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Review

Protein kinase C: Its role in RTK processing

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ABSTRACT

Protein kinase C (PKC) affects signal transduction, vesicle trafficking, chemotactic activity, and cell polarity. It is the target of phorbol ester tumor promoters, but the phorbol esters have a variety of effects on cell growth – even opposite effects. Despite the importance of tumor promotion in cancer, and PKC in tumor promotion, it remains unknown how PKC affects signal transduction in a way that has major consequences for cancer development. PKC has a well-known role in downregulating or desensitizing receptors to further input at the earliest stages of signaling. The signalosomes assembled on receptor tyrosine kinases (RTKs) undergo progressive refinement, and there is some evidence suggesting PKC intervenes at these stages of signal processing. New light may be shed on the complexity of receptor processing by identifying signaling intermediates affected by PKC. It is possible to infer the composition of protein assemblies (signalosomes) on the epidermal growth factor receptor (EGFR) complex by integrating knowledge from proteomics studies with results from cell/ molecular research. Although the full complexity of such complexes cannot be conveyed, the results allowed identification of inflection points where the signalosome composition is altered. Inflections can be anticipated due to Src and Cbl-mediated activities, as both are substrates of PKC and dock on the receptor. In this context, it is essential to develop analogies to convey the complexity of signaling downstream of receptor activation. We propose an analogy for temporal intervention in signalosome composition, similar to the concept of temporality of PKC-receptor interactions proposed by others. It likens PKC to service workers in the economy. To fulfill their roles, such workers, for example, caterers and taxi drivers, can be mobilized at different times and places. PKCs maintain a presence in the cytoplasm, and wherever and whenever they are needed, they are called up in numbers that have a relationship to the strength of the signaling input.

KEYWORDS: actin-binding, clathrin-mediated endocytosis, protein degradation, proteomics, receptor recycling, signaling network, sorting vesicle.

1. Introduction

Despite five decades or more of research, it is still unclear how tumor promoters cause tumors in cells exposed to a carcinogenic insult that would otherwise be negligible. The role of PKC in cellular regulation is complex, but new perspectives are emerging from the field of proteomics. These techniques allow receptor interactomes to be identified by immunoprecipitation of the associated proteins or peptides, followed by mass spectrometry. The methods have been used frequently to study the receptors binding epidermal growth factor (EGF) and related ligands. This receptor family, also known as avian erythroblastosis oncogene B (ErbB) or human EGF (HER), comprises four protein species having different ligand binding characteristics, each also having differentially spliced isoforms. Here, they are referred to

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collectively as the EGF receptors (EGFRs). When occupied by a ligand, the receptors form homodimeric or heterodimeric complexes with other receptor molecules. Then, each member of the dimer is transactivated by having phosphate added to tyrosine residues in the intracellular domain by the other member. The complex can undergo enlargement as dimeric receptors aggregate into tetrameric and higher orders (reviewed in [1, 2]). Although there are innumerable reports on the biochemical nature of the complex, many of the constituents found in EGFR complexes by proteomics methods were not known, from previous reports, to be receptor-associated. Here, we interrogate results of proteomics research in the light of current concepts about signal transduction and especially in relation to PKC's role in receptor processing. This may advance our understanding of how the phorbol ester tumor promoters can have such variable effects, so that they often decrease normal [3] and neoplastic cell proliferation [4], but paradoxically accelerate tumor production.

1.1. PKC's role in receptor-ligand binding and receptor processing

The PKC family of serine-threonine kinases comprises some 11 isoforms. Two classes of isoforms, α, β, γ, δ, called the conventional PKCs, and δ, ε, η, θ, called novel PKCs, are activated downstream of membrane-bound RTKs and G-protein coupled receptors (GPCRs). The RTKs make direct contact with phospholipase C (PLC)γ whereas the GPCRs are coupled to members of the G alpha-Q and alpha-11 classes of GTPases, which activate PLCβ. The consequence is hydrolysis of phosphatidylinositol(4,5)bisphosphate (PIP2) in the plasma membrane, which in turn causes the release of second messengers diacylglycerol (DAG) and calcium. The novel isoforms do not bind to calcium and are activated by DAG, cis-unsaturated fatty acids, phosphatidic acid, or phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 is produced by the activity of phosphatidylinositol 3-kinase (PI3-K) on PIP2. Members of a third class of PKCs, the atypical isoforms, are activated only by free fatty acids, acidic lipids, or PIP3. All PKCs exist in a folded form, which is enzymatically competent but inactive, and all have phosphatidylserine-binding domains that are concealed in the folded form. Upon occupancy of the calcium- and/or DAG-binding domains, the conventional and novel classes of PKCs translocate to membranes causing the proteins to unfold. As PKC unfolds, a phosphatidylserine-binding site is revealed, which allows the molecule to dock on the membrane [5-7]. This allows the kinase domain to have access to physiological substrates on or near the membrane. The PKCs are closely related to the family of PKDs, also known as PKCμ and PKCν, which are also regulated by DAG.

Several PKCs have been recovered by immunoprecipitation of EGFR complexes [8-10]. Although PKCδ was reportedly required for ErbB2-driven mammary gland carcinogenesis [10], the role of PKCs in carcinogenesis is undefined. The EGFRs are among the many substrates of PKCs [11], but they are part of a complex network including multiple substrates of PKC. Using a chimeric receptor combining the extracellular portion of HER1 with the intracellular domains of HER2, Seedorf and coworkers showed that PKC overexpression increased receptor degradation over 6 hours [8]. On the contrary, others found that PKC activation by tumor promoters, which are degraded slowly and therefore cause persistent activation of the PKCs, inhibited degradation of the receptor complex [12-14]. Treatment with the tumor promoter, phorbol 12-myristate 13-acetate (PMA), slowed the rate of ligand-induced disappearance of EGFR from the surface [13]. A recent study elaborated on the mechanisms by showing that PKC mediated the escape of EGFR from ubiquitination and degradation [14]. Those receptors that were internalized were diverted from the degradative path and recycled to the plasma membrane contributing even more EGFR to the amount retained on the plasma membrane [13, 14]. It should be noted that this sequence of events contrasts markedly with nonreceptor-mediated endocytosis, which is increased by phorbol ester treatment (see for review [15]).

One mechanism affecting ErbB1 trafficking involves a site in the juxtamembrane domain, namely Thr654, that is phosphorylated by PKCμ/PKD and other PKC isoforms. Phosphorylation reduces the receptor's affinity for ligand and its dimerization [16, 17], which in turn attenuates signaling [18]

and causes negative cooperativity within the receptor complex (for review, see [19, 20]). The inhibition of EGFR endocytosis by PMA was partially reversed in cells expressing a mutant receptor with alanine substituted at the Thr654 phosphorylation site [13]. The mutant receptors also underwent ligand-induced ubiquitination and degradation in PMA-treated cells [14, 21]. Despite results suggesting that PKCs generally redirect trafficking of the EGFR complex, the mechanisms are still unclear. PKCα was needed for EGFR endocytosis in a squamous cell carcinoma line. Here, both the phosphorylation of Thr654 and the internalization of the EGFR depended on the assembly of a membrane domain containing ganglioside, tetraspanin, EGFR, caveolin, and PKC [9]. Recycling of platelet-derived growth factor receptors was likewise dependent on PKCα [22].

The phosphorylation at Thr654 may introduce negative cooperativity simply by decreasing the receptor's ability to interact with docking sites, including those mediating receptor-receptor interactions. There are two classes of EGFRs at the cell surface, based on their EGF-binding characteristics. The high-affinity sites account for less than 10% of receptors, and treatment of the cells with phorbol esters converts them to low-affinity sites. Signal transduction only occurs through high-affinity receptors [23], which are capable of assembling into dimeric and higher-order complexes. Upon ligand binding, the high-affinity EGFRs are routed into the cell by coordinated interactions between vesicle trafficking and ubiquitination. The Casitas B-lineage lymphoma protein (Cbl) is docked on them and is phosphorylated by several Src family kinases, probably including Src [24-26]. Cbl is recruited to EGFR at a phosphotyrosine site created by Src, and it rapidly adds monoubiquitin to the receptor complex [27-29]. Despite differences in constituents reflecting the different cell types or different internalization mechanisms, this ubiquitin addition creates markers that are used for receptor trafficking [29-31]. In the course of receptor processing, the RING (really interesting new gene) finger E3 ubiquitin Cbl is supplemented or displaced on the EGFR by another RING finger [28, 32] or HECT (homologous to E6-AP carboxyl terminus) E3 ligase [33-35]. The HECT ligases, which include WW1 (WW domain containing E3 ubiquitin protein ligase 1) and Nedd4 (neuronal precursor cell-expressed developmentally down-regulated 4), undergo extensive interactions with RTKs, as does Nedd4 family member, Aip4/Itch (atrophin-1 interacting protein 4) [34]. The experimental overexpression of an E3 ligase usually leads to increased degradation of receptor complexes, but there are systems in which such a ligase can rescue receptors from degradation [36, 37]. In addition to the E3 ligases mentioned, RING finger (RNF) family members, RNF126, and RNF115 (breast cancer associated gene 2), and a protease-associated transmembrane (PA-TM-RING) family member, RNF13, modulate trafficking in endosomal or sorting compartments [38-40].

There is some debate as to where PKC engages in the process, and thus, whether the EGFRs are internalized with their kinase activities intact or diminished by PKC-mediated phosphorylation [41]. PKC is known to be translocated to the plasma membrane within minutes after activation with either endogenous or exogenous agents, and its phosphorylation of EGFR has been found to occur on the membrane [13, 14] or within microdomains containing annexin A6 [42] or caveolin [9]. In direct contradiction, however, other laboratories have found that phosphorylation on the Thr654 residue was contingent on EGFR's transfer to the endosomal compartment [21, 43], (see for review [44]). The receptors were mobilized along with conventional PKCs to perinuclear vesicles through a PLD-dependent mechanism [45]. From these compartments, they were directed into recycling endosomal compartments [21]. It has been shown that other conventional PKCs were mobilized to perinuclear vesicles after 30-60 minutes of phorbol ester treatment [46]. In some reports, phosphorylation was found to be mediated by PKD [47], which was in turn activated by a novel PKC [16]. The results in the aggregate suggest that there is a sequence of events, and different PKC isoforms are involved in different steps of the process. Other interpretations are possible, however, as will be mentioned in the section below.

1.2. Problems in interpretation

It is possible that the balance of activated PKC isoforms is a code dictating how the signals will be

interpreted in a cell. DAG and calcium synergistically activate the conventional PKCs downstream of receptors, but PIP3 activates only the novel and atypical PKCs, and it is produced downstream of PI-3K. The advantages to the cell of regulating physiological functions in this way is clear – different cell types may select a different subset of proteins to execute signaling from a single receptor type. As we have begun to understand that numerous isoforms and regulators of PKCs exist, the degree of complexity has become more apparent. This complexity requires that signaling networks be considered as a system, rather than a sequence of individual steps each exerting a positive or negative effect on signaling outcomes.

There are several additional problems contributing to the lack of consensus about PKC's role in receptor processing. One is the artificial nature of procedures used to perform biochemical experiments. Cells in culture must be used to facilitate experimentation, but to remove ligands that would mediate signaling, the cells are deprived of serum for a prolonged period. This appears to maximize receptor display on the cell surface but causes some signal-processing proteins to move to the surface of the endoplasmic reticulum. With receptor activation, they migrate to the plasma membrane and peripheral cytoplasm [48]. Thus, the procedure used to remove the ligands may alter subsequent receptor trafficking by limiting the availability of signaling intermediates. Another problem with the conditions is that the usual procedure follows a time course of events after providing a ligand. This prohibits performing an experiment under equilibrium conditions. Finally, when investigators use phorbol esters to alter receptor processing, they are often added in vast molar excess over any endogenous activator. The same problem exists with regard to EGF or other ligands which are used to start a time course. They are often in excess of the biologically relevant concentrations.

There are also nontrivial problems. One is that different cell types are used by different investigators, and it is not known that they process receptor complexes in a similar fashion. Finally, studies analyzing internalization and recycling fail to consider the kinetics of receptor trafficking. Whereas it is known that measurements taken over a time scale longer than a few seconds reflect both the rates of internalization and recycling [15], investigators rarely measure the instantaneous rate of internalization. As noted elsewhere, all rates except instantaneous uptake are nonlinear and are governed by the rate of uptake [15]. The practical implication of this is that, any condition causing receptors to be trapped in internal compartments will decrease the surface display, and this may be interpreted as enhanced internalization. Conversely, an increase in recycling relative to passage into successively later compartments makes the receptor display appear greater, and this is often interpreted as inhibition of endocytosis. Moreover, the kinetic rates are treated as though they are constant over the course of treatment. On the contrary, it is likely that there is a progressive modification of the kinetics because the composition of the complex attached to receptors is changing continuously following their activation.

2. Changes in the receptor complex following receptor-ligand interaction

It is clear from section 1.1 that PKC may work at multiple levels of regulation during its interaction with EGFR. Due to the problems identified above (section 1.2), however, the complexity of the system may be more apparent than real. It is equally possible that different isoforms interact with EGFR to inactivate some of its functions, but the exact PKC isoform driving the interaction and its timing is specific to the cell type.

For the sake of argument, we assumed that PKC interacts with the EGFR complex at multiple times and/or subcellular sites. If the concept is valid, it might be possible, through an analysis of progressive changes in the receptor complex, to identify points where progress is affected by the appearance or disappearance of a substrate or binding partner of PKC. A great deal of knowledge of the complex assembly of proteins on receptors has been gained from studies of RTK interactomes isolated by immunoprecipitation of the associated proteins or peptides, followed by mass spectrometry. Following transphosphorylation of the EGFR, constituents are recruited to newly created sites on the receptors' intracellular portion. Here, we select some of the proteins that make direct contact with the EGFR to represent the core constituents (Figure 1). It has become clear that the RTKs can be phosphorylated on tyrosine at

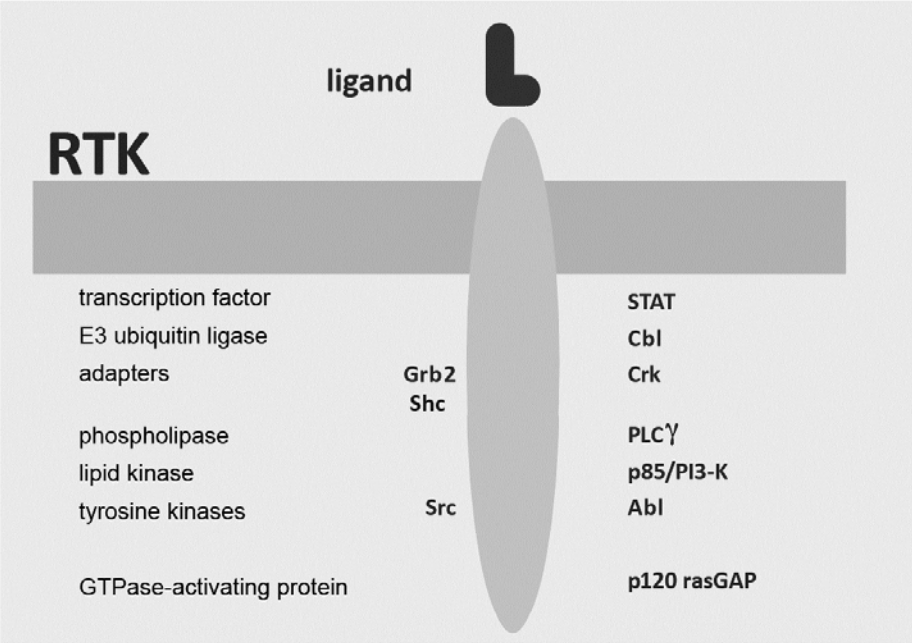

Figure 1. Core constituents activated early after ligand binding to RTKs. The constituents include the enzymes: Abl, Src, and PLC, a lipid phosphatase upstream of PKC. Other core proteins are: GTPase-activating protein, p120 Ras-GAP, E3 ubiquitin ligase, Cbl, and transcription factor, STAT. Not all of the proteins shown have binding sites on all four receptors of the EGFR family. Abbreviations: Abl, Abelson tyrosine kinase; Cbl, Casitas B-lineage lymphoma; Crk, CT10 (chicken tumor virus no. 10) regulator of kinase; Grb2, growth factor receptor-bound protein 2; p85/PI3-K, phosphoinositide 3'-kinase regulatory subunit; PLC, 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma; p120 Ras-GAP, 120 kDa Ras GTPase-activating protein; Shc, Src homologous and collagen-like protein; Src, Rous sarcoma virus protooncogene; STAT, signal transducer and activator of transcription. The core is mainly from references [49, 50] and KEGG pathway (http://www.genome.jp/kegg/pathway/hsa/hsa04012.png, last accessed July 24, 2018). Constituents assembled on other RTKs differ in details but represent similar functions as those shown.

numerous sites, and hence Figure 1 represents just one possible assembly of a core of common signaling components. The proteins shown are nearly universal in tissues of the human body [51, 52]. The exact residues where binding occurs are not relevant to the analysis, and the reader is referred to the literature for further information [2, 49, 53-55].

Some of the core constituents (Figure 1), e.g. Cbl, Shc, PLC, and possibly Ras-GAP, appear to be substrates of EGFR. Disentangling the sequence of phosphorylation is difficult, in part because a priming phosphorylation on a substrate by a nonreceptor tyrosine kinase increases the accessibility of substrate to phosphorylation by the receptor kinase [56]. Priming is discussed in greater detail in the next section (section 2.2). PLC and Shc are known to be phosphorylated immediately after receptor activation [57]. Figure 2 illustrates arrangements of proteins that appear to be bound more transiently or indirectly to the EGFR. Except for p120 rasGAP, all of the core proteins, Abl, Crk, Grb2, p85/PI3-K, PLCγ [58], Shc1, Src, and STAT [59], bind to Cbl.

The many published reports on the EGFR interactome may be useful as a basis for analysis of signaling complexes. Two teams of investigators found interactomes consisting of 183 proteins and 300 proteins, including 31 [60] and 74 [54], respectively, whose phosphorylation status was altered by receptor activation. In studies of cultured mammary epithelial cells, similar interactomes were represented by 58 [61] and 160 proteins [62], respectively. The interactome of HeLa cells consisted of 81 proteins with altered

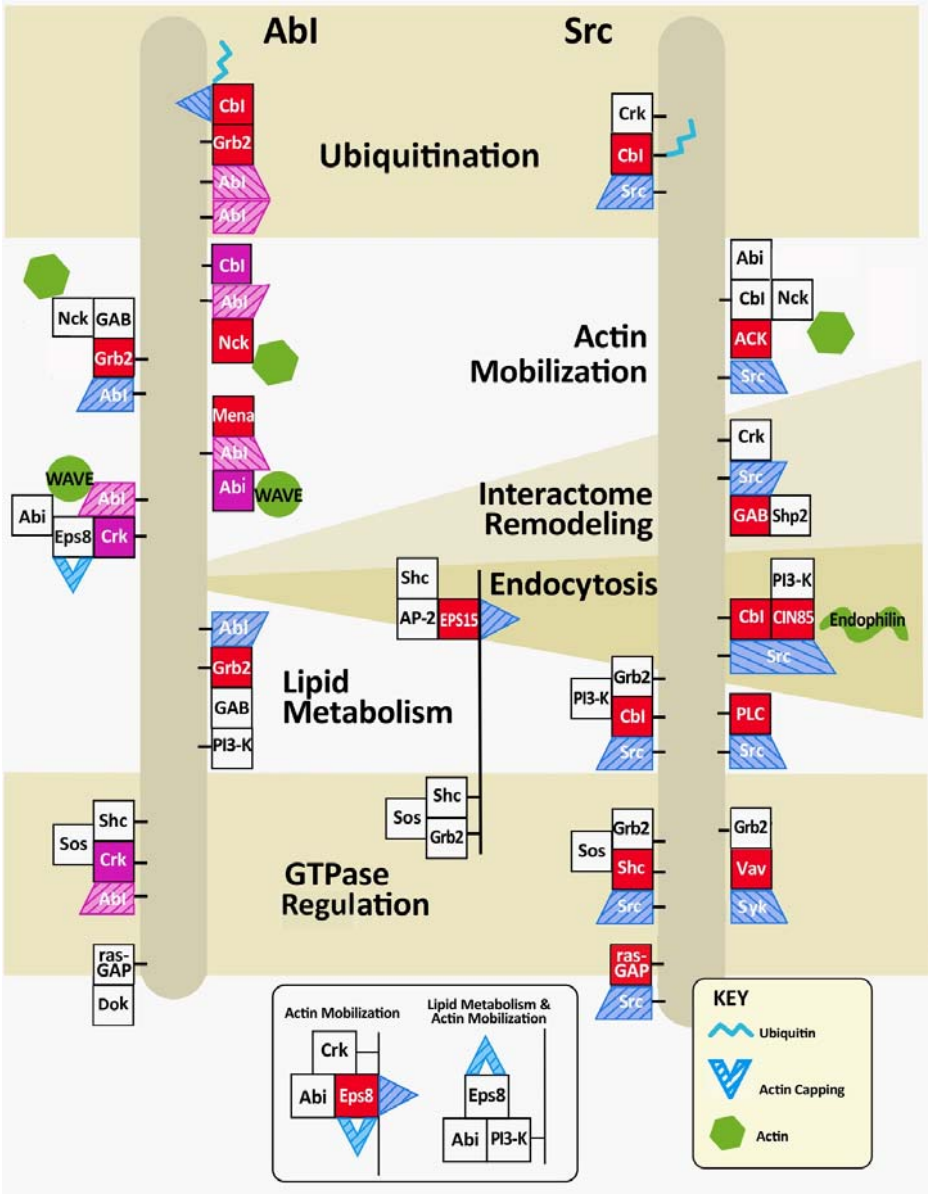

Figure 2. Signalosomes consisting of receptor-enzyme-adaptor complexes and similar clusters of proteins. Constituents with anchors into the receptor are core constituents. About half of the Abl-initiated signalosomes (left) are implicated in actin recruitment. Actin organization (green hexagon) is driven by Nck or Abi through WAVE (green circle). The number of signalosomes assembled on the Src kinases and the receptor itself suggest that they are implicated in receptor internalization. Receptor complex remodeling is represented as a Src function, but Abl also interacts with protein tyrosine phosphatases (PTPs) including Shp2 (not shown). Both kinases also initiate ubiquitination, changes in lipid metabolism, and interactions between core constituents and GEFs. In addition to initiation by one of these tyrosine kinases, signalosomes may be initiated by receptor-associated constituents acting as adaptors, or by the receptors themselves. Abbreviations: Abi, Abl interactor; ACK, activated Cdc42-associated kinase; AP-2, adaptor protein complex 2; Dok, downstream of tyrosine kinase; Eps8, EGFR substrate 8; Eps15, EGF pathway substrate 15; GAB, Grb2-associated binder; Mena, mammalian homolog of Ena (enabled)/VASP (vasodilator stimulated phosphoprotein); Nck, non-catalytic region of tyrosine kinase adaptor protein; Shp2, Src homology phosphotyrosyl phosphatase 2 tyrosine phosphatase; Sos, Son of sevenless; Syk, spleen tyrosine kinase; Vav, Hebrew letter resembling a tent peg, WAVE, WASP (Wiskott-Aldrich syndrome protein) family verprolin-homologous protein. The core components are defined in the legend to Figure 1.

phosphotyrosylation patterns [63]. The typical constituent showed an increase in phosphotyrosylated sites. Only rarely was a phosphosite negatively regulated following receptor activation in these cell types, although it often occurred in embryonic fibroblasts [64]. Abl is not normally phosphorylated on tyrosine [65] and was rarely found among phosphotyrosylated peptides recovered for mass spectrometry. Crk was typically absent from the receptor-associated complex but was found in some studies, e.g. lung adenocarcinoma cells [66]. Grb2 was usually found if high and unphysiological concentrations of EGF were used in experiments [67]. Some proteins are seldom represented in proteomic surveys for technical reasons [68].

To understand the possible consequences of the cell's assembling so many signalosomes, and of its varying the order of assembly or activation, we estimated the number of states the receptor could occupy during its maturation. Let us assume that each signalosome appearing in Figure 2 could be present or absent and that each could exist in two states. The native state would be that shown in Figure 2, and a second state would be achieved by a posttranslational modification (phosphorylation, ubiqitination, sumoylation, acetylation, etc.). Then, that would be 3^{21} or about 10 billion states. This illustrates the problem encountered when trying to predict the state of a receptor complex while it is undergoing progressive changes in the cell.

2.2. Src and Abl recruit constituents to the EGFR complex

Src family kinases (Fyn, Lyn, Src, Yes) were phosphotyrosylated constituents of the EGFR complex [62, 64, 67, 69, 70]. The Src family affects the assembly of signalosomes by its ability to phosphorylate Abl [71], which may be a prerequisite for Abl being phosphorylated by the receptor [72]. Activation of Abl may then cause positive feedback on Src by Abl-mediated phosphorylation and activation of PTP, Shp2 [73] (see also Figure 2). It is known that Shp2 knockdown causes hyperactivation of C-terminal Src kinase (Csk), the kinase that suppresses Src by phosphorylating it on the Tyr 527 residue [74]. Moreover, the Src kinases were responsible for altering the interactions between other proteins at early stages of receptor processing by phosphorylating Cbl, Shc and the EGFR itself [67]. Adaptors that are phosphorylated on tyrosine serve as platforms for binding to both Abl and Csk [75]. Thus, Abl may mediate the interaction of both Csk and Shp2 with Src. Because it is generally not known which proteins are present on an adaptor simultaneously, the results of the interaction are not predictable. There are also indications that Shp2 binds to Src directly. This caused a slight increase in activity, which was not mediated by dephosphorylation of the Src Tyr 527, as Shp2 binding to the Src SH3 domain was sufficient [76].

Abl enzymatic activity is autoinhibited by interactions between the Src-homologies 2 or 3 (SH2 and SH3) domains and the kinase domain, which in turn depend on an N-terminal myristoyl modification of the molecule. Autoinhibition is common to many kinases but is relieved in an unusual way in Abl. The activation loop is phosphorylated by an autocatalytic mechanism in trans. Thus, at high concentrations, Abl enhances its own enzymatic activity by intermolecular interactions. Further elevations of its activity depend on the ability of tyrosine-phosphorylated substrates of Abl to disrupt docking between its SH2 domain and the kinase domain. Abl-mediated phosphorylation of such substrates further enhances the interactions between the partners. Docking of such 'primed' substrates on the SH2 domain of Abl positions them favorably for further phosphate addition. Many substrates that activate Abl at high concentration are thought to have this effect (see for review [65] and [77, 78]). For a few of them, Abl interactor (Abi), Cbl, and Crk [79-81], illustrated in Figure 2, the priming effect on Abl activity is indicated by coloring both substrate and Abl purple. Otherwise, enzymes are represented in blue and substrates in red. Other Abl substrates, e.g. Grb2, Nck, and WAVE, are not shown in purple but may act similarly [82-84]. Adding to the complexity of these interactions, Abl phosphorylates PKC δ under conditions of oxidative stress [85]. Thus, PKC δ was sometimes recovered with the EGFR complex [62, 69]. Conversely Abl is phosphorylated by PKC. Although this phosphorylation doesn't change the enzyme kinetics of Abl, it may alter some aspects of its activation by its binding partners [86].

The Abl kinases are the only known tyrosine kinases with an actin-binding domain. They are able to mobilize actin on membranes and so may affect trafficking in several ways. Abrogation of Abl delayed capping and, consequently, internalization of the B cell antigen receptor. Thus, Abl appeared essential for some aspects of endosome and phagosome formation in B cells and macrophages [87, 88]. In direct contradiction to these findings, constitutively active Abl caused EGFR to be confined to the cell surface in anchorage-dependent mammalian cells. It also reduced Cbl's interaction with the EGFR and decreased Cbl recruitment in response to EGF stimulation [89]. In experiments on constitutively recycled receptors, the knockout of Abl prevented recycling of transferrin and low-density lipoprotein receptors to the cell surface and caused internalized receptors to be routed into degradative pathways [90]. Experiments on different receptor classes may give opposite results, in part because Abl interacts extensively with Src. Abl and Src mutually regulate one another and both also regulate the Cbl-EGFR interaction. Abl's importance in regulating recycling is supported by studies on Rab5, a GTPase that regulates endocytosis. The Rab5 guanine nucleotide exchange factor (GEF), Ras and Rab interactor (RIN), is associated with the EGFR but also binds to Abl's SH2 domain. A mutant form of RIN with less GEF function allowed the EGFR to be recycled, whereas a mutant unable to stimulate Abl activity prevented EGFR recycling [91].

Since conflicting experimental results were obtained, it is possible that Abl works at multiple levels of processing. Although it might be hypothesized that some Abl-mediated activities could be switched on or off by its phosphorylation of PKC, the only isoform known to be phosphorylated by Abl is PKC δ. This isoform is not known to mediate receptor trafficking, and those that do so, e.g. PKCα, PKC ε, and PKC θ, are rarely recovered with the EGFR complex (see also section 4). There are 11 curated sites of tyrosine phosphorylation in PKC δ (https://www.phosphosite.org). Four are conserved as tyrosine in PKC ε but they are seldom found to be phosphorylated.

As mentioned above (section 1), the EGFR interactomes are a rich source of information that can be mined to infer how cells construct signalosomes. Randomly selected constituents recruited by Src and Abl are shown in Figure 2, along with constituents recruited by the receptor itself. The signalosomes are consistent with networks proposed elsewhere [92, 93], but there are many more known combinations than shown here. Activation of a Src family kinase may precede the assembly of Abl-dependent signalosomes, and so the signalosomes shown would not be assembled over the same time course. One example of how constituents are recruited is provided by Cbl interacting protein of 85 kDa (CIN85)/SH3KBP1 (SH3 domain kinase binding protein 1), also called CD2AP (CD2-associated protein). CIN85 is an Abl binding partner [94] and Src substrate [95]. As its name implies, it binds to Cbl and thereby influences the ubiquitination of the EGFR [95]. CIN85 affects trafficking by recruiting endophilin, which is an important accessory protein in endocytosis (see for review [96]). The function and binding partners of CIN85 are discussed further in section 2.5.1.

2.3. Progressive refinement of the interactome

There is abundant evidence that the receptor complex changes over time, a pattern that is referred to here as refinement. Sites on the receptor itself and the Shc Tyr427 site underwent phosphotyrosylation within 10 seconds in MCF-10A and HeLa cells [57, 67]. PLC and PI3-K were among the constituents phosphorylated early in cells exposed to a ligand. Phosphorylation of these proteins was exquisitely sensitive to EGF concentrations over the range of 0.2 to 2.5 nM [67]. Several other proteins underwent phosphorylation early after cells were exposed to ligand. The phosphotyrosine content of Cbl, Dok1, Eps15, SHIP2, Src family kinases, and Vav rose gradually over 1 min [63, 67]. Cbl and Cbl-b were represented in the EGFR complex at the cell surface, whereas the HECT ligase, Aip4/Itch, was present on the internalized complex [54]. This was consistent with the concept that Cbl is displaced by other E3 ligases during receptor processing, as discussed above (section 1.1). Early phosphorylation of Shc, PI3-K and PLC was typically found even in different cell lines. As noted in the very first analysis of the time course, however, kinetic curves of phosphorylation varied

widely among the signalosome constituents. Moreover, different sites on the same protein often showed divergent patterns [63, 70].

An analysis of phosphotyrosylated peptides over a longer time course revealed that PI3-K and Src peptides were unchanged after an initial increase, whereas the phosphotyrosylated Cbl, Crk, PLC, Shc, and STAT peptides declined after the first 8 mins [69]. There are some interesting discrepancies in such time courses. For example, a phosphotyrosylated peptide from p130Cas (Crk-associated substrate) in mammary epithelial cells decreased after EGF treatment [61], whereas investigators working on HeLa cells found a slight increase in overall phosphotyrosylation [63].

The second-rank constituents that are actin-binding proteins, Eps8 and Mena/Ena/VASP, are discussed with third-rank proteins below. Actin appears to play roles in receptor trafficking, both in endocytosis and recycling, but despite rapid progress in the field, there is still little understanding of these roles. Recent studies suggest that there are several mechanisms of internalizing receptor complexes. At ligand concentrations of around 1 ng/ml (0.167 nM), activated EGFRs were reportedly taken up by clathrin-mediated endocytosis (CME). ACK has a clathrin-binding domain and is implicated in CME [97], as well as in receptor processing [98]. With high ligand concentrations, it is thought that the internalization machinery may become saturated, and so receptor internalization occurs through other mechanisms, collectively called nonclathrin-mediated endocytosis (NCE). This may have different consequences for signaling. According to some interpretations, NCE is the main pathway leading to receptor ubiquitination and degradation.

2.4. Temporality of PKC effects on the EGFR complex

The persistence of a signalosome will depend on the rate of inactivation of the complex by disaggregation of its constituents. This may occur by reducing or reversing EGFR activation and the docking of constituents initiated by it. Of the core constituents, four, Cbl, Src, STAT, and the EGFR itself, which is phosphorylated at both Thr654 and Thr693, are known PKC substrates [99]. PKC probably phosphorylates a fifth core constituent, p120 RasGAP, in a way dependent on phosphotyrosylation of RasGAP itself or a binding partner [100]. PKC θ added phosphate to Cbl-b at Ser282, a residue that is conserved in other Cbl isoforms. This phosphorylation may have physiological significance, because PKC downregulates the phosphorylation of Cbl on tyrosine and inhibits its ability to recruit PI3-K and Crk to the EGFR [101]. Cbl is phosphorylated by Src, as mentioned above. This suggests that signaling from the activated EGFR could be affected by the rate at which PKC phosphorylates EGFR and inhibits the binding of Cbl and subsequent recruitment of its binding partners. These rates are regulated by the activators upstream of PKC, specifically by the products released downstream of PLC activation, calcium and/or DAG (see section 1.1). As more PLC molecules are activated, there are more PKC molecules mobilized to the plasma membrane. EGFRs also enable PI3-K activation to differing extents depending on the receptor isoform, and the production of PIP3 may create an additional activator of novel and atypical isoforms. This could result in biased activation of the novel and atypical isoforms relative to the conventional isoforms following ligand binding (see section 1.2).

Where investigators have made constructs of PKC with a fluorescent protein and visualized their localization in cells treated with DAG or a bioactive phorbol ester [102-104], they have been found at the plasma membrane at varying times from 10 seconds to 20 minutes (see for review [105]). That the mobilization of PKC takes place over varying times means that PKC could intervene at different times in a process stream. This is called temporality in studies of the abundant mucin-like protein, CD43, that regulates T cell receptor trafficking. CD43 is essential for regulation of T-cell activation and proliferation, and in its absence, the cell is hyper-responsive to stimuli. CD43 regulates the T-cell receptor by inhibiting Cbl tyrosine phosphorylation in a PKC θ-dependent manner [106]. An early interaction upstream of Cbl means that receptors remain available at the cell surface. Slower intervention by PKC allows signalosome assembly by Cbl and subsequent removal of receptors from the cell surface. A similar PKC θ-mediated downregulation of Cbl phosphotyrosylation is mentioned above.

The proteins referred to as second-order constituents are recruited in a manner not as well understood as for core constituents. Table 1 shows the binding partners and functions of these second-rank constituents and, where known, the kinases, phosphatases, and ubiquitin ligases that carry out relevant posttranslational modifications. Only two PKC substrates are known among these constituents. PKC phosphorylates Shp2 on Ser576 and Ser591 (Table 1). It also phosphorylates VASP on Ser157 [107, 108]. These constituents are important for remodeling the complex, e.g. Shp2, or for integrating it with the actin-mediated force for vesicle trafficking, e.g. Ena/VASP, and these functions could be profoundly altered by their phosphorylation. As yet, however, there is little information on the physiological consequences or timing of the phosphorylation. There are also several substrates of PKC among constituents of the third-rank, as indicated in Table 2. These are discussed further in sections below (sections 2.5.1 and 2.5.2).

Table 1. Functions, upstream regulators, and binding partners of the second-rank EGFR complex constituents shown in Figure 2*†.

Constituent	Posttranslational modification by	Function	Additional binding partners	Second or non-enzymatic activity	Review articles
Abi	Abl	Scaffold	Integrin α4, Mena/Ena/VASP, N-WASP, Sos [109-112]	Actin organization	[65, 113, 114]
ACK/Tnk2	Src [115]	Kinase	Cdc42, clathrin heavy chain, Hsp90, Nck, Nedd4-2, N-WASP, p130Cas, SNX9, [116, 117]	GTPase effector	[118]
AP-2	AP2-associated kinase, coated vesicle associated kinase of 104 kDa	Trafficking	Subunits AP2A1/AP2A2, AP2B2, AP2M1, Eps15, epsin	Adaptor	[119, 120, 121]
CIN85/CD2AP	Src	Adaptor	Cortactin, Crk, dynamin, FAK, Grb2, IQGAP, p130Cas, polyubiquitin, SHIP2, Sos	Actin capping [122]	[123]
Dok1	IGF-I receptor, Src family kinase	Adaptor	Abl, EGFR, Grb2, Nck, p120 RasGAP	Sequesters Grb2 from Shc/Sos complex	[124]
Eps15	PTPN3, Cdc2 [125, 126]	Scaffold	AP2, Crk, epsin, Nck	Cargo sorting, links ubiquitinated receptor to AP180	[121, 127, 128]

Table 1 continued..

Constituent	Posttranslational modification by	Function	Additional binding partners	Second or non-enzymatic activity	Review articles
Eps8	MST3 (Mammalian Sterile 20 (Ste20)-like kinase 3), Src [129]	Adaptor	IRSp53 (Insulin receptor substrate p53), PIP2 5-phosphatase A/ INPP5J, Shp2, Sos, Vav [130-132]	Actin bundling and capping [133]	
GAB	Akt, ERK, Src	Scaffold	Crk, EGFR, Grb2, Nck, p120 rasGAP, paxillin, PI3-K, PLC, SHIP2, Sos, Vav	Adaptor	[124]
Mena/Ena/VASP	Abl, PKA, PKC, PKD, PKG [99, 134]	Subunit addition on actin filaments	Abi, actin, cortactin, IRSp53, profilin, tripartite motif-containing E3 ubiquitin ligase	Anti-capping	[135]
Nck	Abl [136]	Adaptor	Abi, Abl, ACK, ARHGEFs, cortactin, filamin, GAB, N-WASP, PAK, p120 RasGAP, SNX8/12/17, Sos, WAVE [137]		[137, 138]
Shp2	Abl, PKA, PKC [73, 139, 140]	PTP	Csk, Grb2, p130Cas, Src	Adaptor	[141, 142]
Sos	Abl, ERK/MAPK [143]	GEF for Ras and Rho GTPases	Crk, Eps8, GAB, Grb2, Nck, PI3-K, PLC, SNX9	Adaptor	[144]
Vav	EGFR, Shp2, spleen tyrosine kinase (Syk) [145, 146]	GEF for Rac, RhoA, and RhoG	Abi, Eps8, GAB, Grb2, PLC, talin, vinculin [147]	Adaptor	[148]

*Binding partners already shown in Figure 2 are not included.
†For databases relied upon see Table 2.

2.5. Third-rank constituents of the RTK interactome

Additional proteins are found in the EGFR interactome whose mechanisms of recruitment to the EGFR are unknown. Figure 3 shows a selection of such proteins. For both the secondary and tertiary constituents, the approximate frequency of their recovery in different cell types is indicated. Many of these constituents are substrates of the tyrosine kinases, EGFR, Src, or Abl as indicated in Table 2. Others are substrates of enzymes listed in the second-rank constituents (Figure 3). For example, sorting nexin (SNX9) is a substrate of ACK and paxillin a substrate of Shp2. Unlike the second-order constituents, several constituents of this more distantly associated group contribute to focal contact organization.

Table 2. Functions, upstream regulators, and binding partners of third-rank constituents shown in Figure 3*.

Annexin	Abl, EGFR, PKC, Src [99, 149]	Scaffold, endocytosis, exocytosis [150, 151]	Actin, phospholipids, p120GAP, PKC [152, 153]	Inhibits filament elongation at barbed ends, bundles filaments [154]	[155-158]
(Arf)GAP GIT1/2	PKD, Src [159, 160]	Scaffold, focal contact structure, trafficking	ARHGEFs, GPCR kinase, paxillin	Adaptor, PLC activation [160]	[161-163]
ARHGEFs	Abl, PAK, PKC [149, 164, 165]	Receptor trafficking [166-168]	Cbl, Cdc42, GIT1/2, PAK, Rac [169]	Activates and inhibits guanine nucleotide exchange	[170]
Caveolin	Src	Structural protein of caveolae	Filamin, GPCR kinase, PKC [171-175]	Trafficking, inhibits GPCR kinase [174]	[176, 177]
Cortactin	Abl, ACK, ERK/MAPK, PAK, PKC, PKD, Src [99, 178-182]	Filament initiation, receptor trafficking [183, 184]	Actin, Arp2/3, CIN85, Mena/Ena/VASP, Nck, N-WASP [185, 186]	Actin assembly by displacing WASP or VASP family protein on filament [183, 186]	
Filamin	Abl, PAK, PKC [187, 188]	Actin cross-linking	Actin, caveolin, Nck	Filament cross-linking at large angles	[189]
Hsp90	Abl, casein kinase 2 [149, 190]	Chaperone	ACK, cAMP-dependent protein kinase catalytic subunit beta, Rapidly accelerated fibrosarcoma (Raf) kinase, p50 cell division cycle protein, N-WASP, PKC [191]	Actin nucleation and cross-linking [192]	[193]
N-WASP	Abl, ACK, casein kinase 2, Src family kinases [117, 194]	GTPase effector	Arp3, Cdc42, cortactin, FNBP, Grb2, Hsp90, Nck, RNF8, SNX9, SNX33	Adaptor	[195, 196]
p130Cas/BCAR	Abl, Src [149, 197]	Scaffold	ACK, β-COP, FAK, PTP1B, SHIP2	Adaptor	[198]
Paxillin	Abl, FAK and/or Src, Shp2 [199-201]	Scaffold	Csk, FAK, GAB, GIT1/2, RNF5	Adaptor	[202]

Table 2 continued..

SH3PX1/SNX9†, SNX2‡, SH3PX3/SNX33	ACK, Itch, Src [203, 204]	Scaffold, retromer formation	Crk, dynamin, N-WASP, Sos, SNX6, SNX9	Adaptor	[205, 206]
Shb	Frk (Fyn-related kinase) [207]	Adaptor	Abl, Crk, Grb2, Vav [208, 209]		
SHIP2	Src family kinase [210]	Lipid phosphatase	CIN85, GAB, Grb2, p130Cas	Adaptor	[211]

*Databases relied upon:
https://www.phosphosite.org/homeAction.action (last accessed June 28, 2018).
https://www.uniprot.org/ (last accessed July 23, 2018).
https://reactome.org/content/detail/R-HSA-5218820 (last accessed July 23, 2018).
†SNX9 (SH3PX1) interacts with SNX33 (SH3PX3).
‡SNX2 interacts with SNX6.

2.5.1. Proteins organizing actin

Proteins regulating aspects of actin organization include annexin, cortactin, filamin, N-WASP, Eps8, and Mena/Ena/VASP. Eps8 has an actin capping activity that is activated by Abi, as well as bundling activity (Table 1). Proteins of the WASP and VASP families bind to actin through large domains housing the closely related WASP-homology 2 (WH2) and globular (G)-actin-binding motifs [212]. N-WASP also binds to actin indirectly through Arp2/3 [213]. Although Arp2/3 nucleates addition of actin monomers to form a branch on the filament, it is incapable of initiating actin polymerization on its own. Its binding partners, N-WASP and cortactin, enable the creation of sites for actin monomer addition. For reviews of actin filament initiation, see [214, 215].

Cortactin binds directly to actin and enhances filament assembly by binding to Arp2/3 and displacing a mutual binding partner, N-WASP, on the filament [186]. Cortactin is phosphorylated at residues Ser405 and Ser418 by p21-activated kinase (PAK), and this enhances its affinity for N-WASP and hence actin polymerization [179]. Cortactin is also a substrate of PKCα and its homolog, PKC μ/PKD. The PKCs target different residues than PAK, resulting in a reduction in cortactin's actin-binding and -crosslinking activity and consequently endocytosis. Thus, the rate of actin filament assembly can be regulated by N-WASP in cooperation with cortactin. This is thought to be important for generating the forces in vesicle fission and fusion, which depend on multiprotein assemblies built up around actin filaments. Cortactin also interacts with CIN85 downstream of EGFR-Cbl complex formation [185]. The CIN85 signalosome is organized on EGFR-Cbl complexes and engages endophilin (Figure 2). Endophilin is implicated in both CME and NCE mechanisms of receptor internalization [216]. By binding to actin *via* their respective actin-binding domains, CIN85 and cortactin may interact with filaments around the endocytic vesicle, resulting in forces that favor vesicle fission. Cortactin was typically turned over at the early endosome, but a PKC-cortactin complex could be trapped in early endosomes in cells treated with calmodulin inhibitor, W13 [184]. Whereas one would expect cortactin knockdown by siRNA to inhibit the internalization and degradation of the EGFR, the opposite is observed. Reducing cortactin content in a non-small cell lung cancer line accelerated EGFR degradation [217]. Because cortactin also participates in sorting of internalized molecules, cortactin knockdown may prevent the sorting of EGFR in the endosomal pathway and thereby prevent receptors recycling to the plasma membrane [218]. Alternatively, the role of cortactin in exocytosis may be more important in deciding the destination of EGFR.

Although they both engage in actin organization, neither Nck nor Abi has an actin-binding motif.

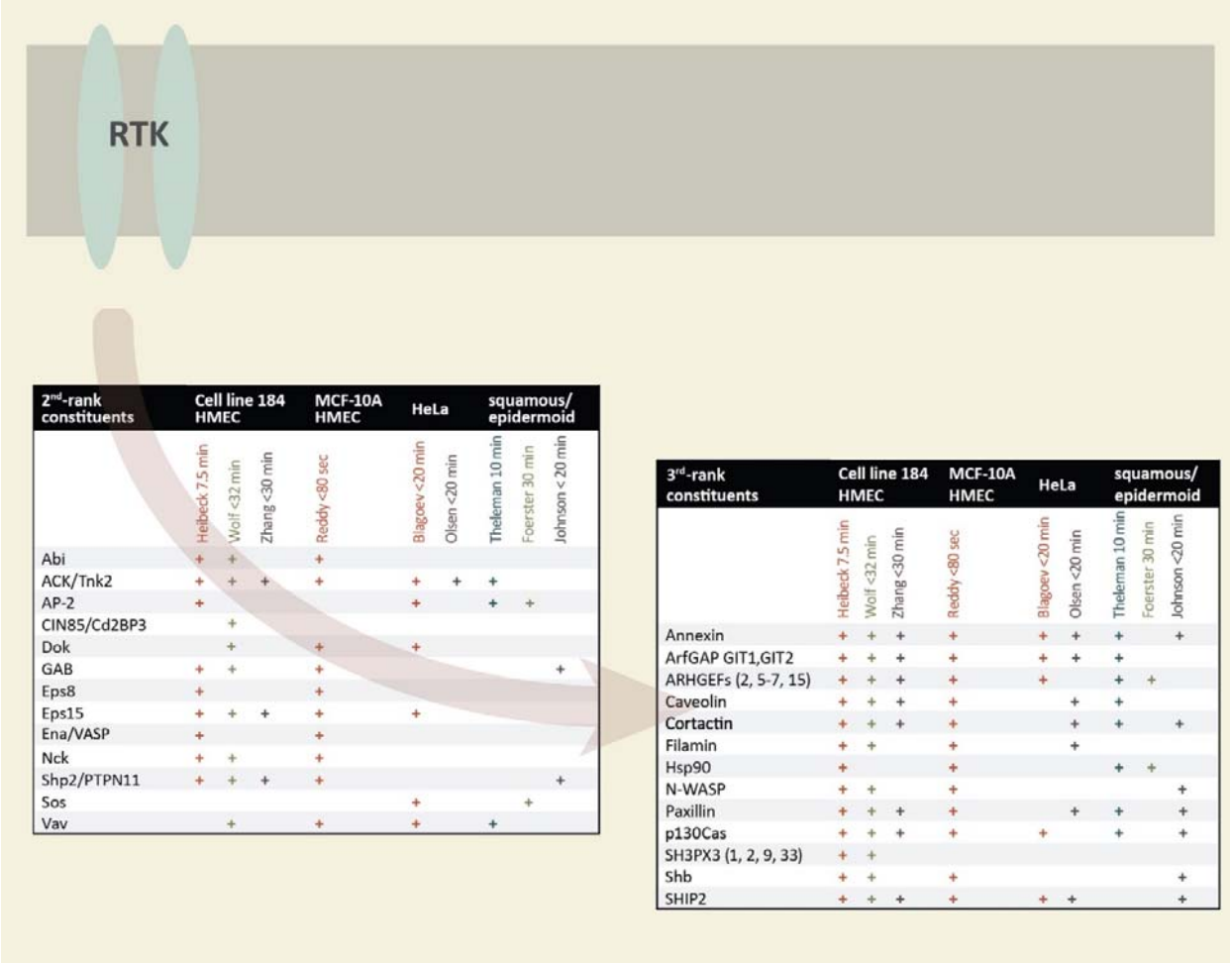

Figure 3. Proteins associated with the EGFR, as identified by peptides recovered for mass spectrometry. Some second-rank constituents are captured by immunoprecipitation in one cell type and not others, e.g. Eps8, Ena/VASP or Mena, and Nck (Left). The third-rank constituents consist largely of three classes of proteins, those mediating actin organization (annexin, cortactin, filamin, and N-WASP), those involved in trafficking (caveolin, and sorting nexins), and those implicated in focal contact regulation (ArfGAP, ARHGEFs, paxillin, and p130Cas) (Right). Abbreviations: ArfGAP, ADP-ribosylation factor GTPase-activating protein; GIT, GPCR kinase-interacting target; ARHGEF, Rho guanine nucleotide exchange factor; Hsp90, heat shock protein 90; N-WASP, neural Wiskott-Aldrich syndrome protein; p130Cas, Crk-associated substrate of 130 kDa size; SH3PX3, SH3 and PX domain-containing protein (sorting nexin); Shb, SH2 domain-containing adapter protein B; SHIP2, PIP3 5-phosphatase also called INPPL1. Second-rank constituents are defined in the legend to Figure 2.

Nck organizes actin nucleation by binding directly to N-WASP [219]. In general, WASP and VASP family members, N-WASP, WAVE, and Mena, make Nck undergo a phase transition resulting in the formation of puncta on phosphorylated receptors [220]. Nck's interaction with N-WASP and WAVE [137] appears to cause elongation and stabilization of filaments associated with RTKs [221].

The annexins comprise a large family of proteins, most of which are PKC substrates and serve as scaffolds for PKC recruitment to membranes, including the plasma membrane, secretory vesicles, and caveolae and lipid rafts. Certain isoforms, e.g. annexin A2, are prominently associated with macropinocytic vesicles (see for review [157, 158]). By binding to phospholipids and PIP2, the annexins form a coat that locally

reorganizes phospholipids in the membrane. Their binding to membranes is sensitive to sub-micromolar calcium levels [222]. Some annexin isoforms have affinities for specific isoforms of PKC, including the novel PKCs which are not calcium-dependent. For example, isoform A2 interacts with PKCs α, β, and ε, and annexin A6 interacts with PKCα. Upon activation of the PKCs associated with annexins, EGFRs are inactivated, presumably because PKC phosphorylates the receptors. PKC knockdown restores EGFR phosphotyrosylation and internalization [42], as does annexin A6 knockdown [223]. These experiments suggest that annexins can enhance signaling from EGFRs by inhibiting the endocytosis of the receptors. The negative effects on signaling, due to PKC-mediated phosphorylation of receptors, may be outweighed by the retention of EGFR on membranes. With prolonged retention, the EGFRs may recover their competency for signaling. The annexins are multifunctional proteins, and so their functions are not easily rationalized in terms of cancer or tumor promotion.

Annexins may also participate in exocytosis of recycled EGFRs. When phosphorylated *in vitro* by PKC, annexin A2 is able to reconstitute secretion of chromaffin granules in permeabilized cells, suggesting that this is the form active in exocytosis [224]. Annexin A2 exists in two main forms: a monomer and a heterotetrameric complex with two A2 molecules bound to a homodimer of the 10 kDa protein S100A10. Phosphorylation of A2 by PKC disbands the complex. The bundling activity associated with the A2-S100A10 complex appears integral to the annexin-dependent exocytosis of chromaffin granules [150]. This, however, is contrary to the evidence that phosphorylation by PKC favors exocytosis. Indeed, exocytosis can be blocked by a synthetic peptide of A2 that competes with the endogenous PKC phosphorylation site [151]. Exocytosis may rely on the actin bundling activity of A2 more than on its phospholipid-binding properties, but there is little concordance among the results. How the process is regulated by PKC is as yet unknown. Annexin levels can be increased or reduced in transformed cells relative to normal cells, and hence the dualism found with regard to signaling is also reflected in relation to growth control (see for review [156-158]).

Abl and its substrate, filamin, both bind to actin through calponin homology (CH) domains. Filamin has the structural protein of the caveolae, caveolin, as a binding partner and the filamin-caveolin complex anchors caveolae in a specific arrangement near actin stress fibers [171]. When phosphorylated by one of several serine-threonine kinases, including PKC, filamin can engage in internalization of the caveolae, but a number of additional proteins are needed, including Abl [225]. Receptors and channels in the caveolae are internalized and downregulated in a caveolin- and PKC-dependent fashion [9, 174, 175]. Annexin is also present in caveolae. PKC phosphorylates both Abl and its two substrates, filamin and annexin (Table 2), but how this contributes to internalization of these organelles is unclear, as the PKCs are bound to caveolin in their inactive form [17], (see for review [226]).

A total of three PKC substrates are included among the third-rank proteins organizing actin. An additional two, if the inclusion of PKD in the PKC family is allowed, are found among third-rank proteins implicated in focal contact dynamics (see below).

2.5.2. Proteins associated with focal contacts

Some of the third-rank constituents are components of focal contact sites. The fact that adhesive structures are recovered with the RTK complex has no obvious explanation. Identification of the binding partners of several core constituents by immunoprecipitation suggests that there is a closer relationship between receptors and focal contacts than previously thought. When the interactome is recovered using an anti-EGR antibody rather than anti-phosphotyrosine, however, there is a noticeable lack of focal contact constituents [54, 60].

One possible way these constituents appear in the interactome is that both of these multiprotein assemblies contain Src, and it migrates between the receptor complexes and focal contacts. p130Cas binds constitutively to many nonreceptor tyrosine kinases, including Src family kinases. The Crk interaction with p130Cas is an interesting example of how an adaptor may participate in both structures. *Via* its SH2 domain, the several isoforms of Crk bind to exposed phosphotyrosine

residues of p130Cas. For the longer isoform of Crk, the interaction with p130Cas is negatively regulated by Abl-mediated phosphate addition to a residue located C-terminal to its SH2 domain, i.e. Tyr221 (see for review [227]). Upon phosphorylation, the molecule rearranges itself to let the SH2 domain dock on its own phosphotyrosine, which blocks the surface of the intervening SH3 domain. Thus, phosphorylation of Crk-II would release it from the focal complex-associated p130Cas but also inhibit its ability to dock on the receptor through its SH3 domain (see for review [228]). Any site with the PTP that dephosphorylates Crk, thought to be PTP1B, can render the adaptor available for docking once again.

Among this group of constituents are several substrates of PKC. The ARHGEFs, including p21-activated kinase (PAK)-interactive exchange factor (PIX) isoforms, ARHGEF6 and ARHGEF7, play a role in both focal complexes and receptor trafficking. The β–PIX (ARHGEF7) isoform is phosphorylated by unknown serine-threonine kinases at several sites in the GTPase-binding domain. One tyrosine phosphorylation site was identified in the same domain [229]. PIX is also phosphorylated on Ser583 by PKC. PIX forms a constitutive complex with ADP-ribosylation factor (Arf)GAP, GIT1, which is implicated in both focal contact dynamics and vesicle trafficking. GIT is phosphorylated by PKC μ/PKD at sites that share sequence similarity other GAPs that are also PKC substrates. These GTPase regulators interact with other proteins, PAK and Nck, as discussed elsewhere [230-232]. PAK is thought to be phosphorylated on Ser199/204 (PAK1) or Ser192/197 (PAK2), enabling its dissociation from PIX at focal contacts. As PIX functions as an exchange factor for Cdc42 and Rac, this enables it to regulate Rac activity. PAK is implicated in both the deposition and dissolution of the structural constituents of the focal contacts, and it is thought that this activity is regulated by the active GTPases supplied by PIX. PKC and PKD may cooperate somehow with PIX and GIT1/2 in focal contact regulation by PAK, but to date no physiological significance can be attributed to this arrangement.

3. EGFR endocytic and post-endocytic trafficking

Internalization pathways were discussed above (sections 2.3 and 2.5.1), and several of the third-rank constituents function in these pathways. The endocytic machinery was identified in studies of clathrin-coated pits from dynamin knockout cells. It contained activated Cdc42-associated kinase (ACK), sorting nexin 9 (SNX9), and epsin [233]. Epsin is an adaptor that bridges the polyubiquitin moiety of a transmembrane protein to adaptor protein complex (AP-2) and clathrin [234]. Epsin binding partner, Eps15, likewise attaches ubiquitin moieties of EGFR to its ubiquitin-interacting motifs (UIMs) and forms a bridge to subunits of AP-2 [235]. Epsin also induces membrane curvature and binds to actin, which stabilizes the clathrin-coated pit and facilitates vesicle fission [236]. Epsin, Eps15, and ACK all have UIM or ubiquitin-associated (UBA) domains. Eps15 and ACK are constituents of signalosomes that interact with the receptor and Src, respectively (Figure 2).

It can be inferred that ubiquitin association is an important property for proteins that sort cargo in clathrin-coated structures (see for review [237]). There is some debate about whether polyubiquitination is required for the endocytosis of receptors or whether monoubiquitination is sufficient. In embryonic kidney cells, mutation of EGFR Tyr998 to an alanine residue depressed the interaction of the receptor with adaptins, AP-1 and AP-2. As EGF-stimulated ubiquitination of the receptor was decreased, some of the internalization machinery may be implicated in the ubiquitination [54]. As in the case of debates surrounding other timing issues in EGFR trafficking, there are conflicting opinions. Another interesting feature of EGFR endocytosis is that the E3 ligases mediate the degradation of receptors along with their accessory proteins, including Src, ACK, Grb2, Shc, and Cbl/Cbl-b itself [32, 238], (see for review [239]). Cbl is replaced on the EGFR by other RING or HECT ligases, suggesting that they process a cluster of proteins including the receptor, and sort them together into the lysosomal degradation pathway.

4. Is PKC in the RTK interactome?

PKCα was recovered twice as a constituent with phospho-serine residues following EGFR exposure

to ligand, once from HeLa cells [70] and again from pro-B Ba/F3 cells [240]. This occurred well downstream of receptor activation, as was apparent from the fact that neither the receptor nor Src or Abl tyrosine kinases would add phosphate to serine residues. It suggests a relationship to the receptor complex resembling second- or third-rank constituents. Indirect associations with the receptor, mediated by cortactin and/or annexin, may suffice to recruit PKC to the complex, but the timing of any such interaction may be crucial to the fate of the complex. The formation of complexes of PKC, cortactin, and/or annexin would represent the third rank of constituents associated with the receptor. Their recruitment may obviously occur later than the constituents associated early, e.g. Shc, PLC, PI3-K, Cbl, Dok1, Eps15, SHIP2, Src family kinases, and Vav (section 2.3). Although the data imply that PKC makes contact with the EGFR complex, the annexin-mediated interaction may be regulated by the local level of calcium.

At least one PKC isoform must make contact with the complex, because it is known to phosphorylate the Thr654 residue. Ordinary enzyme-substrate interactions would be transient, and so it is important to determine whether PKC is associated in a more persistent way with the receptor. Unfortunately, there is little evidence beyond that cited above (section 1.1). Although PKC lacks the typical features of an adaptor, it has docking sites for receptors for activated C kinase (RACKs) which enable it to prolong its presence at a site. The RACK is outside the scope of this review, and the reader is referred to other reviews for information on these topics [241, 242]. Thus, it must be concluded that the question of how persistent PKC is in the interactome cannot be answered at present. Another question of perhaps greater importance is whether activated PKC functions similarly to Src and Abl and remodels the receptor complex through its substrates Cbl, Ena/VASP, p120 RasGAP, Shp2, and/or Src itself?

The importance of timing has been overlooked in the past, but it is now apparent that PKCs play a role in both endocytosis and exocytosis (section 2.5.1). Certain experimental studies mentioned above showed that PKC overexpression increased EGFR degradation, but contradictory results were also obtained. Phorbol ester treatment delayed their degradation, presumably by activating PKC (section 1.1). It is clear that the current knowledge of protein and receptor sorting is inadequate to understand how it is affected by PKC activation. Thus, analogies for the role of PKCs might be productive. Here, we consider the analogy to on-call workers in the economy. Let us consider workers, for example taxi drivers, caterers, or day laborers, in an economic and geographical area, e.g. a city. If they have no work, they advertise their availability to employers directly or by assembling at a pick-up location. The employers then seek out these workers and employ them. Like the workers, the PKCs are readily available. This model differs radically from the model of first and second messengers. Their activities are initiated at specific locations, from which they may diffuse away to meet binding partners. In contrast, PKCs maintain a presence in the cytosol and are recruited to the sites required. This means that the immediacy of PKC availability is essential to their functions. In experimental studies showing the importance of temporality [106], the timing of PKC recruitment was the inflection point that determined whether T cell receptors were internalized. An implication of the analogy is that, because the cell would rely on having on-call PKC, PKC would never be limiting for the signaling mechanism.

The immediate availability of PKC means that negative feedback on the signal transduction process can be instigated earlier or later, to change the direction of the signaling process. This is shown conceptually in Figure 4. The opportunities for regulating the system are summarized on the right side, while sites where the modification would take effect are shown on the left. The inflection points that are most problematic for experiments, because their mechanisms are difficult to study, are indicated by curved arrows (Figure 4).

5. Receptor cross-talk

Each ligand-receptor interaction at the cell surface is processed through a network of feedback mechanisms that edit and refine the incoming

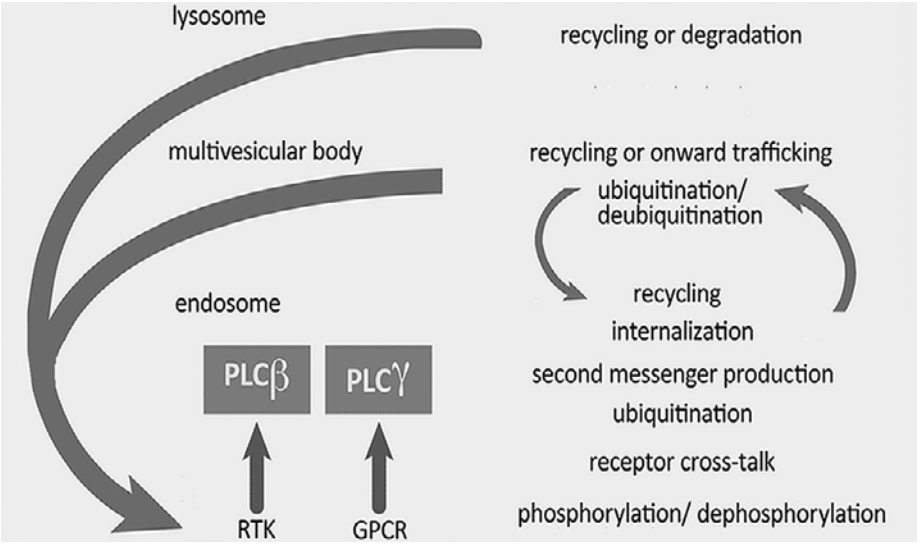

Figure 4. Schematic diagram showing the effect of temporality on signaling. The opportunities for PKC to affect the signaling pathways are listed (right) along with the approximate cellular sites where the effect would be observed (left). The return of receptors to the plasma membrane is indicated by curved arrows (left). At right, the unknown variables' values, such as the sizes of the different, internal pools of vesicles and the rates of trafficking in and out of the pools, are represented by small curved arrows. Aspects of receptor cross-talk with GPCRs are also shown.

signal and integrate it into a communications stream. The role of PKC in the process is chiefly to desensitize receptors to further signal input. Such negative signaling mechanisms have been worked out for GPCRs, where PKC mediates phosphorylation of the β-adrenergic receptor kinase, a GPCR kinase, and causes it to phosphorylate the receptor [243]. This prevents the receptor from activating the G proteins. For some GPCRs, e.g. the δ-opioid receptor [244], PKC-mediated phosphorylation of the receptor directly mediates its desensitization. Whereas this inhibits internalization, GPCR desensitization by β-adrenergic kinase is accompanied by internalization [245]. With activation by PMA, PKC ε can mimic this feedback loop and, by preventing the receptor from coupling to G proteins, downregulate the production of cAMP. Thus, it inhibits GPCRs responsive to histamine or isoproterenol [246]. The opposite also occurs, however. PKCε causes a shortening of the long desensitization of acetylcholine receptors [247]. This is not surprising given the fact that the timing of intervention by the PKCs, as mentioned, routes the trafficking of receptors into different pathways.

6. Summary

The consequences of PKC activity are many and varied, as it affects signal propagation [248], vesicle trafficking [249], chemotactic activity [250], and cell polarity [251, 252]. A better paradigm is needed to understand PKC's role. In one respect, PKC resembles the nonreceptor tyrosine kinases, Abl and Src, because it can alter the course of signalosome assembly on ligand-activated receptors. Temporality plays a significant role in this process, setting the inflection points for functional receptor inactivation. The experimental studies have not revealed the exact timing or locations of these inflection points, even for Abl or Src. It is becoming clear that PKC may affect the fate of internalized receptors by changing the patterns of vesicle trafficking. The PKCs appear to have effects at multiple levels, however, beginning with receptors displayed at the cell surface. There are some suggestions in work done to date, that they also act on compartments where the receptors are sorted into vesicles for recycling or, alternatively, routed to the lysosome (Figure 4).

Regulation of the RTK complexes by PKC differs from regulation by Abl and Src in some important

respects. PKC is more distant in association with the receptor and is probably present in excess of many other constituents. We propose that this defines PKC as a temporal or third messenger, which works in reverse to first and second messengers. It maintains a presence in the cytoplasm and responds to the communications stream coming into the cell to reprogram the signaling patterns. This concept may be oversimplified, but it offers a model that will enable researchers to interrogate signaling in a more incisive way.

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DISCLOSURES

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to disclose.

ABBREVIATIONS

β-COP : coatomer protein
Abl : Abelson tyrosine kinase
Abi : Abl interactor
ACK/Tnk2 : activated Cdc42-associated kinase/ tyrosine kinase non-receptor
Aip4/Itch : atrophin-1 interacting protein 4
Akt : AKT8 virus oncogene cellular homolog
AP180 : adaptor protein of 180 kDa
AP-2 : adaptor protein complex 2
ArfGAP : ADP-ribosylation factor GAP
ARHGEF : guanine nucleotide exchange factor
Cbl : Casitas B-lineage lymphoma protein
CD2AP : CD2-associated protein
CH : calponin homology
CIN85 : Cbl interacting protein of 85 kDa
CME : clathrin-mediated endocytosis
Crk : CT10 (chicken tumor virus no. 10) regulator of kinase
Csk : C-terminal Src kinase
DAG : diacylglycerol
Dok : downstream of tyrosine kinase
EGF : epidermal growth factor
Ena : Enabled protein
Eps8 : EGFR substrate 8
Eps15 : EGF pathway substrate 15
ErbB1 : avian erythroblastosis oncogene B1
ERK/MAPK : extracellular signal-regulated kinase/mitogen-activated protein kinase
FAK : focal adhesion kinase
Fyn : proto-oncogene of the Src kinase family
GAB : Grb2-associated binder
GAP : GTPase-activating protein
GEF : guanine nucleotide exchange factor
GIT : GPCR kinase-interacting target
GPCR : G protein-coupled receptor
Grb2 : Growth factor receptor-bound protein 2
GTPase : guanine triphosphate hydrolase
HECT : homologous to E6-AP carboxyl terminus
HER : human EGF receptor
Hsp90 : heat shock protein of 90 kDa
IGF-I : insulin-like growth factor 1
IQGAP : IQ motif–containing-GTPase activating protein
IRSp53 : insulin receptor substrate of 53 kDa
Lyn : proto-oncogene of the Src kinase family
Mena : mammalian homolog of Ena/VASP
N-WASP : neural homolog of Wiskott-Aldrich syndrome protein
NCE : nonclathrin-mediated endocytosis
Nck : non-catalytic region of tyrosine kinase adaptor protein
Nedd4 : neuronal precursor cell-expressed developmentally down-regulated 4
p120 Ras-GAP: 120 kDa Ras GTPase-activating protein
p130Cas : Crk-associated substrate of 130 kDa
p85/PI3-K : phosphoinositide 3’-kinase regulatory subunit
PA-TM-RING: protease-associated, transmembrane family member
PAK : p21-activated kinase

PI3-K	:	phosphoinositide 3'-kinase
PIP2	:	phosphatidylinositol 4,5-bisphosphate
PIP3	:	phosphatidylinositol-3,4,5-trisphosphate
PIX	:	PAK-interactive exchange factor
PKD	:	protein kinase D
PLC	:	phospholipase C
PMA	:	phorbol 12-myristate 13-acetate
PTP	:	protein tyrosine phosphatase
Rac	:	Ras-related C3 botulinum toxin substrate
RACK	:	receptor for activated C kinase
RIN	:	Ras and Rab interactor
RING	:	really interesting new gene
RNF	:	RING finger protein
RTK	:	receptor tyrosine kinase
SH3KBP	:	SH3 domain kinase binding protein
SH3PX3	:	SH3 and PX domain-containing protein (sorting nexin)
Shb	:	SH2 domain-containing adaptor protein B
Shc	:	Src homologous and collagen-like protein
SHIP2	:	PIP3 5-phosphatase
Shp2	:	Src homology phosphotyrosyl phosphatase 2 tyrosine phosphatase
SNX	:	sorting nexin
Sos	:	Son of sevenless
Src	:	Rous sarcoma virus protooncogene
STAT	:	signal transducer and activator of transcription
Syk	:	spleen tyrosine kinase
UBA	:	ubiquitin-associated
UIM	:	ubiquitin-interacting motif
VASP	:	vasodilator stimulated phosphoprotein
Vav	:	Hebrew letter resembling a tent peg
WASP	:	Wiskott-Aldrich syndrome protein
WAVE	:	WASP family verprolin-homologous protein
WH2	:	WASP-homology 2 domain
WW1	:	WW domain containing E3 ubiquitin protein ligase 1
Yes	:	proto-oncogene of the src kinase family

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