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SPECIFICITY IN SIGNALING BY c-YES

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1. ABSTRACT

c-Yes and c-Src are the two most closely related members of the Src family of nonreceptor tyrosine kinases. Although there is much evidence to support redundancy in signaling between these two kinases, there is also a growing body of evidence to indicate specificity in signaling. In this review, we summarize c-Yes, its potential functions and its ability to modulate signals that are distinct from c-Src.

2. INTRODUCTION AND SIGNIFICANCE

p62 c-Yes and p60 c-Src are two of the nine members of the Src family of non-receptor tyrosine kinases (c-Src, c-Yes, Fyn, Lyn, Lck, Hck, Blk, Fgr, and Yrk). Src family kinases are linked by a common structural architecture, each with four Src homology domains (SH1-4) and a Unique domain. The Src family kinases have been implicated in signaling pathways that regulate a vast array of cellular processes, including cytokine and growth factor responses, cytoskeleton dynamics, cell proliferation, survival and differentiation (1). While c-Src has been extensively studied, comparatively less is known of the biological functions of c-Yes. c-Yes and c-Src are two of the most widely expressed and homologous members of the Src family. Both kinases are present in many of the same tissues and are activated in response to many of the same stimuli. The frequent activation of c-Src and c-Yes in human cancers, coupled with the tumorigenic potential of their viral homologues, v-Src and v-Yes, suggests that they may contribute to the onset or progression of the malignant phenotype. Thus, c-Src and c-Yes represent potential targets for rational drug design. In order to optimize the value of these kinases as therapeutic targets, it will not only be important to understand the biological functions of c-Yes and c-Src, both in normal and cancerous tissues, but also to understand how they are capable of sending specific signals. As the biology and functions of c-Src have been extensively studied and reported, this review will focus on the functions of c-Yes and how c-Yes may be able to participate in specific signaling pathways. Special emphasis will be on differences between c-Yes and cSrc signaling.

3. V-YES AND V-SRC

The Yes kinase was originally discovered as the oncogenic protein encoded by the Yamaguchi 73 and Esh sarcoma viruses (2-6). Initial interest in v-Yes arose as a result of its similarity to v-Src. Both oncoproteins are tyrosine kinases capable of inducing sarcomas in chickens and transforming fibroblasts in culture (5,7). v-Src and v-Yes are 82% similar, with particularly strong homology

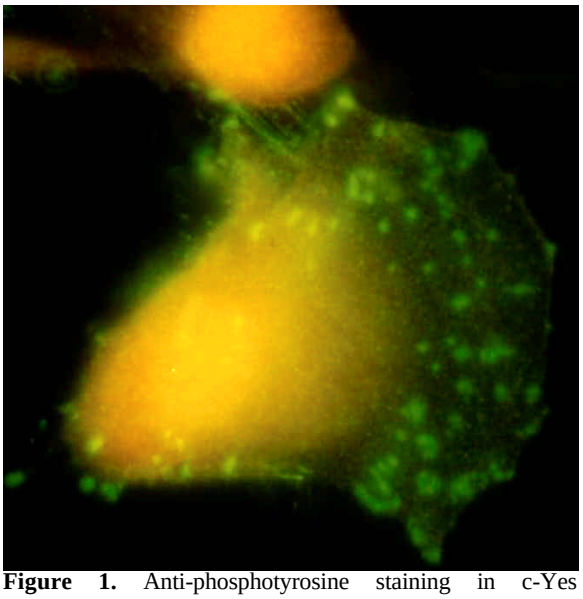

Figure 1. Anti-phosphotyrosine staining in c-Yes overexpressing MDCK cells. Overexpression of c-Yes correlates with increased cellular tyrosine phosphorylation in distinct cellular structures that resemble focal adhesions. The cYes oncoprotein was overexpressed in MDCK cells, fixed and labeled with anti-phosphotyrosine (green).

(90%) within the tyrosine kinase domain (8). The amino acid similarity between the two kinases allows immunorecognition of v-Yes by monoclonal antibodies raised against v-Src (9), and the two proteins release overlapping subsets of tyrosine-phosphorylated peptides upon tryptic digestion and two-dimensional electrophoresis (10). In addition to their structural homology, biochemical and functional similarities have been observed between v-Src and v-Yes in chicken embryo fibroblast culture. Both proteins fractionate with the detergent-insoluble cytoskeleton and localize to focal contacts, where the cell and extracellular matrix meet (Figure 1) (11,12). Many of the same proteins are phosphorylated in cells transformed by either v-Src or v-Yes (13,14). v-Src and v-Yes also appear to utilize shared downstream signaling pathways, as both proteins induce activation of PI3 kinase and require the activator protein 1 (AP-1) transcription factor complex (composed of heterodimers of various isoforms of the c-Jun and c-Fos transcription factors) for cell transformation (15,16).

Homology between v-Src and v-Yes can be extended further to include the mechanisms by which these kinases have been rendered transformation-competent. Both kinases are constitutively active due to loss or replacement of sequence in their carboxy-termini (17). Src family kinases contain a conserved tyrosine residue (Tyr^{527} in c-Src, Tyr^{535} in c-Yes) in their carboxy-terminal tail sequence. This tyrosine residue is phosphorylated by members of the Csk (C-terminal Src kinase) family and forms an intramolecular interaction with the SH2 domain (18). The SH2/tail interaction forms the linchpin of the "closed" or "assembled" conformation (19,20). The SH3 domain contributes to the stability of the closed conformation through formation of an intramolecular interaction with the kinase linker, a short stretch of amino acids bridging the SH2 and kinase domains (19,20). In the assembled state, the conformation of the kinase active site is such that ATP and substrates are prevented from entering, resulting in repressed kinase activity (21). Although recent evidence indicates that autophosphorylation within the active site (Tyr^{416} in c-Src, Tyr^{424} in c-Yes) may allow kinase activity in the presence of phosphorylation at the regulatory tyrosine (21,22), Src family kinases must be released from the closed conformation in order to achieve their full transforming potential. The ability of v-Src, v-Yes, Src^{527F} (in which Tyr^{527} is mutated to Phe), and various SH3 and SH2 domain mutants of c-Src to transform cells illustrates this point (17,23,24).

4. C-YES EXPRESSION PATTERNS: COMPARISON AND CONTRAST WITH C-SRC

Phylogenetically, c-Yes expression has been confirmed in simple vertebrates such as Xenopus (25) and teleost fish (26). Although there is a single report of a c-Yes homolog in drosophila (27), this observation was not verified. The potential absence of c-Yes from Drosophila is interesting given that there is a c-Src ortholog present in fly (28-30). Thus, it is possible that c-Yes may have evolved along a vertebrate line, although this has not been examined in great detail. The Xenopus and teleost sequences, as well as the avian, murine and human sequences of c-Yes have been deposited in the GenBank database. The accession numbers for c-Yes sequences in GenBank are: Rat (BAB21451), Xenopus (P10936), human (P07947), chicken (P09324), dog (Q28923), mouse (Q04736) and fish (P27447).

The YES gene was discovered as the cellular homologue of the v-*yes* oncogene (31-33). Initial reports localized the YES gene to chromosome 18 at locus q21.3 in humans (34-35). However, a refinement of this observation was reported via sequencing of YAC's, which indicated that the YES gene localizes nearby, to chromosome 18p11.32 (36). There does appear to be a pseudogene for YES, sometimes referred to as c-*yes*-2, found on chromosome 22q11.2 (37). Interestingly, the c-Yes related kinase, Yrk, has been reported to be present only in fowl (38). The significance of c-*yes*-2 is unknown; however, it may be noteworthy that c-yes-related sequences may be present as "fossils" in the genome. The YES gene encodes a 62 kDa tyrosine kinase that displays extensive similarity with the c-Src protein and, like c-Src, is expressed across a wide range of normal cells and tissues (39). c-Yes expression is found in epithelial tissues (lung, kidney, gastrointestine, liver, skin, etc.), connective tissue, vascular endothelial cells, and smooth muscle; expression levels are particularly high in platelets, neurons (particularly Purkinje cells of the cerebellum), and spermatid acrosomes (35,40-45). While c-Src is also expressed in many of these tissues, the expression patterns of the two proteins are not identical, thus providing evidence that c-Yes may perform unique functions. Mapping of the c-*yes* promoter region revealed a sequence that was reminiscent of other oncogenes and likely dictates its broad expression pattern (46). Gessler and Barnekow observed that the c-*yes* and c-*src* genes were differentially expressed during chicken embryogenesis

(47). In these studies, c-*src* message was expressed at high levels in brain throughout embryogenesis and displayed an age-dependent decrease in muscle tissue. Message levels of c-*yes* were initially low in brain, muscle, and heart, but increased throughout embryogenesis. Bixby and Jhabvala noted differential developmental expression of c-Src and c-Yes in the brain (48). Peak c-Src expression was observed between days 10 and 12, followed by a gradual decrease in expression, whereas c-Yes expression did not peak until day 20 and remained at high levels into adulthood. Sudol *et al.* noted a similar age-dependent decrease in chicken cerebellar c-Src expression, in contrast to increased c-Yes expression (49). Collectively, these data imply that c-Src and c-Yes may perform different functions in the developing embryo and adult.

5. INVOLVEMENT OF C-YES IN CELLULAR SIGNALING PATHWAYS

The ability of v-Yes to induce cellular transformation suggested that c-Yes may be involved in the control of cell proliferation and cell shape changes associated with the transformed phenotype. However, the fact that many of the cells in which c-Yes is highly expressed are post-replicative (i.e. neurons, platelets) and fully differentiated suggests a possible role in differentiation or the facilitation of cell-specific processes. In fact, evidence exists for involvement of both c-Yes, and Src family kinases in general, in a variety of signal transduction pathways that regulate cell division, differentiation, survival, motility, adhesion, spreading, and vesicular transport (1). In serum-starved NIH 3T3 fibroblasts, serum stimulation results in activation of c-Yes, along with c-Src and Fyn, during the G_2/M cell cycle transition (50). c-Yes and c-Src are both activated during proliferation and differentiation of rat trophoblast cells (51). In keratinocytes, calcium treatment results in decreased c-Yes kinase activity, coinciding with a shift from cellular proliferation to differentiation (52,53). Upon calcium treatment of epithelial cells, c-Yes, c-Src, and Fyn localize to sites of cell/cell contact, where their kinase activity is necessary for disruption of cell/cell junctions (54,55). In cultured neuronal cells, c-Yes, c-Src, and Fyn are concentrated and activated in growth cones (56). c-Yes, like c-Src, is also activated in response to stimulation of receptor tyrosine kinases (PDGF-R, CSF-1-R, Neu, FGF-R, Sky, Flt 1) (57-62), G-protein coupled receptors (angiotensin II receptor, thrombin receptor, endothelin receptor) (63-65), cytokine receptors (oncostatin M, interleukin-11, GM-CSF) (66-68), the FceRI receptor, and the polyimmunoglobulin (pIg) receptor (69-70). Both c-Src and c-Yes will induce phosphorylation of common substrates (13-14). In addition, both c-Src and c-Yes will bind to common substrates, such as p120GAP or p38 (71-72). Collectively, these data indicate that c-Src and c-Yes have common and overlapping signaling functions. As previously hypothesized, it is quite likely that both c-Src and c-Yes function as amplifiers of receptor signaling (73).

6. SPECIFICITY IN SIGNALING BETWEEN C-YES AND OTHER SRC FAMILY KINASES

While the above list represents what is likely to be only a sampling of the pathways in which c-Yes is involved, it illustrates the potential for diversity in the function of this kinase. However, as c-Src and other Src family kinases are activated in response to many of the same cellular signals, it is not always clear where c-Yes performs unique functions and where it functions redundantly with other Src family kinases. The answer appears to vary from system to system. c-Yes may function redundantly with c-Src and Fyn in serum-induced fibroblast cell cycle progression. Microinjection of antibodies immunoreactive against all three proteins induces a cell cycle block at the G_2/M phase transition, whereas microinjection of a c-Src-specific antibody does not interfere with cell cycle progression unless no other Src family kinases are expressed (50). c-Yes may also function redundantly with c-Src and Fyn in PDGF-receptor signaling (74-75).

The phenotypes of the Src family kinase knockout mice provide further evidence for shared functions between Src family members. Whereas c-*src* -/-, *fyn* -/-, and c-*yes* -/- individual knockout mice develop distinct phenotypes, they typically survive for extended periods after birth. The loss of both the c-*src* and c-*yes* genes, however, invariably leads to perinatal lethality (76). Mice lacking both the *fyn* and c-*yes* genes develop degenerative renal damage, leading to diffuse segmental glomerulosclerosis (76). Interestingly, mice harboring a disruption of the c-*yes* gene do not display significant abnormalities in the cells or tissues in which c-Yes is most highly expressed (platelets, neurons, and spermatid acrosomes), suggesting that c-Src and/or Fyn are capable of compensating for the lack of c-Yes activity in these cells.

Despite the evidence for functional overlap, data also exist that indicate c-Yes- specific signaling. Much of this data is obtained from studies of c-*src* -/- mice and cells derived from them. Several of these studies indicate that c-Yes is unable to compensate for c-Src in processes that are dependent on the dynamic regulation of the actin cytoskeleton. Despite the presence of c-Yes, osteoclasts from c-*src* -/- mice are unable to reabsorb bone, resulting in an osteopetrotic phenotype (77). This deficiency correlates with an inability of the osteoclasts to form membrane ruffles and actin ring structures (78). Additionally, cells from the c-*src* -/- mice fail to spread properly on fibronectin and demonstrate reduced motility (79,80). Neuronal cells derived from c-*src* -/- mice are deficient in neurite extension on NCAM-L1 (81). Mice lacking the c-*src* gene also fail to develop Middle T antigen-induced mammary tumors, whereas c-*yes* -/- mice develop mammary tumors at a normal rate (82). Despite the apparent deficiency of c-Yes in eliciting these actin-dependent processes, active variants of c-Yes are localized to detergent insoluble cytoskeletal fractions (11,191). It is possible that c-Yes may associate with and/or regulate other components of the cytoskeleton. Indeed, it was observed by Ciesielski-Treska *et al.* that c-Yes co-localizes specifically with vimentin intermediate filaments in amoeboid microglia (83). Interestingly, c-Yes associates with adherens junctions (54). Nusrat et al. demonstrated that the tight junction associated protein, occludins, uniquely associates with c-Yes and not c-Src (84).

Occludins are transmembrane proteins that regulate extracellular interactions in tight junctions. Activation of Raf-1 is associated with down-regulation of occludins expression (85). Interestingly, chimeric constructs of c-Yes/Src^{527F} that contain the c-Yes SH3 domain (Y3^{527F}) fail to activate Raf-1, while chimeric constructs that contain the c-Src SH3 domain can activate Raf-1 (191). These data are consistent with a role for activated c-Yes as a binding signaling partner for occludins, while activation of c-Src might be predicted to direct activation of Raf-1 and downregulation of occludins. Thus, it is tempting to speculate that activated c-Yes may play a key role in participating in the maintenance of tight junction interactions, whereas activation of c-Src is known to cause their dissociation.

The inability of c-Yes to function as a "molecular backup" for c-Src is not limited exclusively to regulation of the actin cytoskeleton. In rat aortic smooth muscle cells, c-Src is specifically necessary for angiotensin II-induced phosphorylation of p120 Ras GAP and p190 Rho GAP (86). In rat ventricular myocytes, c-Yes, c-Src, and Fyn are activated in response to endothelin, however, only c-Src is able to drive elevated transcription from the atrial natriuretic peptide promoter, a hallmark of hypertrophy in ventricular cardiomyocytes (65). c-Src and c-Yes also differ in their respective abilities to participate in the hypoxia response. Of the three ubiquitously expressed Src family members, c-Src exclusively is activated under hypoxic conditions (87). This is in agreement with recent data that indicates that Src^{527F}/c-Yes chimeras with the c-Yes SH4 and Unique domains are deficient in upregulation of Heme Oxygenase 1 expression, as normally occurs in response to cellular stresses, including hypoxia (88).

Specificity between c-Yes and c-Src is not limited to pathways in which c-Yes fails to compensate for c-Src. While both c-Yes and c-Src are able to induce phosphorylation of the adaptor protein Cbl when overexpressed in COS cells, only c-Yes is able to efficiently co-immunoprecipitate with Cbl (89). During trophoblast cell proliferation, c-Yes associates with several tyrosine-phosphorylated proteins that are not found in complex with other Src family kinases (51). c-Yes is inactivated in keratinocytes upon calcium and phorbol ester treatment (53). In this system, c-Yes inactivation correlates with association with unknown proteins of 110 and 220 kDa (53). In 3T3 L1 mouse pre-adipocytes, both c-Src and c-Yes are activated upon IL-11 stimulation; however, only c-Yes is detected in a receptor-associated signaling complex including JAK2, PP2A, and gp130 (67). In angiotensin II-treated pulmonary vein endothelial cells, of the three ubiquitously expressed Src family kinases, only c-Yes induces phosphorylation of and association with the calcium-sensitive kinase Pyk2 (90). Unfortunately, the importance of co-association between c-Yes and the proteins mentioned above remains unknown. However, they do illustrate that c-Yes is capable of forming distinct protein/protein interactions and is thus capable of initiating or participating in unique signaling pathways.

In addition to differences in intermolecular interactions, signaling specificity between c-Src and c-Yes has been observed at the level of kinase activation. c-Yes is the predominant Src family kinase activated upon engagement of the Fcε receptor in the TF-1 mast cell line (69) and upon stimulation of neutrophils with oncostatin M (66). By virtue of its localization to lipid raft fractions, c-Yes may be selectively involved in the renal tubular cell internalization of Shiga toxin (Stx), produced by *Shigella dysenteriae* and enterohemorrhagic *Escherichia coli.* c-Yes associates with the globotriaosylceramide Gb3, to which Stx binds. Association of Stx with Gb3 induces c-Yes-specific kinase activation (91); however, the importance of c-Yes activity for Stx entry was not determined. In rat liver epithelial cells, c-Yes and Fyn are specifically activated in response to angiotensin II (63). In this system, c-Yes and Fyn are essential for the induction of DNA synthesis and c-Fos expression, as both responses are inhibited by microinjection of antibodies against either protein (63). Both c-Src and c-Yes, but not Fyn, appear to be required for VEGF-induced vascular permeability in mice, as cells lacking either protein are deficient in this response (92). Finally, c-Yes specifically associates with the polyimmunoglobulin (pIg) receptor and is activated upon receptor engagement (70). c-Yes appears to be required for pIg receptor-mediated transcytosis, as mice lacking the c-*yes* gene are deficient in transcytosis of IgA (70). Examples of cellular signals uniquely associated with c-Yes and not c-Src are summarized in Table 1. Taken together, these results indicate that in normal cells, c-Yes is able to send unique and specific signals.

7. CONTRIBUTIONS OF THE C-YES FUNCTIONAL DOMAINS TO SIGNALING SPECIFICITY

As a member of the Src family, c-Yes shares the functional domain architecture common to all Src family kinases. As mentioned above, this consists of an SH4 motif responsible for membrane localization, a Unique domain, followed by SH3 and SH2 domains, the protein tyrosine kinases or SH1 domain and a carboxy terminal regulatory region (17). While the c-Yes functional domains, specifically, have not been studied extensively, a great deal has been learned about the roles of Src family functional domains in general, and this knowledge will be important in the elucidation of how the c-Yes functional domains are likely to contribute to signaling specificity.

7.1. SH4 motif

The amino terminal 7-14 amino acids, sometimes referred to as the SH4 domain (98) or SH4 motif, is a region necessary and sufficient for localization to cellular membranes. All Src family members contain a glycine in the second position from the amino terminus. This glycine is myristoylated co-translationally and targets Src family members to cellular membranes. While the addition of myristic acid is necessary for membrane localization, it is not sufficient (98). All Src family members, with the exception of c-Src and Blk, additionally undergo palmitoylation at one or more cysteine residues downstream of the myristoylation site (98). Palmitoylation occurs post-translationally, and potentially spontaneously, as cysteine-acylation has been shown to occur

Table 1. Functions uniquely associated with c-Yes and not c-Src

Functions	Ref
c-Yes binds to a unique 85-87 kDa tyrosine phosphorylated protein	93
c-Yes binds to occludins	84
c-Yes binds to CD36 in human platelets	94
c-Yes regulates CD46 tyrosine phosphorylation in response to N. gonorrhoeae infection of epithelial cells	95
In keratinocyte hemidesmosomes, c-Yes binds to integrin a6ß4.	96
c-Yes mediates Et-1 stimulated glucose transport in 3T3 L1 adipocytes	97
c-Yes co-immunoprecipitates with Cbl	89
In trophoblasts, c-Yes associates with proteins not found in complex with c-Src	51
In keratinocytes, c-Yes associates with a novel 110 kDa and 220 kDa protein, unlike c-Src	53
In mouse pre-adipocytes, c-Yes binds to JAK2, PP2A, and gp130, unlike c-Src	67
In angiotensin II-treated pulmonary vein endothelial cells, c-Yes induces phosphorylation of and associates with the calcium-sensitive kinase Pyk2	90
c-Yes specifically associates with the pIg receptor and is activated upon receptor engagement.	70
c-Yes appears to be required for pIg receptor-mediated transcytosis, as mice lacking the c-yes gene are deficient in transcytosis of IgA	70
Ca^{2+} induces downregulation of c-Yes.	52
Activated c-Yes fails to alter actin filament integrity in CEF	191
c-Yes fails to compensate for c-Src in c-src -/- cells	77

spontaneously in the presence of acyl-CoA *in vitro* (99). However, prior myristoylation is required for palmitoylation (100). It has been suggested that palmitoylation is a dynamic process and may serve as a means of regulating the sub-cellular localization of Src family members and their access to substrates (101). Palmitoylation targets Src family members to lipid rafts, regions of the plasma membrane rich in glycolipids, sphingolipids, cholesterol, and glycosylphosphatidyl inositol-linked proteins, that can be isolated by flotation on sucrose gradients (102,103). The importance of the SH4 domain in raft localization has not been demonstrated specifically for c-Yes but has been shown for other Src family members, including Lck and Hck (102). However, since c-Yes is palmitoylated and is found in lipid rafts (104,105), it is likely that palmitoylation also serves to target c-Yes to these membrane microdomains.

Several recent studies have revealed the importance of palmitoylation and lipid raft localization in Src family kinase signaling. A functional palmitoylation signal is required for the ability of Src family kinases to efficiently transduce signals from the T cell receptor and Fc receptors (106-108). It has been speculated that the presence of c-Yes in lipid raft fractions in MDCK cells may be indicative of a role for c-Yes in vesicular trafficking or proper sorting of GPI-linked proteins (104,105).

7.2. Unique Domain

Following the SH4 domain is the Unique domain, a 60 to 90 amino acid sequence that is completely heterogeneous across the Src family. Very little is known about the role of the Unique domain in c-Yes signaling, and due to the lack of sequence homology in this region, it is difficult to infer a function for the c-Yes Unique domain based upon what is known of other Src family kinases. c-Src is phosphorylated on serine and threonine residues in the Unique domain during mitosis, and these phosphorylation events correlate with elevated kinase activity (109-111). Upon stimulation of the PDGF receptor in fibroblasts, Fyn is phosphorylated on tyrosine residues in its Unique domain, via both autophosphorylation and PDGF receptor phosphorylation (112). As with the c-Src Unique domain, phosphorylation of the Fyn Unique domain also correlates with increased kinase activity. An *in vitro* autophosphorylation site was mapped to tyrosine 32 in the c-Yes Unique domain, however, the importance of this phosphorylation event remains unclear (113). Phosphorylation of Unique domain residues may serve to facilitate protein/protein interactions or to alter the global conformation of the protein in such a fashion that the inactive "closed" conformation is destabilized, thus increasing kinase activity. In addition to serving as a site of phosphorylation, the Unique domain may also direct protein/protein interactions. The Unique domain is responsible for association of Lck with CD4 and CD8 in T cells, and association of Lyn with the Fcε receptor (114,115).

7.3. SH3 Domain

Located carboxy-terminally to the Unique domain are the SH3 and SH2, two modular protein-protein interaction domains. These domains direct c-Yes signaling in several ways. First, SH3 and SH2 domain binding partners disrupt the intramolecular interactions that maintain the protein in the assembled or inactive conformation and enhance the accessibility of the kinase active site for ATP and substrates, thus increasing the specific activity of the kinase (116,117). Additionally, proteins binding to the SH3 or SH2 domains may be presented for tyrosine phosphorylation by c-Yes. Thus the SH3 and SH2 domains may contribute to signaling specificity through differential substrate selection and presentation for processive phosphorylation (118-122). Finally, stable complexes between c-Yes and SH3 or SH2 domain binding partners may serve as sites of activation or inactivation of downstream signaling pathways or target c-Yes to distinct sub-cellular locations (15).

The SH3 domain is approximately 60 amino

acids in length and directs protein/protein interactions through association with left-handed type II helical proline-rich sequences within SH3 domain binding partners (17,123,124). Domain swapping studies and *in vitro* binding assays have demonstrated overlapping ligand specificity between Src family SH3 domains but c-Yes SH3 domain swaps were not analyzed (120,125). Interestingly, studies utilizing phage display analysis have revealed subtle differences in the ligands selected by Src family kinase SH3 domains, including c-Src and c-Yes (126,127). In addition, differential protein binding has been demonstrated between the c-Yes and c-Src SH3 domains *in vitro* and *in vivo.* In comparison to the c-Src SH3 domain, the c-Yes SH3 domain is deficient in binding the 110 kDa actin filament associated protein (AFAP-110) *in vitro* (93). The significance of these observations is supported by *in* vivo studies in which Src^{527F}/c-Yes SH3 chimeras were unable to form a stable complex with AFAP-110, as demonstrated by a lack of co-immunoprecipitation between the proteins (93). Conversely, the c-Yes SH3 domain readily binds the 65 kDa Yes Associated Protein (YAP 65), a weak c-Src SH3 domain binding partner (128).

Another example of signaling specificity and the role of the SH3 domains is illustrated by the calcium-mediated activation and inactivation of c-Src and c-Yes, respectively (52,53). A systematic domain swap analysis between c-Src and c-Yes, including the Unique, SH2 and SH3 domains revealed that their respective SH3 domains are responsible for the opposite regulation by calcium (Monteiro and Hanafusa; unpublished data). In summary, despite redundant and overlapping functions with other Src family SH3 domains, the Yes SH3 domain is critical to confer signaling specificity.

7.4. SH2 Domain

Immediately following the SH3 domain is the SH2 domain. The SH2 domain is comprised of 90-100 amino acids that form a modular phosphotyrosine-binding motif (17). SH2 domain interactions are high affinity, with dissociation constants in the nanomolar range. The SH2 domain is comprised of a central β-barrel structure, flanked by two α-helices and a smaller β-sheet (129,130). The tertiary structure of the SH2 domain forms two binding pockets, the first for phosphotyrosine and the second for the pY+3 amino acid (129,130). SH2 domain binding specificity is dictated by the sequence of amino acids surrounding the phosphorylated tyrosine residue, particularly those on the carboxy-terminal side (131,132). The optimal c-Yes SH2 domain ligand sequence has not been specifically determined, however, all Src family SH2 domains appear to select the sequence pYEEI with maximal affinity (132). In support of the peptide-binding data, SH2 domain redundancy has also been demonstrated with regard to full-length protein binding. c-Src, Fyn, and c-Yes bind the same sites within the PDGF and CSF-1 receptors via their SH2 domains (116), and the c-Yes and c-Src SH2 domains appear to bind the same sites within the Neu protein (59).

Little data exists to suggest signaling specificity between Src family members at the level of the SH2 domain, however, it was recently noted that Src^{527F}/c-Yes chimeras with the c-Yes SH2 domain display enhanced co-immunoprecipitation with an unknown phosphoprotein of approximately 87 kDa (pp87) (93). As the identity of this protein is currently unknown, the significance of complex formation between pp87 and the c-Yes SH2 domain is unclear. An 85 kDa tyrosine phosphorylated protein identified by Parker et al., as a co-immunoprecipitating protein with GPI-1 anchored protein from pancreatic acinar cells, in an immunecomplex which also included c-Yes and caveolin proteins, and was hypothesized to regulate endocytosis (133). It is has not been determined whether the reported pp87 and p85 proteins are related. These data suggest that c-Yes-specific signaling events may be generated through SH2 domain interactions. A recent study by Bradshaw *et al.* revealed that SH2 domain specificity between Src family members may arise as a result of differences within the phosphotyrosine binding pocket (134). It was noted in this study that the amino acid in the βC3 position of the pTyr-binding pocket contributes considerable energy to phosphotyrosine binding. c-Src contains a cysteine in this position, whereas all other Src family kinases, including c-Yes, contain a serine. Mutation of the cysteine to serine resulted in a four-fold increase in binding affinity for pTyr. This may have implications for the relative abilities of Src family kinase SH2 domains to associate with their cognate regulatory phosphotyrosines or intermolecular binding partners.

Homology modeling of human c-Yes on c-Src (PDB ID entry 2SRC) with SwissModel reveals the remarkable similarity between the two proteins (Figure 2) (190). The surface of the SH3 domain that recognizes the polyproline helix of binding partners is identically conserved, as is the core phosphopeptide binding surface of the SH2 domain. Additionally, the interdomain interfaces that stabilize the closed, inactive form of the enzyme are largely conserved. Most amino acid substitutions in c-Yes relative to c-Src are in surface residues that are not known to be important for regulation or binding interactions. However, there are differences in regions flanking the SH3 and SH2 binding surfaces that could reasonably be expected to alter binding specificity relative to c-Src. In particular, several substitutions in the SH3 domain cluster in or near the RT loop. These include E97 > T107 and T98 > E108 (in the RT loop) and T129 > N139, L120> E130, H122 > R132, and V111 > I121 which form a surface adjacent to the RT loop. The RT loop is often important for coordination of basic residues flanking the core PXXP recognition motif.

Additionally, there are a few differences in the SH3-linker-kinase and SH2 kinase interfaces which might alter the activation properties of the molecule. In Src, Gln 324 in the kinase domain hydrogen bonds with the linker; the corresponding residue in Yes is Pro 334, which cannot make an equivalent interaction. In the SH2 kinase interface, Glutamic acid residues 157 and 320 are both replaced by Aspartic acid in c-Yes, and Arg 156 corresponds to Lys 166 in c-Yes. Although these substitutions are conservative, they will alter the electrostatic environment of the interface and could

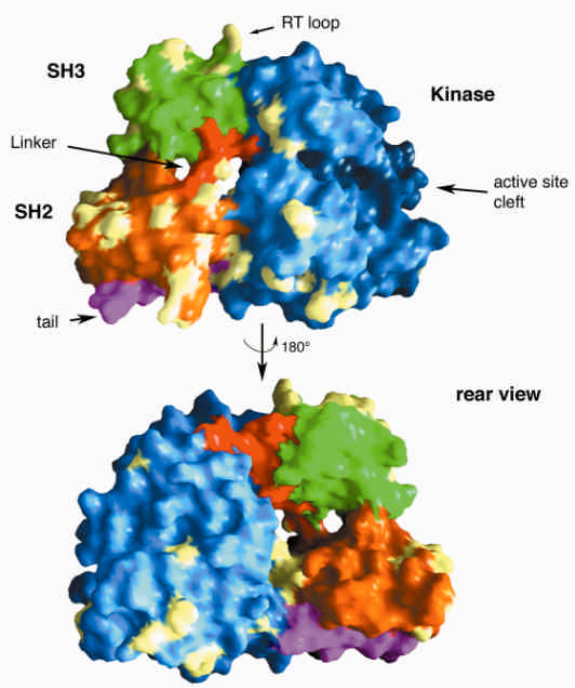

Figure 2. Molecular Model of the cYes SH3-SH2-Kinase domains. Domain organization of the auto inhibited Src kinase and analysis of surface residues that are different in Yes. The molecular surface representation of Src in the auto inhibited conformation (with Tyr 527 phosphorylated) is shown with the SH3 domain colored green, the SH2 domain orange, and the tyrosine kinase domain blue. The SH3 and SH2 domains pack against the "back" of the kinase domain, opposite the active site cleft, and help to lock the kinase in the inactive conformation. The linker (red), which connects the SH2 and kinase domains, packs between the SH3 and kinase domains and helps to maintain the inhibited conformation. Likewise, the phosphorylated C-terminal tail (purple) binds to the SH2 domain to lock the enzyme in the auto inhibited conformation. The portions of the surface formed by residues that differ in human Yes (from the corresponding residues in human c-Src) are colored yellow. Note that the differences are scattered among all domains, but are notably absent from the region of the kinase active site and the c-terminal tail. Divergent residues in and near the RT loop, which forms part of the recognition surface of the SH3 domain, may affect the binding specificity of the SH3 domain. Also, substitutions in the interfaces between the kinase and SH3 and SH2 domains may subtly alter the regulation of the kinase (see text).

therefore influence disassembly and activation of the closed form of the kinase. Additionally, substitutions within the hydrophobic core of the SH3, SH2 and Kinase domains may subtly affect their structure and therefore their binding properties as well. Thus, although there appears to be great conservation in the structure of the SH2 and SH3 domains of c-Src and c-Yes, there are sufficient differences to indicate mechanisms for differential interactions with distinct binding partners.

7.5. SH2/Kinase Linker

Following the SH2 domain is a short stretch of 15 amino acids that spans the gap between the SH2 and tyrosine kinase domains, referred to as the SH2/kinase linker. The primary function of the linker sequence is association with the SH3 domain in the closed conformation. In the closed conformation, the linker takes on the left-handed helical structure characteristic of SH3 domain binding partners. Mutations in this region can enhance kinase activity through destabilization of the closed conformation (135). Thus any contributions to signaling specificity made by the linker sequence would likely be reflected in the ease of enzymatic activation. Only one amino acid difference exists between the linker sequences of c-Yes and c-Src: the fourth position in the sequence is occupied by a serine residue in c-Src and a valine in c-Yes. The role of this amino acid in maintenance of the closed conformation has not been investigated, and thus, at this point no accurate assessment of its contribution to c-Yes signaling specificity can be made.

7.6. Tyrosine Kinase Domain

Following the linker sequence is the tyrosine kinase domain. This is the most highly conserved region across the Src family. As mentioned above, the kinase domains of c-Yes and c-Src are approximately 90% similar. The extremely high similarity between c-Yes and c-Src, and members of the Src family in general, in the kinase domain suggests that this region contributes little to signaling specificity. Differences in the optimal substrate sequences phosphorylated by Src and Lck have been demonstrated (136), however, these differences were subtle, and Src and Hck have been demonstrated to select the same peptide sequences for phosphorylation *in vitro* (137).

8. SPECIFICITY IN SIGNALING DICTATED BY C-YES FUNCTIONAL DOMAINS

There are differences in substrate specificity as modulated by the SH3 and SH2 domains between c-Yes and c-Src, as evidenced by different affinities for AFAP-110 and pp87 (or pp85). Differential protein binding between the c-Src and c-Yes SH3 domains was not unexpected as differences in SH3 domain ligand specificity, to the amino or carboxy terminal sides of the core PXXP motif, between Src family members, including c-Src and c-Yes, have been shown previously using *in vitro* peptide binding studies (126,127,138). The c-Src and c-Yes SH3 domains are very similar differing at only 11 amino acid positions (19,33); however, these amino acids have not been definitively identified as crucial for ligand binding. In lieu of the homology between the c-Src and c-Yes SH3 domains, it is of little surprise that ligand specificity detected between the two was subtle (LXXRPLPXΨP for Src, ΨXXRPLPXLP, where Ψ represents an aliphatic residue, and X represents any amino acid) (127). It was not determined if these differences in ligand specificity corresponded to actual differences in the ability of the c-Src and c-Yes SH3 domains to associate with full-length polypeptides, either *in vitro* or *in vivo.* The

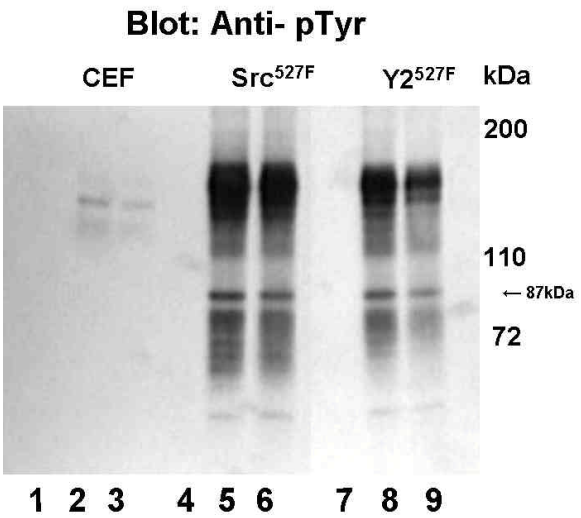

Figure 3. The c-Yes SH2 domain does not affect tyrosine phosphorylation of c-Src *in vitro* SH2 domain binding partners. (A) Mock-transfected CEF or cells expressing Src^{527F}, $Y3^{527F}$, $Y2^{527F}$, $Y32^{527F}$, or $Y4U32^{527F}$ were lysed in RIPA buffer, and five hundred μg of cell lysates were absorbed with GST, GST-SH2Src, or GST-SH2Yes bound to glutathione-sepharose beads. Bound proteins were eluted by boiling in Laemmli's sample buffer (LSB), and resolved by 8% SDS-PAGE. After transfer to PVDF membrane, proteins were blocked in 1% BSA/TBS-T and probed with a rabbit anti-phosphotyrosine antibody. Results are shown for mock-transfected CEF and cells expressing Src^{527F} and $Y2^{527F}$. Lanes 1, 4, 7 = GST; Lanes 2, 5, 8 = GST-SH2Src; Lanes 3, 6, 9 = GST-SH2Yes.

SH3 domains of c-Src, Fyn, and Lyn have previously demonstrated differential protein binding capacities *in vitro* (120), however this was not observed for the c-Src and c-Yes SH3 domains.

The data of Summy et al. (93) indicated that the differences in specificity between the c-Src and c-Yes SH3 domains are sufficient for differential protein/protein interactions both *in vitro* and *in vivo.* The individual amino acids responsible for ligand specificity, however, remain to be determined. Although systematic mutagenesis was not carried out to identify the individual residues responsible for imparting ligand specificity, they are likely to be localized to regions harboring non-conservative amino acid changes between the two SH3 domains. Glu^{97} and Thr^{98} in the c-Src SH3 domain, which correspond to Thr^{105} and Asp^{106} in the c-Yes SH3 domain, represent two non-conservative amino acid differences between the c-Src and c-Yes SH3 domains. These residues lie within the RT loop, a region that connects two of the core β sheets and contributes to ligand specificity (142,143), thus identifying these amino acids as potentially important for directing SH3 domain specificity between c-Src and c-Yes. Further studies, in which these residues in the c-Src and c-Yes SH3 domains are mutated, will be necessary to fully assess their importance for ligand specificity between the two proteins.

While differences in ligand specificity were expected between the c-Src and c-Yes SH3 domains, the evidence for differential protein binding between the c-Src and c-Yes SH2 domains was a surprise. *In vitro* binding assays indicate that Src family kinases select the same cognate peptide ligand sequences (132). The c-Src and c-Yes SH2 domains in particular have been demonstrated to bind the same sites within their cognate ligands (59,144). While no obvious differences in ligand specificity were detected between the c-Src and c-Yes SH2 domains *in vitro* (Figure 3), an 87 kDa tyrosine-phosphorylated protein (pp87) was observed to co-immunoprecipitate preferentially with Src^{527F}/c-Yes chimeras with the c-Yes SH2 domain. This is strongly suggestive that pp87 represents either a direct or indirect SH2 domain binding partner. Again, the question remains as to which amino acids dictate SH2 domain specificity between c-Src and c-Yes.

The SH2 domain is composed of two binding pockets separated by a large central β sheet (129,130). The two binding pockets accommodate phosphotyrosine and downstream residues within the cognate ligand respectively (129,130). In the case of Src family kinases, the second ligand-binding pocket is typically occupied by a hydrophobic amino acid three residues downstream of the phosphotyrosine (pY+3) (130). As phosphotyrosine binding is a conserved feature of all SH2 domains, and differences in the downstream binding pocket dictate ligand specificity between the various SH2 domain sub-classes, the pY+3 pocket initially seemed the more attractive candidate for directing specificity between Src family kinases.

There are 27 amino acid differences between the c-Src and c-Yes SH2 domains, however, the most notable non-homologous differences between the SH2 domains, within the pY+3 pocket, occur in the B helix, which forms the bottom of the pocket. These differences occur at Gln^{223}, Ala^{228}, and Tyr^{229} in Src^{527F}, corresponding to Lys^{231}, Lys^{236}, and His^{237} in c-Yes, respectively (amino acid numbers correlate with chicken c-Yes). However, the existing crystal structures of Src family SH2 domains (19,20) suggest that only His^{237} is likely to be directed toward the interior of the ligand-binding pocket (129). In a recent paper by Bradshaw *et al.*, it was reported that Cys^{185} in the c-Src SH2 domain phosphotyrosine pocket, occupied by serine in all other Src family kinases (Ser^{193} in c-Yes), imparts a reduced affinity for phosphotyrosine relative to serine (134). Differences in phosphotyrosine affinity may represent a mechanism for dictating ligand specificity between the c-Src and c-Yes SH2 domains, as a non-consensus SH2 domain binding partner may be more readily accommodated by a higher affinity for phosphotyrosine.

Differential phosphotyrosine affinity between the c-Src and c-Yes SH2 domains may also have implications for the relative abilities of these kinases to be activated in response to cellular signals. A higher affinity for phosphotyrosine may allow more efficient association between the phosphorylated tail and the SH2 domain, thus stabilizing the inactive, closed conformation. Interestingly, it was observed that c-Src/c-Yes chimeras with the c-Yes

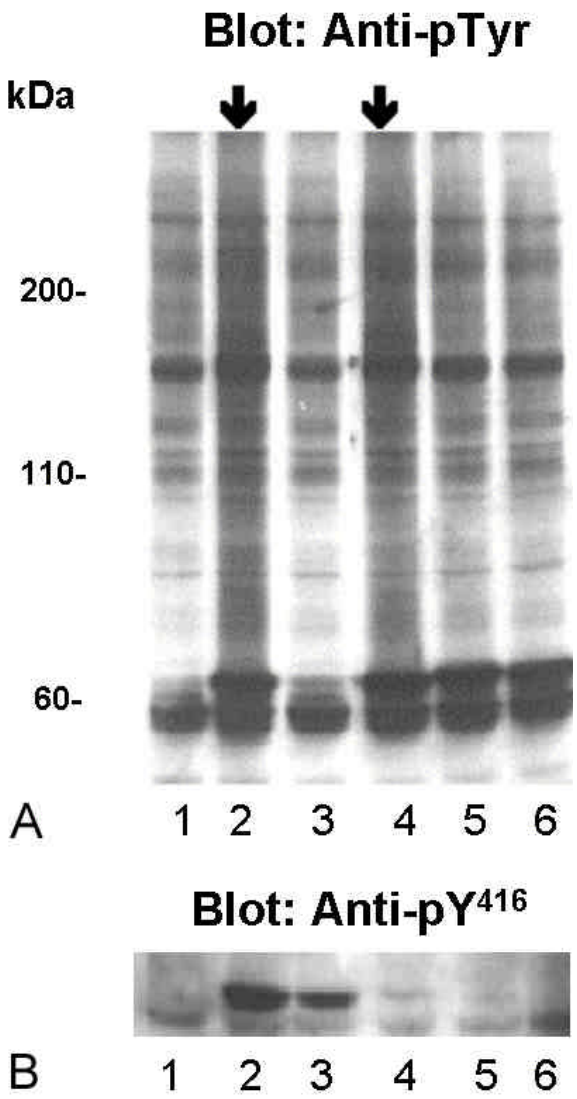

Figure 4. Induction of cellular phosphotyrosine and autophosphorylation of c-Src/c-Yes chimeras. Mock-transfected CEF or cells expressing c-Src, c-Yes, or c-Src chimeric constructs expressing c-Yes SH3 domain (Y3), c-Yes SH2 domain (Y2), both the c-Yes SH2 and SH2 domains (Y32), or the c-Yes SH4/Unique/SH3/SH2 domains (Y4U32) were lysed in RIPA buffer, and 30 µg of lysates were resolved by 8% SDS-PAGE, transferred to PVDF, blocked in 1% BSA/TBS-T, and probed with a rabbit anti-phosphotyrosine antibody. Lane 1 = CEF; Lane 2 = c-Src; Lane 3 = c-Yes; Lane 4 = Y3; Lane 5 = Y2; Lane 6 = Y32; Lane 7 = Y4U32. (B). CEF, c-Src, Y3, Y2, Y32, or Y4U32 lysates were prepared for western blot analysis as described above and probed with a rabbit anti-phospho-Y416 antibody. Lane 1 = CEF; Lane 2 = c-Src; Lane 3 = Y3; Lane 4 = Y2; Lane 5 = Y32; Lane 6 = Y4U32.

SH2 (Y2) domain induced much lower levels of cellular phosphotyrosine and autophosphorylation than corresponding constructs with the c-Src SH2 domain (Figure 4). Mutation of Ser^{185} to Cys in Y2, however, did not increase cellular phosphotyrosine or autophosphorylation of this kinase (Figure 5A).

In an effort to determine why c-Src/c-Yes chimeric constructs with the c-Yes SH2 domain displayed reduced levels of cellular phosphotyrosine and autophosphorylation, the possibility that the closed conformation was stabilized by an ionic interaction between a positive-charged amino acid in the c-Yes SH2 domain (His^{237}) and a negative-charged residue in the regulatory tail (Glu^{531} in c-Src, Asp^{539} in c-Yes) was explored. Again, however, mutation of these residues to uncharged amino acids (His^{237} to Tyr, as in c-Src and Glu^{531} to Gln) did not result in increased kinase activation, as evidenced by autophosphorylation (Figure 5A, leftl) and phosphorylation of the regulatory tyrosine Tyr^{527} (Figure 5B, right panel). It thus appears that additional residues are involved in directing specificity between the c-Src and c-Yes SH2 domains. Nevertheless, the results indicate that the c-Src and c-Yes SH2 domains may contribute to signaling specificity between these proteins and may play a larger role in directing signaling specificity between Src family kinases than previously believed.

What are the functional implications of ligand specificity between the c-Src and c-Yes SH3 and SH2 domains? The results to date do not suggest a strong influence on substrate selection. The tyrosine phosphorylation of AFAP-110 and other tyrosine-phosphorylated SH3 domain binding partners induced by $Y3^{527F}$ (a Src^{527F} chimera containing the c-Yes SH3 domain) was surprising given the inefficient co-immunoprecipitation and *in vitro* binding between these proteins. As mentioned above, SH3 domain interactions are necessary for Src-induced tyrosine phosphorylation of AFAP-110 and other SH3 domain binding partners (118,120,121). Mutations in either the SH3 domain binding site of AFAP-110 or the Src SH3 domain impair the ability of Src^{527F} to induce tyrosine phosphorylation of AFAP-110 (118,121). Due to the failure of the c-Yes SH3 domain to bind AFAP-110 efficiently *in vitro* or *in vivo*, a corresponding deficiency in the ability of $Y3^{527F}$ to induce AFAP-110 tyrosine phosphorylation was expected. This was not the case, however, as $Y3^{527F}$ and Src^{527F} induced comparable levels of AFAP-110 tyrosine phosphorylation (93). How are these seemingly contradictory results explained? One possible explanation is that the c-Yes SH3 domain, as opposed to a c-Src SH3 domain in which residues critical for ligand binding have been mutated, is capable of low-level association with AFAP-110. As seen by Summy et al., there is modest association of the c-Yes SH3 domain with AFAP-110 *in vitro*, and this correlates with inefficient yet still detectable co-immunoprecipitation between $Y3^{527F}$ and AFAP-110 in RIPA lysates. This relatively weak association between the c-Yes SH3 domain and AFAP-110 may allow a transient association between AFAP-110 and $Y3^{527F}$ that is sufficient to permit tyrosine phosphorylation of AFAP-110 but not detectable co-immunoprecipitation between the two proteins. Conversely, deletion of amino acids 92-95 in the Src SH3 domain may have abrogated any interaction with AFAP-110, resulting in significantly reduced tyrosine phosphorylation of AFAP-110 by $Src^{527F/dl92-95}$ (121).

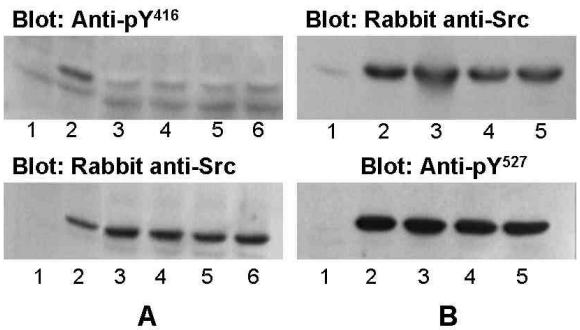

Figure 5. Activation state of c-Src, Y2, and Y2 mutants in CEF. Mock-transfected CEF or cells expressing c-Src, Y2, Y2S185C, Y2H229Y, or Y2E531Q were lysed in RIPA buffer, and 50 µg of lysates were resolved by 8% SDS-PAGE, transferred to PVDF, blocked in 5% milk/TBS-T, and probed with either a rabbit anti-phospho-Y416 antibody (A, top panel) or a rabbit anti-phospho-Y529 antibody (B, top panel). In the bottom panels, blots were stripped and re-probed with rabbit anti-Src, which reacts with a conserved carboxy terminal peptide sequence common to c-Src and c-Yes. (A) Lane 1 = CEF; Lane 2 = c-Src; Lane 3 = Y2; Lane 4 = Y2S185C; Lane 5 = Y2H229Y; Lane 6 = Y2E531Q. (B) Lane 1 = CEF; Lane 2 = Y2; Lane 3 = Y2S185C; Lane 4 = Y2H229Y; Lane 5 = Y2E531Q.

The benefit of the more stable association between the c-Src or Src^{527F} SH3 domain and AFAP-110 remains unclear, as it does not result in a significant enhancement in tyrosine phosphorylation. Higher affinity association between the c-Src SH3 domain and AFAP-110 may allow formation of longer lived signaling complexes with distinct functions, including sub-cellular targeting, recruitment of signaling molecules, or alterations in the multimeric status of AFAP-110 itself. AFAP-110 has an intrinsic ability to bind actin filaments (146), and phosphorylation of AFAP-110 by PKCα activates the ability of AFAP-110 to induce actin filament bundling *in vitro* (95). Thus, AFAP-110 may play a key role in remodeling the actin filament cytoskeleton in response to cellular signals, as occurs during embryonic development, cell division, and metastasis of cancer cells. Src^{527F}/c-Yes chimeras with the c-Yes SH3 domain are capable of inducing actin filament remodeling in CEF comparable to Src^{527F}, thus it does not appear that stable association between Src^{527F} and AFAP-110 is a requirement for induction of actin filament rearrangement. However, it is noteworthy that $Src^{527F/dl92-95}$ exerts different effects on cells, whereby the cells are fusiform without the formation of actin filament rosettes (118). AFAP-110 association with active Src is also not sufficient to target Src to focal adhesion structures, as Fincham *et al.* demonstrated that mutants of Src that failed to localize to focal adhesions were still able to associate with AFAP-110 (147).

Regardless of the biological importance of stable association between activated c-Src and AFAP-110, the inefficient association of c-Yes with AFAP-110 would presumably preclude the involvement of c-Yes in these signaling pathways. Conversely, stable association between pp87 and Src^{527F}/c-Yes chimeras with the c-Yes SH2 domain, and theoretically activated c-Yes itself, may allow these proteins to participate in signaling pathways that do not involve c-Src. This may occur, as described above for Src/AFAP-110 interactions, through sub-cellular localization or formation of c-Yes-specific signaling complexes. Unfortunately, in comparison to c-Src, little is known about the biological functions of c-Yes, and there are few intermolecular interactions between c-Yes and its cellular binding partners that have demonstrated definitive functional implications. Association of c-Yes with YAP65 may facilitate localization of c-Yes to the apical surface in airway epithelial cells (148), and it was recently demonstrated that recruitment of c-Yes to a signaling complex including β-arrestin 1 and the endothelin type-A receptor might allow c-Yes to participate in endothelin-stimulated glucose transport (97). The only phenotype reported thus far as a result of loss of the c-*yes* gene is a deficiency in transcytosis mediated by the pIg receptor (70). Expression of the Src^{527F}/c-Yes chimeras in c-*yes* -/- cells may prove useful in uncovering why c-Src is unable to compensate for c-Yes in pIg receptor function and what role c-Yes-specific binding partners may play in this pathway.

The affinity of the c-Src and c-Yes SH3 and SH2 domains for their cognate ligands also has implications for the activation of these kinases in response to cellular signals. The HIV-1 Nef protein is able to bind and activate Hck through SH3 domain interactions (117). Greenway and colleagues found that SH3 domain specificity between Src family kinases allows HIV-2 and SIV Nef to target the SH3 domains of c-Src and Fyn, as opposed to Hck (149). Baisden *et al.* demonstrated that a mutant of AFAP-110, in which the leucine zipper motif is deleted, is able to activate c-Src in an SH3 domain-dependent fashion, most likely through displacement of the intramolecular interaction between the SH3 domain and the kinase linker (150). The low affinity interaction between AFAP-110 and the c-Yes SH3 domain may be insufficient to permit AFAP-110-induced activation of c-Yes in response to cellular signals. The functions of AFAP-110, both downstream and upstream of c-Src, remain the subject of ongoing investigations by others. It remains possible that pp87 binding contributes to the activation of c-Yes in a similar fashion, however, this will be difficult to determine experimentally until pp87 is cloned and identified.

Thus, the known c-Src substrates p130 CAS and AFAP-110, as well as several unidentified tyrosine phosphorylated SH3 and SH2 domain binding partners, have not provided evidence for SH3 or SH2 domain-mediated substrate specificity between activated c-Src and c-Yes. While these results should not be over-interpreted to imply that SH3 and SH2 domain-mediated substrate selection between c-Src and c-Yes do not exist, the results imply that they may not be the primary means of generating signaling specificity. The SH3- and SH2-mediated specificity in protein binding observed in these experiments may contribute more to overall signaling specificity through differential signaling complex formation than through substrate selection.

In contrast to the differences in protein/protein interactions, differences in transcriptional activation and cell biological responses were primarily found to be due to differences in the Src^{527F} and c-Yes amino terminal regions, including the SH4/Unique domain. Chimeric proteins with the c-Yes amino terminal SH4/Unique/SH3/SH2 domains, $Y4U32^{527F}$, or $Y4U^{527F}$, which only contains the cYes SH4/Unique domain, were deficient in upregulation of the *heme oxygenase* 1 (HO-1) gene product (88) and induction of the morphological and cytoskeletal changes that occur concomitant with overexpression of constitutively activated c-Src despite the fact that they were fully active, as evidenced by anti-phosphotyrosine and anti-phospho-Y^{416} western blot analysis (191). The decreased HO-1 induction and failure of these chimeras to induce rearrangement of the actin cytoskeleton was attributed to the presence of the c-Yes amino terminus, as $Y32^{527F}$ (Src^{527F} chimera containing the c-Yes SH3/SH2 domains) was fully functional in regard to both of these processes. Both the SH4 and Unique domains were necessary in order to obtain these results, as chimeric proteins in which only the SH4 or Unique domain of Src^{527F} was replaced by that of c-Yes were unable to induce actin filament rearrangement and efficient upregulation of HO-1 expression in CEF.

In recent years, it has become clear that the amino terminal regions of Src family kinases do not simply localize these proteins to cellular membranes; they also regulate the compartmentalization of these kinases within cellular membranes. Specifically, it has been demonstrated that palmitoylation of Src family members dictates their inclusion into triton X-100 resistant membrane fractions, referred to as lipid rafts (103). These are formed by aggregates of bulky lipids, including sphingolipids and cholesterol, and glycosylphosphatidyl inositol (GPI)-linked proteins (151). Several acylated signaling proteins, including many Src family kinases, are recruited to lipid raft fractions by virtue of their long chain fatty acid modifications. Fatty acylation is not necessarily a requirement for inclusion into lipid raft fractions, however, as several transmembrane receptors localize to lipid rafts, including the PDGF and Fcε receptors (107,151).

Recruitment to lipid rafts is important for the participation of Src family kinases in several signaling pathways. The detection of c-Yes in caveolae, a sub-class of lipid rafts that are defined by the presence of caveolin, was among the first reports of Src family kinases in lipid rafts (43). The importance of c-Yes localization to caveolae, however, was not determined. Shenoy-Scaria *et al*. were among the first to observe that association between Src family kinases and a cellular binding partner was dependent on palmitoylation and localization to lipid rafts (102,105). At a functional level, co-localization of the Fcε receptor and Src family kinases in lipid raft fractions is essential for Fcε receptor signaling (107,108). In this system, it was demonstrated that raft localization serves as a means of achieving signaling specificity, as c-Src was unable to reconstitute Fcε receptor signaling unless a palmitoylation signal was included in the amino terminus (107,108). Palmitoylation-dependent signaling is also observed for Lck, which is unable to transmit signals from the T-cell receptor in the absence of palmitoylation (101).

The results obtained by Summy et al., (191) were particularly novel, in that the entire amino terminal region, including both the SH4 palmitoylation signal and the Unique domain, was required in order to achieve the differential effects on induction of Heme oxygenase I (HO-1) transcription and changes in the actin cytoskeleton. This is of note in that previous studies have traditionally reported the functions of the SH4 and Unique domains individually, with little consideration given to the possibility that these domains may act in concert. The heterogeneity of the Unique domain across the Src family renders it virtually impossible to assign a generalized function. Interestingly, computer-based secondary structure predictions of the Unique domains from different Src family kinases predict a relative conservation of structure (Flynn, unpublished observation). This could indicate that the Unique domains could have a conserved structure without conservation of sequence, not unlike that seen with PH domains. Lck associates with the CD4 and CD8 T cell co-receptors by virtue of its Unique domain (114,152), whereas the Unique domain of Lyn is responsible for mediating its association with the Fcε receptor (115). The Unique domains of Fyn, c-Src, and c-Yes harbor phosphorylation sites that demonstrate some correlation with activation, however, the full importance of these phosphorylations remains unknown (109-113). Bijlmakers and colleagues reported that the Unique domain of Lck is sufficient for membrane localization, however, the construct used in those studies included the Lck SH4 region (153).

Unlike the Unique domain, the function of the SH4 domain has been well established. Myristoylation, in conjunction with either palmitoylation or positive-charged amino acids, localize Src family kinases to cellular membranes (98). Palmitoylation then localizes Src family kinases to lipid raft fractions, allowing them to participate in signaling pathways originating in these membrane microenvironments. The partial requirement of the c-Yes SH4 domain for the inability of Src^{527F}/c-Yes chimeras to induce actin filament rearrangement and elevated HO-1 expression suggests that localization to lipid rafts is important in these results. However, the additional requirement for the c-Yes Unique domain complicates the issue. These results indicate that either the Unique domain participates in localization or that it, in conjunction with the SH4 domain, facilitates interaction with a protein or proteins that inhibit the ability of $Y4U32^{527F}$ and $Y4U^{527F}$ to influence signaling pathways that induce HO-1 expression and actin filament rearrangement. Alternatively, the c-Yes Unique domain, again in conjunction with the SH4 domain, may prevent interaction with a Src substrate or binding partner that is necessary for initiation of these signaling events. Regardless, it is clear that both the c-Yes SH4 and Unique domains prevent induction of HO-1 expression and actin filament rearrangements. Thus, evidence presented that indicate a synergistic effect of the SH4 and Unique domains in c-Yes

signaling could indicate that these two regions may actually function as one domain.

What do these results reveal about the biological functions of c-Yes and the signaling pathways it utilizes? Unfortunately, they suggest more about what activated c-Yes cannot do than what it can. However, they may also provide insight as to why c-Yes is frequently unable to compensate for the absence of c-Src in c-*src* -/- cells. The c-Yes amino terminal region is necessary and sufficient to inhibit the ability of Src^{527F}/c-Yes chimeras to participate in pathways that effect elevated induction of HO-1 and rearrangement of actin stress fibers into rosettes, lamellipodia, and filopodia. These results correlate well with data in the literature that indicate that c-Yes is unable to participate in related signaling pathways. Heme oxygenase 1 is an enzyme that cleaves the heme molecule into iron, carbon monoxide, and biliverdin (140,154). HO-1 is expressed in a wide range of tissues in response to diverse cellular stimuli, including hypoxia (140), heavy metals (155), Ras (156), MAP kinases (139,157), NFκB (139), Hypoxia-Inducible Transcription Factor 1 (HIF-1α) (158), and, Src activation (88). The ability of activated Src to induce HO-1 expression was not surprising in lieu of the fact that tyrosine kinase inhibitors block HO-1 induction in response to heavy metals (155) and given the ability of v-Src to induce HIF-1α expression (159). In fact, the reduced induction of HO-1 by $Y4U32^{527F}$ and $Y4U^{527F}$ may be due to a failure to induce expression of HIF-1α.

Chimeric proteins with the c-Yes amino terminus failed to induce activation of the PI3K/Akt pathway (191). Several recent studies have indicated that activation of PI3K and/or Akt are necessary for induction of HIF-1α (160-162). Failure to activate PI3K may prevent $Y4U32^{527F}$ and $Y4U^{527F}$ from efficiently activating an oxidative stress response pathway that involves HIF-1α expression and subsequent expression of HO-1. These results suggest that c-Yes, by virtue of its amino terminal region, may not participate in this oxidative stress response pathway. In agreement with this hypothesis, it was previously observed that c-Src, but not c-Yes, is activated in response to hypoxia, and results in expression of vascular endothelial growth factor, which is also induced in response to HIF-1α (87).

The inability of $Y4U32^{527F}$ and $Y4U^{527F}$ to induce actin cytoskeletal rearrangement and morphological changes is also in agreement with results reported previously. Cells lacking the c-*src* gene are deficient in several processes that are dependent on the dynamic regulation of the actin cytoskeleton. These include cell migration (80), cell spreading (79), neurite extension (81), membrane ruffle and ring formation (78), and osteoclast-mediated bone resorption (77). Recent results suggest that the c-Yes amino terminus may prevent $Y4U32^{527F}$ and $Y4U^{527F}$, and by extension activated c-Yes, from inducing remodeling of the actin cytoskeleton (191). Cells transfected with $Y4U32^{527F}$ or $Y4U^{527F}$ retained a normal CEF morphology, characterized by intact focal adhesions and long actin stress fibers. In contrast, cells expressing Src^{527F} and the other activated Src^{527F}/c-Yes chimeras displayed a fully transformed morphology and a repositioning of actin from stress fibers into rosettes and actin-based membranous motility structures, such as lamellipodia and filopodia.

9. C-YES IN CANCER AND DISEASE

The ability of v-Yes to induce cell transformation piqued interest in the study of the normal cellular homologue, as it was inferred that c-Yes might be involved in the onset or progression of human cancers. As with c-Src, several lines of evidence have in fact pointed to this possibility. The evidence is largely circumstantial, however, and consists primarily of observations that c-Yes is activated in several transforming, cancerous, and pre-cancerous conditions. Nevertheless, the data discussed below have laid the groundwork for future studies of c-Yes in human cancers. Both c-Yes and c-Src are frequently activated in human colon carcinoma cells and pre-malignant lesions of the colon (141,163-166). The degree of c-Yes and/or c-Src kinase activation in these cells correlates with their malignant potential (141,165). In colorectal carcinoma liver metastases, the kinase activity of either c-Src or c-Yes decreases relative to the parent tumor (167). However, activation of c-Yes in colon carcinoma liver metastases is associated with more aggressive disease and a poorer prognosis than c-Src activation (167). Interestingly, Park and Cartwright demonstrated that while c-Src activity increases during mitosis of human colon carcinoma cells, c-Yes protein expression and activation decrease (168). c-Yes protein levels and kinase activity are elevated 5-10 fold in malignant melanoma cells, in comparison to normal melanocytes, whereas c-Src activity and expression are unchanged (169). The c-yes proto-oncogene has also been reported to be amplified in human gastric cancer (170). c-Yes kinase activity correlates with the brain metastatic potential of melanoma cell lines, as the most highly metastatic melanoma cell lines display the highest levels of c-Yes kinase activity (171). c-Yes activity is further elevated upon stimulation with NGF or neurotrophin 3 (171). The neurotrophin-induced activation is specific for c-Yes, as c-Src activity is not affected. Despite the apparent dispensability of c-Yes kinase activity for Middle T antigen-induced murine breast tumors, the ability of Middle T to transform cells correlates well with its ability to associate with and activate both c-Src and c-Yes (172-177). While c-Yes activity does not appear to be necessary for transformation of fibroblasts by Middle T, it is important for Middle T-induced transformation of endothelial cells (82). In human breast cancer cells, overexpression of members of the EGFR family, specifically erbB-2 (Neu), is associated with poor clinical prognosis. When expressed in mice, under control of the mouse mammary tumor virus (MMTV) promoter, the Neu protein induces transformation of breast epithelial cells. In this system, the Neu protein associates with and activates both c-Src and c-Yes through SH2 domain interactions (59). Moasser *et al.* demonstrated up-regulation of c-Src and/or c-Yes kinase activity in a panel of human breast cancer cell lines, and inhibition of Src family kinase activity in this system resulted in cell cycle arrest during mitotic prophase (178). Together, the above data illustrate

that not only is c-Yes activated in select human cancers, but its contributions to the transformed phenotype may differ from those of c-Src. Furthermore, the differential activation of c-Yes and c-Src in some human cancers suggests that it may be beneficial to selectively target these kinases individually using rational drug design. In order to accomplish this, however, it is necessary to understand how these kinases send specific signals.

The results in this review raise questions about the relative contributions of c-Src and c-Yes to the onset and progression of human cancers. The transition of a cell from the normal to the cancerous state is a multi-step process, and while the biochemical pathways utilized to achieve full transformation may vary, there are certain phenotypic changes that are almost universally present. The experiments outlined in these studies directly and indirectly touch on four of these: cell proliferation, resistance to apoptosis, rearrangement of the cytoskeletal architecture, and the ability to survive in the face of oxidative stress. Cancerous cells are characterized by unchecked cell division (179), and remodeling of the actin cytoskeleton allows them to invade surrounding tissue and metastasize (145). Resistance to apoptosis allows the continued survival of the tumor in the face of DNA damage and removal from its normal cellular milieu (180). Resistance to oxidative damage allows cancerous cells to survive oxidative threats from the body's defense system and hypoxic conditions encountered in the interior of a tumor in the absence of vascularization (181). Many studies directly demonstrate or suggest that c-Src activation is able to induce all of these effects.

Src^{527F} expression results in activation of the MAPK pathway, which in turn induces cell cycle progression (182). It was also shown that Src^{527F} induces efficient rearrangement of the actin cytoskeleton, repositioning actin from stress fibers to actin based motility structures (183,184). c-Src activation also induces activation of the Akt/PI3K pathway, which in addition to its effects on the cytoskeleton, is an important mediator of cell survival and resistance to apoptosis (185). Finally, it was demonstrated that Src^{527F} induces high levels of HO-1 expression, a protein that is involved in the resistance to hypoxic stress (140).

The data of Summy et al. (191) indicated that chimeric constructs of Src^{527F} with the c-Yes amino terminus were only able to induce one of the effects mentioned above, at levels that approached those achieved by Src^{527F}: activation of the MAPK pathway. Unfortunately, due to the lack of a constitutively activated c-Yes, there is currently no data available in the literature on the phenotype of cells expressing this protein and how they differ from cells expressing active c-Src. $Y4U32^{527F}$ should functionally approximate constitutively active c-Yes (Yes^{535F}), as the only differences between the two proteins occur in their highly homologous tyrosine kinase domains. c-Yes is frequently activated in a subset of human cancers, most notably colon carcinoma and melanoma (141,164,169,171). In these cancers, the level of c-Yes activation correlates with the metastatic potential of the cells. How then does activated c-Yes contribute to the cancerous phenotype? Activation of the MAPK pathway may allow c-Yes to induce mitogenesis and thereby contribute to cell transformation. Tsygankova *et al.* demonstrated the importance of c-Yes in activation of the MAPK pathway in response to angiotensin II treatment of liver epithelial cells (63). Recent evidence also suggests that c-Yes may be important in regulation of cell adhesions (55) and VEGF-induced vascular permeability (92). c-Yes is most frequently activated in cancers of epithelial origin, thus c-Yes activation may contribute to metastasis by regulating tight junctions and/or through an increase in vascular permeability that would enhance angiogenesis.

While the data indicate that the c-Yes amino terminus prevents its participation in pathways that regulate actin filament rearrangement, it should be noted that palmitoylation of Src family kinases is a reversible process (99,101). As the SH4 domain is essential for the loss of function associated with the c-Yes amino terminus, regulated de-palmitoylation of c-Yes in cancerous cells may allow the protein to move out of lipid raft fractions and participate in signaling pathways that were previously inaccessible. As mentioned above, v-Yes is not palmitoylated and is capable of inducing PI3K activation, CEF transformation, and tumor formation in chickens (5,15,186). The palmitoylation status of activated c-Yes in cancerous cells has not been reported. Nevertheless, the results presented here indicate that the c-Yes amino terminus, including the SH4 palmitoylation signal and the Unique domain, are crucial for the inability of activated Src/Yes chimeras to induce actin filament rearrangement, activation of the PI3K/Akt pathway, and induction of HO-1 expression. The amino terminal region may thus limit the ability of c-Yes itself to contribute to the onset and progression of the metastatic phenotype. Thus, if activated c-Yes plays a role in cancer, we would predict that it would have to be displaced from interactions governed by the SH4/Unique domain before it could exert activation of PI3-kinase and changes in actin filament integrity that are a hallmark of transformation.

The experiments outlined in this review have examined the contributions of the non-catalytic functional domains to signaling specificity between the c-Src and c-Yes tyrosine kinases. The results indicate that each non-catalytic functional domain contributes to some aspect of signaling specificity. These results allow the proposal of a working model for specificity in signaling between c-Src and c-Yes (Figure 6). In this model, after synthesis, the two proteins are rapidly localized to cellular membranes. Upon membrane localization, c-Src and c-Yes differentially partition into detergent soluble and insoluble regions, respectively, as dictated by differences in their amino termini. This may be the most important contribution to signaling specificity between c-Src and c-Yes, as it determines the subset of proteins available for intermolecular interactions. Protein/protein interactions mediated by the Unique domain may contribute to membrane compartmentalization or stable complex formation with intermolecular binding partners that allow initiation of downstream signaling events. Protein/protein

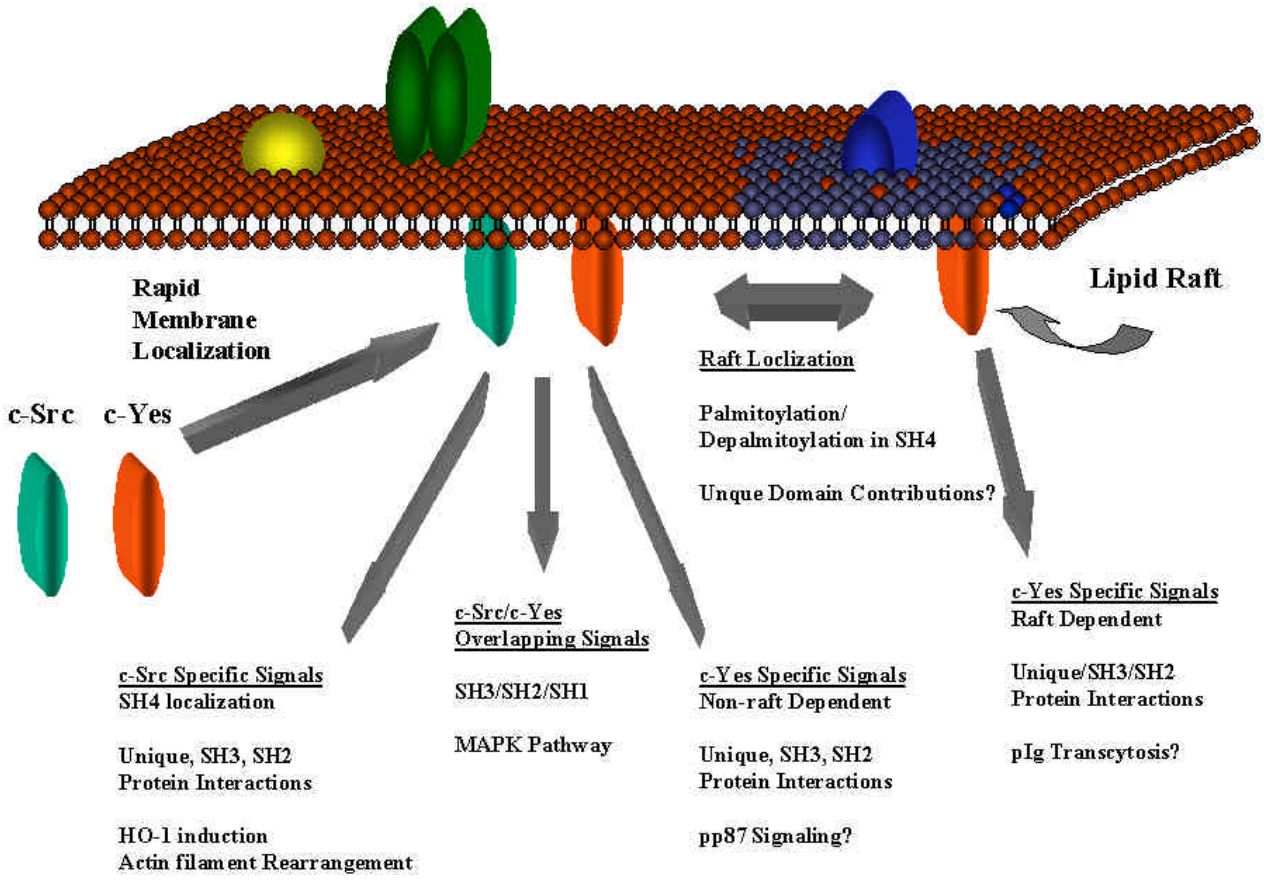

Figure 6. Model for Signaling Specificity between c-Src and c-Yes. In this model, after synthesis, c-Src and c-Yes are rapidly localized to cellular membranes. At the membrane, subtle differences in signaling between the two proteins may be mediated by differential protein/protein interactions through the SH3, SH2, and Unique domains. These protein/protein interactions may serve to affect subcellular localization, direct stable signaling complex formation, or select substrates for phosphorylation. The SH3, SH2, and SH1 (the tyrosine kinase domain) domains may also serve to transmit overlapping signals between the kinases. After membrane localization, c-Yes partitions into lipid raft fractions. The reversible nature of palmitoylation may allow c-Yes to traffic in and out of lipid rafts. Sequestration into lipid rafts may contribute significantly to c-Yes specific signaling by dictating the sub-set of proteins available for intermolecular interactions. The Unique domain may also contribute to localization. Once inside lipid rafts, c-Yes-specific signals may be dictated by protein/protein interactions mediated through the Unique, SH3, and SH2 domains.

interactions or phosphorylation events mediated by the Unique domains may also contribute to differential activation of c-Src and c-Yes, through destabilization of the closed conformation. Signaling specificity is further fine-tuned through intermolecular interactions mediated by the SH3 and SH2 domains. These regions serve to select substrates, and perhaps more importantly, direct stable associations with protein binding partners that may influence sub-cellular localization, activation state, and formation of multi-protein signaling complexes through which specific signals are sent. This model for signaling specificity does not exclude the ability of c-Src and c-Yes to function redundantly. Src family kinases are rapidly (5 min) localized to cellular membranes after synthesis, however, it was observed for Fyn that localization to detergent resistant lipid rafts occurs after a 10-20 min lag time (187), indicating potentially different mechanisms for membrane association. c-Yes and c-Src may thus interact with overlapping subsets of proteins before c-Yes partitions into lipid rafts. Additionally, regulated de-palmitoylation of c-Yes, and partitioning of other proteins between raft and non-raft fractions, may bring c-Src and c-Yes into contact with overlapping sets of proteins.

This model for signaling specificity between c-Src and c-Yes may be more broadly applicable to signaling specificity between Src family kinases in general. Understanding how these kinases are capable of sending specific signals will be important in the overall understanding of the function of these proteins, and in their possible usage as targets for rational drug design. Src family kinase inhibitors have shown promise as anti-cancer drugs due to their ability to block cell proliferation, however, most of these compounds do not distinguish

between Src family members (178,188,189). Studies in mice lacking one or more Src family members have revealed that loss of individual Src family kinases is more readily tolerated than loss of multiple Src family members (76). Thus, non-specific inhibition of Src family kinases may have deleterious effects as a result of disruption of multiple signaling pathways. A more ideal treatment strategy would involve inhibition of only the kinase or kinases that were abnormally activated in a particular tumor. A comprehensive knowledge of the roles of the functional domains in generating signaling specificity may allow the design of drugs specific for individual Src family kinases that would avoid the potentially deleterious effects of global Src family kinase inhibition.

10. SUMMARY

While the information available on c-Yes still lags behind that of c-Src and other members of the Src family, over the past decade, knowledge of the regulation and functions of the c-Yes tyrosine kinase have increased dramatically. It has become clear that c-Yes is capable of sending specific signals, however, the manner by which c-Yes accomplishes this remains a mystery. The works presented in this review have summarized c-Yes and the issue of signaling specificity between c-Src and c-Yes, with particular emphasis placed on the contributions of the functional domains to specificity in signaling between these closely related proteins. It is now clear that c-Src plays a key role in regulating a number of cellular signals associated with cell growth and changes in the cytoskeleton that are associated with transformation and cell motility. A function for c-Yes is less clear; however, several important observations point to a potential role in regulating cell-cell interactions and vesicle trafficking, particularly in polarized cells. Each of the functional domains present in c-Yes appears to play some role in dictating specificity in signaling. A role for c-Yes in cancer is less clear, especially given the results of Summy et al., indicating that activated c-Yes does not direct changes in actin filaments that are normally associated with transformation (191). However, it is possible that distinct signals or changes that occur in cancer cells could direct activated c-Yes away from membrane microdomains and permit c-Yes to stimulate signaling cascades associated with transformation. With regards to cancer, the data of Summy et al., (191) indicate that activated c-Yes would be unable to induce activation of signals that alter actin filament integrity – a hallmark for transformation. This inability is based on the function of the SH4/Unique domain, which likely positions c-Yes in membranous regions of the cell that sequester it away from PI3-kinase, or permit it to interact with signaling proteins that preclude interactions with signaling partners that would direct activation of PI3-kinase and subsequent changes in actin filament integrity. Here, it was also noted that activate c-Yes is predicted to be unable to induce motility or invasion of chick embryo fibroblast cells. It was noteworthy that activated c-Yes was able to induce increased phosphorylation of Erk1/3, but unable to activate c-Raf. Thus, at first glance, it would appear that activated c-Yes would be unable to induce activation of cellular signals that direct transformation. However, in cancer cells, it is possible that activated c-Yes could be moved to different subcellular regions and stimulate transformation. This is evident based on that observation that chimeric constructs that replace either the SH4 region or Unique domain with the equivalent domains/regions of c-Src enable activated c-Yes to induce a transformed phenotype. Thus, activated c-Yes has the potential to induce transformation; however, we would hypothesize that SH4/Unique domain interactions prevent this.

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