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FUNCTIONAL IMPLICATION OF THE INTERACTION BETWEEN EGF RECEPTOR AND C-SRC

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1. ABSTRACT

EGF receptor (EGFR) and c-Src are the prototypes that individually represent the receptor and nonreceptor tyrosine kinases respectively. Accumulated evidence reveals the association between EGF-activated EGFR and c-Src that leads to activation of both kinases. Importantly, their mutual interaction is required for many EGFR-mediated cellular functions including proliferation, migration, survival and EGFR endocytosis. Interestingly, activation of c-Src and its association with transactivated EGFR is also observed in cells stimulated with non-EGF agonists. This review will not only discuss the structure, function and regulation of these two tyrosine kinases, but also will summarize our current knowledge of the molecular mechanisms depicting the cellular events that require their participation.

2. INTRODUCTION

The protein tyrosine kinases are a large family of proteins that contain highly conserved catalytic domains.

Based on their localization within cells, they can further be divided into receptor- and nonreceptor tyrosine kinases. As reflected by their designation, receptor tyrosine kinases are transmembrane glycoproteins that can bind growth factors and become activated to mediate normal cellular functions such as growth, differentiation, motility, and survival. In contrast, nonreceptor tyrosine kinases are either membrane-anchored or cytosolic. Following the delineation of the various signaling pathways, compelling evidence revealed the cooperation between the receptor and nonreceptor tyrosine kinases. In this review, we are going to focus the cross-talk between EGFR (the representative of receptor tyrosine kinases) and c-Src (the representative of nonreceptor tyrosine kinases) and discuss the mechanism and functional implication of their interplay.

3. THE EGFR FAMILY TYROSINE KINASES

The epidermal growth factor receptor (EGFR) family comprises 4 related members that include EGFR, HER2/Neu, HER3 and HER4 (1). They all possess two cysteine-rich repeats at their extracellular domain, an uninterrupted kinase region and a C-terminal tail where multiple autophosphorylation sites are clustered. Unlike the other 3 relatives, HER3 does not retain any kinase activity, though it is capable of binding ATP (2). While EGF, transforming growth factor-α (TGFα), amphiregulin, epiregulin, heparin-binding EGF and betacellulin are identified as EGFR ligands, a family of alternatively spliced heregulins (HRGs) are defined as the ligands for HER3 and HER4. In contrast, no ligand has been assigned for HER2 yet (2).

3.1. The EGFR structure and autophosphorylation

The human EGFR is a 170 kDa transmembrane glycoprotein with 1186 amino acids. Dissecting its amino acid sequence reveals the following structural features: (1) the extracellular domain (amino acids 1-621), (2) a single transmembrane domain (amino acids 622-644), (3) a juxtamembrane domain (amino acids 645-682), (4) a tyrosine kinase domain (amino acids 683-958), and (5) the C-terminal tail (amino acids 959-1186) where all the autophosphorylation sites are located. In response to EGF stimulation, dimerization of EGFR occurs that results in activation of EGFR tyrosine kinase activity as well as its autophosphorylation that mediated by cross-phosphorylation. To date, among all the well-established EGFR autophosphorylated residues, Tyr-1068, -1148, and –1173 are major sites, whereas Tyr-992 and –1086 are minor sites (2). Their phosphorylation confers the binding sites for a variety of SH2-containing proteins that lead to the formation of Shc-Grb2-SOS complex, trigger Ras activation, and activate Raf/MEK/ERK cascade (3). Interestingly, recent studies indicate that EGFR autophosphorylation can also alter its conformation. To what extent can this conformational change affect the intracellular signaling and the function of the EGFR remains to be elucidated (2).

3.2. Identification of EGFR substrates

Tremendous efforts have been focused to identify and characterize the substrates for EGFR. By utilizing the ^{32}P-labeled C-terminal tail of EGFR as a probe to screen the cDNA expression library, Skolnik *et al*. obtain various putative EGFR substrates, all of which contain SH2 domain. The prominent examples are Grb2, p85 subunit of PI-3K and PLCγ (4). Another approach employs pTyr (phosphotyrosine)-immunoaffinity chromatography, purifies a set of EGF-induced tyrosyl phosphorylated proteins. These proteins are then utilized as antigens to generate antisera that are in turn applied in the immunological screening. With this method, cDNAs encoding putative EGFR targets including Eps8 and Eps15 are isolated (5).

3.3. Downregulation of EGFR

Caveolae are the specialized plasma membrane microdomains enriched in gangliosides, cholesterol, sphingomyelin and caveolin (6). Studies of the EGFR trafficking reveal that prior to EGF stimulation, EGFR is localized in caveolae and non-caveolae plasma membrane domains where EGFR becomes dimerized following EGF binding. Then, the dimerized EGFR exits from caveolae, migrates in the bulk plasma membrane and is captured and endocytosed in clathrin-coated pits (6). To accomplish the first step in this process, EGFR with intact kinase domain must be occupied by EGF and is at least phosphorylated at one of the autophosphorylation sites (7). Once within the subsequently formed clathrin-coated intracellular vesicles and clathrin-free endosomes, the endocytosed receptor can be either targeted to lysosomal degradation or recycled back to the cell surface. It is noteworthy that various determinants such as the identity of the receptor and its dimeric partner can decide the fate of the internalized receptor (8). In the case of EGFR homodimers, they are destined to lysosomal degradation. Downregulation or attenuation of the EGF-induced signal transduction can be achieved by this receptor-mediated endocytosis. However, accumulated evidence indicates that during EGFR desensitization, the Ras/MEK/ERK cascade and the PLC-mediated hydrolysis of PI-4,5-P_2 still occur in the trafficking compartments (i.e. caveolae, clathrin-coated pits and various endosomes) prior to EGFR degradation in lysosomes (6). Thus, the prolonged EGF-induced signaling that originates at a high level in caveolae but sustains at a lower level within the intracellular compartments provides an explanation for the long-term requirement of EGF to provoke a mitogenic response (6).

4. THE SRC FAMILY TYROSINE KINASES

As the cellular counterpart of *v-src*, the transforming gene of Rous sarcoma virus, *c-src* encodes a 60-kDa protein with tyrosine kinase activity. Src is the prototype membrane of a family of nonreceptor tyrosine kinases including Src, Yes, Fyn, Lyn, Lck, Hck, Fgr, Blk and Yrk. While Src, Yes, Fyn are ubiquitously expressed, the distribution of the remaining members exhibited a more tissue-specific fashion, mainly in hematopoietic cells. Alignment of the amino acid sequence of all the Src family members reveals their structural similarity. The conserved regions include: 1) an N-terminal myristylation signal that directs the association of proteins with the plasma membrane; 2) a unique region where the greatest sequence

divergence among family members occurs; 3) an SH3 domain and an SH2 domain that mediate protein-protein interaction; 4) an SH2/kinase linker; 5) a kinase domain; and 6) a C-terminal regulatory region (9).

4.1. The Src structure and regulation of Src activity

Tyr-416 and –527 are the well-documented Src phosphorylation sites that located within the kinase domain and the C-terminal regulatory region respectively. While self-mediated phosphorylation of the former enhances the catalytic activity of Src, phosphorylation of the latter that mediated by CSK (a C-terminal Src kinase) downregulates Src activity (10). Counteracting the action of CSK, RPTPα (a receptor tyrosine phosphatase) is speculated to dephosphorylate Tyr-527 (11). Why Tyr-416 and –527 phosphorylation (pTyr-416 and pTyr-527) influences Src activity so differently? Based on the mutational studies and X-ray crystallographic analyses, the proposed model that delineates the structure-activity relationship of Src is described as the following. Due to the association between SH2 domain and pTyr-527 as well as SH3 domain and the SH2/kinase linker, Src is held in an inactive conformation (12, 13). Disruption of these intramolecular interactions will drive Src to become active and lead to pTyr-416 that in turn not only exposes the substrate binding site that has previously been shielded but also alters the conformation of Src that results in its activation (13).

4.1.1. Regulation of Src activity by mutation

The major difference between viral and cellular Src is the lack of Tyr-527 in v-Src (14). The absence of the intramolecular interaction between pTyr-527 and SH2 domain renders v-Src to be constitutively active. And the same interpretation can also be applied to illustrate why 527F-Src possesses high intrinsic kinase activity. Interestingly, a mutant *c-src* encoding a truncated human c-Src that ends with Tyr-530 (analogous to Tyr-527 in chicken c-Src) has been identified in colon carcinoma with high metastatic potential (15). Though this c-Src mutant still retains pTyr-530 in vivo, surprisingly, its kinase activity has not been deteriorated. This finding implicates that the absence of the amino acid residues C-terminal to Tyr-530 jeopardizes the intramolecular interaction between pTyr-530 and SH2 domain. Finally, the elevated kinase activity detected in c-Src devoid of SH3 domain echoes the inhibitory role of the association between SH3 domain and the SH2/kinase linker on Src activation (16).

4.1.2. Regulation of Src activity by modification other than pTyr-416 and -527.

Modification at some other residues could also modulate c-Src activity. It has been demonstrated that pThr-34, pThr-46 and pSer-72 occurred in mitosis (17) can destabilize the interaction between pTyr-527 and SH2 domain and facilitate the dephosphorylation of Tyr-527 mediated by a tyrosine phosphatase (18, 19). Tyr-138 and Tyr-213 are located within Src SH3 and SH2 domains respectively. In response to PDGF, they become phosphorylated and reduce the ligand-binding activity of these domains (20, 21). Conceivably, the inhibitory intramolecular interactions within the Src molecule will be disturbed and result in Src activation.

4.1.3. Regulation of Src activity by interacting with proteins

Accumulated evidence indicates that proteins with Src binding activity can disturb the intramolecular interaction and release the constraints, permitting activation of Src. By competing with pTyr-527 for the association of Src SH2 doamin domain, autophosphorylated PDGFR (22) and focal adhesion kinase (FAK) (23) have been demonstrated to lead to Src activation. Nef (24) and Sin (25) that individually bound to the SH3 domain of Hck and Src are also proved to activate Hck and Src respectively. Besides, by interacting with Src SH2 and SH3 domains, $p130^{Cas}$ can also result in Src activation (26). However, unlike Src SH2 and SH3 domains, proteins that are capable of binding with either the SH2/kinase linker or pTyr-527 have not been reported yet. It is believed that if such proteins did exist, they should be participated in Src regulation. In addition to SH2 and SH3 domains, the SH2/kinase linker and pTyr-527 that described, the kinase region turned out to be a new choice for Src regulatory molecules. Ma *et al.* has demonstrated that by interacting with the Src catalytic domain, Gαs and Gαi are able to alter the conformation of Src, allowing the substrates of Src easier to access the active site (27).

4.2. The targets of c-Src

To date, a variety of proteins have been identified and characterized as the substrates for c-Src. Since c-Src is important in cellular functions such as mitogenesis, migration and cell survival, thus, define the phosphorylation sites of the c-Src substrates and disclose their functional implication become an important issue. Among the numerous c-Src targets, those that are known to participate in signaling conveyed from EGFR/Src interaction will be described below.

4.2.1. Focal adhesion kinase

By immunological screening of the cDNA expression library with a panel of monoclonal antibodies directed against the putative v-Src substrates, a novel cytoplasmic tyrosine kinase that located in the focal contact is identified and thus designated as focal adhesion kinase (FAK) (28). FAK is a prototype for a family of tyrosine kinases including itself and PYK2 (or designated as CAK-β, RAFTK, or CADTK) (29). Unlike the more restricted expression pattern of PYK2, FAK is expressed in most tissues. In response to integrin engagement, FAK becomes activated and autophosphorylated at Tyr-397 (30). pTyr-397 is a strong binding site for Src SH2 doamin domain, therefore, c-Src is recruited to focal contact where it can access focal complex-associated proteins such as $p130^{Cas}$ and paxillin. Through complex with FAK, c-Src mediates the phosphorylation of FAK at Tyr-407, -576/577, -863, and –925 (31-34**).** While pTyr-576/577 can enhance the kinase activity of FAK (31), pTyr-925 provides the binding site for Grb2 SH2 domain and triggers the Ras signaling (33, 34). The formation and activation of this Src-FAK bipartite kinase complex turns out to be important in cell spreading, cell migration, and cell survival (29).

4.2.2. Clathrin

Clathrin, the major component of the coated pit

and vesicle, is a three-legged molecule consisting of three 190-kDa heavy chains (CHC), each with an associated 25-kDa light chain (35). Like a spider, 3 CHCs radiated from a central hub named triskelion. The antiparallel interaction of the CHCs from triskelions centered on adjacent vertices of the lattice determines assembly of the coated vesicle (35). Early studies indicate that clathrin is highly tyrosyl posphorylated in v-Src transformed cells and its Tyr-1477 is identified as a c-Src phosphorylated residue that located in a domain critical for clathrin assembly (36). How CHC phosphorylation promotes clathrin recruitment is an intriguing issue and needs further study.

4.2.3. Cortactin

Cortactin, a major tyrosyl phosphorylated protein in v-Src transformed cells, is an F-actin binding and cross-linking protein (37). The structural features of cortactin contain six and a half tandem repeats of 37 amino acids at its N-terminus, and an SH3 domain near its C-terminal end. As a F-actin cross-linker, overexpression of cortactin in fibroblasts does not display any changes in morphology or cellular proliferation, but does significantly enhance cell migration and invasive capacity in vitro (38). Enhanced tyrosyl phosphorylation of cortactin in cells overexpressing c-Src (39) as well as in cells deficient of CSK (40) implicates that cortactin is a substrate for c-Src. To date, Tyr-421, -466, and –482 have been defined as the sites phosphorylated by Src both in vitro and in vivo. Notably, tyrosyl phosphorylation of cortactin can result in its reduced F-actin cross-linking activity. And consistent with this notion is the abrogation of cellular movement in endothelial cells expressing tyrosyl phosphorylation deficient cortactin (41). Thus, through phosphorylating cortactin, c-Src can modulate cytoskeleton reorganization and cell migration.

4.2.4. Cas

Initially identified as a tyrosyl phosphorylated protein in cells transformed by v-Src or v-Crk (42), Cas (Crk-associated substrate) turns out to be a crucial adapter in signal transduction. Cas encompasses an SH3 domain at its N-terminus, followed by a strikingly long "substrate-binding domain" where YXXP repeats can be frequently found and served as the phosphorylation sites for tyrosine kinases (43). Once phosphorylated, they provide the binding sites for SH2 domain and pTyr binding (PTB) domains contained in a variety of proteins including Crk. It is established that by linking different molecules together, Cas is able to propagate and elicit signaling (44). And this can be evidenced by the increased activity of c-Src that interacts with Cas as described above (26). Studies of the phenotypes of Cas null cells indicate that though Cas is important in regulation of actin cytoskeleton, its absence does not hamper the formation of focal contacts (45). Besides, reduced cell mobility detected in Cas-deficient cells is similar to that observed for cells devoid of Src or FAK (46-48). Thus, these three proteins might work in concert to promote cell migration. Indeed, Src and FAK have been implicated in Cas phosphorylation in response to integrin engagement (23, 34, 47). Besides, by interacting with Src, FAK, and Crk, Cas is also involved in cell proliferation (26).

4.2.5. STAT3

Signal transducers and activators of transcription (STATs) are a family of latent cytoplasmic transcription factors that become activated and tyrosyl phosphorylated in response to growth factors and cytokines (49, 50). Due to their possession of SH2 domain, tyrosyl phosphorylation thus allows their dimerization. Homo- or heterodimers of STAT molecules then translocate into the nucleus where they recognize specific DNA sequences and activate transcription (51). The constitutive activation of STAT3 in v-Src transformed cells as well as the enhancement of its tyrosyl phosphorylation by the induced expression of Src (52, 53), it is established that STAT3 is a substrate for Src family kinases.

5. THE INTERACTION BETWEEN THE EGFR AND C-SRC

The interaction between receptor and nonreceptor tyrosine kinases is first described in NIH3T3 fibroblasts stimulated with PDGF. Three Src family members, c-Src, c-Yes and c-Fyn, are demonstrated to associate with activated, autophosphorylated PDGF receptor (PDGFR) that results in their activation (22). In addition to PDGFR, other receptor tyrosine kinases including colony-stimulating factor receptor (CSFR), EGFR, nerve growth factor receptor (NGFR), fibroblast growth factor receptor (FGFR) also convey their signaling through c-Src (54). In this paper, we are going to focus mainly on the interaction between the EGFR and c-Src and investigate its functional implication.

5.1. C-Src has a synergistic effect in both EGF-induced cellular growth and tumorigenesis

In an attempt to elucidate the role of c-Src in EGF-dependent signaling, artificially engineered cells that overexpress c-Src are generated. By comparison of the EGF-induced mitogenic effects between control and c-Src overexpressors, Luttrell *et al.* reported that c-Src could potentiate EGF-induced cell proliferation (55). And this potentiation required c-Src with intact myristylation, kinase and SH2 domains (56). The abrogation of EGF-induced DNA synthesis by microinjection of Src antibody also verified the participation of c-Src in EGF-dependent mitogenesis (57). As an extension, a series of experiments were performed to corroborate the involvement of c-Src in EGF-induced tumorigenesis. Maa *et al.* have demonstrated that cells overexpressing both EGFR and c-Src resulted in a synergistic increase in EGF-induced DNA synthesis, growth in soft agar, and tumor formation in mice, as compared to cells overexpressing EGFR or c-Src alone (58). Obviously, c-Src cooperated with the EGFR in both proliferation and tumorigenesis.

5.2. The association between Src SH2 and the autophosphorylated EGFR

By binding with the EGFR and augmenting its activity was one of the mechanisms underlying the above synergy. However, unlike the readily detected complex formation between autophosphorylated PDGFR and c-Src, tremendous efforts have been dedicated to studying the EGF-dependent complex formation between c-Src and

EGFR. And only by utilizing cells overexpressing both EGFR and c-Src could this endeavor be fulfilled (58). It was plausible that the binding between these two kinases might be of a low affinity or short-lived. Consistently, only when low-stringency detergents were employed could the EGFR/c-Src complex be visualized (58). What is the nature of the interaction between EGFR and c-Src? Many lines of evidence indicated that autophosphorylated EGFR could form heteromeric complexes with a variety of signaling proteins via pTyr-SH2 interactions. Since Src contains an SH2 domain, thus, it becomes the most likely region to contact with EGFR. Indeed, *in vitro* affinity precipitation as well as the "far Western" overlay experiment confirmed this speculation (59). To identify the tyrosine residues whose phosphorylation rendered Src SH2 domain binding ability, a subset of peptides corresponding to the potential tyrosyl phosphorylation sites in the C-terminal tail of EGFR were screened. Peptides encompassing Tyr-992, -1086, -1101, and –1148 were demonstrated to retain Src SH2 domain binding activity (60). It was noteworthy that except Tyr-1101 (the c-Src mediated site on EGFR, see below), the remaining three were EGFR autophosphorylation sites.

5.3. The events occurred accompanied with the formation of EGFR/c-Src complex

Increasingly compelling data indicated the activation of c-Src in cells stimulated with EGF. Since EGFR autophosphorylated tyrosine residues can serve as the ligands for Src SH2 domain, thus, their competition with Src pTyr-527 for Src SH2 domain binding destabilizes the inactive conformation of Src and causes its activation. Activated c-Src then mediates EGFR phosphorylation further. While Tyr-891 and –920 are detected on the EGFR from DLD-1 and MCF7 cells (61), Tyr-845 and –1101 are identified and characterized as the Src-mediated sites both *in vitro* and *in vivo* in murine fibroblasts overexpressing EGFR and c-Src both. Unlike Tyr-1101 whose phosphorylation is not absolutely dependent on c-Src kinase activity, phosphorylation of Tyr-845 is completely attributable to c-Src (62).

Phosphorylation of these EGFR nonautophosphorylation residues plays significant roles in EGFR-mediated mitogenesis and oncogenesis. Although lack of functional implication, phosphorylation of Tyr-891 and Tyr-920 confers the binding sites for the SH2 domains of Src and the p85? regulatory subunit of PI-3K, respectively (61). As a homologue of Src Tyr-416 whose phosphorylation leads to activation of c-Src, pTyr-845 results in EGFR hyperactivation that correlates with elevated level of tyrosyl phosphorylated EGFR substrates such as PLC? and Shc, and promotes growth and tumor formation (58). Consistently, reduced EGF-induced proliferation is detected in cells expressing 845F-EGFR (59). Besides, the absence of Tyr-845 phosphorylated EGFR in cells co-expressing EGFR and kinase-impaired c-Src correlates with reduced growth and tumor development (62). These studies support the notion that pTyr-845 promotes the mitogenic and oncogenic capacity of EGFR.

6. CELLULAR EVENTS THAT REQUIRE THE COOPERATION BETWEEN EGF-ACTIVATED EGFR AND C-SRC

As described above, we have discussed the nature of the interaction between the EGFR and c-Src and its implication in proliferation and tumorigenesis. However, the interplay between these two kinases is also involved in a variety of cellular events such as EGFR endocytosis, cell migration and cell survival. The participation of c-Src in these EGFR-dependent functions will be discussed below.

6.1. EGFR endocytosis

According to previously described association of c-Src with endosome memebrane (63), one may speculate the role of c-Src in EGFR trafficking. Indeed, c-Src increases the rate of endocytosis of EGF-EGFR complex until the endocytotic apparatus is saturated and becomes rate limiting (64). In agreement with this finding, PP2 (a tyrosine kinase inhibitor that was more specific for Src) is reported partially blocking EGFR exit from caveolae, the first step in EGFR internalization.

Convincing evidence indicates that EGFR-mediated endocytosis is associated with recruitment of clathrin to the plasma membrane. And increased tyrosyl phosphorylation of clathrin heavy chain (CHC) is correlated with its ability of membrane redistribution in v-Src transformed cells. To study how EGF binding modulates the properties of clathrin that lead to EGFR endocytosis, Wilde *et al.* (36) demonstrate that in response to EGF binding, activated EGFR causes the activation of c-Src, which phosphorylates CHC at Tyr-1477 and induces clathrin redistribution to the cell periphery. The effect of c-Src on the EGFR endocytic machinery is further supported by the delay of EGF uptake in cells lacking Src kinase or cells treated with a specific Src inhibitor. Thus, based on this model, we tempt to speculate that the increased internalization rate of EGFR observed in c-Src overexpressors might be attributable to the enhancement of CHC phosphorylation mediated by c-Src.

6.2. EGF-induced cell migration

The actin cytoskeleton not only provides the structural framework to maintain cellular shape but also plays a pivotal role in many cellular functions including cell mobility. In response to PDGF stimulation, c-Src is activated and translocated from inner plasma membrane to cytoskeleton where it mediates the phosphorylation of its substrates that results in alteration of the cytoskeletal architecture and increased cell migration (65). The correlation between the level of c-Src expression and the extent of the PDGF-induced migration implicates the importance of c-Src in cell mobility (65). And this notion is further confirmed by the requirement of c-Src in FGF-1-mediated cell migration. It is well established that cortactin is a modulator for the actin cytoskeleton since its F-actin cross-linking activity is attenuated by Src-mediated phosphorylation (37). Therefore, its failure of being tyrosyl phosphorylated in FGF-1-stimulated Src knockout cells is attributable to impaired cell migration detected (66). In addition to PDGF and FGF-1, c-Src is also demonstrated to

regulate EGF-induced actin reorganization in cells overexpressing c-Src (67). Furthermore, significant increase in c-Src activity is observed in highly metastatic cells as compared to cells with poor metastatic potential (68). And this metastasis-associated activation of c-Src is regulated by a variety of receptor tyrosine kinases including EGFR (68).

The implication of c-Src in cell migration can further be substantiated by the migration defect observed in FAK null cells. Due to FAK can promote focal-contact turnover, thus FAK-/- cells form an increased number of cell-substractum contacts that retrain cell motility (46). By accelerating focal contact turnover, the kinase activity of Src family members is able to increase cell migration and compensate for the loss of FAK in fibronectin-stimulated cell migration (69). Importantly, Src/FAK interactions are also demonstrated to be crucial for EGF-stimulated cell motility (70). As a receptor-proximal component of RPTK and integrin signaling, Sieg *et al.* demonstrate the recruitment of FAK to sites of EGFR and integrin clustering in intact cells (71). And this event can further provoke focal-contact remodeling and initiate migratory signals. To our surprise, it is the pTyr-397, but not the kinase activity, of FAK is needed in EGF-stimulated cell mobility (71). Therefore, via the pTyr-397 and Src SH2 domain interaction, formation of a productive signaling complex between FAK and Src family members seem to be indispensable for both integrin- and EGF-induced cell migration.

6.3. EGF-dependent cell survival

It is well established that cells stimulated with growth factors exhibit not only increased proliferation but also suppressed cell death (72). And abrogation of apoptosis seems to be prerequisite for growth factors to induce mitogenesis. Since members of EGFR can homo- and heterodimerize and relay the signals, therefore, many events attributed to EGFR are actually mediated by heterodimer partners. In an attempt to get rid of the interference from the endogenous ErbB proteins, the murine hemopoietic cell line BaF/3, which lacks all the EGFR family members becomes an ideal system (73). By expressing wt- or various EGFR mutant alone in BaF/3 cells, signaling mechanisms intrinsic to the EGFR or its mutant can thus be analyzed. Interestingly, with this assay system, Walker *et al.* demonstrate that ligand binding to EGFR can provoke both mitogenic and survival signals. Remarkably, activation of Src family members is involved in EGF-initiated anti-apoptosis (73).

Of potential importance is that a reduction of expression or activity of Src in cells overexpressing EGFR results in apoptosis as well as a concomitant decline in tyrosyl phosphorylated STAT3 and the amount of Bcl-X_L, an anti-apoptotic protein (74). Since STAT3 mediates c-Src-dependent Bcl-X_L transcription, therefore, c-Src not only acts as a mitogenic molecule, but also as an anti-apoptotic element. Conceivably, combination of these two properties renders c-Src to be oncogenic.

7. THE INTERPLAY BETWEEN EGFR AND C-SRC IN THE ABSENCE OF EGF

There is now overwhelming evidence indicating that stimuli other than the cognate ligands can provoke EGFR tyrosyl phosphorylation (75). And unlike its traditional role, EGFR acts as a switch point in the signaling network to regulate various cellular functions. Among all the reported growth factor-independent pathways, only those in which EGFR tyrosyl phosphorylation may be directly or indirectly attributable to Src family kinases will be described below.

7.1. Integrin activation

Adhesion of cells to the extracellular matrix (ECM) is important in a variety of biological processes including proliferation, differentiation and migration (76). Engagement of the integrins, the cell surface adhesive receptors composed of α and β subunits, can stimulate multiple signaling pathways that regulate the above cell physiology. Due to the absence of the intrinsic kinase activity and the short cytoplasmic domains of both integrin subunits, assembly of transduction machinery encompassing integrins and other proteins has been proposed to promote intracellular signaling. FAK and caveolin are the two molecules that have been reported to cooperate with integrins under this context (29). Interestingly, integrins induce tyrosyl phosphorylation of EGFR in the absence of its natural ligand. This event depends on the transient *bona fide* activation of the EGFR's intrinsic kinase activity and is involved in proliferation and cell survival in response to extracellular matrix (77). However, the underlying mechanisms remain to be defined. Since (1) FAK is activated and autophosphorylated at Tyr-397 when cells attach to ECM, (2) pTyr-397 of FAK creates a docking site for Src SH2 domain and results in Src activation, and (3) Src can mediate EGFR phosphorylation at Tyr-845 and cause EGFR hyperactivation, we tempt to speculate that the crosstalk between integrin and EGFR pathways may be mediated by Src family kinases. Experiments that show the abolishment of integrin-initiated EGFR tyrosyl phosphorylation in cells expressing kinase-defective Src or in cells pre-treated with Src-specific inhibitor (i.e. PP1) can clarify this issue.

7.2. Membrane depolarization

Tyrosyl phosphorylation is an important post-translational modification observed in various proteins participated in multiple signaling pathways. Studies aimed to address the effect of membrane depolarization on the profile of tyrosyl phosphorylated proteins reveal the increased level of EGFR tyrosyl phosphorylation that leads to MAPK activation in response to calcium influx (78). Similar results are also obtained when PC12 cells were treated with calcium ionophore (79). Obviously, EGFR signal transduction can be stimulated by ion channel activation. It is noteworthy that PYK2, a FAK family member, becomes activated and tyrosyl phosphorylated in response to calcium influx (80) and is capable to interact as well as activate Src (81). Therefore, it is likely that through tyrosine kinase complex PYK2/Src, membrane depolarization-induced calcium influx can evoke EGFR

signal transduction.

7.3. Oxidative stress stimulation

Abundant evidence indicates that reactive oxygen species (ROS) including H_2O_2 are important chemical mediators that regulate signal transduction. Exposure of H_2O_2 to endothelial cells results in transphosphorylation and activation of EGFR that finally leads to JNK activation (82). And interestingly, these H_2O_2-initiated processes are Src dependent (82). Since Src kinase activity can be induced by a variety of oxidative events (83, 84) and Src can transphosphorylate EGFR and cause its hyperactivation, thus, it is plausible that H_2O_2-induced JNK activation involves the previously described Src-mediated EGFR phosphorylation.

7.4. Epoxyeicosatrienoic acid stimulation

As the products of cytochrome P450 epoxygenase-mediated epoxidation of arachidonic acid and the proposed second messengers for growth factors and hormones in the proximal tubule, the epoxyeicosatrienoic acids (EETs), have been implicated in a variety of cellular functions including regulation of mitogenesis (85). To one's surprise, EET exposure results in not only c-Src activation, but also the complex formation between the EGFR and c-Src (86). Thus, this intriguing observation implicates that through activation of c-Src, the lipid mediators are also capable to utilize the EGFR as a scaffold for transmitting mitogenic signals.

7.5. GPCR activation

Structurally consisted of seven transmembrane domains with extracellular N-terminus as well as intracellular C-terminus, G protein-coupled receptors (GPCRs) comprise the largest group of cell surface receptors. By stimulating with a diverse array of external agonists such as neurotransmitters, hormones, ordants, and light, GPCRs associated with the heterotrimeric G proteins that lead to the exchange of GDP for GTP bound to G_α and the dissociation of $\beta\gamma$ heterodimers. Then, this dissociated G_α and $G_{\beta\gamma}$ trigger intracellular signaling by affecting effectors including adenyl cyclases, phospholipases, ion channels and kinases. Based on their sequence and functional similarities, the α subunits of G proteins are divided into four classes: α_s, α_i, α_q and α_{12}. Besides, different β and γ subunits are also identified. Therefore, G proteins subunit composition, their coupling specificity to the receptor, and the employed downstream effector make the GPCRs the most diverse and complex signal transduction system in eucaryotic cells.

It has been perceived that through agonist-stimulated dissociation of trimeric G proteins, activated GPCRs transmit their signals via small molecule second messengers. However, considerable evidence demonstrates that EGFR is an important signaling element in GPCR-mediated Ras/MAPK signaling cascade and mitogenesis since overexpression of a dominant negative EGFR or exposure of EGFR specific inhibitors to cells can abrogates these effects (87). Conceivably, through intracellular crosstalk, ligand-independent transactivation of EGFR is required for GPCR mitogenic signaling. Due to inhibition of Src activity ablates GPCR-induced EGFR transactivation, the involvement of c-Src in this process is verified (88). To date, mediators in GPCR signaling pathway that have been proposed and demonstrated to activate c-Src in response to agonists will be summarized and described below.

7.5.1. G_s and G_i

In an attempt to study the effect of G proteins on c-Src, the enzymatic activities of downregulated, Tyr-527 phosphorylated c-Src incubated with various purified G proteins are compared. Interestingly, only $G_{\alpha s}$ and $G_{\alpha i}$ directly stimulate c-Src catalytic activity (27). Further delineation of the mechanisms reveals that $G_{\alpha\sigma}$ and $G_{\alpha i}$ bind to c-Src catalytic domain via their switch II region and cause a conformational change of c-Src that lead to its activation. These findings establish c-Src as a novel direct effector of $G_{\alpha s}$ and $G_{\alpha i}$. Thus, activation of c-Src in cells stimulated with agonists whose receptors coupled to G_s or G_i (i.e. lysophosphatic acid, endothelin-1) is likely mediated by this mechanism.

7.5.2. b-Arrestin

EGFR transactivation has been reported in isoproterenol-stimulated cells (89). Previous studies indicate that following agonist stimulation, βARK mediates the phosphorylation of β_2 adrenergic receptor (β_2 AR) and renders its association with β-arrestin. Since c-Src is recruited to the GPCR/arrestin complex and becomes activated (90), thereby, we envision that c-Src executes the EGFR transactivation initiated by β_2 AR.

7.5.3. G_q

Stimulation of intestinal epithelial cells with carbachol (CCh), an agonist for the G_q-coupled muscarinic M3 receptor (91), elicits EGFR transactivation (92). Due to G_q activates PLC and causes elevation in intracellular Ca^{+2}, therefore the mechanism responsible for membrane depolarization-induced EGFR phosphorylation should be applicable to CCh-initiated EGFR transactivation. Therefore, Ca^{+2} influx, PYK2 and Src are all involved in this G_q-mediated signaling pathway (93).

7.5.4. G_{bg}

Studies performed in cells artificially overexpressing $G_{\beta\gamma}$ subunits exhibit the activation of c-Src, and inhibition of Src family kinase activity by expression of a dominant negative c-Src abolishes $G_{\beta\gamma}$-mediated EGFR phosphorylation (94). Thereby, $G_{\beta\gamma}$ is implicated in Src activation. Furthermore, PI-3Kγ, a PI-3K isoform, has been implicated as an intermediate in the process of $G_{\beta\gamma}$-induced Src activation (95).

8. THE IMPLICATION OF EGFR-SRC INTERACTION IN HUMAN TUMORS

Overexpression of EGFR and/or c-Src has been reported in a variety of human tumors. Considering the biology synergy between these two kinases as well as the involvement of c-Src in EGFR-mediated proliferation, tumorigenesis, cell migration and antiapoptosis,

overexpression of either kinase can deteriorate the cellular transformation not to mention overexpression of both. For detailed information about the study of EGFR and c-Src in human tumors, readers are encouraged to read Biscardi's review (9). Of potential importance, the interplay between EGFR and c-Src can also occur in response to a variety of stimulation including GPCR agonists other than EGF. Substantial evidence corroborates the contribution of GPCR-initiated signaling pathways in aberrant cell proliferation. The oncogenes *gsp* and *gip*2 discovered in human endocrine tumors are the genes encoding GTPase-impaired $G_{\alpha s}$ and $G_{\alpha i}$ (96, 97) and their artificial expression in NIH3T3 cells are highly tumorigenic (98). Since $G_{\alpha s}$ and $G_{\alpha i}$ can directly interact with c-Src and induce its activity, one might envision that the EGFR signaling pathway evoked by EGFR/Src interaction can propagate constitutively in the tumors harboring oncogenic mutations in G_{α} genes. Similarly, activation of G_q and $G_{\beta\gamma}$ in cells exposed to various stimuli is also capable to induce EGFR transaction and transmit EGFR signaling. Thus, the convergence of EGFR/Src and GPCR signaling pathways not only introduce complexity into signaling network, but also provides novel insights in oncogenesis of human tumors.

9. CONCLUSIONS AND FUTURE PERSPECTIVES

EGFR and c-Src are originally viewed as the representatives for receptor and nonreceptor tyrosine kinases respectively. Substantial evidence implicates the involvement of c-Src in EGFR-induced signaling. Indeed, c-Src has been verified as an important mediator in EGFR endocytosis and EGFR-initiated cellular functions such as mitogenesis, tumorigenesis, cell migration, and cell survival. Consistently, cells artificially overexpressed these two kinases exhibit enhanced mitogeneic effects and increased tumor formation relative to cells overexpressing either kinase alone. Interestingly, this biological synergy between the EGFR and c-Src is associated with the formation of a heterocomplex between these two kinases. The accompanied tyrosyl phosphorylation of EGFR on the nonautophosphorylation residues might result in its increased kinase activity and enhanced signaling transduction. However, to date, phosphorylation of EGFR at these Src-mediated sites has not been directly detected in human cancers yet. Confirmation of their presence in a variety of human tumors thus becomes an imminent goal to substantiate its role in human tumorigenesis. Besides, if the extent of Src-mediated EGFR phosphorylation correlated with the progression stage of tumors, then its specific antibodies might be applied as novel markers for tumor diagnosis. Furthermore, peptidomimetics (99-101) corresponding to these Src-mediated sites on EGFR can be utilized as drugs for cancer treatment. All these goals merit further investigation.

The family of GPCR comprises more than 1000 members that can be activated by a diverse array of stimuli that contribute to a variety of functions including mitogenesis, chemotaxis, embryogenesis, exocytosis, neurotransmission, and differentiation. Recent advance reveals the interplay between the EGFR and c-Src in response to a plethora of GPCR agonists. Several lines of evidence indicate that EGFR/Src cross-talk not only participates in GPCR-initiated mitogenesis, but also involves in prevention of carbacol-stimulated chloride secretion (93). Since EGFR/Src interplay has been reported in diverse systems, its functional outcome might be different depending on the cellular context and deserved intensive study. Also, we believe that the widespread occurrence of EGFR/Src interaction in diverse cell types in response to EGF or non-EGF stimulation discloses its integral role in cell physiology.

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Abbreviations: pTyr: phosphorylated Tyrosine, EGFR: epidermal growth factor receptor, STAT: signal transducer and activator of transcription, GPCR: G protein-coupling receptor, PI-3K: phosphatidylinositol 3-kinase, PI-4,5-P_2: phosphatidylinositol-4,5-bisphosphate

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