Tyrosine kinases: modular signaling enzymes with tunable specificities

Cytoplasmic tyrosine kinases are composed of modular domains; one (SH1) has catalytic activity, the other two (SH2 and SH3) do not. Kinase specificity is largely determined by the binding preferences of the SH2 domain. Attaching the SH1 domain to a new SH2 domain, via protein–protein association or mutation, can thus dramatically change kinase function.

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Protein kinases are one of the largest protein families identified to date; over 45 new members are identified each year. It is estimated that up to 4 % of vertebrate proteins are protein kinases [1]. The protein kinases are categorized by their specificity for serine/threonine, tyrosine, or histidine residues. Protein tyrosine kinases account for roughly half of all kinases. They occur as membrane-bound receptors or cytoplasmic proteins and are involved in a wide variety of cellular functions, including cytokine responses, antigen-dependent immune responses, cellular transformation by RNA viruses, oncogenesis, regulation of the cell cycle, and modification of cell morphology (Fig. 1). Although new tyrosine kinases are rapidly identified by homology, only a handful of authentic *in vivo* substrates of tyrosine kinases have been unequivocally identified. This is a result of the overlapping substrate specificities of many tyrosine kinases, which makes it difficult to dissect the individual signaling pathways by scanning for unique target motifs [2].

The apparent promiscuity of individual tyrosine kinases is a result of their unique structural organization. Enzyme specificity is typically programmed by one binding site, which recognizes the substrate and also contains exquisitely oriented active-site functional groups that help to lower the energy of the transition state for the conversion of specific substrates to products. Tyrosine kinases instead segregate catalysis and substrate binding into separate domains. This segregation allows rapid modulation of the target specificity of tyrosine kinases in

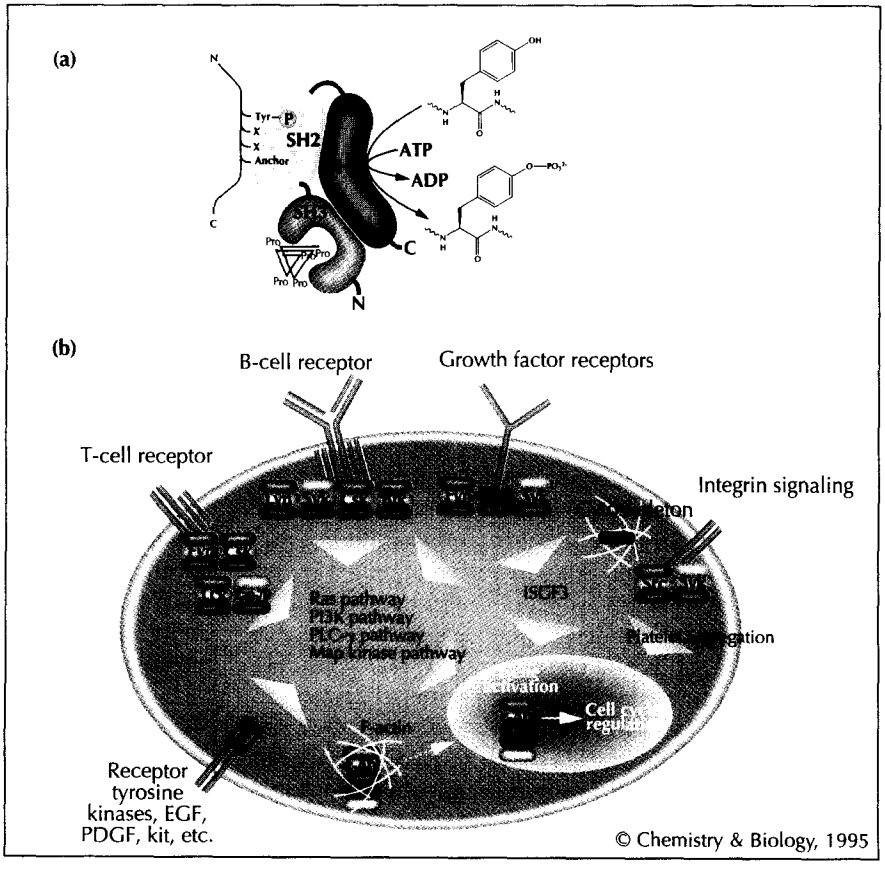

Fig. 1. Functions of tyrosine kinases in receptor signaling. **(a)** Domain structure of a Src-family tyrosine kinase. The SH1 domain phosphorylates proteins on tyrosine; the SH2 domain binds to phosphotyrosine (Tyr-P)-containing sites; the SH3 domain binds to proline-rich sequences in a type II helix. **(b)** Receptor complexes known to have tyrosine kinase activity (i.e., receptor tyrosine kinases) or known to associate with cytoplasmic tyrosine kinases. These tyrosine kinases in turn activate a number of signaling pathways directly or indirectly, including the Ras, phosphatidylinositol 3 kinase (PI3K), phospholipase C-γ (PLC-γ), and mitogen-activated protein (MAP) kinase pathways. The ultimate result of the activation of these pathways is selective gene regulation. One important class of tyrosine kinases, the Jak family, directly phosphorylate cytoplasmic targets, which then translocate to the nucleus and serve directly as transcription factors. Integrin signaling leads to changes in cell morphology and cell–cell aggregation due to activation of kinases associated with the cell cytoskeleton. c-Abl is a tyrosine kinase known to associate with F-actin in the cytoplasm and with DNA in the nucleus. The activity of c-Abl is controlled by several nuclear factors including the Ser/Thr kinase Cdc2.

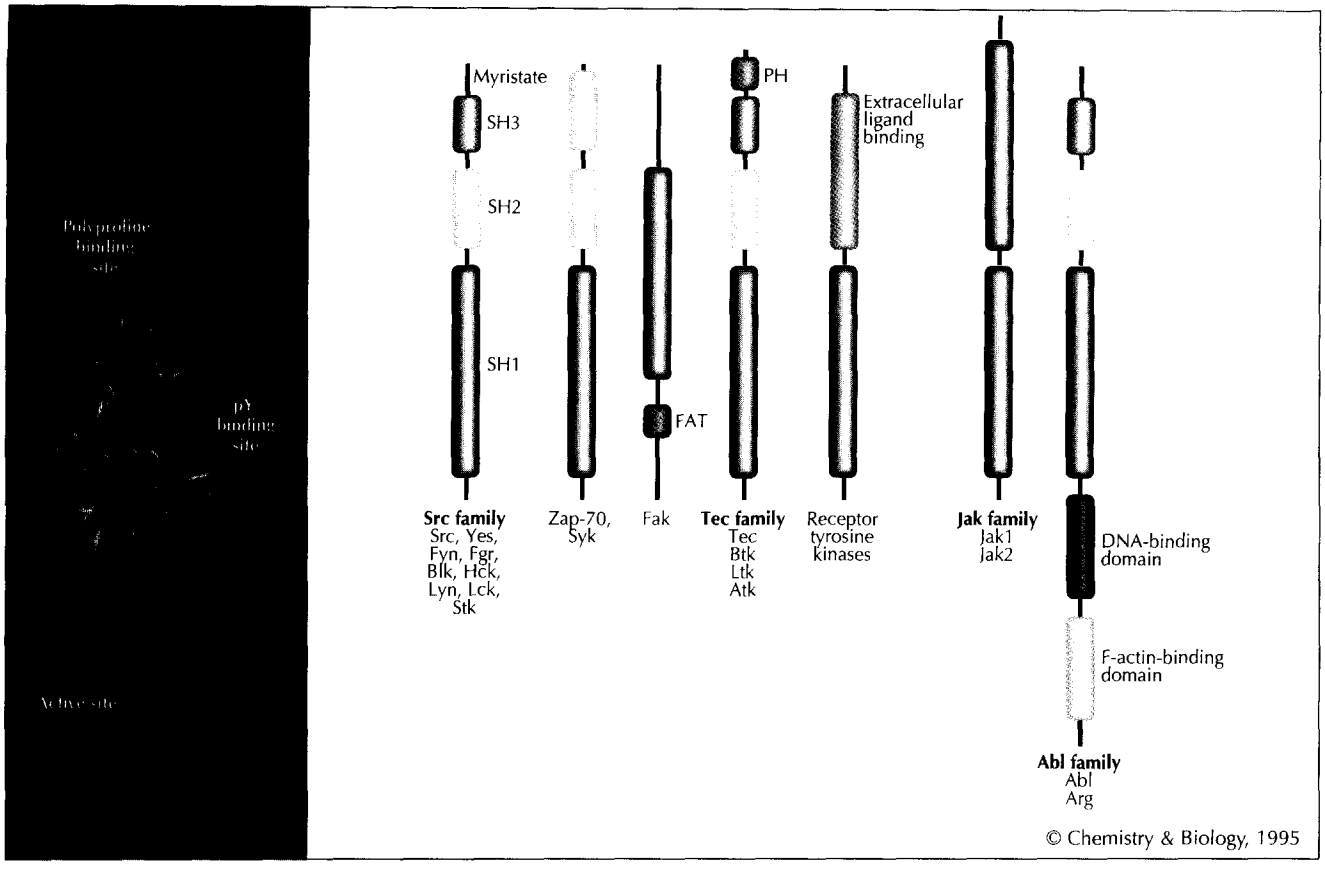

Fig. 2. Tyrosine kinases are made up of modular domains. Left: ribbon diagrams of the structures of SH3 [11] (top), SH2 [15] (middle) and SH1 [22] (bottom) domains (not to scale). Right: schematic representations of the domain structure of tyrosine kinases. Cytoplasmic tyrosine kinases contain at least one domain that mediates protein–protein association, such as the SH2 domain (color-coded yellow), SH3 domain (blue), pleckstrin homology domain (PH) or the focal adhesion targeting (FAT) domain of Fak. Src family kinases also carry a site for myristoylation,allowing their incorporation into the inner leaflet of the membrane bilayer. Abl also has a DNA-binding domain. The receptor tyrosine kinases, such as the EGF receptor, or the PDGF receptor, carry a domain for extracellular ligand binding. Hormone binding to this domain generally crosslinks the receptor molecules, causing the internal SH1 domains to associate and phosphorylate each other.

response to external signals and appears to provide a powerful strategy for transmitting signals within cells. As well as this, tyrosine kinases are localized in different parts of the cell, which is a powerful determinant of substrate availability and greatly affects the spectrum of *in vivo* kinase substrates. This review will focus primarily on the substrate specificity of cytoplasmic tyrosine kinases and how their specificity can be modulated *in vivo* (for other recent tyrosine kinase reviews see [3–7]).

Domain structure of kinases

Most cytoplasmic tyrosine kinases are organized into Src homology 1 (SH1), SH2, and SH3 domains which are defined by sequence homology, structural studies of isolated single domains, and domain-swapping experiments (Fig. 2) [6]. The domains are named after those found in the first tyrosine kinase identified, the protein product of the v-*src* gene of Rous sarcoma virus [8]. The SH1 domain is responsible for catalyzing phosphate transfer to protein tyrosine residues, and is relatively promiscuous. The SH3 and SH2 domains have no catalytic activity, but are largely responsible for determining cellular localization and protein–protein association, and thus largely determine substrate specificity. In what follows, I will briefly review the binding specificities of the individual domains, before discussing how these different specificities are integrated to determine the overall substrate selectivity of the tyrosine kinase.

The SH3 domain recognizes proline-rich sequences that can adopt a polyproline type II (PPII) helix conformation. The binding interaction was identified both by screening of peptide libraries [9] and by analysis of a number of X-ray [10] and NMR [11] structures of SH3 domains with bound peptides. Interestingly, SH3 domains can bind peptides in either the amino→carboxy orientation or the carboxy→amino orientation [12,13]; binding affinities are in the low micromolar range. The interaction between SH3 domains and their ligands is now sufficiently well understood that the binding specificity of a given SH3 domain for a given proline-rich peptide can be predicted.

The binding of SH2 domains to their targets is tighter, with affinities in the low nanomolar range, and the specificity of the SH2 domain of a tyrosine kinase appears to be the major determinant of its substrate specificity. The SH2 domain binds phosphotyrosine-containing protein

or peptide sequences with high specificity. NMR [14] and X-ray [15,16] structures of the free SH2 domain and of the complex with phosphotyrosine-containing ligands have been determined, and show that the SH2 domain is composed of two key binding pockets, one for the phosphotyrosine side chain, the other for the third residue on the carboxy-terminal side of the tyrosine. Peptide binding to the SH2 domain has been compared to a two-pronged plug fitting into a two-holed socket [17].

The target sequence preferences of many SH2 domains have been determined using a random peptide library approach [18]. The majority of SH2 domains tested have non-overlapping sequence preferences. An important exception, however, is the Src kinase family whose nine members all have SH2 domains that recognize the pYEEI motif. Substrate selectivity between Src family kinases may be guided instead by the SH3 domain [6]. Several tyrosine kinases contain tandem SH2 domains (see Fig. 2) and have been shown to bind selectively to proteins with appropriately spaced tandem phosphotyrosine motifs [19]. The presence of two SH2 domains provides a mechanism for increasing the target selectivity of a kinase while using the same set of modular elements. The common occurrence of SH2 domains in kinases, phosphotyrosine phosphatases, and in proteins possessing no catalytic activity (so-called adapter proteins, see below) suggests that this module is highly versatile and can confer useful recognition properties on a number of proteins [6]. The SH2 domain may localize the tyrosine kinase to a particular receptor complex (e.g. the B-cell receptor complex) and allow the SH1 domain to phosphorylate a number of substrates in the complex. Thus, the SH2 domain does not necessarily bind to the same protein that the SH1 domain phosphorylates.

The carboxy-terminal domain (SH1) is the catalytic domain responsible for ATP binding and transfer of the γ-phosphate to tyrosine residues. Several crystal structures of the highly homologous Ser/Thr kinases [20,21] and a recent structure of the SH1 domain of the insulin receptor tyrosine kinase [22] have provided detailed structural information about the catalytic domain. The SH1 domain is composed of a 120 amino acid amino-terminal domain and a 150 amino acid carboxy-terminal domain with the domain interface forming the active site of the enzyme and the ATP binding site. Information from many sources has led to the conclusion that the SH1 domain is extremely promiscuous in its substrate specificity [1]. For example, Lawrence and coworkers [23] showed that the SH1 domain of Src will phosphorylate many divergent substrates; D-Tyr-containing peptides are phosphorylated almost as efficiently as the corresponding L-Tyr-containing peptides, serine residues can be phosphorylated with modest efficiency, and even aliphatic alcohols containing 2–10 methylene groups between the phosphate acceptor and the peptide chain are phosphorylated. The SH1 domain of Abl can even accept nucleotides other than ATP as phosphate donors with modest efficiency (the K_Ms for the different nucleotide triphosphates tested are: ATP, 18 μM; GTP, 1.6 mM; CTP, 5.5 mM; and UTP, 6.9 mM [24]).

In vitro specificity of tyrosine kinases

The *in vitro* specificities of a number of tyrosine kinases have been determined using peptide libraries [25,26]. Sonjyang *et al.* [26] synthesized a library of 15-amino-acid peptides containing a fixed Tyr at position seven. The four residues preceding and following this residue were randomized among all amino acids except for Trp, Cys, Tyr, Ser and Thr for a total possible degeneracy of $15^8 = 2.56 \times 10^9$. Aliquots (1 mg, 6.7×10^{-7}M) of the peptide library were phosphorylated *in vitro* by individual tyrosine kinases, and the phosphorylated sequences (<1%) were separated and analyzed by amino-terminal sequence analysis. After an adjustment to control for different coupling efficiencies, it was possible to assign a scale of preferences in which a value of 15 denotes absolute specificity for one residue over the 14 other possible amino acids and a value of 1 indicates no sequence preference. The most specific tyrosine kinase tested (c-Fps) had a maximal preference of 4.5 for Glu next to the phosphotyrosine, on the carboxy-terminal side. Most of the tyrosine kinases tested showed preference values of 2–3 for residues flanking the phosphorylated tyrosine. The Ser/Thr-specific protein kinases were also studied by the same method [27], and found to have much greater target selectivity. The most specific Ser/Thr kinase in the study was cyclin B–Cdc2 which preferred proline carboxy-terminal to the phosphoserine, with a value of 11.2. Other Ser/Thr kinases generally gave preference values in the range of 5 to 9.

Control of tyrosine kinase specificity *in vivo*

This sophisticated peptide library approach as well as many *in vivo* studies have demonstrated that the tyrosine kinases do not display nearly the same levels of intrinsic substrate specificity as the Ser/Thr-specific protein kinases. Caution must therefore be used when applying *in vitro* methods for assessing substrate specificity of tyrosine kinases to *in vivo* target specificities. Since the level of intrinsic specificity is small, selectivity must be determined by other factors, such as competition for a limited number of targets. This would allow small differences in substrate specificity *in vitro* to give much higher selectivities *in vivo*. The intracellular localization of tyrosine kinases is also critical in determining substrate specificity. For example, mutations that abrogate amino-terminal myristoylation of Src family members, preventing them from localizing to the cell membrane, block their ability to transform cells [28,29]. In addition to tyrosine kinase activity, the specificity and activity of protein tyrosine phosphatases in cells is critical for the control of the steady-state level of tyrosine phosphorylation [1].

Another mechanism for determining substrate selectivity is regulation of the pleiotropic catalytic SH1 domain by the SH3 and SH2 domains. For example, in the case of Src and Abl it has been shown that the SH3 domains

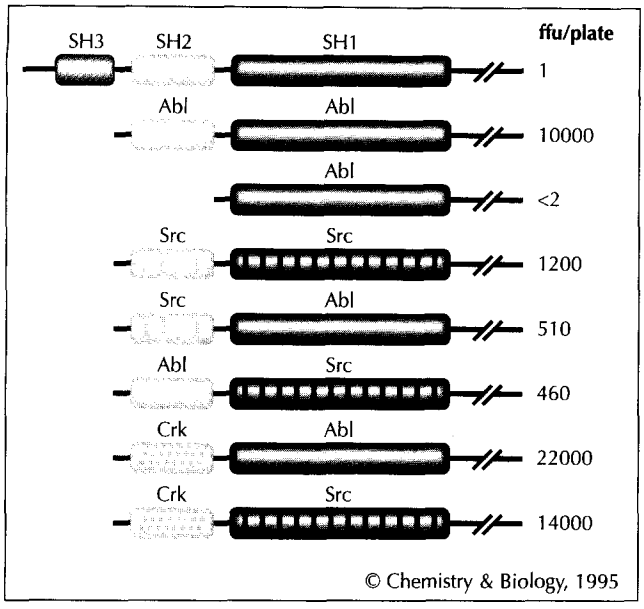

Fig. 3. Varying the combinations of SH2 and SH3 domains attached to an SH1 domain can have large effects on its transforming activity in cells. Wild-type Src or Abl (top) are not transforming (transforming activity is measured as focus-forming units per plate in a lawn of transfected 3T3 cells [31]). When the SH3 domain is deleted, the transfected cells are transformed 10 000 (Abl) or 1200 (Src) times more often. Deleting the SH2 domain reduced transforming activity in both cases; replacing it with the Crk SH2 domain enhanced transforming activity.

negatively regulate kinase activity, either by recruiting an inhibitor to the protein, or by binding to a carboxy-terminal region of the kinase, causing inhibition [3,30]. Viral tyrosine kinases often lack the SH3 domain, and are therefore constitutively active and highly transforming. Deletion of the SH3 domain of c-Abl causes an increase in transformation efficiency of five orders of magnitude. The SH3 domains in these two kinases can be viewed primarily as on/off switches for catalytic activity [31].

Deletion of the SH2 domain of Src or Abl, in contrast, results in mutants that are less able to transform cells, although their *in vitro* kinase activity, assayed on synthetic substrates, is not affected [32,33]. Thus, an SH2 domain is necessary to guide the catalytic SH1 domain to the targets responsible for the transformation of normal cells. To examine this requirement further, hybrid kinases with SH2 domains from one kinase and SH1 domains from another were constructed (Fig. 3) [31]. Hybrids between Src and Abl have diminished transforming activities (Fig. 3). But when Src or Abl SH1 domains were coupled to the SH2 domain derived from the protein Crk, the transforming activities were increased. The protein Crk is a so-called adapter protein. It possesses one SH2 and two SH3 domains but no SH1 domain and thus has no catalytic activity [34]. Thus it appears that the Crk SH2 domain is even better than the Src or Abl SH2 domains at directing the SH1 domain of either of these kinases to the targets responsible for transforming activity. Clearly, the phosphotyrosine-binding domain (SH2) is the determining factor in specifying the function of the catalytic (SH1) domain.

Modulation of tyrosine kinase specificity by associated proteins

What is the physiological significance of the highly transforming hybrid kinase composed of the Crk SH2 domain and Abl SH1 domain? To answer this question Mayer *et al.* [33] looked *in vitro* at the phosphorylation of a substrate of c-Abl — a protein called p130. Upon transformation by Abl or by v-Crk, p130 is hyperphosphorylated at a number of tyrosine residues. But wild-type Abl does not significantly hyperphosphorylate p130 *in vitro*, suggesting that a cellular factor is required to bring Abl close to p130. A hybrid kinase composed of the Crk SH2 domain and the Abl SH1 domain does hyperphosphorylate p130 *in vitro*, however, suggesting that the Crk SH2 domain was itself sufficient to localize the hybrid protein to p130 (Fig. 4). Strikingly, wild-type Abl could hyperphosphorylate p130 in the presence of Crk in a processive manner. These data and the finding that Crk and Abl are stably associated *in vivo* (via the central SH3 domain of Crk and a proline-rich sequence in Abl) strongly suggest that the substrate specificity of Abl is modulated by non-covalent association with Crk [35]. This finding dramatically shows that modules from non-covalently associated proteins can tune the specificity of these versatile catalysts. In terms of catalyst design, if substrate specificity needs to be modulated in response to other signals (i.e. Crk or other adapters) it is critical for the SH1 domain to have little intrinsic substrate specificity.

Wang and coworkers have identified the carboxyl-terminal repeated domain (CTD) of mammalian RNA polymerase II as a true *in vivo* substrate of c-Abl [36]. Strikingly, the CTD is hyperphosphorylated by c-Abl in

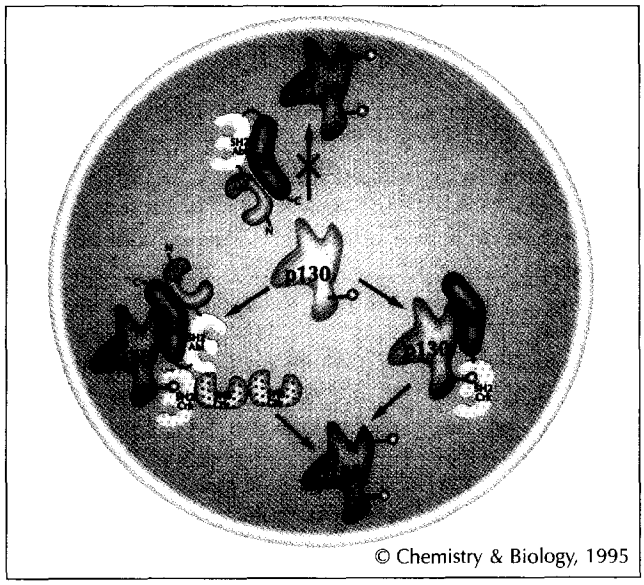

Fig. 4. Protein–protein interactions alter the specificity of Abl [33]. Abl does not bind directly to p130, but Abl and Crk can associate due to interactions between the central Crk SH3 domain and a proline-rich sequence in Abl (left). When Crk recognizes phosphorylated p130, Abl is also recruited to the complex and phosphorylates it at a new site. Right: in a cell transfected with a hybrid protein comprising the Abl SH1 domain and the Crk SH2 domain, binding and phosphorylation occurs directly, without requiring protein–protein association. Green circles, phosphate groups.

a processive manner on up to 52 tyrosine residues [37]. Importantly, the SH2 domain of Abl is necessary to mediate the processive phosphorylation of CTD. Although it is necessary, the SH2 domain of Abl is not sufficient to mediate phosphorylation of CTD; a hybrid kinase composed of the Abl SH2 domain and the Src SH1 domain does not phosphorylate CTD in a processive manner. These studies suggest that it is the combination of the Abl SH1 and SH2 domains that is essential for processive phosphorylation of physiological substrates. It has been proposed that, as a general rule, the specificity of the SH2 domain is coordinated with the modest specificity of the SH1 domain in cytoplasmic tyrosine kinases [26]. Thus, the SH2 domain of Abl would be expected to bind the product of the phosphorylation catalyzed by the SH1 domain on CTD, allowing processive phosphorylation. But how is CTD initially phosphorylated? Wang and coworkers have shown that another domain of Abl, carboxy terminal to SH1, contains a binding site for unphosphorylated CTD [37].

Conclusions

The tyrosine kinases are one of the largest and most important families of proteins in vertebrates. They function in a fundamentally different manner from most enzymes. Rather than having a single active site, the tyrosine kinases have separate domains for substrate recognition (SH3 and SH2) and chemical catalysis (SH1). The segregation of these two functions allows the specificity of kinases to be rapidly modulated, for example by association with other SH2/SH3-containing adapter proteins.

The segregation of catalysis and binding into different domains and the poor intrinsic specificity of tyrosine kinases *in vitro* has made assignment of the precise *in vivo* targets of protein tyrosine kinases difficult to determine. New strategies for identifying cellular substrates of individual tyrosine kinases would greatly facilitate the study of tyrosine kinase signaling. By identifying each kinase's substrate under true *in vivo* signaling conditions, the molecular logic of these signaling cascades could be more fully understood. Furthermore, identification of the *in vivo* targets of oncogenic or viral kinases will provide critical targets for rational drug design. Our laboratory and others are currently developing novel chemical approaches for identifying *in vivo* substrates of tyrosine kinases.

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