

REVIEW

**Structure-Function Relationships of Two
Protein-Tyrosine Kinases; the *src* Gene Product
and the Fibroblast Growth Factor Receptor**

Masahiro SATO*

Received November 22, 1991

KEY WORDS: Receptor type/ Nonreceptor type/ pp60^{src}/ *bek*/ Signal transduction

I. Introduction

Protein phosphorylation plays essential roles as intracellular regulatory mechanisms at several different levels of signal transduction pathways from cell surface receptors to nuclear proteins in eukaryotic cells. These reactions are catalyzed by a group of enzymes called protein kinases, and studies on these kinases represent the major contribution to our understandings on the malignant transformation of cells as well as the control of normal cell growth (for reviews, see Refs. 1-8).

Based on the species of amino acid residues to be phosphorylated, protein kinases can be classified into two groups (1, 2, 5); one is protein-tyrosine kinase which phosphorylates tyrosine residues on substrate proteins and the other is serine/threonine kinase which phosphorylates serine or threonine residues or both. In contrast to serine/threonine kinases whose activities have been identified early and found to be distributed abundantly in various types of cells (2), the tyrosine kinase activity was identified rather recently in the product of the *src* gene which is a transforming gene (oncogene) of an avian retrovirus, Rous sarcoma virus (RSV) (10, 11, 22). This finding established a unique protein kinase with activity of phosphorylating the tyrosine residue which is directly involved in the neoplastic tumor formation. The importance of the protein-tyrosine kinase was further emphasized by the identification of this enzymatic activity in a variety of oncogene proteins, including *fes*, *fgf*, *fms*, *ros*, and *yes* (5, 14-21). Several of these genes were found to encode receptors for growth factors, and confirmed that tyrosine kinases play essential roles for the control of cell growth. Accumulation of the information on such genes also made it possible to classify the protein-tyrosine kinases into two groups based on their structural and functional features: one is nonreceptor tyrosine kinases which are localized in cytosolic fraction and the other is receptor tyrosine kinases which are transmembrane molecules (1, 5).

Structural and functional characteristics of protein-tyrosine kinases identified originally

* 佐藤仁彦: Laboratory of Molecular Biology, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611

as viral oncogenes are twofold; first, primary structures of oncogenes are altered from those of normal cellular counterparts from which viral genes derived, and second, the protein kinase activity is found to be enhanced constitutively, otherwise regulated in response to growth conditions. Structure-function relationships of oncogenes have been extensively analyzed and through these studies, the regulatory mechanisms of the tyrosine kinase activities have become elucidated.

The author has been interested in the signal transduction pathways through the protein-tyrosine kinases and engaged in the characterization of the *src* protein (a nonreceptor tyrosine kinase) and the fibroblast growth factor (FGF) receptor (a receptor tyrosine kinase). In this article, experiments which have been carried out to clarify the structure-function relationships of the two protein kinases will be described; these include i) introduction of mutations into the *src* protein, ii) biochemical and biological characterizations of the mutated *src* proteins, and iii) isolation and identification of the isoforms of the FGF receptors. Finally, the significance of these findings will be discussed in perspective of receptor and nonreceptor tyrosine kinases.

II. Structure-function relationships of the *src* protein

1) The *src* protein

pp60^{v-src} is encoded by the transforming gene (the *v-src* gene) of an avian retrovirus RSV, and numerous biochemical and genetical studies showed that the expression of pp60^{v-src} is primarily responsible for both tumor formation *in vivo* and cellular transformation *in vitro* caused by the infection of RSV (for reviews, see Refs. 9–12). pp60^{c-src} is the product of its cellular homolog (the *c-src* gene), and comparison of the predicted amino acid se-

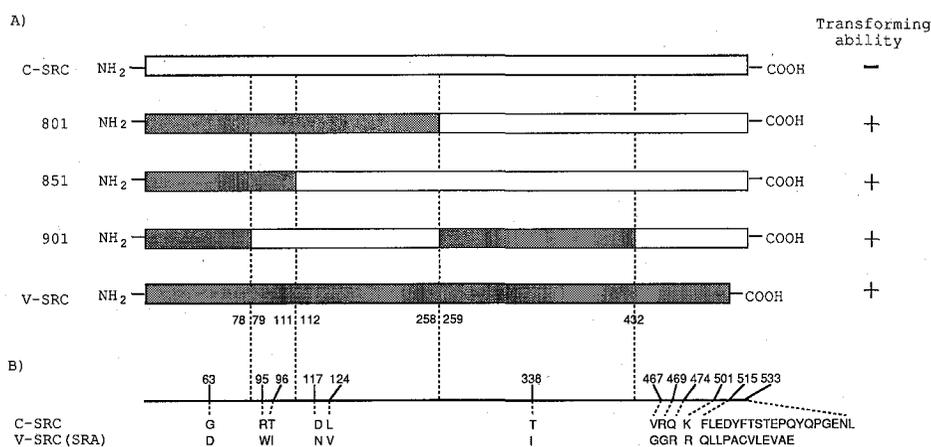


Fig. 1 Construction of the chimeric *src* gene between *v-src* and *c-src*⁽⁵³⁾. A) shaded and open box code for *v-src* and *c-src* sequence, respectively. Transforming ability (+, positive; -, negative) is also indicated. B) Summary of the differences in the primary structures between pp60^{c-src} and pp60^{v-src}.

Protein-Tyrosine Kinases

quences of the two molecules revealed their strong similarities in the primary structures except for both scattered point mutations and total replacement at their carboxy-termini; the last 19 residues of pp60^{c-src} (amino acids 515 to 533) are replaced by 12 unrelated residues in pp60^{v-src} (Fig. 1B) (23). Both pp60^{v-src} and pp60^{c-src} are membrane-bound phosphoproteins that exhibit protein-tyrosine kinase activity. Despite these similar characteristics, pp60^{c-src} cannot transform avian and mammalian cells even when expressed to the same level as that of pp60^{v-src} (24–26). This observation attracted attentions on the analysis on the structure-functional relationship of the *src* protein.

2) Structural and functional domains of the *src* protein

The overall structure of the *src* protein can be viewed as a series of interactive structural and functional domains of which boundaries have been defined by the amino acid sequence comparison with other protein kinases and the results of biochemical and genetical analyses; they are a) myristylation domain, b) modulatory domain, c) kinase domain and d) regulatory domain (Fig. 2) (11).

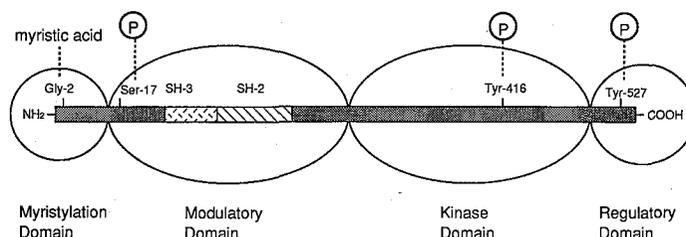


Fig. 2 Schematic diagram of the functional domains of pp60^{c-src(10)}. pp60^{c-src} consists of 533 amino acids and the domain structure is predicted based on sequence comparison and interpretation of genetic data (see text). Possible sites of serine and tyrosine phosphorylation are indicated as P. Gly-2 is a myristylation site.

a) Myristylation domain

pp60^{v-src} as well as pp60^{c-src} is associated with the inner face of the plasma membrane through myristic acid covalently attached to Gly-2 (26–29). This association is critical in viral transformation; mutants of pp60^{v-src} which do not associate with membranes do not transform cells, notwithstanding these mutants retain wild type kinase activity (28). Substitution of amino acid residues 11–15 of pp60^{v-src} does not alter the transforming potential nor the membrane association of the mutated *src* protein through the myristylation (29), suggesting that sequences required for efficient myristylation reside within the amino-terminal 10 residues of pp60^{v-src} of which sequence is conserved in pp60^{c-src} as well.

b) Modulatory domain

The region downstream to the myristylation domain of the *src* protein (amino acids 84 to 241) has been identified to be fairly conserved among the *src* protein and most of other nonreceptor tyrosine kinases, and divided further into two subdomains based on structural and functional features; SH-2 (*S*rc *H*omology-2) and SH-3 (*S*rc *H*omology-3) (31, 32). The SH-2 domain encompasses about 100 amino acids immediately amino-terminal to the cata-

lytic domain (see below), whereas the SH-3 domain encompasses about 50 amino acids amino-terminal to the SH-2 domain. Several lines of evidence showed the possible involvement of these two domains in cellular transformation somehow (11); i) CEF infected by pp60^{v-src} with a deletion of amino acids 116–141 showed fusiform morphology compared to round refractile one by wild type pp60^{v-src} (37), ii) Introduction of longer deletion in this region (e.g., amino acids 100–200) impaired the transforming ability of pp60^{v-src}, although the kinase activity was still retained (30), and iii) amino acid substitutions at amino acids 95 and 96 of pp60^{c-src} conferred the transforming activity to pp60^{c-src} (53). These results further suggest that the SH-2 domain is a positive regulatory domain and in contrast to the SH-2 domain, the SH-3 domain is a negative regulatory domain. The sequences related to either the SH-2 or SH-3 domain have been identified recently in several other proteins with or without protein kinase activity (31–36), and this will be discussed below.

c) Kinase domain

Accumulation of the DNA sequences of protein kinases made it possible to compare the deduced primary structures among them, and revealed the presence of highly conserved sequences in individual kinase including Ser/Thr kinases (5). This sequence spans in the region of amino acid residues 270 to 516 in the *src* protein. In fact, a carboxy-terminal 30kDa proteolytic fragment containing this region was shown to exhibit tyrosine kinase activity *in vitro* confirmed that the catalytic domain of pp60 is contained within this region (38). The well conserved sequence for ATP binding site (GXGXXG: G, glycine; X, any amino acid: amino acids 274–279) is seen in this region, and is followed by a lysine residue (amino acid 295) 16 residues downstream which is believed to be an acceptor site of γ -phosphate. Analysis of temperature sensitive mutants of RSV directly showed that mutations within the catalytic domain actually affect on kinase activity and transforming potential as well (39). Taken together, these results and observations mutually contributed to establish a concept of the “kinase domain”.

d) Regulatory region

The carboxy-terminal sequence of pp60^{c-src} is totally replaced by an unrelated sequence in pp60^{v-src} as described (23). By using chimeric molecules of pp60^{c-src} of which carboxy-terminal region is replaced by the corresponding region of pp60^{v-src}, the structural difference in this region solely was shown to be sufficient to convert nontransforming pp60^{c-src} to a transforming protein (53), suggesting that this carboxy-end region plays an important role for the regulation of biological activity. The critical residue(s) has been identified directly by using a mutant molecule of which Tyr-527, major phosphorylation site of pp60^{c-src} *in vivo*, was substituted with Phe (40–42); the mutated pp60^{c-src} molecules showed enhanced kinase activity, caused morphological transformation of CEF or mouse cells, and formed foci in soft agar medium.

Since the sequences in the carboxy-terminal regions including the residue corresponding to Tyr-527 of pp60^{c-src} are fairly conserved among nonreceptor tyrosine kinases, the negative regulation of the kinase activity by phosphorylation of the tyrosine residue seems to be common (12).

3) pp60 as a phosphoprotein

a) Phosphorylation sites *in vivo*

Both pp60^{c-src} and pp60^{v-src} are known to be phosphorylated at multiple sites *in vivo*.

Major sites on pp60^{c-src} are Ser-17 and Tyr-527, whereas Ser-17 and Tyr-416 on pp60^{v-src} (43-47). Ser-17 is next to the consensus sequence for the reported recognition site of cAMP-dependent protein kinase (A-kinase) and the phosphorylation by A-kinase has been proven by *in vitro* reaction (47). Tyr-416 is believed to be phosphorylated by an either inter- or intramolecular phosphotransferase activity of pp60^{v-src} by itself (48). On the other hand, Tyr-527 is believed to be phosphorylated in two ways; one is by intermolecular phosphotransfer reaction of pp60^{c-src} and the other is exogenously by another PTK distinct from pp60^{c-src}. Recently, a protein-tyrosine kinase which might be responsible for the latter reaction has been isolated from rat brain and the gene has also been molecularly cloned (49, 50). This enzyme, named CSK (c-src kinase), was shown to contain the SH-2 and SH-3 domains similar to the *src* protein. However, the amino acid identity to the *src* protein is not more than 47%, and moreover, neither putative phosphorylation sites corresponding to Tyr-416 and Tyr-527 nor the site for myristylation signal was not found.

b) Amino acid substitution and activation of pp60^{c-src}

Overexpressed *c-src* is unable to transform CEF or mouse cells *in vitro* (25-27). pp60^{c-src} has been shown to possess lower kinase activity than that of pp60^{v-src} (51, 52), possibly accounting for the lack of transforming activity. With regard to this, it is feasible that the structural differences described above (II-1) somehow contribute to give rise to the difference in their kinase activities as well as their biological activities.

More precise experiments by using chimeric molecules between pp60^{c-src} and pp60^{v-src} showed that replacement of amino acids 1 to 432 (the variant NY801) or 432 to 533 of pp60^{c-src} (the variant BB4) with the corresponding sequences of pp60^{v-src} resulted in hybrid proteins with transforming activity (53). Both of these proteins showed high levels of protein kinase activity. In contrast to the latter chimeric molecule which was constructed solely by exchanging the carboxy-terminal region (II-2-d), the former chimeric molecule contained scattered single amino acid substitutions between pp60^{v-src} and pp60^{c-src}. Kato *et al.* further dissected the *v-src* sequence of NY801 (Fig. 1) and examined the amino acid substitution(s) that might be involved in conferring the transforming activity to pp60^{c-src} protein. As a result, they found that either the change from Thr to Ile at position 338 (NY901) or the replacement of Arg-95 and Thr-96 with Trp-95 and Ile-96 (NY851) was sufficient to convert the nontransforming pp60^{c-src} protein into a transforming protein by the criteria of focus formation, anchorage-independent growth and tumor formation in new born chicken. The resulted molecules showed in elevation of the protein kinase activity as well.

c) Characterization of activated pp60^{c-src} molecules

While the RSV variants carrying these chimeric genes (NY801, NY851, and NY901; Fig. 2) were transforming viruses, they gave somewhat lower titers than that of wild-type RSV when infected onto CEF, and the morphology of the foci produced was not so distinct as that of foci produced by wild type RSV. For example, many flat cells were still observed in NY801-infected cultures (NY851 and NY901-infected cultures as well) after several passages at the time when the RSV-infected cultures showed complete morphological changes. Furthermore, pp60s encoded by these variants showed weaker kinase activities *in vitro* than did pp60^{v-src}.

Sato *et al.* characterized these RSV variants further to understand precisely the biolog-

ical features unique to these variants in terms of the behaviors of the chimeric pp60 proteins (54). First, protease V8 and tryptic peptide mapping analysis of ^{32}P -labeled chimeric molecules revealed the three major phosphorylation sites in each construct: Ser-17, Tyr-416 and Tyr-527. This was a quite contrast to normal pp60^{c-src} and even to the NY701T7 pp60, another activated form of pp60^{c-src}, which had been identified in a transforming virus from the cells infected by the RSV variant carrying the c-src gene instead of the v-src gene (55). The phosphorylation site of the NY701T7 pp60 was reported to be solely on Tyr-416 instead of Tyr-527.

On the other hand, pp60 in colonies in soft agar which composed of fully transformed cells was found to be phosphorylated exclusively at Tyr-416 and Ser-17 in contrast to pp60 in the variant virus-infected cells. These observations strongly suggest that the difference in transforming ability correlates with difference in Tyr-416 and Tyr-527 phosphorylation and further that the Tyr-416-phosphorylated form is an activated form of the chimeric pp60 and a limited population of pp60 is activated in the variant-infected cells.

Secondly, the affinity of the chimeric molecules were examined for the detergent-insoluble cellular matrix. pp60^{v-src} has been shown to be associated with a detergent-insoluble cellular matrix containing the cellular cytoskeleton. pp60^{c-src}, in contrast, is not tightly bound to the detergent-insoluble matrix (56-58). Together, the association of the c-src protein to the cytoskeleton is somehow related with its transforming activity. CEF infected with either NY801, NY851 or NY901 and cells subcultured from colonies were labeled with (^{35}S)-methionine, and pp60s were extracted with non-ionic detergent buffer after exposing for various times. The difference of the distribution between the soluble and resistant fractions after short exposure was distinct in the NY801 pp60 (as well as the NY851 and NY901 pp60s); about 40 to 45% of the chimeric pp60s were already solubilized within 1 min after exposure, while only less than 20% remained in the resistant fraction after 10 min in the case of the virus-infected cells. On the other hand, pp60 remained in detergent-resistant fraction at 1 min of extraction, and still about 40% remained after up to 10 min. According to Hamaguchi and Hanafusa, about 90% of pp60^{c-src} is solubilized within the first 2 min of extraction, whereas more than 70% of pp60^{v-src} remains in the resistant fraction after extraction for up to 10 min (56). Thus, the NY801 pp60 in which only Tyr-416 is phosphorylated, associates with the detergent-insoluble cytoskeleton more tightly than does pp60^{c-src}, but less tightly than does wild-type pp60^{v-src}.

The correlation between the phosphorylation site of the chimeric pp60 protein and the stability against the detergent extraction was examined further in NY801-infected cells. It appears that the phosphorylation of Tyr-527 was much more intense than that of Tyr-416 in detergent-soluble fraction and vice versa in detergent-insoluble fraction. Thus pp60 of which Tyr-527 was phosphorylated had less affinity for the detergent-insoluble cellular matrix than did the NY801 pp60 protein phosphorylated on Tyr-416. Therefore, phosphorylation of Tyr-416 of pp60^{c-src}, not Tyr-527, is favored for the affinity with detergent-insoluble cytoskeleton, but it is not sufficient for tight association as pp60^{v-src} shows.

In summary, the NY801 pp60 (NY851 and NY901 as well) showed a difference in the affinity for the detergent-insoluble cellular matrix depending on its tyrosine-phosphorylation site. This indicated that either the tyrosine phosphorylation site *per se* determines the stability of the association or the alteration of the phosphorylation-site reflects a conformation-

Protein-Tyrosine Kinases

al change that may result in the different mode of association. In other words, other factor(s) than the primary sequence seems to determine the stability of the association of pp60 and the cellular matrix.

III. SH-2 domain of pp60^{c-src}; implication for the unique phosphorylation profile in the chimeric molecules

The results of numerous experiments support the idea that the SH-2 domain is primarily involved in the interaction with a phosphorylated tyrosine residue (33–35). Substitution of Arg residue which is highly conserved within the SH-2 domains results in the loss of proper function (59), suggesting that an ionic interaction between phosphate group and basic amino acid residue may play a role in this interaction. The scheme on such mode of action was mainly deduced from the works on pp60^{c-src} and can be summarized as follows. Tyr-527 is believed to be phosphorylated by another PTK, possibly CSK, and phosphorylated Tyr-527 interacts with the SH-2 domain of pp60^{c-src} itself, resulting in a conformation that might have an inhibitory effects on its intrinsic kinase activity. When the phosphorylation of Tyr-527 is disturbed somehow and thereby Tyr-527 is not associated to the *src* SH-2 domain, the resulting conformation is presumed to be favorable for revealing its kinase activity fully, and thus induces autophosphorylation of Tyr-416 (Fig. 3). In this case, Tyr-

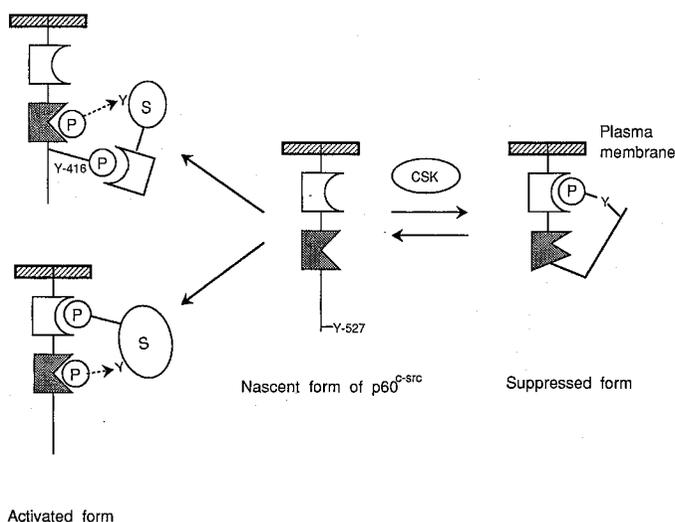


Fig. 3 Model for regulation of the kinase activity of pp60^{c-src} by phosphorylation on tyrosine residue^(7,33). Try-527 phosphorylated by CSK (c-src kinase) associates with the SH-2 region of the same pp60^{c-src} molecule, leading the molecule to the suppressed form with low kinase activity. Dephosphorylation of phosphotyrosine-527 is needed for taking the activated form. Tyr-416 will be phosphorylated by auto-phosphorylation in this form. □, Sh-2 region; ◼, Active site; Y, tyrosine residue; S, substrate.

416 could associate with other protein through the SH-2 domain. The chimeric pp60 molecules described above are presumed to be unable to be fully phosphorylated by CSK due to amino acid substitutions, giving rise to a population of unphosphorylated form of pp60 proteins on Tyr-527 which would be phosphorylated on Tyr-416 eventually. This could explain the presence of two species of pp60 in NY801 (NY851 and NY901 as well) infected CEF. The NY801 pp60 of which Tyr-416 is phosphorylated, which possesses a modest kinase activity, can associate with putative substrates in cytoskeletal matrix, but it is not so tight as pp60^{v-src} due to the remaining amino acid substitutions.

IV. Receptor tyrosine kinases vs. nonreceptor tyrosine kinases

Many of peptide hormones or growth factors mediate their pleiotropic actions by binding to and activating cell surface receptors (1, 3-6). The receptor for epidermal growth factor (EGF) was found to possess the protein-tyrosine kinase activity first (60), and the en-

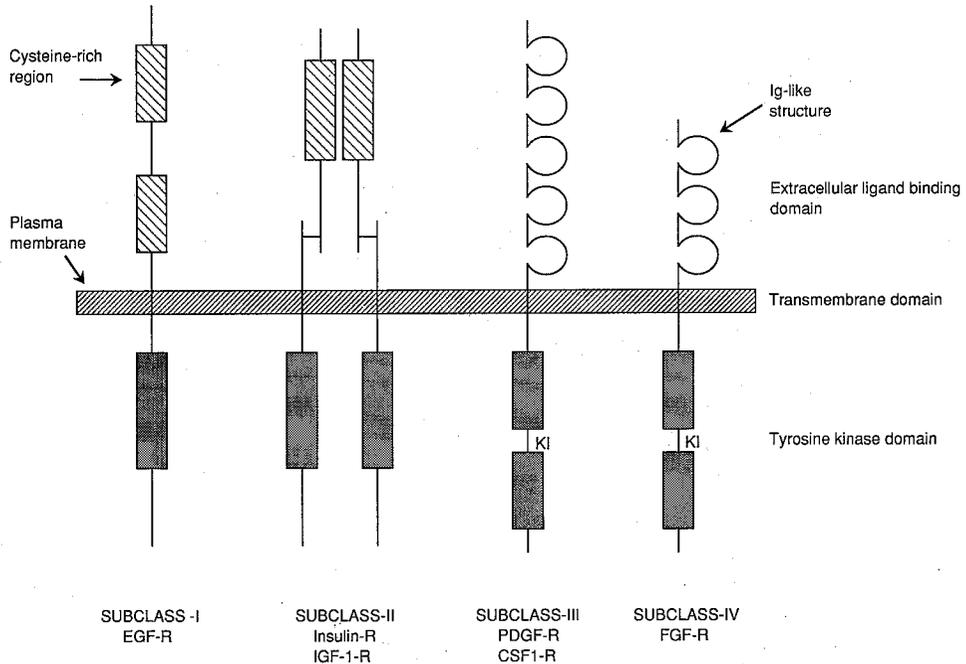


Fig. 4 Organization of receptor-tyrosine kinases^(6,8). These receptors consist of amino-terminal extracellular ligand binding domains, transmembrane domains and carboxy-terminal intracellular tyrosine kinase domains. The extracellular domains of receptors in subclasses I and II include cysteine-rich regions and those of receptors in subclasses III and IV include immunoglobulin-like repeats. Receptors in subclass II are composed of two subunits. The tyrosine kinase domains of receptors in subclasses III and IV are interrupted by an insertion of several amino acids (kinase insert: KI).

zymatic activity was shown to be stimulated by EGF binding (61), suggesting that the kinase activity plays an important role in the signal transduction. This was further confirmed by a parallel observation that *erbB*, a transforming gene of avian erythroblastosis virus (AEV), encodes a truncated form of EGF receptor lacking the extracellular domain with a constitutively elevated kinase activity (62, 63). In addition to these findings, several other growth factor receptors including the receptors for platelet-derived growth factor (PDGF), insulin, insulin-like growth factor (IGF-1), and fibroblast growth factor (FGF) were similarly found to have protein-tyrosine kinase activity (64–70). Receptor protein-tyrosine kinases were thus implicated as signal transducers in the response of cells to normal growth factors as well as in neoplastic transformation.

Comparison of the amino acid sequences of the EGF, PDGF, insulin, IGF-1, FGF, and CSF-1 receptors clarified the related structural organizations consisting of three major domains distinct in their functions (4, 6). First, the ligand binding domain is encoded in the amino-terminal extracellular portions of the proteins. There is then a stretch of nearly 24 hydrophobic amino acids which span the plasma membrane (the transmembrane domain). Finally, the tyrosine kinase catalytic domain is on the cytoplasmic side of the membrane, encoded in the carboxy-terminal portions of the proteins beginning almost 50 amino acids from the transmembrane region.

More precisely, however, on the basis of sequence similarities and distinct structural characteristics, it is possible to classify these receptors into 4 subclasses (Fig. 4) (6). Characteristic structural features of each subclasses are the following; I) two cysteine-rich repeat sequences in the extracellular domain of monomeric receptors, II) disulfide-linked heterotetrameric structures with similar cysteine-rich sequences, III) five immunoglobulin (Ig)-like repeats, and IV) three Ig-like repeats, respectively. The tyrosine kinase domain of the latter two is interrupted by hydrophilic insertion sequences of varying length (called KI).

V. FGF-receptor family and isoforms

1) Structural features of FGF receptor

Among the receptor tyrosine kinases, the FGF receptor (FGF-R) is unique in that three repeats of an Ig-like motif constitute the extracellular region (6). The details of this primary structure was revealed first by DNA sequence analysis of a cDNA clone encoding chicken basic FGF receptor (70). This Ig-like motif has the following structural features (13): 1) the presence of two characteristic cysteine residues, 2) the presence of a consensus tryptophan residue 11 or 12 amino acids from and on the carboxy-terminal side of the first cysteine residue, and finally 3) the presence of the consensus sequence DXGX₁YXC (D, Asp; G, Gly; Y, Tyr; C, Cys; X, any amino acid) on the amino-terminal side of the second cysteine residue.

2) Chicken FGF receptor and isoforms

By screening chicken embryo fibroblast cDNA expression library with anti-phosphotyrosine antibodies, Sato *et al.* isolated several cDNA clones encoding basic FGF-R related receptor molecules and two of them were named *bek* and *brk*, respectively (73). Of the two genes, the *bek* gene was expected to encode 824 amino acids carrying the structural characteristics including the three Ig-like repeats. By using the similar approach, cDNA

clones containing three FGF-R related genes, *Cek1*, 2 and 3, have been cloned independently by Pasquale (71, 72). The overall primary structure of *bek* was found to be almost identical to that of *Cek3*. However, despite this similarity, a significant structural difference was detected between the two genes; 48 amino acids between residue 316 and 363 of the *Cek3* protein are replaced by 49 amino acids in the *bek* gene (Fig. 5). This variable region, referred to AS (alternative sequence) hereafter, is in the last of the three Ig-like repeats and all the characteristics to the repeat were shown to be completely conserved.

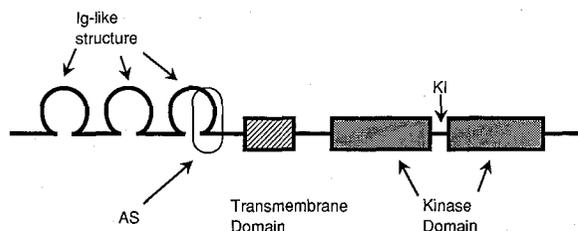


Fig. 5 Predicted primary structure of the FGF receptor is schematically shown.⁽⁷³⁾ AS: structural variation between *bek* and *Cek3*.

3) Exon-intron structure

Structural analysis of the genomic counterpart of AS gave direct evidence that ASs for *bek* and *Cek3* (*Bek-AS* and *Cek3-AS*) are located closely on the chicken genome. Structural features in this region are as follows. First, two units of sequences exactly corresponding to *bek-AS* and *Cek3-AS* respectively were found to be localized 1.3 kb apart each other, and *bek*-specific and *Cek3*-specific sequences were shown to be separated from common upstream and downstream exons at a distance of 0.7 kb and 2.3 kb, respectively. Second, considering each unit is surrounded by the consensus sequence for splice donor and acceptor sites, two units seem to be functional exons. Finally, besides these two putative internal exons, no significant sequence for another potential AS was found in this region (data not shown). Taken together, *bek* and *Cek3* specific genomic sequences could function as internal exons, and *bek* mRNA and *Cek3* mRNA could be produced by alternative splicing.

The structural alteration identical to the chicken genes has also been detected in the human-*bek* gene; they have been named human-*bek* and *K-sam*. Both *bek* mRNA and *K-sam* mRNA were reported to be derived from the same pre-messenger (76). Sequencing of the genomic region for human-*bek* gene revealed the presence of two internal exons which are specific to *K-sam* (upstream) and *bek* (downstream), respectively. Overall organization of internal and common exons seems to be well conserved between human and chicken, although sizes of putative introns are not identical.

4) Expression of *bek*- or *Cek3*-specific sequence in chicken tissues

The expression of the chicken-*bek* mRNA was examined in several tissues with a probe common to both *Cek3* and *bek* and found to be expressed predominantly in lung and brain (73). A major transcript of 4.0 kb was found to be expressed to the similar level in these tissues. A *bek*-specific probe which was derived from the *bek-AS* hybridized exclusively

Protein-Tyrosine Kinases

with 4.0 kb mRNA from lung, whereas *Cek3*-specific probe, *Cek3*-AS, hybridized predominantly with brain RNA and, to less extent, with lung RNA, strongly suggesting that the expression of *bek* and *Cek3* mRNAs are regulated in a manner of tissue-specific.

The significance of this tissue specificity was examined more precisely by *in situ* hybridization histochemistry in brain and lung using digoxigenin-labeled RNA probes. Both *bek* and *Cek3* probes detected the homogeneous expression in lung, whereas in brain, *Cek3* probe detected the expression in corpora medulla of cerebellum as well as in cerebrum. Any significant signal was hardly detected with *bek* probe in consistent with the results of northern blot analysis.

5) Physiological significance of the existence of isoforms

In addition to the *bek*-isoforms described above, several isoforms have been identified in FGF-receptor family. Structural alterations generating those isoforms can be mapped at five sites within the receptor molecule (Table 1); 1) the first of the three Ig-like repeats is missing or deleted in several genes related to *flg* and *bek* (69, 70, 75–84), 2) two amino acids insertion or deletion in the two regions was identified in *flg* and *bek* (76, 78, 80), 3)

Table 1. FGF receptor family and isoforms (Refs.: see text).

| FGF-R subfamily | Genes in subfamily | Structural features of isoforms | Ligand |
|-----------------|---|---|----------------------|
| 1 | <i>flg</i> <i>Cek1</i> | 3 Ig-like repeats type 2 Ig-like repeats type secreted type 2 amino acids insertion/deletion type kinase domain deletion type | aFGF bFGF KFGF |
| 2 | <i>bek</i> <i>K-sam</i> <i>Cek3</i> <i>KGF-R</i> | 3 Ig-like repeats type 2 Ig-like repeats type 2 amino acids insertion/deletion type | aFGF bFGF KGF |
| 3 | <i>Cek2,brk</i> | 3 Ig-like repeats type | |
| 4 | FGF-R 3 | 3 Ig-like repeats type | aFGF, bFGF |
| 5 | FGF-R 4 | 3 Ig-like repeats type | aFGF |

deletion in the carboxy-terminal region of kinase domain has been reported in human-*flg* (80), 4) a stretch of amino acid residues has been replaced with distantly related sequence in *bek* as described in this article (72, 73, 79, 82), and finally 5) truncated forms consisting of a part of extracellular domain and thus could be secreted from the cell have been identified in *flg* and *bek* (78, 84). Several lines of evidence indicate that alternative splicing is primarily involved in generating such diverse FGF receptor isoforms.

The biological significance of the existence of diverse isoforms and the physiological role of each receptor molecule are mostly unclear. With regard to this, two isoforms of *flg* consisting of two and three Ig-like motifs respectively have been reported to show no significant difference in the binding activity of either acidic FGF or basic FGF, indicating that at least the amino-terminal first Ig-like motif seems to have no significant role for the binding of ligand of *flg* (84). At present, the following possibilities can be listed in considering the significance of isoforms; 1) structural variations may be involved in tissue specific expression

as described above, 2) structural differences are directly involved in the ligand specificity, 3) the structural change close to transmembrane domain may affect the localization or dimerization in plasma membrane, 4) some of the isoforms are inactive form and the formation of heterodimer between active and inactive form is responsible for the inhibitory effect, 5) the truncated form could be a noncompetitive inhibitor for the ligand binding, and finally 6) the structural alteration in the kinase domain could affect on the tyrosine kinase activity as well as the substrate specificity, modulating the signal transduction pathway in the cell.

Recently, it appears that proteoglycans such as heparin or heparan sulfate play important roles for growth factor activities by binding to both extracellular proteins and growth factors, suggesting that the role of structural variations identified within extracellular domain could affect the interaction with such modulators (86–89).

VI. Conclusions

Both the receptor and nonreceptor protein-tyrosine kinases can be viewed as transducers of information within the cell. They respond to activating stimuli by increasing their catalytic activities and transfer information to target molecules by phosphorylation, raising two important questions; how their activities are regulated and which proteins are their substrates.

The enhancement of catalytic activity of the receptor is initiated by the binding of the genuine growth factor to its extracellular domain. This induces dimerization (tetramerization in case of subgroup II) of homologous monomer, and the resulting conformational change is believed to enhance its intrinsic kinase activity somehow, leading to the autophosphorylation on tyrosine residue(s) within the kinase domain (6, 90, 91). The phosphorylated receptor molecules are now able to associate with other proteins carrying the SH-2 domain and phosphorylate them to transduce signals. Among proteins to be recognized by receptors, several proteins functioning in the metabolism of second messengers are known to be regulated in such a manner; for example, such as phospholipase C- γ (PLC- γ), GTPase activating protein (GAP) and phosphatidylinositol 3-phosphate kinase (PI-3 kinase) (6, 7, 92–97).

In contrast, nonreceptor tyrosine kinases are not exposed to the external environment of the cell, and are regulated either by other cytosolic protein kinases or by linking noncovalently to receptors with or without protein kinase activity. Indeed, several nonreceptor tyrosine kinases (*fyn*, *lyn*, and *lck*), which belong to *src* family, are reported to interact with cell surface receptor proteins, such as CD4, CD8, T-cell receptor and sIgM, which have no intrinsic kinase activity (98–101). In this case, the nonreceptor kinases would be functionally equivalent to the catalytic domains of the receptor kinases and thus the activation mechanisms seem very similar between two types of PTKs. Furthermore, the association of several *src* family proteins with PDGF receptor has also been reported (102).

An important subject which was practically untouched in this review, mainly due to limited space, is presenting a picture of the physiologically relevant substrates for protein-tyrosine kinases, especially for the nonreceptor tyrosine kinases. Transformation by the oncogenes encoding protein-tyrosine kinases, as well as physiological stimulation of receptor tyrosine kinases results in a substantial increase in the level of phosphotyrosine in cells. A

Protein-Tyrosine Kinases

large number of proteins to be specifically phosphorylated on tyrosine residues in transformed or growth factor stimulated cells have been reported; previously identified proteins include major cytoskeletal proteins which are related to lipocortins, vinculin and the glycolytic enzymes enolase, phosphoglycerate mutase, and lactate dehydrogenase (103–105). However, none of these represent molecules that have been clearly implicated in the induction of a mitogenic response. In addition, these putative substrates have also been reported to be phosphorylated in nontransformed cells expressing mutant *src* proteins which are transformation defective in spite of being active kinases (106). Recently, by using different approaches with anti-phosphotyrosine antibodies and more refined cell fractionation procedures, a set of proteins containing tyrosine residues of which phosphorylation were fairly correlated with other transformation parameters have been identified. Precise analyses on these proteins should give us useful information to understand the signal transduction pathways involved in normal cells as well as transformed cells.

Acknowledgements

I greatly thank Prof. M. Takanami for his encouragement through my work cited in this review. I also thank Dr. T. Takeya for helpful discussions and critical reading of this manuscript.

REFERENCES

- (1) T. Hunter and J.A. Cooper, *Annu. Rev. Biochem.*, **54**, 897 (1985).
- (2) A.M. Edelman, D.K. Blumenthal and E.G. Krebs, *Annu. Rev. Biochem.*, **56**, 567 (1987).
- (3) G. Carpenter, *Ann. Rev. Biochem.*, **56**, 881 (1987).
- (4) Y. Yarden and A. Ullrich, *Annu. Rev. Biochem.*, **57**, 443 (1988).
- (5) S.K. Hanks, A.M. Quinn and T. Hunter, *Science*, **241**, 42 (1988).
- (6) A. Ullrich and J. Schlessinger, *Cell*, **61**, 203 (1990).
- (7) L.C. Cantley, K.R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller and S. Soltoff, *Cell*, **64**, 281 (1991).
- (8) G.M. Cooper, *In "Oncogenes."* Jones and Bartlett Publishers (1990).
- (9) H. Hanafusa, H. Iba, T. Takeya and F. Cross, *In G.F. Vande Woude, A.J. Levine and J.D. Watson. (Ed.), "Cancer cells, vol. 2."* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1984), p 1.
- (10) R. Jove and H. Hanafusa, *Annu. Rev. Cell Biol.*, **3**, 31 (1987).
- (11) J.T. Parsons and M.J. Weber, *In P.K. Vogt (Ed.), "Current Topics in Microbiology and Immunology 147"*: Springer-Verlag, (1989), p 79.
- (12) J.A. Cooper, *In B.E. Kemp. (Ed.), "Peptides and protein phosphorylation."*, CRC Press., (1991), p 85.
- (13) A.F. Williams and A.N. Barclay, *Ann. Rev. Immunol.*, **6**, 381 (1988).
- (14) M. Barbacid, M.L. Breitman, A.V. Lauver, L.K. Long and P.K. Vogt, *Virology*, **110**, 411 (1981).
- (15) W.J.M. Van de Ven, A.S. Khan, F.H. Reynolds, K.T. Mason and J.R. Stephenson, *J. Virol.*, **33**, 1034 (1980).
- (16) G. Naharro, K.C. Robbins and E.P. Reddy, *Science*, **223**, 63 (1984).
- (17) L. Donner, L.A. Fedele, C.F. Garon, S.J. Anderson and C.J. Sherr, *J. Virol.*, **41**, 489 (1982).
- (18) A. Hampe, M. Gobet, C.J. Sherr and F. Galibert, *Proc. Natol. Acad. Sci. USA*, **81**, 85 (1984).
- (19) R.A. Feldman, L.-H. Wang, H. Hanafusa and P.C. Balduzzi, *J. Virol.*, **42**, 228 (1982).
- (20) L.-H. Wang, H. Hanafusa, M.F. Notter and P.C. Balduzzi, *J. Virol.*, **41**, 833 (1982).

- (21) S. Kawai, M. Yoshida, K. Segawa, H. Sugiyama, R. Ishizaki and K. Toyoshima, *Proc. Natl. Acad. Sci. USA*, **77**, 6199 (1980).
- (22) T. Hunter and B.M. Sefton, *Proc. Natl. Acad. Sci. USA*, **77**, 1311 (1980).
- (23) T. Takeya and H. Hanafusa, *Cell*, **32**, 881 (1983).
- (24) H. Iba, T. Takeya, F.R. Cross, T. Hanafusa and H. Hanafusa, *Proc. Natl. Acad. Sci. USA*, **81**, 4424 (1984).
- (25) R.C. Parker, H.E. Varmus and J.M. Bishop, *Cell*, **37**, 131 (1984).
- (26) D. Shalloway, P.M. Coussens and P. Yaciuk, *In* G.F. Vande Woude, A.J. Levine and J.D. Watson. (Ed.), "Cancer cells, vol. 2." Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1984), p 10.
- (27) J.E. Buss, M.P. Kamps, K. Gould and B.M. Sefton, *J. Virol.*, **58**, 468 (1986).
- (28) M.P. Kamps, J.E. Buss and B.M. Sefton, *Proc. Natl. Acad. Sci. USA*, **82**, 4625 (1985).
- (29) D. Pellman, E.A. Garber, F.R. Cross and H. Hanafusa, *Proc. Natl. Acad. Sci. USA*, **82**, 1623 (1985).
- (30) V.W. Raymond and J.T. Parsons, *Virology*, **160**, 400 (1987).
- (31) I. Sadowski, J.C. Stone and T. Pawson, *Mol. Cell. Biol.*, **6**, 4396 (1986).
- (32) M.L. Stahl, C.R. Ferenz, K.L. Kelleher, R.W. Kriz and J.L. Knopf, *Nature*, **332**, 269 (1988).
- (33) C.A. Koch, D. Anderson, M.F. Moran, C. Ellis and T. Pawson, *Science*, **252**, 668 (1991).
- (34) M.F. Moran, C.A. Koch, D. Anderson, C. Ellis, L. England, G.S. Martin and T. Pawson, *Proc. Natl. Acad. Sci. USA*, **87**, 8622 (1990).
- (35) M. Matsuda, B. Mayer and H. Hanafusa, *Mol. Cell. Biol.*, **11**, 1607 (1991).
- (36) P.-G. Suh, S.H. Ryo, K.H. Moon, H.W. Suh and S.G. Rhee, *Cell*, **54**, 161 (1988).
- (37) D.J. Fujita, J. Bechberger and I. Nedic, *Virology*, **114**, 256 (1981).
- (38) J.S. Brugge and D. Darrow, *J. Biol. Chem.*, **259**, 4550 (1984).
- (39) B. Mayer, R. Jove, J.F. Krane, F. Poirier, G. Calothy and H. Hanafusa, *J. Virol.*, **60**, 858 (1986).
- (40) H. Piwnica-Worms, K.B. Saunders, T.M. Roberts, A.E. Smith and S.H. Cheng, *Cell*, **49**, 75 (1987).
- (41) T.E. Kmiecik, and D. Shalloway, *Cell*, **49**, 65 (1987).
- (42) C.A. Cartwright, W. Eckhart, S. Simon and P.L. Kaplan, *Cell*, **49**, 83 (1987).
- (43) M.S. Collett, E. Erikson and R.L. Erikson, *J. Virol.*, **29**, 770 (1979).
- (44) J.A. Cooper, K.L. Gould, C.L. Cartwright and T. Hunter, *Science*, **231**, 1431 (1986).
- (45) F.R. Cross and H. Hanafusa, *Cell*, **34**, 597 (1983).
- (46) J.E. Smart, H. Oppermann, A.P. Czernilofsky, A.F. Purchio, R.L. Erikson and J.M. Bishop, *Proc. Natl. Acad. Sci. USA*, **78**, 6013 (1981).
- (47) T. Patschinsky, T. Hunter and B.M. Sefton, *J. Virol.*, **59**, 73 (1986).
- (48) J.A. Cooper and A. MacAuley, *Proc. Natl. Acad. Sci. USA*, **85**, 4232 (1988).
- (49) M. Okada and H. Nakagawa, *J. Biol. Chem.*, **264**, 20886 (1989).
- (50) S. Nada, M. Okada, A. MacAuley, J.A. Cooper and H. Nakagawa, *Nature*, **351**, 69 (1991).
- (51) H. Iba, F.R. Cross, E.A. Garber and H. Hanafusa, *Mol. Cell. Biol.*, **5**, 1058 (1985).
- (52) P.M. Coussens, J.A. Cooper, T. Hunter and D. Shalloway, *Mol. Cell. Biol.*, **5**, 2753 (1985).
- (53) J. Kato, T. Takeya, C. Grandori, H. Iba, J.B. Levy and H. Hanafusa, *Mol. Cell. Biol.*, **6**, 4155 (1986).
- (54) M. Sato, J. Kato and T. Takeya, *J. Virol.*, **63**, 683 (1989).
- (55) J.B. Levy, H. Iba and H. Hanafusa, *Proc. Natl. Acad. Sci. USA*, **83**, 4228 (1986).
- (56) M. Hamaguchi and H. Hanafusa, *Proc. Natl. Acad. Sci. USA*, **84**, 2312 (1987).
- (57) J.G. Burr, G. Dreyfuss, S. Penman and J.M. Buchanan, *Proc. Natl. Acad. Sci. USA*, **77**, 3484 (1980).
- (58) D.M. Loeb, J. Woolford and K. Beemon, *J. Virol.*, **61**, 2420 (1987).
- (59) M.C. O'Brien, Y. Fukui and H. Hanafusa, *Mol. Cell. Biol.*, **10**, 2855 (1990).
- (60) H. Ushiro and S. Cohen, *J. Biol. Chem.*, **255**, 8363 (1980).
- (61) Y. Yarden, I. Harari and J. Schlessinger, *J. Biol. Chem.*, **260**, 315 (1985).
- (62) J. Downward, Y. Yarden, E. Mayes, G. Scrace, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger and M.D. Waterfield, *Nature*, **307**, 521 (1984).

Protein-Tyrosine Kinases

- (63) M.J. Hayman, *TIG*, **2**, 260 (1986).
- (64) Y. Yarden, J.A. Escobedo, W.-J. Kuang, T.L. Yang-Feng, T.O. Daniel, P.M. Tremble, E.Y. Chen, M.E. Ando, R.N. Harkins, U. Francke, V.A. Fried, A. Ullrich and L.T. Williams, *Nature*, **323**, 226 (1986).
- (65) A. Ullrich, J.R. Bell, E.Y. Chen, R. Herrera, L.M. Petruzzelli, T.J. Dull, A. Gray, L. Coussens, Y.-C. Liao, M. Tsubokawa, A. Mason, P.H. Seeberg, C. Grunfeld, O.M. Rosen and J. Ramachandran, *Nature*, **313**, 756 (1985).
- (66) Y. Ebina, L. Ellis, K. Jarnagin, M. Edery, L. Graf, E. Clauser, J.-H. Ou, F. Masiarz, Y.W. Kan, I.D. Goldfine, R.A. Roth and W.J. Rutter, *Cell*, **40**, 747 (1985).
- (67) A. Ullrich, A. Gray, A.W. Tam, T. Yang-Feng, M. Tsubokawa, C. Collins, W. Henzel, T.L. Bon, S. Kathuria, E. Chen, S. Jacobs, U. Francke, J. Ramachandran and Y. Fujita-Yamaguchi, *EMBO J.*, **5**, 2503 (1986).
- (68) S. Kornbluth, K.E. Paulson and H. Hanafusa, *Mol. Cell. Biol.*, **8**, 5541 (1988).
- (69) M. Ruta, R. Howk, G. Ricca, W. Drohan, M. Zabelshansky, G. Laureys, D.E. Barton, U. Francke, J. Schlessinger and D. Givol, *Oncogene*, **3**, 9 (1988).
- (70) P.L. Lee, D.E. Johnson, L.S. Coussens, V.A. Fried and L.T. Williams, *Science*, **245**, 57 (1989).
- (71) E.B. Pasquale and S.J. Singer, *Proc. Natl. Acad. Sci. USA*, **86**, 5449 (1989).
- (72) E.B. Pasquale, *Proc. Natl. Acad. Sci. USA*, **87**, 5812 (1990).
- (73) M. Sato, T. Kitazawa, T. Iwai, J. Seki, N. Sakato, J. Kato and T. Takeya, *Oncogene*, **6**, 1279 (1991).
- (74) M. Ruta, W. Burgess, D. Givol, J. Epstein, N. Neiger, J. Kaplow, G. Crumley, C. Dionne, M. Jaye, and J. Schlessinger, *Proc. Natl. Acad. Sci. USA*, **86**, 8722 (1989).
- (75) A. Safran, A. Avivi, A. Orr-Urtreger, G. Neufeld, P. Lonai, D. Givol and Y. Yarden, *Oncogene*, **5**, 635 (1990).
- (76) P. Champion-Arnaud, C. Ronsin, E. Gilbert, M.C. Gesnel, E. Houssaint and R. Breathnach, *Oncogene*, **6**, 979 (1991).
- (77) C.A. Dionne, G. Crumley, F. Bellot, J.M. Kaplow, G. Searfoss, M. Ruta, W.H. Burgess, M. Jaye and J. Schlessinger, *EMBO J.*, **9**, 2685 (1990).
- (78) A. Eisemann, J.A. Ahn, G. Graziani, S.R. Tronick and D. Ron, *Oncogene*, **6**, 1195 (1991).
- (79) Y. Hattori, H. Odagiri, H. Nakatani, K. Miyagawa, K. Naito, H. Sakamoto, O. Katoh, T. Yoshida, T. Sugimura and M. Terada, *Proc. Natl. Acad. Sci. USA*, **87**, 5983 (1990).
- (80) J. Hou, M. Kan, K. McKeehan, G. McBride, P. Adams and W.L. McKeehan, *Science*, **251**, 665 (1991).
- (81) A. Mansukhani, D. Moscatelli, D. Talarico, V. Levytska and C. Basilico, *Proc. Natl. Acad. Sci. USA*, **87**, 4378 (1990).
- (82) T. Miki, T.P. Fleming, D.P. Bottaro, J.S. Rubin, D. Ron and S.A. Aaronson, *Science*, **251**, 72 (1991).
- (83) H.H. Reid, A.F. Wilks and O. Bernard, *Proc. Natl. Acad. Sci. USA*, **87**, 1596 (1990).
- (84) D.E. Johnson, P.L. Lee, J.Lu and L.T. Williams, *Mol. Cell. Biol.*, **10**, 4728 (1990).
- (85) K. Keegan, D.E. Johnson, L.T. Williams and M.J. Hayman, *Proc. Natl. Acad. Sci. USA*, **88**, 1095 (1991).
- (86) J. Partanen, T.P. Makela, E. Eerola, J. Korhonen, H. Hirvonen, L. Claesson-Welsh and K. Alitalo, *EMBO J.*, **10**, 1347 (1991).
- (87) A. Yayon, M. Klagsbrun, J.D. Esko, P. Leder and D.M. Ornitz, *Cell*, **64**, 841 (1991).
- (88) A. Rapraeger, A. Krufka and B.B. Olwin, *Science*, **252**, 1705 (1991).
- (89) K. Sakaguchi, M. Yanagishita, Y. Takeuchi and G.D. Aurbach, *J. Biol. Chem.*, **266**, 7270 (1991).
- (90) L.T. Williams, *Science*, **243**, 1564 (1989).
- (91) J. Schlessinger, *Trends Biochem. Sci.*, **13**, 443 (1988).
- (92) D.A. Kumjian, M.I. Wahl, S.G. Rhee and T.O. Daniel, *Proc. Natl. Acad. Sci. USA*, **86**, 8232 (1989).
- (93) J. Meisenhelder, P.-G. Suh, S.G. Rhee and T. Hunter, *Cell*, **57**, 1109 (1989).
- (94) B. Margolis, S.G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein and J. Schlessinger, *Cell*, **57**, 1101 (1989).
- (95) B. Margolis, F. Bellot, A.M. Honegger, A. Ullrich, J. Schlessinger and A. Zilberstein, *Mol. Cell.*

- Biol.*, **10**, 435 (1990).
- (96) C.J. Molloy, D.P. Bottaro, T.P. Fleming, M.S. Marshall, J.B. Gibbs and S.A. Aaronson, *Nature*, **342**, 711 (1989).
- (97) L. Varticovski, B. Druker, D. Morrison, L. Cantley and T. Roberts, *Nature*, **342**, 699 (1989).
- (98) L.E. Samelson, A.F. Phillips, E.T. Luong and R.D. Klausner, *Proc. Natl. Acad. Sci. USA*, **87**, 4358 (1990).
- (99) Y. Yamanashi, T. Kakiuchi, J. Mizuguchi, T. Yamamoto and K. Toyoshima, *Science*, **251**, 192 (1991).
- (100) C.E. Rudd, J.M. Trevillyan, J.D. Dasgupta, L.L. Wong and S.F. Schlossman, *Proc. Natl. Acad. Sci. USA*, **85**, 5190 (1988).
- (101) A. Veillete, M.A. Bookman, E.M. Horak and J.B. Bolen, *Cell*, **44**, 301 (1988).
- (102) R. Kypka, Y. Goldberg, E.T. Ulug and S.A. Courtneidge, 1990. *Cell*, **62**, 481 (1990).
- (103) K. Radke and G.S. Martin, *Proc. Natl. Acad. Sci. USA*, **76**, 5212 (1979).
- (104) B.M. Sefton and T. Hunter, *Cell*, **24**, 165 (1981).
- (105) J.A. Cooper, N.A. Reiss, R.J. Schwartz and T. Hunter, *Nature*, **302**, 218 (1983).
- (106) M.P. Kamps, J.E. Buss and B.M. Sefton, *Cell*, **45**, 105 (1986).