Regulation of the mammalian cell cycle: a model of the G1-to-S transition

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Qu, Zhilin, James N. Weiss, and W. Robb MacLellan. Regulation of the mammalian cell cycle: a model of the G1-to-S transition. Am J Physiol Cell Physiol 284: C349–C364, 2003. First published October 9, 2002; 10.1152/ajpcell.00066.2002.—We have formulated a mathematical model for regulation of the G1-to-S transition of the mammalian cell cycle. This mathematical model incorporates the key molecules and interactions that have been identified experimentally. By subdividing these critical molecules into modules, we have been able to systematically analyze the contribution of each to dynamics of the G1-to-S transition. The primary module, which includes the interactions between cyclin E (CycE), cyclin-dependent kinase 2 (CDK2), and protein phosphatase CDC25A, exhibits dynamics such as limit cycle, bistability, and excitable transient. The positive feedback between CyclE and transcription factor E2F causes bistability, provided that the total E2F is constant and the retinoblastoma protein (Rb) can be hyperphosphorylated. The positive feedback between active CDK2 and cyclin-dependent kinase inhibitor (CKI) generates a limit cycle. When combined with the primary module, the E2F/Rb and CKI modules potentiate or attenuate the dynamics generated by the primary module. In addition, we found that multisite phosphorylation of CDC25A, Rb, and CKI was critical for the generation of dynamics required for cell cycle progression. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

THE EUKARYOTIC CELL CYCLE regulating cell division is classically divided into four phases: G1, S, G2, and M (35, 38). Quiescent cells reside in the G0 phase and are induced to reenter the cell cycle by mitogenic stimulation. In the S phase, the cell replicates its DNA, and at the end of the G2-to-M transition the cell divides into two daughter cells, which then begin a new cycle of division. This critical biological process is orchestrated by the expression and activation of cell cycle genes, which form a complex and highly integrated network (24). In this network, activating and inhibitory signaling molecules interact, forming positive- and negative-feedback loops, which ultimately control the dynamics of the cell cycle. Although many of the key cell cycle regulatory molecules have been cloned and identified, the dynamics of this complicated network are too complex to be understood by intuition alone.

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MATHEMATICAL MODELING

Figure 1 summarizes the key interactions between regulators of the G1-to-S transition in the mammalian cell cycle that have been identified experimentally over the last two decades and have been incorporated into our model.

CycE and CDK2 Regulation

Increased CDK2 activity marks the transition from the G1 to the S phase. CDK2 activity is regulated by at least two mechanisms, including 1) transcriptional regulation of its catalytic partner, CycE, and 2) posttranslational modification of the CycE-CDK2 complex itself. For the purposes of our model, we will assume that CycE transcription is primarily regulated by two mechanisms (step 1). Mitogenic stimulation promotes Myc-dependent transcription of CycE (4, 34, 45), which we assume occurs at a constant rate ($k_1$). In addition, E2F is also an important transcription factor for CycE synthesis (4, 9). We assume that it induces CycE synthesis at a rate proportional to E2F concentration ($k_{3, e}$ in Eq. 1a). Thus the total CycE synthesis rate is $k_1 + k_{3,e}$. For CycE and CDK2 binding and activation, we adopted the scheme proposed by Solomon et al. (51, 52). CycE and CDK2 bind together, forming an inactive CycE-CDK2 complex (step 3), with CDK2 phosphorylated at Thr$^{14}$, Tyr$^{15}$, and Thr$^{160}$. CDC25A dephosphorylates Thr$^{14}$ and Tyr$^{15}$ and activates the kinase (step 5) (18, 47, 51, 52). The complex is activated by ubiquitin-mediated degradation of CycE in its free form (step 2) or in the active bound form (CycE-CDK2, step 7) (66, 67). We assume that the rates of degradation through both pathways are proportional to their concentrations ($k_{2,y}$ for free CycE and $k_{7,x}$ for active CycE-CDK2 in Eq. 1a).

CDC25A Regulation

CDC25A is a phosphatase that dephosphorylates and activates CDK2 by removing inhibitory phosphates from Thr$^{14}$ and Tyr$^{15}$. CDC25A is a transcriptional target of Myc (28) and E2F (64). We assume that Myc induces CDC25A synthesis at a constant rate ($k_{11}$) and that E2F induces CDC25A synthesis in proportion to E2F concentration ($k_{3,e}$ in Eq. 1a). CDC25A is also degraded through ubiquitination, which we assume is proportional to its concentration ($k_{9,y}$ in Eq. 1a). For CDC25A to become active, it must itself be phosphorylated. This phosphorylation is catalyzed by the active CycE-CDK2 complex (18), which forms a key positive-feedback loop in CycE-CDK2 regulation. It has been shown that CDC25C is highly phosphorylated at the G2-to-M transition and has five serine/threonine-proline sites: Thr$^{48}$, Thr$^{97}$, Ser$^{122}$, Thr$^{130}$, and Ser$^{214}$ (17, 25, 32). The number of functionally important phosphorylation sites on CDC25A has not been determined experimentally, but we assume that CDC25A has a total of L phosphorylation sites and that its multisite phosphorylation occurs sequentially, with each phosphorylation step catalyzed by CycE-CDK2 (Fig. 1B). We also assume that highly phosphorylated CDC25A is degraded through ubiquitination (step 10).

E2F/Rb Regulation

E2F is a transcription factor for a number of cell cycle genes that are critical for G1-to-S transition in mammalian cells (49, 68). This family of transcription factors binds to and is inactivated by a second family of proteins known as pocket proteins, the prototypical member being the Rb gene product. In G0 or early G1, E2F is complexed to Rb and is inactive. E2F is freed by Rb phosphorylation, which occurs sequentially first by CycD-CDK4/6 and subsequently by CycE-CDK2, forming another positive-feedback loop. E2F is also synthesized de novo as the cell progresses from G0 to G1 and autocatalyzes its own production (9, 27). E2F can be inactivated by binding to dephosphorylated Rb and by degradation through ubiquitination after phosphorylation by CycE-CDK2 (9). In the model, we assume that E2F is synthesized at a constant rate $k_{11}$ and a rate related to free E2F concentration [$k_{11-e}g(e)$ in Eq. 1b] and degraded at a rate proportional to its concentration ($k_{12,e}$ in Eq. 1b) and CycE-CDK2 ($k_{12,e}$ in Eq. 1b). Although it is known that Rb has 16 phosphorylation sites (14), the number of functionally important sites is unknown. Therefore, we assume that Rb has a total of $M'$ phosphorylation sites and that E2F is dissociated from Rb when a certain number ($M$) of sites are phosphorylated and Rb is hyperphosphorylated by phosphorylation of the other phosphorylation sites ($M' - M$) on Rb. In our model, we vary $M$ and $M'$ to study the effects of multisite phosphorylation.

CycD and CDK4/6 Regulation

Mitogenic stimulation of cells in the G0 phase triggers synthesis of CycD (28). CycD interacts with CDK4 or CDK6 to form a catalytically active CycD-CDK4/6 complex, which phosphorylates Rb to free active E2F (9, 49). It also potentiates CDK2 activity indirectly by titrating away inhibitory CKIs from CycE-CDK2 (50).

CKI Regulation

CKIs, such as p21 or p27, bind to CycD-CDK4/6 or CycD-CDK2 to form trimeric complexes. CKI activity is high during the Go phase but decreases during the cell cycle (49). Because factors regulating CKI synthesis are not well understood, we assume that CKI is synthesized at a constant synthesis rate ($k_{18}$) and degraded at a rate proportional to its concentration ($k_{19}$ in Eq. 1d), which is low enough to ensure a high CKI level at the beginning of the G1-to-S transition. It has been shown that p27 binds to CycE-CDK2 and has to
Fig. 1. Schematic model of molecular signaling involved in regulation of the G1-to-S transition. A: signaling network divided into functional modules for the G1-to-S transition. Cyclin E (CycE), cyclin-dependent kinase (CDK) 2 (CDK2), and protein phosphatase CDC25A form module I (red box), retinoblastoma protein (Rb) and transcription factor E2F form module II (green box), cyclin D (CycD) and CDK4/6 form module III (orange box), and CDK inhibitor (CKI) forms module IV (blue box). B: reaction scheme for CDC25A multisite phosphorylation catalyzed by active CycE-CDK2 complex. C: E2F-Rb pathway, in which multisite phosphorylation of Rb is catalyzed by CycD-CDK4/6 and active CycE-CDK2. When Rb in the Rb-E2F complex is phosphorylated at M sites, E2F is freed. Free Rb can then be phosphorylated at the rest of its phosphorylation sites (M’. M). D: multisite phosphorylation of the trimeric complex CycE-CDK2-CKI is catalyzed by active CycE-CDK2. When CKI has N sites phosphorylated, it binds to F-box protein for ubiquitination and degradation (step 25), and CycE-CDK2 is freed. See Table 1 for rate constants.
be phosphorylated on Thr\textsuperscript{187} by active CycE-CDK2 for ubiquitination and degradation (31, 48, 65). Ishida et al. (19) showed that p27 is phosphorylated on many sites, including Ser\textsuperscript{10}, Ser\textsuperscript{178}, and Thr\textsuperscript{187}, and showed that Ser\textsuperscript{10} was phosphorylated-dephosphorylated in a cell cycle-dependent manner and contributed to p27 stability. Although it is known that Thr\textsuperscript{187} phosphorylation is required for p27 ubiquitination, it is not clear whether phosphorylation and dephosphorylation of any other sites are also needed for p27 ubiquitination in the mammalian cell cycle. A recent study (33) in yeast has shown that Sic1, a homolog of p27, has nine phosphorylation sites, including Ser10, Ser178, and Thr187, and showed that Ser10 was phosphorylated-dephosphorylated in a cell cycle-dependent manner and contributed to p27 stability. Although it is known that Thr187 phosphorylation is required for p27 ubiquitination, it is not clear whether phosphorylation and dephosphorylation of any other sites are also needed for p27 ubiquitination in the mammalian cell cycle.

On the basis of the model outlined in Fig. 1, we constructed differential equations (see Table 1 for definitions of symbols and parameters), which are listed separately for each module as follows.

For module 1 (CycE + CDK2 + CDC25A)

\[
y = k_1 + k_{1e} - k_2 y - k_3 y + k_x x_1 \\
x_1 = k_3 y - k_4 x_1 - [k_5 + f(z)] x_1 + k_x x \\
x = [k_5 + f(z)] x_1 - k_x x - k_4 x - k_{23} x_i + k_{24} x_0 + k_{25} x_N \\
z_0 = k_8 + k_8 e - k_9 z_0 + k_{1z} z_1 - k_{1z} z_0 \\
z_i = k_{1z} z_{i-1} - k_{1z} z_i + k_{1z} z_{i+1} - k_{1z} z_i, \quad i = 1, L - 1 \\
z_L = k_{1z} z_{L-1} - k_{1z} z_L - k_{10} z_L
\]

where \( f(z) = \sum_{i=1}^{L} \sigma_i z_i \) represents the catalyzing strength of CDC25A on CDK2 and \( \sigma_i \) is a weighing parameter. \( L \) is the total number of phosphorylation sites of CDC25A. \( k_{1z} = b_x + c_x x \) is the rate constant for CycE-CDK2-catalyzed phosphorylation of CDC25A, and \( k_{1z} = a_x \) is the rate constant for dephosphorylation of CDC25A. In Eq. 1a, we assumed that CDK2 concentration was much higher than cyclin concentration (3) and, thus, set CDK2 concentration to be constant 1.

### Table 1. Definitions of variables in Eq. 1 and default parameter set

<table>
<thead>
<tr>
<th>Variable</th>
<th>Definition</th>
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<td>( x )</td>
<td>Active CycE-CDK2</td>
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<tr>
<td>( y )</td>
<td>Free CycE</td>
</tr>
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<td>( x_1 )</td>
<td>Inactive CycE-CDK2</td>
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<td>( z_i )</td>
<td>( l )-Site-phosphorylated CDC25A (( l = 0, L ))</td>
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<tr>
<td>( e )</td>
<td>Free E2F</td>
</tr>
<tr>
<td>( P_m )</td>
<td>( m )-Site-phosphorylated Rb-E2F (( m = 0, M ))</td>
</tr>
<tr>
<td>( r_m )</td>
<td>( m )-Site-phosphorylated Rb (( m = 0, M' ))</td>
</tr>
<tr>
<td>( d )</td>
<td>Free CycD</td>
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<tr>
<td>( i_d )</td>
<td>CycD-CDK 4/6</td>
</tr>
<tr>
<td>( i_n )</td>
<td>( n )-Site-phosphorylated CycE-CDK2-CKI (( n = 0, N ))</td>
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<table>
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<tr>
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**Constant for CDC25A, Rb, and CKI phosphorylation and dephosphorylation in Fig. 1, B-D**

<table>
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<th>Parameter</th>
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<tr>
<td>( c_x )</td>
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<td>( a_r )</td>
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<tr>
<td>( b_r )</td>
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</tr>
<tr>
<td>( c_r )</td>
<td>1</td>
</tr>
<tr>
<td>( a_t )</td>
<td>50</td>
</tr>
<tr>
<td>( b_t )</td>
<td>1</td>
</tr>
<tr>
<td>( c_t )</td>
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</tr>
</tbody>
</table>

**Rate constant for E2F and Rb affinity and dissociation in Fig. 1C**

<table>
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</tr>
<tr>
<td>( k_m^{-} )</td>
<td>0.5</td>
</tr>
<tr>
<td>( k_M^{+} )</td>
<td>(( m = 0, M - 1 ))</td>
</tr>
<tr>
<td>( k_M^{-} )</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**Total Rb concentration**

\( R_0 = 100 \)

Parameters served as default set. Any parameter not given in corresponding figure legend is set to default value. CycE and CycD, cyclins E and D; CDK, cyclin-dependent kinase; Rb, retinoblastoma protein; CKI, CDK inhibitor.
For module II (Rb + E2F)

\[ \dot{e} = k_{11} + k_{12} g(e) - (k_{12} + k_{13} x) e \]

\[ + \sum_{m=0}^{M} k_{m}^p p_m - \sum_{m=0}^{M} k_{m}^e r_m \]

\[ p_0 = k_r p_1 - k_r p_0 - k_0 p_0 + k_0 e r_0 \]

\[ \vdots \]

\[ p_m = k_r p_{m-1} - k_r p_m - k_r^a p_m + k_r^a r_{m-1} + k_m^p p_m \]

\[ + k_m^e r_m, \quad m = 1, M - 1 \]

\[ \vdots \]

\[ p_M = k_r p_{M-1} - k_r p_M - k_M^p p_M + k_M^e r_M \]

\[ r_0 = k_0^p p_0 - k_0^e r_0 - k_r^a r_0 + k_r^a r_1 \]

\[ \vdots \]

\[ r_m = k_m^p p_m - k_m^e r_m + k_r^a r_m - k_r^a r_m \]

\[ + k_r^a r_{m+1}, \quad m = 1, M - 1 \]

\[ \vdots \]

\[ r_M = R_0 - \sum_{m=0}^{M} p_m - \sum_{m=0}^{M} r_m \]

where \( g(e) \) is a function representing E2F synthesis by E2F itself and will be defined later. \( M \) is the number of phosphorylation sites on Rb needed for dissociation of E2F from Rb. \( M' \) is the total number of phosphorylation sites on Rb. \( k_m^p \) and \( k_m^e \) \((m = 0, M)\) are the rate constants for E2F dissociation from \( m \)-site-phosphorylated Rb and for association with \( m \)-site-phosphorylated Rb, respectively. \( k_r^a = b_r + c_x + c_d a_4 \) is the combined rate constant for CycD-CDK4/6- and CycE-CDK2-catalyzed phosphorylation of Rb, and \( k_r^a = a_r \) is the rate constant for Rb dephosphorylation. For simplicity, here we assumed that CycD-CDK4/6 and CycE-CDK2 have the same catalytic effects on Rb and that the phosphorylation and dephosphorylation are not state dependent; i.e., we used the same rate constant for each phosphorylation or dephosphorylation step. \( R_0 \) is the total Rb concentration.

For module III (CycD + CDK4/6)

\[ \dot{d} = k_{13} - k_{14} d - k_{15} d + k_{16} d_4 \]

\[ \dot{d}_4 = k_{15} d - k_{16} d_4 - k_{17} d_4 - k_{20} d_4 + k_{21} i_d \]

For module IV (CKI)

\[ \dot{i} = k_{18} - k_{19} i - k_{20} d_4 + k_{21} i_d + k_{22} i_d - k_{23} i + k_{24} i_0 \]

\[ \dot{i}_d = k_{20} i d_4 - k_{21} i d - k_{22} i d \]

\[ \dot{i}_x = k_{22} i x - k_{23} i x_0 - k_{1} i x_0 + k_{1} i_1 x_1 \]

\[ \vdots \]

\[ \dot{i}_n = k_{1} i_{n-1} - k_{1} i_n - k_{1} i_{n} + k_{1} i_{n+1}, \quad n = 1, N - 1 \]

\[ \dot{i}_{nN} = k_{1} i_{nN-1} - k_{1} i_{nN} - k_{25} i_{nN} \]

where \( k_r^a = b_1 + c_x \) is the rate constant for CycE and CDK2-catalyzed CKI phosphorylation and \( k_r^a = a_r \) is the rate constant for dephosphorylation. \( N \) is the total number of phosphorylation sites on CKI. We also assumed that the rate constants are the same for each phosphorylation step.

When we simulate one module or a combination of some modules, we indicate that we remove all the interaction terms in Eq. 1 from the modules that are not involved. In numerical simulation, we used the fourth-order Runge-Kutta method to integrate Eq. 1. The time step we used in simulation was \( \Delta t = 0.005 \).

**RESULTS**

**Dynamics of Module I (CycE + CDK2 + CDC25A)**

The full model shown in Fig. 1 is too complex for a complete analysis of its dynamical properties. We therefore divided the major components of the G1-to-S transition into modules and examined their individual behaviors before reintroducing them into the full model to determine their combined effects. We consider the primary module (module I in Fig. 1A) as comprised of CycE, CDK2, and CDC25A, with CDC25A driving a positive-feedback loop catalyzing active CyclinE-CDK2 production.

Figure 2 shows the case for two functional phosphorylation sites on CDC25A \((L = 2)\) and \( f(z) = zL \) (hereafter defined as the default conditions for module I, unless otherwise indicated), in which the steady state of active CyclinE-CDK2 is plotted as a function of the CyclinE synthesis rate \( k_1 \). Figure 2, C and D, also shows the corresponding bifurcations vs. \( k_1 \). Depending on the stability of the steady state and the other parameter choices, the system exhibits the following dynamical regimes.

**Regime 1: monotonic stable steady-state solution.** For any CyclinE synthesis rate \( k_1 \), there is only one steady-state solution, and it is stable (Fig. 2A).

**Regime 2: bistable steady-state solutions.** There are three steady-state solutions over a range of \( k_1 \) \((k_1 = 418–612 in Fig. 2B)\): two are stable, and one is a saddle (unstable). As \( k_1 \) is increased from low to high, CyclinE-CDK2 remains low until \( k_1 \) exceeds a critical value, at which point there is a sudden increase in CyclinE-CDK2 (upward arrow in Fig. 2B). When \( k_1 \) decreases from high to low, however, the transition occurs at a differ-
Fig. 2. Steady-state solutions and bifurcations for module \( I \). Parameters are default values in Table 1, unless otherwise indicated. \( A \): steady state of active CycE-CDK2 vs. CycE synthesis rate \( k_1 \), with \( k_2 = 1.0 \). \( B \): steady state of active CycE-CDK2 vs. \( k_1 \), with \( k_2 = 5.0 \). \( C \): steady state and bifurcation of active CycE-CDK2 vs. \( k_1 \), with \( k_2 = 0.5 \). \( D \): steady state and bifurcation of active CycE-CDK2 vs. \( k_1 \), with \( k_2 = 1.25 \). \( E \): free CycE and active CycE-CDK2 vs. time for a limit cycle in \( C \) at \( k_1 = 30.0 \). \( F \): free CycE and active CycE-CDK2 vs. time for an excitable case in \( D \) for \( k_1 = 210.0 \). At \( t = 50.0 \), we held active CycE-CDK2 at 6 for a duration of 0.02 to stimulate the large excursion.

Regime 3: limit cycle solution. The steady state in the parameter window \(( k_1 = 90.0–290.0 \) in Fig. 2C) is an unstable focus, and CycE and CycE-CDK2 oscillate spontaneously (Fig. 2E, with \( k_1 = 150.0 \)). The transition from the stable steady state to the limit cycle is via Hopf bifurcation.

Regime 4: multiple steady-state and limit cycle solutions. There are three steady-state solutions over a range of \( k_1 (k_1 = 192.0–245.0 \) in Fig. 2D): a stable node, a saddle, and an unstable focus. For a range of \( k_1 \) just beyond the triple steady-state solution \(( k_1 = 245.0–320.0 \) in Fig. 2D), a limit cycle solution exists. In this case, the system undergoes a saddle-loop (or homoclinic) bifurcation (54).

Regime 5: excitable transient. Suprathreshold stimulation causes a large excursion that gradually returns to the stable steady state.

Role of CDC25A

The dynamics shown in Fig. 2 are critically dependent on CDC25A phosphorylation. Figure 3 shows steady-state fully phosphorylated CDC25A (Fig. 3A) and total CDC25A (Fig. 3B) vs. active CycE-CDK2. As the number of phosphorylation sites increases, the fully phosphorylated CDC25A increases more steeply and at a higher threshold as active CycE-CDK2 increases. This steep change in CDC25A is critical for instability, leading to limit cycle, bistability, and other dynamical behaviors. When CDC25A had only one phosphorylation site \(( L = 1)\), the steady state was always stable, regardless of other parameter choices (Fig. 4, A and B). It can be demonstrated analytically that the steady state can become unstable and lead to interesting dynamics only when CDC25A has more than one phosphorylation site and requires phosphorylation of both sites to become active (see Appendix). In Fig. 2, we show various dynamics for \( L = 2 \); only biphosphorylated CDC25A is active. In Fig. 4, A and B, we also show bifurcations for \( L = 3 \). When only triphosphorylated CDC25A is active, i.e., \( f(z) = z_3 \) in Eq. 1a, the range of the limit cycle is \( k_1 = 300.0–520.0 \) (Fig. 4A) and the bistability occurs at \( k_1 = 675.0–2,385.0 \) (dashed-dotted line in Fig. 4B), which is a much higher range of \( k_1 \) than for \( L = 2 \). If we assume that bi- and triphos-
phosphorylated CDC25A are equally active, i.e., \( f(z) = (z_2 + z_3)/2 \) in Eq. 1a, the limit cycle (open circle in Fig. 4A) and the bistability (dotted line in Fig. 4B) occur at much lower \( k_1 \), very close to the case of \( L = 2 \).

Figure 4, C and D, shows the effects of CDC25A synthesis rate on limit cycle and bistability. Decreasing the synthesis rate of CDC25A shifts the limit cycle and bistable regions to higher but wider \( k_1 \) ranges. For example, when \( k_8 = 25 \), the limit cycle occurred at \( k_1 = 195-480 \) (Fig. 4C, cf. \( k_1 = 90-290 \) in Fig. 2C), and bistability occurred at \( k_1 = 860-1,400 \) (Fig. 4D, cf. \( k_1 = 418-612 \) in Fig. 2B). In Figs. 2 and 4, we assumed no degradation of phosphorylated CDC25A; i.e., \( k_{10} = 0 \). If we assume that fully phosphorylated CDC25A is degraded at a certain rate (\( k_10 > 0 \)), the limit cycle region is widened and the bistable region is shifted to a higher range of \( k_1 \). For example, the range of the limit cycle regime shown in Fig. 2C was \( k_1 = 90-365 \) and the range of bistability in Fig. 2B was \( k_1 = 475-640 \) after we set \( k_{10} = 5 \).

In Figs. 2 and 4, we assumed that the CDC25A phosphorylation and dephosphorylation rates were fast. If these rates were slow, the steady state did not change and bistable behavior was not altered, but limit cycle behavior was affected. Slowing the phosphorylation and dephosphorylation rates caused narrowing of the \( k_1 \) range over which limit cycle behavior occurs, and eventually limit cycle behavior disappeared (Fig. 4E).

**Role of E2F**

The results presented thus far show that the primary module of the G1-to-S transition by itself exhibits multiple dynamical regimes. We now examine how Rb and E2F (module II in Fig. 1), known to play crucial roles in mammalian cell cycle progression, regulate the dynamics of G1-to-S transition.

With total E2F constant. If there is no E2F synthesis and degradation, i.e., steps 11 and 12 are absent in module II, then the total E2F is constant (for simplicity, we set it equal to total Rb). Figure 5A shows the steady state for free E2F concentration vs. active CycE-CDK2 or CycD-CDK4/6. As the number of Rb phosphorylation sites \( M \) required to free E2F, as well as the total number of phosphorylation sites \( M' \) increases, the threshold for E2F dissociation and the steepness of the response increased.

A steep sigmoidal function due to multisite phosphorylation of Rb as shown in Fig. 5A, coupled with the positive-feedback loop between CycE and E2F, might be predicted to generate instability. To test this, we removed the CDC25A from module I and simulated Eq. 1, \( a-c \), together. We assumed that dephosphorylation of Thr\(^{14} \) and Tyr\(^{15} \) was carried out by a phosphatase (possibly CDC25A) at a constant rate and, thus, set \( k_5 = 1 \) and \( f(z) = 0 \) in Eq. 1a. In this system, we found that bistability could be generated, but no other dynamics such as limit cycle occurred. Figure 5B shows an example of a bistable steady state of active CycE-CDK2 vs. CycE synthesis rate \( k_1 \), with \( (k_{13} = 50) \) or without \( (k_{13} = 0) \) CycD for \( M = 2 \) and \( M' = 16 \). The presence of CycD caused the bistability to occur at lower \( k_1 \), because CycD phosphorylates Rb and frees E2F, which can then promote the E2F-dependent synthesis of CycE. In Fig. 5C, we show the conditions in the \( M - M' \) space under which bistability occurs. This demonstrates that multisite phosphorylation of Rb is critical for the CycE-E2F positive-feedback loop to generate bistability.

We then added the CDC25A function back to module I and simulated Eq. 1, \( a-c \), to study how E2F modulates the dynamics of module I. With module I in the limit cycle regime (Fig. 2C), Fig. 5D shows two bifurcations with \( (k_{13} = 50) \) and without \( (k_{13} = 0) \) CycD. Without CycD, the limit cycle range occurred at \( k_1 = 90-250 \) (compared with \( k_1 = 90-290 \) in Fig. 2C) and a period 2 oscillation occurred at around \( k_1 = 230 \) (Fig. 5D, inset). With CycD, the range decreased to \( k_1 = 75-220 \). We then set module I in the bistability regime as in Fig. 2B. Without CycD (Fig. 5E), the range of the bistable region was \( k_1 = 405-610 \) (compared with \( k_1 = 420-612 \) in Fig. 2B); with a low CycD level \( (k_{13} = 50) \) in Fig. 5E), the range was \( k_1 = 385-598 \); with a high CycD level \( (k_{13} = 200) \) in Fig. 5E), the range was \( k_1 = 320-514 \). Because in our model the CycD module (mod-
ule III) does not include any feedback, it did not lead to any novel dynamics. CycD-CDK4/6 is simply proportional to CycD. The major effect of CycD-CDK4/6 is to phosphorylate Rb and, thus, produce more free E2F. Greater free E2F promotes more CycE synthesis, which causes the Hopf or saddle-node (SN) bifurcations at lower $k_1$. Another effect of CycD-CDK4/6 shown in Fig. 5 is the removal of period 2 oscillation. This can be explained as follows: because CycD-CDK4/6 frees a certain amount of E2F from the Rb-E2F complex, the availability of Rb-E2F for active CycE-CDK2 to phosphorylate is reduced. This makes the steady-state response curve of free E2F vs. CycE-CDK2 less steep than is the case without CycD. The reduction of steepness of the free E2F response to active CycE-CDK2 thus causes the period 2 behavior to disappear. In fact, even in the case of no CycD, if we reduce the hyperphosphorylation sites $(M - M)$ of Rb, this period 2 will also disappear because of the reduction of steepness of the response of free E2F to active CycE-CDK2.

With E2F synthesis and degradation. If we introduce E2F synthesis and degradation (steps 11 and 12) into the E2F-Rb regulation network, then the steady-state concentration of E2F as a function of active CycE-CDK2 is simply determined by steps 11 and 12 and satisfies the following equation

$$k_{11} + k_{11e}g(e_0) - (k_{12} + k_{12e}x)e_0 = 0$$

(2)

If $g(e) = e$, then $e_0 = k_{11}/(k_{12} + k_{12e}x - k_{11e})$. If $k_{11e} > k_{12e}$, no steady-state solution of E2F exists when $x$ is small. If $k_{11e} < k_{12e}$, the steady state is always stable for any $x$ and will always decrease as $x$ decreases. For
g(e) = e/(α + e), the situation is similar. This does not agree with the experimental observation that free E2F is low in G0 or early G1 but high during the G1-to-S transition. With g(e) = e/(α + e), Eq. 2 results in a bistable solution. Figure 6A shows two bistable solutions for k_{11} = 0.02 and 0.1. Symbols in Fig. 6, A and B, are values of free E2F and the total Rb-E2F complex for k_{11} = 0.02 by simulating module II and setting active CycE-CDK2 (x) as a control parameter. In Fig. 6, A and B, x is shown changing from high to low and from low to high, with E2F being initially set on the upper branch. Even at very low free E2F, the complexed Rb-E2F (Fig. 6B) is high when active CycE-CDK2 (x) is low. With the bistability feature of free E2F, the observation that free E2F is low in G0 and early G1 but high during the G1-to-S transition can be explained as follows: in G0 or early G1, free E2F is at the lower branch of the bistable curve and most of the E2F is stored as the Rb-E2F complex. As CycD increases, E2F freed from Rb-E2F brings free E2F into the upper branch.
Alternatively, increasing the E2F synthesis ($k_{11}$) could also shift the bistable region into a higher $x$ range ($k_{11} = 0.1$ in Fig. 6A), causing E2F transit to the upper branch at low $x$.

Except for the bistability generated by the positive feedback of E2F on its own transcription rate, the positive feedback between CycE and E2F does not generate any new dynamics without the CDC25A feedback loop in module I. When the CDC25A feedback loop is present, high E2F increases the transcription of CycE, which causes limit cycle and bistability of module I at lower $k_1$. Figure 6, C and D, shows active CycE-CDK2 and free E2F vs. time for $k_1 = 75$ and $k_{11} = 0.1$ and for $k_1 = 100$ and $k_{11} = 0.1$, respectively, with module I in the limit cycle regime as in Fig. 1C. At $k_1 = 75$, the oscillation is slower and the maximum E2F is much higher than the steady-state value shown in Fig. 6A. At $k_1 = 100$, the oscillation becomes much faster and E2F decays to a much lower level, indicating that the dynamics are governed more by module I.

Role of CKI

Experiments in yeast have shown (33) that six of its nine phosphorylation sites have to be phosphorylated for Sic1 ubiquitination. Although it has been shown that phosphorylation of p27 on Thr-187 by CycE-CDK2 is required for p27 ubiquitination in the mammalian cell cycle (31, 48, 65), whether additional phosphorylation sites may also be important for p27 stability is unknown (19). To assess the possible dynamics caused by multisite phosphorylation, we assume that a certain number of sites have to be phosphorylated (or dephosphorylated) by active CycE-CDK2 for ubiquitination (module IV, Fig. 1, A and D). We first simulated the steady-state responses of fully phosphorylated CKI and total CKI vs. active CycE-CDK2 ($x$). As more phosphorylation sites were assigned to CKI, the fully phosphorylated CKI had a steeper response to $x$ and at a higher threshold (Fig. 7A). The total CKI increased first to a maximum and then decreased to a very low level as $x$ increased (Fig. 7B). This indicates that CKI and CycE-CDK2 were first buffered in the incompletely phosphorylated CKI states. As $x$ increased beyond the threshold, positive feedback caused the sharp decrease of total CKI, which may cause instability and lead to interesting dynamics.

Dynamics caused by CKI phosphorylation by CycE-CDK2. To study the dynamics caused by the positive-feedback loop between CycE-CDK2 and CKI alone, we simulated Eq. 1, a and d, with CDC25A removed from module I ($\beta z = 0$ in Eq. 1a). When CKI had only one phosphorylation site ($N = 1$), the steady state was stable for any $k_1$ (Fig. 7C). When CKI had more than one site ($N > 1$), the steady state became unstable and led to a limit cycle over a range of $k_1$. At larger $N$, the limit cycle occurred at a higher $k_1$ threshold and had a larger oscillation amplitude. In a previous study, Thron (58) showed that the positive-feedback loop between CycE-CDK2 and CKI caused bistability. In our present model, there is no bistability, because the steady state of the active CycE-CDK2 is simply proportional to $k_1$. The difference between our model and Thron’s model is that in the latter the total CycE-CDK2 remained constant, whereas in our model it varied.

![Fig. 6. Role of E2F when total E2F is not constant. $g(e) = e^2/(50 + e^2)$. A: steady-state free E2F vs. active CycE-CDK2 of module II. Dashed line, $k_{11} = 0.02$; solid line, $k_{11} = 0.1$. Circles, simulation results of module II, with $x$ as control parameter: O, $x$ from small to large; C, $x$ from large to small. B: total Rb-E2F vs. $x$ when module II was simulated as described in A. C: active CycE-CDK2 and free E2F vs. time. Simulation was done with modules I and II, with $k_{11} = 0.1$. Parameters for module I are the same as in Fig. 2C, with $k_1 = 75$. D: same as C, with $k_1 = 100$.](image)
CKI modulation of the dynamics of module I. We next investigated how CKI modulates the dynamics of module I by simulating Eq. 1, a and d, with CDC25A in module I. Because the CKI module does not change the steady state of active CycE-CDK2, we studied only the case for module I in the limit cycle regime (Fig. 2C). Figure 7D shows bifurcations for different numbers of total phosphorylation sites on CKI. With one site (N = 1), the limit cycle occurred at k₁ = 97–300 (compare k₁ = 90–290 in Fig. 2C), showing that CKI had a little effect on the dynamics. When N = 2, the limit cycle occurred at k₁ = 112–326, and when N = 6, k₁ = 268–350. Thus increasing the number of phosphorylation sites of CKI caused the instability to occur at a higher k₁ threshold and narrowed the range of the instability.

CKI mutation. Finally, we examined the effects of a simulated mutation of CKI on the dynamics of the G₁-to-S transition. We assume that CKI is mutated so that it cannot bind to F-box protein for degradation by setting k₂₅ = 0 in our simulation. Figure 7E shows a bifurcation diagram for N = 0, 1, and 5 at high and low CKI expression (k₁₈ = 100 and 25, respectively). At high CKI, the steady state was always stable for any N (solid line in Fig. 7E). In other words, the limit cycle dynamics generated by module I were blocked by CKI.

Fig. 7. Effects of CKIs on stability and bifurcations. A: steady-state fully phosphorylated CKI vs. active CycE-CDK2 for N = 1 (1p), 2 (2p), and 6 (6p). Results were obtained from module IV, with x as a control parameter. B: total CKI vs. active CycE-CDK2. C: limit cycle bifurcation generated by CKI and CycE-CDK2 feedback loop. Simulations used modules I and IV with f(z) = 0, k₂ = 0.5, k₅ = 1, and k₇ = 1. D: modulation of dynamics of module I by CKI. Simulations used modules I and IV. Parameters for module I are the same as in Fig. 2C. E: effects of mutating CKI on dynamics of module I. Simulations were done as in D, but with k₂₅ = 0. Low CKI, k₁₈ = 25; high CKI, k₁₈ = 100.
mutation. However, if CKI was low, the limit cycle still occurred for \( N = 0, 1, \) and 5, but the range was narrower with more phosphorylation sites. Contrary to the control case shown in Fig. 7D, the mutation had little effect on the \( k_1 \) threshold of instability.

**DISCUSSION**

We have presented a detailed mathematical model of regulation of the G1-to-S transition of the mammalian cell cycle. Our approach was to divide the full G1-to-S transition model into individual signaling modules (15) and then analyze the dynamics in a stepwise fashion. Our major findings are as follows. 1) Multisite phosphorylation of cell cycle proteins is critical for instability and dynamics. 2) The positive feedback between CycE-CDK2 and CDC25A in the primary module generates limit cycle, bistable, and excitable transient dynamics. 3) The positive feedback between CycE and E2F can generate bistability, provided total E2F is constant and Rb is phosphorylated at multiple sites. 4) The positive feedback between CKI and CycE-CDK2 can generate limit cycle behavior. 5) E2F and CKI modulate the dynamics of the primary module.

Although all the dynamical regimes manifested by the primary module in this study have been described in previous models, there are several important advantages to the present formulation. 1) The full G1-to-S transition model is reasonably complete with respect to incorporating the state of knowledge about experimentally determined physiological details. 2) All the relationships between components were modeled according to biologically realistic reaction schemes, rather than phenomenological representations, as in many prior models. 3) Despite its complexity, we achieved a reasonably complete description of the dynamics of the full model by breaking it down into modules and systematically examining the dynamical consequences of recombining the individual modules. 4) The G1-to-S transition model exhibits a wide range of dynamical behaviors, depending on the parameter choices. This is a powerful aspect, since it provides a wide degree of flexibility for fitting the model to experimental observations. Specifically, experimental perturbations that alter G1-to-S transition features may correspond to transitions between dynamical regimes.

The dynamics responsible for the checkpoint and cell cycle progression at the G1-to-S transition or during the entire cell cycle are not clearly understood (61). A Hopf or an SN bifurcation can mimic the G1-to-S transition or other checkpoint transitions. Here we cannot distinguish unequivocally the dynamics responsible for the G1-to-S transition, so we consider both dynamics as possible candidates and discuss the biological implications of our modeling results.

**CycE Expression and Degradation**

Proper CycE regulation is important for normal cell cycle control. Insufficient CycE results in cell arrest in the G1 phase, whereas overexpression of CycE leads to premature entry into the S phase (41, 42, 44), genomic instability (53), and tumorigenesis (8, 21). In our model (Figs. 3 and 4), insufficient CycE expression keeps CycE-CDK2 activity very low, and the cell remains in the G1 phase. As CycE expression increases, CycE-CDK2 moves into the bistable or limit cycle regimen. As CycE expression further increases, CycE-CDK2 stays stably high. However, CycE-CDK2 has to be downregulated for stable DNA replication (43). Therefore, the stable high CycE-CDK2 caused by overexpression of CycE might be the cause of genomic instability and tumorigenesis.

Our simulations show that high degradation of free CycE and CycE bound to CDK2 makes active CycE-CDK2 very low, whereas a low degradation rate keeps CycE-CDK2 stably elevated. Recent studies (22, 30, 55) showed that the failure to degrade CycE stabilized CycE-CDK2 activity and was tumorigenic, similar to overexpression of CycE.

**CDC25A**

CDC25A is a key regulator of the G1-to-S transition and is highly expressed in several types of cancers (6, 7). Overexpression of CDC25A accelerates the G1-to-S transition (5). It is a target of E2F and is required for E2F-induced S phase (64). It is also the key regulator of the G1 checkpoint for recognizing DNA damage (4, 11, 29). CDC25A is downregulated and, thus, delays the G1-to-S transition. In our modeling study, CDC25A is required for limit cycle and bistability. At low CDC25A expression levels, these dynamics require a high CycE synthesis rate (Fig. 4C). In other words, overexpression of CDC25A makes the dynamics occur at a lower CycE synthesis rate or stabilizes CycE-CDK2 at a high level. These results may explain the experimental observations described above.

**Role of CKI**

Overexpression of CKIs, such as p27, causes G1 cell cycle arrest (50). According to our model, the presence of CKIs makes the limit cycle occur at higher CycE synthesis rates, which agrees with the observation that G1 cell cycle arrest by p27 can be reversed by overexpression of CycE (26). Mutation of CKI so that it cannot be degraded is predicted in the model to prevent the limit cycle regime in the CycE-CDK2-CDC25A network and to maintain active CycE-CDK2 at a high level. This agrees with the experimental observation that overexpression of nondegradable CKI permanently arrests cells in G1 (33, 46, 63).

**E2F-Rb Pathway**

Rb was the first tumor suppressor identified. Blocking Rb’s action shortens the G1 phase, reduces cell size, and decreases, but does not eliminate, the cell’s requirement for mitogens (49). According to our simulations, if total E2F is conserved, E2F-Rb has little effect on the threshold for limit cycle and bistability when CycD-CDK4/6 is absent. However, when CycD-CDK4/6 is present, the threshold for these regimes shifts to a lower CycE synthesis rate (\( k_1 \)). If we increase Rb syn-
thesis or reduce E2F, these bifurcations occur at higher
$k_1$, and vice versa if Rb synthesis is reduced or E2F
synthesis is increased. These simulation results agree
with the observation that overexpressing E2F acceler-
ates the G1-to-S transition, whereas overexpression of
Rb delays or blocks the G1-to-S transition. One impor-
tant consequence is that overexpressing E2F or delet-
ing Rb causes CycE-CDK2 to remain stably high,
which may promote tumorigenesis.

Importance of Multisite Phosphorylation

Multisite phosphorylation is common in cell cycle
proteins. Multisite phosphorylation has two effects: it
sets a high biological threshold and causes a steep
response (33). A steep response is well known to be
critical for instability and dynamics in many systems,
including cell cycle models, and here we identify mul-
tsite phosphorylation as the biological counterpart re-
ponsible for this key feature. In our G1-to-S transition
model, the elements involved in feedback loops,
CDC25A, Rb, and CKI, had to be phosphorylated at
two or more sites to become active. For Rb, an even
greater number was required. A caveat for CDC25A is
that, in our model, we assumed that the dephosphory-
lations of Thr$^{14}$ and Tyr$^{15}$ occurred simultaneously.
If we assume that these dephosphorylations occur
sequentially, then first-order phosphorylation of
CDC25A may be enough to generate the dynamics.
Nevertheless, experiments have shown that CDC25 is
highly phosphorylated during the cell cycle and has
multiple phosphorylation sites (17, 25, 32). To our
knowledge, the point that multisite phosphorylation
may be the biological mechanism critical for cell cycle
dynamics has not been explicitly appreciated.

Summary and Implications

Although without additional experimental proof we
cannot identify the specific dynamical regime(s) in-
volved in the G1-to-S transition, the model described in
our study provides a means to approach this goal
systematically. The model is consistent with most of
the available experimental observations about the G1-
to-S transition, including the checkpoint dynamics reg-
ulating the G1-to-S transition under physiological con-
ditions and the loss of checkpoint under certain
pathophysiological conditions, and the cycllical changes
in G1-to-S transition cell cycle proteins. In concert with
experimental approaches to define more precisely the
dynamical regimes under which this physiologically
detailed G1-to-S transition model operates, the next
major goal is to develop analogously detailed models
for the G2-to-M and other transitions. These models
can then be coupled to reconstruct a complete formu-
lation of the mammalian cell cycle as a modular sig-
aling network, the underlying dynamical behavior of
which has been thoroughly investigated.

Limitations

In this study, we constructed a network model de-
scribing regulation of the G1-to-S transition and ana-
lyzed its dynamics and the biological implications.
However, there are some limitations in our study. The
parameters were chosen arbitrarily to investigate pos-
sible dynamical behaviors of the model and were not
based on any experimental data. There are so many
parameters in this model that it is impossible for us to
analyze the model completely, and this may prevent us
from identifying other dynamics, such as high period-
icty and chaos. There are other regulatory interact-
ations, such as wee1 phosphorylation (52) by active CDK
and CDC25 phosphorylation by enzymes other than
active CDK (20), which we did not incorporate into our
model. These interactions may have new consequences
to the dynamics of the G1-to-S transition. Another
ceaveat of this study is that we assumed that cell cycle
proteins were distributed uniformly throughout the
cell, but actually they distribute nonuniformly and
dynamically inside the cell (56), which should be ad-
dressed in future studies.

APPENDIX

If we assume that steps 3 and 4 occur very fast, i.e., $k_3$ and
$k_4$ are very large, we can remove $x_1$ from Eq. 1a. In addition,
if we assume that phosphorylation and dephosphorylation of
CDC25A is also very fast, we can treat CDC25A as a function of
active CycE-CDK2 ($x$). We have the following two-variable
simple model for module $I$

\[
\begin{align*}
\dot{x} &= [k_5 + f(x)]y - k_2x - k_2x
\end{align*}
\]

\[
\begin{align*}
\dot{y} &= k_1 - [k_5 + f(x)]y + k_2y - k_2y
\end{align*}
\]

where $f(x)$ represents the catalytic effect of phosphorylated
CDC25A. If we assume that $k_{10} = 0$, then from Eq. 1a

\[
\begin{align*}
A &= \sum_{i=1}^{4} \sigma_i z_i = \sum_{i=1}^{4} \sigma_i \frac{(b_i + c_i x) k_i}{a_i^2 k_i}
\end{align*}
\]

By setting $\dot{x} = 0$ and $\dot{y} = 0$, the steady-state solution $(x_0, y_0)$
of Eq. $A1$ satisfies

\[
\begin{align*}
x_0 &= \frac{(k_3 + k_4 x_0)}{k_1 + f(x_0)} \quad k_1 = [k_2 + k_3 + f(x_0)] y_0 - k_6 x_0
\end{align*}
\]

Following the standard method of linear stability analysis
(54), we have for the steady state of Eq. $A1$

\[
\begin{align*}
\lambda_1 &= -\alpha + \sqrt{\frac{(\alpha^2 - 4\beta)}{2}}
\end{align*}
\]

\[
\begin{align*}
\lambda_2 &= -\alpha - \sqrt{\frac{(\alpha^2 - 4\beta)}{2}}
\end{align*}
\]

where $\lambda_1$ and $\lambda_2$ are the two eigenvalues from the linear
stability analysis and $\alpha$ and $\beta$ are

\[
\begin{align*}
\alpha &= k_2 + k_3 + k_4 + f(x_0) - f_\alpha y_0 \\
\beta &= k_2[k_6 + f(x_0) + k_2 - f_\alpha y_0]
\end{align*}
\]

\[
\begin{align*}
f_{\alpha} = df(x)/dx |_{x=x_0} \quad \text{Three types of steady-state behaviors de-}
\end{align*}
\]

\[
\begin{align*}
\text{pend on the values of $\alpha$ and $\beta$:}
\end{align*}
\]

\[
\begin{align*}
1) \quad (\alpha^2 - 4\beta) < 0. \quad \text{The eigenvalues are a pair of conjugate}
\end{align*}
\]

\[
\begin{align*}
\text{complex numbers. The steady state is a focus. When $\alpha > 0$, it is}
\end{align*}
\]

\[
\begin{align*}
\text{a stable focus. When $\alpha < 0$, it is an unstable focus.}
\end{align*}
\]

\[
\begin{align*}
2) \quad (\alpha^2 - 4\beta) > 0 \text{ but $\beta > 0$, the steady state is a node. When}
\end{align*}
\]

\[
\begin{align*}
\alpha > 0, \text{ it is a stable node. When $\alpha < 0$, it is an unstable node.}
\end{align*}
\]

\[
\begin{align*}
3) \quad (\alpha^2 - 4\beta) > 0, \text{ but $\beta < 0$, for whatever $\alpha$, one of the two}
\end{align*}
\]

\[
\begin{align*}
\text{eigenvalues is positive. Then the steady state is a saddle.}
\end{align*}
\]

For $f(x) = ax$ or $ax(b + x), (k_0 + k_7 - f_\alpha y_0)$ in Eq. $A5$
becomes $k_0(k_6 + k_7)[k_5 + f(x_0)]$ or $(k_6 + k_7)[k_5 + ax^2(b +
Fig. 8. Phase diagrams in various parameter spaces for simplified model in the APPENDIX. BS, region in which 2 of the triple steady-state solutions are stable; TS, region of triple steady-state solutions in which 1 is a stable node, 1 is a saddle, and 1 is an unstable focus; LC, region of limit cycle. Unmarked regions have only steady-state solution that is stable. Solid lines, SN bifurcation; dashed lines, H bifurcation. In region between 2 dashed lines, steady state or 1 of the steady states is an unstable focus. In region between 2 solid lines, triple steady-state solutions exist. A: $k_6 = 1, k_7 = 9$. B: $k_5 = 0.1, k_6 = 1$. C: $k_5 = 1, k_7 = 9$. D: $k_2 = 0.5, k_7 = 9$. 

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parameters of the rate constants and for phosphorylation sites to create a higher-order response in the system. Therefore, to cause instability in another nullcline from Eq. A1, we show in Fig. 8 the phase diagram for various parameters of the rate constants and for $f(x) = (b_0 + c_0x^2) \alpha k_2 k_3 k_4 \sin \theta$. Limit cycle occurred at small $k_2$ and large $k_7$, whereas bistability occurred at large $k_2$. This can intuitively be understood as follows. If we let $x = y + z$ and combine the two equations in Eq. A1, we have $\dot{z} = k_1 (x - k_2 x) - k_2 z$. The nullcline for this equation is $z = k_1 k_2 / (k_1 - k_2) x$; another nullcline from Eq. A1 is $z = (k_2 + k_3 x) / (k_1 + k_4 x) + x$. The second nullcline is a typical N-shaped curve; the first is a straight line. For these curves to have three intersections, $k_1 k_2$ and $k_1 k_4$ have to be in a certain range.

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