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Receptor Tyrosine Kinase Signaling Mechanisms: Devolving TrkA Responses with Phosphoproteomics

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Abstract

Receptor tyrosine kinases (RTKs) function through protein kinase entities located in the intracellular domain of each protomer. Following activation by ligand binding, they selectively form phosphotyrosine residues by autocatalytic modification. Some of these sites are involved in maintaining the active conformation of the kinase, while others become docking sites for various adaptor/effector/scaffold proteins, which, after complexing with the receptor, then initiate further responses through cascades of post-translational modifications and the generation of lipid second messengers. Although there is substantial overlap in the pathways and activities stimulated by this superfamily, the molecular features of the endodomains of the sub-families and the moieties that they interact with to perpetrate their signals are surprisingly distinct, which may play a significant role in the regulation and responses of the individual RTK types. Some use large scaffold proteins as the basis for most, if not all, of their signal-generating interactions, while others have numerous receptor endodomain phosphotyrosine sites that are quite overlapping in specificity. The members of the Trk family of receptors each have several tyrosine residues that are phosphorylated following stimulation, including those in the kinase activation loop, but there are only two established sites (Y490 and Y785 on TrkA) that are known to be directly involved in signal propagation. Taking advantage of this limited repertoire of docking sites, we have applied phosphoproteomic methods to dissect the signaling responses of both the native protein and derivatives that have had these two sites modified. Interestingly, a clear subset that was not dependent on either docking site was identified. A comparison with a similar set of data for EGFR indicates a considerable degree of similarity in the downstream signaling profile between these two RTKs.

Introduction

The phosphorylation of tyrosine is a key protein modification in the propagation of extracellular stimuli to effect intracellular responses. Both the receptor tyrosine kinases (RTKs) and the cytokine receptor family make use of this alteration(Bradshaw and Dennis, 2009). The former utilize kinases that are an inherent structural element of the receptor protomer while the latter bind their tyrosine kinase effectors non-covalently. In either case,

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the binding of a ligand specific for that receptor results in activation and the subsequent formation of phosphotyrosine derivatives on intracellular parts of the receptor and on signaling proteins that become associated with it.

The human RTK superfamily (Blume-Jensen and Hunter, 2001) contains 58 members grouped into 20 sub-families (not all of which may function as ligand binding entities). With the exception of the insulin receptor (IR) (and the other two members of this sub-family: insulin-like growth factor 1- receptor and insulin-related receptor), they are all expressed as single protomers that aggregate to form stable non-covalently associated dimers. The insulin receptor family is also expressed as a single subunit, but it undergoes processing to form two polypeptide chains that are assembled into a heterotetramer stabilized by disulfide bonds.

RTK protomers are organized as membrane-spanning proteins where the N-terminal extracellular domain is generally composed of various structural sub-domains, contains multiple intrachain disulfide bonds and has numerous N-linked glycosylation sites. It provides the binding site for the activating ligand(s) and participates in dimer formation. Other than the use of homologous but quite distinct structural motifs, the 20 subfamilies are unrelated in terms of their ectodomains. The ectodomains are joined to the endodomains by a transmembrane segment (TM) that is embedded in the plasma membrane in the mature receptor. Although significantly discounted in earlier studies, the TM may play an important role in the formation and stabilization of the dimer(Arkin, 2002). The intracellular domains each have a tyrosine kinase (of substantial sequence similarity) flanked by sequence between it and the membrane (the juxtamembrane (JM) domain) and a C-terminal extension (CT) region. The JM and CT regions differ markedly in size and tyrosine content among superfamily members and these differences reflect significant differences in the mechanisms employed to generate and propagate intracellular signals.

RTK Signaling Mechanisms

A comparison of the endodomains of representative members of six of the sub-families of the human RTK superfamily is illustrated in Fig 1. In panel A, these are shown in linear fashion, aligned on the boundaries of the kinase domains, with the full complement of tyrosine residues of each one indicated. Those residues known to be phosphorylated are indicated in red and the aligned activation loop regions are highlighted.

This projection readily demonstrates that while the size of the kinase domain is relatively constant (albeit some members of the superfamily do have kinase inserts, most notably the platelet-derived growth factor receptor (PDGFR)), the size of the two regions that straddle the kinase domain can be significantly different in length, making the overall size of the intracellular domains distinct. For example, TrkA contains only 356 residues while PDGFRβ has 552 amino acids; the others depicted falling in between (Table I).

The residues comprising the JM and CT regions are dramatically different. Both the PDGF and epidermal growth factor (EGF) receptors have large CT domains (144 and 231 residues, respectively) while the TrkA and the discoidin domain receptors (DDR) have only 15 and 8 amino acids, respectively. In contrast, DDR1 (isoform1) has the largest JM region in the RTK superfamily: 171 residues.

The total number of tyrosines found in each endodomain and those that have been shown to be modified are substantially different (Table I) and their distribution (both modified and unmodified) is quite variable (See Fig 1A). For example, the PDGFRβ endodomain contains 27 tyrosines, while the TrkA endodomain has only 11. Quite clearly only a subset of the total tyrosines present in any given receptor are actually converted to their phosphorylated derivatives following stimulation and, of those converted, only a subset actually play an

active role in signaling. These differences are depicted schematically in Fig 1B, where the positions of only the tyrosines known to be involved in downstream signaling are shown. These assignments are based for the most part on direct identification of the germane phosphorylated peptide by proteomic techniques. In some cases they are based on comparisons among similar family members. One modification that is commonly found in the RTK superfamily is the phosphorylation of a pair of tyrosines located closely adjacent to each other in the activation loop (Fig 1A). In many cases a third tyrosine located a few residues upstream is also included in this group. The pair of phosphotyrosines are required for function and mutation of one or both inactivates the kinase domain. Only EGFR of the commonly studied members of the superfamily is an exception to this rule. It has only a single tyrosine in this position and although it is phosphorylated on activation by EGF it is not required for kinase function. The role of the activation loop tyrosines is to stabilize the loop in an open conformation so that both ATP and the substrate peptide can be bound(Hubbard and Till, 2000).

The function of the other essential phosphotyrosines is to provide binding sites for the soluble (or membrane-anchored) proteins that are recruited following receptor activation(Pawson, 2002). These include scaffolding proteins, which provide single or multiple sites for additional docking interactions and individual effectors. In some cases more than one docking protein is known to interact with a site, e.g. Shc and FRS2 with Y490 in TrkA(Kaplan and Miller, 2000); in others the same entity may bind to more than one site on the receptor, e.g. Gab1 to Y1068 and 1086 of the EGFR(Liu and Rohrschneider, 2002). Not surprisingly, given the substantial differences in organization of the RTK endodomains, these interactions are quite different. The insulin and FGF receptors mainly function by binding large scaffolding proteins that are themselves tyrosine phosphorylated and subsequently dock multiple adaptors/effectors to amplify and propagate the signal. In some cells, TrkA can also utilize the FRS scaffold that is utilized by FGFR. Interestingly, this interaction requires that Y490 be modified, while in FGFR, the interaction is constitutive (no phosphorylated tyrosine is required)(Ong *et al.*, 2000). Indeed, FGFR1 has only a single phosphotyrosine docking site, located at the border between the kinase and C-terminal domain; all of the remaining tyrosines (including some that can become phosphorylated) are not required for signal transduction as measured in a PC12 cell assay(Foehr *et al.*, 2001). In contrast, the PDGF and EGF receptors have multiple sites that are modified and utilized. Although some are more significant than others, large deletions or multiple substitutions still leave the receptor able to signal, albeit with less potency. For example, EGFR mutants with the 5 principal tyrosine sites in the CT domain converted to phenylalanine or with the excision of the entire CT domain can still activate the Ras/ERK pathway in PC12 cells(Tyson *et al.*, 2003) or NIH 3T3 cells(Gotoh *et al.*, 1994). This multiplicity makes dissection of the roles of individual sites within these receptors difficult.

Both Trk and DDR1 present a more simplified structure. In TrkA, there are only two tyrosine docking sites that have been defined and mutation of these sites gives measureable changes in various assays. Thus, TrkA is an excellent candidate for further analysis of downstream changes in the PTM profile generated by NGF, its ligand. DDR1 also seems to have a relatively simple signaling structure, but this has not yet been defined as clearly as TrkA (Vogel *et al.*, 2006)**.**

RTKs are known to activate several pathways that are linked to cellular phenotypic responses that have been extensively described(Schlessinger, 2000). Primarily these involve the activation of the ERKs, usually via Ras and a cascade of subsequent kinase activations; phospholipase Cγ, which cleaves phosphoinositides to produce diacylglycerol and inositol trisphosphate; and the Akt-linked pathways, which are initiated by the activation of phosphoinositide-3-kinase (PI-3-K). While the initiating events that immediately follow the

receptor activation extensively (if not exclusively) involve tyrosine phosphorylation, the longer term propagation of the signal generates (and certainly requires) a much greater number of serine/threonine modifications. In most cellular paradigms, by approximately 20 minutes, the majority of the phosphotyrosine modifications have been replaced by a much larger number of serine and threonine phosphorylations, along with the expansion of N^{ϵ}-acetylation, ubiquitination and O-GlcNAc modifications(Choudhary and Mann, 2010). All of these modifications are reversible in nature and specific enzymes to remove these PTMs are well known. However, it has been shown by numerous proteomic experiments that the there is a substantial basal level of all of these modifications, necessitating careful quantitative measurements to detect those PTMs that have significantly changed as a result of receptor stimulation. It is those entities with increased (or decreased) stoichiometries of modification that are eventually instrumental in producing the cellular/phenotypic responses driven in large part by transcriptional modulation. This provides many potential targets for therapeutic/diagnostic applications, some of which have already been exploited.

TrkA stimulated serine/threonine phosphoproteome of PC12 cells

Although the signals generated by NGF stimulation of TrkA involve many of the same pathways that are stimulated by other RTKs, its more simplified endodomain structure with only two defined docking sites makes it an attractive target to devolve how individual signaling pathways are regulated in terms of downstream responses. To accomplish this, chimeric receptors containing the extracellular domain of the PDGF receptor were fused to the transmembrane and intracellular domains of the TrkA receptor and then stably transfected into the NGF-responsive PC12 cell line. This pheochromocytoma cell line responds to stimulation by adopting a sympathetic neuron-like morphology, including neurite extension. Since PC12 cells do not express PDGF, the chimeras can be stimulated with PDGF to the exclusion of endogenous TrkA and P75, the pan neurotrophin receptor, which would confound the results. It also allows the two tyrosines to be mutated to phenylalanine singly or together to assess their relative contributions to the overall activation response.

The changes in the levels of individual phosphorylation sites upon stimulation were ascertained by the stable isotope labeling of amino acids in cell culture (SILAC) technique (Fig 2)(Ong *et al.*, 2002). The stimulated sample yielded 4315 unique phosphopeptides as compared to 3452 in the unstimulated (untransfected) control, at a false discovery rate of ~0.5% for peptides and ~3.5% for proteins. A total of 2035 phosphorylated peptides (806 proteins) were indentified and quantified in both datasets. Using an arbitrary cutoff of 1.8 fold change, 424 phosphopeptides on 259 proteins were found to be up-regulated and 392 sites on 206 proteins were down regulated upon receptor stimulation(Biarc *et al.*, 2012). Among the up-regulated sites were several kinases and phosphatases, while the down-regulated sites were much less represented in these overall classes of proteins (Fig 3). There was a good sampling of signaling effectors, including Erk1/2 and others. An evaluation of kinase motifs showed that there was a down-regulation of the CK2 class of kinases, while Erk phosphorylation was statistically up-regulated. A comparison of the up-regulated motif profile observed to that reported by Olsen et al. (Olsen *et al.*, 2006) for EGFR in human HeLa cells at the same time point showed a considerable amount of similarity, supporting the view that RTK transduction pathways and downstream modifications are likely to be extensively overlapping (Fig 4b). Interestingly, there were several clusters of proteins, known to interact, that were found to have mutually enhanced (or decreased) phosphorylations, suggesting that multiple phosphorylations are often coordinated to control more complex physiological responses.

The serine/threonine phosphoproteomes of TrkA signaling mutants

Utilizing the same approach as described above, two additional stably transfected PC12 lines were analyzed following stimulation for 20 min: PTR Y490F and PTR Y490F, Y785F. The former shows very weak phenotypic responses (neurite outgrowth) upon stimulation, while the latter is essentially inactive by this measure. Utilizing SILAC quantification, each sample (labeled with light isotopes) was mixed with a heavy labeled sample consisting of PC12 cells stably transfected PTR and stimulated with PDGF-BB for 20 min(Biarc *et al.*, 2012). There were 988 phosphopeptides corresponding to 501 proteins that could be identified in all 4 conditions (PTR, PTR Y490F, PTR Y490F, Y785F and control). Among them, 208 (21%) were up-regulated after stimulation of the wild-type receptor compared to the control cells and 172 (17%) were down-regulated (Biarc, J., Chalkley, R.J., Burlingame, A.L. and Bradshaw R. A., manuscript submitted).

The data were analyzed in the same way as the native PTR results(Biarc *et al.*, 2012) and four classes of responses were extracted depending on the behavior of the mutants relative to the stimulated native and the control samples: 'Y490 dependent'(55); 'Y785 dependent' (77); 'Y490 dependent / 785 rescue'(24); and 'other'(41) (where the number in parentheses is the number of phosphopeptides found in each category). All of these phosphorylations were affected by PTR stimulation. The 'Y490 dependent' group represents proteins whose phosphorylation increased/decreased relative to stimulated PTR in both mutants and therefore were dependent on Y490 (common to both) as compared to proteins that only changed in the double mutant ('Y785 dependent'), indicating that they were dependent on the phospholipase Cγ activation associated with Y785.

Of particular interest is the group of phosphoproteins regulated independently of Y490 and Y785 (labeled 'other'). Several studies have suggested other possible regions could lead to the recruitment of signaling molecules. For example, it has been shown that the adapter proteins Grb2 (McDonald *et al.*, 2000), SH2B and rAPS (Qian *et al.*, 1998) can bind the three phosphotyrosines present in the activation loop (see Fig 1A) of the catalytic domain of the receptor. Residue Y751 has also attracted considerable attention. It is present in a motif YXXM that is considered a canonical sequence to bind the p85 subunit of PI3-K and evidence has been presented, using inhibition of binding as measured by immunoprecipitation by a phosphopeptide bearing this sequence(Obermeier *et al.*, 1993), for such an association. In addition, elimination of this site abolishes the Src-mediated phosphorylation of the heat- and capsaicin gated ion channel TRPV1 via the activation of PI3-K(Zhang *et al.*, 2005). The activation of PI3-K by TrkA has been demonstrated independently (Raffioni and Bradshaw, 1992). However, it has previously been shown that modifying this site has no effect on neurite formation(Obermeier *et al.*, 1993) and direct evidence that this site is phosphorylated in the activated receptor is lacking. The guanine nucleotide exchange factor RasGrf1 has been shown to bind TrkA through its HIKE domain, corresponding to the region 507-518 of the receptor(Robinson *et al.*, 2005), and there remains the possibility that other non-phosphotyrosine binding sites may exist, such as seen in the association of FRS2 to FGFR1 (Ong *et al.*, 2000).

Figure 5 summarizes the 'dependent' changes in phosphorylation relative to the general cellular/phenotypic responses deduced by these analyses. These classifications are certainly not exclusive, but they do provide broad indications of the pathways induced by the two identified sites as well as some insight into what other sites might control. Thus, the PLCγ site (Y785) is involved in cell/cycle events whereas Y490 is manifested in transcriptional events and in this paradigm, is essential for promoting neurite proliferation. The Y490 dependent/785 rescue group is characterized by ERK1/2 activation which is consistent with the fact that the Y785 site can give a weak response in PC12 cells in the absence of the

potent Y490 site(Obermeier *et al.*, 1993). Finally, the 'other' category, which may involve several sites, is apparently important in regulating CK2. Importantly, the phosphorylations observed in this grouping do not suggest a significant involvement of PI3-K. It may be that this is not a major activity of TrkA in PC12 cells, unlike in nocioceptive neurons or other NGF target tissues.

Summary

RTKs are found ubiquitously in nature and are involved in a broad range of physiological processes. Although there is a significant amount of overlap in the major pathways that most stimulate, they display significant differences in their organization and how signals are propagated following activation. Using the unbiased technologies of mass spectrometry-based proteomics, it has been possible to dissect the signaling output of stably transfected chimeric TrkA receptors that have been mutated in key docking sites. These studies have expanded the signaling profile of this RTK and provide new insight into the heretofore unknown involvement of a new site(s) for signaling in this receptor. However, it needs to be stressed that the profile observed is only a sampling of the total downstream phosphorylations induced and deeper analyses may yet reveal even more novel aspects to TrkA signaling. It is also important to remember that there are many other PTMs that are changing at the same time and their interaction with the phosphorylations will be important to document and correlate before a complete picture of TrkA signaling is achieved.

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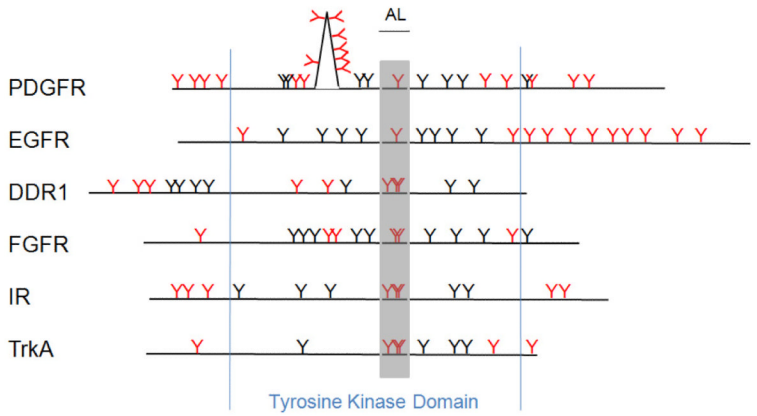

Figure 1.

Comparison of six different RTK endodomains, illustrating the diversity in organization and molecular signaling mechanisms. (A) Linear plot showing the location of the tyrosine residues; those that have been shown to be phosphorylated (according to UniprotKB annotation) are highlighted in red. The activation loop region is shaded and annotated by AL. (B) Schematic representation of these six RTKs that emphasizes the differences in the juxtamembrane, kinase and C-terminal extension domains and highlights the major signaling and docking sites.

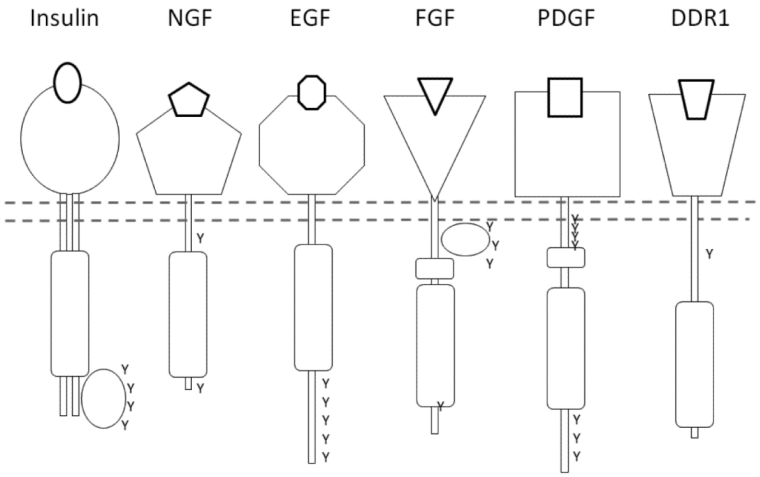

Figure 2.

Experimental strategy for quantifying phosphopeptides. PC12 cells, with or without stably transfected PTR or mutant versions of PTR, were cultivated in an isotopically unlabelled medium containing 200 mg/ml of unlabelled proline and either stimulated or not stimulated with human PDGF-BB (50 ug/mL) for 20 minutes. As a standard for the relative quantification in all comparisons, PC12-PTR were cultivated in medium containing heavy lysine and arginine and unlabelled proline and stimulated with PDGF-BB (50 ug/mL) for 20 minutes was used. Three mg of each sample was mixed with 3 mg of the common heavy sample and were reduced, alkylated and digested with trypsin. The phosphopeptides were enriched on a TiO2 column. The phosphopeptides and the non-phosphopeptides (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. Adapted from (Biarc *et al.*, 2012)with permission.

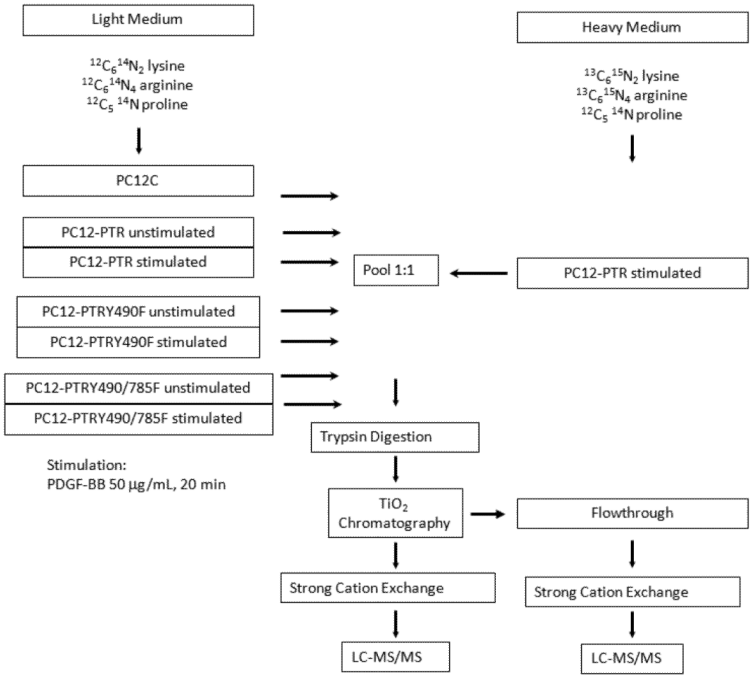

Figure 3.

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 2 fold (square), between 1.8 and 2 fold (triangle), and between 1.5 and 1.8 fold (circle). Lipid kinases are indicated in gray. Copied from (Biarc *et al.*, 2012) with permission.

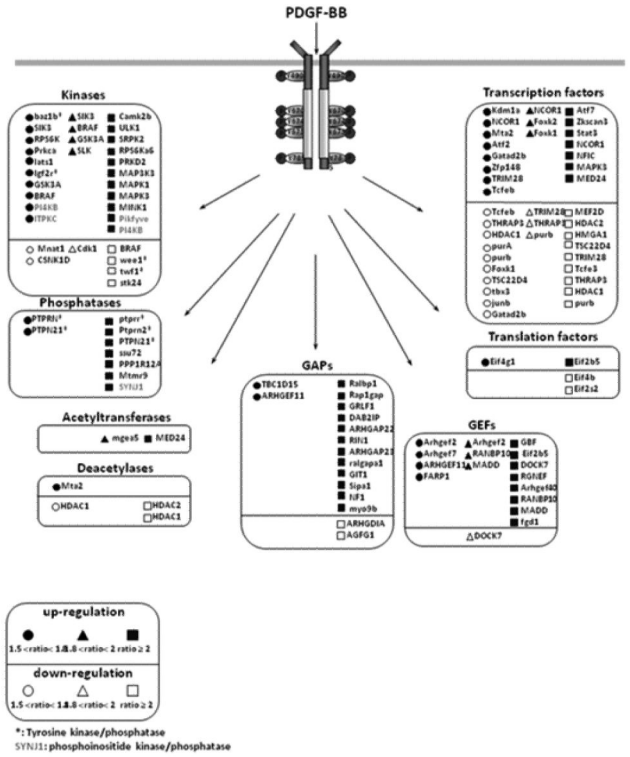

Figure 4.

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 (frequency of observation of a motif compared to how often it would be expected at random) in each population. The upper panel represents the enrichment factor (see text for definition) of each motif in the up (dark grey bars) and down (light grey bars) regulated phosphopeptides upon stimulation of PTR. The lower panel shows the same analysis on up-regulated phosphopeptides upon stimulation of PTR (dark grey bars) and EGFR in Hela cells [9](white bars). Copied from (Biarc *et al.*, 2012)with permission.

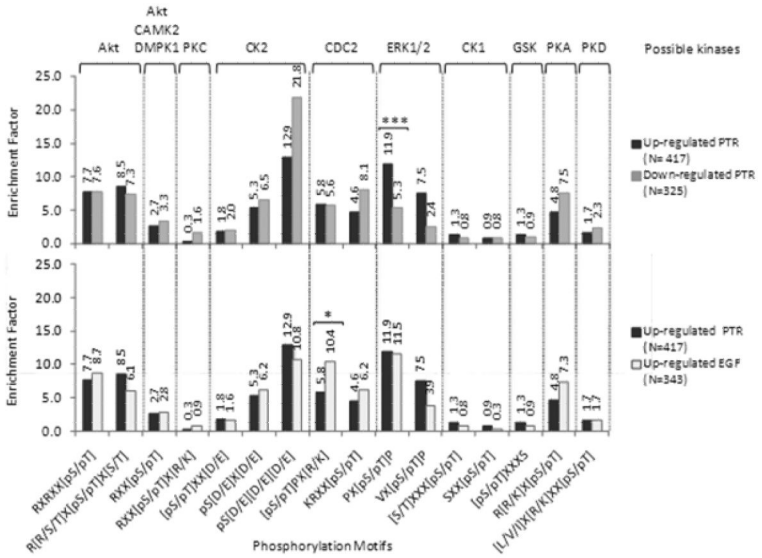

Figure 5.

Summary of activities regulated through different phosphotyrosine docking sites (or combinations) in the TrkA receptor.

Table I

Comparison of cytoplasmic domains of RTKs.

RTK	Length of Cytoplasmic Domain (total AA)	Length of Kinase Domain	#Y	#phosphoY
PDGFRb	552	362	27	19
EGFR	542	268	20	12
DDR1	475	295	15	8
FGFR1	425	290	15	6
IR	403	276	13	8
TrkA	356	271	11	6