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Negative feedback and ultrasensitivity can bring about oscillations in the mitogen-activated protein kinase cascades

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Functional organization of signal transduction into protein phosphorylation cascades, such as the mitogen-activated protein kinase (MAPK) cascades, greatly enhances the sensitivity of cellular targets to external stimuli. The sensitivity increases multiplicatively with the number of cascade levels, so that a tiny change in a stimulus results in a large change in the response, the phenomenon referred to as ultrasensitivity. In a variety of cell types, the MAPK cascades are imbedded in long feedback loops, positive or negative, depending on whether the terminal kinase stimulates or inhibits the activation of the initial level. Here we demonstrate that a negative feedback loop combined with intrinsic ultrasensitivity of the MAPK cascade can bring about sustained oscillations in MAPK phosphorylation. Based on recent kinetic data on the MAPK cascades, we predict that the period of oscillations can range from minutes to hours. The phosphorylation level can vary between the base level and almost 100% of the total protein. The oscillations of the phosphorylation cascades and slow protein diffusion in the cytoplasm can lead to intracellular waves of phospho-proteins.

Keywords: signal transduction; protein phosphorylation; MAPK cascades; bistability; sustained oscillations.

The mitogen-activated protein kinase (MAPK) cascades are widely involved in eukaryotic signal transduction, and these pathways are conserved in cells from yeast to mammals (reviewed in [1,2]). The MAPK cascades relay extracellular stimuli from the plasma membrane to targets in the cytoplasm and nucleus, initiating diverse responses involving cell growth, mitogenesis, differentiation and stress responses in mammalian cells. The MAPK pathway consists of several levels (usually three), where the activated kinase at each level phosphorylates the kinase at the next level down the cascade. MAPKs are the kinases of the terminal level of the cascades (Fig. 1). The MAPKs are activated by the MAPK kinases (MKKs) that phosphorylate MAPKs at two sites, conserved threonine and tyrosine residues. Dephosphorylation of either residue is thought to inactivate MAPKs, and mutant kinases lacking either residue are almost inactive. At one level upstream, MKKs are themselves phosphorylated at serine and threonine residues by the MAPK kinase kinases (MKKKs). The kinases of the first level, MKKKs, are activated by several mechanisms involving (in the case of Raf) phosphorylation at a tyrosine residue. At each cascade level, the protein phosphatases inactivate the corresponding kinases (Fig. 1).

One physiological function of kinase cascades could be amplification of a signal, in the sense that a small number of signaling molecules causes the conversion of a large number of target molecules. However, this type of amplification was shown to be quite modest in the MAPK cascade of *Xenopus* eggs, and the signal transfer from MKK (MEK1) to p42 MAPK even caused the amplification to decrease [3]. In contrast, the signal propagation through the MAPK pathway brings about a remarkable increase in the sensitivity of the target to the signal: a graded stimulus is converted into a sigmoidal or 'ultrasensitive' switch-like response [3–6]. Ultrasensitivity arises from the property of phosphorylation cascades to multiply the responses of individual levels into the overall cascade response [4], which in *Xenopus oocytes* extracts is described by a Hill curve with a Hill coefficient of 5 [3].

A simple linear phosphorylation cascade can already exhibit the ultrasensitive response to the signal. Yet, positive feedback from the bottom to the top of a cascade increases dramatically the steepness of the response [4]. In fact, in *Xenopus* oocytes the MAPK cascade (Mos/MEK1/p42 MAPK) was found to be embedded in a positive feedback loop [7,8]. The activation of Mos results in the activation of p42 MAPK, but in turn, p42 MAPK brings about stimulation of Mos. The positive feedback is active only in intact cells and it does not operate in oocyte extracts. In the absence of feedback, the response of p42 MAPK to Mos is already sigmoidal or ultrasensitive, but the positive feedback increases a Hill coefficient from 5 to more than 35, producing essentially an 'all-or-none' response [7,8]. Importantly, positive feedback can endow a cascade with another remarkable property, i.e. bistability or the coexistence of two different stable steady states [7–10]. When the signal increases (or decreases) over the trigger value only transiently, bistability allows the system to switch to an alternative steady state, at which it may remain, when the signal returns to its initial value.

In mammalian cells, one of the best characterized signal transduction pathways links the activation of receptor tyrosine kinases (RTKs) to the MAPK cascades. In response to mitogenic stimuli, phosphorylated RTKs complexed with adapter proteins (Grb2 or Shc-Grb2), recruit the cytoplasmic guanine nucleotide exchange protein Son of Sevenless homolog protein (SOS) to the cell membrane, where it activates the small

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Abbreviations: MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MKKK, MKK kinase; MKKKK, MKKK kinase; RTK, receptor tyrosine kinase; Grb2, growth factor receptor binding protein 2; Shc, src homology and collagen domain protein; SOS, Son of Sevenless homolog protein; ERK, extracellular signal regulated kinase.

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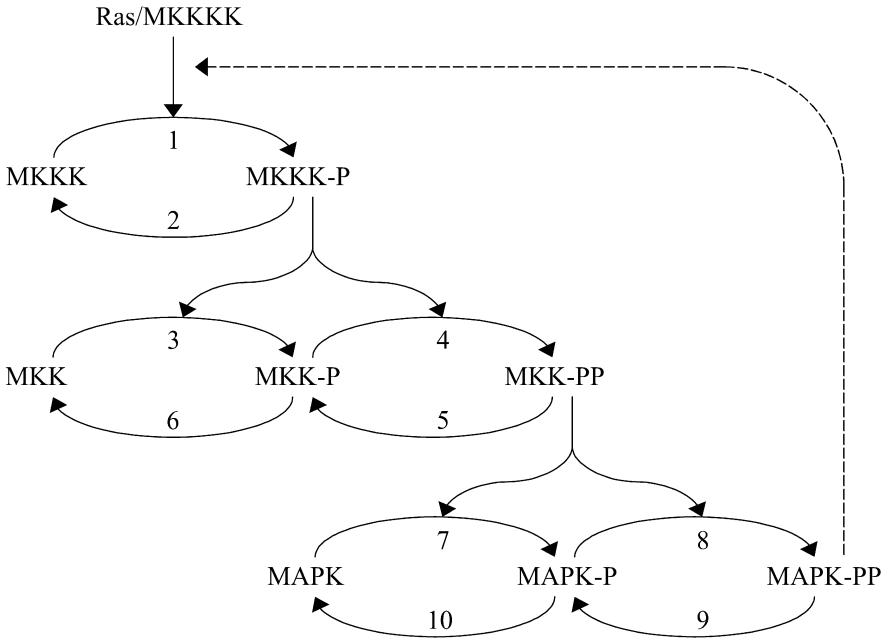

Fig. 1. Kinetic scheme of the MAPK cascade. Feedback effect of MAPK on the rate of MKKK phosphorylation is shown schematically by the dashed line. Numbering of individual steps corresponds to kinetic equations in Tables 1 and 2.

membrane-bound GTPase Ras. SOS catalyzes the conversion of Ras from its inactive GDP-bound state to the active GTP-bound state. Ras-GTP promotes the activation of Raf, the first protein kinase of the MAPK cascade by recruiting Raf to the plasma membrane, where Raf is phosphorylated at a tyrosine residue by an unknown protein kinase [1,11,12]. Thus, membrane-bound Ras together with the unknown membrane kinase (playing a role of MKKKK) control Raf (i.e. MKKK) activity [13].

Inhibitory phosphorylation of SOS by p42/p44 MAPK (an extracellular signal regulated kinase; ERK) provides a mechanism for switching off Ras signaling [14–16]. This inhibition creates a negative feedback in the MAPK cascade, as shown schematically in Fig. 1. Indeed, whereas tyrosine-phosphorylated Raf brings about ERK activation, ERK-mediated inhibition of Raf stimulation by SOS decreases ERK phosphorylation. In this paper, we demonstrate that the combination of a negative feedback and ultrasensitivity can bring about yet another property of the MAPK cascades, sustained biochemical oscillations. We analyze this in two ways: first, as the general emerging property of phosphorylation cascades embedded into a negative feedback loop and second, using a computational model which takes into account the available information about the kinetics of MAPK cascade reactions. The hypothesis about MAPK cascade oscillations is awaiting experimental verification.

MATERIALS AND METHODS

Kinetic modeling

The quantitative computational model of the MAPK cascade used here resembles the model developed by Huang and Ferrell [3], but involves explicitly a negative feedback from MAPK-*PP* to the MKKK activating reaction (as shown schematically in Fig. 1). The time-dependent behavior of the MAPK cascade is described by a set of differential kinetic equations derived from the reaction scheme (Fig. 1) and presented in Table 1. Three moiety conservation relations derived from the stoichiometry, correspond to the total concentrations of MKKK, MKK and MAPK (Table 1).

Rate equations

In vitro enzymatic studies have demonstrated that dual-specificity kinases (MEK1) follow the Michaelis–Menten mechanism. Monophosphorylated products are released into the solution, from which they interact with a new enzyme molecule [17]. Because only biphosphorylated kinases are fully active [18], both dual-specificity protein tyrosine phosphatases (e.g. VHR [19]) and single specificity phosphatases are able to inactivate the MAPK cascade kinases. Table 2 presents the rate expressions of the reactions of the MAPK cascade. The total concentrations of MKKK, MKK and MAPK were reported to be in the range 10–1000 nM ($[MKKK]_{total}$ is less than $[MKK]_{total}$, and the latter is roughly equal to $[MAPK]_{total}$), and the K_m values for kinase phosphorylation were estimated to be in the same range [3,10,17,20]. Estimates for the k_{cat} values of the protein kinases and phosphatases range from 0.01 to 1 s^{-1} [3,10,21,22]. Table 2 lists the assumed values for the K_m, k_{cat} and V_{max} and the total concentrations of the kinases. We

Table 1. Kinetic equations comprising the computational model of the MAPK cascade.

$d[MKKK]/dt = v_2\text{-}v_1$
$d[MKKK\text{-}P]/dt = v_1\text{-}v_2$
$d[MKK]/dt = v_6\text{-}v_3$
$d[MKK\text{-}P]/dt = v_3 + v_5 - v_4 - v_6$
$d[MKK\text{-}PP]/dt = v_4 - v_5$
$d[MAPK]/dt = v_{10}\text{-}v_7$
$d[MAPK\text{-}P]/dt = v_7 + v_9 - v_8 - v_{10}$
$d[MAPK\text{-}PP]/dt = v_8 - v_9$

Moiety conservation relations:
$[MKKK]_{total} = [MKKK] + [MKKK\text{-}P]$
$[MKK]_{total} = [MKK] + [MKK\text{-}P] + [MKK\text{-}PP]$
$[MAPK]_{total} = [MAPK] + [MAPK\text{-}P] + [MAPK\text{-}PP]$

Table 2. Rate equations and parameter values of the MAPK cascade model. Concentrations and the Michaelis constants (K_1–K_{10}) are given in nM. The catalytic rate constants (k_3, k_4, k_7, k_8) and the maximal enzyme rates (V_1, V_2, V_5, V_6, V_9, V_{10}) are expressed in s^{-1} and nM·s^{-1}, respectively.

Reaction number	Rate equation	Parameter values
1	V_1·[MKKK]/((1 + ([MAPK-*PP*]/K_I)^n)·(K_1 + [MKKK]))	$V_1 = 2.5$; $n = 1$; $K_I = 9$; $K_1 = 10$;
2	V_2·[MKKK-*P*]/(K_2 + [MKKK-*P*])	$V_2 = 0.25$; $K_2 = 8$;
3	k_3·[MKKK-*P*]·[MKK]/(K_3 + [MKK])	$k_3 = 0.025$; $K_3 = 15$;
4	k_4·[MKKK-*P*]·[MKK-*P*]/(K_4 + [MKK-*P*])	$k_4 = 0.025$; $K_4 = 15$;
5	V_5·[MKK-*PP*]/(K_5 + [MKK-*PP*])	$V_5 = 0.75$; $K_5 = 15$;
6	V_6·[MKK-*P*]/(K_6 + [MKK-*P*])	$V_6 = 0.75$; $K_6 = 15$;
7	k_7·[MKK-*PP*]·[MAPK]/(K_7 + [MAPK])	$k_7 = 0.025$; $K_7 = 15$;
8	k_8·[MKK-*PP*]·[MAPK-*P*]/(K_8 + [MAPK-*P*])	$k_8 = 0.025$; $K_8 = 15$;
9	V_9·[MAPK-*PP*]/(K_9 + [MAPK-*PP*])	$V_9 = 0.5$; $K_9 = 15$;
10	V_{10}·[MAPK-*P*]/(K_{10} + [MAPK-*P*])	$V_{10} = 0.5$; $K_{10} = 15$;
Total concentrations: $[MKKK]_{total} = 100$; $[MKK]_{total} = 300$; $[MAPK]_{total} = 300$		

initially assumed the lowest estimates for the K_m values (Table 2). We subsequently varied these values over a 10-fold range to analyze numerically the conditions under which the oscillations may or may not occur (see below).

RESULTS

Emerging properties of phosphorylation cascades with feedback loops.

Steady-state properties. Kinase cascades (e.g. MAPK cascades) include several cycles, where each cycle consists of two or more interconvertible forms of some kinase (e.g. a dephosphorylated, monophosphorylated and dual-phosphorylated form, which is assumed to be fully active). There is a simple way to quantify how sensitive the response of a cascade is. The overall cascade sensitivity (R) equals the steady-state fractional change in the level of the activated terminal kinase (E_n, e.g. MAPK) divided by the fractional change in the signal (E_0, e.g. MKKKK),

$$R = \mathrm{d}\ln[E_n] / \mathrm{d}\ln[E_0] \quad (1)$$

The overall sensitivity R is essentially equal to the percentage change in E_n brought about by a 1% change in E_0 [23]. If R is much greater than 1, the response curve (E_n versus E_0) is steeply sigmoidal. If the response is approximated by the Hill equation, R can be related to a Hill coefficient.

Within a cascade, the response of an individual level (E_i, the concentration of activated kinase) to the preceding level (E_{i-1}, the corresponding kinase kinase) can be quantified as above, just as if there were a single level cascade, $r_i = \mathrm{d}\ln[E_i]/\mathrm{d}\ln[E_{i-1}]$. We will call r_i the local sensitivity of level i to the immediately preceding level $i - 1$. This simply quantifies the sensitivity of the phosphorylation of some kinase (e.g. MKK) to the phosphorylation of the kinase kinase (MKKK). From the chain rule of differentiation, it then follows that the overall sensitivity of a cascade with no feedback (R) is equal to the product of the local sensitivities at each level [4,24]. For example, for a cascade with three levels, $R = r_1 \cdot r_2 \cdot r_3$. Therefore, if the sensitivity at each level is more than 1, than merely having more levels can greatly increase the overall sensitivity of a cascade to the stimulus.

How feedback changes steady-state responses. When the terminal kinase (E_n, e.g. MAPK) immediately or indirectly affects some reaction(s) at the initial cascade level, this is referred to as a positive or negative feedback depending on whether E_n enhances or inhibits the activation of the initial kinase (E_1, e.g. MKKK). The strength of the feedback can be quantified as the percentage change in the reaction rate brought about by a 1% change in E_n. As shown in Fig. 1, we assume that the initial level consists of two interconvertible kinase forms, inactive (MKKK) and active (MKKK-*P*), and that the rate of the activation conversion (v_1, catalysed by MKKKK) is affected by E_n. Then, the feedback strength is given by:

$$f = \mathrm{d}\ln v_1/\mathrm{d}\ln[E_n] \quad (2)$$

In the case of activation, f is positive, and in the case of inhibition, f is negative. If a phosphorylation cascade is embedded into a feedback loop, the overall sensitivity (R_f) changes dramatically compared to the same cascade with no feedback (R) [4,25],

$$R_f = R / (1 - f \cdot R) \quad (3)$$

From this equation, it then follows that a negative feedback ($f < 0$) decreases the overall sensitivity of a cascade, whereas a positive feedback ($f > 0$) brings about an increase in the sensitivity, as indeed was observed in *Xenopus* eggs [7,8].

Dynamic properties

Positive feedback: bistability and switches. A phosphorylation cascade with a strong positive feedback can have three different steady states, stable 'off' and stable 'on' states with low and high phosphorylation levels, respectively, separated by an unstable state that corresponds to a threshold level [7]. When the kinase activity E_0 exceeds the threshold, this triggers the feedback and the cascade switches to its on state. If the cascade with no feedback was already sensitive to the stimulus ($R > 1$), then from Eqn (3) it follows that the steady-state sensitivity R_f can be huge ($R_f \gg 1$, as $f > 0$). Importantly, the term $f \cdot R$ cannot be more than 1 and, therefore R_f could not become negative at any stable steady state. The reason for this is that prior to an increase in $f \cdot R$ over 1, the corresponding stable state merges with an unstable state leaving the system with only one stable state, where $f \cdot R$ is less than 1. Such an abrupt change in the system's dynamic picture (in this case disappearing of bistability) is called bifurcation.

Negative feedback can bring about oscillations in the kinase activities. Following a hormone-induced activation of the initial kinase and a subsequent activation of the terminal cascade

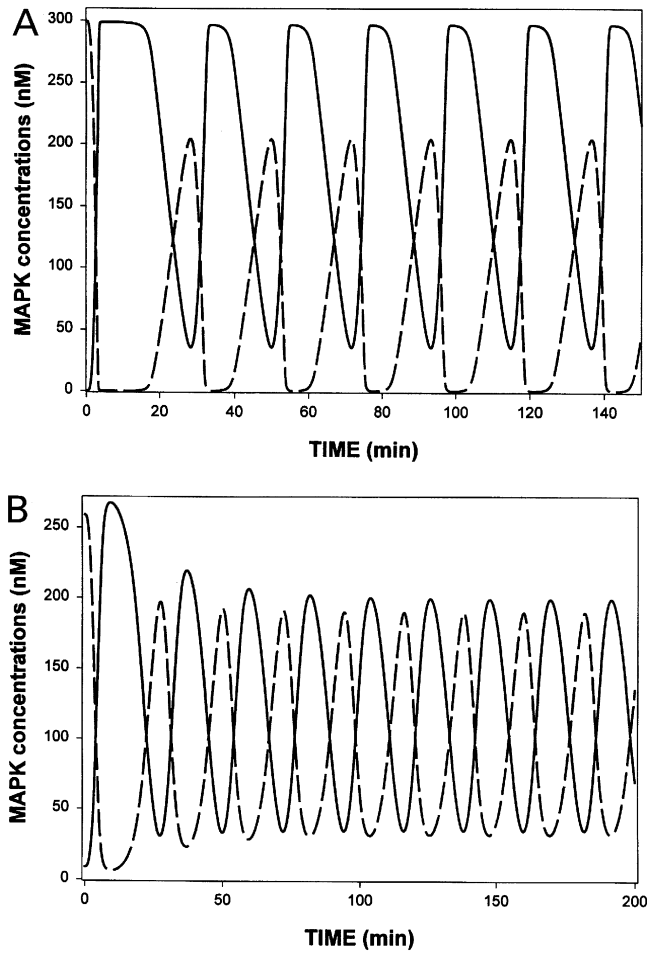

Fig. 2. Oscillations in the MAPK concentrations. At zero time point, the input stimulus (V_1, Table 2) increased from the basal level of 5% to 100% (2.5 nM·s^{-1}). Solid line, active biphosphorylated MAPK-*PP* (ERK-*PP*); dashed line, inactive unphosphorylated MAPK (ERK). (A) The kinetic parameters are listed in Table 2. (B) 'Cooperative' inhibition ($n = 2$) of the activation of the initial kinase by MAPK-*PP*. $K_I = 18$, $K_1 = 50$, $K_2 = 40$, $K_3 = K_4 = K_5 = K_6 = K_7 = K_8 = K_9 = K_{10} = 100$, $V_9 = V_{10} = 1.25$. Other kinetic parameters are the same as in (A).

kinase, a strong negative feedback can be operational in turning off the activation of a cascade. This dynamic picture corresponds to a transient response to a stimulus, as opposed to a sustained activation [26]. Yet, implementation of a strong negative feedback may have effects other than turning off the cascade response. The Appendix shows that a threshold increase in the feedback strength causes the system steady state to lose its stability. Not only is there no other stable state, but the phosphorylation level of cascade kinases starts to oscillate in a sustained manner. This dramatic change in the system's dynamic behavior is known as a Hopf bifurcation. Sustained oscillations are an emerging property of ultrasensitive cascades with negative feedback, as there is always a range of kinetic constants in which oscillatory behavior is observed. Therefore, the question arises whether the oscillations occur in MAPK cascades of a living cell, and if so, how the occurrence and disappearance are regulated. To answer this question we engage in numerical analysis of the dynamics of the MAPK cascades.

Sustained oscillations in MAPK cascades

Using the computational model (see Materials and methods), we calculated the time course of the active and inactive forms of the MAPK cascade kinases following an abrupt increase in the input stimulus (i.e. active MKKKK concentration) at zero time point. At low basal stimulus (a 5% activation of MKKKK), the kinases of the cascade remained predominantly in the inactive forms, and the corresponding steady state (off state) was stable. A threshold stimulus switches the kinases into the active forms. However, the on state with high activities appeared to be unstable at the assumed values for kinetic parameters (Table 1). The cascade kinases did not remain phosphorylated for a prolonged period of time (Fig. 2). Due to the negative feedback from MAPK-*PP*, the rate of activating phosphorylation of MKKK (Fig. 1, step 1) decreased with an increase in MAPK-*PP*. As the phosphatase continues to operate (step 2), the rate of MKKK-*P* dephosphorylation began to exceed the phosphorylation rate and the concentration of MKKK-*P* decreased. A decrease in the MKKK-*P* caused the kinase activity down the cascade (MKK) to drop. Finally, the concentration of MAPK-*PP* decreased, and a new oscillation cycle began. Figure 2A illustrates this dynamics and demonstrates that the amplitude of oscillation in the concentrations of the active biphosphorylated MAPK-*PP* (ERK-*PP*) and inactive MAPK (ERK) can be large. The period of oscillation is about 20 min, but it can be in the range 2–100 min within the range of kinetic parameters available in literature. Importantly, the oscillations appear to be stable to changes in the initial distribution of active and inactive kinase forms (random variations in the phosphorylation levels between 1 and 90% of the total kinase concentrations were tested). Therefore, if the initial conditions differ from those used in Fig. 2A, the variation will be observed for only one or two oscillation cycles; the sustained oscillations will be the same.

One of the reasons for the MAPK cascade ultrasensitivity is partial saturation of the kinases and phosphatases by their substrates [4,5,27]. We initially assumed the total kinase concentrations to be significantly greater than the K_m values for their phosphorylation/dephosphorylation. The total concentration/K_m ratio for MKKK, MKK and MAPK was 10, 20 and 20, respectively (Table 2). When this ratio was decreased substantially, the oscillations disappeared and the stable on state appeared in our calculations. Importantly, stronger feedback inhibition could restore the oscillations. The computational model describes the negative feedback loop from MAPK as noncompetitive inhibition of MKKK phosphorylation. However, dual serine/threonine phosphorylation of SOS by ERK providing a negative feedback [14,15] can be roughly equivalent to a cooperative inhibition that rises as the square of the inhibitor concentration, $V_1/(1 + ([ERK]/K_I)^2)$. Figure 2B illustrates that with the stronger negative feedback, sustained oscillations were observed at the total kinase concentration/K_m ratio of 2–3 (with the K_m values of 100 nM). Interestingly, the period of oscillations did not change significantly, and the oscillation amplitude decreased slightly. This numerical study suggests a rather wide range of kinetic parameters and the conditions under which sustained oscillations might occur in MAPK cascades.

DISCUSSION

Oscillations in cellular biochemical pathways were discovered more than 30 years ago in cell-free extracts and in suspensions

of intact yeast cells [28–30]. In many pathways, the major source of oscillations is a negative feedback loop due to time delays in feedback circuits [31,32]. Negative feedback has been suggested to define a circadian clock [33,34] and to cause the mitotic oscillations [35]. Along with the negative feedback loop, the network of protein interactions that controls the cell cycle involves additional oscillatory mechanisms such as the positive feedback loops involved in autocatalytic activation of M-phase promoting factor [36,37]. The present paper demonstrates that the negative feedback loop in the MAPK cascades of mammalian cells can bring about sustained oscillations in the kinase activities, e.g. in ERK activity. The activation of the kinase cascades is initiated at the cell membrane and then transferred further into the cell at subsequent levels of the cascade. Sustained oscillations in the kinase activities and slow protein diffusion in the cytosol may result in traveling waves of phospho-proteins in the cell and this may be one reason for the existence of cascades [22].

Cells process and encode external information in terms of the temporal and spatial pattern of the activation of signaling proteins [38]. For many cell types, the temporal pattern of ERK activation was shown to determine the fate of the cell (reviewed in [26]). For example, transient activation of ERK by epidermal growth factor, mediated by Ras/Raf/MEK/ERK pathway, stimulates proliferation of PC12 cells, whereas a sustained activation of ERK causes these cells to differentiate [26,39]. The reason for this difference is that sustained ERK activation leads to translocation of ERKs to the nucleus, whereas transient activation does not cause massive nuclear translocation. Depending on their period, sustained oscillations of ERK activity may mimic either transient or sustained activation, the difference being critical for cell signaling decisions. It should be noted that instabilities in the MAPK cascades may affect other signaling pathways, giving rise to oscillatory behavior at different levels, including gene expression.

Emerging evidence indicates that the MAPK cascade kinases can bind to scaffolding proteins, known as MP1 and JIP-1 in mammalian cells (reviewed in [40]). Scaffold proteins bring together the cascade kinases for selective activation and localization, thereby reducing 'cross-talk' between different signaling pathways. Moreover, the organization of the MAPK module by a scaffold may well change the system's kinetics. When a kinase and its kinase kinase are bound to a scaffold, the activated kinase kinase may phosphorylate the downstream kinase without diffusion in the bulk aqueous phase, effectively decreasing the reaction order. This reduction in the system nonlinearity can lead to elimination of sustained oscillations and this may be one reason that scaffolds exist.

The observation of sustained oscillations in a cell population implies some mechanism of synchronization. For glycolytic oscillations in yeast cells, such a mechanism was identified as the extracellular acetaldehyde concentration that couples individual cell oscillations [41]. Whether some synchronization mechanism exists for MAPK oscillations remains unknown. Therefore, experimental verification of possible oscillations in MAPK phosphorylation will require examination of individual cells.

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APPENDIX

Without loss of the generality we consider a cascade of cycles, where each cycle consists of two interconvertible forms of a kinase, the active phosphorylated form E_i and inactive dephosphorylated form $\boldsymbol{E}_i^{\mathrm{I}}$ (the conclusions below are valid for more than two forms, but the proof becomes more complicated). The dynamic behavior of a cascade is described by the following system of nonlinear differential equations:

$$\mathrm{d}E_i/\mathrm{d}t = v_i - w_i, v_i = E_{i-1}\cdot u_i \; i = 1, \ldots, n \qquad \text{(A1)}$$

Here v_i and w_i are the rates of the activating and inactivating conversions (i.e. the protein kinase and protein phosphatase reactions), u_i and w_i depend on the concentrations E_i and $\boldsymbol{E}_i^{\mathrm{I}}$. The rate of phosphorylation of the initial kinase (v_1) depends also on the concentration (E_n) of the activated terminal kinase (see Eqn 2 of the main text). Since the total concentration, $[E_i]_{\mathrm{total}} = E_i + \boldsymbol{E}_i^{\mathrm{I}}$ remains constant, only the phosphorylated form E_i will be considered. The stability of the steady state ($E_i^{st,st}$) is determined by the roots of the characteristic equation of the linearized system,

$$\prod_1^n(\tau_i\cdot\lambda + 1) - f\cdot\prod_1^n \boldsymbol{r}_i = 0, \; \tau_i = E_i\cdot\boldsymbol{r}_i/\boldsymbol{v}_i^{st,st} \qquad \text{(A2)}$$

Here λ are the eigenvalues of the linearized matrix of Eqn (A1), r_i is the local sensitivity of level i defined in the main text. Importantly, the r_i value is determined by the kinetic properties of the protein kinase and phosphatase reactions at cycle i and does not depend on the feedback strength f (expression of r_i in terms of the K_{m} and E_i values is given elsewhere [4]). Thus, the parameter τ_i plays a role of the characteristic time of cycle i.

As shown for equations such as Eqn (A2) [31,32], if n exceeds 2, a threshold increase in the absolute value of f causes two eigenvalues to cross the imaginary axis, $\lambda_{1,2} = \pm\,\mathrm{i}\omega$ ($\omega > 0$). This is known as a Hopf bifurcation, which leads to the appearance of a periodic solution of Eqn (A1) (sustained oscillations) with a period close to $2\pi/\omega$. If the number of cascade levels (n) equals 2, it follows from Eqn (A2) that the eigenvalues cannot have positive real parts. Therefore, only damped oscillations can be observed. In order for sustained oscillations to occur, the number of cascade levels must equal or exceed three. Using an approach developed in [32], a threshold strength of the negative feedback ($f < 0$) can be estimated in terms of the characteristic time and local sensitivity values (τ_i and r_i). In the simplest case, when all τ_i are equal, the threshold feedback strength reads:

$$f_{threshold} = -\frac{\sec^n(\pi/n)}{\prod_1^n \boldsymbol{r}_i} \qquad \text{(A3)}$$

It was demonstrated for metabolic pathways with a negative feedback loop, that the stability range widens significantly if the pathway contains one or two (but not more) slow reactions with high τ_i [32]. However, for signaling cascades, an increase in the local sensitivity and therefore in the characteristic time τ_i at any cascade level does not prevent the loss of dynamic stability. This difference is related to the fact that there is a mass flow through a metabolic pathway, whereas there is only information flow through a cascade of protein kinases.

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