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Regulation and targets of receptor tyrosine kinases

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Abstract

Ligand-mediated activation of receptor tyrosine kinases (RTKs) results in autophosphorylation of both the receptor catalytic domain and noncatalytic regions of the cytoplasmic domain. Catalytic domain phosphorylation leads to activation and potentiation of receptor kinase activity. Noncatalytic domain phosphorylation creates docking sites for downstream cytoplasmic targets, which bind to specific receptor phosphotyrosine residues. Downstream signaling pathways are constructed in a modular fashion. In addition to SH2 and PTB (phosphotyrosine binding) domains, downstream signal proteins also contain domains that recognize other protein and phospholipid motifs. The arrangement and re-arrangement of various combinations of modular domains in different signaling proteins (combinatorial use) has allowed for the creation of complex signaling networks and pathways. In addition to performing catalytic functions, signaling proteins serve as scaffolds for the assembly of multiprotein signaling complexes, as adaptors, as transcription factors and as signal pathway regulators. Recent results show that the juxtamembrane region of Eph receptors is important in receptor autoregulation. Mutations in the juxtamembrane region of several RTKs have been shown to play a role in oncogenesis. It is likely that dysregulation of other modular components of signaling pathways also plays a role in oncogenic transformation.

Keywords: Phosphotyrosine; SH2 domains; Protein interactions

1. Introduction

The receptor tyrosine kinases (RTKs) are membrane-spanning cell surface proteins that play critical roles in the transduction of extracellular signals to the cytoplasm. The approximately 60 RTKs identified to date can be divided into 20 subfamilies defined by a prototypical receptor and/or ligand [1,2]. As shown in Fig. 1, these include the platelet-derived growth factor receptor (PDGFR)-KIT and ephrin receptor (EphR) subfamilies. The Eph receptors make up the largest family of mammalian RTKs, probably because they control a variety of complex cell interactions [3,4]. RTKs contain an extracellular ligand-binding domain, usually glycosylated, connected to the cytoplasmic domain by a transmembrane helix. The cytoplasmic domain consists of a juxtamembrane region, a conserved protein tyrosine kinase (PTK) core and additional regulatory sequences also subject to phosphorylation [2]. Recent research has provided increasing insight into the regulation of RTK activity and the nature of RTK interactions with their downstream cytoplasmic targets in both normal and transformed cells.

1.1. Activation of RTKs leads to interaction with modular downstream targets

Fig. 2 illustrates the basic scheme of RTK activation and function. A growth factor binds to the RTK and induces receptor clustering (or, if the receptor is preclustered, reorientation of the receptor in the membrane). Clustering leads to autophosphorylation within (1) the catalytic domain, leading to activation and potentiation of kinase activity and (2) noncatalytic regions of the cytoplasmic domain, creat-

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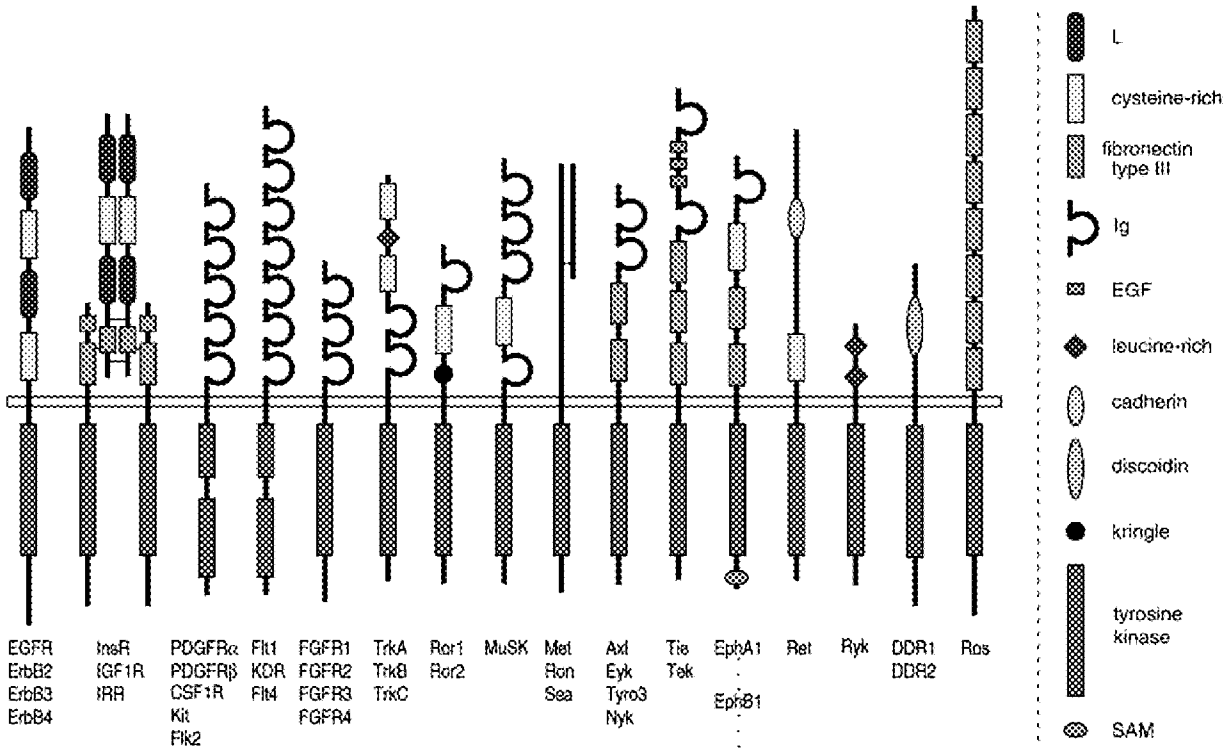

Fig. 1. Schematic representation of structural components of receptor tyrosine kinase (RTK) subfamilies.

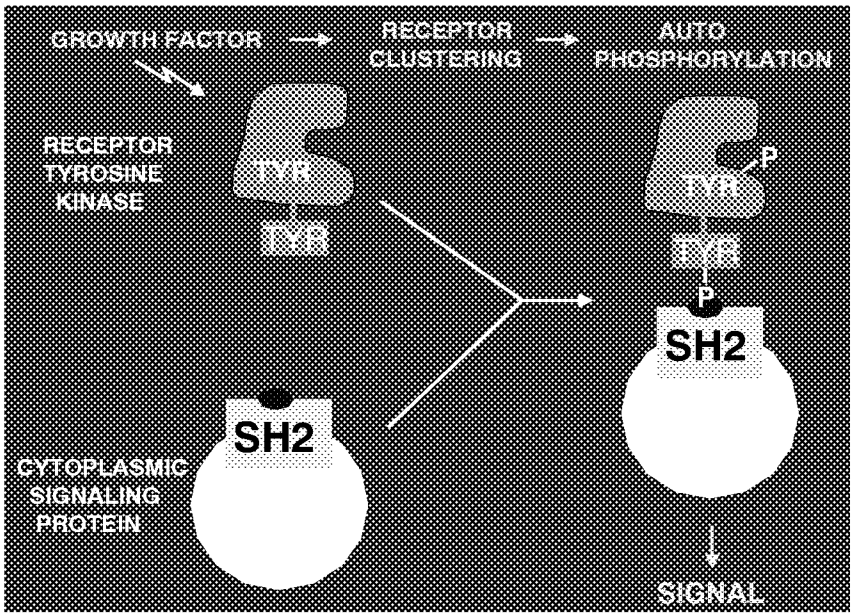

Fig. 2. Sequence of events following RTK activation.

ing docking sites for downstream cytoplasmic targets that are diverse biologically and biochemically but which share the presence of an SH2 and/or phosphotyrosine binding (PTB) domain [5]. Autophosphorylation explains the initially somewhat surprising finding that following stimulation of a cell with growth factor, the growth factor RTK itself is often the most highly tyrosine-phosphorylated cellular protein.

In general, and particularly in tyrosine kinase signaling pathways, downstream signal transduction pathways are constructed in a modular fashion [6]. As shown in Fig. 3, in addition to SH2 and PTB binding domains that recognize phosphorylated tyrosine residues, there are domains such as SH3 that recognize protein-rich motifs [5]. Domains that recognize phosphoserine and phosphothreonine sequences have also been identified, indicating that phosphorylation of

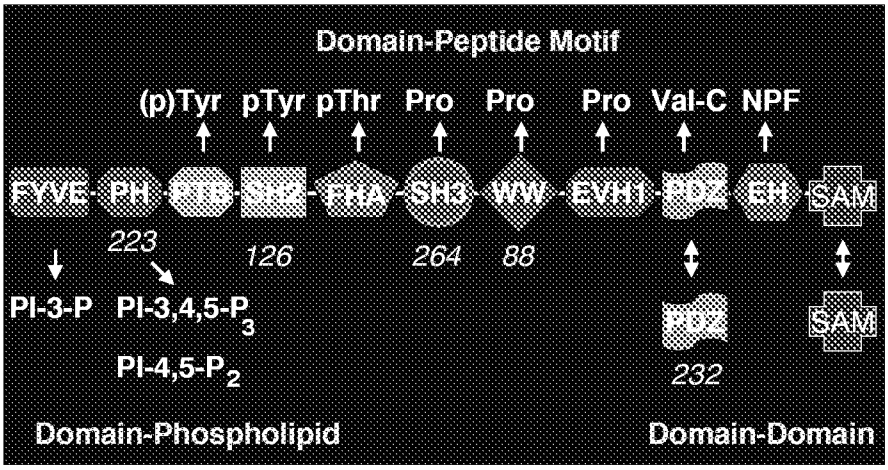

Fig. 3. Modular domains of signaling proteins: interactive functions and numbers encoded in the human genome (PH, SH2, SH3, WW, PDZ).

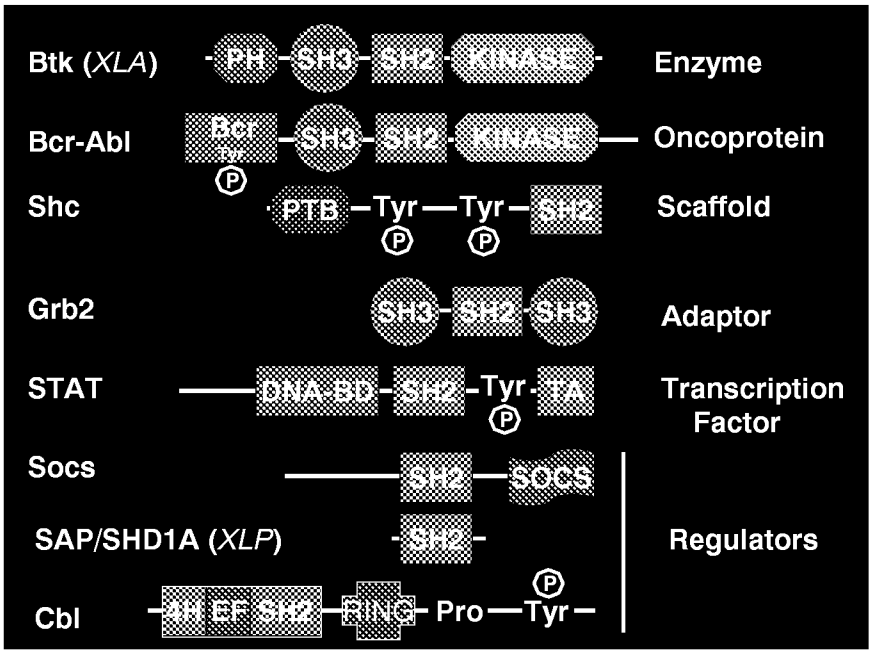

Fig. 4. Modular structures and functions of SH2 domain-containing signaling proteins.

serine and threonine also plays a role in mediating protein–protein interactions [5,7,8]. Other domains show specificity for non-protein molecules. PH recognizes specific membrane-associated phosphoinositides, allowing juxtaposition of a signaling protein alongside both the receptor and additional downstream intracellular targets. Multiple diverse domains are often linked together in a single signaling protein, allowing for initiation of multiple protein–protein and protein–phospholipid interactions that target specific receptor signals to specific intracellular signaling pathways [5,6]. Modular domains occur in the genome in large numbers (Fig. 3). There are over 200 PH domains, over 110 SH2 domains, and nearly 300 SH3 domains. The repeated use of the domains in modular cassette-like fashion in multiple proteins provides a means to build complex signaling networks out of fairly simple binary interactions [9].

Fig. 4 shows the activities associated with a variety of SH2 domain signaling proteins according to their differing patterns of modular construction. The cytoplasmic tyrosine kinase Btk has a kinase domain linked to an SH2 domain, an SH3 domain and a PH domain. The catalytic activity of Btk depends on recruitment to the membrane through the PH domain, followed by interaction of the SH2 and SH3 domains with downstream targets. Btk is expressed in B-cells and plays a role in B-cell antigen receptor signaling; mutations in Btk, not only in the kinase domain but also in the SH2 and PH domains, are associated with an X-linked lymphoproliferative disorder characterized by agammaglobulinemia [10]. Other SH2 proteins, such as Shc, lack a catalytic domain and serve to nucleate the formation of multiprotein complexes. Shc interacts with activated receptors through its PTB or SH2 domain, can itself be-

come tyrosine-phosphorylated and, once phosphorylated, combines with other SH2-containing proteins, such as the adaptor Grb2. Grb2, through its SH3 domain, then links to further downstream targets. The STAT proteins are latent transcription factors that interact through their SH2 domain with a cytokine receptor at the membrane and then become phosphorylated, leading to dimerization through a mutual SH2–phosphotyrosine interaction. Dimerization is followed by translocation to the nucleus and activation of gene expression. Finally, a growing number of SH2-containing regulatory proteins have been identified. For example, Cbl is an E3 protein ubiquitin ligase that binds to an activated receptor through its SH2 domain and to an E2 ubiquitin ligase through its ring domain. Binding to ubiquitin ligase induces ubiquitination, leading to proteosomal degradation and downregulation of the activated receptor [11]. By contrast, the SHZDIA/SAP/DSHP protein is composed almost entirely of a single SH2 domain and appears to regulate the kinetics of signaling in activated T cells [12]. Mutations in human SH2DIA cause X-linked lymphoproliferative.

Disturbances in the activation and regulation of such carefully orchestrated signaling networks can lead to the transformation of normal proteins into oncoproteins. Aberrations in RTK-associated protein–protein interaction and catalytic domain activation have been identified in many cancers [1,7,9].

1.2. The PDGF β receptor: control of activation and signaling specificity

Fig. 5 shows the array of SH2-containing proteins that bind to the multiple tyrosine-phosphorylated sites expressed on the activated PDGF β receptor. Each phosphorylated tyrosine site is associated with a distinct sequence motif that drives binding to a specific SH2-containing protein. The Grb2 SH2 domain binds to a phosphotyrosine followed two amino acids later by an asparagine. PI-3 kinase binds to a phosphotyrosine with a methionine at the plus-3 position. PLC-gamma binds to a phosphotyrosine followed by a run of hydrophobic amino acids. The sequence context of a receptor's autophosphorylation sites can thus determine to a significant degree the receptor's signaling specificity.

The phosphotyrosine motif in the juxtamembrane region of the PDGF β receptor may play a dual role. Once phosphorylated, it mediates interaction with the SH2-containing Src protein, but in its unphosphorylated state, it may also play an inhibitory role by repressing receptor kinase activity [13]. This dual consequence of juxtamembrane tyrosine phosphorylation raises the question of whether there is coordination between receptor kinase activation on the one hand and exposure of phosphorylated docking sites for downstream receptor targets on the other. As a corollary, it raises the question of whether this is a more general role for the juxtamembrane region, since mutations of the juxtamembrane domain of the KIT protein have been shown to play a transforming role in several tumor types, including gastrointestinal stromal tumors (GISTs) [14], acute myelogenous leukemia (AML) [15], systemic mastocytosis [16], and small cell lung cancer [17].

1.3. The unphosphorylated EphB2 juxtamembrane region autoinhibits the EphB2 receptor tyrosine kinase

Following ligand binding, the activity of many RTKs is stimulated by autophosphorylation within a region of

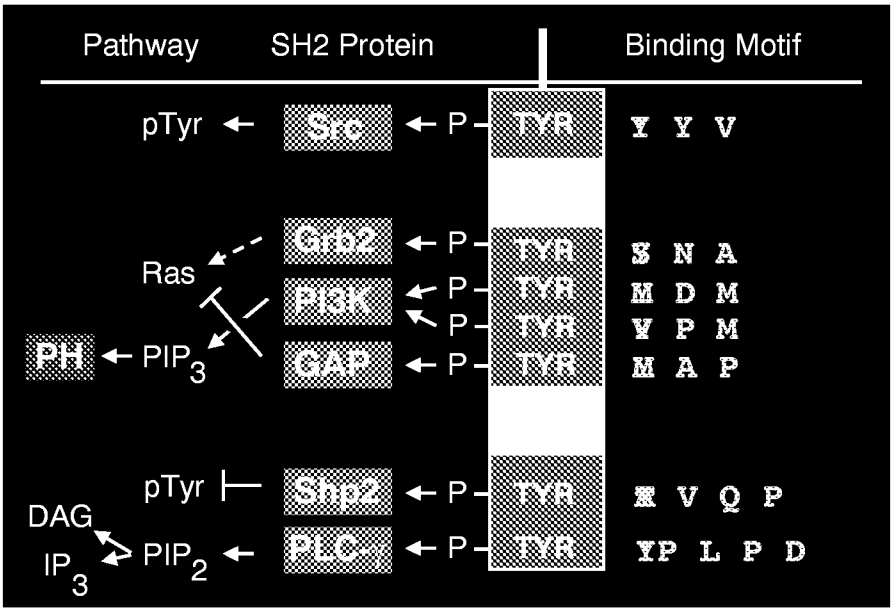

Fig. 5. Sequence contexts of β-PDGFR phosphotyrosine SH2-binding sites.

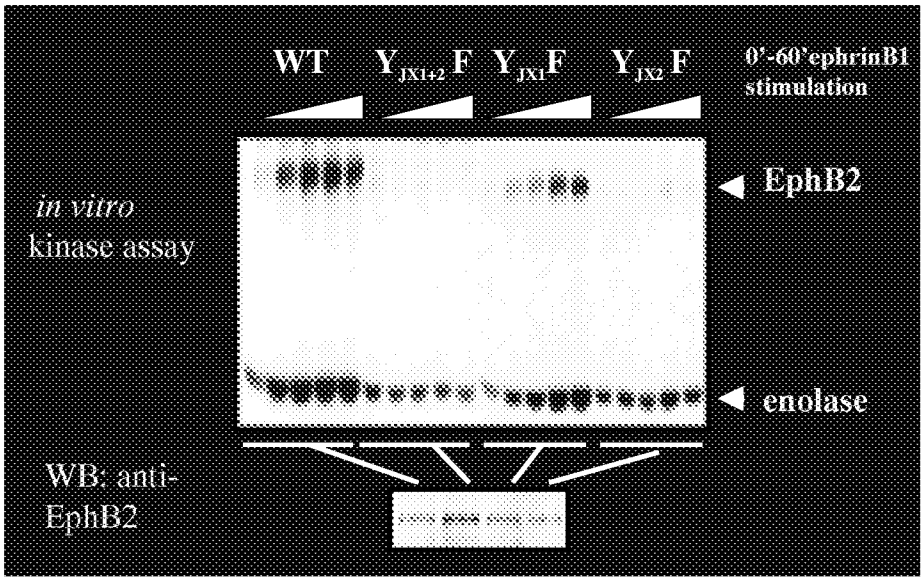

Fig. 6. Juxtamembrane mutations reduce tyrosine phosphoryalation of EphB2 and enolase in response to ephrin-B1 stimulation. Wild-type (WT) or mutant receptors (as indicated) were stimulated with 2 μg of soluble clustered Fc-ephrin-B1 per ml.

the kinase domain termed the activation segment [18]. Structural analysis has shown how the activation segment controls tyrosine kinase activity [19]. Additional observations suggest that polypeptide regions outside the catalytic domain may also play a regulatory role. In addition to findings that several cancers are linked to mutations in the juxtamembrane domain of c-KIT (see above), recent biochemical and mutational analysis has suggested that Eph receptors, the largest class of vertebrate RTKs, may be regulated by their juxtamembrane region [20].

Eph receptors have a typical RTK structure, including a fairly long juxtamembrane region, and a C-terminal sterile alpha-motif (SAM) domain that may assist in oligomerization [21]. They have multiple functions in embryonic development, including formation of the nervous system and cell movement and compartmentalization during angiogenesis [3,4]. Activation of receptors such as EphB2 or EphA4 leads to tyrosine autophosphorylation on multiple residues, including two tyrosines within a highly conserved juxtamembrane motif and a tyrosine in the activation segment [4]. As shown in Fig. 6, stimulation of wild type EphB2 receptor with ligand results both in autophosphorylation and phosphorylation of exogenous substrate. Substitution of the two juxtamembrane tyrosines with phenylalanine abolishes autophosphorylation of these sites in vitro (predictably) and also abrogates EphB2-mediated morphological remodeling in neuronal NG108-15 cells, consistent with a failure to engage SH2-containing cytoplasmic targets. However, the phenylalanine substitution also abolishes the ability to induce phosphorylation of exogenous substrate in response to ligand stimulation. This suggests that in addition to binding SH2 domain signaling proteins, the EphB2 juxtamembrane region acts as a regulator of receptor tyrosine kinase activity [20].

Analysis of the kinase activity of the highly related EphA4 receptor in vitro has suggested that Eph receptor activation requires a two-component mechanism involving autophosphorylation of both the kinase domain activation segment and the juxtamembrane tyrosines [20]. Although substitution of the juxtamembrane tyrosines with phenylalanines does not influence ATP binding, the mutant proteins show a 10-fold decrease in the ability to phosphorylate substrate compared with wild-type [20]. Recently, we have solved the X-ray crystal structure of the EphB2 kinase domain and juxtamembrane region in the autoinhibited state. Fig. 7 shows a schematic model for Eph RTK activation based on the crystallographic structural data. In its unphosphorylated state, the juxtamembrane region adopts a helical structure that impinges on the ordering of the activation segment and distorts the conformation of the small lobe, thereby disrupting the active site. The nucleotide binding site is influenced in such a way that ATP is not productively positioned for phosphate transfer. Phosphorylation of juxtamembrane tyrosines 604 and 610, resulting in charge repulsion and steric clashes with the pockets accommodating the tyrosines, is proposed to move the juxtamembrane region away from the kinase domain and relieve the autoinhibition [22].

Why might EphB2 employ this complex mechanism of autoregulation? One reason could be to block juxtamembrane region signaling activity until the receptor is activated, since Tyr604/610 phosphorylation in fact creates docking sites for SH2 domain proteins. Another could be to establish a threshold of kinase activity required for receptor activation: the use of at least two distinct phosphoregulatory steps (the activation segment and the juxtamembrane region) may preclude adventitious Eph receptor activation resulting from basal levels of kinase ac-

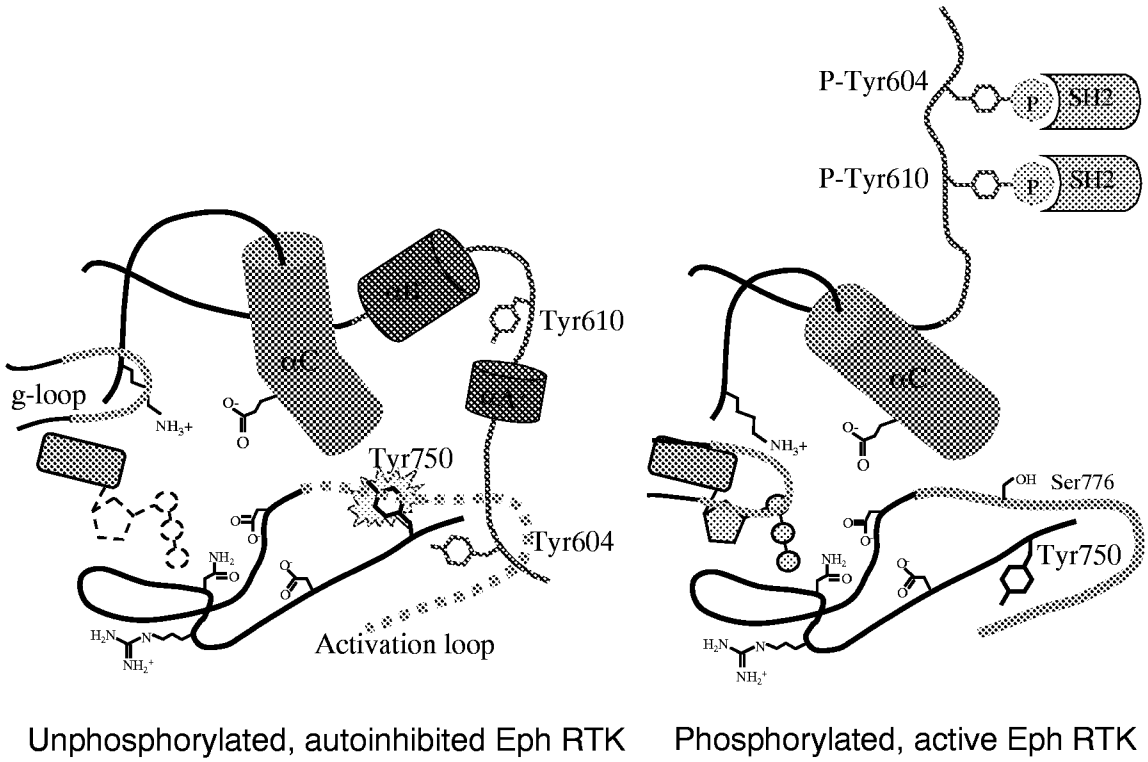

Fig. 7. Schematic diagram of the difference between the autoinhibited and active states of the Eph receptor tyrosine kinase.

tivity. Given the importance of the juxtamembrane region in PDGFR activity[13] and c-KIT-mediated oncogenesis, the findings for the Eph receptor may represent a more general phenomenon in receptor tyrosine kinases.

1.4. Shc modulates the mitogen-activated protein kinase core signaling pathway

The Grb2 adaptor protein has a central SH2 domain and two flanking SH3 domains. As shown in Fig. 8, the SH2 domain can bind to pTyr-X-Asn motifs on autophosphorylated RTKs at the same time as the SH3 domains engage proline-rich motifs of Sos, which activates the Ras GTPase and thus the mitogen-activated protein (MAP) kinase pathway. The Grb2 SH3 domain can also interact with Gab1, allowing recruitment of PI-3 kinase, which then interacts with PH domain-containing downstream targets, such as Akt, that control growth, differentiation and survival [23].

This highly conserved, multifunctional core signaling pathway can be modulated through interaction with the docking protein ShcA. ShcA proteins associate physically with a variety of activated RTKs and become phosphory-

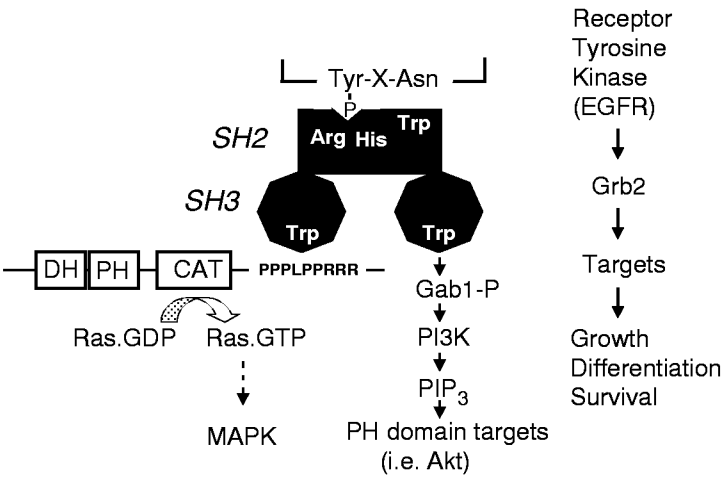

Fig. 8. Grb2 binding to activated EGFR and Grb2-mediated downstream activation sequences.

lated at sites that bind to the SH2 domain of Grb2 [24]. Although knockout of the ShcA gene induces a cardiovascular defect in mice, mutant cells are still able to activate MAP kinase in response to high concentrations of growth factor but are much less responsive to low levels of growth factor [25]. This suggests that one function of ShcA is to render cells more sensitive to low concentrations of growth factor, to which they would otherwise be relatively nonpermissive. The ability of ShcA to amplify signaling through the Grb2 pathway may be related to the presence on ShcA of multiple Grb2-binding sites. ShcA may also mediate access of the Grb2 pathway to novel phosphotyrosine-containing sites, such as Asn-Pro-X-pTyr sites recognized by the ShcA PTB domain.

1.5. ShcA-mediated signal modulation promotes oncogenesis in polyomavirus middle T antigen-induced hemangiomas

The MAP kinase pathway is highly conserved in evolution. Its numerous biological functions are modulated and regulated by many factors, including the ShcA protein. It seems possible that oncoproteins may also engage this core modular interaction-based pathway.

The polyomavirus middle T antigen (PymT) is a potent oncogene that transforms endothelial cells and causes an endothelial cell malignancy (lethal hemangioma) when introduced into a retrovirus and injected into newborn mice [26]. PymT binds to the Src family of tyrosine kinases, leading to its phosphorylation on multiple sites. Phosphorylation of an NPXY motif at Tyr 250 leads PymT to bind the PTB domain of ShcA [27]. ShcA is then phosphorylated by the Src tyrosine kinase and recruits Grb2, which as described above in turn activates not only the MAP kinase pathway, but PI-3 kinase as well.

We investigated whether endothelial cell transformation and the development of lethal hemangiomas is dependent on the interaction of PymT with the PTB domain of ShcA, and downstream signaling through Grb2 [27]. If the PTB-binding phosphotyrosine sites of PymT are deleted or mutated to phenylalanine, the development of lethal hemangiomas is strikingly attenuated. To test whether Shc is signaling through Grb2, we inserted the two Grb2-associated tyrosine-based motifs of the Shc protein directly into the PymT deletion mutant, allowing the sites to be tyrosine-phosphorylated on PymT directly, leading to recruitment of Grb2. This restored the ability of PymT to induce disease. Fig. 9 shows the poor survival of animals injected with a retroviral vector encoding wild-type PymT. When all PymT tyrosine phosphorylation sites are substituted with phenylalanine, survival is no longer compromised. Mutation of only the ShcA-binding site also significantly attenuates the disease, but replacement of the ShcA PTB-binding site with Grb2-binding sites restores the lethal effect, with survival essentially indistinguishable from that of animals implanted with wild-type PymT. ShcA modulation of the MAP kinase pathway thus appears to be necessary for the expression of the malignant phenotype [27].

Extensive work showing that the Bcr-Abl oncoprotein affects multiple signaling pathways indicates that the findings in the PymT-lethal hemangioma model are relevant to the pathogenesis and understanding of human cancer as well [28]. For example, phosphorylation of the Bcr region of Bcr-Abl creates a binding site for Grb2, thereby potentiating Bcr-Abl-transforming activity [29].

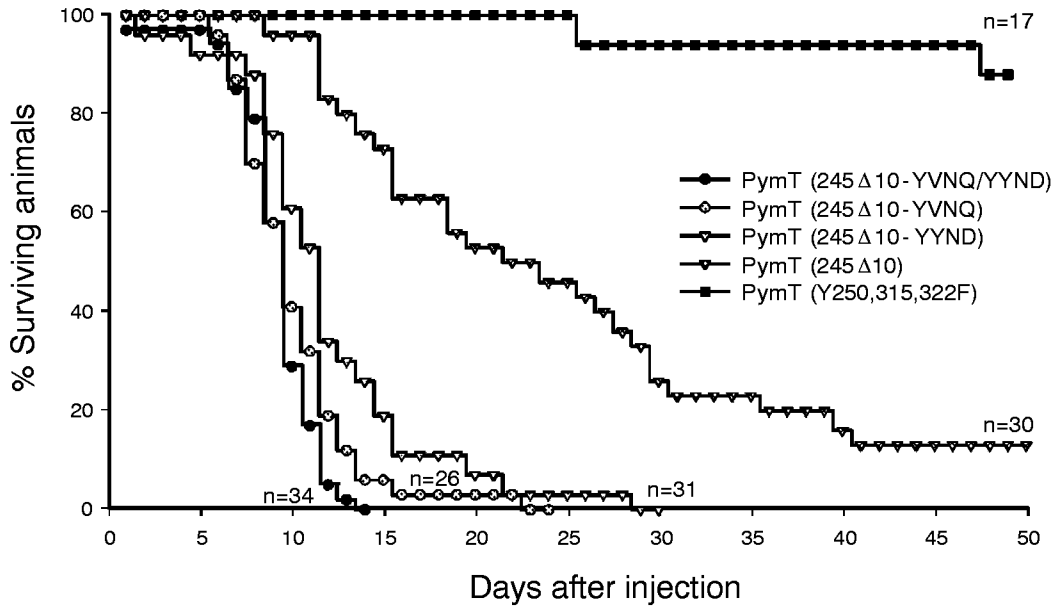

Fig. 9. Survival of animals after injection with wild-type and mutant PymT variants.

2. Conclusions

Multiple facets of cellular behavior depend on modular protein–protein, protein–phospholipid, and protein–nucleic acid interactions. Protein interaction domains and motifs provide a basic mechanism to organize signaling pathways and other forms of information transfer in cells (cell cycle, protein trafficking, gene expression, DNA repair). The combinatorial use of interaction domains has allowed the creation of novel signaling pathways and networks in various species over the course of evolution. Modulatory and regulatory features allow for both control and diversity. The juxtamembrane region of the Eph RTK binds and inactivates the receptor tyrosine kinase in the unphosphorylated state. Mutations that disrupt the juxtamembrane-kinase interface lead to constitutive activation. Additional aberrant interactions induced by pathogenic viruses and bacteria or mutant cellular proteins (oncoproteins) can also disrupt the cell's interaction network, leading to oncogenic transformation and disease.

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