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Article

The Robustness of Proofreading to Crowding-Induced Pseudo-Processivity in the MAPK Pathway

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ABSTRACT Double phosphorylation of protein kinases is a common feature of signaling cascades. This motif may reduce cross-talk between signaling pathways because the second phosphorylation site allows for proofreading, especially when phosphorylation is distributive rather than processive. Recent studies suggest that phosphorylation can be pseudo-processive in the crowded cellular environment, since rebinding after the first phosphorylation is enhanced by slow diffusion. Here, we use a simple model with unsaturated reactants to show that specificity for one substrate over another drops as rebinding increases and pseudo-processive behavior becomes possible. However, this loss of specificity with increased rebinding is typically also observed if two distinct enzyme species are required for phosphorylation, i.e., when the system is necessarily distributive. Thus the loss of specificity is due to an intrinsic reduction in selectivity with increased rebinding, which benefits inefficient reactions, rather than pseudo-processivity itself. We also show that proofreading can remain effective when the intended signaling pathway exhibits high levels of rebinding-induced pseudo-processivity, unlike other proposed advantages of the dual phosphorylation motif.

INTRODUCTION

Cells must sense and respond to their environment, and external signals must be transmitted from cell-surface receptors to the interior. Eukaryotic signal transmission often involves phosphorylation cascades of mitogen-activated protein kinases (MAPKs) (1–3). Phosphorylation, the addition of a phosphate group to a residue (typically serine, threonine, or tyrosine), is a common posttranscriptional protein modification. Kinases catalyze phosphorylation, and a kinase cascade involves the successive phosphorylation of downstream kinases by upstream counterparts, with each kinase becoming enzymatically active after phosphorylation. Phosphatases catalyze the release of inorganic phosphate and enzymatic deactivation (4–7). The result is a characteristic push-pull motif in which competition between phosphatases and upstream kinases sets the activation level of a downstream kinase, the first kinase having been activated directly or indirectly by the receptor.

MAPKs typically require phosphorylation at two residues for activation (4–8). Each stage necessitates the breakdown of an ATP molecule, the cell's fuel source. The need for two phosphorylation events is thus potentially costly and time-consuming, and it is reasonable to assume that such a motif would only survive by conferring a biological advantage. Several possible uses of dual phosphorylation have been proposed:

1. Kinases that require double phosphorylation can respond more sensitively, i.e., ultrasensitively, to changes in phosphatase and upstream kinase concentrations (4). When the upstream enzymes are saturated, it is even possible to achieve bistability (9,10).
2. Dual phosphorylation allows for more discrimination between substrates (6). All signaling pathways will experience some degree of cross-reactivity, and the need to perform two phosphorylations rather than one allows for an extra stage of discrimination (or proofreading).
3. Some kinase cascades involve scaffolding proteins that bind to upstream and downstream kinases simultaneously (11,12). Such a motif could enhance signaling and improve insulation of pathways (11,12). It has been claimed (7) that this enhancement is only effective when coupled with dual phosphorylation, since the scaffold allows for a single upstream kinase to perform both modifications, rather than requiring two separate interactions in the cytosol.

The effectiveness of these motifs depends on whether phosphorylation in the cytosol is naturally processive (a single enzymatic molecule can perform both phosphorylations during one interaction) or distributive (two separate interactions are required). The third motif mentioned above obviously requires phosphorylation to be naturally distributive in the cytosol. A reduction in ultrasensitivity with processivity has also been demonstrated elsewhere (13,14). In their original article on proofreading, Swain and Siggia (6) considered partially processive kinase operation, in which a certain fraction of phosphorylation events leads directly to the doubly phosphorylated state and the rest cause single

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phosphorylation via a discard-pathway. In the limit that they considered, Swain and Siggia (6) showed that specificity is compromised by increasing processivity, and that this decrease is due to a drop in the discrimination at the second stage of phosphorylation.

Reactants that physically separate after phosphorylation may nonetheless show pseudo-processive behavior due to finite rates of diffusion (14,15), as shown in Fig. 1. If diffusion is slow enough compared to the intrinsic binding rate, two protein molecules can rebind after the first phosphorylation, allowing effectively processive phosphorylation if the kinase can also catalyze the second step. Recent experiments (8,16) and theory (16,17) suggest that molecular crowding (which slows diffusion relative to intrinsic reaction rates) can cause pseudo-processivity in conditions similar to those found in the cell. Rebinding due to slow diffusion is also relevant in a wide range of biophysical systems; examples include T-cell fate decisions (18), signaling involving membrane-bound clusters (19), the accuracy with which surface receptors can sense ligand concentrations (20), and the dynamics with which transcription factors search DNA for their binding sites (21).

Given these insights, characterizing the robustness of dual phosphorylation-based motifs to rebinding-driven processivity is essential. We study a simple model of pseudo-processivity in the limit of unsaturated reactants. We analyze the consequences of rebinding and pseudo-processivity for the selective phosphorylation of one substrate over another. Our results are consistent with the simpler model of Swain and Siggia (6) for parameters that allow comparison, but our approach reveals key features that arise when rebinding drives pseudo-processivity. High binding probabilities when in close proximity rather than pseudo-processivity per se are generally responsible for low specificity, and specificity is lost at both stages of phosphorylation. Further, the relative increase in discrimination from adding a second phosphorylation site can remain appreciable with significant pseudo-processivity. Finally, we argue that pseudo-processivity does not limit proofreading as it does other uses of dual phosphorylation, which can also be understood through the same simple model.

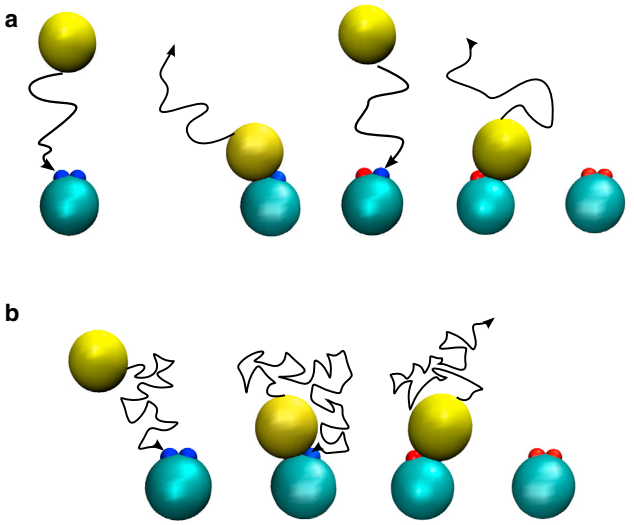

FIGURE 1 Diffusion-induced pseudo-processivity. (*a*) Conventional distributive phosphorylation of two residues by two distinct kinase molecules. (*b*) When diffusion is slow compared to intrinsic reaction rates, the same kinase molecule can rebind and modify the second site, resulting in a pseudo-processive scheme. To see this figure in color, go online.

MODEL AND METHODS

Our model of diffusion and catalysis is based on that of Dushek et al. (22). We model the system at the level of molecular concentrations. Upstream kinases can bind to and unbind from substrates, with catalysis and rapid release possible when bound. Substrates can also be dephosphorylated by a phosphatase. Importantly, the model includes states representing configurations in which two proteins are in close proximity, but unbound (22). These states permit rapid rebinding of molecules, since reactants remain in close proximity for some time after separating. Rebinding either occurs rapidly or the reactants diffuse apart and all memory is lost—such a picture is consistent with theoretical analyses of rebinding in dilute solution (20,23). Transitions between states are quantified by rate constants.

The primary system studied in this article is illustrated in Fig. 2, which also defines rate constants. Here, the substrate A exists in unphosphorylated (A), singly phosphorylated (A_p), and doubly phosphorylated (A_{pp}) states, and a single kinase K and single phosphatase P can catalyze reactions for both phosphorylation sites. We use $\bigcirc$ to indicate close proximity. This system allows for pseudo-processivity, since rebinding and a second catalysis event can occur immediately after the first. In the language of Swain and Siggia (6), reactants that diffuse apart after the first phosphorylation follow a discard-pathway. We will later introduce a substrate B with different underlying rate constants, and consider the specificity with which A is activated over B. We will also apply the model to alternative systems in which substrates have only one phosphorylation site, or enzymes can only act on one phosphorylation site.

The close proximity state is assumed to be equally close to both phosphorylation sites, so proteins have no memory of previous binding in that state. This is reasonable if the phosphorylation sites are close to each other, as is typical (24–26), and pseudo-processivity is due to reattachment following failure to escape the local environment. A second assumption is that our model has only one singly phosphorylated state, rather than explicitly considering phosphorylation on either residue. Technically, this assumes an ordered, or sequential, phosphorylation of the sites. This simplification is common in the literature (4,6,14,27). To check that our results are not overly sensitive to this assumption, we consider independent phosphorylation sites in Section S11 in the Supporting Material.

For simplicity, we assume that reactants are unsaturated; i.e., most molecules of each species are not in complexes at any time. States such as KA and $K \bigcirc A$ must therefore be short-lived compared to the time taken for a given reactant to come into close proximity with a reactant partner. For the first stage of phosphorylation, this limit is obtained when

$$\frac{1}{k_D[A_0]}, \frac{1}{k_D[K]} \gg \frac{k_d + k_{\mathrm{cat}} + k_a}{k_{\mathrm{esc}}k_d + k_{\mathrm{esc}}k_{\mathrm{cat}} + k_a k_{\mathrm{cat}}}, \qquad (1)$$

in which $[A_0]$ is the total concentration of substrate A. Similar inequalities must hold for all reactions. The right-hand side of Eq. 1 is the average time taken for either escape or catalysis to occur once the reactants are in close

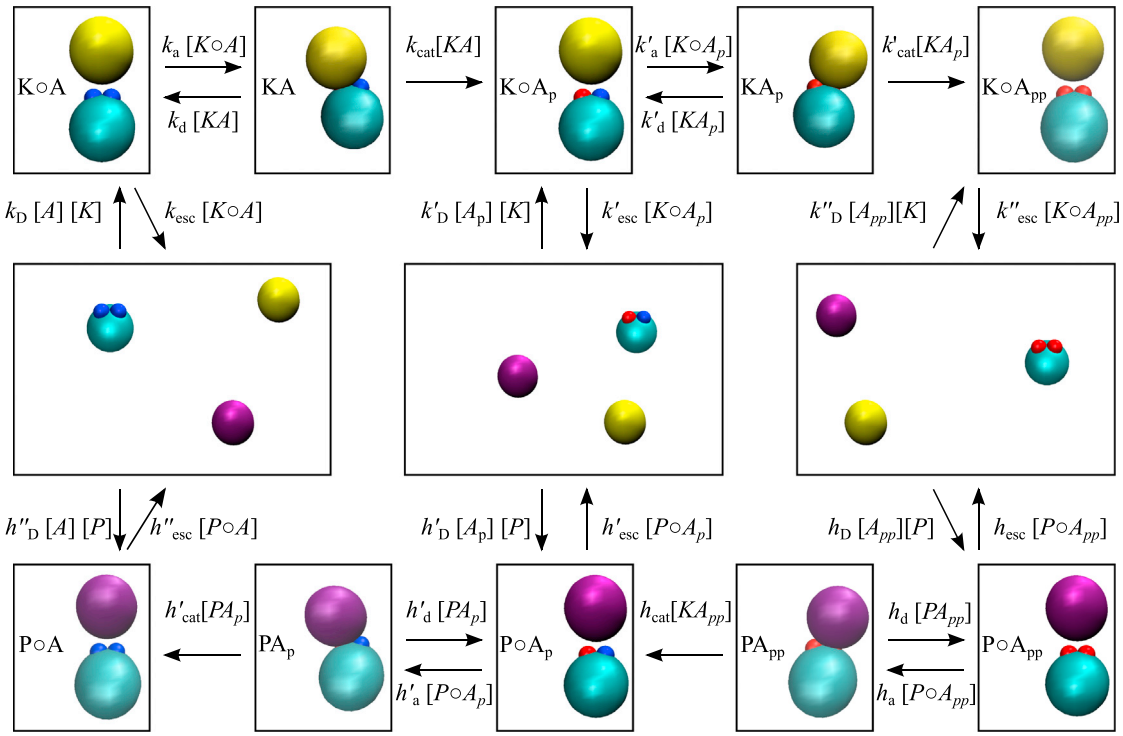

FIGURE 2 A simple model for pseudo-processive phosphorylation. A is phosphorylated in two stages by a kinase (*yellow*). First, K and A diffuse into close proximity, a state labeled by $K \bigcirc A$. The two can then bind (KA), at which point phosphorylation and release can occur, leaving the kinase and substrate in close proximity but with the substrate singly phosphorylated $K \bigcirc A_p$. From here, the two can diffuse apart (escape), leaving an isolated A_p. Alternatively, the kinase can rebind and perform a second phosphorylation. The reverse process can be observed for the phosphatase (*purple*). Reaction arrows are labeled with rates per unit volume at which reactions occur.

proximity. It is derived in Section S1 in the Supporting Material, where we also show that the right-hand side of Eq. 1 is $\leq$ max ($1/k_{cat}$, $1/k_{esc}$). Thus, fast catalysis and escape compared to diffusive encounter is a sufficient (but not necessary) condition to ensure that our approximation holds. Note that the saturation of reactants, which we preclude, should not be confused with mechanisms by which the yield of product can become saturated. For example, the yield of A_{pp} can become saturated when $[A_{pp}] \approx [A_0]$.

In the unsaturated (low concentration) limit, and assuming fixed total concentrations, the model reduces to an effective first-order interconversion of substrates between phosphorylation states (Fig. 3). The rate constants defined in Fig. 2 determine the probabilities of various reaction outcomes; the key collective variables that emerge are the effective rate constants (e.g., k_{eff}), and f_α (f_β), which is the probability that phosphorylation of A (dephosphorylation of A_{pp}) leads to modification of both sites rather than just one. Large f_α and f_β values indicate substantial pseudo-processivity.

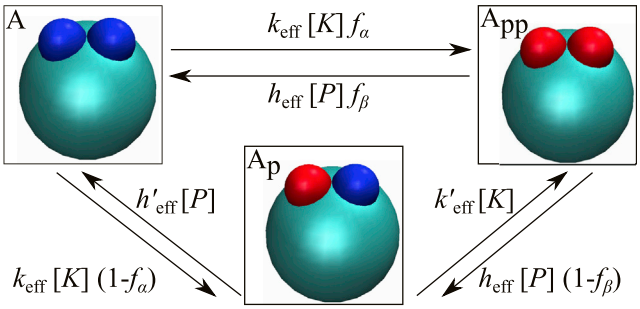

FIGURE 3 Effective first-order description of the conversion of A between its phosphorylation states that results from the assumption of unsaturated kinetics. Arrows are labeled with effective rate constants. To see this figure in color, go online.

The effective rate constants, f_α, and f_β, can be expressed via the probabilities that reactants bind given close proximity, and that catalysis occurs given binding. The relevant probabilities are

$$\begin{aligned}
P_{cat} &= \frac{k_{cat}}{k_{cat}+k_d}, \\
P'_{cat} &= \frac{k'_{cat}}{k'_{cat}+k'_d}, \\
P_{on} &= \frac{k_a}{k_{esc}+k_a}, \\
P'_{on} &= \frac{k'_a}{k'_a+k'_{esc}}, \\
Q_{cat} &= \frac{h_{cat}}{h_{cat}+h_d}, \\
Q'_{cat} &= \frac{h'_{cat}}{h'_{cat}+h'_d}, \\
Q_{on} &= \frac{h_a}{h_{esc}+h_a}, \\
Q'_{on} &= \frac{h'_a}{h'_a+h'_{esc}}.
\end{aligned} \tag{2}$$

Primed probabilities relate to the second stage of phosphorylation (or dephosphorylation), and unprimed probabilities to the first, as in Fig. 2.

The rate constant at which K phosphorylates A is given by the rate constant for K and A coming into close proximity multiplied by the probability that a successful reaction occurs after n binding events, summed over n:

$$k_{\mathrm{eff}} = k_D \sum_{n\geq 1} P_{\mathrm{on}}^n (1-P_{\mathrm{cat}})^{n-1} P_{\mathrm{cat}} = k_D P_{\mathrm{react}}.$$

P_{react} is the probability that phosphorylation of the first site occurs given that an A molecule is in close proximity to a kinase capable of catalyzing the $A \rightarrow A_p$ transition. This sum is a simple geometric progression,

$$k_{\mathrm{eff}} = k_D P_{\mathrm{react}} = k_D \frac{P_{\mathrm{cat}} P_{\mathrm{on}}}{1-P_{\mathrm{on}}(1-P_{\mathrm{cat}})}. \quad (3)$$

Similar quantities can be calculated for other reactions,

$$\begin{aligned} k'_{\mathrm{eff}} &= k'_D P'_{\mathrm{react}} = \frac{k'_D P'_{\mathrm{cat}} P'_{\mathrm{on}}}{1-P'_{\mathrm{on}}\left(1-P'_{\mathrm{cat}}\right)}, \\ h_{\mathrm{eff}} &= h_D Q_{\mathrm{react}} = \frac{h_D Q_{\mathrm{cat}} Q_{\mathrm{on}}}{1-Q_{\mathrm{on}}(1-Q_{\mathrm{cat}})}, \\ h'_{\mathrm{eff}} &= h_D Q'_{\mathrm{react}} = \frac{h'_D Q'_{\mathrm{cat}} Q'_{\mathrm{on}}}{1-Q'_{\mathrm{on}}\left(1-Q'_{\mathrm{cat}}\right)}. \end{aligned} \quad (4)$$

P'_{react} is the probability that the second site will be modified given that an appropriate kinase is close to an A_p molecule. A kinase that has just modified the first site will be in close proximity to the substrate A_p. If this kinase can also catalyze the phosphorylation of the next site, i.e., if both sites are modified by the same kinase species (as assumed hitherto), then the fraction of pseudo-processive modifications is $f_\alpha = P'_{\mathrm{react}}$. Similarly, $f_\beta = Q'_{\mathrm{react}}$. We will later consider a system in which two distinct kinases and phosphatases are needed, in which case this identification is inappropriate; we thus retain distinct variables.

Neglecting noise, Fig. 3 implies differential equations for the concentrations of $[A]$, $[A_p]$, and $[A_{pp}]$. The steady-state solution is simple since the equations are linear. The results are easiest to express in terms of the ratios $\theta = h_{\mathrm{eff}}/h'_{\mathrm{eff}}$, $\phi = k_{\mathrm{eff}}/k'_{\mathrm{eff}}$, and $\phi = k_{\mathrm{eff}}/h_{\mathrm{eff}}$, and $Y = [K]/[P]$. Low ϕ would imply that the second stage of phosphorylation is faster than the first. The value of θ has the same meaning for dephosphorylation, and ψ and Y values simply quantify the relative activity and concentrations of kinases and phosphatases. In terms of these variables,

$$\begin{aligned} \frac{[A]}{[A_0]} &= \frac{\phi + (Y\psi)\theta f_\beta}{\phi + (Y\psi)\left(\theta f_\beta + \phi f_\alpha + \theta\phi\left(1-f_\alpha f_\beta\right)\right) + (Y\psi)^2\theta}, \\ \frac{[A_p]}{[A_0]} &= \frac{(Y\psi)\theta\phi\left(1-f_\alpha f_\beta\right)}{\phi + (Y\psi)\left(\theta f_\beta + \phi f_\alpha + \theta\phi\left(1-f_\alpha f_\beta\right)\right) + (Y\psi)^2\theta}, \\ \frac{[A_{pp}]}{[A_0]} &= \frac{(Y\psi)\phi f_\alpha + (Y\psi)^2\theta}{\phi + (Y\psi)\left(\theta f_\beta + \phi f_\alpha + \theta\phi\left(1-f_\alpha f_\beta\right)\right) + (Y\psi)^2\theta}. \end{aligned} \quad (5)$$

Although the model is simple, it maps to the results of a previous analysis of rebinding based on continuum diffusion (15). When the standard diffusion equation is a good description of particle motion, and by neglecting behavior on short timescales (15), phosphorylation can be treated as a second-order reaction involving a diffusion-influenced rate constant with a finite probability that more than one phosphorylation event occurs during an encounter. In Section S2 in the Supporting Material, we show that our model is consistent with this result and reproduces the rates at which different products form. In this analogy, k_D is the diffusion-limited rate constant and $k_a P_{\mathrm{cat}} k_D/k_{\mathrm{esc}}$ is the rate constant in the limit of infinitely fast diffusion (the reaction-limited rate constant). To understand this assignation, note that k_D/k_{esc} quantifies the probability that enzyme and substrate are in close proximity, and $k_a P_{\mathrm{cat}}$ is a reaction rate given close proximity. We emphasize, however, that our model does not rest upon a particular description of diffusion. In the cell, crowding molecules mean that reactants do not diffuse as they would in a simple solution, tending to show subdiffusive behavior on short timescales. In Section S3 in the Supporting Material, we show that a lattice model also produces results that support our simple finite-state analysis. Dushek et al. (22) also verified that explicit lattice simulations reproduced results obtained with a similar model.

RESULTS

To explore specificity, we introduce a substrate B that is less efficiently phosphorylated by the kinase but obeys similar differential equations to A. Our model has many parameters; we wish to explore system behavior, but because the parameters are so varied, there are too many to allow us to do so exhaustively. We therefore assume that all diffusion rates are identical, and that encounter rates are described by a single k_D, and escape rates by a single k_{esc}. As in Swain and Siggia (6), we shall assume that differential catalytic activity is entirely due to variations in unbinding rates k_d. We consider alternatives in Section S10 in the Supporting Material. Finally, we shall assume that the phosphatases do not discriminate between substrates. The reduction in free parameters is summarized as follows:

$$\begin{aligned} &k_D^{A,B},\ k_D^{A\prime,B\prime},\ k_D^{A\prime\prime,B\prime\prime},\ h_D^{A,B},\ h_D^{A\prime,B\prime},\ h_D^{A\prime\prime,B\prime\prime} = k_D, \\ &k_{\mathrm{esc}}^{A,B},\ k_{\mathrm{esc}}^{A\prime,B\prime},\ k_{\mathrm{esc}}^{A\prime\prime,B\prime\prime},\ h_{\mathrm{esc}}^{A,B},\ h_{\mathrm{esc}}^{A\prime,B\prime},\ h_{\mathrm{esc}}^{A\prime\prime,B\prime\prime} = k_{\mathrm{esc}}, \\ &k_a^{A,B},\ k_a^{A\prime,B\prime},\ h_a^{A,B},\ h_a^{A\prime,B\prime} = k_a, \\ &k_{\mathrm{cat}}^{A,B},\ k_{\mathrm{cat}}^{A\prime,B\prime},\ h_{\mathrm{cat}}^{A,B},\ h_{\mathrm{cat}}^{A\prime,B\prime} = k_{\mathrm{cat}}, \\ &h_d^{A,B} = h_d, \text{ and } h_d^{A\prime,B\prime} = h'_d. \end{aligned} \quad (6)$$

As a result of this simplification,

$$P_{\mathrm{on}}^{A,B} = P_{\mathrm{on}}^{A'B'} = Q_{\mathrm{on}}^{A,B} = Q_{\mathrm{on}}^{A'B'} = P_{\mathrm{on}}.$$

Here, P_{on} is the probability of binding given close proximity, and hence the probability of rebinding after dissociation. The terms "P_{on}" and "frequent rebinding" are used synonymously in this work.

A and B then differ only in their binding free energies with K:

$$\Delta\Delta G = kT\ln\left(k_d^A/k_d^B\right),$$

$$\Delta\Delta G' = kT\ln\left(k_d^{A\prime}/k_d^{B\prime}\right).$$

The maximum possible discrimination factor is $\exp(-(\Delta\Delta G + \Delta\Delta G')/kT)$. However, this discrimination is not necessarily manifested. We can define kinetic selectivity factors S and S',

$$S = \frac{k^A_{\text{eff}}}{k^B_{\text{eff}}} = \left(\frac{P^A_{\text{cat}}}{P^B_{\text{cat}}}\right)\left(\frac{1-P_{\text{on}}\left(1-P^B_{\text{cat}}\right)}{1-P_{\text{on}}\left(1-P^A_{\text{cat}}\right)}\right),$$
$$S' = \frac{k^{A\prime}_{\text{eff}}}{k^{B\prime}_{\text{eff}}} = \left(\frac{P^{A\prime}_{\text{cat}}}{P^{B\prime}_{\text{cat}}}\right)\left(\frac{1-P_{\text{on}}\left(1-P^{B\prime}_{\text{cat}}\right)}{1-P_{\text{on}}\left(1-P^{A\prime}_{\text{cat}}\right)}\right). \quad (7)$$

The value S is the ratio (see Eq. 3) of rates for going from $K \bigcirc A \rightarrow K \bigcirc A_p$ and $K \bigcirc B \rightarrow K \bigcirc B_p$ (regardless of whether another phosphorylation occurs immediately). S' is the equivalent for the second step, and $S, S' \geq 1$ as A is the intended substrate. We have $S \leq \exp(-\Delta\Delta G/kT)$; selectivity is reduced when P_{on} and P^A_{cat} are large. Note

$$S = S_0\left(1-P^A_{\text{react}}\right) + P^A_{\text{react}},$$
$$S' = S'_0\left(1-P^{A\prime}_{\text{react}}\right) + P^{A\prime}_{\text{react}}. \quad (8)$$

Here, $S_0 = P^A_{\text{cat}}/P^B_{\text{cat}}$ and $S'_0 = P^{A\prime}_{\text{cat}}/P^{B\prime}_{\text{cat}}$ are the selectivities in the limit of no rebinding. We also define a metric for the overall specificity of

$$X = \lg\left(\left[A_{pp}\right]/[A_0]\right) - \lg\left(\left[B_{pp}\right]/[B_0]\right)$$

(here lg stands for $\log_{10}$). Using Eq. 5, and

$$f^B_\alpha = f^A_\alpha/S',\ f^B_\beta = f^A_\beta,\ \theta^B = \theta^A,\ \phi^B = (S/S')\phi^A$$

and

$$\psi^B = \psi^A/S,$$

then

$$X = \lg(SS') + \lg\left(\frac{\phi^A + \frac{Y\psi^A}{S'}\left(\frac{f^A_\alpha\phi^A}{S} + f^A_\beta\theta^A + \frac{S' - f^A_\alpha f^A_\beta}{S}\theta^A\phi^A\right) + \frac{\left(Y\psi^A\right)^2}{SS'}\theta^A}{\phi^A + Y\psi^A\left(f^A_\alpha\phi^A + f^A_\beta\theta^A + \left(1 - f^A_\alpha f^A_\beta\right)\theta^A\phi^A\right) + \left(Y\psi^A\right)^2\theta^A}\right). \quad (9)$$

The two terms in X describe separate contributions. The first term represents the difference in effective phosphorylation rates of A and B (see Section S7 in the Supporting Material). The second determines whether that difference in rates is manifest in the overall yield of A_{pp} and B_{pp}.

The low kinase activity limit

To understand Eq. 9, we first consider the limit in which phosphatases dominate over kinases ($Y\psi^A = [K]k^A_{\text{eff}}/[P]h^A_{\text{eff}} \rightarrow 0$). The second term of Eq. 9 then tends to zero; using Eq. 8, the first term is

$$X = \lg\left(S_0\left(1-P^A_{\text{react}}\right) + P^A_{\text{react}}\right) + \lg\left(S'_0\left(1-P^{A\prime}_{\text{react}}\right) + P^{A\prime}_{\text{react}}\right). \quad (10)$$

In the previous section, we argued that $f^A_\alpha = P^{A\prime}_{\text{react}}$ when a single kinase catalyzes both phosphorylation steps. Thus specificity drops, inasmuch as $f^A_\alpha \rightarrow 1$; one might naïvely say that proofreading is compromised by pseudo-processivity (although it is independent of f^A_β). This argument, however, is misleading in two ways, as follows.

Low specificity is correlated with pseudo-processivity, but not caused by it (increased pseudo-processivity does not lead mechanistically to a decrease in specificity)

Frequent rebinding (due to high P_{on}) is itself responsible for the low specificity. To understand the distinction, note that rebinding only causes pseudo-processivity if a kinase is physically capable of catalyzing phosphorylation at both sites, as we have assumed hitherto. Instead, we could consider a system with two chemically distinct kinase species (of equal concentration) and two chemically distinct phosphatase species (of equal concentration) that each can only interact with one of the two residues in question. Here, pseudo-processivity is impossible; $A \rightarrow A_{pp}$ requires the action of two distinct kinases. The new system is still governed by the differential equations implied by Fig. 3, but primed rate constants (and underlying reaction probabilities) now refer to the action of the second enzyme, and $f_\alpha = f_\beta = 0$ in this necessarily distributive system. Equation 5, with $f^A_\alpha = f^A_\beta = 0$, solves this system. Equation 10 still holds, but now $P^{A\prime}_{\text{react}} \neq f^A_\alpha = 0$, as $P^{A\prime}_{\text{react}}$ is a property of the second kinase and f^A_α is a property of the first. If the parameters are otherwise identical to the original single-kinase, single-phosphatase system, $P^{A\prime}_{\text{react}}$ and X are unchanged (in the low yield limit considered here), despite the fact that now $f^A_\alpha = f^A_\beta = 0$.

Thus, pseudo-processivity itself is not required for the drop in specificity. Why, then, does X drop as $P^{A\prime}_{\text{react}} \rightarrow 1$? $P^{A\prime}_{\text{react}}$ is the likelihood of a successful reaction given proximal K and A_p. For $P^{A\prime}_{\text{react}} \rightarrow 1$, we require $P_{\text{on}} \rightarrow 1$; Eq. 4 shows that $P^{A\prime}_{\text{react}} < P_{\text{on}}$ and $P^{A\prime}_{\text{react}} \rightarrow 1$ as $P_{\text{on}} \rightarrow 1$. We note that $P'_{\text{cat}} \rightarrow 1$ is neither sufficient nor necessary; even with $P'_{\text{cat}} = 1$, catalysis is largely distributive if rebinding is rare, and even inefficient catalysis can be pseudo-processive at high P_{on}. When $P_{\text{on}} \rightarrow 1$, there can be many rounds of dissociation and rebinding before modification occurs, favoring inferior substrates that are less likely to be catalyzed the first time. Mathematically

(Eq. 4), we see that when $P'_{\text{react}} \to 1$, the dependence on the factor which distinguishes A and B, P'_{cat}, is lost. In this low kinase activity limit, therefore, frequent rebinding (due to high P_{on}) reduces specificity and can also coincidentally cause pseudo-processive behavior if an enzyme can modify both sites.

The efficacy of proofreading is not X, but the increase in X due to the second site

Equation 10 shows that the contribution from the first site is just as vulnerable to P_{on}-driven increases in P^A_{react} as the contribution from the second site is to increases in $P^{A\prime}_{\text{react}}$. A single-site substrate with the same properties as the first site of the two-site system has specificity

$$X_{\text{ss}} = \lg(S) + \lg\left(\frac{1 + Y\psi^A\theta^A/S}{1 + Y\psi^A\theta^A}\right), \tag{11}$$

in which $\psi^A\theta^A = k^A_{\text{eff}}/h^{A\prime}_{\text{eff}}$ is the ratio of effective rate constants for phosphorylation and dephosphorylation. In the limit of low kinase activity, $Y\psi^A > 0$,

$$X_{\text{ss}} \to \lg(S) = \lg\left(S_0\left(1 - P^A_{\text{react}}\right) + P^A_{\text{react}}\right),$$

and the additional specificity due to the second site is

$$X - X_{\text{ss}} \to \lg\left(S'_0\left(1 - P^{A\prime}_{\text{react}}\right) + P^{A\prime}_{\text{react}}\right).$$

Clearly, the contribution of the first site is compromised by $P^A_{\text{react}} \to 1$ in the same way as the contribution of the second site is by $P^{A\prime}_{\text{react}} = f^A_\alpha \to 1$. It too suffers a loss of selectivity due to rebinding; Eqs. 3 and 4 show that P^A_{react} and $P^{A\prime}_{\text{react}}$ have equivalent dependencies on P_{on}. Thus, the contribution of the second site does not systematically fall off faster than the first as rebinding becomes more common (the site with larger P^A_{cat} is more sensitive).

We note that $X - X_{\text{ss}}$ can remain substantial even when pseudo-processivity is high ($f^A_\alpha \geq 1/2$). For example, if $S'_0 = 10$ (the intrinsic selectivity without rebinding is a factor of 10), $X - X_{\text{ss}}$ drops from 1 in the limit $f^A_\alpha \to 0$, to 0.70 at $f^A_\alpha = 1/2$, and only drops to 0.50 when $f^A_\alpha = 0.760$ (at which point the specificity is halved in the logarithmic sense; $[A_{pp}]$ and $[B_{pp}]$ are distinguished by a factor of $\sqrt{10}$ rather than 10). For lower values of S'_0, this halving occurs at lower f^A_α, but for higher values it occurs even later. Robustness of specificity is therefore clearly dependent on the intrinsic specificity at low P_{on}, but, importantly, pseudo-processive reactions do not necessarily preclude proofreading.

We now compare our results to the original work of Swain and Siggia (6). The main results (Eqs. 4 and 6 of their article) look quite different, because they considered a distinct limit. They also considered a system with weak kinase activity, but treated the two stages of phosphorylation asymmetrically. They assumed that the success rate of phosphorylation once the kinase and substrate are bound is low for the first stage (the reaction is close to equilibrium), but potentially not for the second stage. This assumption was made because the authors reasoned that it would be optimal in allowing the full selectivity from the first stage to be manifested, while permitting possible processive behavior. Thus, when Swain and Siggia (6) allowed processive phosphorylation, they observed that the selectivity arising from the second stage was compromised whereas that arising from the first was not. In our case, however, processivity arises from rebinding events that increase the probability of successful phosphorylation for both stages, compromising both S and S' and incidentally leading to pseudo-processivity. This symmetry does not arise naturally unless rebinding is explicitly modeled as the cause of pseudo-processivity.

Swain and Siggia (6) state that proofreading is optimized at low processivity (f^A_α small). While we do not contradict this result, we find that proofreading is more robust than this statement suggests. Specificity can be relatively high even when the majority of phosphorylations are pseudo-processive (in the low kinase activity limit, processivity of dephosphorylation reactions is irrelevant). Proofreading discriminates between two substrates, A and B; even when phosphorylation of A is moderately pseudo-processive, B can still be less efficiently phosphorylated. Furthermore, the second stage of phosphorylation is not more strongly affected than the first. This conclusion is the central result of this work. In what follows, we explore the consequences of finite kinase activity for this result, and then compare to other proposed uses of the dual phosphorylation motif.

Finite kinase activity for distributive systems

For finite kinase activity ($Y\psi^A > 0$), the second term in Eq. 9 is nonzero. We first consider the distributive limit of $f^A_\alpha = f^A_\beta = 0$, which is obtained if $P_{\text{on}} \to 0$ or by considering a system with two distinct kinases and two distinct phosphatases. In this case,

$$X_{\text{dis}} = \lg(SS') + \lg\left(\frac{\phi^A + \left(Y\psi^A\right)\theta^A\phi^A/S + \left(Y\psi^A\right)^2\theta^A/SS'}{\phi^A + \left(Y\psi^A\right)\theta^A\phi^A + \left(Y\psi^A\right)^2\theta^A}\right). \tag{12}$$

The second term in Eq. 12 is always negative. It captures the fact that finite concentrations of A_p and A_{pp} tend to suppress specificity, since the phosphorylation transitions $A_p \to A_p$ and $A_p \to A_{pp}$ become saturated for A but not for B. If $[A_{pp}] \approx [A_0]$, then a substantial change in

$Y = [K]/[P]$ can hardly change $[A_{pp}]$, whereas the smaller $[B_{pp}]$ will still grow toward $[B_0]$, reducing the difference in yields. Similarly, if $[A_p] > [A]$, then increasing Y can do little to convert more A into A_p, whereas it will have a larger effect on the $B \rightarrow B_p$ transition: large $[A_p]$ thus reduces the difference between substrates due to the first phosphorylation stage.

Specificity can therefore be compromised by high yields of phosphorylated products. But the efficacy of proofreading is perhaps best represented by $X_{\rm dis} - X_{\rm ss}$. As is clear from Eq. 11, high kinase activity in a single-site system also suppresses specificity. To make a fair comparison, we therefore consider the two-site and single-site systems at the same yield of product g ($g = [A_p]/[A_0]$ for the single-site system, $g = [A_{pp}]/[A_0]$ for the two-site system) rather than at the same $Y = [K]/[P]$, as the yield of activated product is after all the output of the system. Below, we express specificity in terms of the overall yield g and parameters that depend only on the microscopic rate constants (eliminating $[K]$ and $[P]$):

$$\phi^A = k^A_{\rm eff}/k^{A\prime}_{\rm eff},\ \theta^A = h^A_{\rm eff}/h^{A\prime}_{\rm eff},\ S, \text{ and } S',$$

$$X_{\rm ss} = \lg(S) + \lg\left((1-g) + \frac{g}{s}\right),$$

$$X_{\rm dis} = \lg(SS') + \lg\left((1-g)\left(\frac{1 + Y_d\psi^A\theta^A/S}{1 + Y_d\psi^A\theta^A}\right) + \frac{g}{SS'}\right),$$

$$Y_d\psi^A\theta^A = \frac{g\theta^A\phi^A + \sqrt{\left(g\theta^A\phi^A\right)^2 + 4g(1-g)\theta^A\phi^A}}{2(1-g)}. \quad (13)$$

As $g \rightarrow 1$, the value of S required to achieve a given specificity $X_{\rm ss}$ rises. When g is large, $S = 1/(1-g)$ is required to give $X_{\rm ss} \approx \lg 2$. This implies $S \geq 1/(1-g)$ is needed to discriminate between substrates by a factor of 2 at high yield, quantifying the magnitude of S required to distinguish substrates at a given g.

Whether the second site's specificity $X_{\rm dis} - X_{\rm ss}$ is more strongly affected by g than $X_{\rm ss}$ depends on

$$Y_d\psi^A\theta^A = [K]k^A_{\rm eff}/[P]h^{A\prime}_{\rm eff},$$

which is $[A_p]/[A]$ in the two-site system (Eq. 5). If it is negligible, then $X_{\rm dis} - X_{\rm ss} > X_{\rm ss}$ for equal intrinsic selectivities $S = S'$ (see Section S4 in the Supporting Material). Indeed, if $S \geq 1/(1-g)$, $X_{\rm dis} - X_{\rm ss} > \lg S' - \lg 2$ (see Section S4 in the Supporting Material), so the specificity of the second site is weakly affected by g when $[A_p]/[A]$ is small. However, if $Y_d\phi^A\theta^A = [A_p]/[A] >> 1$, and g is not close to unity, $X_{\rm dis} - X_{\rm ss} \approx \lg(S'/S)$. This is disastrous—adding the second site eliminates the specificity from the first. When $[A_p]/[A] >> 1$, the fully unphosphorylated states are almost unoccupied, so we essentially have a single-site system based on the second stage $[A_p] \rightarrow [A_{pp}]$. Equation 13 shows that, at fixed yield g, $\theta^A\phi^A = k^A_{\rm eff}h^A_{\rm eff}/k^{A\prime}_{\rm eff}h^{A\prime}_{\rm eff}$ determines $Y_d\psi^A\theta^A$. Lower $\theta^A\phi^A$ is advantageous, since A_p is rapidly converted into either A_{pp} or A, keeping its concentration low. For $\theta^A\phi^A = 1$, $X_{\rm dis} - X_{\rm ss}$ is compromised marginally more by g than the $X_{\rm ss}$ (see Section S4 in the Supporting Material). In Fig. 4, we show how $X_{\rm dis} - X_{\rm ss}$ falls off with g for some representative values of $\theta^A\phi^A$, in comparison to $X_{\rm ss}$, illustrating this dependency of $X_{\rm dis} - X_{\rm ss}$ on $\theta^A\phi^A$.

Overall, finite kinase activity in distributive systems reduces specificity, and the second site's contribution can be more vulnerable to high product yields than that of the first site. In the next section, we will consider pseudo-processivity. As a preliminary, we study the effects of $P_{\rm on}$ without pseudo-processivity by considering a system with two distinct kinases and two distinct phosphatases. From the previous section, increasing $P_{\rm on}$ tends to reduce S and S' and hence specificity; here we instead examine the effect of finite $P_{\rm on}$ on the sensitivity of one- and two-site systems to finite g. Equation 13 shows that whether the two-site system suffers more from finite yield as $P_{\rm on}$ increases depends on whether $\theta^A\phi^A = k^A_{\rm eff}h^A_{\rm eff}/k^{A\prime}_{\rm eff}h^{A\prime}_{\rm eff}$ grows or shrinks with $P_{\rm on}$.

High values of $P_{\rm on}$ tend to make all reactions equally fast by allowing multiple attempts for intrinsically inefficient reactions (Eqs. 3 and 4). Consequently, $\theta^A\phi^A \rightarrow 1$ as $P_{\rm on} \rightarrow 1$. Rebinding thus makes systems that are intrinsically robust to finite g with low $\theta^A\phi^A$ as $P_{\rm on} \rightarrow 0$ becomes less so, but makes systems that are intrinsically vulnerable to finite g with high $\theta^A\phi^A$ as $P_{\rm on} \rightarrow 0$ becomes more robust. Rebinding makes it more challenging to

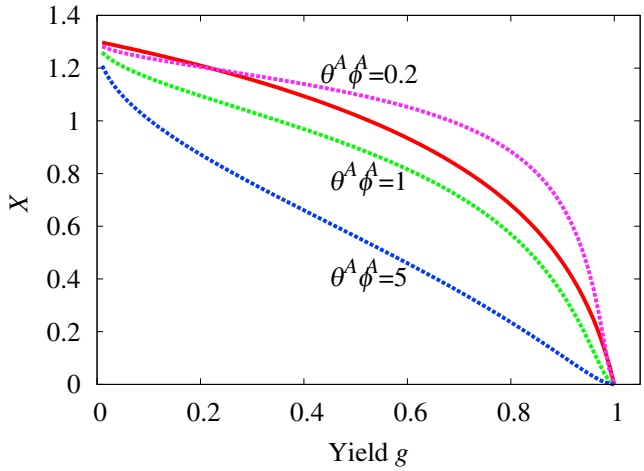

FIGURE 4 Drop in specificity of a single-site system ($X_{\rm ss}$, *solid line*) and the additional specificity of the second site in a distributive system ($X_{\rm dis} - X_{\rm ss}$, *dotted lines*) with yield g. We use intrinsic selectivities $S,S' = 20$. For the distributive system, we plot several values of $\theta^A\phi^A = k^A_{\rm eff}h^A_{\rm eff}/k^{A\prime}_{\rm eff}h^{A\prime}_{\rm eff}$ (defined in Fig. 2). $X_{\rm dis} - X_{\rm ss}$ is more robust to high yields when $\theta^A\phi^A$ is small. To see this figure in color, go online.

evolve a system with low $\theta^A\phi^A$ (and therefore a low concentration of A_p), and the consequences of rebinding for specificity can be substantial if the intrinsic ($P_{on} \rightarrow 0$) value of $\theta^A\phi^A$ is very different from unity. Nonetheless, proofreading can remain effective for systems with $\theta^A\phi^A \approx 1$ at high yields, as discussed in Section S4 in the Supporting Material, provided the selectivity is not as small as $S' \sim 1/(1-g)$.

Finite kinase activity for pseudo-processive systems

We now consider finite kinase activity for systems with the potential for pseudo-processivity. X_{proc}, the specificity with $f_\alpha^A, f_\beta^A \neq 0$, can be written in terms of g and parameters that depend only on the rate constants $\phi^A = k_{eff}^A/k_{eff}^{A\prime}$, $\theta^A = h_{eff}^A/h_{eff}^{A\prime}$, S, S', f_α^A, and f_β^A,

$$X_{proc} = \lg(SS') + \lg\left((1-g)\left(\frac{1+\frac{Y_p\psi^A\theta^A}{S}\left(1+\frac{f_\beta^A S}{\phi^A S'}-\frac{f_\alpha^A f_\beta^A}{S'}\right)}{1+Y_p\psi^A\theta^A\left(1+\frac{f_\beta^A}{\phi^A}-f_\alpha^A f_\beta^A\right)}\right)+\frac{g}{SS'}\right),$$

$$Y_p\psi^A\theta^A = \frac{g\theta^A f_\beta^A + g\theta^A\phi^A\left(1-f_\alpha^A f_\beta^A\right)-(1-g)\phi^A f_\alpha^A}{2(1-g)} + \frac{\sqrt{\left(g\theta^A f_\beta^A + g\theta^A\phi^A\left(1-f_\alpha^A f_\beta^A\right)-(1-g)\phi^A f_\alpha^A\right)^2 + 4g(1-g)\theta^A\phi^A}}{2(1-g).} \quad (14)$$

The expression reduces to X_{dis} if $f_\alpha^A = f_\beta^A = 0$, so we need only study the consequences of f_α^A $f_\beta^A > 0$. Here, $\partial X_{proc}/\partial f_\alpha^A \geq 0$ (with g, S, S', θ^A, ϕ^A, and f_β^A fixed; see Section S5 in the Supporting Material). Thus, finite f_α^A reduces the effect of finite yield g; it is always better to have a single (potentially pseudo-processive) kinase than two distinct kinases (implying $f_\alpha^A = 0$) with otherwise identical parameters. This is because converting A directly to A_{pp} helps to avoid the buildup of A_p, which was seen to reduce X_{dis} in the previous section. Note that $\partial X_{proc}/\partial f_\alpha^A \geq 0$ does not imply that higher P_{on}, which will cause increased f_α^A, is always beneficial provided $f_\beta^A = 0$; increased rebinding will also tend to reduce S and S', and will influence θ^A and ϕ^A.

The value f_β^A, the degree of pseudo-processivity in dephosphorylation, is more ambiguous. When it appears in $-f_\alpha^A$ f_β^A terms, it too reduces the buildup of A_p. When it appears separately from f_α^A, however, it reduces X_{proc}. The physical explanation, discussed in detail in Section S6 in the Supporting Material, is subtle. Here, we simply note that pseudo-processivity in the dephosphorylation pathway, rather than in the phosphorylation pathway or rebinding (high P_{on}) itself, can compromise specificity and proofreading when $Y_p\psi^A\theta^A f_\beta^A/\phi^A$ and f_β^A/ϕ^A are large. We outline the parameter space for which this sensitivity to processive dephosphorylation is strong in Section S6 in the Supporting Material, where we show that having phosphatases that are intrinsically less efficient than kinases is sufficient (but not necessary) to inhibit this sensitivity. Although interesting, we focus on the majority of parameter space where this unwanted behavior is weak.

Overall, we find that finite f_α^A never reduces specificity relative to otherwise equivalent systems with distributive phosphorylation. Processive dephosphorylation can compromise specificity, but outside of a regime of strong sensitivity to f_β^A, potentially pseudo-processive systems are not worse than distributive systems with equivalent microscopic parameters. The specificity provided by the second site in either case is generally more sensitive to higher yield g than that of the first site, due to the possibility of saturating the $A \rightarrow A_p$ transition before the $A_p \rightarrow A_{pp}$ transition. It is also harder to avoid this saturation through low values of $\theta^A\phi^A = k_{eff}^A h_{eff}^A/k_{eff}^{A\prime}h_{eff}^{A\prime}$ when P_{on} is high. However, in general, the earlier results still hold: the loss of specificity with increased P_{on} is primarily associated with rebinding itself (and hence high reaction probabilities), rather than pseudo-processivity. The selectivity of both the first and second sites is compromised by rebinding, and the additional contribution from the second site can remain significant even when the system is substantially pseudo-processive ($f_\alpha^A, f_\beta^A \geq 1/2$), particularly if intrinsic ($P_{on} \rightarrow 0$) specificities are high.

We plot characteristic behavior in Figs. 5 and 6. Conceptually, we consider a system with fixed microscopic enzymatic rate constants, but in which the rate of diffusion with respect to binding can be modulated (by adding crowders, for example). In this picture, all catalysis probabilities (P_{cat}^A, etc.) are constant, and P_{on} is variable. We then ask how X varies with P_{on} at fixed g (to provide a fair comparison). We take

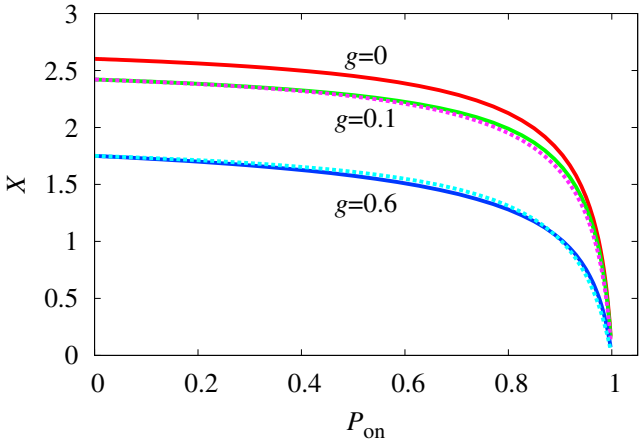

FIGURE 5 Drop of specificity with P_{on} and yield g for the system outlined in the text, showing the similarity between pseudo-processive systems (*solid lines*) and distributive systems with two kinases and phosphatases (*dotted lines*). We consider activated substrate yields of $g = 0$, 0.1, and 0.6. The two systems give identical results for $g = 0$, and so only a single solid line is plotted. To see this figure in color, go online.

$$P^{A}_{cat},\ P^{A\prime}_{cat},\ Q_{cat},\ Q'_{cat} = 0.2,$$

$$P^{B}_{cat},\ P^{B\prime}_{cat} = 0.01$$

to provide representative plots. Other parameter choices are shown in Section S9 in the Supporting Material. We consider yields $g = 0$, 0.1, and 0.6. In Fig. 5, we plot X_{proc} and X_{dis} as a function of P_{on}, showing that although specificity drops with P_{on} and increased g, it also does so when each stage requires its own kinase and phosphatase, and pseudo-processivity is impossible.

In Fig. 6, we plot $X_{proc} - X_{ss}$ and X_{ss} parametrically against the ratio of processive to nonprocessive reactions, $\alpha^A = f^A_\alpha/(1 - f^A_\alpha)$. Both contributions to specificity drop with increased α^A (which itself rises with P_{on}), and increased g, but the additional specificity of the second site is somewhat more sensitive to finite g. Fig. 6 further demonstrates that the contribution of the second site to specificity can remain appreciable at $\alpha^A \geq 1$ ($f^A_\alpha \geq 1/2$), even at fairly high yields of A_{pp}. For illustrative purposes, we have chosen $S = S' = 20$ as $P_{on} \to 0$. Higher values would make specificity at both stages more robust to increased α^A and yield g.

Phosphorylation kinetics, as well as the steady state, could also be important. After a sudden activation of upstream kinases, $[A_{pp}]/[A_0]$ and $[B_{pp}]/[B_0]$ always initially rise in a ratio SS' (see Section S7 in the Supporting Material). Thus, finite kinase activity does not compromise the difference in phosphorylation rates—only whether this difference is manifested in the steady-state yield.

Comparison with other proposed advantages of dual phosphorylation

As outlined in Section S8 in the Supporting Material, the robustness to pseudo-processivity of ultrasensitivity and the use of dual phosphorylation to favor scaffold-mediated pathways can be treated with the same simple model. We can show (as others have (13,14)) that ultrasensitivity arising from dual phosphorylation is always small when f_α and $f_\beta \gtrsim 1/2$. Ultrasensitivity can be fairly robust when either f_α or $f_\beta \gtrsim 1/2$ individually, provided that the second stage of the processive reaction is intrinsically faster than the first. With regard to the use of dual phosphorylation to favor scaffold-mediated pathways, the ratio of scaffold-derived A_{pp} to that produced without a scaffold is limited to $1/f_\alpha$, unless factors independent of dual phosphorylation are relevant. If the mechanism in the cytosol is purely distributive, the scaffold-derived yield can be arbitrarily larger, but $f_\alpha \gtrsim 1/2$ almost completely eliminates the advantage of scaffolds in this context.

Unlike proofreading, these alternative uses are generally compromised by pseudo-processivity itself, rather than rebinding (high P_{on}). As with proofreading, we can imagine a system with identical parameters, but containing two distinct species of phosphatases and upstream kinases that each can only catalyze one step. For the alternatives uses, the effects of increasing P_{on} are substantially alleviated if pseudo-processivity is prohibited in this way (see Section S8 in the Supporting Material). Ultrasensitivity (and scaffold-mediated enhancement) require kinases and phosphatases to compete against each other at two separate stages when activating/deactivating substrates in the cytosol, whereas proofreading requires two stages at which substrate A can be discriminated from B. The first requirement can be met even when P_{on} is high by having distinct kinases and phosphatases for each stage, whereas this does not help to discriminate A from B.

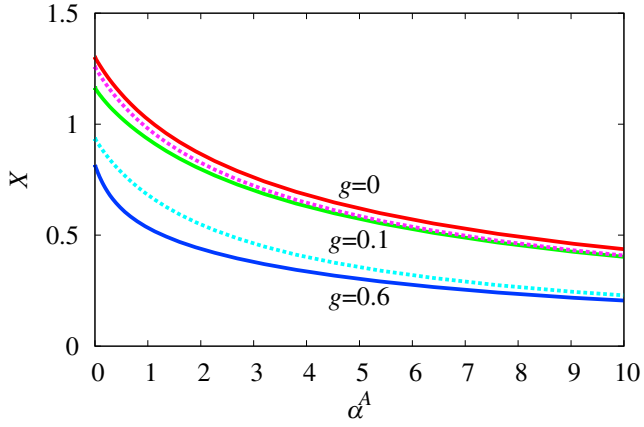

FIGURE 6 Contributions to X of the first and second sites against $\alpha^A = f^A_\alpha/(1 - f^A_\alpha)$, the ratio of processive to nonprocessive phosphorylations for the system outlined in the text. We plot X_{ss} (*dotted lines*) and $X_{proc} - X_{ss}$ (*solid lines*) for activated substrate yields of $g = 0$, 0.1, and 0.6. X_{ss} is the specificity in a system with only the first phosphorylation site. The two curves are identical for $g = 0$, so we plot only a single solid line. The contribution of the second site is somewhat more sensitive to g, but sensitivity to α^A is similar. Specificity, and the contribution of the second site, can remain significant at $\alpha^A > 1$. To see this figure in color, go online.

DISCUSSION

We have studied the effect of rebinding-induced pseudo-processivity on proofreading via dual phosphorylation in the linear regime. While specificity drops as pseudo-processivity increases, this is generally due to a loss of enzymatic selectivity through rebinding, rather than pseudo-processivity itself. High binding probabilities leading to multiple rebinding events reduce the difference in phosphorylation rates between good and poor substrates, and can incidentally increase pseudo-processivity. We contrast this with other proposed advantages of dual phosphorylation, namely ultrasensitivity and the ability to enhance scaffold-mediated signaling pathways. These alternative uses for dual phosphorylation are specifically compromised by pseudo-processivity.

This distinction is not academic—it might be easier for a cell to prevent pseudo-processive behavior (e.g., via a finite refractory period of a kinase after phosphorylation (14,22)) than to prevent rebinding after a failed reaction. We find that eliminating pseudo-processive phosphorylation in this way would always be beneficial for ultrasensitivity and the ability to enhance scaffold-mediated signaling, but never for proofreading (see Sections S5 and S8 in the Supporting Material). However, pseudo-processivity itself in the dephosphorylation pathway can compromise specificity under certain conditions. Although rebinding might be difficult to control through evolution, the reduction in both ultrasensitivity and specificity with P_{on} could be tested in vitro by varying the concentration of an inert crowding agent (8). The distinction between P_{on} and pseudo-processivity would also be testable with kinases modified to reduce nucleotide release rates.

The efficacy of proofreading is primarily related to the additional benefit in specificity obtained by adding a second site. We find that even when reactions become significantly pseudo-processive due to rebinding, the addition of the second site can still provide a substantial relative boost to specificity, meaning that proofreading is still useful. In fact, because pseudo-processivity can only occur in parallel with a reduced intrinsic selectivity for a single site, proofreading via multisite phosphorylation is even more important in maintaining specificity.

Proofreading is based on the difference between two pathways, and a poor substrate can still be less efficiently phosphorylated even if some discrimination is lost through rebinding. The degree to which this is true depends, of course, on the intrinsic discrimination without rebinding. The other uses of dual phosphorylation considered here depend on the properties of a single pathway and are fundamentally limited by moderate pseudo-processivity in that pathway. We would therefore argue that if a signaling cascade is observed to be significantly pseudo-processive in experiment, its dual phosphorylation motif is most likely used for proofreading.

We have considered an extremely simple model without spatial resolution. To test this simplification, we simulate a lattice model in Section S3 in the Supporting Material, reproducing basic results. We also demonstrate that our approach is consistent with limits of a reaction-diffusion description (see Section S2 in the Supporting Material). We have also neglected long-lived enzyme/substrate complexes due to the increased number of relevant parameters and nonlinearities in equations. Analytic results in the unsaturated linear regime are valuable for three reasons:

1. The biophysical principles underlying our conclusions are still relevant in the nonlinear regime; our basic findings are thus likely to be widely applicable. Indeed, we have considered finite complex concentrations for a few systems in Section S12 in the Supporting Material; moderate concentrations of enzyme/substrate complexes have only a weak effect, and we do not see evidence that reactant saturation invalidates our previous findings on proofreading.
2. Detailed analysis of the linear regime is an important first step in comprehending the full nonlinear system, and the analytic results presented here will help to frame the findings of future work into rebinding and pseudo-processivity in the general case.
3. Although some authors have argued for substantial saturation in kinase cascades (4,5), recent work has suggested that MAPK cascades can function in regimes in which the reactants are not strongly saturated (8). Our analysis in the linear regime is therefore not only instructive, but of direct biological relevance.

Nonetheless, the effect of pseudo-processivity in nonlinear systems remains an important open question. To explore the accuracy of our modeling of a crowded environment, explicit simulations (analogous to recent work on transcription factors (21)) would also be beneficial.

SUPPORTING MATERIAL

Ten figures and supplemental information containing explicit derivations and examples of additional results are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)01071-6.

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SUPPORTING CITATIONS

References (28–30) appear in the Supporting Material.

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