in each intersection of Fig. 4 were hierarchically clustered according to their behavior to the different mutations of the receptor PTR by calculating the Euclidean distance using Cytoscape v_2.7.0 (22).

Western Blots—Twelve μg of PC12c, PTRu, PTRs, PTR Y490Fu, PTR Y490s, PTR Y490/785Fu, and PTR Y490/785s (treated as described above) were separated on a 4–20% Tris-HCl ready gel (Bio-Rad)) and transferred onto a PVDF membrane (Polyscreen®). The blocking step was performed using 5% BSA in TBS, 0.5% Tween 20 buffer for 1 h. The first antibody was used at 1:1,000 dilution for 1 h followed by three washes of 5 min each in TBS, 0.5% Tween 20 buffer. The secondary antibody coupled to HRP (Bio-Rad) was incubated for 30 min. After three washes of 5 min with TBS, 0.5% Tween 20, the substrate ECL-plus (GE Healthcare) was added to the membrane and the detection performed.

RESULTS

TrkA Intracellular Domain Mutations Affect Downstream Signaling Differently—In previously reported experiments (15), changes to the phosphoproteome introduced by a 20-min exposure to activating ligand (PDGF BB, 50 ng/ml) in PC12 cells and PC12 cells stably transfected with the chimeric receptor PTR were analyzed by LC MS/MS. In this study, the phosphoproteomes of mutant PTR Y490F and PTR Y490F/Y785F (Fig. 1), also in stably transfected cells, have been determined under the same conditions and compared with those of the native receptor (PTR). The phenotypic response of both of these mutants to ligand stimulation was disrupted and generally resulted in incomplete differentiation, albeit that the double mutant was more severely compromised. The wild-type receptor yielded neurite outgrowth characteristic of the stimulation of PC12 cells by NGF through TrkA, although the mutant Y490F showed a decreased response, and the double mutant Y490F/Y785F was not able to significantly extend neurites at all. Relative expression of the chimeric receptors, as judged by Western blotting and previously performed radioligand measurements (data not shown), indicated that levels of receptor expression were within a factor of 2 of the wild-type levels. Western blots (data not shown) of the activated forms of several substrates involved in neurite outgrowth underscore that these phenotypic differences can be explained by alterations in the protein phosphorylation patterns stimulated by PTR and its mutant equivalents. The phosphorylation of PLCγ, which is activated by docking to Tyr-785 of the TrkA intracellular domain, was increased by the stimulation of PTR and PTR Y490F but was missing after stimulation of the double mutant PTR Y490F/Y785F (14). The phosphorylation on Tyr-490 was absent in both mutants after stimulation, so the decrease in the phosphorylation of AKT on Thr-308 and Ser-473 relative to that seen for the wild-type PTR was as expected. The same pattern was observed for GSK3β (Ser-21) and ERK1/2 (Thr-202/Tyr-204 and Thr-183/Tyr-185), where both mutations dramatically decrease the signal. A longer exposure was able to show there was some activation of ERK1/2 even in the double mutant, suggesting that limited activation of these key kinases can also arise through events not controlled by these two docking sites.

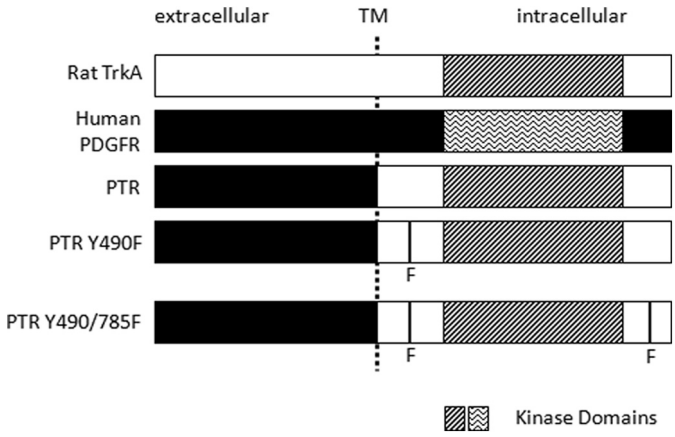

FIGURE 1. **Chimeric receptor construction.** The chimeric PTR receptor consists of the intracellular and transmembrane (*TM*) domains of the rat TrkA receptor fused to the extracellular region of human PDGF receptor-β. *Black* and *white bars* indicate human PDGF receptor sequence and rat TrkA sequence, respectively. The mutant receptors annotated PTR Y490F and PTR Y490F/Y785F contain mutations of tyrosine 490 and 490/785, respectively.

Comparison of the Phosphoproteomes of the Native and Mutated Receptors—As in the previous study (15), the increases and decreases in phosphorylation site stoichiometry were measured by mixing each sample with the same reference standard: PC12 cells transfected with the wild-type chimeric receptor PTR, grown in media containing heavy isotope-labeled lysine and arginine residues, and stimulated with PDGF-BB for 20 min (Fig. 2). After enrichment for phosphopeptides using TiO_2 and then fractionation by strong cation

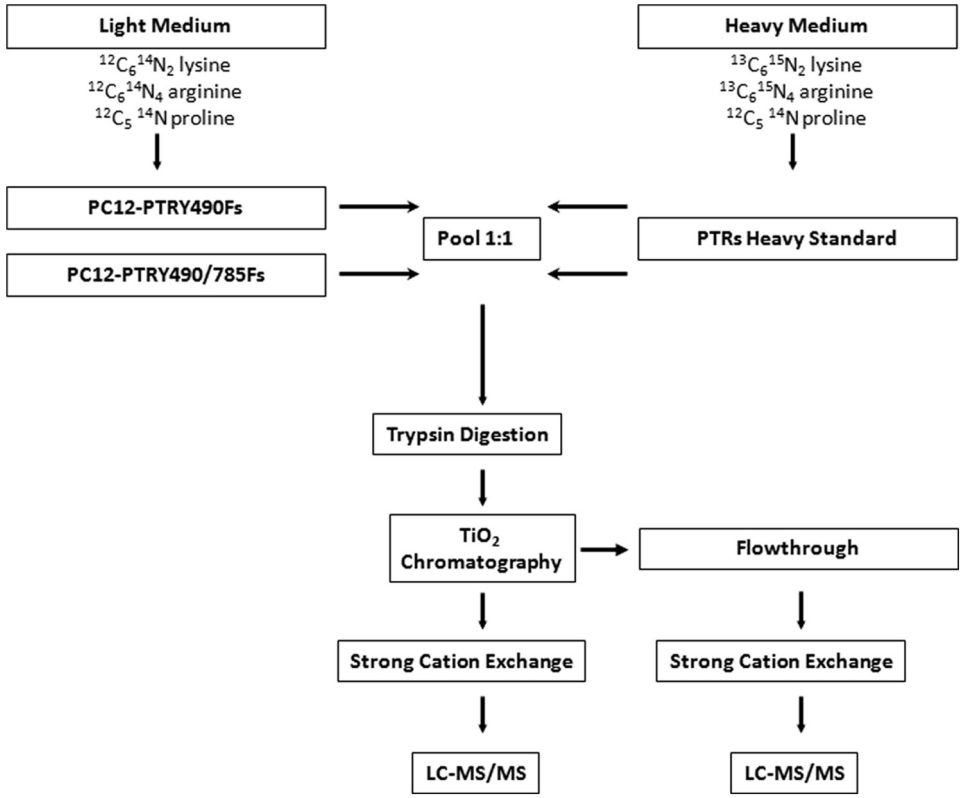

FIGURE 2. **Experimental strategy.** PC12 cells stably transfected with chimeric receptors PTR Y490F and PTR Y490F/Y785F were cultivated in an isotopically unlabeled medium containing 200 mg/ml unlabeled proline and stimulated with PDGF-BB (50 ng/ml) for 20 min. As a heavy standard for the relative quantification, PC12-PTRs were cultivated in medium containing heavy lysine and arginine and unlabeled proline and stimulated with PDGF-BB (50 ng/ml) for 20 min. 3 mg of each sample were mixed with 3 mg of the common heavy sample and were then reduced, alkylated, and digested with trypsin. The phosphopeptides were enriched on a TiO_2 column. The phosphopeptides and the nonphosphopeptides (flow-through) were separated on a strong cation exchange column (polysulfoethyl). Samples were analyzed by LC-MS/MS for 90 min on a LTQ-Orbitrap XL.

exchange chromatography, 4,152 and 3,931 unique quantifiable phosphopeptides were identified in the samples PTR Y490F(s) and PTR Y490F/Y785F(s), respectively, at an estimated false discovery rate of ≈0.5% for peptides and ≈3.5% for proteins, according to target-decoy database searching (17). When combined with the previous results from PC12(c) cells and PTR(s), a total of 988 phosphopeptides, corresponding to 903 phosphorylation sites on 501 proteins, could be identified in all four conditions (Fig. 3*A*). Among them, 208 (21%) were up-regulated after stimulation of the wild-type receptor compared with the control cells, and 172 (17%) were down-regulated. The threshold selected to determine a significant change in phosphorylation was set at a log ratio of +0.25 and −0.25 for down- and up-regulation, respectively; the threshold selected to determine an absence of change was set between +0.125 and −0.125 (Fig. 3*B*) (see "Experimental Procedures"). Using these parameters, different populations from the four data sets could be extracted (Fig. 4). Each of the four *solid colored circles* and the corresponding *boxes* in Fig. 4 (also solid color-coded in the same way) represent a group of phosphopeptides displaying the same pattern of responses to each PTR receptor type (wild type or mutant). For example, the group labeled *Mutant Y490F-dependent* in Fig. 4 (*yellow circle* and *box*) indicates a phosphopeptide up- or down-regulated by the stimulation of the wild-type receptor that shows a different response with the mutation Y490F with a log ratio PTR490(s)/PTR(s) greater than 0.25 or lesser than −0.25. Thus, in this case the mutation (Tyr-490) either prevented the change caused by PTR stimulation or it exacerbated the effect.

The *numbers* in each intersection of the central Venn diagram in Fig. 4 correspond to the number of phosphopeptides common to the overlapping categories, and their profiles are found in the *boxes with colored borders*. For example, there are 76 phosphopeptides that are affected by the single mutation Y490F and differently by the double mutation Y490F/Y785F. Among them, some peptides are affected by the mutation Y490F and by the double mutant (*i.e.* by Y785F), but in an opposite way. To simplify this group for further analysis, these peptides were separated into a group of 55 phosphopeptides (57 phosphorylation sites) from 44 proteins that are similarly affected by the single and double mutations, and these are plotted in the top box in Fig. 4 (*orange border*). Of particular interest are the 41 phosphopeptides (41 phosphorylation sites on 37 proteins) that do not show any effect after both mutations, indicating that they are stimulated independently of either of the mutated tyrosine residues (Fig. 4, *blue frame*). As an alternative representation, heatmap plots of each of the intersection groups (Fig. 4, *open boxes with colored borders*), after performing hierarchical clustering (see "Experimental Procedures"), show the relative changes in phosphorylation of these peptides according to their behavior to the different receptor mutants (supplemental Fig. 1).

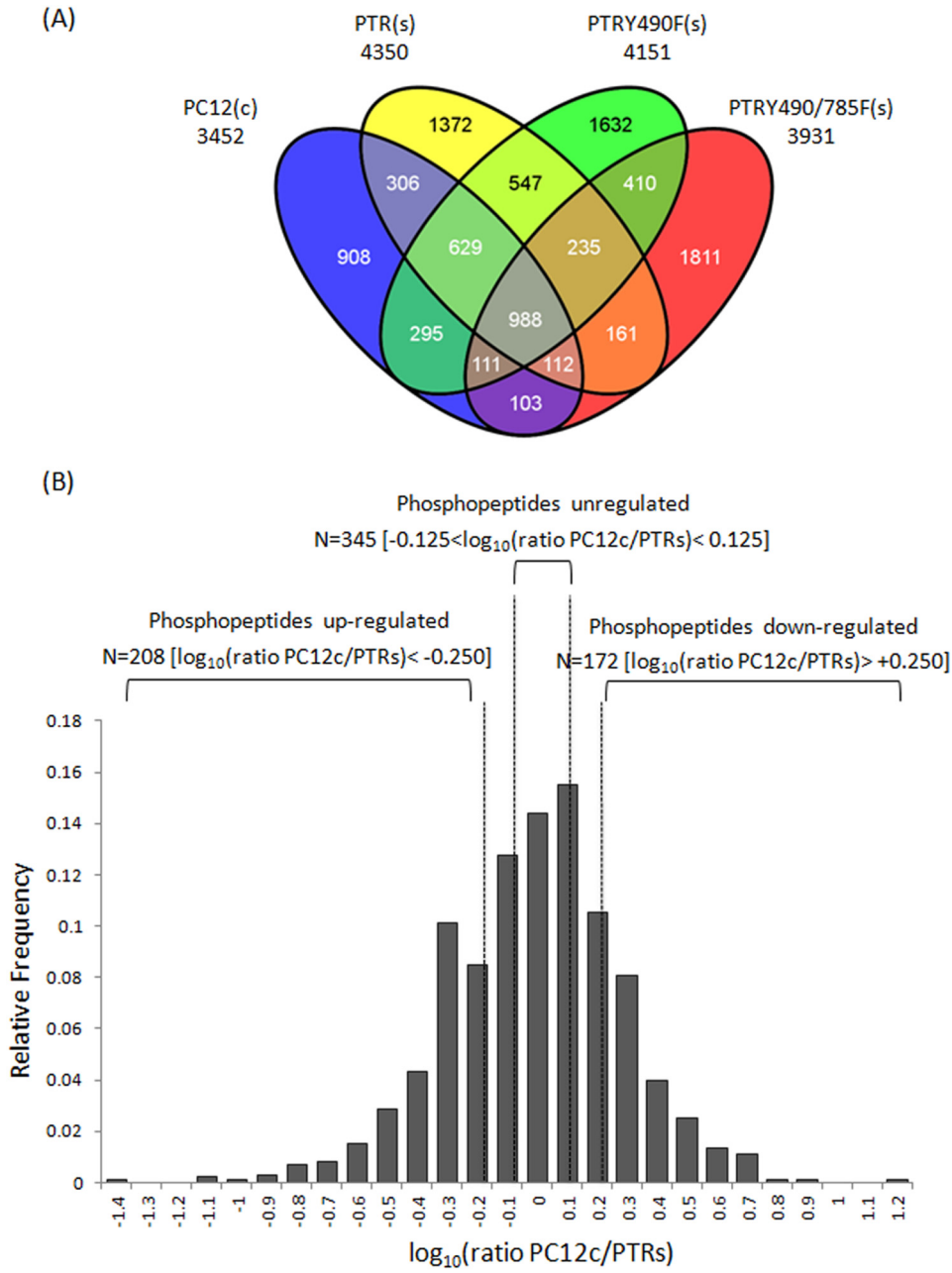

FIGURE 3. **Overlap of phosphopeptides identified and the distribution of their changes upon receptor stimulation.** *A,* Venn diagram describing the phosphopeptides identified in PC12 cells (*PC12c*), PC12 cells stably transfected with chimeric receptor PTR stimulated for 20 min with PDGF-BB (PTRs), PTR Y490F stimulated (PTR Y490F(s)) or PTR Y490F/Y785F stimulated with a peptide false-positive rate of 0.5%. 988 phosphopeptides (*gray part*) were identified in all four conditions. (Data were produced using Venny.) *B,* distribution of the log(ratio) of the sample PC12c normalized against the log(ratio) of the peptides from the PTRs sample. Each phosphopeptide with a log(ratio) above 0.25 or below −0.25 represents down-regulated or up-regulated stimulation modification due to stimulation, respectively, and phosphopeptides with a log(ratio) between −0.125 and 0.125 represent peptides that are not regulated.

Regulation of Protein Kinase Motifs through Tyr-490 and Tyr-785—To characterize the possible classes of kinases involved in the signals induced by the chimeric receptor PTR and to determine the contributions of Tyr-490 and Tyr-785 to them, an analysis was conducted of the phosphorylation motifs of the phosphopeptides identified. The motifs queried were listed in the human protein reference database (19) and were analyzed with respect to each group described in Fig. 4. In a previous study, it was shown that the motifs related to AKT, casein kinase 2, ERK1/2, and PKA were enriched after stimulation of the wild-type receptor PTR (15). Fig. 5 shows the behavior of the 16 queried motifs in response to the two mutations studied. The two motifs that can be phosphorylated by AKT (R*X*R*XX*(pS/pT) and R(R/S/T)*X*(pS/pT)*X*(S/T)) seem to be shared between being Tyr-490- and Tyr-785-responsive. The motif potentially phosphorylated by PKA (R(R/K)*X*(pS/pT)) was more affected by the mutation on Tyr-490. Many phosphorylations in the motif targeted by casein kinase 2 (CK2) (pS(D/E)(D/E)(D/E)) were affected by the Y785F mutation, but even more appear to be regulated by another part of the receptor. The ERK1/2 motifs (P*X*(pS/pT)P and V*X*(pS/pT)P) were not particularly associated with a specific mutation and seem to be

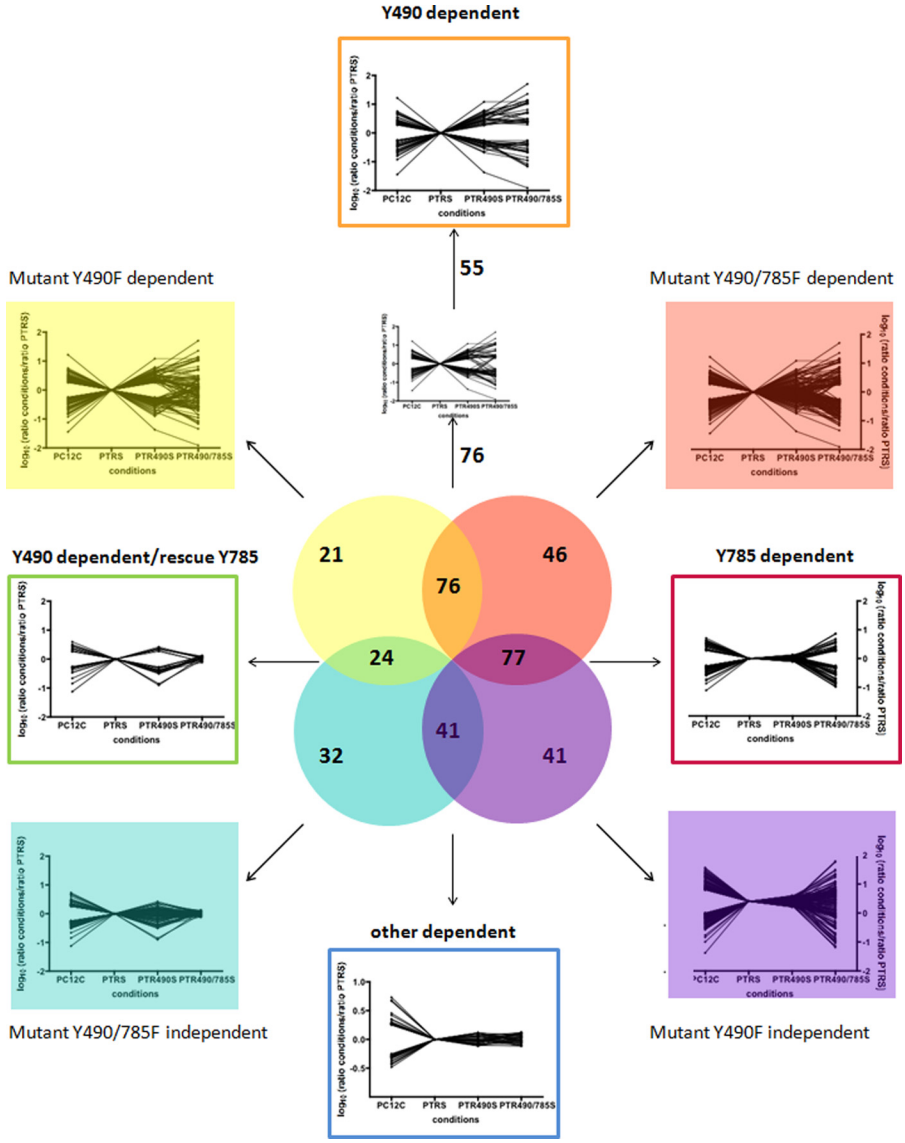

FIGURE 4. **Phosphorylation patterns in extracted populations.** The threshold selected to determine a significant change in phosphorylation was set at a log of ratio of +0.25 and −0.25 for down- and up-regulation, respectively, and the threshold selected to determine an absence of change was set between −0.125 and 0.125. Using these parameters, different populations can be extracted from the phosphopeptides affected by PTR stimulation. Each *circle* corresponds to a group of phosphopeptides displaying the same responses to each version of the PTR receptor. The *numbers in each intersection* correspond to the number of phosphopeptides common to both categories. 55 phosphopeptides are affected by the mutation Y490F (still visible with the mutation Y490F/Y785F) (*orange border*); 77 phosphopeptides are affected by the mutation Y785F only (*red border*), and 41 phosphopeptides do not show any changes after both mutations (*blue border*). 24 phosphopeptides show a change after mutation Tyr-490 that seems to be rescued by the mutation of the second tyrosine Tyr-785 (*green border*).

shared by the four different populations, again indicating, as seen on the Western blots, that these sites can be stimulated by signals emanating from both the classic docking sites as well as additional site(s).

GO Annotations of Proteins Regulated by Tyr-490 and Tyr-785—The biological processes and the cellular components of the phosphoproteins identified in each population were characterized using the DAVID Bioinformatics Resources 6.7 (david.abcc.ncifcrf.gov) and are presented in supplemental Fig. 2. No large variation between the different groups was observed, but the results seemed to suggest that the phosphoproteins regulated by Tyr-490 are in a large part nuclear (12/44 proteins) and are involved in transcription regulation (9/44 proteins) and RNA splicing (4/44 proteins). The phosphoproteins regulated by Tyr-785 seem to play an important role in mitosis (5/57 proteins) and cell cycle (7/57 proteins) compared with the other groups. Finally, the population of phosphoproteins controlled independently of Tyr-490 and Tyr-785 of the receptor are less represented in the nuclear region (2/37 proteins).

DISCUSSION

The RTK family members initiate their signaling activities through the autocatalytic phosphorylation of intracellular domain tyrosine residues. These are important for either maintaining the active conformation of the kinase or for providing docking sites for downstream effector/adaptor/scaffold proteins. In the case of the Trk family, there are two principal

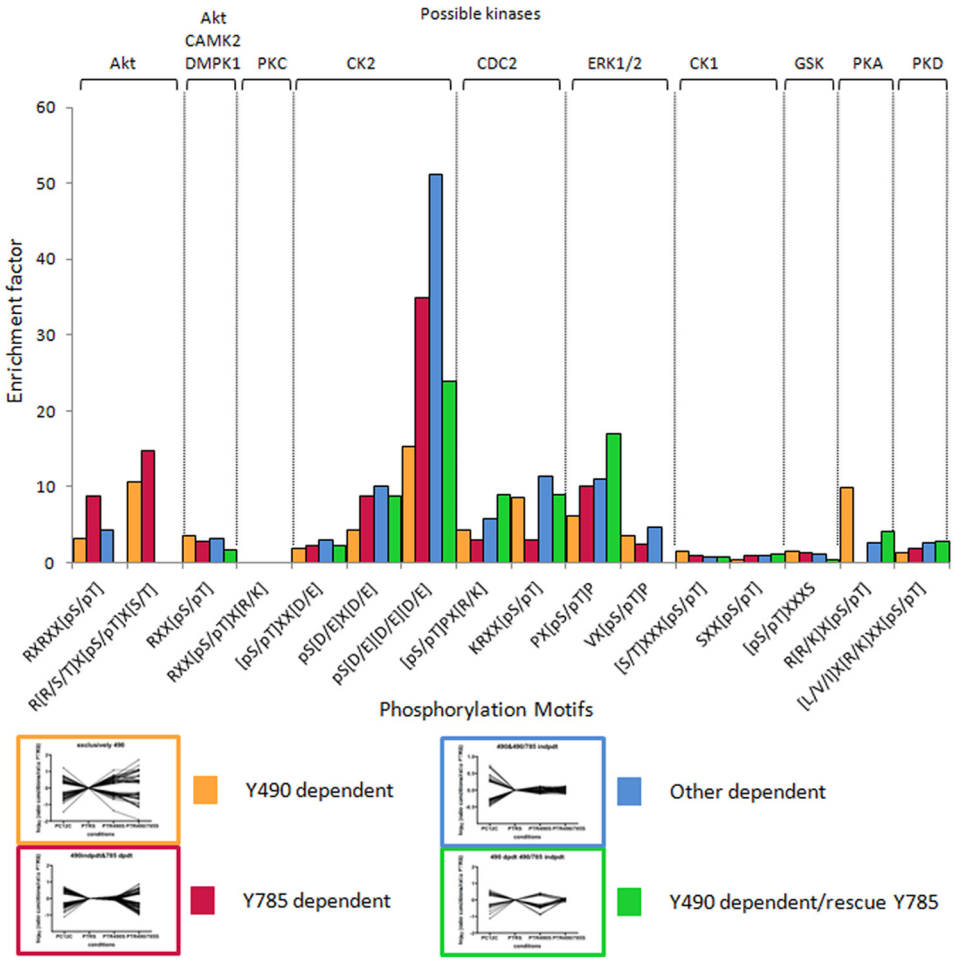

FIGURE 5. **Regulation of phosphorylation motifs.** 16 phosphorylation motifs modified by different kinases (among others), which are represented at the *top* of the figure, were analyzed by determining their enrichment in the populations of phosphopeptides displaying the same regulation by each version of the PTR receptor: *Y490 dependent* represents phosphorylations affected by the mutation of the Tyr-490 (*yellow bars*); *Y785 dependent* represents phosphorylations affected by the mutation of the Tyr-785 (*red bars*); *other dependent* represents phosphorylations not affected by the either mutation of the Tyr-490 and Y785F (*blue bars*), and *Y490 dependent/rescue Y785* represent phosphorylations affected by the mutation of the Tyr-490 and rescued by the mutation on Y785F (*green bars*). Specificity of each motif has been designated according to the Human Protein Reference Database (54).

tyrosines in the latter category, Tyr-490 and Tyr-785 (14). Tyr-490, when phosphorylated, binds FRS2 and Shc, whereas Tyr-785 binds phospholipase Cγ. The conversion of these sites to phenylalanine prevents their modification and obliterates the signaling arising from any association with signaling partners. These downstream signals are mainly protein phosphorylations, primarily on serine/threonine residues, that reach a peak at about 20 min after receptor activation. Using a model system (PC12 cells stably transfected with a chimeric form of TrkA, where the extracellular domain has been replaced by that of the human PDGF receptor, which has no natural ligand in PC12 cells), over 4,000 unique phosphopeptides at this time point with more than 800 showing significant changes relative to unstimulated cells were previously identified (15). The current report extends this study and presents a quantitative analysis of the phosphoproteome induced by TrkA chimeras in two mutant forms (PTR Y490F and PTR Y490F/Y785F), also after 20 min of stimulation. This study quantified 988 phosphopeptides from 501 proteins in all four conditions, of which 40% (380) were regulated by TrkA. A similar number of phosphopeptides were up- and down-regulated, as was seen in the previous study (15).

As expected, the two tyrosine mutations differentially affected the phosphorylation patterns. However, importantly, the double mutant, with both known docking sites eliminated, still elicited downstream phosphorylation changes, suggesting the existence of another site(s) in the receptor that can activate signal transduction responses independently of Tyr-490 and Tyr-785. The clustering of the phosphopeptides regulated by PTR into different groups, categorized by their behavior to the mutations, revealed four major classes of phosphoproteomic modifications as follows: those dependent on Tyr-490 (14%, 55 phosphopeptides on 44 proteins), those dependent on Tyr-785 (21%, 78 phosphopeptides on 57 proteins), those not dependent on either (11%, 41 phosphopeptides on 37 proteins), and those where phosphorylation was dependent on Tyr-490 but were rescued by the additional mutation of Tyr-785 (7%, 23 phosphopeptides, 20 proteins).

Fig. 6 presents a subset of proteins for which the phosphorylation is regulated by the stimulation of PTR, as described in our previous study (15). The *colored frames* in Fig. 6 indicate the sensitivity of these phosphorylations to the mutations Y490F and Y785F. In Fig. 6, *no frame* surrounding a protein either means that the phosphoprotein could not be identified in all

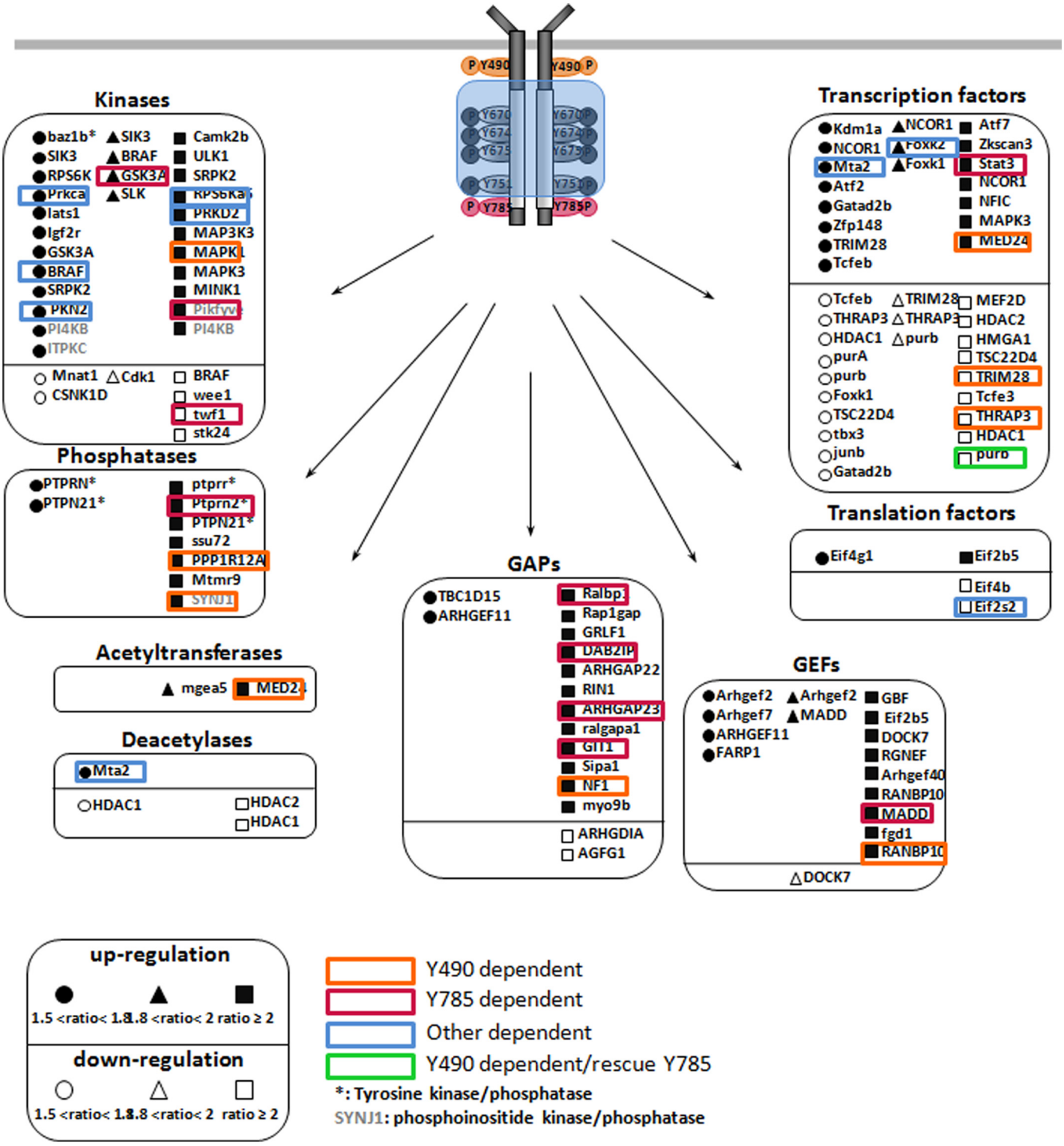

FIGURE 6. **Regulated phosphoproteins grouped by molecular function.** Selected phosphoproteins regulated after 20 min of stimulation by the chimeric receptor PTR were classified according to their molecular function. Each protein was manually assigned using the Gene Ontology terms found in the DAVID National Institutes of Health database (21). The *closed* and *open symbols* indicate up-regulated and down-regulated phosphorylations, respectively. The changes are classified according to their magnitude as follows: more than 2-fold (*square*), between 1.8- and 2-fold (*triangle*), and between 1.5- and 1.8-fold (*circle*). The *colored frames* indicate the sensitivity of these phosphorylations to the different tyrosines of TrkA: Tyr-490 (*orange frame*), Tyr-785 (*red frame*), other (*blue frame*), or affected by Tyr-490 and rescued by Tyr-785 (*green frame*). *No frame* surrounding a protein either means that the phosphoprotein could not be identified in all four conditions or that its behavior to the mutations was not above the significance thresholds selected for grouping. *GAPs,* GTPase-activating proteins; *GEFs,* guanine nucleotide exchange factors.

four conditions or that its behavior to the mutations was not above the significance thresholds selected for the grouping. Those displaying sensitivity to the mutation of Y490F and the same sensitivity to the double mutant are surrounded by an *orange frame* in Fig. 6. For example, in the kinase MAPK1, the phosphatases PPP1R12A and SYNJ1, the guanine nucleotide

exchange factors/GTPase-activating proteins NF1 and RANBP10, and the transcription factors MED24, TRIM28, and THRAP3 show a lack of phosphorylation after mutation of Tyr-490. Interestingly, GSK3β (Ser-20) seems to be more affected by Tyr-785 than Tyr-490 that is responsible for the phosphorylation on Ser-21 as shown on the Western blot. Several kinases (RPS6K, PRKD2, Prkca, and B-Raf) and transcription/translation factors (Mta2, Foxk2, and Eif2s2) were found to be still regulated in receptors containing mutations of both Tyr-490 and Tyr-785.

Gene Ontology analysis of the Tyr-490-dependent group showed strong representation of nuclear proteins involved in several complexes related to the regulation of transcription and mRNA processing. For example, phosphorylations on CHD3 (chromodomain helicase DNA-binding protein) and on TRIM28 (transcription intermediary factor 1-β) that are part of, or interact with, the histone acetylase containing complex NuRD, suggest a role for Tyr-490 in the repression of transcription by altering the modifications of histones (23). Another protein, MED24 (mediator of RNA polymerase II transcription subunit 24), is part of the mediator complex that plays an important role in transcription by controlling the recruitment of polymerase II to genes and by regulating its activity during transcription initiation and elongation (24, 25). This protein is also an acetyltransferase and its phosphorylation, regulated by Tyr-490, could contribute to the regulation of transcription through remodeling histone modifications along with CHD3 and TRIM28 cited above. The complex SNARP (SNIP1/SkIP-associated RNA processing) contains THRAP3 (thyroid hormone receptor-associated protein 3) and BCLAF (Bcl-2-associated transcription factor 1), whose phosphorylations were both found to be regulated by Tyr-490 and has been reported as targeting cyclin D1 RNA processing (26). These two proteins are also coactivators of a nuclear receptor and its transcriptional activity (THRAP3 (27)) and a repressor of transcriptional activity (BCLAF (28)). DDX54 (ATP-dependent RNA helicase DDX54) has been shown to repress the transcriptional activity of nuclear receptors, notably estrogen receptors (29). MYBBP1A also regulates transcription via interaction with DNA binding factors (30). Finally, several phosphorylations regulated by Tyr-490 could play an important role in RNA processing as modulated sites were found on srrm2, sf3b1, sf4, POM121, Cpsf7, and Ppp1r12a, members of the nuclear pore/spliceosome complexes whose signaling pathways are activated by TrkA (15).

The group of phosphorylations dependent on Tyr-785 contains a larger proportion of proteins involved in cell cycle/mitosis and morphogenesis/cytoskeleton compared with the other groups. Tyr-785 is known to recruit PLCγ, which cleaves phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate, leading to PKC activation and Ca^{2+} release in the cell. The PKC family contains several isoforms as follows: the classic PKCs (α, β, and γ) that are activated by calcium/DAG; the nonconventional PKCs (δ, ϵ, η, and θ) that are activated by DAG but insensitive to calcium; and atypical PKCs (ζ and λ) that are insensitive to both calcium and DAG. NGF has been described as regulating the activity of all classes (31–34). The phosphorylation of the transcription factor STAT3 was found to be regulated by Tyr-785, which could be the result of the stimulation of PKCγ, among other possibilities (35). This phosphorylation on Ser-727 has been described as inhibiting the DNA binding activity of STAT3, as contributing to mitotic arrest, and may play a role in regulating the onset and progression of M-phase during the cell cycle (36). Other phosphoproteins involved in cell cycle and mitosis seem also to be regulated by Tyr-785. PP6R3 is a regulatory subunit of the protein phosphatase PP6 complex and plays an important role in mitosis by dephosphorylating Aurora Kinase A (AURKA), an essential mitotic kinase. It has been shown that the loss of function of PP6, by depleting the catalytic or regulatory subunit, interferes with spindle formation and chromosome alignment through increased AURKA activity (37). The protein Cdc26, whose dephosphorylation is regulated by Tyr-785, is part of the anaphase-promoting complex. This complex is responsible for the ubiquitination and targeting for proteasomal degradation of numerous regulatory proteins, including AURKA, to control key events in mitosis. Anaphase-promoting complex activity seems to be regulated by phosphorylation, as this complex shows hyper-phosphorylation during mitosis (38), and phosphatase treatment results in loss of ubiquitination activity. Thus, the dephosphorylation observed after stimulation of PTR could play an important role in the arrest of the cell cycle necessary to start differentiation. MADD (MAPK-activating death domain protein) exhibited a 3-fold increase in phosphorylation after stimulation of PTR on a site that was dependent on Tyr-785 for modification. Phosphorylation of this protein has been shown to abolish spontaneous apoptosis in cancer cells and to be sufficient and necessary for cancer cell survival (39). Another protein Zwint, part of the MIS12 complex, is required for kinetochore formation and spindle checkpoint activity. NIPBL (Nipped-B-Like) helps the cohesin complex to control sister chromatid cohesion during S-phase to obtain appropriate segregation of chromosome to daughter cells (40). NUCKS (nuclear ubiquitous casein and cyclin-dependent kinases substrate) is a protein containing a lot of modifications that change between cell cycle phases (41). For example, the two phosphorylations on Ser-19 and Thr-34 of the human protein have been shown to be 8-fold higher during G_2/M-phase compared with asynchronous cells. In our study, an increase in phosphorylation on Ser-19 of 4-fold was observed, but Thr-34 does not exist in the rat protein. Finally, proteins regulated by Tyr-785 are involved in morphogenesis and cytoskeleton. For example, Ralbp1 has been reported as promoting neurite branching (42).

Potentially the most interesting group is that containing phosphoproteins regulated independently of Tyr-490 and Tyr-785. Even though these two sites are required for many of the functions of the receptor TrkA, several studies have suggested other possible regions could lead to the recruitment of signaling molecules. For example, it has been shown that the adaptor proteins Grb2 (43), SH2B, and rAPS (44) can bind the phosphotyrosines present in the activation loop (Tyr-670, Tyr-674, and Tyr-675) of the catalytic domain of the receptor. Tyr-751 is present in a motif Y*XX*M that is considered a canonical sequence to bind the p85 subunit of PI3K. However, it has previously been shown that modifying this site has no effect on neurite formation (14). The guanine nucleotide exchange fac-

tor RasGrf1 has been shown to bind TrkA through its HIKE domain, corresponding to the region ^{507}**HIK**RQDIILKW**E**^{518} of the receptor (45), providing another potential location that could induce downstream phosphorylation independent of Tyr-490 and Tyr-785.

The adaptor Nck1 showed reduced phosphorylation on Ser-85 upon PTR stimulation, and this was not changed in either mutant. A study has reported that overexpression of Nck in PC12 cells caused continued proliferation even in the presence of NGF and inhibited neurite outgrowth (46), providing evidence for its involvement in signaling. This protein, and its homolog Nck2, have been described as interacting with TrkB, another member of the neurotrophin receptor family (47). This association, linked to the activation of the receptor, led to tyrosine phosphorylation of Nck and was abolished by mutating the tyrosines in the activation loop of the kinase domain or mutating the tyrosine equivalent to Tyr-751 in TrkA but not by mutating Tyr-490 and Tyr-785. Nck-1 has been demonstrated to bind eIF2β (eukaryotic initiation factor 2 subunit β) after its translocation to ribosomes upon stimulation with insulin, regulating protein translation (48). The protein eIF2β was dephosphorylated upon PTR stimulation, and this was also unaffected by both mutations. This event could explain the decreased activity of CK2; 50% of the down-regulated phosphopeptides from this group present a CK2-targeted motif of phosphorylation. Indeed, eIF2γ is known to interact with and be phosphorylated by CK2 (49), regulating its activity (50). Two other proteins with down-regulated phosphorylation in a CK2 motif are part of the ribosome as follows: 60 S acidic ribosomal protein 1, which interacts with eIF2β, and Nascent polypeptide-associated complex subunit α. The protein RGD1564319, a homolog of PDCD5 (Programmed cell death protein 5), showed decreased phosphorylation on Ser-120, targeted by CK2 to promote apoptosis (51). This process is consistent with a differentiation and survival signal driven by the TrkA receptor.

The last group (Fig. 4, *green bordered box*) contains proteins whose phosphorylations are regulated by Tyr-490 but seem to be rescued by the mutation on Tyr-785. This group did not show any particular association in terms of biological processes or cellular components, but it did show a relatively significant enrichment of the phosphorylation motif targeted by ERK1/2. This could be due to subtle regulation of ERK1/2 by the modification of several kinases and phosphatases that are not canonically associated with the ERK1/2 pathway, as described previously (52).

Although some biological processes could be associated to a particular tyrosine residue, the phosphorylation motifs could not be assigned to one population, revealing the complexity of the signal regulation and the lack of specificity of the different kinase motifs queried. For example, the described AKT motifs (R*X*R*XX*(pS/pT) and R(R/S/T)*X*(pS/pT)*X*(S/T)) were not specific to the population affected by the mutation Tyr-490, even though the activation of AKT is largely inhibited by the mutation on Tyr-490. The remaining phosphorylations of Ser-473 and Thr-308 visible on a Western blot, albeit largely reduced, were completely inhibited in the double mutant. This suggests that several kinases targeting the same motifs are being activated but are being regulated through different phosphotyrosine-docking sites. In the same way, the phosphorylation motifs assigned to ERK1/2 (P*X*(pS/pT)P and V*X*(pS/pT)P) are also present in the group that showed independence of Tyr-490 and Tyr-785 and are for the most part up-regulated. It has been shown that ERK1/2 are largely stimulated through Tyr-490 and Tyr-785. We have demonstrated that the remaining activity, measured as a double phosphorylation on ERK1 and ERK2, was still visible by Western blotting, despite the two mutations on PTR. This result is consistent with a study that showed that stimulation of the mutant TrkA Y490F/Y785F with NGF induced a 2-fold increase in the phosphorylation of myelin basic protein compared with a 20-fold increase with the wild-type receptor or a 10-fold increase with the mutant Y490F or Tyr-785 (53). Increased phosphorylation on B-Raf, a proto-oncogene serine/threonine-protein kinase, upon receptor stimulation was still observable in the two mutants. This kinase could be responsible for the remaining activation of the MAPK pathway independent of Tyr-490 and Tyr-785 that could lead to the increased phosphorylation of RSK4 (RPS6Ka6). The lack of representation of these motifs in the group dependent on Tyr-490 may also be due to negative feedback regulation through phosphatases and inhibitors of the Ras/MAPK pathway, such as neurofibromin-1, whose phosphorylation seems to be regulated by Tyr-490.

Our previous study (15) characterized phosphorylation changes after 20 min of activation of TrkA. In this follow-up study, the use of two mutant chimeric receptors, PTR Y490F and PTR Y490F/Y785F, allowed the dissection of the dependence of these phosphorylation changes on Tyr-490 and Tyr-785, the principal docking sites of the receptor. While this study focused on phosphorylation, evidence was also unearthed of regulation of other post-translational modifications as follows: phosphorylation of acetylases, deacetylases, and the *O*-GlcNAcase were all altered following stimulation of the TrkA receptor. Large scale proteomic analyses of these other post-translational modifications in the same model (native/mutant receptors PTR) would therefore produce a more complete and accurate picture of TrkA signaling in terms of various pathways affected.

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