The Role of Proofreading in Signal Transduction Specificity

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ABSTRACT Many intracellular signaling proteins such as MAP kinases and transcription factors require multiple covalent modifications before activating downstream targets. This property suggests that signaling pathways are organized to facilitate proofreading, which expends energy to enhance the specificity of the pathway for the appropriate effector. Focusing on MAP kinases, we show that each phosphorylation of the kinase can act as an independent specificity test for that kinase. This is independent of whether MAP kinase activation is distributive, processive, or confined to a protein scaffold. We also highlight the importance of phosphatases in developing and maintaining specificity. Support for our proposals can be drawn from the existing literature.

INTRODUCTION

Although there has been considerable progress in identifying the architectures of signaling networks, the mechanisms by which signaling specificity is maintained are not so well understood. Information transfer is often accomplished through a cascade of covalent modifications as upstream molecules phosphorylate downstream targets. Perhaps surprisingly, many molecules require more than one phosphorylation to become activated. In this paper, we argue that these multiple phosphorylations act to significantly improve signaling specificity.

To illustrate our argument, consider a MAP kinase kinase (MAPKK) that has been activated by a signaling cascade and is now primed to phosphorylate MAP kinase (MAPK). Although MAPKK will bind with highest specificity to MAPK, given the dense protein concentration in the cytosol, one can easily imagine a second protein, a kinase, X say, from another signaling route, which MAPKK will also phosphorylate. Examples include human MKK4, an MAPKK, which phosphorylates the two MAPKs, c-Jun amino-terminal (JNK) kinase, and p38 MAPK (Derijard et al., 1995); and yeast Ste7 MAPKK, which phosphorylates Fus3 and Kss1 MAPKs (Madhani et al., 1997). Although Fus3 is activated by pheromone, Kss1 normally regulates filamentation and invasion in response to nitrogen starvation. Cross talk can lead to the erroneous activation of a pathway even though it receives no input signal. In the absence of Fus3 in yeast, pheromone leads to filamentation-specific gene expression and the mating response (Madhani et al., 1997). In this particular example, localization of the MAPK has been suggested as a means to reduce cross talk (Madhani et al., 1997). We are concerned with an additional mechanism that may have evolved to minimize erroneous activation of the individual kinases themselves.

Although the reduced binding energy between MAPKK and X (compared to MAPKK and MAPK) will certainly favor the phosphorylation of MAPK over X, the known enzymology of MAPK activation points toward the existence of a proofreading scheme that significantly enhances specificity. MAPK undergoes two phosphorylations (Canagarajah et al., 1997) and requires both of them before becoming competent to activate the next step of the signaling pathway (Anderson et al., 1990). As mentioned above, it is this double phosphorylation that we believe is a strong indicator that MAPKK improves specificity by proofreading its substrates.

HYPOTHETICAL SCHEME: MAPK ACTIVATED BY ONE PHOSPHORYLATION

First of all, consider a simple hypothetical model in which only one phosphorylation by MAPKK is required for MAPK to become activated, as shown in Fig. 1. Activated kinases are dephosphorylated by a phosphatase that need not discriminate between K_1 and X_1 and here acts on both with the same rate. A measure of specificity for this scheme, i.e., how efficient MAPKK is in activating MAPK and MAPK alone, is given by ρ, defined as

$$\rho = \frac{\text{concentration of error product at steady state}}{\text{concentration of product at steady state}}, \quad (1)$$

where the error product is activated X in this case. From Fig. 1, we wish to calculate the ratio of X_1 to K_1 (note that here we use X_1, etc., interchangeably as a concentration and as a symbol for a chemical species) at steady state. The smaller this ratio the less erroneous information transfer has taken place. For example, ρ is zero when no decoy substrate, X, exists. In fact, to provide a better illustration of the virtues of different reaction schemes, it is useful to set initially equal concentrations of X and MAPK so that any

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Submitted September 27, 2001 and accepted for publication February 11, 2002.

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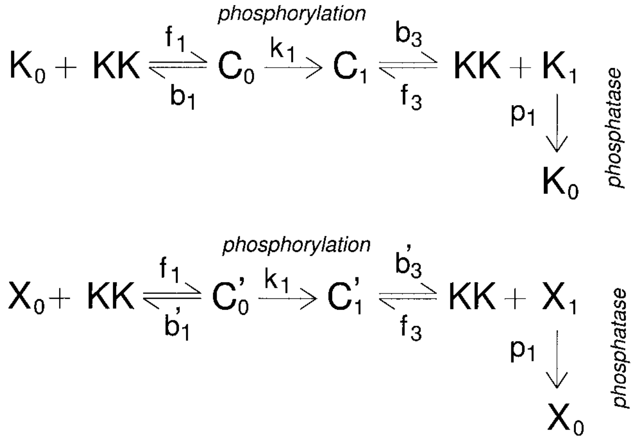

FIGURE 1 A hypothetical scheme of MAPK activation by MAPKK. Just one phosphorylation is required for MAPK to be switched. Subscripts denote the degree of phosphorylation, and primes indicate all complexes formed between MAPKK and X, a decoy substrate from another pathway. For simplicity, only the b_i rate constants are assumed to change ($b_i' > b_i$) when X becomes the substrate (which is certainly true for diffusion-limited reactions). Phosphorylated kinases are dephosphorylated by a phosphatase with rate p_1.

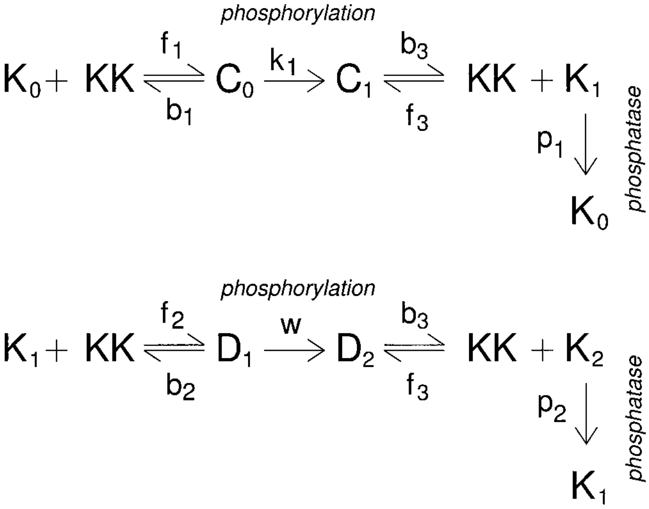

FIGURE 2 Proofreading scheme of distributive MAPK activation by MAPKK. Two phosphorylations are required for MAPK to be switched. X undergoes an identical scheme with, again, only the backward, b_i, rate constants assumed altered. Subscripts denote the degree of phosphorylation.

competition between them for MAPKK is not trivially determined by having more of one present than the other.

The system of mass action equations describing Fig. 1 can be written down and solved under steady-state conditions. Assuming $b_1 \gg k_1$, i.e., that the first reaction is close to equilibrium, and that the phosphatase concentration is such that $p_1 \gg k_1$, then the specificity ρ obeys

$$\rho = \frac{X_1}{K_1} \simeq \frac{b_1 + f_1 KK}{b'_1 + f_1 KK}, \tag{2}$$

with KK the steady-state concentration of MAPKK. Hence,

$$\rho \geq \frac{b_1}{b_1'} \simeq \exp(-\Delta G/T), \tag{3}$$

where ΔG is the difference in binding energies of K_0 and X_0 to MAPKK, and T is temperature in suitable units. Therefore, for a scheme in which MAPK requires only one phosphorylation to be activated, given equal initial concentrations of MAPK and a decoy kinase, the optimum specificity ρ is set by the binding energy difference ΔG.

MAPK ACTIVATED BY TWO PHOSPHORYLATIONS

In reality, MAPK requires two phosphorylations before it is activated (Anderson et al., 1990) and competent to switch downstream targets. Its activation by MAPKK can therefore be either processive (MAPK, once bound to MAPKK, can be phosphorylated twice directly) or distributive (MAPK is phosphorylated once by MAPKK, released, and then has to re-find MAPKK before being phosphorylated a second time). In vitro evidence (Ferrell and Bhatt, 1997; Burack and Sturgill, 1997), indicates that p42 MAPK/ERK2 is activated distributively in both *Xenopus laevis* oocytes and mammalian cells. The presence of protein scaffolds in vivo (Garrington and Johnson, 1999), for example, ERK1 and MEK1 (its MAPKK) are believed to interact with MP1 (Schaeffer et al., 1998), may however, depending on the "on" and "off" rates of the kinases to the scaffold, necessitate processive MAPK activation. In any case, for either activation mechanism, proofreading schemes can increase signaling specificity significantly above the equilibrium limit set by ΔG (see Eq. 3).

Distributive proofreading

Distributive activation of MAPK is shown in Fig. 2. The unphosphorylated kinase, K_0, is first phosphorylated to K_1 through complexes C_0 and C_1, and then only on rebinding to MAPKK is phosphorylated again (through D_1 and D_2) to form the final activated state, K_2. The decoy kinase, X, undergoes an identical scheme to form error product, X_2, though with the b_i rates higher so that $b_i' > b_i$. The specificity ρ obeys, at steady state,

$$\rho = \frac{X_2}{K_2} \simeq \frac{b_1 + f_1 KK}{b_1' + f_1 KK} \times \frac{b_2 + w}{b_2' + w} \tag{4}$$

where the first reaction is again assumed, for clarity, to be close to equilibrium, $b_1 \gg k_1$, and p_1 is such that $p_1 \gg k_1$.

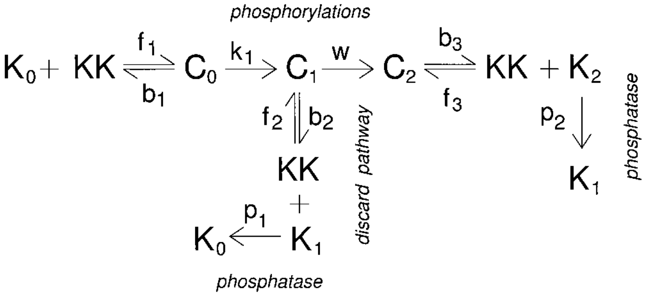

FIGURE 3 Proofreading scheme of processive MAPK activation by MAPKK. A postulated discard pathway has been included (the breakdown of C_1 and the dephosphorylation of K_1), which recycles substrates to be retested. X is activated equivalently.

Therefore, if the concentration of MAPKK is small at steady state, $f_1KK \ll b_1$ and $b_2 \gg w$ (and also $p_2 \gg w$), then

$$\rho \simeq \frac{b_1 b_2}{b_1' b_2'} \simeq \exp(-2\Delta G/T). \tag{5}$$

The specificity is the square of the singly-phosphorylated case, Eq. 3, and is set now not by the binding energy difference of MAPK and X for MAPKK but by twice that difference. The distributive scheme forces each MAPKK substrate to undergo two specificity tests (Ferrell and Bhatt, 1997) and so the effective binding energy difference is doubled.

Eq. 5 is unchanged if the phosphatases act processively, i.e. convert K_2, for example, directly to K_0, though the additional inequality $f_2\mathrm{KK} \ll p_1$ must hold. This while keeping ρ low, also reduces the output of the system as less K_2 is produced for a given amount of MAPKK.

Processive proofreading

If MAPK activation is processive, for example, it occurs on a protein scaffold, then a kinetic proofreading scheme (Hopfield, 1974), first used to account for the fidelity of translation, is appropriate. Figure 3 illustrates this. The first phosphorylation of MAPK leads to its complex with MAPKK (C_1) becoming more unstable and to a finite probability of that complex breaking down, releasing MAPKK and a phosphorylated MAPK. The latter is dephosphorylated by a phosphatase. These side reactions provide a "discard" pathway that irreversibly breaks down C_1 (and C'_1, the complex between X and MAPKK). As pointed out first by Hopfield (1974) and Ninio (1975) for a biosynthetic reaction, such a one-way chute immediately allows specificity to be enhanced.

The steady-state specificity ρ can again be calculated

$$\rho = \frac{X_2}{K_2} \simeq \frac{b_1 + f_1KK}{b'_1 + f_1KK} \times \frac{b_2 + w}{b'_2 + w}, \tag{6}$$

which is identical to Eq. 4. Each MAPKK substrate again undergoes two specificity tests; first, competing to bind to MAPKK, and second, avoiding being recycled through the discard pathway. Both tests favor MAPK over the less strongly binding decoy X and so, in the same limit that Eq. 4 goes to Eq. 5,

$$\rho \simeq \exp(-2\Delta G/T). \tag{7}$$

If one assumes, however, that $w \ll b_2$ and that all the other reactions in Fig. 3 are reversible, then thermodynamics dictates that ρ must equal $\exp(-\Delta G/T)$. In reality, the reactions are held out of equilibrium by the energy bought into the system from ATP via phosphorylations. This energy is used productively to improve, in an inherently kinetic process, ρ below this value (hence the term kinetic proofreading).

In Figs. 2 and 3, we have presented only the minimal model needed for proofreading. This captures the essential processes required for the scheme to function. Most biochemical examples of proofreading will include many additional chemical steps (for example, degradation). These could be added, but because they should not interfere with the ability of a particular molecule to proofread, are not necessary for our purposes.

The importance of phosphatases

For both proofreading schemes, the phosphatases that recycle the MAPKK substrates are crucial as they control the absolute concentrations of K_2 and X_2. In the limits of $p_1 \ll k_1$ and $p_2 \ll w$, the specificity for Figs. 2 and 3 becomes

$$\begin{aligned} \rho &= \frac{X_2}{K_2} \\ &\simeq \frac{X_{\mathrm{tot}}}{K_{\mathrm{tot}}} \times \frac{b'_3(b_3 + f_3KK)}{b_3(b'_3 + f_3KK)} \simeq \frac{X_{\mathrm{tot}}}{K_{\mathrm{tot}}}, \end{aligned} \tag{8}$$

and proofreading is completely degraded with the steady-state value of ρ being determined purely by initial concentrations of MAPK and X (K_{tot} and X_{tot}, respectively). If only the reaction given by rate p_2 (in Figs. 2 and 3) is inhibited, then a better measure of specificity is the initial ratio of the rate of formation of X_2 to the rate of formation of K_2. Given a steady influx of substrates, one can show that this ratio of rates is given by Eq. 7.

Numerical results

To confirm Eq. 3 and Eq. 7, numerical solutions for the various reaction schemes are shown in Fig. 4. The two

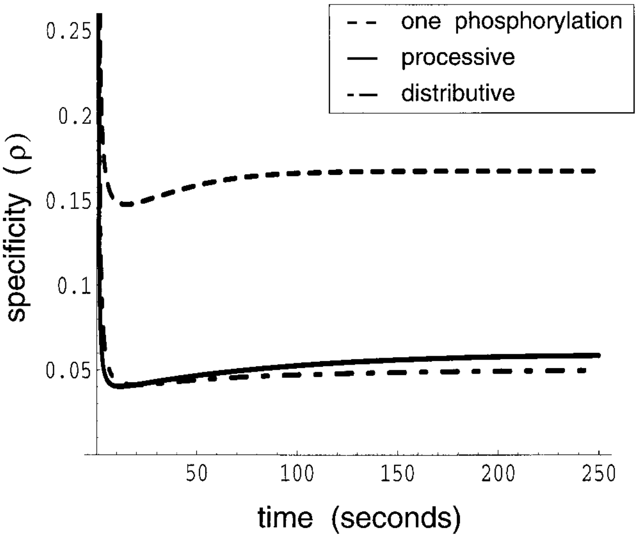

FIGURE 4 Numerical solution for the specificity ρ as a function of time. Parameters: $f_1 = 1.62 \times 10^7$ $M^{-1}s^{-1}$, $b_1 = 0.6$ s^{-1}, and $k_1 = 0.15$ s^{-1} (Bhalla and Iyengar, 1999). For simplicity, $f_2 = f_1$, $b_2 = b_1$, and $k_2 = k_1$. $f_3 = 1.0 \times 10^4$ $M^{-1}s^{-1}$ and $b_3 = b_3' = 6.0$ s^{-1} to favor the formation of K_2 and X_2. $p_1 = p_2 = 0.05$ s^{-1}, $w = 1.0$ s^{-1}, $b_1' = 10b_1$ and $b'_2 = 10b_2$. Initial concentrations: activated MAPKK, 0.27 μM, unactivated K_0 and X_0, 2.83 μM (Ferrell, 1996).

backward rates b_1' and b_2' are $b_1' = 10b_1$ and $b_2' = 10b_2$, which roughly corresponds to a ΔG difference in MAPKK binding energies of 1.4 kcal/mol. The value of $\rho \simeq 0.055$ for both the proofreading cases is close to (considering $w > b_2$ for this example) the square of the steady-state specificity reached in the singly phosphorylated case, $\rho \simeq 0.18$. Figure 5 shows the actual concentrations of K_2 and the error product, X_2, for the processive proofreading case (distributive activation is similar). One can see that almost 32% of MAPK, K, is activated compared to less than 2% of X. Note that K_1, K_2, X_1, and X_2 are all dephosphorylated at the same rate; there is no need for specificity at the level of the phosphatases.

MAPKK specificity can be increased further by raising the value of p_1. For example, if $p_1 = 0.15s^{-1}$ (and p_2 is unchanged), ρ drops to $\simeq 0.04$. However, the faster rate of phosphatase action leads to more MAPK being trapped in the proofreading loop and only 19% of K_0 is activated at steady state. The cell must therefore reach a compromise between the degree of specificity and the efficiency of the activation process.

DISCUSSION

We have shown that the multiple covalent modifications required before a molecule can be switched by the intracellular signal transduction machinery can plausibly act to increase signal specificity. For MAPK, the necessary phosphorylations on both a threonine and a tyrosine residue before activation can effectively double the binding energy difference between it and a rival substrate for MAPKK. Whether the activation reaction is processive, distributive, or confined to a protein scaffold, the known enzymology points toward improved specificity through proofreading.

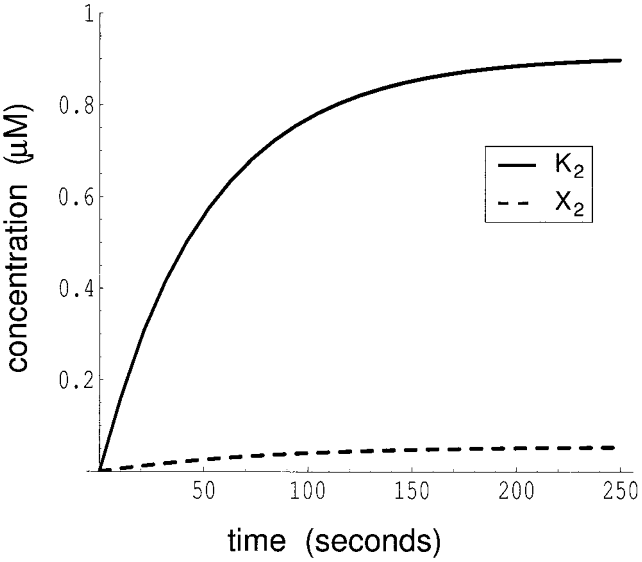

FIGURE 5 The concentration of K_2 and X_2 for the processive proofreading scheme. Parameter values are given in the caption of Fig. 4 and initial concentrations are as before: MAPKK, 0.27 μM; K_0 and X_0, 2.83 μM.

The two required phosphorylations force each MAPKK substrate to undergo two specificity tests. For a distributive mechanism, MAPK has to find MAPKK twice (see Fig. 2) and each time there is competition between it and any rival kinases. Having $b_1 \gg k_1$ and $b_2 \gg w$ ensures that both these reactions are close to equilibrium so that the full binding energy difference between the two competing substrates can be exploited. If the activation of MAPK by MAPKK occurs processively, then a kinetic proof-reading scheme involving a discard pathway provides two specificity tests. The first occurring again as direct competition between rival substrates for MAPKK (see Fig. 3) and the second a measure of ability to bypass the discard pathway (favoring the stronger binding substrate) to go on to be fully activated. Proofreading is optimized by a choice of constants favoring discardment over acceptance; $b_1 \gg k_1$, $p_1 \gg k_1$, $f_1KK \ll b_1$, $p_2 \gg w$, and $b_2 \gg w$.

For both proofreading schemes, phosphatases are essential. These would be expected to be constitutively expressed and do not need to be specific. In particular, for the processive case, a phosphatase catalyzing $K_1 \rightarrow K_0$ in Fig. 3 is of fundamental importance. If this reaction were reversible, then substrates could move up the discard route and so undergo only one specificity test. More generally, the recycling actions of the phosphatases enables specificity to be determined by the rates of the individual reactions (and so by the binding energies to MAPKK) and not simply by the initial concentration difference of rival substrates. In vivo, proteins are being constantly made and degraded but on much longer time scales than the minutes involved here (see Fig. 4), and so it is a reasonable approximation to assume that signaling molecule concentrations are mainly controlled by activation and deactivation processes.

One could argue that, by extending the schemes of Figs. 2 and 3 to include an additional phosphorylation before activation so that MAPK now requires three phospho-residues, the specificity would be increased still further. In fact, it can be shown that, for *n* phosphorylations (and *n* discard pathways for processive activation), the specificity, ρ, is given by Eq. 3 raised to the *n*th power. However, a necessary consequence of the recycling of substrates is a slowing down of the activation process—in the example of Fig. 4, it takes approximately 270 s to reach the steady-state value for the processive proofreading case compared to just 110s for the simple system of Fig. 1 (where just one phosphorylation confers activation). The more phosphorylations, the longer it takes to reach a given threshold value of activated MAPK. Perhaps then two phosphorylations (for MAPK, at least) is a compromise value, chosen by evolution to give good specificity coupled with acceptable response times.

In fact, the MAPK cascade has a number of competing design features: it must amplify initial inputs and do so reasonably quickly, and, it must activate only on the correct signal. Proofreading ensures that, once activated, MAPKK only goes on to switch the appropriate MAPK but this increase in specificity comes at the price of reduced amplification. Raising the efficiency of proofreading (by increasing the phosphatase rates in Figs. 2 and 3, for example) significantly reduces the amount of erroneously activated decoy kinase but, at the same time, decreases the steady state levels of activated MAPK. Proofreading does not interfere with an additional scheme (Ferrell and Machleder, 1998; Bagowski and Ferrell, 2001) that exists to ensure that the whole MAP kinase cascade only activates after the input at the top of the cascade exceeds a threshold value. This all-or-none switch leads to MAPK activation being highly sigmoidal, and arises due to a positive feedback loop (Ferrell and Machleder, 1998; Bagowski and Ferrell, 2001) acting on the cascade. Proofreading acts in parallel to this scheme and does not interrupt positive feedback or any other ultrasensitive (Huang and Ferrell, 1996) mechanisms.

Experimentally, to the best of our knowledge, no direct competition between two substrates for one type of signaling enzyme has been examined. A possible in vitro verification would be, after isolation of a MAPKK, MAPK, X (perhaps Ste7, Fus3, and Kss1 is the best example) and the necessary phosphatases (Zhan et al., 1997), to measure the specificity ρ (the ratio of X_2 to K_2) and the ratio of X_1 to K_1. Distinguishing the two phosphorylated forms of MAPK can be done by, for example, tryptic peptide analysis (Ferrell and Bhatt, 1997). The value of ρ should be much higher than X_1/K_1 (ideally it should be the square of the latter) because it is determined by two, as opposed to one, specificity tests.

We believe that the examples shown here are not isolated exceptions but are part of a more general principle consistently chosen by evolution to increase specificity. Receptor tyrosine kinases often undergo multiple phosphorylations before being fully activated (Schlessinger, 2000), and one can quite easily imagine a kinetic proofreading scheme (akin to that of Fig. 3) with the partially phosphorylated receptor complex begin prone to dissociate through a discard pathway. This would allow the receptor to proofread the various ligands binding to it. A similar scheme has already been proposed to account for the high specificity with which T-cells distinguish foreign from self antigens (McKeithan, 1995). Furthermore, some MAP kinase phosphatases undergo a phosphorylation themselves before dephosphorylating their substrate (Pulido et al., 1998). If this phosphorylation leads to the phosphatase/MAPK complex becoming unstable or occurs distributively, one can argue that the phosphatase proofreads its kinase substrates to ensure that it only dephosphorylates the one it binds to most strongly. In some cases, MAPKK itself (Zheng and Guan, 1994) undergoes two phosphorylations before becoming activated and again could be proofread by MAPKK kinase. Similarly, many of the transcription factors activated by MAPK are multiply phosphorylated by the same kinase: examples include the ternary complex factor Sap-1a (Janknecht and Hunter, 1997), c-Myc (Noguchi et al., 1999), c-Jun (Pulverer et al., 1991), and Elk-1 (Marais et al., 1993).

In conclusion, we have argued that the multiple phosphorylations required by a signaling protein to become activated is one way to improved specificity in the signaling pathway. Molecules that are phosphorylated more than once by upstream proteins can be proofread by these proteins. The molecular species that binds most strongly to the upstream protein will overwhelmingly be the one selected for activation. This reduces crosstalk between signaling routes. The probability that a decoy protein from another pathway, rather than the intended protein, is activated is not determined by the difference in binding energy of these molecules to the upstream protein but by an effective binding energy difference that is much greater. Evidence of proofreading has even now been found in directed vesicle transport at the Golgi (Goldberg, 2000), and it is tempting to think that it is a strategy adopted in many systems to ensure the specificity of their signal transduction.

We are grateful to A. Benecke, J. E. Darnell, A. Hemmati-Brivanlou, N. Rajewsky, and particularly to Jim Ferrell, Daniel Besser and Curt Horvath for conversation and correspondence.

P.S.S. acknowledges financial support from the National Institutes of Health.

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