Mathematical Models of Protein Kinase Signal Transduction

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Summary

We have developed a mathematical theory that describes the regulation of signaling pathways as a function of a limited number of key parameters. Our analysis includes linear kinase-phosphatase cascades, as well as systems containing feedback interactions, crosstalk with other signaling pathways, and/or scaffolding and G proteins. We find that phosphatases have a more pronounced effect than kinases on the rate and duration of signaling, whereas signal amplitude is controlled primarily by kinases. The simplest model pathways allow amplified signaling only at the expense of slow signal propagation. More complex and realistic pathways can combine high amplification and signaling rates with maintenance of a stable offstate. Our models also explain how different agonists can evoke transient or sustained signaling of the same pathway and provide a rationale for signaling pathway design.

Introduction

An enormous amount of information has accumulated about the components of various signaling pathways, their interplay, and their final output. However, the complex nature of these pathways renders it extremely difficult to understand how they are regulated and which parameters determine their dynamics. For example, how do the magnitudes of signal output and signal duration depend on the kinetic properties of pathway components, such as kinases or phosphatases? Can high signal amplification be coupled with fast signaling? How are signaling pathways designed to ensure that they are safely off in the absence of stimulation, yet display high signal amplification following receptor activation? How can different agonists stimulate the same pathway in

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distinct ways to elicit either a sustained or a transient response, which can have dramatically different consequences (Marshall, 1995)? These and other questions can be addressed by a mathematical description of signaling pathways.

Mathematical modeling is a powerful method for the analysis of complex biological systems. Modeling has been used extensively to study metabolic networks, resulting in a theory, termed control analysis, that describes in quantitative terms the role of metabolic enzymes in the regulation of pathway fluxes and metabolite concentrations (Kacser and Burns, 1973; Heinrich and Rapoport, 1974; Westerhoff and Chen, 1984; Reder, 1988; Hofmeyr et al., 1993; Fell, 1997; Cornish-Bowden and Cárdenas, 2000). Modeling is useful for describing experimental data, deducing regulatory principles, and understanding more complex dynamic phenomena such as oscillations in metabolic pathways (see Heinrich and Schuster, 1996).

Although some specific pathways and reaction schemes have been analyzed (e.g., Huang and Ferrell, 1996; Kholodenko et al., 1999; Brightman and Fell, 2000; Asthagiri and Lauffenburger, 2001), mathematical modeling of signal transduction is at an early stage. A particularly thorough simulation was performed on a signaling network using experimentally determined rate constants and component concentrations (Bhalla and Iyengar, 1999; Bhalla, 2001). In many other cases, such quantitative kinetic data are unavailable, but even without these data, mathematical models can enhance our understanding of regulatory principles and pinpoint critical parameters for further experimentation.

We have developed mathematical models for the large class of receptor-stimulated kinase/phosphatase signaling cascades. We analyze key features, such as the amplitude of the signal output, and the rate and duration of signaling. Our results provide insight into the regulatory roles of the signaling components and may help to explain the design of pathways.

Results

Overall Rationale

To simplify the analysis, we first consider a simple linear signaling cascade in which stimulation of a receptor leads to the consecutive activation of several downstream protein kinases (Figure 1). The signal output of this pathway is the phosphorylation of the last kinase which, in turn, can elicit a cellular response (e.g., activation of a transcription factor). Signaling is terminated by phosphatases, which dephosphorylate the kinases, and by inactivation of the receptor, which can involve receptor dephosphorylation, internalization of the receptor-ligand complex, and/or degradation of the receptor or ligand.

This general scheme is representative of many signaling pathways. For example, growth factors such as EGF, PDGF, or NGF stimulate a receptor tyrosine kinase (RTK), which leads to the activation of three or four consecutive

receptor (R) $\xrightarrow{\lambda}$

kinase$_1$ $\overset{\alpha_1}{\underset{\beta_1}{\rightleftharpoons}}$ kinase$_1$~P(X$_1$)

kinase$_2$ $\overset{\alpha_2}{\underset{\beta_2}{\rightleftharpoons}}$ kinase$_2$~P(X$_2$)

kinase$_n$ $\overset{\alpha_n}{\underset{\beta_n}{\rightleftharpoons}}$ kinase$_n$~P(X$_n$)

cellular response
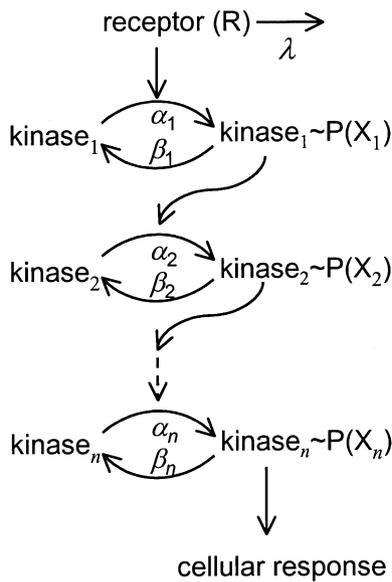
Figure 1. Simple Signal Transduction Cascades

In this scheme, R and X_i indicate the activated receptor and kinases, respectively. λ is the rate constant for inactivation of the receptor, and α_i and β_i are rate constants for the kinase and phosphatase reactions, respectively.

downstream kinases (e.g., Raf, MEK, ERK, and RSK) (Cobb, 1999). Growth factor signals are terminated by protein-tyrosine phosphatases (PTPs), RTK endocytosis and degradation, protein serine-threonine phosphatases, and dual-specificity and tyrosine-specific MAP kinase phosphatases (Keyse, 2000). The same type of scheme can model pathways that include lipid kinases, such as PI3K, whose reaction products (3-phosphoinositides) help to activate downstream kinases such as PDK1 and Akt (Toker, 2000; Vanhaesenbroeck and Alessi, 2000).

Typically, real signaling pathways are more complicated than this simple scheme. Therefore, in later stages of our analysis, we will consider the effects of crosstalk between signaling pathways, the binding of kinases to scaffolding proteins, the requirement that multiple sites be phosphorylated to activate a kinase, and the participation of G proteins.

Linear Signaling Cascades

To derive equations that describe the dynamics of the signaling cascade schematized in Figure 1, we retain the essentials of reactions while neglecting their mechanistic details. Each phosphorylation step is described as a reaction between the phosphorylated form X_{i-1} of kinase $i-1$ in the pathway and the nonphosphorylated form $\tilde{X}_i$ of a downstream kinase (i). The phosphorylation rate for each reaction in the pathway is given by the expression $v_{p,i} = \tilde{\alpha}_i X_{i-1} \tilde{X}_i$, where $\tilde{\alpha}_i$ is the second order rate constant for phosphorylation by the ith kinase. These equations implicity assume that the concentration of each kinase-substrate complex is small compared with the total concentration of the reaction partners. Assuming that the concentration of active phosphatase is constant, dephosphorylation can be modeled as a first order reac-

tion; thus, the dephosphorylation rate of the ith kinase is given by $v_{d,i} = \beta_i X_i$, where β_i is the rate constant for dephosphorylation by ith phosphatase.

For all but the first activated kinase in the pathway, the concentration of each activated kinase i as a function of time, i.e., $X_i(t)$, is given by the solution of the family of differential equations:

$$\frac{dX_i}{dt} = v_{p,i} - v_{d,i} = \tilde{\alpha}_i X_{i-1} \tilde{X}_i - \beta_i X_i. \tag{1}$$

If we define $C_i = \tilde{X}_i + X_i$ as the total concentration of kinase i (i.e., the sum of the phospho- and dephospho-forms) and $\alpha_i = \tilde{\alpha}_i C_i$ as a pseudo-first order rate constant, Equation 1 becomes

$$\frac{dX_i}{dt} = \alpha_i X_{i-1}\left(1 - \frac{X_i}{C_i}\right) - \beta_i X_i. \tag{2}$$

For the first kinase (X_1), activation occurs via the stimulated receptor, and inactivation is mediated by phosphatase 1. Therefore, in place of Equation 2, we have

$$\frac{dX_1}{dt} = \alpha_1 R(t)\left(1 - \frac{X_1}{C_1}\right) - \beta_1 X_1 \tag{3}$$

where $R(t)$ is the concentration of the activated receptor as a function of time.

For our initial analysis, we assume that all pathway components are inactive basally and then undergo rapid stimulation, which is modeled by setting the concentration of active receptor at $t = 0$ to R. Receptor inactivation by various processes (see above) is approximated by the equation $R(t) = \exp(-\lambda t)$, where $1/\lambda$ is the characteristic time of the active receptor. The smaller the value of λ, the longer the receptor will be in the activated state. In the limiting case of $\lambda \to 0$, the pathway is permanently activated.

For any signal transduction system, three key questions are: (1) How fast does the signal arrive at its destination? (2) How long does the signal last? and (3) How strong is the signal? To provide answers to these questions, we introduce three key parameters: (i) the signaling time, τ_i, is the average time to activate kinase i; (ii) the signal duration, ϑ_i, is the average time during which kinase i remains activated; and (iii) the signal amplitude, S_i, is the average concentration of activated kinase i. Occasionally, we also refer to the signaling rate, defined simply as the reciprocal of the signaling time ($v = 1/\tau_n$).

The signaling time τ_i is given by

$$\tau_i = \frac{T_i}{I_i}, \text{ where } I_i = \int_0^\infty X_i(t)dt, \text{ and } T_i = \int_0^\infty tX_i(t)dt. \tag{4}$$

I_i, the integrated response of X_i, is the total amount of active kinase i generated during the signaling period, and thus can be used to characterize signal output (Asthagiri et al., 2000). If one plots X_i versus time (Figure 2), I_i corresponds to the area under the curve (shaded). The ratio T_i/I_i is an average, analogous to the mean value of a statistical distribution.

The signal duration is given by

$$\vartheta_i = \sqrt{\frac{Q_i}{I_i} - \tau_i^2}, \text{ where } Q_i = \int_0^\infty t^2 X_i(t)dt. \tag{5}$$
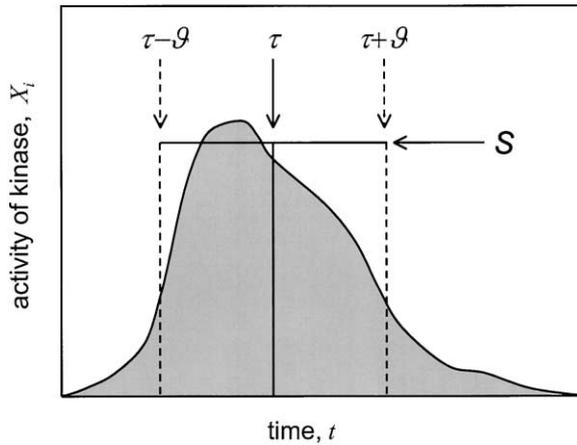
Figure 2. Geometric Representation of the Signaling Time τ, signal duration ϑ, and signal amplitude S.

Shown is the hypothetical time course of the activity of kinase i in a signaling pathway following receptor stimulation. Geometric interpretations of the definitions of τ, ϑ, and S were based on Equations 4–6. Note that the definition of S leads to a value that is not identical with the maximum of the curve.

ϑ_i is similar to the standard deviation of a statistical distribution; thus, ϑ_i gives a measure of how extended the signaling response is around the mean time (Figure 2).

The signal amplitude, S_i, is given by

$$S_i = \frac{I_i}{2\vartheta_i}. \tag{6}$$

In a geometric representation, S_i is the height of a rectangle whose length is $2\vartheta_i$ and whose area equals the area under the curve $X_i(t)$ (Figure 2).

These definitions can apply to a wide range of signal shapes, including those that display more than one maximum. They require only that the signal eventually returns to its initial ground state (otherwise the integrals in Equation 4 would not reach a finite value). If the ground state is attained only at infinite time (i.e., in a permanently activated pathway; $\lambda \rightarrow 0$), signaling time and duration both tend to infinity, but the amplitudes still have finite values (derivation not shown; see also below). The above definitions are most appropriate if the rates of signal activation and inactivation are of the same order of magnitude. If kinase activation is much faster than inactivation, the decay of the activated kinase can be used to characterize signal duration (see below).

Weakly Activated Pathways
A pathway is termed "weakly activated" if all of its component kinases are phosphorylated to a low degree $(X_i << C_i)$. This may occur when the concentration of activated receptor is low, when the receptor is rapidly inactivated, and/or when the kinases are present at high concentrations. Under these conditions, Equation 2 simplifies to

$$\frac{dX_i}{dt} = \alpha_i X_{i-1} - \beta_i X_i, \tag{7}$$

and the key parameters can be calculated explicitly (Ap-

pendix). For the signaling time through the entire pathway one obtains

$$\tau = \frac{1}{\lambda} + \sum_{j=1}^{n} \frac{1}{\beta_j}, \tag{8}$$

i.e., τ is the sum of the characteristic times (and thus, of inverse of the rate constants; see above) for receptor decay and phosphatase action. Remarkably, under these conditions τ is independent of the kinase rate constants, and all phosphatases have the same effect on τ, regardless of their position in the pathway. This is in contrast to metabolic pathways or most other reaction schemes, including "strong activation" of signaling pathways (see below), in which all kinetic parameters help to determine signaling time.

For signal duration one obtains

$$\vartheta = \sqrt{\frac{1}{\lambda^2} + \sum_{j=1}^{n} \frac{1}{\beta_j^2}}. \tag{9}$$

Note that this too is independent of the kinase rate constants, and all phosphatases have the same effect.

Finally, for signal amplitude one obtains

$$S = \frac{S_0 \prod_{k=1}^{n} \frac{\alpha_k}{\beta_k}}{\sqrt{1 + \lambda^2 \sum_{j=1}^{n} \frac{1}{\beta_j^2}}} \tag{10}$$

where S_0 is defined as $R/2$. In contrast to the signaling time and duration, S depends on the kinetic properties of all pathway components (i.e., receptor, kinases, and phosphatases). The above formula shows that high signal amplitudes are obtained with fast kinases and slow phosphatases; moreover, the kinase rate constants have a stronger effect than those of the phosphatases. Thus, in a weakly activated pathway, kinases regulate only signal amplitude, not signaling time or duration. In contrast, phosphatases affect all of these parameters, and in the same direction; i.e., high amplitude can be achieved only with long signal duration and slow signaling rate. Analogous expressions for τ, ϑ, and S can be defined for any intermediate step i. The equations above show that the signaling time (τ) and duration (ϑ) increase with the position in the cascade (i.e., $\tau_i > \tau_{i-1}$, $\vartheta_i > \vartheta_{i-1}$), but signal amplitudes can increase or decrease along the pathway, as discussed below.

Amplification and Dampening
At any step in a signaling cascade, a signal can be amplified ($S_i > S_{i-1}$), dampened ($S_i < S_{i-1}$), or remain constant ($S_i = S_{i-1}$). From Equation 10, one can derive that amplification at step i will occur if

$$\beta_i < \alpha_i \sqrt{1 - \frac{1}{\alpha_i^2 \vartheta_{i-1}^2}}. \tag{11}$$

Thus, amplification requires that the phosphatase rate constant (β_i) for a given reaction is small compared to the kinase rate constant (α_i). Amplification at step i also depends on signal duration at the preceding step (i.e., ϑ_{i-1}); when signal duration at step $i-1$ is long (i.e., large ϑ_{i-1}), amplification can be achieved even when the phosphatase rate constant (for step i) is high. Since signal

duration increases along the cascade (see above), amplification is favored at later steps in a signaling pathway.

Since the expression within the square root of Equation 11 must be positive, there is a lower limit for ϑ_{i-1} at which amplification can occur ($\vartheta_{i-1} > 1/\alpha_i$); i.e., signal duration at step $i-1$ must be longer than the characteristic time ($1/\alpha_i$) of the next kinase. For $\vartheta_{i-1} \rightarrow \infty$, which corresponds to the case in which the receptor and/or an upstream kinase remain permanently activated, Equation 11 simplifies to $\beta_i < \alpha_i$. Thus in a permanently activated pathway (e.g., as might occur in the presence of a constitutively activated RTK), signal amplification can be achieved at higher β values than in a time-dependent situation.

Examples of how our model predicts that a signal will traverse a pathway under conditions of weak activation are shown in Figure 3. If the amplification condition (Equation 11) is fulfilled for all steps (Figure 3A), the peak activity (i.e., signal amplitude) of each succeeding kinase (kinases 1 to 4 in the example) increases. In contrast, if the amplification condition is not met at any step, the amplitudes of successive steps decrease (Figure 3B). Alternatively, the amplification condition may be fulfilled only at later steps, owing to the increase in signal duration ϑ_i along the pathway (see above). In that case, kinase amplitude could decrease in the upper part of the signaling cascade, while increasing in the lower part (inset; Figure 3B).

To evoke an appropriate biological response, the ultimate signal produced by a pathway must be of sufficient magnitude. Equation 10 implies that the same overall signal can be achieved with different combinations of kinase and phosphatase rate constants.

Longer Signaling Cascades Can Give Sharper and Faster Signals

Our analysis reveals a surprising effect of the length of the signaling pathway. On the one hand, longer pathways tend to increase signaling time and duration (see Equations 8 and 9). However, longer pathways also permit amplification to be distributed over more steps (Equation 10), which allows the same signal output to be achieved with faster individual phosphatase reactions. Together, these effects can result in a minimum for signaling time and duration at a certain chain length (Figure 4A; curves for 1- and 10-fold amplification). Figure 4B shows the signal output over time of pathways of different lengths (2–4 steps), each with the same overall amplification (10-fold). Clearly, as the number of steps in the pathway is increased, a signal can travel faster and be of shorter duration. In very short pathways (e.g., only two steps), signal amplification can be achieved only with slow signal propagation and prolonged signal duration. This is probably biologically undesirable, potentially explaining why signaling cascades generally consist of multiple steps.

Strongly Activated Pathways

"Strong activation" occurs if one or more kinases are converted essentially completely to the phosphorylated, activated state. This can occur as a consequence of extensive stimulation of the receptor or the existence
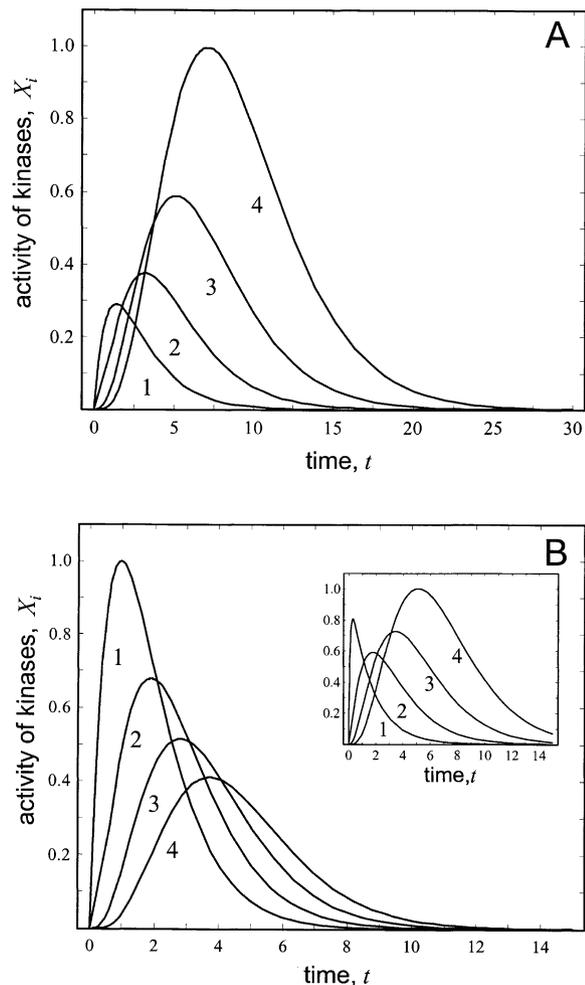
Figure 3. Amplification and Dampening in a Weakly Activated Signaling Cascade

(A) Signal amplification along a pathway consisting of four steps. The parameters used for these calculations were: $\alpha_i = 1$, $\beta_i = 0.5$, $\lambda = 1$, and $C_i \gg 1$. The relative concentrations X_i of the individual activated kinases in the pathway were plotted versus time. The activities were normalized to the highest amplitude achieved by any kinase (set as 1.0).

(B) Signal dampening along a pathway in which $\alpha_i = 1$, $\beta_i = 1.1$, $\lambda = 1$, and $C_i \gg 1$. The inset shows a case in which signal dampening occurs in the upper part and signal amplification in the lower part of the cascade ($\alpha_i = 1$, $\beta_i = 0.6$, $\lambda = 10$).

of an extremely slow phosphatase or fast kinase in a cascade. In such cases, the simplification $X_i \ll C_i$ is no longer valid.

We first consider the properties of a system that is permanently in a strongly activated state. From the condition $dX_i/dt = 0$ one derives from Equation 2:

$$X_i = \frac{C_i X_{i-1}}{\frac{\beta_i}{\alpha_i} C_i + X_{i-1}}, \tag{12}$$

which relates the concentration of the activated form of kinase (X_i) to that of its upstream activator (X_{i-1}). This formula is similar in form to the Michaelis-Menten equation $[V_{max}S/(K_m + S)]$ with C_i being the maximum concen-
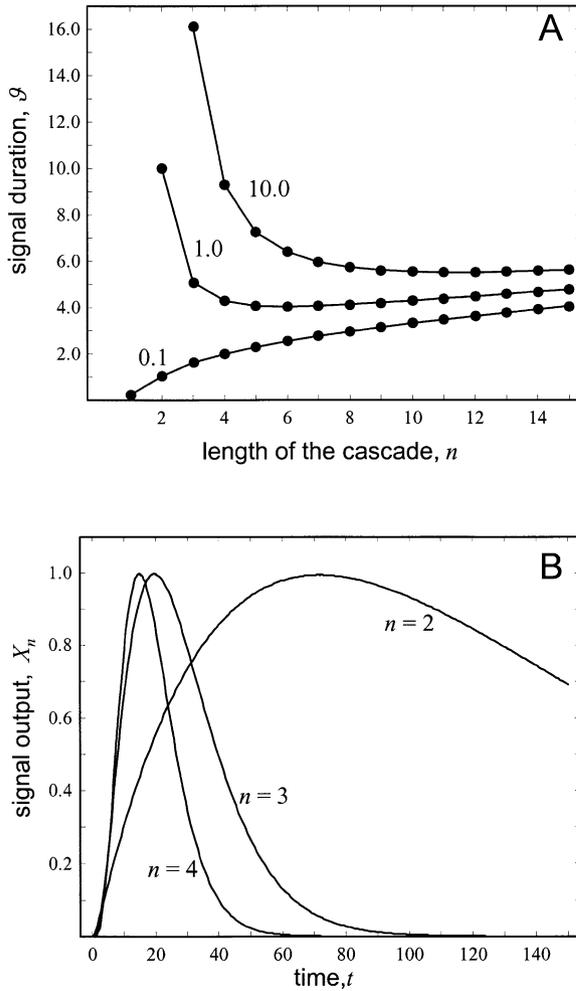
Figure 4. Effect of the Length of a Signaling Pathway on Signal Output

(A) The signal duration ϑ was calculated as a function of the length of the pathway (n) for overall amplifications of $S_n/S_0 = 0.1$, 1.0, and 10.0, respectively. Other parameters were: $\alpha_i = 1$, $\lambda = 5$, and $C_i \gg 1$.

(B) Shown is the time-dependence of the signal output, calculated for $n = 2$, 3, and 4 and an overall amplification of $S_n/S_0 = 10$. These calculations were performed with $\alpha_i = 1$, $\lambda = 5$, and $C_i \gg 1$, and different β values to keep the amplification constant; $n = 2$, $\beta = 0.0141$ ($\triangle\vartheta = 100.0$); $n = 3$, $\beta = 0.1076$ ($\triangle\vartheta = 16.1$); $n = 4$, $\beta = 0.2166$ ($\triangle\vartheta = 9.3$).

tration of X_i (analogous to V_{max}) and $C_i\beta_i/\alpha_i$ being the half saturation constant (analogous to K_m).

From Equation 12, it follows that signal amplification at step i (i.e., $X_i > X_{i-1}$) occurs if

$$X_{i-1} < C_i\left(1 - \frac{\beta_i}{\alpha_i}\right). \tag{13}$$

As in a weakly activated pathway (see above), $\beta_i < \alpha_i$ is a *necessary* condition for amplification (lest the term on the right side of condition [13] be negative). However, in a strongly activated pathway, this condition is not *sufficient*. Instead, condition (13) shows that the concentration of the activated kinase X_{i-1} also must be lower than the total concentration of the next kinase (C_i); indeed, it may have to be considerably lower unless the
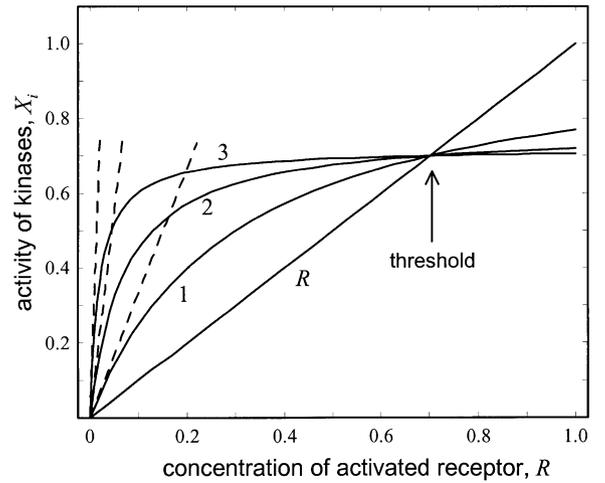
Figure 5. Signal Amplification in a Strongly Activated Pathway

The concentrations of the activated kinases (1–3) in a pathway were calculated for a permanently activated pathway ($\lambda = 0$) as a function of the concentration of activated receptor. The parameter values were $\alpha_i = 1$, $\beta_i = 0.3$, and $C_i = 1$. For comparison, the concentration of the activated receptor, R, is shown. Amplification (visualized as the distance between the curves 1, 2, and 3 at given R) occurs below the indicated threshold, $R = C(1 - \beta_i/\alpha_i)$. Above this threshold, the signal is dampened along the pathway to reach a limiting value. Calculations were also performed for $C_i \gg 1$, corresponding to the weakly activated case (dashed lines).

values of β_i and α_i are similar. Thus, signal amplification is less pronounced if a pathway is strongly activated.

Equation 12 can be applied repeatedly to relate the concentration of kinase i to the concentration of the activated receptor. Written in reciprocal form, one obtains

$$\frac{1}{X_i} = \sum_{j=1}^{i}\frac{1}{C_j}\prod_{k=j+1}^{i}\frac{\beta_k}{\alpha_k} + \frac{1}{R}\prod_{k=1}^{i}\frac{\beta_k}{\alpha_k}. \tag{14}$$

Equation 14 is again a Michaelis Menten-like equation of X_i as a function of the receptor concentration (R):

$$X_i = \frac{X_i^{max}R}{K_{M,i} + R}, \tag{15}$$

where $K_{M,i}$ is the effective Michaelis constant for the receptor and X_i^{max} is the maximum concentration.

These equations imply that the behavior of a signaling system depends on the degree of its stimulation (Figure 5). For weak activation and $\beta_i < \alpha_i$, gradual signal amplification along the pathway is observed, as discussed before (Figure 3A and compare the dashed lines for kinases 1 to 3 at a given value of R in Figure 5). In contrast, strong activation (Figure 5, solid curves) gives lower levels of amplification, and amplification occurs only if the activated receptor R does not exceed a threshold concentration. If R is equal to the threshold level, the signal is propagated without amplification or dampening. Above the threshold, the signal decreases along the pathway to reach a limiting value.

Weakly and strongly activated pathways also differ when time-dependent signaling is introduced (i.e., when the pathway is not permanently activated as above). Consider the three possible routes to a strongly acti-

vated pathway: (1) strong receptor stimulation, (2) a particularly slow phosphatase, or (3) a particularly fast kinase in the cascade. When the receptor is strongly stimulated and the phosphatases are fast compared to receptor inactivation, the cascade will quickly attain a quasi-steady state, during which kinase activation will decay slowly. Since this is a highly asymmetrical time course, we can define the signal duration as the time ϑ at which the concentration of the activated form of the last kinase (X_n) has declined to half of its initial quasi-steady-state value.

Inserting an expression for exponential decay of the activated receptor into Equation 15, one obtains

$$\vartheta = \frac{1}{\lambda}\ln\left(2 + \frac{R}{K_M}\right). \tag{16}$$

The $(1/\lambda)$ term also applies to a weakly activated pathway with fast phosphatases (see Equation 8), but the additional term is specific for strong activation. The latter can increase the signal duration drastically if the receptor concentration is high and the effective K_M value (as defined in Equation 15) is small. The effective K_M value, in turn, depends on the rate constants and the length of the chain. To illustrate the property of the solutions of Equations 14 and 15, we set all α's equal, all β's equal, and all C's equal ($\alpha_i = \alpha$, $\beta_i = \beta$, and $C_i = C$). This allows a simplification of Equation 14 by applying the rules of geometric progression

$$K_M = \frac{C(\beta/\alpha)^n(\beta/\alpha - 1)}{(\beta/\alpha)^n - 1}. \tag{17}$$

Thus, long signal duration, caused by small K_M (Equation 16), will be seen if $\beta < \alpha$ and n is large (Equation 17). In the extreme case of $\beta \ll \alpha$, the signal duration is linearly dependent on the number of steps in the cascade. Hence, signal duration can be much longer in a strongly activated (Equation 16), compared to a weakly activated (Equation 9), pathway. Moreover, in contrast to weak activation, the rate constants of both phosphatases *and* kinases (not phosphatases alone; see above) determine signal duration.

Similar analyses can be performed for pathways containing a slow phosphatase or a fast kinase. Assuming that the rate constant β_j for phosphatase j is much smaller than that of the other phosphatases and setting the latter all equal ($\beta_i = \beta$ for $i \neq j$), signal duration can be approximated by the formula

$$\vartheta \cong \frac{(n - j)}{\beta_j}\ln\left(\frac{\alpha}{\beta}\right). \tag{18}$$

For pathways with a fast kinase, the approximate solution is

$$\vartheta \cong \vartheta_{j-1}\ln\left(\frac{\alpha_j}{\beta}\right), \tag{19}$$

where ϑ_{j-1} is the signaling time up to step $j-1$ and α_j is the rate constant for the fast kinase (for derivation of Equations 18 and 19, see supplemental data at http:www.molecule.org/cgi/content/full/9/5/957/DC1).

As is the case for strong receptor activation (and again, unlike for weak activation), with either a slow phosphatase or a fast kinase, the signal duration for a pathway is dependent on the rate constants of the kinases as well as the phosphatases. However, the impact of the kinases remains lower, since they affect signal duration logarithmically (see Equations 18 and 19). Also, in a strongly activated pathway, upstream phosphatases have a greater effect on signaling time and duration than downstream phosphatases (Equation 18), whereas with weak activation, phosphatase effects are position independent (Equation 8). It may be shown that the basic conclusions based on Equations 18 and 19 do not depend on the specific simplifying assumptions, as long as there is no other particularly slow phosphatase or fast kinase in the pathway (data not shown).

In our analysis so far, we assumed that all downstream kinases are inactive prior to receptor stimulation. We have also considered the possibility that a downstream kinase(s) (j) may be active constitutively, by assuming that j can autoactivate either in a mono- or bimolecular reaction (for details, see supplemental data at http:www.molecule.org/cgi/content/full/9/5/957/DC1). In realistic pathways, the basal activity should be low, and the system will behave similarly to those considered above, except that the effects caused by receptor stimulation will be less pronounced. Similar conclusions apply if the unphosphorylated form of a kinase has some activity.

Crosstalk between Signaling Pathways
Binding of an agonist to its receptor may activate several different signaling cascades, which may influence one another by crosstalk. For example, PDGF receptor stimulation leads to the activation of the Ras/ERK and PI3 kinase/Akt pathways; the latter may inhibit an ERK-specific phosphatase, contributing to sustained ERK activation (Grammer and Blenis, 1997).

We model crosstalk by considering the perturbation of a given signaling cascade by component Y of another pathway (Figure 6A). If component Y inhibits phosphatase i in the pathway, one may write

$$\beta_i = \frac{\beta_i^0}{1 + \dfrac{Y}{K_I}}, \tag{20}$$

where β_i^0 is the rate constant of the phosphatase i in the unperturbed situation and K_I is the inhibition constant. The concentration of component Y is assumed to be proportional to the concentration of the activated receptor R.

To obtain explicit solutions and a qualitative description of the system, we assume that component Y inhibits all phosphatases and set the parameters of the different steps equal ($\alpha_i = \alpha$, $\beta_i = \beta$, and $C_i = C$). Substituting expression (20) for β_i in Equation 12, the concentration of active receptor at which the signal is propagated at constant level through the pathway can be calculated from the following quadratic equation:

$$R^2 + (K_I - C)R - K_IC\left(1 - \frac{\beta^0}{\alpha}\right) = 0. \tag{21}$$

The solution of this equation gives values of R that separate signal dampening from signal amplification. If this equation has only one positive solution, the system behaves identically to the unperturbed pathway analyzed
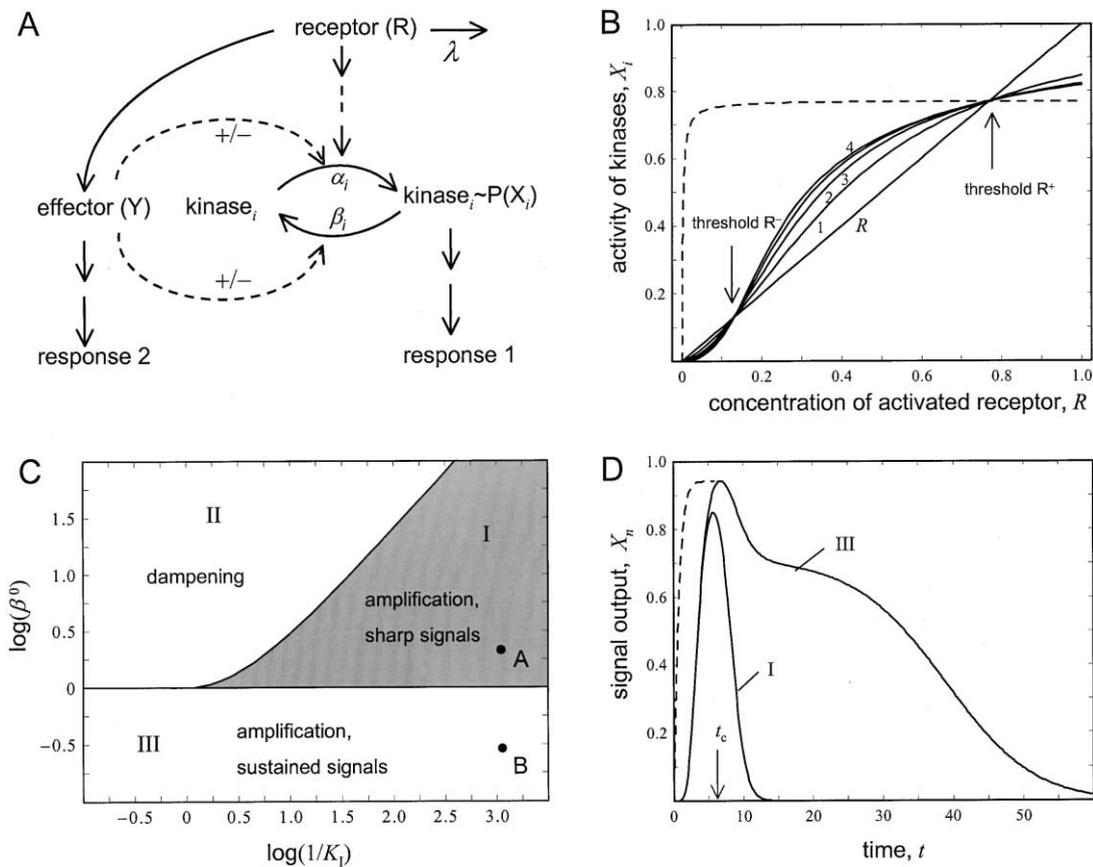
Figure 6. The Effect of Crosstalk on a Signaling Cascade

(A) Shown is a scheme in which a pathway similar to that in Figure 1 is affected by a component Y of another pathway. Both pathways are downstream of the same receptor. Component Y can act either on the kinase or phosphatase of step i in a positive or negative manner.

(B) All phosphatases of a signaling pathway are assumed to be inhibited by component Y. Shown are the concentrations of the activated kinases i calculated as a function of the concentration of a permanently activated receptor ($\lambda = 0$). Parameter values: $n = 4$, $\beta^0/\alpha = 2$, $C = 1$, $K_I = 0.1$. Amplification occurs only between the thresholds R^- and R^+, and outside this region dampening is observed. For comparison, the dashed line shows the signal output (X_4) in the absence of crosstalk with $\beta = 0.23$.

(C) Parameter regions corresponding to the different behavior of a cascade with crossinhibition of the phosphatases. The rate constant for the phosphatases (β^0) in the absence of inhibition is plotted against the inverse of the inhibition constant in a double logarithmic manner. These calculations were performed with $\alpha_i = 1$. Parameter region I corresponds to amplification and gives an S-shaped curve, such as that shown in (B). Under these conditions, the time-dependence shows a sharp signal (see [D]). Parameter region II results in signal dampening along the pathway. Region III results in curves without a lower threshold and with sustained signaling (D).

(D) The time course of the signal output is calculated for parameter values corresponding to the points A and B in parameter regions I and III (C). For point A we choose $\beta^0 = 2$ and for point B $\beta^0 = 3$. Other parameters are: $K_I = 8.0 \times 10^{-4}$, $\lambda = 1$. t_c is the time up to which the phosphatase inhibition is effective (see Equation 22). For comparison, calculations were performed for the case in which the kinases are activated by a component of another pathway ($K_A = 8.0 \times 10^{-4}$, $\beta^0 = 0.3$, dashed line). Only the initial phase of the time course is shown; at later time points the curve essentially coincides with the solid line.

before. However, if this equation has two positive solutions, there are two threshold values for R (R^- and R^+; Figure 6B), as in the case of strong activation without crosstalk (see Figure 5 and dashed line in Figure 6B). Consequently, there are three distinct regions in which the system displays different properties: below R^-, there is signal dampening along the pathway, between R^- and R^+ there is amplification, and above R^+, there again is dampening. The resultant system has two novel properties. First, it filters out small signals (i.e., below R^-); only signals above this noise will be amplified. Second, the system remains sensitive over a wide range of receptor activation (Figure 6B), whereas in an unperturbed pathway, the signal output is extremely responsive at

low but insensitive at high concentrations (Figure 5 and dashed curve in Figure 6B).

Different combinations of β^0 and K_I lead to distinct types of system behavior of the system (Figure 6C). In shaded area I, corresponding to strong inhibition (i.e., low K_I), Equation 21 has two positive solutions. For parameter combinations outside this shaded area, the system behaves as if there were no crosstalk: in area II, dampening occurs for all R values, whereas in area III, amplification occurs for R values below and dampening for R values above a certain threshold value (see Figure 5). Although these calculations were performed for a drastically simplified systems (α's, β's, and C's are equal), qualitatively similar results are obtained as long

as the respective kinase and phosphatase rate constants are not extremely different from one another (not shown). The most interesting consequence of crossinhibition of phosphatases is seen in the time-dependent behavior of the system. Figure 6D shows plots of the signal output of a four component pathway as a function of time. Both curves are calculated for the same low K_I, i.e., for strong inhibition of the phosphatases. Curve III is derived using the parameter combination corresponding to point B in Figure 6C (area III, low phosphatase activity) and shows long signal duration. Curve I is calculated from the parameter combination corresponding to point A in Figure 6C (area I, high phosphatase activity) and shows spike-like behavior. After receptor stimulation, the kinases are initially fully activated by the inhibition of the inactivating phosphatases, but the subsequent release of inhibition results in rapid termination of signaling. The duration of the phase during which the phosphatases are strongly inhibited ($R(t_c)/K_I > 1$) can be approximated by

$$t_c = \frac{1}{\lambda} \ln\left(\frac{R}{K_I}\right). \qquad (22)$$

Beyond t_c, the signal decays as in an unperturbed pathway, fast for parameter combinations corresponding to area I in Figure 6C and slow for those represented by area III.

This spike-like behavior is a qualitatively novel behavior of the system, which cannot be observed without crosstalk. In an unperturbed system, short signals (high phosphatase rates) are always linked to low amplitude. With crosstalk, however, amplification and short signal duration can coexist. Interestingly, transition from a prolonged to a spike-like response can occur with relatively minor changes in the rate constants of the system (see legend to Figure 6D). This may explain how relatively moderate alterations of the conditions can lead to markedly different qualitative responses by the same signaling pathway.

Although thus far we have assumed that all phosphatases are inhibited simultaneously by component Y, qualitatively similar (although less pronounced) effects are seen if only one phosphatase is inhibited (not shown). The further upstream in a pathway that phosphatase is located, the stronger these effects (see Equation 18). This predicts that phosphatases regulated by crosstalk may be preferentially located at the beginning of a signaling cascade.

Qualitatively similar results are obtained when crosstalk with another pathway leads to strong activation of a kinase $\alpha_i = \alpha_i^0(1 + Y/K_A)$. The same three parameter areas as in Figure 6C exist. The kinetics differ only at early time points, during which activation of kinases leads to immediate stimulation of the pathway (see dashed curve in Figure 6D). At later times, the signal output remains unchanged.

These effects of crosstalk can be seen only if a phosphatase is strongly inhibited or a kinase strongly activated (by a factor of 100–1000). Since such drastic changes are difficult to achieve with activations, inhibition of phosphatases may be more relevant physiologically. Notably, the two other possible effects of cross-

talk, i.e., leading to activation of a phosphatase or inhibition of a kinase, cause signal dampening (not shown). Although such interactions may be important for the detailed response of a system, the more decisive crosstalk mechanisms are activation of kinases and, particularly, inhibition of phosphatases.

Stability of Signaling Pathways

Signaling pathways must be stable in the off-state; i.e., gratuitous kinase stimulation should be dampened, rather than amplified. In mathematical terms this requires that, upon an infinitesimal change in the concentration of an activated kinase, the system must return to its ground state.

One source of instability can be a positive feedback loop. Consider the case in which the last kinase in a pathway activates the first kinase. The term $\alpha_1 R$ in Equation 3 is then replaced by $\alpha_1 R + \epsilon X_n$, where ϵ is the rate constant that characterizes the positive feedback. The conditions under which the ground state ($X_i = 0$) exhibits dynamic instability can be derived from the Eigenvalues of the Jacobi matrix:

$$\beta_1 \beta_2 ... \beta_n < \epsilon \alpha_2 ... \alpha_n . \qquad (23)$$

If this relation is fulfilled, the signaling cascade may be activated in the absence of receptor stimulation and cannot return to the ground state following receptor inactivation. Notably, the conditions for instability (Equation 23) are identical to the amplification conditions $\beta_1 \beta_2 ... \beta_n < \alpha_1 \alpha_2 ... \alpha_n$ derived from Equation 10 for permanent pathway activation $\lambda \to 0$, except that α_1 is replaced by ϵ. Thus, high amplification also is potentially destabilizing. Indeed, a pathway can show amplification and be stable only if the feedback parameter ϵ is smaller than α_1. These considerations suggest that positive feedback interactions may be infrequent in signal transduction pathways. However, some signaling systems are bistable, i.e., they can switch from one stable state at low level activation to another at high level activation, and such a behavior can be caused by positive feedback interactions (Ferrell, 2002; Höfer et al., 2002). As the switch-like behavior is required only for signal output, e.g., for a transcriptional response, the positive feedback loop may be restricted to downstream portions of a pathway.

Another cause of instability may be insufficient kinase specificity. Consider the case in which kinase j can phosphorylate not only its normal substrate $j + 1$ but also kinase i with the rate constant α_{ij}. The differential equations then read

$$\frac{dX_i}{dt} = \left(\sum_{j=1}^{n} \alpha_{ij} X_j\right)\left(1 - \frac{X_i}{C_i}\right) - \beta_i X_i . \qquad (24)$$

The consequences of kinase promiscuity for the stability of the system can be best appreciated if one assumes that each kinase phosphorylates its immediate downstream target with the rate constant α and all other kinases in the pathway with the rate constant ϵ. The solution of Equation 24 results in parameter plots with
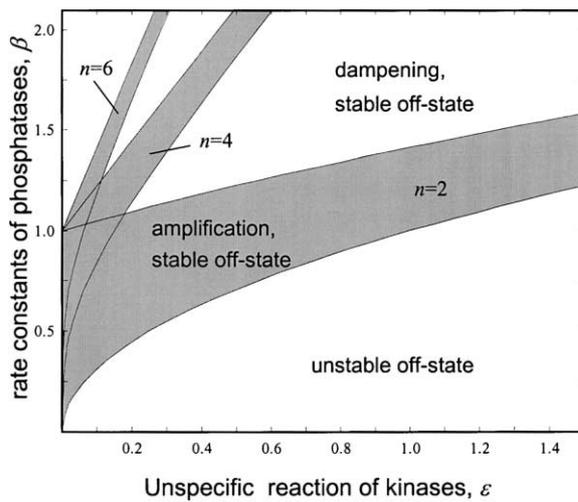
Figure 7. Kinase Promiscuity Results in Signaling Pathway Instability

The rate constant of the phosphatases β is plotted versus the rate constant ϵ that describes the crossphosphorylation of kinases outside the actual pathway. These calculations were performed for $\alpha = 1$ and different pathway lengths ($n = 2$, 4, or 6). Shaded areas correspond to parameter regions in which the system has a stable off-state and displays amplification when activated. Above this region there is a stable off-state and dampening along the pathway after activation, whereas below this region, the off-state is unstable.

three possible outcomes (Figure 7). Below the shaded area, i.e., for low phosphatase activity (β small), the off-state of the pathway is unstable. Within the shaded area (intermediate β values), the pathway is stable and signal amplification occurs. Finally, with high phosphatase activity (high β), the system is stable but displays signal dampening. The higher the rate constant ϵ for nonspecific phosphorylation (i.e., the more promiscuous the kinases), the stronger the tendency toward instability. Increasing the number of kinases in the system also increases the tendency toward instability (Figure 7; note the increase in the area below the shaded region with increasing n). Thus, the kinases in a system need to be very specific to achieve stability, particularly if one takes into account the large number of kinases in cells. A further implication is that the broadening of the substrate range of a kinase, either by mutation or overexpression, may lead to instability. This may help explain the pathogenic consequences of some oncogenic mutations (Songyang et al., 1995; Piao et al., 1996).

The Role of Scaffolding Proteins

Many signaling pathways utilize scaffolding proteins that bind multiple kinases in the pathway (for review see Pawson and Scott, 1997). Several potential functions for scaffolding proteins have been proposed.

One possibility is that scaffolding proteins serve to activate a group of kinases. We consider a pathway in which the component kinases are active only when bound to a scaffolding protein, F. Assume that F binds three consecutive kinases (e.g., the JIP proteins in the stress-activated JNK pathway; Whitmarsh and Davis, 1998). The following equilibria can be written

$$\frac{F \cdot T_i}{(FT_i)} = D_i, \quad \frac{(FT_i)T_j}{(FT_iT_j)} = D_{ij},$$

$$\frac{(FT_iT_j)T_l}{(FT_iT_jT_l)} = D_{ijl}, \quad (25)$$

where T_i, T_j, and T_l are the concentrations of the free kinases, the species in brackets are binary, ternary, and quaternary complexes of F with its kinase binding partners, and the D's are dissociation constants. Using these equations, the concentration of the active species can be calculated from the total concentrations of the kinases and the scaffolding protein F_{tot}. Figure 8A shows the concentration of the active species as a function of F_{tot}, assuming that the active complex contains the scaffolding protein and all three kinases ($FT_iT_jT_l$). The solid line ($n = 3$) illustrates the system's behavior when the kinases bind tightly to F (i.e., low dissociation constants): the active complex increases linearly to reach a maximum at which the concentrations of scaffolding protein and kinases are about equal, and thereafter, decreases steeply. The sharp decline reflects the decreasing probability that all three kinases will bind to the same scaffolding protein simultaneously (a situation analogous to decreased immune complex formation in the presence of antibody excess). If only two kinases are required to generate the active complex, the decrease is less pronounced (Figure 8A, solid line; $n = 2$). Alternatively, if the kinases bind to the scaffolding protein weakly (high D's), only a small fraction of the total population of kinases is found in the active complex under any condition (dashed curve; $n = 3$). These results show that the scaffolding protein allows efficient signaling only below a critical concentration. However, a large fraction of the kinases remains unutilized by the pathway. Obviously, such a situation would only be meaningful if the unbound kinases were essentially inactive. The fact that scaffolding proteins may either stimulate or inhibit signaling, depending on their concentration, may explain differences in the observed effects of scaffolding protein overexpression (e.g., Gu et al., 1998; Zhao et al., 1999).

Although more speculative, another possible role for scaffolding proteins could be to sterically hinder phosphatases from acting on bound kinases. To analyze this situation, we assume again that the scaffolding protein can bind three kinases, resulting in a total of eight species in which the scaffold-bound kinases have different phosphorylation states (indicated in Figure 8B by "0" or "P" at the three positions within brackets). If phosphatases cannot act on the bound kinases (scheme I in Figure 8B), dephosphorylation is possible only after release of all kinases (dashed arrow). If the phosphatases can act on the bound kinases, there are many potential pathways for phosphorylation-dephosphorylation (see arrows in scheme II of Figure 8B).

For both schemes, we have calculated that the signaling rate and signal duration, assuming that the amplitude of the signal output, species (PPP), has a given value. In Figure 8C, the signaling rate is plotted versus the rate constant of receptor inactivation (λ). For both schemes, signaling becomes extremely slow if receptor inactivation is slow (λ close to zero). If receptor inactivation is fast (λ close to 1), the rate constants for the phosphatases have to be small to achieve a given amplitude,
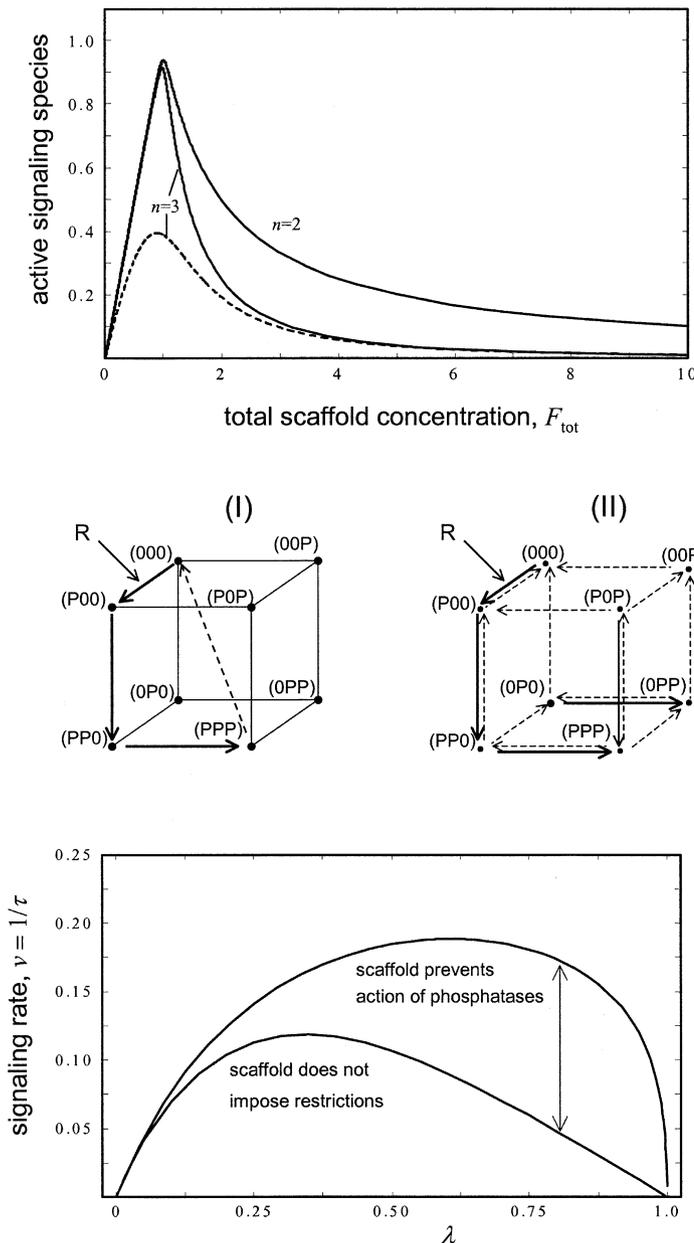
Figure 8. Signaling Involving a Scaffolding Protein

(A) A scaffolding protein, F, was assumed to bind three kinases ($n = 3$) to give an active signaling complex. The concentration of this complex is plotted versus the total concentration of F (F_{tot}). The solid and dashed lines were calculated for strong $D = 0.001$ and weak $D = 0.1$ binding, respectively. Also shown are the results of calculations performed for a scaffolding protein that binds only two kinases ($n = 2$; $D = 0.001$).

(B) Reaction schemes describing the possible effects of phosphatases on kinases bound to a scaffolding protein. "P" and "0" denote phosphorylated and nonphosphorylated kinases, respectively. The pathway is assumed to be stimulated by phosphorylation of the first kinase, and its output is the state (PPP). In scheme I, phosphatases are prevented from acting on bound kinases and therefore can inactivate the kinases only after their release from the scaffolding protein (dashed arrow). In scheme II, the phosphatases can act on each activated kinase bound to the scaffold. Solid and dashed arrows indicate possible phosphorylation and dephosphorylation steps.

(C) Signaling rate is plotted as a function of the rate constant for receptor inactivation for the case in which the amplification factor is $S(PPP)/R = 1$. These calculations were performed for a weakly activated pathway by solving three systems of linear algebraic equations for the integrals defined in Equation 4. For scheme I, explicit expressions for the signaling rate are obtained. For scheme II, the corresponding linear equation systems are much more complex and were solved numerically. The calculations were performed with $\alpha = 1$ and different β values to keep the amplification constant. The upper curve corresponds to scheme I in (B), whereas the lower curve corresponds to scheme II. The arrow shows where the difference between the two schemes is maximal.

again leading to a long-lasting signal. When the two schemes are compared, the one with restricted phosphatase steps (scheme I) has a faster signaling rate (particularly in the region indicated by the double-headed arrow). Thus, by preventing the inactivation of bound kinases, a scaffolding protein may allow faster signaling at a given signal output.

A third possible function of a scaffolding protein is to allow transport of the bound kinases to the receptor at a certain site at the plasma membrane, away from the unbound kinases in bulk solution. In this case, signaling would occur only locally, and the kinases need not increase their activity upon binding to the scaffold. Because this situation is intuitively obvious, we have not performed specific calculations.

Kinase Activation by Multi-Site Phosphorylation
Some kinases require multiple phosphorylation events for full activation. For example, the MAP kinase ERK is activated by phosphorylation at closely spaced Thr and Tyr residues by the dual-specificity enzymes MEK1 or MEK2 (Cohen, 2000). Here, we consider the general case in which a kinase phosphorylates a downstream kinase at two different sites without intermediate dissociation (processive phosphorylation; Burack and Shaw, 2000; Levchenko et al., 2000). If there is such a double phosphorylation step in a signaling pathway, the signal duration (τ) can be calculated by a method similar to that described in the Appendix:

$$\tau = \tau^* + \frac{1}{\beta'} + \frac{1}{\beta''} + \frac{\alpha''}{\beta'\beta''}, \qquad (26)$$

where τ^* is the signal duration caused by the rest of the pathway and β' and β'' are the rate constants for the two phosphatases that inactivate the kinase regulated by multisite phosphorylation. In contrast to cascades with only single phosphorylation steps, Equation 26
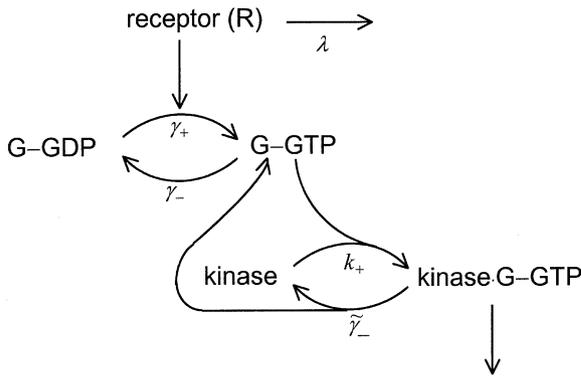
Figure 9. The Effect of G Proteins

The scheme illustrates the activation of a G protein by receptor-stimulated guanine nucleotide exchange (rate constant γ_+). The activated G protein may bind to a kinase (rate constant k_+). Inactivation occurs by dissociation of the GTP-form of the G protein from the kinase ($\tilde{\gamma}_-$) followed by nucleotide hydrolysis (γ_-).

shows that kinases that perform double phosphorylation do affect signaling time, even under weakly activating conditions. Interestingly, only the rate constant for the second phosphorylation step (α'') plays a role; activation of this step increases the signaling time.

The Effect of G Proteins

Many signaling pathways contain steps in which kinase activation involves a G protein (Van Aelst and D'Souza-Schorey, 1997; Kjoller and Hall, 1999; Symons and Settleman, 2000). We consider a simple scheme (Figure 9) in which receptor stimulation leads to the activation of a G protein by conversion from its GDP to its GTP form (rate constant γ_+). The activated G protein then activates a kinase (with rate constant k_+). Inactivation of the kinase is described by the rate constant $\tilde{\gamma}_-$. The GTP form of the G protein dissociates from the kinase and is subsequently inactivated by hydrolysis of GTP (rate constant γ_-). In this scheme, we assume that the GTPase-activating enzyme (GAP) and the kinase compete for binding to the G protein, so that GTP hydrolysis only occurs on the free G protein (Zhang et al., 1993; Warne et al., 1993). The reactions described by the rate constants k_+ and $\tilde{\gamma}_-$ may include several individual steps that lead to activation and inactivation of the kinase, respectively.

To allow explicit solutions, we consider a weakly, permanently activated pathway. For the amplitude X of the G protein-activated kinase, one obtains

$$X = \frac{\gamma_+ k_+}{\gamma_- \tilde{\gamma}_-} R. \tag{27}$$

This equation is similar to the one derived for a cascade consisting of two kinases and two phosphatases:

$$X_2 = \frac{\alpha_1 \alpha_2}{\beta_1 \beta_2} R. \tag{28}$$

For the signaling time of the G protein-containing pathway (Figure 9), one obtains

$$\tau = \frac{1}{\lambda} + \left\{ \frac{1}{\gamma_-} + \frac{1}{\tilde{\gamma}_-} + \frac{k_+}{\gamma_- \tilde{\gamma}_-} \right\}, \tag{29}$$

which is similar to the formula derived for a kinase requir-

ing two phosphorylation events for activation:

$$\left(\triangleq \frac{1}{\beta'} + \frac{1}{\beta''} + \frac{\alpha''}{\beta' \beta''} \right). \tag{30}$$

The signaling time in a G protein-containing pathway obviously depends on several parameters, including the rate constants for GTP hydrolysis and for dissociation of the G protein/kinase complex. Like kinases that perform double phosphorylation, G protein-activated kinases in a pathway can also affect the signaling time, even in a weakly activated situation. However, in reality, the dissociation of the G protein/kinase complex is fast ($\tilde{\gamma}_-$ large; Sydor et al., 1998), and therefore the signaling time is mostly determined by the hydrolysis rate of the free G protein/GTP complex. In any case, however, G protein-mediated reactions appear to be kinetically equivalent to kinase-phosphatase steps.

Discussion

We have modeled the kinetics of protein kinase signaling pathways. To derive analytic expressions that allow transparency of the regulatory principles, we considered pathways that are simplified versions of those found in nature. Some included cascades only of kinases and their corresponding phosphatases, but we have also analyzed some more complex situations, including crosstalk with parallel pathways, feedback interactions, multisite phosphorylation, the effects of scaffolding proteins, and the inclusion of G proteins. Although more detailed simulations are required to describe realistic systems, our basic models allow general conclusions that likely apply to a large number of pathways. These models also provide a framework for evaluating quantitative kinetic data on signaling pathway components.

We focused on three key parameters that describe the behavior of signal transduction pathways: the amplitude of signal output, the signaling time (or its inverse, the signaling rate), and the signal duration. These quantities may not account for the detailed kinetics of all signaling systems (e.g., oscillations), but they should be sufficient to describe most responses. Moreover, each of these parameters may have biological impact. For example, a critical signal amplitude may be needed to evoke a biological effect. Signal amplification may not always be required, so long as the signal arrives at its final target (e.g., a specific transcription factor). Most likely, fast signaling is desirable in all signal transduction pathways. However, signal duration may have to be short in some cases, for example, in a metabolic response, and longer in others, such as in a transcriptional response.

Our analysis reveals that these signaling parameters are determined by different components of signaling systems. For example, the signal amplitude is influenced more by kinases than by phosphatases. Signaling rate and duration, on the other hand, are mostly regulated by phosphatases. In the simplest pathway undergoing weak activation, the kinases have no role in determining signaling rate or duration. In more complicated systems, kinases can have a moderate effect, but one still less than the phosphatases. A recent numerical simulation supports these conclusions for the specific example of

the ERK MAP kinase pathway (Asthagiri and Lauffenburger, 2001). Obviously, the amplitude of signal output is limited; its maximum is reached when the final target is fully phosphorylated. In contrast, signal duration can, at least in principle, be unlimited. Thus, phosphatases, which largely determine signal duration, can have a significantly stronger effect than kinases on the biological outcome of a pathway. This critical role of the phosphatases is limited to normal signaling, in which the system returns to an inactive ground state. In contrast, when a signaling pathway is permanently on, kinases can play a major role (see Equations 10 and 12). Indeed, many known oncogenic mutations cause constitutive activation of a kinase.

Our results also suggest that phosphatase inhibition is the most effective way that one signaling pathway can influence another. Phosphatases generally have high catalytic efficiency, with rate constants much higher than kinases. Our analysis shows that signal amplification can occur only when the kinase rate constants exceed those of the phosphatases (i.e., $\alpha_i > \beta_i$). The optimal situation should occur when a phosphatase at the beginning of a signaling pathway has a high basal activity and is inhibited during early stages of signaling. Such a system would maintain a stable off-state, permit signal amplification after receptor stimulation, and allow fast signal termination following receptor inactivation. An example of such regulation is provided by the tyrosine phosphatase PTP1B, which regulates signaling from several RTKs. PTP1B has a high turnover number (Tonks et al., 1988; Zhang et al., 1994), and recent evidence suggest that it is transiently inhibited by reactive oxygen species generated upon RTK activation (Lee et al., 1998; Mahadev et al., 2001). In simple signaling systems, we find that high amplification can occur only with slow signaling rates and long signal duration. More complex systems, such as those involving crosstalk with other pathways, gain flexibility, allowing amplification to be combined with fast and transient signaling. Crosstalk also allows small parameter variations to cause drastic changes in signal duration. This may help explain why the same pathway can respond differently when stimulated by two different receptors; for example, EGF stimulates the MAP kinase pathway in PC12 cells only transiently, leading to proliferation, whereas NGF causes a sustained response that evokes neuronal differentiation (Traverse et al., 1992; Marshall, 1995).

We studied two principle regimes at which a signaling pathway may function: weak versus strong activation. Highly activated pathways have the disadvantage of displaying lower amplification potential and long signal duration. Under physiological conditions, most pathways are likely to be weakly activated. However, in certain pathological states, such as in a cancer cell, high level activation may exist and be sustained. A transiently high phosphorylation state of a kinase may occur even under physiological conditions, if regulatory mechanisms exist that result in its fast decay.

Our analysis reveals multiple mechanisms that regulate signaling pathway stability. If the kinases are not exquisitely specific or if positive feedback interactions occur, random stimulation of a kinase can lead to activation of the pathway in the absence of receptor stimulation, which may have catastrophic consequences. The longer the pathway, the more severe this problem becomes. Phosphatases play a critical role in preventing gratuitous pathway activation. Spurious activation of a cascade can also be suppressed if at least one of the kinases in a pathway requires dual signals for activation (mathematical analysis not shown). For example, p70 S6 kinase I requires binding of the activated small G proteins Cdc42 or Rac, as well as multisite phosphorylation, before it can be fully activated by the kinase PDK1 (Chou and Blenis, 1996). A stable off-state is maintained because neither the random activation of the G protein nor the upstream kinases alone can activate the pathway. Scaffolding proteins also may ensure stability because they may prevent bound kinases from phosphorylating random targets. In addition, amplification of random noise can be minimized if signal amplification is distributed over several steps in a pathway.

Analysis of more complicated signaling pathways suggests that scaffolding proteins may serve several, not mutually exclusive functions. One possibility is that kinases become activated only when bound to a scaffolding protein. In this case, the kinases either are not utilized efficiently for the given pathway or are prevented from interacting with each other, depending on the relative concentrations of kinases and scaffolding protein; similar conclusions were reached from a numerical simulation (Levchenko et al., 2000). A second possibility is that scaffolding proteins might allow faster signaling in the pathway by preventing phosphatases from acting on the bound kinases. Finally, and likely most important, a scaffolding protein may allow a complex of consecutive kinases to be recruited to an activated receptor at the plasma membrane, leaving the unbound kinases inactive in bulk solution. The complex could even be transported to a distant location, as is the case for JIP, which is transported by kinesin to the tips of neurites (Verhey et al., 2001).

Signaling cascades that contain G proteins turn out to be surprisingly similar to those consisting only of kinases and phosphatases. Our analysis shows that the rate of GTP hydrolysis of the free G protein/GTP complex largely determines signal duration. This is consistent with the fact that oncogenic mutations in the G protein Ras decrease its ability to interact with RasGAP and thus its ability to undergo GTP hydrolysis (Bos, 1997).

Finally, our analysis reveals striking differences in the regulation of signaling and metabolic systems. Signaling cascades can show amplification; indeed, amplification is a key feature of most signal transduction systems. In contrast, metabolic systems typically exhibit dampening along the pathway. In addition, all components of a signaling system determine the amplitude of signal output, whereas in a metabolic pathway control by individual enzymes is generally small (Heinrich and Rapoport, 1974; Kahn and Westerhoff, 1991; Mazat et al., 1996). Because signal transduction pathways are more sensitive to the properties of their components, they require a special design that permits both stability of the ground state and high signal output upon stimulation.

Appendix
Calculation of Signaling Time, Signal Duration, and Amplitudes for a Weakly Activated Pathway
The quantities may be calculated without using the explicit solutions $X_i(t)$ of the differential equation system (7). Taking into account that the signaling pathway is in

an off-state at the beginning of the process ($t = 0$) as well as after decline of the receptor activity ($t \rightarrow \infty$), one derives from Equation 7 for the integrated response of all kinases the following recurrent formula:

$$I_i = \frac{\alpha_i}{\beta_i} I_{i-1}. \tag{31}$$

Note that $R(t) = R \exp(-\lambda t)$ implies $I_0 = R/\lambda$. Then, Equation 31 yields the following expression for the integrated response for any kinase

$$I_i = \frac{R}{\lambda} \prod_{j=1}^{i} \frac{\alpha_j}{\beta_j}. \tag{32}$$

Multiplication of Equation 7 by t and subsequent integration yields

$$I_i = -\alpha_i T_{i-1} + \beta_i T_i \tag{33}$$

where the left sides of the equations result from integration by parts. Dividing Equation 33 by I_i gives

$$1 = -\alpha_i \frac{T_{i-1}}{I_{i-1}} \frac{I_{i-1}}{I_i} + \beta_i \frac{T_i}{I_i}, \tag{34}$$

which simplifies with the definitions of τ and Equation 31 to

$$1 = -\alpha_i \tau_{i-1} \frac{\beta_i}{\alpha_i} + \beta_i \tau_i. \tag{35}$$

Obviously, the rate constants α_i cancel out, resulting in a recurrent formula for τ that only depends on the constants β_i:

$$\tau_i = \tau_{i-1} + \frac{1}{\beta_i}. \tag{36}$$

Since $\tau_0 = 1/\lambda$, this gives immediately expression (8) for the signaling time.

The derivation of the formula for the signal duration is done in a similar way. Multiplication of Equation 7 by t^2 and subsequent integration yields

$$2T_i = -\alpha_i Q_{i-1} + \beta_i Q_i. \tag{37}$$

Again, the left hand sides of these equations result from integration by parts. Combination of Equations 31–37 yields the recurrent formula

$$\vartheta_i^2 = \vartheta_{i-1}^2 + \frac{1}{\beta_i^2}. \tag{38}$$

With $\vartheta_0^2 = 1/\lambda^2$ repeated application of Equation 38 gives expression (9) for the signal duration.

By using definition (6), an explicit expression for the amplitude S_i follows directly from the expressions for the integrated response I_i and for the signal duration ϑ_i.

Although not mentioned in the text, the present treatment allows for the calculation of ATP consumption during signaling. Assuming that each kinase step is coupled to consumption of one molecule of ATP, one derives from Equation 2

$$\Delta ATP = \sum_{i=1}^{n} \alpha_i \int_0^\infty X_{i-1}\left(1 - \frac{X_i}{C_i}\right) dt. \tag{39}$$

In the case of a low level activation, integration can be easily performed, and one obtains

$$\Delta ATP = \sum_{i=1}^{n} \alpha_i I_{i-1} \tag{40}$$

i.e., ATP consumption is directly related to the integrated response (Equation 4) of all kinases.

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