

Molecular Signal Integration. Interplay Between Serine, Threonine, and Tyrosine Phosphorylation

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Tyrosine phosphorylation has emerged as a dominant theme in cellular regulation, owing largely to the finding that activation of a tyrosine kinase is the initial event in many signaling pathways. The oncogenes of several different retroviruses encode tyrosine kinases, and transformation of cells by these retroviruses is the direct result of unregulated tyrosine kinase activity. In addition to oncoproteins, several trans-membrane growth factor receptors possess tyrosine kinase activity that is required for proper signaling (reviewed by Ullrich and Schlessinger, 1990; Cantley *et al.*, 1991). Although tyrosine phosphorylation is an integral component of regulated cell growth, the effects of tyrosine kinases are at least in part mediated through the increased phosphorylation of the aliphatic hydroxy amino acids, serine and threonine. Stimulation of transmembrane tyrosine kinase growth factor receptors causes a robust quantitative increase in the phosphorylation of many cell proteins at serine and threonine, and tyrosine phosphorylated proteins remain a small minority. This pattern is consistent with the evolution of kinase cascades as a means to amplify and transduce the initial signal.

It should be noted that the flow of information does not always start with a tyrosine kinase. Cell transformation may result from deregulation of serine kinases, and some cell-surface receptors have kinase domains that phosphorylate serine and threonine, not tyrosine, residues. High level overexpression of protein kinase C (PKC) in some cell types induces a transformed phenotype and enables them to grow in soft agar (Housey *et al.*, 1988; Persons *et al.*, 1988). In addition, the products of the viral oncogenes *v-raf*, *v-mos*, and *v-akt* are serine kinases that induce cell transformation (Bellacosa *et al.*, 1991; Cantley *et al.*, 1991). Cell-surface receptors for transforming growth factor, (TGF) β and activin are serine/threonine kinases (Mathews and Vale, 1991; Lin *et al.*, 1992). The signal transduction pathways leading from these receptors are largely unexplored.

Unraveling chains of phosphorylation events can be problematic. For a given substrate whose phosphorylation is stimulated under specific conditions, it is necessary to identify a candidate kinase, to show that it phosphorylates the substrates at the physiological sites

and with appropriate affinity *in vitro*, and to show that the kinase is regulated in the expected way by extracellular stimuli. *In vivo* approaches, such as expression of interfering or activated mutant genes, can corroborate *in vitro* results or suggest alternative pathways. In this essay, we first discuss one pathway that connects tyrosine kinases to increases in serine and threonine phosphorylation. In this pathway, tyrosine kinases activate serine/threonine kinases indirectly, by way of phospholipid-derived second messengers. The end result is the activation of a cascade of serine/threonine kinases, including PKC, mitogen-activated protein (MAP) kinase, Rsk, and Raf. Recent evidence that the low molecular weight GTP-binding protein, Ras, may participate in this pathway will be presented. The second part of the review discusses the opportunities for cross-talk between serine/threonine and tyrosine phosphorylation signals that are afforded by the multisite phosphorylation of key regulatory enzymes and by the existence of kinases and phosphatases that act on all three hydroxyamino acids.

A PATHWAY BY WHICH TYROSINE KINASES CAN ACTIVATE SERINE/THREONINE KINASES

Phospholipase C and PKC

The best understood mechanism by which tyrosine kinases increase serine and threonine phosphorylation is by stimulation of members of the PKC family (Nishizuka, 1984). The PKC family comprises seven or more structurally-distinct isozymes, which are expressed in different tissues. At least four isozymes (α , β I, β II, and γ) are stimulated by the second messenger, diacylglycerol (DG). DG provides the link between tyrosine kinases and PKC, because the intracellular DG concentration is increased as a result of tyrosine kinase activation. The change in DG level appears to be a consequence of the activation of phospholipase C (PLC), an enzyme that makes DG (Majerus *et al.*, 1986) (Figure 1).

There is evidence that two different types of PLC are activated with different kinetics in response to mitogens

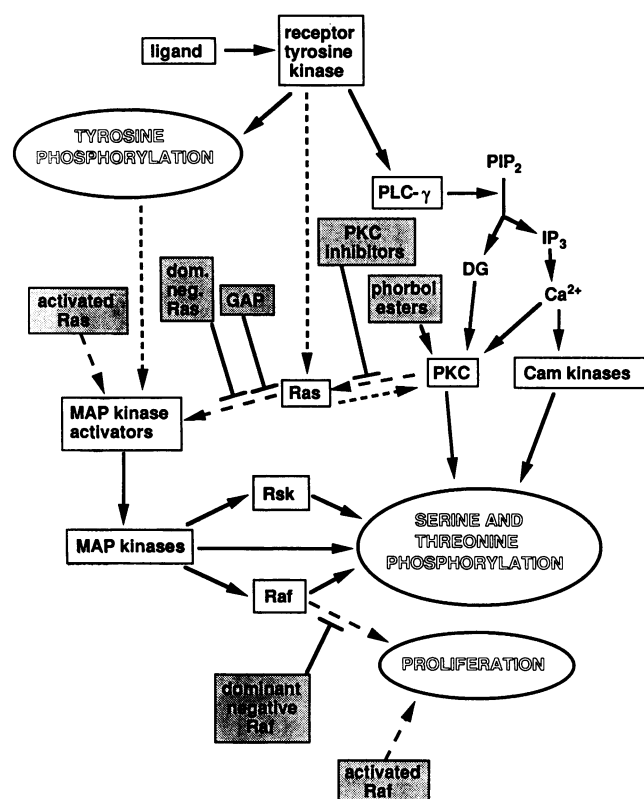


Figure 1. A speculative pathway by which receptor tyrosine kinases may activate serine/threonine phosphorylation and cell proliferation. Arrows indicate direct enzyme/substrate relationships, demonstrated *in vitro*, that are believed to occur *in vivo*. Each broken arrow indicates a pathway containing an unknown number of steps and preceding by unknown biochemical mechanisms, deduced from *in vivo* experiments. Shaded boxes show tools used to deduce *in vivo* dependencies. Besides the pathway shown, additional, redundant, or essential pathways may also exist (dotted arrows). For example, active tyrosine kinases may activate Ras through pathways that do not involve PKC. Ras can also activate PKC. *In vivo* experiments indicate that Raf lives downstream of Ras, but the position of Raf relative to MAP kinase is based only on *in vitro* studies. Commitment to proliferation almost certainly normally involves additional pathways to the one shown. For details and references, see text.

that act through tyrosine kinase receptors. The most rapid effect is on phosphoinositide-specific PLCs (PI-PLC), which cleave phosphatidylinositol 4,5-bisphosphate (PIP₂), producing inositol 1,4,5-trisphosphate (IP₃) and DG (Majerus *et al.*, 1986). IP₃ is the trigger for the release of stored intracellular calcium, which is an activator of calmodulin-regulated serine/threonine kinases and a coactivator of PKC. A phosphatidylcholine-specific PLC is also activated, with slower kinetics, producing additional DG but no IP₃ (Nakashima *et al.*, 1991). This enzyme could be important for maintaining PKC activity later after stimulation.

Only one of the PI-PLCs is thought to be activated directly by tyrosine kinases. Platelet-derived growth factor (PDGF) or epidermal growth factor (EGF) stim-

ulation of cells induces tyrosine phosphorylation of PLC- γ (Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989). Kinase-inactive PDGF or EGF receptor mutants do not induce PLC- γ phosphorylation and do not stimulate inositol phosphate production (Escobedo *et al.*, 1989; Margolis *et al.*, 1990). The sites of PDGF-induced tyrosine phosphorylation in PLC- γ have been mapped to Y771, Y783, and Y1254 (Kim *et al.*, 1991). Over-expression of wild-type PLC- γ increases PDGF-induced PI degradation. This increase is not seen if PLC- γ mutants with Y783 or Y1254 changed to phenylalanine are expressed (Kim *et al.*, 1991). This suggests that phosphorylation stimulates activity, a conclusion that is supported by *in vitro* studies. Purified nonphosphorylated PLC- γ is active when assayed with solutions of PIP₂. However, most of the PIP₂ in the cell is probably sequestered by binding either to proteins, such as profilin, and or to membrane lipids. Hydrolysis of PIP₂ *in vitro* by PLC- γ is inhibited by either profilin or detergent micelles (which may mimic the lipid environment of the cell membrane). Tyrosine phosphorylation of PLC- γ relieves inhibitory effects of both profilin and detergents (Nishibe *et al.*, 1990; Goldschmidt-Clermont *et al.*, 1991). Whether cellular PIP₂ is bound to profilin, membrane lipids, or both, the *in vitro* experiments support the idea that phosphorylation stimulates PLC activity.

Like several other cellular enzymes, PLC- γ binds tightly to certain activated tyrosine kinase transmembrane receptors (Anderson *et al.*, 1990a; Cantley *et al.*, 1991). This binding may facilitate PLC- γ phosphorylation by the receptor and may also modify PLC- γ activity directly. Anchoring PLC- γ to the membrane, via a receptor tyrosine kinase, may promote access to substrate PIP₂ contained in the cell membrane, and thus increase PIP₂ hydrolysis. The properties of PLC- γ mutants that do not bind to active tyrosine kinase receptors have not been investigated.

Once tyrosine kinases stimulate PLC- γ , DG and calcium levels increase and PKC is activated (Nishizuka, 1984). The importance of PKC for mitogenic signal transduction is shown both by the mitogenic effects of phorbol esters, which activate PKC, and by the ability of PKC inhibitors to block gene induction and mitogenesis in many cell types. Although many substrates for PKC have been detected, those substrates that relay the mitogenic signal have not been identified. Some experiments implicate the low molecular weight GTP-binding protein, Ras, in signaling from PKC, although Ras is probably not a substrate for PKC *in vivo*. The mechanism by which Ras may be regulated by PKC and tyrosine kinases is obscure.

Ras

Ras is thought to be essential for signaling from both tyrosine kinases and PKC. Microinjection of antibodies

to Ras blocks the mitogenic effects of the tyrosine kinase activators, PDGF and EGF, tyrosine kinase oncogenes, and phorbol esters (Mulcahy *et al.*, 1985; Smith *et al.*, 1986; Yu *et al.*, 1988). Overexpression of dominant-negative Ras mutants, or of an inhibitor of Ras (the Ras-GTPase activator protein [Ras-GAP]), inhibits transformation by oncogenic tyrosine kinases and phorbol ester-induced mitogenic and differentiation responses (Cai *et al.*, 1991; DeClue *et al.*, 1991; Kremer *et al.*, 1991; Nori *et al.*, 1991). These types of experiments do not indicate whether Ras is required for an early or late step in mitogenesis, transformation, or differentiation, but biochemical studies suggest that Ras is activated rapidly by mitogenic stimuli. The GTP content of Ras, presumably a measure of the amount of Ras in the active state, increases rapidly when fibroblasts are stimulated with growth factors (Gibbs *et al.*, 1990; Satoh *et al.*, 1990). The Ras GTP content is also elevated in cells transformed by oncogenic tyrosine kinases (Gibbs *et al.*, 1990; Satoh *et al.*, 1990). On the other hand, there has been only one report of phorbol esters inducing an increase in Ras GTP content, in T lymphocytes (Downward *et al.*, 1990), and there is no evidence that Ras activation by growth factors proceeds via PKC. The issue is contentious because Ras can activate PKC (Morris *et al.*, 1989), and transformation by activated mutants of Ras seems to require PKC (Lacal *et al.*, 1987), indicating that Ras can act via PKC. Ras could have more than one mechanism of activation and more than one effect. Even a cycle of interdependent events is possible. Notwithstanding the uncertainty over the order of Ras and PKC in the mitogenic signaling pathway, studies described below suggest that Ras mediates the PKC-dependent activation of other serine kinases.

MAP Kinases and Rsk

MAP kinases, also known as extracellular signal-regulated kinases (ERKs) (Cobb *et al.*, 1991), are serine/threonine kinases that are stimulated by a broad range of mitogenic and differentiation stimuli, including agents that act via tyrosine kinases, G-proteins, PKC, and Ras (Cobb *et al.*, 1991; L'Allemain *et al.*, 1991a; Leever and Marshall, 1992). MAP kinases are activated by one or more "activators" that are themselves protein kinases and are described in more detail later.

Activation of MAP kinase by growth factors appears to depend on Ras. A dominant-negative Ras mutant blocks MAP kinase activation by tyrosine kinase receptors, and Ras-GAP blocks MAP kinase activation by PKC (Nori *et al.*, 1992; Thomas *et al.*, 1992; Wood *et al.*, 1992). MAP kinase activation by growth factors also depends at least partly on PKC, although there may also be PKC-independent pathways (L'Allemain *et al.*, 1991b). This suggests that Ras lies between PKC and MAP kinases (Figure 1). However, inactive forms of Ras may block signaling from PKC to the MAP kinase

activator by other mechanisms, and stimulation may not normally involve Ras.

Many substrates for MAP kinases are being identified. Rsk (ribosomal S6 kinase)-family serine kinases are substrates for, and are activated by, MAP kinase (Sturgill and Wu, 1991). Therefore, Rsk is activated by the same range of signals that activate MAP kinases. Rsk has a number of interesting *in vitro* substrates, including a regulatory subunit of a serine/threonine protein phosphatase, PP1. Rsk-catalyzed phosphorylation of the PP1 G-subunit redirects its dephosphorylating activity toward alternate substrates (Dent *et al.*, 1990; Lavoigne *et al.*, 1991). Changes in this phosphatase explain many of the effects of insulin on protein phosphorylation in muscle. MAP kinases and Rsk are found in both the cytoplasm and nucleus (Chen *et al.*, 1992). Both enzymes can phosphorylate, and may regulate, transcription factors (Pulverer *et al.*, 1991; Seth *et al.*, 1991; Chen *et al.*, 1992). Thus, MAP kinases may provide a node through which Ras-dependent signaling pathways stimulate serine/threonine phosphorylation events in the nucleus and cytoplasm (Figure 1).

Raf

Raf is a serine kinase that is implicated genetically in signal transduction (Morrison, 1990). The substrates it phosphorylates *in vivo* are unknown. Sequences amino terminal to the Raf catalytic domain appear to play a regulatory role in controlling kinase activity, and deletion of these sequences creates a deregulated kinase that delivers an oncogenic signal (Stanton *et al.*, 1989; Heidecker *et al.*, 1990). As a corollary, expression of mutants of Raf in which the kinase has been inactivated generate a dominant negative effect, blocking proliferation signals originated by serum, phorbol esters, and oncogenic mutants of Ras (Kolch *et al.*, 1991). Assuming that the kinase-inactive Raf mutant blocks only Raf-dependent pathways, it seems that Raf is necessary for normal mitogenic and oncogenic signaling and lies downstream of tyrosine kinases, PKC and Ras. Accordingly, Raf transformation is not reversed by microinjection of antibodies to Ras (Smith *et al.*, 1986).

Raf kinase activity is increased three- to five-fold by treating cells with PDGF, phorbol ester, insulin, or cytokines, consistent with a function for Raf in signal transduction (Morrison *et al.*, 1988; Blackshear *et al.*, 1990). Because stimulation correlates well with phosphorylation of Raf and treatment of activated Raf with phosphatases decreases its activity, Raf may be activated by phosphorylation. However, the phosphorylation sites in Raf differ according to the stimulus: EGF, M-CSF, and insulin induce the serine phosphorylation and activation of Raf (Kovacina *et al.*, 1990; Baccarini *et al.*, 1991a,b), yet erythropoietin, interleukin 2, and PDGF induce Raf phosphorylation at tyrosine (Morrison *et al.*, 1989; Carroll *et al.*, 1991; Turner *et al.*, 1991) (Tyrosine

phosphorylated Raf is not always detected in PDGF-treated cells [Baccarini *et al.*, 1991b].) The detection of phosphotyrosine in Raf suggests it may be activated directly by certain receptor tyrosine kinases (Morrison *et al.*, 1989). However, direct activation of Raf by tyrosine kinases is unlikely, because tyrosine kinases require Ras for signaling but Raf does not (Smith *et al.*, 1986). Possibly, the tyrosine phosphorylation of Raf is not involved in activation. It will be important to determine whether inactivating Ras, with antibodies or GAP, blocks Raf activation by tyrosine kinases and PKC.

In vitro, Raf is a substrate for MAP kinase, and phosphorylation occurs at one of the major phosphopeptides whose phosphorylation is stimulated in vivo by insulin (Anderson *et al.*, 1991). Raf was not activated detectably as a consequence of this phosphorylation, however. Thus, one model is that Raf is regulated by Ras, perhaps indirectly via MAP kinase (Figure 1).

DUAL REGULATION BY TYROSINE AND THREONINE PHOSPHORYLATION

Two kinases, MAP kinase and p34/cdc2, seem to be designed to integrate signals from both serine/threonine and tyrosine kinases and phosphatases.

MAP Kinase

As mentioned above, MAP kinases are activated in response to mitogens that activate tyrosine kinases or PKC. Active MAP kinases are phosphorylated on tyrosine and threonine, and removal of phosphate from either residue obviates MAP kinase activity (Anderson *et al.*, 1990b). This initially suggested that MAP kinases may integrate signals arising from tyrosine kinases, such as growth factor receptors, and serine/threonine kinases, such as PKC. However, the tyrosine kinase signal is routed, at least partly, through PKC, and somehow PKC can induce the tyrosine, as well as threonine, phosphorylation of MAP kinase (Cobb *et al.*, 1991). It now seems that MAP kinases are activated not by the combined actions of tyrosine and serine/threonine kinases but by the action of a single dual specificity kinase.

Mammalian cells have two MAP kinases, ERK1 (MAP kinase 2, 45 kDa) and ERK2 (MAP kinase 1, 42 kDa), that are activated by a common mechanism. A third MAP kinase (54 kDa) may be regulated in the same way (Kyriakis *et al.*, 1991). In ERK1 and 2, the critical phosphotyrosine and threonine are separated by only one residue (glutamic acid) and lie in catalytic subdomain VIII, a common site of autophosphorylation in protein kinases (Figure 2) (Payne *et al.*, 1991). Indeed, MAP kinases can autophosphorylate. Intriguingly, although the activity of MAP kinases toward exogenous substrates is restricted to serine or threonine residues, they autophosphorylate slowly on tyrosine (Crews *et al.*, 1991; Seger *et al.*, 1991). Autophosphorylation oc-

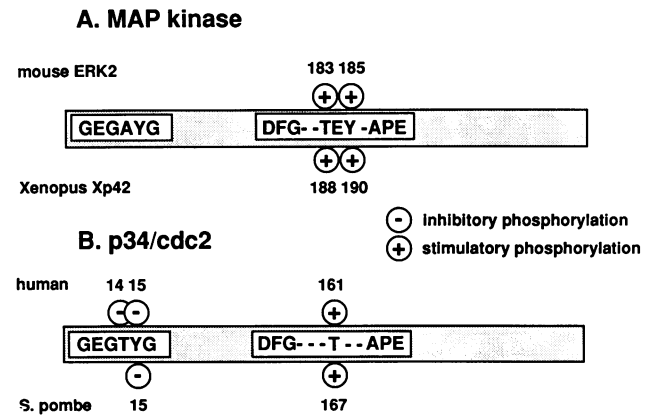


Figure 2. Regulatory phosphorylation sites in MAP kinase and p34/cdc2. The kinase domains of MAP kinase and p34/cdc2 are diagrammed, with the landmark sequences in subdomains I, VII, and VIII indicated. Positions of activating and inhibiting phosphorylations are shown.

cur at the activating tyrosine residue, but the key threonine residue is not phosphorylated and the autophosphorylated enzyme has only very weak activity toward added substrates (Wu *et al.*, 1991). This finding raised the possibility that MAP kinase could be activated by a hypothetical noncatalytic activator of autophosphorylation (Seger *et al.*, 1991).

Several laboratories are in the process of purifying MAP kinase activators from different sources. The properties of the purified activators are sufficiently similar that one suspects there may only be one or two activators induced by diverse stimuli. At the purity obtained, each activator is able to fully activate dephosphorylated MAP kinase by inducing phosphorylation at both the tyrosine and threonine residue (Ahn *et al.*, 1991; Gomez and Cohen, 1991; Matsuda *et al.*, 1992). Tyrosine phosphorylation in the presence of activator is much faster than autophosphorylation in its absence. Evidence that the activator is not working by stimulating autophosphorylation comes from use of mutant MAP kinases that are enzymatically inactive. Crude and purified activators can induce the double phosphorylation of the inactive mutants at the appropriate sites (L'Allemain *et al.*, 1992; Posada and Cooper, 1992). Therefore, the activator may be a single kinase with dual specificity (see below) or a complex of a threonine kinase and a tyrosine kinase. The small size of the activator (~50 kDa) argues in favor of the former. The purified MAP kinase activator was inhibited by treatment with a serine/threonine protein phosphatase (Gomez and Cohen, 1991), suggesting that the activator is a kinase that is stimulated by phosphorylation.

Why did this system evolve? It would seem simplest to have a monospecific activating kinase and a MAP kinase that is activated by phosphorylation at a single residue. Indeed, this scenario may hold true for a MAP

kinase relative purified from sea star, where the threonine residue is absent from the subdomain VIII sequence and single phosphorylation at tyrosine appears to be sufficient for activation (Ettahadieh *et al.*, 1992). If the same were true for vertebrate MAP kinases, their ability to autophosphorylate would allow them to autoactivate, albeit inefficiently. By requiring double phosphorylation, autoactivation is prevented, as is activation by chance phosphorylation by a nonspecific tyrosine kinase. Instead, the only means of activation may be by the highly-specific activator kinase (Figure 3). Double phosphorylation also has the potential advantage of allowing two pathways for MAP kinase inactivation. Although physiological inactivators have not been identified, MAP kinases can be inactivated *in vitro* by either serine/threonine or tyrosine protein phosphatases. Thus, the system seems to be designed to ensure that MAP kinases are kept inactive unless specifically stimulated (Figure 3).

p34/cdc2

Another serine/threonine kinase that is regulated by tyrosine as well as threonine phosphorylation is p34, the catalytic subunit of maturation (or M-phase) promoting factor. The gene encoding p34 is required for cell-cycle progression in both *Schizosaccharomyces pombe* (where p34 is encoded by *cdc2*) and *Saccharomyces cerevisiae* (*CDC28*). Entry into both S-phase (DNA synthesis) and M-phase (mitosis) requires these genes (Hartwell *et al.*, 1974; Nurse and Bisset, 1981; Beach *et al.*, 1982). Different alleles of *cdc2/CDC28* can either block cell-cycle progress or cause premature entry into M-phase, depending on the specific mutation. These different phenotypes indicate that p34 contains both positive and negative regulatory regions. Mutating a region of p34 required for enzymatic activity or for positive control causes cell-cycle arrest. Conversely, mutating a region needed for negative control speeds up the cell cycle, sometimes with lethal consequences.

Regulation of p34 is achieved by specific noncatalytic subunits, the cyclins, and by phosphorylation. The functions of the cyclins will not be discussed here. The

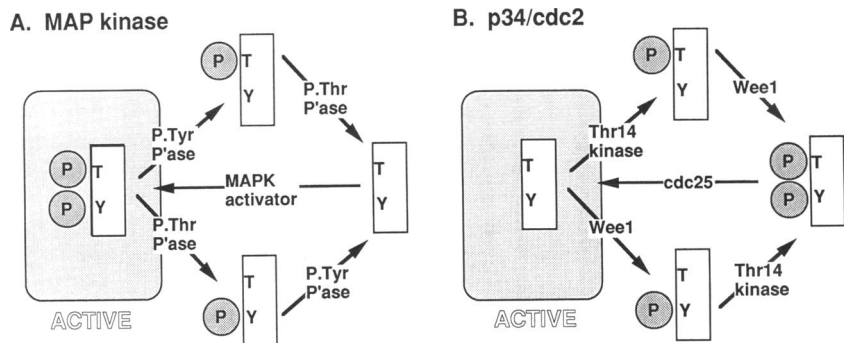
sites of p34 phosphorylation and the effects of phosphorylation are complex, and details vary between organisms (Figure 2).

Mutation of T167 in *S. pombe* p34 (T161 in other p34s) to a residue other than serine has the same effect as mutating essential catalytic residues, creating a null allele (Booher and Beach, 1986; Gould *et al.*, 1991). This residue lies in subdomain VIII in the homologous position to the activating phosphothreonine residue of MAP kinase (Figure 2). Indeed, T161/167 is a phosphorylation site in p34s from *S. pombe* and vertebrates (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Krek and Nigg 1991a; Solomon *et al.*, 1992). Phosphorylation accompanies p34 activation at M-phase, and replacement of T161 with an acidic residue causes premature mitosis (Ducommun *et al.*, 1991). Phosphorylation of T161 involves another kinase, because a nonfunctional p34 kinase domain is phosphorylated normally (Solomon *et al.*, 1992). This "cdc2 activating kinase" (CAK) may require a p34/cyclin complex as a substrate, because phosphorylation in a crude cell-free system is cyclin dependent. CAK has not been purified or cloned. Inactivating mutations in its gene would be expected to cause M-phase arrest.

Another phosphorylation site in *S. pombe* p34 is Y15 in subdomain I (Gould and Nurse, 1989). In vertebrate p34, both Y15 and the adjacent T14 are phosphorylation sites (Krek and Nigg, 1991a; Norbury *et al.*, 1991; Solomon *et al.*, 1992). Mutation of Y15 in *S. pombe* or vertebrate p34s allows early activation, resulting in premature M-phase entry (Gould and Nurse, 1989; Krek and Nigg, 1991b). This suggests that phosphorylation of Y15 is inhibitory. Double mutations in vertebrate p34, replacing both T14 and Y15, cause a more severe effect than replacing Y15 alone, suggesting that phosphorylation of T14 may also be inhibitory (Krek and Nigg, 1991b; Norbury *et al.*, 1991). Curiously, in *S. cerevisiae* the Y15 homolog (Y19) is phosphorylated, but phosphorylation does not seem to regulate activity (Amon *et al.*, 1992; Sorger and Murray, 1992).

Like T161, phosphorylation of Y15 and T14 seems to be catalyzed by another kinase. Phosphorylation is cyclin dependent, suggesting that the physiological sub-

Figure 3. Parallels between the activation/inactivation controls on MAP kinase and vertebrate p34/cdc2. Both MAP kinase and p34 have two routes to inactivation, but one (known) route to activation. The activation route requires addition (MAP kinase) or removal (p34) of two phosphates. In each case, this is achieved by a specific activator. Chance phosphorylation of MAP kinase by a tyrosine or threonine kinase, or dephosphorylation of p34 by a threonine or tyrosine phosphatase, will not activate the enzyme. Note that p34 is subject to additional controls by cyclins and by phosphorylation at T161.



strate for this inactivating kinase is a p34/cyclin complex (Solomon *et al.*, 1992). In the cell, there can be a time delay between formation of p34/cyclin complexes and phosphorylation of T14 and Y15 (Krek and Nigg, 1991a; Meijer *et al.*, 1991). These complexes may be active, performing the G1- and S-phase functions of p34 before repression by phosphorylation late in S-phase and through G2. Dephosphorylation allows the surge of p34 kinase activity at the start of M-phase (Gould and Nurse, 1989).

The Y15 kinase has been identified genetically in *S. pombe*. Loss of function mutations in the *wee1* gene cause premature mitosis, and overexpression of *wee1* delays mitosis. Wee1 has the properties of a p34 inhibitor. Its sequence predicts a kinase. It is closely related to another *S. pombe* gene product, Mik1, and there seems to be some redundancy between Wee1 and Mik1. Strains containing mutations in both *wee1* and *mik1* enter mitosis precipitately, a so-called mitotic catastrophe (Lundgren *et al.*, 1991). p34 in these cells lacks phosphate at Y15. The Wee1 protein can promote Y15 phosphorylation of a human p34/cyclin complex, produced together in insect cells (Parker *et al.*, 1991). In vitro, Wee1 can phosphorylate a synthetic substrate on tyrosine, although phosphorylation of p34/cyclin in vitro has not been demonstrated (Featherstone and Russell, 1991). Possibly another protein is required for the latter reaction. *wee1* relatives are being cloned from vertebrates, but it is not known whether they perform similar functions. The T14 kinase has not been identified.

Convincing evidence identifies the *S. pombe cdc25* gene product as a phosphatase responsible for dephosphorylation of Y15 (Millar and Russell, 1992). Loss of function mutations in *cdc25* cause mitotic arrest and *cdc25* overexpression causes premature mitosis. In vitro, Cdc25 can dephosphorylate Y15 of vertebrate or sea star p34 present in a p34/cyclin complex (Galaktionov and Beach, 1991; Gautier *et al.*, 1991; Lee *et al.*, 1992). The Cdc25-catalyzed dephosphorylation of p34 appears to be stimulated by cyclin B. The in vitro phosphatase activity of bacterially expressed Cdc25 is increased about fivefold when incubated in the presence of cyclins B1 and B2 but not cyclins A or D1 (Galaktionov and Beach, 1991). The complete phosphatase active site may contain parts of both cyclin B and Cdc25, so that Cdc25 functions as part of a complex rather than as a true catalyst (Jesus and Beach, 1992). The T14 phosphatase has not been convincingly identified, but Cdc25 may dephosphorylate this residue too (Gautier *et al.*, 1991; Strausfeld *et al.*, 1991).

It is curious that phosphorylation of T14 or Y15 should inhibit activity, whereas phosphorylation of T161 is stimulatory. Comparing the primary structures of p34 and the cyclic AMP dependent protein kinase (PKA) allows one to superimpose p34 onto the PKA three-dimensional structure (Knighton *et al.*, 1991). If this is justified, then all three residues would lie in the

active site cleft of the enzyme. Visualizing the cleft as a mouth, T14 and Y15 would lie on the upper lip. Phosphorylation could interfere with ATP or substrate binding, or catalysis. The homolog of T161 in PKA lies next to a phosphothreonine, which stabilizes the lower jaw of the mouth. Phosphorylation could ensure proper folding of the active site.

Like the activation of MAP kinase, the repression of p34 is achieved by phosphorylation of two residues, T14 and Y15. As described above, phosphorylation of these two residues may not have identical effects, but it appears that phosphorylation of either residue can inhibit p34, in that mutation of both residues has a more severe effect than mutation of either alone. This is the opposite of the MAP kinase story, where phosphorylation at two sites simultaneously is required for an effect, but similar in that the system seems to be designed to reduce the likelihood of inappropriate kinase activation (Figure 3). For p34, dephosphorylation of both residues is needed for activity (at least in vertebrates), and chance encounter with either a serine/threonine or tyrosine phosphatase will not activate doubly-phosphorylated p34. Cdc25 may be the only phosphatase that can efficiently activate p34. On the other hand, inhibition of p34/cyclin can be achieved by Y15 or T14 phosphorylation, and other kinases besides Wee1 and Mik1 may be capable of phosphorylating one or other or both residues. Like MAP kinase, p34 seems to be designed with fail-safe switches for inactivation (Figure 3).

Other Mechanisms of Dual Regulation: Dual Specificity Kinases, Phosphatases, and Binding Sites

The activation of MAP kinase involves a dual specificity tyrosine/threonine kinase. MAP kinase itself autophosphorylates on tyrosine and threonine but phosphorylates other substrates on serine or threonine. Wee1 autophosphorylates on serine and tyrosine but phosphorylates synthetic substrates on tyrosine (Featherstone and Russell, 1991). These kinases are examples of a growing group of bifunctional protein kinases (Lindberg *et al.*, 1992). Most of the other examples were identified from screening bacterial expression libraries with antibodies to phosphotyrosine and are able to phosphorylate bacterial proteins on tyrosine. However, their protein sequences suggest that they are not classical tyrosine kinases. The Clk/Sty kinase autophosphorylates on serine and tyrosine (Ben-David *et al.*, 1991), but Spk1 autophosphorylates predominantly on serine and threonine (Stern *et al.*, 1991), even though both proteins phosphorylate some bacterial proteins on tyrosine. Autophosphorylation may not be a reliable indicator of amino acid specificity on exogenous substrates, especially in cases like MAP kinase where autophosphorylation appears to be intramolecular (Crews *et al.*, 1991; Posada, unpublished data). The likely absence of tyrosine protein phosphatases from

bacteria may allow phosphotyrosine to accumulate even if the rate of phosphorylation is very low. Therefore, it is necessary to test the candidate bifunctional protein kinases with appropriate exogenous substrates to determine whether they can phosphorylate tyrosine, serine, and threonine in *trans*. Synthetic polypeptides containing only tyrosine and glutamic acid are popular substrates but may not be appropriate for this purpose because they are phosphorylated weakly by "authentic" serine/threonine kinases (Stern *et al.*, 1991).

In the past, primary sequence comparison of the protein kinase family in subdomain VI of the catalytic domain has been the gold standard for discriminating between serine/threonine and tyrosine kinases (Hanks *et al.*, 1988) (Figure 4). This sequence-based prediction of hydroxyl specificity is cast in uncertain light with the emergence of the dual specificity kinases, which would be categorized as serine/threonine kinases on this basis (Lindberg *et al.*, 1992).

Further links between serine/threonine and tyrosine phosphorylation are provided by dual specificity phosphatases and binding proteins. Protein phosphatases have been grouped into distinct categories as serine/threonine or tyrosine specific, and there does not appear to be any sequence homology between the two. There are now examples of dual specificity protein phosphatases. The cdc25 protein can probably dephosphorylate both T14 and Y15 of p34 and works poorly on both phosphotyrosyl and phosphoseryl model substrates in vitro (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991). A vaccinia virus phosphatase, expressed in bacteria, also dephosphorylates serine and tyrosine residues in vitro, and both activities are dependent on a single catalytic-site cysteine residue (Guan *et al.*, 1991).

Proteins and peptides containing phosphotyrosine bind to related sequences of ~100 amino acids termed Src homology-2 (SH2) domains (Anderson *et al.*, 1990a; Matsuda *et al.*, 1990). These are found in certain signaling proteins, such as PLC- γ , GAP, the oncoprotein v-crk, and cytoplasmic tyrosine kinases (Cantley *et al.*, 1991). SH2 domains now have been found in a tyrosine phosphatase where they may mediate binding to phosphorylated substrates (Shen *et al.*, 1991). The SH2 domain of the Abl kinase also has been shown to bind serine/threonine phosphorylated sequences (Pendergast *et al.*, 1991). Also, sequencing of the v-akt oncogene shows that it encodes a serine kinase that contains an SH2 domain (Bellacosa *et al.*, 1991). SH2 domains may thus act as additional links between serine/threonine and tyrosine phosphorylation.

SUMMARY AND FUTURE DIRECTIONS

We have described the evidence for a cascade of protein kinases that contributes to the induction of protein phosphorylation at serine and threonine residues by ty-

SERINE/THREONINE KINASES

Cam II- α	DLKPEN
PKA- α	DLKPEN
PKC- α	DLKLDN
CDC2	DLKPQN

TYROSINE KINASES

SRC	DLRAAN
Abl	DLAARN
EGFR	DLAARN
PDGR	DLAARN

CANDIDATE DUAL SPECIFICITY KINASES

Wee1	DLKPAN
Clk/Sty	DLKPEN
ERK2 (a MAP Kinase)	DLKPSN

Figure 4. Sequence comparison of protein kinases in subdomain VI of the catalytic domain. The candidate dual-specificity kinases shown are those that autophosphorylate on serine/threonine and tyrosine.

rosine kinases. In this cascade, the transition from tyrosine phosphorylation to serine/threonine phosphorylation involves the direct tyrosine phosphorylation of PLC- γ and the subsequent indirect activation of PKC. The mechanisms of activation of the remaining members of the cascade are less clear. MAP kinase may directly regulate Rsk and perhaps Raf, but the pathway between PKC and MAP kinases is indistinct. Ras is implicated in this pathway, but the biochemical details remain to be worked out. Presently, the pathway is being attacked from both ends: working downstream from Ras and upstream from MAP kinase. The immediate activator of MAP kinase is probably a dual-specificity protein kinase, able to phosphorylate all three hydroxyl amino acids. Further experiments with this kinase may illuminate the connection between MAP kinases and Ras.

Both MAP kinase and p34/cdc2 are controlled by changes in the phosphorylation state of specific tyrosine and threonine residues. In each case, the phosphorylation and dephosphorylation reactions appear to be regulated. Both enzymes appear to be designed to respond to one or more dedicated activators but may be inactivated by several less-specific enzymes. These systems may have evolved to avoid inadvertent activation and to guarantee rapid inactivation.

Additional links between tyrosine and serine/threonine phosphorylation are provided by dual-specificity kinases and phosphatases. The *in vivo* functions of many of these enzymes are not known. In some cases, the identification of good *in vitro* substrates may guide future experiments. The MAP kinase activator is a poor

protein kinase on most substrates tested, yet efficiently phosphorylates MAP kinase. Other dual-specificity kinases may be similarly restricted in their choice of substrates and may play important regulatory roles in vivo.

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