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Mathematical analysis of binary activation of a cell cycle kinase which down-regulates its own inhibitor

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Abstract

In mammalian cells, the heterodimeric kinase cyclin E/CDK2 (EK2) mediates cell cycle progress from G1 phase into S phase. The protein $p27^{Kip1}$ (p27) binds to and inhibits EK2; but EK2 can phosphorylate p27, and that leads to the deactivation of p27, presumably liberating more EK2 and forming a positive-feedback loop. It has been proposed that this positive-feedback loop gives rise to binary (all-or-none) release of EK2 from its inactive complex with p27. Binary release suggests a bistable biochemical system in which a stable steady state with low EK2 activity is extinguished in a saddle-node bifurcation, causing the system to shift abruptly to a stable steady state with high EK2 activity. Two mathematical models are discussed, one in which free EK2 deactivates p27 in the EK2-p27 inhibitory complex as well as free p27, and one in which the rate of EK2-catalyzed deactivation of free p27 has saturable kinetics with respect to free p27. In general, if inhibitory binding is approximately in equilibrium, bistability requires that there be a potential unstable steady state where the reaction order of p27 deactivation is greater with respect to EK2 than with respect to p27.

Keywords: Biochemical switch; Bistability; Saddle-node bifurcation; Cyclin-dependent kinase inhibitor

1. Introduction

In the cycle of cell division in mammalian cells, the heterodimeric kinase cyclin E/CDK2 (EK2) mediates cell cycle progress from G1 phase into phase S phase, the phase of DNA replication [1–4]. The protein $p27^{Kip1}$ (p27) binds to EK2 and inhibits its kinase activity, and appears to be an important mediator of G1 arrest [5–8]. An unusual feature of this system is that EK2 phosphorylates p27, and this leads to the deactivation of p27. This creates a positive-feedback loop, since deactivation of p27 releases more EK2, which tends to accelerate p27 phosphorylation and deactivation. Conceivably this positive-feedback process could sustain itself until all the p27 has been deactivated and all the EK2 released; and therefore it has been suggested [9,10] that the release of EK2 from its inactive complex with p27

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is binary (all-or-none). This paper is a mathematical analysis of the biochemical kinetics required for such a binary activation.

Several investigators have reported that EK2 phosphorylates p27 specifically on threonine 187, and that this phosphorylation is followed by (and is required for) degradation of p27 [9,11–13]. In in vitro studies of the phosphorylation, Sheaff et al. [9] found that addition of EK2 to p27, in the presence of ATP, causes a burst of p27 phosphorylation on T187, followed by continued slower phosphorylation. However, if EK2 and p27 were preincubated for 10 min in the absence of ATP, the addition of ATP produced only slow phosphorylation, with no initial burst. Sheaff et al. [9] suggest that p27 binds to EK2 in two ways, first weakly as a substrate and then tightly as an inhibitor. In accord with this concept are the findings of Vlach et al. [13], who have studied point mutants of p27 deficient in their interactions with EK2, and have found that T187 phosphorylation of p27 by EK2 requires an interaction of p27 with the cyclin E subunit, while inhibition of the kinase activity requires an additional interaction with the CDK2 subunit. Similarly, structural studies on the complex of cyclin A/CDK2 with p27, have suggested that the cyclin interaction is likely to serve as an initial anchor in complex formation [14].

This concept of all-or-none EK2 release has not yet been unequivocally demonstrated experimentally, and there is some evidence against it, including the following: (1) Pérez et al. [10] observed that when EK2 was induced by the transcription factor Myc it did not cause appreciable breakdown of pre-existing EK2-p27 complexes for several hours; (2) Müller et al. [12] observed that p27 did not inhibit its own phosphorylation by EK2; and (3) Müller et al. [12] found evidence that phosphorylation does not reduce the affinity of p27 for EK2. However, these objections do not seem fatal, because they can be answered as follows: (1) Myc eventually causes dissociation of EK2-p27 complexes [12,15], and therefore the observation of Pérez et al. only indicates that the process may be slow or delayed; (2) the EK2 phosphorylation observed by Müller et al. may have corresponded to the transient burst observed by Sheaff et al. [9]; and (3) Müller et al. also found that p27 phosphorylation increases the rate of dissociation from EK2 (and presumably the rate of association); and that effect could facilitate the removal of p27 by an active process of degradation or sequestration (p27 can be sequestered away from EK2 either by the kinase cyclin D/CDK4 [16–18] or by a still uncharacterized activity induced by Myc [10,-12,19]).

In this paper the phosphorylation of p27 by EK2 is considered as an isolated process, but in cells it is part of a system of EK2 activation that has at least two other stages. First, EK2 is produced in an inactive form that must be enzymatically phosphorylated on T160 of the CDK2 subunit in order to become active. p27 binds both forms of EK2 and in binding the inactive form it prevents T160 phosphorylation [20,21]. Secondly, CDK2 is subject to inhibitory phosphorylations on T14 and tyrosine 15, and the corresponding activating dephosphorylations may be effectively autocatalytic [22,23]. It is likely that a full understanding of EK2 activation in cells will eventually require a consideration of the full system comprising all these reactions; but that will not be attempted here. Other factors that will not be considered here are the subcellular distribution of p27 [16,24,25] and the regulation of p27 synthesis and degradation [19,26,27].

The question considered here is the theoretical question whether deactivation of p27 by EK2 can produce binary EK2 release, and if so what biochemical kinetic features are required for this behavior. Binary enzyme activation implies an abrupt switch from a stable steady state with a low level of free active enzyme to a new stable steady state with a high level of free active enzyme. This type of behavior would seem to imply a bistable biochemical system in which, under certain conditions, a small parameter change can cause the low-activity stable steady state to be extinguished in a saddle-node bifurcation [28–31]. The underlying assumption in the following models is that a steady state of p27 is maintained by a balance between continuous biosynthesis and deactivation by EK2-catalyzed and non-EK2-dependent processes.

2. Simple models of the interaction of EK2 and p27

We assume, as proposed by Sheaff et al. [9], that p27 binds to EK2 in two ways, first weakly to form a catalytic complex X, and then tightly in an inhibitory complex Y. The two complexes may be formed as independent alternatives:

$$\mathrm{Y} \underset{k_3}{\overset{k_4}{\rightleftharpoons}} \mathrm{EK2} + \mathrm{p27} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{X} \overset{k_{e1}}{\rightarrow} \mathrm{p27P} + \mathrm{EK2} \qquad \text{(I)}$$

or X may be an obligatory intermediate in the formation of Y:

$$\begin{array}{c} \mathrm{EK2} + \mathrm{p27} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{X} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{Y} \\ \qquad\qquad \downarrow k_{e1} \\ \qquad\quad \mathrm{p27P} + \mathrm{EK2} \end{array} \qquad \text{(II)}$$

Here p27P is phosphorylated p27, which we assume is removed and does not affect the kinetics. The following equations define a general model which includes both of these models as special cases. This general model adds two assumptions to Models I and II, namely (1) that free p27 is biosynthesized at a constant rate k_0; and (2) that p27 is partly removed by a process independent of EK2, with rate constant k_{e2}:

$$\frac{\mathrm{d[p27]}}{\mathrm{d}t} = k_0 + k_2[\mathrm{X}] + k_4[\mathrm{Y}] - [(k_1 + k_3)[\mathrm{EK2}] + k_{e2}][\mathrm{p27}], \qquad (1)$$

$$\frac{\mathrm{d[X]}}{\mathrm{d}t} = k_1[\mathrm{EK2}][\mathrm{p27}] + k_6[\mathrm{Y}] - (k_2 + k_5 + k_{e1})[\mathrm{X}], \qquad (2)$$

$$\frac{\mathrm{d[Y]}}{\mathrm{d}t} = k_3[\mathrm{EK2}][\mathrm{p27}] + k_5[\mathrm{X}] - (k_4 + k_6)[\mathrm{Y}], \qquad (3)$$

$$\frac{\mathrm{d[EK2]}}{\mathrm{d}t} = (k_2 + k_{e1})[\mathrm{X}] + k_4[\mathrm{Y}] - (k_1 + k_3)[\mathrm{EK2}][\mathrm{p27}]. \qquad (4)$$

For Model I, $k_5 = k_6 = 0$, and for Model II, $k_3 = k_4 = 0$.

The total concentration of all forms of EK2 ($[\mathrm{EK2}]_\mathrm{T}$) is equal to [X] + [Y] + [EK2] and is constant (adding Eqs. (2)–(4) gives $\mathrm{d}([\mathrm{X}] + [\mathrm{Y}] + [\mathrm{EK2}])/\mathrm{d}t = 0$). The total concentration of all forms of p27 ($[\mathrm{p27}]_\mathrm{T}$) is equal to [p27] + [X] + [Y]; and adding Eqs. (1)–(3) shows that

$$\frac{\mathrm{d}[p27]_\mathrm{T}}{\mathrm{d}t} = k_0 - (k_{e1}[\mathrm{X}] + k_{e2}[\mathrm{p27}]). \qquad (5)$$

From this it can be seen that if there is no non-EK2-dependent deactivation of p27 (i.e. if $k_{e2} = 0$) then, since the maximum possible value of [X] is $[\mathrm{EK2}]_\mathrm{T}$, there is no steady state if $[\mathrm{EK2}]_\mathrm{T} < k_0/k_{e1}$.

The system of Eqs. (1)–(4) has positive feedback, in that EK2 catalyzes the deactivation of p27 and thereby increases its own rate of liberation; however, the system is not bistable. As shown in Appendix A, Eqs. (1)–(4) have only one steady state solution, which is stable (the system does not show sustained oscillations). The behavior of this system is illustrated qualitatively in Fig. 1. In the absence of EK2, the concentrations [X] and [Y] are 0, and the steady-state $[\mathrm{p27}]_\mathrm{T}$ (= [p27] + [X] + [Y]) is equal to [p27], which equals k_0/k_{e2} by Eq. (A1). The binding of EK2 and p27 as X and Y has been assumed to be fairly strong, and therefore as $[\mathrm{EK2}]_\mathrm{T}$ increases there is at first very little free EK2, and nearly all the EK2 is bound up in X and Y. The increase of the catalytic complex X, with increasing $[\mathrm{EK2}]_\mathrm{T}$, increases the rate of p27 deactivation, and consequently the steady-state $[\mathrm{p27}]_\mathrm{T}$ decreases. When nearly all the p27 has been complexed as X and Y, [X] and [Y] cannot increase appreciably further; and therefore further increase in [EK2] causes no further appreciable change in the rate of p27 deactivation or the steady-state $[\mathrm{p27}]_\mathrm{T}$.

3. A model with bistability

We now assume that EK2 phosphorylates not only free p27 but also p27 in the inhibitory complex Y:

$$\mathrm{Y} + \mathrm{EK2} \overset{k_c}{\rightarrow} \mathrm{p27P} + 2\mathrm{EK2} \qquad \text{(III)}$$

We also assume that the p27 in Y may be removed by a non-EK2-dependent process, with

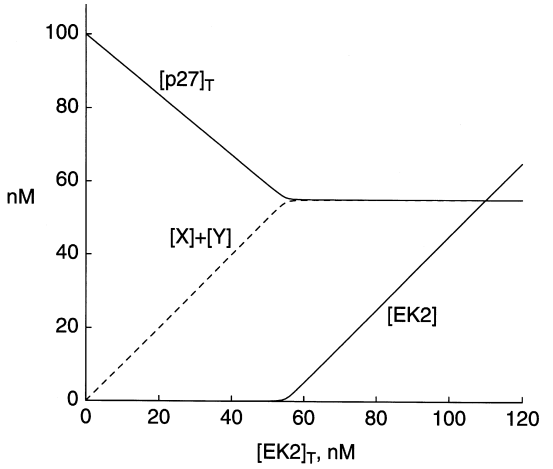

Fig. 1. Effect of total EK2 ($[EK2]_T$) on steady-state total p27 ($[p27]_T$) and free EK2 in the model of Eqs. (1)–(4). Increasing $[EK2]_T$ causes increased [X] and consequently an increased p27 phosphorylation rate and decreased steady-state $[p27]_T$, until p27 is essentially all converted to X and Y, after which further increases of $[EK2]_T$ causes no appreciable further decrease in steady-state $[p27]_T$. Computed from Eq. (A4b) (solved for [EK2]) and Eqs. (A1)–(A3) with $k_0 = 0.5$ nM min^{-1}, $k_{e1} = 0.1$ min^{-1}, $k_{e2} = 0.005$ min^{-1}, $\kappa = 10$, and $C = 0.01$ nM.

liberation of EK2:

$$Y \xrightarrow{k_d} \text{p27 products} + \text{EK2} \qquad \text{(IV)}$$

We simplify the model in two ways: (1) we eliminate the state variable X representing the catalytic complex of EK2 and p27, and assume that the rate of phosphorylation of p27 is simply proportional to the product [EK2][p27], with rate constant k_a; and (2) we assume that the inhibitory binding of EK2 to p27 equilibrates instantaneously (technically we should make a quasi-steady state assumption taking reactions (III) and (IV) into account, but to keep this illustrative model simple we assume that the binding and dissociation reactions are rapid enough to maintain approximate equilibrium). The rate of change of $[p27]_T$ (i.e. [p27] + [Y]) is the difference between a rate of formation F and a rate of deactivation R:

$$\frac{d[p27]_T}{dt} = F - R, \qquad (6)$$

where $F = k_0$ and

$$R = k_a[EK2][p27] + k_b[p27] + k_c[EK2][Y] + k_d[Y], \qquad (7)$$

where $k_b = k_{e2}$. With instantaneous binding equilibrium,

$$K = \frac{[EK2][p27]}{[Y]}. \qquad (8)$$

Substituting $[EK2] = [EK2]_T - [Y]$ and $[p27] = [p27]_T - [Y]$ in Eq. (8), and solving for [Y] gives

$$[Y] = \tfrac{1}{2}\left([EK2]_T + [p27]_T + K - \sqrt{([EK2]_T + [p27]_T + K)^2 - 4[p27]_T[EK2]_T}\right). \qquad (9)$$

The negative sign must be taken for the radical because the positive sign would make [Y] exceed $[EK2]_T$, which is not physically possible.

R is computed as a function of $[p27]_T$ by Eq. (7), with Eq. (9) and the relations $[EK2] = [EK2]_T - [Y]$ and $[p27] = [p27]_T - [Y]$. Fig. 2a shows a graph of R and the rate of $[p27]_T$ formation F, which is constant. The parameter values used are more or less arbitrary, since true values of the concentrations and kinetic constants are not accurately known. We have taken levels of $[EK2]_T$ suggested by various observations in extracts of *Xenopus* eggs and oocytes [32,33], but the effect of subcellular localization has not been taken into account. *Xenopus* eggs lack a G1 phase [32] and may not be representative of other cells. We have taken $k_b = k_d = 0.005$ min^{-1}, which approximately gives the half-life of 2.5 h reported by Hengst and Reed [26] for p27. With this value of k_b the rate of p27 formation F was set to 0.5 nM min^{-1}, so as to set the steady-state $[p27]_T$ at 100 nM in the absence of EK2. The value of k_c was chosen arbitrarily so that the figure would display clearly all the important dynamical features, and k_a was taken equal to k_c. The value of K has been reported as 0.2–0.5 nM [34]; but a smaller value (0.01 nM) was taken here, for reasons which will appear below.

As a function of $[p27]_T$, the rate of p27 deacti-

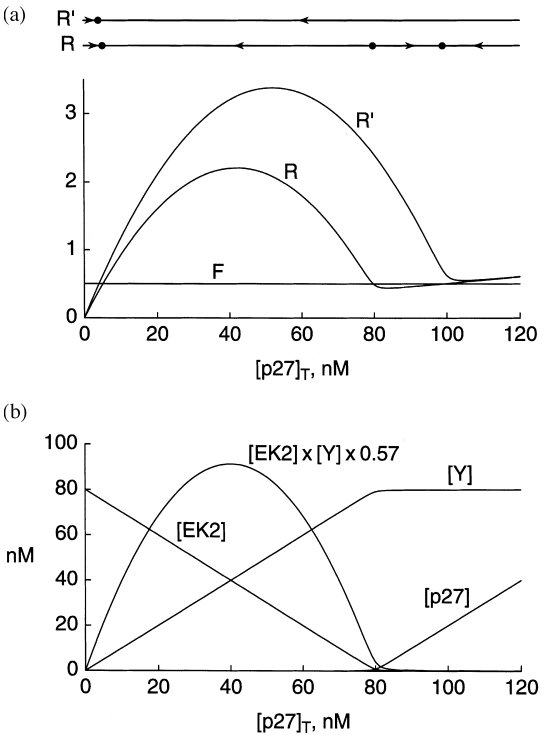

Fig. 2. (a) Rates of formation (F) and deactivation (R, R') of total p27 for the bistable model of Eqs. (6)–(9), as functions of $[p27]_T$. $F = 0.5$ nM min^{-1}, $k_a = k_c = 0.00125$ nM^{-1} min^{-1}, $k_b = k_d = 0.005$ min^{-1}, $K = 0.01$ nM, $[EK2]_T = 80$ nM (curve R), 100 nM (curve R'). The diagrams (phase portraits) above the figure, labelled R and R', indicate by dots the locations of the steady states, and by arrows the direction of spontaneous change of $[p27]_T$. (b) Concentrations of EK2, p27, Y, and the product [EK2][Y] (arbitrarily scaled by a factor 0.57) as functions of $[p27]_T$, with $[EK2]_T = 80$ nM.

vation R shows a maximum and a minimum (Fig. 2a). The maximum occurs where there is free EK2, and it is due to the catalytic effect of free EK2 on p27 phosphorylation. At higher $[p27]_T$, EK2 becomes progressively more inhibited, and consequently R decreases. When essentially all the EK2 is bound to p27, R begins to increase again slowly, because of non-EK2-dependent p27 deactivation (the k_b term in Eq. (7)). With $[EK2]_T = 80$ nM, the curvature of R results in three intersections with curve F, corresponding to three steady states (where $F = R$). These steady states are indicated by dots in the diagram ('phase portrait' [35, pp. 10 ff.]) above the graph. Between the steady states, $[p27]_T$ tends to change spontaneously, increasing if $F > R$ and decreasing if $F < R$, as shown by arrows in the phase portrait. Since the direction of spontaneous change is away from the middle steady state, this steady state is unstable. In contrast, the local direction of spontaneous change in $[p27]_T$ is toward each of the other two steady states, and consequently these steady states are stable, the right-hand one with low free EK2 and the left-hand one with high free EK2.

With 100 nM $[EK2]_T$ (curve R'), the rate of p27 deactivation is higher and there is only one steady state, a stable steady state with high free EK2. Between the $[EK2]_T$ levels of 80 and 100 nM, there is a specific level (between 90.9054 and 90.9055) where the minimum of curve R is just tangental to curve F, at a point where the unstable and the right-hand stable steady states coincide. Any increase of $[EK2]_T$ above this level breaks the contact between curve R and F, obliterates the right-hand stable steady state (and the unstable steady state), and causes the system to shift in an all-or none manner to the left-hand stable steady state with high free EK2. Mathematically, this mutual extinction of a stable and an unstable steady state is called a saddle-node bifurcation ([35], p. 26).

With constant F, it is clear that the slope of R must be negative in the immediate neighborhood of the unstable steady state. Therefore in that region $[p27]_T$ shows in effect a 'substrate inhibition', in that it effectively inhibits its own deactivation. This negative slope of R is caused entirely by the third term in Eq. (7), corresponding to EK2-catalyzed phosphorylation of the inhibitory complex Y. This term is the only term in Eq. (7) that ever decreases with increasing $[p27]_T$, and it accounts for almost all the deactivation rate of p27 to the left of the minimum of the R curve. It contains the product [EK2][Y], which with increasing $[p27]_T$ passes through a maximum because it is the product of approximately linearly increasing and decreasing functions [Y] and [EK2], respectively (Fig. 2b). The near linearity of the functions [Y] and [EK2] results from the fact that if binding is strong an increase in $[p27]_T$ causes an approximately stoichiometric conversion of EK2 to Y.

The EK2-catalyzed phosphorylation of free p27 (the first term in Eq. (7)) does not produce a negative slope in R and does not produce bistability or all-or-none release of EK2. This term contains the product [EK2][p27], which is directly proportional to [Y] by Eq. (8), and [Y] increases with increasing $[p27]_T$ (Fig. 2b). Free EK2 does indeed tend to liberate more EK2, but although this additional EK2 tends to increase the rate of p27 phosphorylation it also tends to convert more free p27 to Y, reducing the free p27 available for phosphorylation and counteracting the positive feedback.

The non-EK2-dependent deactivation of free p27, represented by the second term in Eq. (7), is essential for a stable steady state with low EK2 activity. A stable steady state requires a positive slope in R; but once $[p27]_T$ exceeds $[EK2]_T$, and free EK2 is essentially all converted to Y, none of the other terms in Eq. (7) continue to increase appreciably. Without a stable steady state, $[p27]_T$ would accumulate indefinitely, eventually reaching levels too high to be efficiently removed for activation of EK2.

This effect of increasing $[EK2]_T$ is also shown in Fig. 3, which may be compared with the corresponding figure for the model of the preceding section (Fig. 1). At low $[EK2]_T$ there is just one steady state, with high $[p27]_T$ and low [EK2]. As $[EK2]_T$ increases, there is little change in the steady-state value of either $[p27]_T$ or [EK2] (in contrast to Fig. 1) but $[EK2]_T$ enters a range where each of the curves of $[p27]_T$ and [EK2] has three values at any given $[EK2]_T$, and therefore there are three steady states. For example, the three values of the curve of $[p27]_T$ where $[EK2]_T = 80$ nM are 5.07, 79.8, and 98.9, which are just the three steady states for curve R in Fig. 2a. When $[EK2]_T$ increases beyond the critical value of 90.9055, the stable steady state with low [EK2] ceases to exist, and the system must shift in an all-or none manner to the steady state with high free EK2.

Fig. 4 shows all-or-none release of EK2 from this system, caused by a slow increase in total EK2. At time 0 the system is in a steady state with no EK2. As $[EK]_T$ increases at a constant rate, $[p27]_T$ remains essentially unchanged and

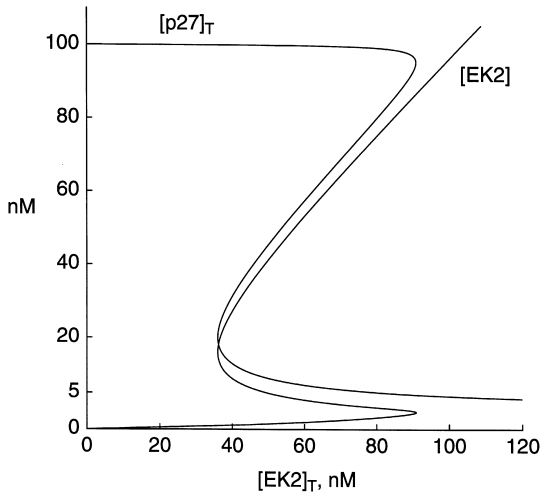

Fig. 3. Steady-state concentrations of total p27 ($[p27]_T$) and free EK2 ([EK2]) for the model of Fig. 2, as functions of $[EK2]_T$. The ordinate scale is slightly non-linear (expanded at the lower end).

there is no detectable free EK2, until $[EK2]_T$ exceeds $[p27]_T$, at which point $[p27]_T$ begins to fall rapidly, and EK2 is released. The release of EK2 is more rapid if the value of k_c is greater (dashed curves, Fig. 4).

It may be noted that the release of EK2 in Fig. 4 does not begin when $[EK2]_T = 90.9055$, i.e. at the saddle-node bifurcation, but only later, when $[EK2]_T > [p27]_T$. This is because the rate processes are very slow near the bifurcation point.

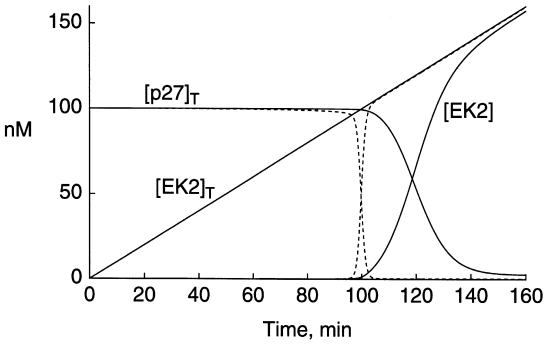

Fig. 4. All-or-none release of EK2 by the model of Fig. 2, caused by gradual increase in $[EK2]_T$ (1 nM min^{-1}). Initial state is a steady state with $[EK2]_T = 0$. Solid curves have parameter values as in Fig. 2. For dashed curves, $k_a = k_c = 0.0125$ nM^{-1} min^{-1}.

The slope of R at the right-hand stable steady state is quite shallow (Fig. 2a), and consequently when curve R is raised the steady state point is shifted substantially to the left, but the difference $R-F$, which drives the change toward the new steady state, is relatively small. Therefore there is delay in establishing a new steady state; and if meanwhile curve R continues to rise then the decrease in $[p27]_T$ lags behind the increase in R. In Fig. 4 the rate processes are so slow that almost no change is detectable when the bifurcation point is passed. Change only becomes apparent when continued increase in $[EK2]_T$ has shifted the minimum of curve R to the right above the level of $[p27]_T$. There is therefore a definite lag in the all-or-none release of EK2. The curvature at the minimum of R depends on the value of K, and we have taken a value that produces only moderate lag. A larger value, such as that reported by Harper et al. [34], would produce a much broader minimum in curve R, and would make the slow equilibration more prominent.

A steeper slope of R at the right-hand stable steady state can be produced if the non-EK2-dependent deactivation of free p27 is actually somewhat faster than that of Y, i.e. if binding to EK2 inhibits non-EK2-dependent p27 deactivation (Fig. 5). Delays in all-or-none EK2 release would then be less. However, this modification also produces another change, namely that in the absence of EK2 the stable steady state has $[p27]_T = 25$ nM, much lower than in the model of Fig. 4. Gradual increase in $[EK2]_T$ will cause a gradual increase in steady-state $[p27]_T$; but lag in $[p27]_T$ will allow premature all-or-none release of EK2 in a relatively small quantity unless the rate of $[EK2]_T$ increase is much slower than in Fig. 4.

Although we have considered only the effect of increasing $[EK2]_T$ as a cause of all-or-none EK2 release, the stability of the steady state with low EK2 activity depends also on the other parameters in Eqs. (6)–(9); and in the course of the cell cycle EK2 release might be caused, for example, by a decrease in F or an increase in k_b.

4. Another model with bistability

In the model just described, it was necessary

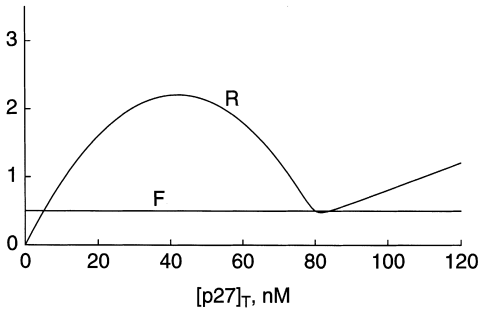

Fig. 5. Rates of formation (F) and deactivation (R) of total p27 for the model of Fig. 2, with parameters as in Fig. 2, except $k_b = 0.02$.

that the enzyme catalyze the deactivation of the inhibitor in the inhibitory complex, and not just free inhibitor, in order to have bistability. This appears not to be true for a similar model proposed by Novak and Tyson [36] for fission yeast cell cycle control. However, that model has an important difference, namely that the enzyme-catalyzed deactivation of free inhibitor is kinetically less than first order with respect to the inhibitor. We modify Eq. (7) to eliminate EK2-catalyzed removal of Y and make EK2-catalyzed deactivation of free p27 saturable with respect to [p27]:

$$R = \frac{k_a[EK2][p27]}{K_M + [p27]} + k_b[p27] + k_d[Y]. \tag{10}$$

Fig. 6 shows graphs of this function with different values of K_M. When $K_M = K$ (curve R) the curve is nearly identical to curve R in Fig. 2a. In fact, if $K_M = K$ the product [EK2][Y] in the third term of Eq. (7) is equal to a function of exactly the same form as the first term in Eq. (10), as can be shown by substituting $[EK2] = [EK2]_T - [Y]$ in Eq. (8) and solving for [Y]:

$$[Y] = \frac{[EK2]_T[p27]}{K + [p27]}. \tag{11}$$

A function of the form of Eq. (10) could arise if the kinase has an obligatory ordered sequential mechanism with competitive high-substrate inhibition ([37], pp. 134–135), in which EK2 must combine first with ATP and then with p27 in

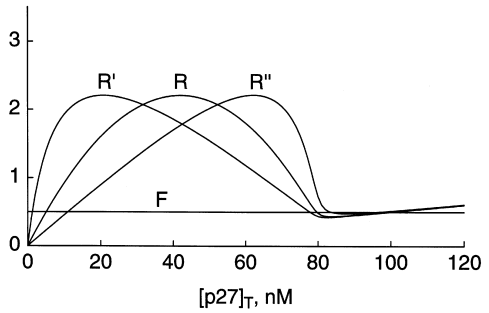

Fig. 6. Rates of formation (F) and deactivation (R) of total p27 for the model with saturable kinetics of EK2-catalyzed deactivation of free p27 (Eqs. (9) and (10)). Parameters as in Fig. 2, except $k_a = 0.1$ min^{-1} and $K_M = 0.01$ nM (curve R), 0.001 nM (curve R'), and 0.1 nM (curve R'').

order to phosphorylate the latter, and the reaction of EK2 with ATP is rate-determining for the phosphorylation of p27:

$$\mathrm{EK2} + \mathrm{ATP} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \mathrm{EK2A} \qquad \text{(V)}$$

$$\mathrm{EK2A} + \mathrm{p27} \overset{k_{e1}}{\rightarrow} \mathrm{p27P} + \mathrm{EK2} \qquad \text{(VI)}$$

Then

$$\frac{\mathrm{d[EK2A]}}{\mathrm{d}t} = k_7[\mathrm{ATP}][\mathrm{EK2}] - (k_8 + k_{e1}[\mathrm{p27}])[\mathrm{EK2A}]. \qquad (12)$$

In the steady state,

$$[\mathrm{EK2A}] = \frac{k_7[\mathrm{ATP}][\mathrm{EK2}]}{k_8 + k_{e1}[\mathrm{p27}]}. \qquad (13)$$

The rate of p27 phosphorylation by EK2A is then

$$k_{e1}[\mathrm{EK2A}][\mathrm{p27}] = \frac{k_7[\mathrm{ATP}][\mathrm{EK2}][\mathrm{p27}]}{k_8/k_{e1} + [\mathrm{p27}]}. \qquad (14)$$

At constant [ATP] this is of the same form as the first term in Eq. (10). [EK2] and [p27] are different, because $[\mathrm{EK2}]_\mathrm{T}$ in Eq. (9) is replaced by $[\mathrm{EK2}]_\mathrm{T} - [\mathrm{EK2A}]$; but if [EK2A] is relatively small then [Y] is given approximately by Eq. (9) and [EK2] and [p27] are approximately $[\mathrm{EK2}]_\mathrm{T} - [\mathrm{Y}]$ and $[\mathrm{p27}]_\mathrm{T} - [\mathrm{Y}]$ respectively.

5. A necessary condition for all-or-none EK2 release if inhibitory binding is approximately in equilibrium

Other models of p27 deactivation might come under consideration, but if the inhibitory binding obeys Eq. (8), and the rate of p27 formation F is constant, there exists a general kinetic requirement for all-or-none EK2 release, namely that the reaction order of p27 deactivation must be greater with respect to EK2 than with respect to p27, over some range of $[\mathrm{p27}]_\mathrm{T}$. This will now be shown.

As noted above, an essential requirement for bistability, if F is constant, is a region of negative slope of R as a function of $[\mathrm{p27}]_\mathrm{T}$. In that region $\partial R/\partial[\mathrm{p27}]_\mathrm{T} < 0$, and also $\partial \log R/\partial \log [\mathrm{p27}]_\mathrm{T} < 0$. Substituting $[\mathrm{EK2}]_\mathrm{T} - [\mathrm{EK2}]$ for [Y] in Eq. (7) or Eq. (10) gives R as a function of [EK2] and [p27], and

$$\frac{\partial \log R}{\partial \log[\mathrm{p27}]_\mathrm{T}} = \frac{\partial \log R}{\partial \log[\mathrm{EK2}]}\frac{\partial \log[\mathrm{EK2}]}{\partial \log[\mathrm{p27}]_\mathrm{T}} + \frac{\partial \log R}{\partial \log[\mathrm{p27}]}\frac{\partial \log[\mathrm{p27}]}{\partial \log[\mathrm{p27}]_\mathrm{T}} < 0. \qquad (15)$$

Differentiating Eq. (8), with $[\mathrm{Y}] = [\mathrm{EK2}]_\mathrm{T} - [\mathrm{EK2}]$, gives

$$\frac{\partial \log[\mathrm{EK2}]}{\partial \log[\mathrm{p27}]_\mathrm{T}} + \frac{\partial \log[\mathrm{p27}]}{\partial \log[\mathrm{p27}]_\mathrm{T}} = \frac{\partial \log([\mathrm{EK2}]_\mathrm{T} - [\mathrm{EK2}])}{\partial \log[\mathrm{p27}]_\mathrm{T}} = -\left[\frac{[\mathrm{EK2}]}{[\mathrm{EK2}]_\mathrm{T} - [\mathrm{EK2}]}\right]\frac{\partial \log[\mathrm{EK2}]}{\partial \log[\mathrm{p27}]_\mathrm{T}}, \qquad (16a)$$

hence

$$\frac{\partial \log[\mathrm{EK2}]}{\partial \log[\mathrm{p27}]_\mathrm{T}} = -\left[\frac{[\mathrm{EK2}]_\mathrm{T} - [\mathrm{EK2}]}{[\mathrm{EK2}]_\mathrm{T}}\right]\frac{\partial \log[\mathrm{p27}]}{\partial \log[\mathrm{p27}]_\mathrm{T}}. \qquad (16b)$$

The only logarithmic partial derivative to the right of the equal sign in Eq. (15) which can plausibly be negative is $\partial \log [\mathrm{EK2}]/\partial \log[\mathrm{p27}]_\mathrm{T}$, and Eq. (16b) shows that this is numerically less

than $\partial\log[p27]/\partial\log\,[p27]_T$. Therefore the inequality in Eq. (15) cannot hold unless $\partial\log R/\partial\log[EK2] > \partial\log R/\partial\log[p27]$. Since these logarithmic partial derivatives are the effective reaction orders (e.g. $\partial\log\,(kS^n)/\partial\log S = n$), this establishes that the reaction order must be greater with respect to EK2 than with respect to p27.

It is easily verified that if R is a sum of several rate processes R_i then the logarithmic partial derivative $\partial\log R/\partial\log S$ is a weighted sum of the individual logarithmic partial derivatives $\Sigma\rho_i$-$\partial\log R_i/\partial\log S$, where $\rho_i = R_i/R$. It follows that the inequality $\partial\log R/\partial\log[EK2] > \partial\log R/\partial\log\,[p27]$ can hold only if the corresponding inequality $\partial\log R_i/\partial\log[EK2] > \partial\log\,R_i/\partial\log\,[p27]$ holds for at least one of the individual processes R_i.

In Eq. (10) of Section 4, the first term is first-order with respect to [EK2], and less than first-order with respect to [p27] [the reaction order with respect to [p27] is $\partial\log[k_a[EK2][p27]/(K_M + [p27])]/\partial\log\,[p27]$, which is equal to $K_M/(K_M + [p27])$]. This is not true for any other term in Eq. (10), and therefore this term is essential to satisfy the necessary condition for a negative slope of R as a function of $[p27]_T$. This is not a sufficient condition, because the fact that $\partial\log R/\partial\log[EK2] > \partial\log R/\partial\log[p27]$ does not guarantee that the inequality in Eq. (15) holds. The slope of R is positive in some regions (Fig. 2), partly because the inequality in Eq. (15) does not hold everywhere for the first term in Eq. (10), and partly because of the other terms in Eq. (10).

Similarly, in Eq. (7), the third term contains the product [EK2][Y], which by Eq. (8) is proportional to $[EK2]^2[p27]$; therefore the reaction order is greater with respect to EK2 than with respect to p27.

These examples illustrate how analysis of reaction orders can help to identify those kinetic mechanisms that can give rise to all-or-none activation of an enzyme that deactivates its own inhibitor.

6. Discussion

This analysis shows that an enzyme that attacks and deactivates its own inhibitor is not released from inhibitor binding in an all-or-none fashion unless certain kinetic features are present in the mechanism. These features are not present in the simplest models (e.g. Models I and II). It is true that a small amount of uninhibited enzyme deactivates some of the inhibitor, releasing more enzyme, and the additional enzyme tends to accelerate the deactivation of the inhibitor. However, the additional enzyme also tends to bind more inhibitor as inhibitory complex, and this reduces the proportion of free inhibitor, and therefore tends to counteract the enzymatic deactivation of free inhibitor. This limitation is absent or less marked in two cases: (1) if free enzyme deactivates inhibitor in the inhibitory complex; and (2) if enzymatic deactivation of free inhibitor has saturable kinetics with respect to the concentration of free inhibitor. In each of these cases bistability is possible; and a bistable model of each kind has been presented here. With regard to the possibility of other bistable models, theoretical analysis shows that, in the case where inhibitory binding is approximately in equilibrium, bistability is possible only if there is a potential unstable steady state where the reaction order of p27 removal or deactivation is greater with respect to free EK2 than with respect to free p27.

It is not now known whether either of the bistable models presented here actually fits the deactivation of p27 by EK2. With regard to the first model, it has not been experimentally established whether p27 in the inhibitory complex with EK2 can be phosphorylated and deactivated by another EK2 heterodimer. The findings of Vlach et al. [13] would suggest that this does not occur, because the site on p27 which interacts with the cyclin E subunit of EK2, and which is necessary for EK2-catalyzed p27 phosphorylation, is apparently utilized in the formation of the inhibitory complex. As to the second model, it has been speculated above that the saturable kinetics might be accounted for by an obligatory ordered sequential enzyme mechanism, in which the kinase must combine with ATP before combining with p27. The findings of Russo et al. [14] indicate that p27 in the inhibitory complex covers the ATP binding site; so it seems possible that p27 bound

only to the cyclin subunit might sterically inhibit ATP binding, so that the EK2/ATP/p27 catalytic complex can only form if ATP binds first to EK2.

Bistability and all-or-none EK2 release also appear to require at least three other things that are not yet established. First, phosphorylated p27 must be either inactive or rapidly inactivated or sequestered. As noted above, phosphorylation may not reduce the affinity of p27 for EK2; and although phosphorylation leads to the degradation of p27, it is not established whether in cells the rate and kinetics of sequestration and degradation could support all-or-none EK2 release. Second, for a stable steady state with low EK2 activity it appears necessary to assume that there is a non-EK2-dependent process of p27 removal or deactivation. It has been pointed out (Fig. 5) that if this process removes free p27 faster than p27 in the complex Y this could improve stabilization of the steady state with low EK2 activity. However, no such process has been clearly characterized. Third, for rapid stabilization and destabilization near the threshold of EK2 release it appears necessary to assume an inhibitor binding affinity considerably higher than has been reported [34]. The kinetic studies of Sheaff et al. [9] also suggest a larger K; but the kinetics are somewhat complex and have not yet been worked out in detail. Larger K is associated with slower equilibration of the system near the threshold for EK2 release, and this may lead to either premature or delayed EK2 release.

It may be that the kinetic role of EK2-catalyzed p27 phosphorylation in cells can only be understood in its interaction with other cellular reactions, such as T160 phosphorylation of EK2, or activation of EK2 by CDC25A. In any case, experimental evidence indicates that deactivation of p27 is necessary for full activation of EK2; and the mechanisms discussed here may contribute something to EK2 activation and release, even if other switching mechanisms require to be involved.

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Appendix: Uniqueness and stability of the steady state of Eqs. (1)–(4)

The following argument shows that Eqs. (1)–(4) have at most one stable steady state. Eq. (5) in the steady state (with the derivative set to zero) gives

$$[\mathrm{p27}] = \frac{k_0 - k_{e1}[\mathrm{X}]}{k_{e2}}. \tag{A1}$$

Eliminating the product [EK2][p27] from Eqs. (2) and (3), with the derivatives set to zero, gives

$$[\mathrm{Y}] = \frac{(k_1 + k_3)k_5 + k_3(k_2 + k_{e1})}{(k_1 + k_3)k_6 + k_1 k_4}[\mathrm{X}] = \kappa[\mathrm{X}]. \tag{A2}$$

From this it follows that at a steady state $[\mathrm{EK2}]_\mathrm{T} = [\mathrm{EK2}] + (1+\kappa)[\mathrm{X}]$, hence

$$[\mathrm{X}] = \frac{([\mathrm{EK2}]_\mathrm{T} - [\mathrm{EK2}])}{(1+\kappa)}. \tag{A3}$$

Substituting from Eqs. (A1)–(A3) for [p27], [X], and [Y] in Eq. (4), with the derivative set to 0, gives

$$0 = (k_2 + k_{e1} + k_4\kappa)\left(\frac{[\mathrm{EK2}]_\mathrm{T} - [\mathrm{EK2}]}{1+\kappa}\right) - \left[\frac{(k_1 + k_3)[\mathrm{EK}_2]}{k_{e2}}\right] \times \left[k_0 - k_{e1}\left(\frac{[\mathrm{EK2}]_\mathrm{T} - [\mathrm{EK}_2]}{1+\kappa}\right)\right], \tag{A4a}$$

$$[\mathrm{EK2}]_\mathrm{T} = [\mathrm{EK2}]\left[1 + \frac{(k_0/k_{e1})(1+\kappa)}{[\mathrm{EK2}] + C}\right], \tag{A4b}$$

where $C = (k_2 + k_{e1} + k_4\kappa)(k_{e2}/k_{e1})/(k_1 + k_3)$. Given that [EK2] is positive, this relationship between $[\mathrm{EK2}]_\mathrm{T}$ and [EK2] is monotonic; and therefore there is only one steady state at any given $[\mathrm{EK2}]_\mathrm{T}$, and there is no bistability. As already noted, the solution is not physically realizable if $k_{e2} = 0$ and $[\mathrm{EK2}]_\mathrm{T} < k_0/k_{e1}$. In that case $C = 0$, Eq. (A4b) becomes $[\mathrm{EK2}]_\mathrm{T} = [\mathrm{EK2}] + k_0/k_{e1}$, and [EK2] must be negative.

To determine the stability of the steady state, the system of Eqs. (1)–(4) can be reduced to Eqs. (1)–(3) with the substitution $[EK2] = [EK2]_T - [X] - [Y]$. The reduced system is stable iff the characteristic roots of the Jacobian matrix all have a negative real part. The Jacobian matrix of the reduced system is

$$J = \begin{bmatrix} -[(k_1+k_3)[EK2] + k_{e2}] & k_2 + (k_1+k_3)[p27] & k_4 + (k_1+k_3)[p27] \\ k_1[EK2] & -(k_2+k_5+k_{e1}+k_1[p27]) & k_6 + k_1[p27] \\ k_3[EK2] & k_5 - k_3[p27] & -(k_4+k_6+k_3[p27]) \end{bmatrix} \quad \text{(A5)}$$

The characteristic roots of J are the roots of the equation

$$\lambda^3 - D_1\lambda^2 + D_2\lambda - D_3 = 0, \quad \text{(A6)}$$

where D_1 is the trace of J, D_2 is the sum of the principal minors of order 2, and D_3 is the determinant ([38], p. 70). The roots all have negative real part if the Hurwitz determinants $-D_1$, $-D_1D_2 + D_3$, and $-(-D_1D_2 + D_3)D_3$ are all positive ([39], pp. 167 ff). It can be verified from Eq. (A5) that $-D_1$ and D_2 are positive. D_3 can be evaluated by considering the matrix J_0 formed by setting $k_{e1} = k_{e2} = 0$ in J. The column sums of J_0 vanish, so the determinant is zero. The determinant of J is equal to the determinant of J_0 plus the sum of products containing k_{e1} or k_{e2}, and may be written:

$$D_3 = -k_{e2}M_{23}^0 - k_{e1}M_{13}^0 - k_{e2}k_{e1}(k_4 + k_6 + k_3[p27]), \quad \text{(A7)}$$

where M_{23}^0 and M_{13}^0 are principal minors of order 2 of J_0. M_{23}^0 and M_{13}^0 are easily shown to be positive, therefore D_3 is negative. D_2 is a sum of terms which include M_{23}^0, M_{13}^0, and $k_{e2}k_{e1}$, together with other positive terms; therefore $-D_1D_2$ contains positive terms numerically equal to all the terms on the right-hand side of Eq. (A7). The second Hurwitz determinant $-D_1D_2 + D_3$ is therefore positive; and since D_3 is negative the third Hurwitz determinant is also positive. It follows that the roots of Eq. (A6) have negative real part.

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