the FEBS Journal

Principles behind the multifarious control of signal transduction

ERK phosphorylation and kinase/phosphatase control

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Keywords
control analysis; kinase; MAPK pathway; phosphatase; systems biology

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Note
The mathematical model described here has been submitted to the Online Cellular Systems Modelling Database and can be accessed free of charge at: http://jjj.biochem.sun.ac.za/database/hornberg/index.html

(Received 16 July 2004, revised 15 September 2004, accepted 24 September 2004)

doi:10.1111/j.1432-1033.2004.04404.x

General and simple principles are identified that govern signal transduction. The effects of kinase and phosphatase inhibition on a MAP kinase pathway are first examined *in silico*. Quantitative measures for the control of signal amplitude, duration and integral strength are introduced. We then identify and prove new principles, such that total control on signal amplitude and on final signal strength must amount to zero, and total control on signal duration and on integral signal intensity must equal −1. Collectively, kinases control amplitudes more than duration, whereas phosphatases tend to control both. We illustrate and validate these principles experimentally: (a) a kinase inhibitor affects the amplitude of EGF-induced ERK phosphorylation much more than its duration and (b) a phosphatase inhibitor influences both signal duration and signal amplitude, in particular long after EGF administration. Implications for the cellular decision between growth and differentiation are discussed.

Much signal transduction occurs through cascades of activation and inactivation. The mitogen-activated protein-kinase (MAPK) cascades are highly conserved examples. They govern many cellular processes, such as proliferation and differentiation (reviewed in [1,2]). They consist of a linear cascade of three kinases that each phosphorylate the next one in line. The kinases are counteracted by phosphatases. The net function of such a pathway, i.e. to decide whether a downstream protein is in the inactive or the active state, is thus the

Abbreviations
EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; ERK-PP, doubly phosphorylated ERK; MAP(K), mitogen-activated protein (kinase); MEK, MAPK/ERK kinase; NRK, normal rat kidney; PTP, protein tyrosine phosphatase; TBS, tris-buffered saline.

result of the concerted action of all kinases and phosphatases [3]. In many human tumors, the MAPK pathway via the extracellular signal-regulated kinases (ERK) 1 and 2, is constitutively active [4]. This is often associated with somatic mutations in genes that encode components that activate the pathway, such as Ras or Raf [5,6]. The magnitude and duration (transient vs. sustained) of MAPK activation are critical for the cellular response [7,8], for instance by influencing different target genes [9]. However, it is not understood completely, to what extent amplitude and duration of signaling are controlled by the kinases or phosphatases in the system, and whether they are controlled differentially by some or all of them. On the basis of the antagonistic action of the kinases and phosphatases one might expect them to control signal transduction precisely in opposite ways, as has been shown for steady-state signal transduction [10].

Experimental possibilities to investigate this issue are limited by the incompleteness of the arsenal of inhibitors of specific kinases or phosphatases. Systems biology approaches that combine mathematical modeling with quantitative experimentation may help in such cases [11–13]. Detailed mathematical models are available that describe and predict the behavior of a few complex signaling networks [14–20]. However, such specific models can be cumbersome when one wishes to track down general principles. Simpler models have led to new suggestions concerning protein kinase signaling, such as the possibility of spatially resolved signaling [21] and have also shed light on the control of kinases and phosphatases on signal transduction [22]. For instance, it was predicted that, in a protein kinase signaling pathway, kinases mainly control signaling amplitude, whereas phosphatases control both signaling amplitude and duration of signaling [22]. Here, we shall first use a simple model of a MAPK cascade. With properties of these as inspiration, we shall then employ mathematics to extend hierarchical control analysis [8] to time-dependent processes and derive general principles of signal transduction cascades. Some of the methodology is similar to that employed in a recent extension of control analysis to the spatial domain [23]. The results confirm and extend predictions of earlier theoretical work, namely that duration of signaling is controlled mainly by phosphatases and that all kinases together control signaling amplitude to the exact same extent as all phosphatases together.

We test these general principles experimentally in the ERK pathway of normal rat kidney (NRK) fibroblasts. These cells can be synchronized relatively easily, causing all cells to behave similarly in response to external stimuli. They are used frequently as a model system to study cellular alterations that accompany oncogenic transformation [24]. Activation of the ERK pathway is required for the proliferation of fibroblasts [25]. The pathway consists of three kinases in succession (Raf, MEK and ERK) and can be activated by various extracellular stimuli, including the epidermal growth factor (EGF).

We determined the effect of kinase and phosphatase inhibitors on the activity of the ERK pathway upon EGF stimulation. Our experimental findings confirm the predictions from the theoretical work, namely that kinases control signaling amplitude rather than the duration of signaling and that phosphatase activity mainly controls duration.

The mathematical model described here has been submitted to the Online Cellular Systems Modelling Database and can be accessed at: http://jjj.biochem.sun.ac.za/database/hornberg/index.html free of charge.

Results

How kinase and phosphatase inhibition affect signal transduction

In order to track down principles governing the control of signal transduction, we analyzed a kinetic

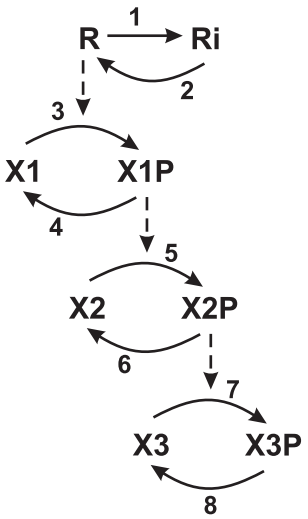
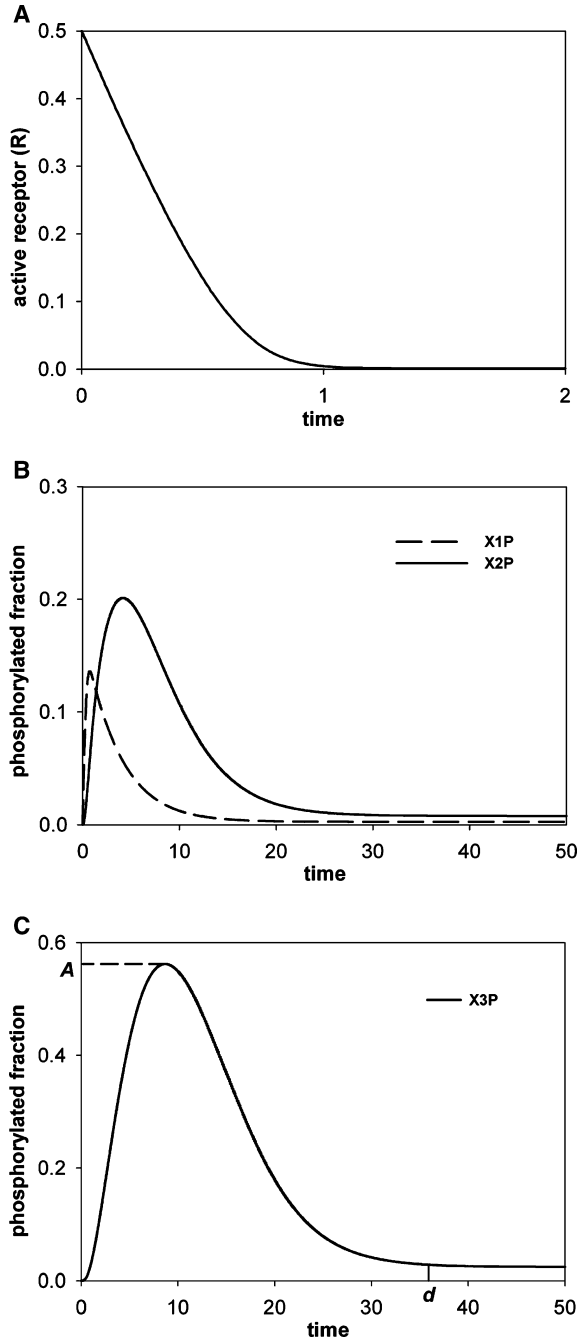


Fig. 1. Schematic representation of the reactions in the model. Active receptor R activates a cascade of three kinase/phosphatase monocycles, by phosphorylating (activating) X1 to X1P (v3). X1P then causes the phosphorylation of X2 to X2P (v5), which, in turn, activates the last monocycle (X3 to X3P; v7). The phosphorylated counterparts are dephosphorylated (v4, v6 and v8, respectively), as is the receptor (to Ri; v1). We modeled the case in which the ligand that activates the receptor remains present and therefore the inactive receptor was taken to slowly recycle to become active again (v2). The architecture of the model was intended to represent a simplified form of a MAPK pathway.

model of a simple linear pathway that consisted of a receptor and three consecutive kinase/phosphatase monocycles (Fig. 1). The activation and inactivation reactions in the model are analogous to phosphorylation (by kinases) and dephosphorylation (by phosphatases) reactions in cellular signaling pathways such as the MAPK cascade. The first kinase was activated by a receptor that switches slowly between an active and an inactive state.



As the duration and amplitude of signaling may be critical for the response evoked [7], we calculated the activation time profile of the signaling molecules (Fig. 2). Receptor activation (e.g. by EGF binding) was instantaneous, at $t = 0$. Whilst the concentration of active receptor R declined over time, the three consecutive kinases (X1, X2 and X3) were activated (i.e. were phosphorylated to become X1P, X2P and X3P, respectively), reached a peak value and subsequently declined to levels that exceeded the level before receptor activation. These time-patterns for activation of the components of the MAPK cascade were commensurate with what has been reported experimentally for many cell types and with the experimental results we will present here. This stimulated us to interrogate the model as to how these time patterns are controlled by the kinases and the phosphatases.

In order to examine how the second kinase in the cascade determines the time dependence of the activity of the third kinase, we varied the V_{max} of the second kinase reaction and recalculated the concentration of the active form of the third kinase as a function of time. (This modulation corresponded to the experiment described below in which MEK, the second kinase of the MAPK pathway, was inhibited by the noncompetitive inhibitor PD98059 [26].) The results show that the peak concentration of X3P decreased substantially (Fig. 3A). The duration (width) of the peak also decreased, but much less so; an inhibition that decreased the peak height by 25%, advanced the time at which the signal returned to below 0.1 by 10%. The final level of X3P also decreased very significantly when calculated in relative terms; the final level was already low before the kinase was inhibited (Fig. 3A). We concluded that in this example, the activity of the second kinase exerted substantial control on signaling amplitude, both in the initial phase of signaling and

Fig. 2. Time profile of the activity of the four proteins in the model. The receptor R is inactivated (relatively) quickly to attain a very low steady state concentration. The kinase/phosphatase cycles are activated slightly after each other to reach a maximal peak concentration, and then decrease to a low steady state concentration. The activity of the pathway was considered to be represented by the X3P concentration, which is in line with MAPK pathways, in which the active third kinase has multiple cytoplasmic and nuclear targets. Calculations on the various aspects of signaling were performed on (a) signaling amplitude *A*, i.e. the maximal X3P concentration that was attained; (b) duration of signaling *d*, i.e. the time point at which the X3P concentration dropped below an arbitrarily chosen value of 0.05; (c) the 'integral signal strength', i.e. the area-under-the-curve until *d* (this represents the total effect X3P would have on a target molecule) and (d) the final signaling amplitude, i.e. the steady state X3P concentration that is attained.

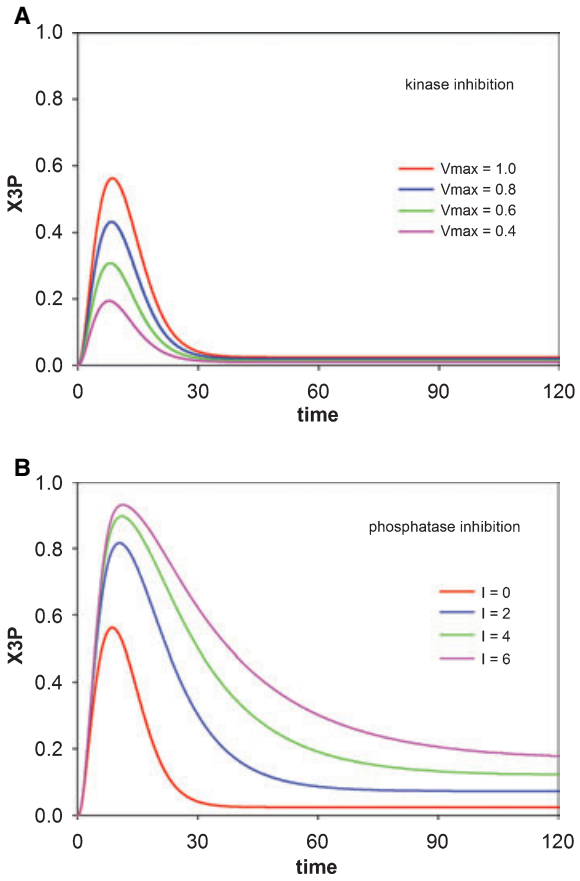


Fig. 3. The effect of kinase and phosphatase inhibition in the X3P time profile. (A) The second kinase reaction in the model (v5) was increasingly inhibited (noncompetitive inhibition by decreasing the V_{max}). This caused a large decrease of the amplitude. Duration was also affected, but not as much. (B) The third phosphatase reaction (v8) in the model was increasingly inhibited (by increasing the concentration of the competitive inhibitor I). This caused an increase in both the amplitude and duration of signaling. Both kinase and phosphatase inhibition also affected the integral signal strength and steady state X3P concentration.

much later, and less control on the duration of signaling. The effect of signal transduction on transcription of downstream genes might not just be a function of the amplitude of X3P. Other aspects of signaling dynamics may be important as well, such as frequency of recurrent pulses [27] or the integrated concentration of an active molecule (e.g. the area under the X3P curve). Accordingly, we also calculated the area-under-the-curve before $t = 50$ and found that kinase inhibition had a considerable effect on this (Fig. 3A).

To examine the influence of phosphatases on signaling kinetics, and in particular whether that role should always be the opposite of that of the corresponding kinases, we introduced an inhibitor, I, that competitively inhibited the dephosphorylation of X3P. In this way we anticipated an experiment (see below) in which protein tyrosine phosphatases were inhibited. We calculated that, with increasing inhibitor concentration, the X3P peak concentration became quite a bit higher (Fig. 3B). In addition, the inhibitor increased the duration of the peak dramatically, prolonging X3P signaling. For instance, an inhibitor concentration that increased the peak height by one-third, doubled the time it took for the X3P concentration to drop below 0.1. Phosphatase inhibition also increased both the final level of X3P and the 'area-under-the-curve' quite substantially (Fig. 3B).

These calculations lead to the hypothesis that phosphatases and kinases were equally important for two characteristics of signal transduction, i.e. the peak amplitude and final amplitude, whereas the duration of signaling and the 'area-under-the-curve' might be more exclusively the control domain of the phosphatases. The latter would confirm a prediction from earlier theoretical work [22]. To corroborate this hypothesis, we systematically modulated the activities of all components in the model, by increasing (and subsequently decreasing) the rate constant by a factor of two for one reaction at a time. The effects on the signaling characteristics specified above were calculated and the results confirmed that the peak amplitude and the final level were controlled both by kinases and by phosphatases, whereas the duration, although influenced by kinases, was mainly controlled by phosphatases (results not shown).

Quantification suggests that all kinases are equally as important for signaling amplitude as all phosphatases

In the absence of any quantification of the strength of control, the above suggestion that kinases and phosphatases exert (opposite) control on amplitudes in signal transduction remains vague. It is not clear whether the effects on amplitudes should be precisely equal but opposite, and whether it should be expected that this be true for the control by a kinase and the phosphatase acting at the same level of the cascade. In order to address these issues, we need to quantify the extents of control exerted by the individual kinases and phosphatases. We do this by asking: 'What is the percentage change in signal strength that is induced by a 1% activation of a kinase or a phosphatase?' This (or rather the variant in which the percentage is infinitesimal) corresponds to the control coefficient as defined for the flux control by enzymes in metabolic pathways [28–31] and for the steady-state amplitudes in signal transduction [10,19]. Here we shall use these control

coefficients to quantify the control on (a) the peak height (i.e. the highest X3P concentration that was attained) and (b) the final signal strength (i.e. the steady state X3P concentration that was attained).

The kinase reactions all had positive control coefficients both with respect to peak height and with respect to final signal strength (Table 1). This was expected as kinases activate the pathway and thus cause higher amplitudes. The phosphatase reactions, on the other hand, all had negative control coefficients, again in line with expectations. However, one might have the expectation that corresponding kinases and phosphatases (e.g. kinase 1 and phosphatase 1) should always have precisely opposite effects on certain aspects of signaling. Indeed, such antagonism is found for the control on the final steady state amplitude (Table 1, bottom row), which is in line with the control analysis for steady states [10]. The expectation is not borne out for signaling before that steady state is attained: controls on maximal signal amplitude, for instance, were opposite in sign, but not always equal in absolute magnitude (e.g. compare column kin1 with column pho1 in Table 1). Our calculations therefore reject the intuition purporting that the kinase in a certain monocycle should have precisely the same control strength as the corresponding phosphatase in that monocycle. Further inspection of Table 1 shows that, both for the maximum signal amplitude and for its final amplitude, the total control by the kinases and the receptor reactivation was almost equal to the total control by the phosphatases and receptor inactivation ('Total' in Table 1). This could have been a coincidence, or it could reflect a general principle. To shed light on this, we calculated the control coefficients of all processes together on the amplitude and final signal strength (by simultaneously perturbing all processes ('all' column in Table 1). We found that both coefficients were 0, which indeed shows that the total control of all activating processes equals the total control of all inactivating processes.

Phosphatases are more important for signal duration and integral signal intensity than kinases

Figure 3 suggested that duration and (possibly) the integrated signal are controlled more by phosphatases than by kinases. To be more precise, we define duration of signaling as the time point at which the X3P concentration declined below the (arbitrarily chosen) low value of 0.5% of total X3 (= 0.05). The integral signal strength, which is a measure for the total number of downstream molecules that are affected by the signal, was calculated as the total area under the

Table 1. Control of any out of four characteristics of the time dependent signal by any of the molecular processes in the cascade. The signal is taken to reside in the extent of phosphorylation of the third kinase in the model MAPK pathway (Fig. 1). The characteristics of that signal are its maximum (amplitude), its ultimate value (final strength), its duration and its time-integrated concentration. Controlling processes are receptor activation and inactivation and the three consecutive kinase and phosphatase processes. Control is quantified in terms of the control coefficients defined in the text. A control coefficient equals the derivative towards a reaction rate. Here, we have calculated the control coefficients by increasing the rate of a reaction by 1% and then divide the relative effect on the characteristics by the relative change in reaction rate. The calculated control coefficients depicted here therefore slightly deviate from the actual control coefficient (numerical error). All the individual control coefficients were summed (Total). Furthermore, the control coefficient for all reactions together was calculated (All), by simultaneously increasing all reaction rates by 1%. Conclusions that can be drawn from the table are discussed in the text. R. inact., receptor inactivation reaction; R. act., receptor activation reaction.

	Reaction															
	v1 R. inact.	v2 R. act.	v1 + v2	v3 kin 1	v4 pho 1	v3 + v4	v5 kin 2	v6 pho 2	v5 + v6	v7 kin 3	v8 pho 3	v7 + v8	Activating processes	Inactivating processes	Total	All	Law
Amplitude	−1.20	0.03	−1.17	1.21	−0.81	0.40	1.15	−0.77	0.38	1.06	−0.64	0.41	3.45	−3.43	0.02	0.00	0
Duration	−0.44	0.17	−0.27	0.44	−0.60	−0.16	0.42	−0.62	−0.21	0.38	−0.70	−0.32	1.40	−2.37	−0.96	−0.99	−1
Integral	−1.41	0.12	−1.30	1.43	−1.25	0.18	1.35	−1.24	0.12	1.22	−1.16	0.06	4.12	−5.06	−0.94	−0.99	−1
Final strength	−1.03	1.04	0.01	1.03	−1.02	0.01	1.03	−1.02	0.01	1.02	−1.01	0.01	4.12	−4.08	0.04	0.00	0

'signal strength' vs. time curve until the X3P concentration declined below 0.05. We shall again use control coefficients to quantify the control of the duration of signaling and 'integral signal strength' (Table 1).

The inactivation reaction (i.e. phosphatase or receptor inactivation) always had a more negative control than the corresponding activation reaction (i.e. kinase or receptor reactivation) had positive control. Signal duration and integral signal strength were not controlled equally by corresponding kinases and phosphatase. For these two signaling characteristics, the total precise antagonism of all kinases (activating enzymes) combined and all phosphatases (inactivating enzymes) combined was not obtained either. So, are there no general principles for these aspects of signal transduction? We did calculate that all phosphatases together must exert higher negative control on duration and on the integral signal strength than all kinases together exert positive control. Also, the difference in control, i.e. the sum of all control coefficients on duration (and the integral) was not zero, but −1 or close to that ('Total' in Table 1). Perhaps this −1 also reflects a general principle, which should then be the quantitative underpinning of the greater average importance of phosphatases than kinases for duration and integral signal strength.

Of course, these findings can be no more than suggestions, as they were obtained for a certain set of kinetic parameters and a certain type of kinetics of the kinases and phosphatases. When we next repeated the above calculation for different magnitudes of the kinetic parameters, both the curves and the individual control coefficients varied with the parameters that were set in the model (Fig. 4 and results not shown). Figure 4 shows a case where the activation reactions were more active, leading to a higher peak in X3P phosphorylation; in fact at the peak most X3 was phosphorylated. Figure 4B shows that, as expected for this case, the phosphatase inhibitor had little effect on the peak height. As all X3 was already X3P at the peak, little more X3P could be generated. Accordingly, the control by the phosphatases on the peak height was smaller (Table 2). Table 2 shows that also for this case, the following features were observed: (a) corresponding kinases and phosphatases did not exert equal opposite control; (b) control of all activating enzymes combined equaled control by all inactivating enzymes with respect to signal amplitude and (c) control of all inactivating enzymes exceeded the control by all activating enzymes with respect to both duration and integral signal strength.

The control for all processes together equaled 0 for peak amplitude and final signal strength and it equaled −1 for duration and integral signal strength ('All' in Table 2). Therefore, even though the changes in the parameters caused the individual control coefficients to change significantly, the total control always produced the same result. This led us to hypothesize that there are laws that govern the totals of control, just as there are for total control of metabolic fluxes and concentrations [29]. Proving such general laws, however, requires more than the calculation of a number of examples; the general case must be addressed.

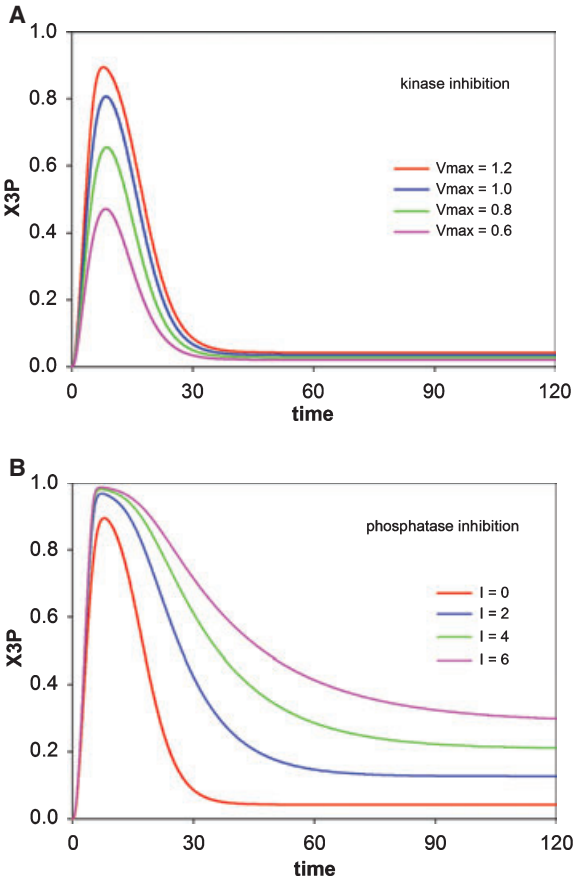


Fig. 4. The calculations were repeated with different magnitudes of the kinetic parameters. The activation reactions were more active, leading to a higher peak X3P concentration. Inhibition of the second kinase reaction (v5) again decreased the amplitude of signaling (A). Inhibition of the third phosphatase reaction (v8) had some effect on the amplitude (B), but this was significantly smaller than in the case of Fig. 3, as now virtually all X3 was phosphorylated in the peak. Duration and integral signal strength were again affected by both kinase and phosphatase inhibition, although inhibition of the phosphatase appeared to have more effect on these signaling characteristics.

Summation laws for control on signal transduction

The above definition of the extent to which a process controls an aspect of signal transduction, i.e. in terms

Table 2. Control of any reaction on any signaling characteristic in a case with a different set of parameters. The individual control coefficients are different from those in Table 1, but the summation laws remain. See the text for the conclusions and further discussion of this Table.

	Reaction																
	v1 R. inact.	v2 R. act.	v1 + v2	v3 kin 1	v4 pho 1	v3 + v4	v5 kin 2	v6 pho 2	v5 + v6	v7 kin 3	v8 pho 3	v7 + v8	Activating processes	Inactivating processes	Total	All	Law
Amplitude	−0.42	0.01	−0.41	0.40	−0.27	0.13	0.38	−0.25	0.13	0.34	−0.23	0.11	1.12	−1.17	−0.04	0.00	0
Duration	−0.83	0.71	−0.12	0.86	−1.00	−0.13	0.84	−1.03	−0.19	0.80	−1.16	−0.36	3.21	−4.02	−0.81	−0.99	−1
Integral	−0.86	0.19	−0.68	0.86	−0.90	−0.03	0.81	−0.91	−0.10	0.71	−0.88	−0.17	2.57	−3.55	−0.97	−0.99	−1
Final strength	−1.05	1.06	0.01	1.05	−1.04	0.01	1.05	−1.04	0.01	1.04	−1.03	0.01	4.20	−4.15	0.05	0.00	0

of a corresponding control coefficient, enables the application of mathematical methodologies. As shown in the Appendix, this makes it possible to prove a summation law for: the maximum signal strength; the duration of the signal; the final signal strength and the integral signal strength. The summing is over all the processes in the system, and its results are given in the final column of Table 1.

We present herein the principle behind the proof. This is the concept that equal activation of all reactions has the same effect as accelerating time. Starting at $t = 0$, we consider the situation that all processes become 1% more active. This has the effect that everything happens 1% faster than in the control situation, but in precisely the same way. Consequently, the maximum signal strength and the final signal strength will be the same, each signal magnitude will be reached 1% earlier and the integral signal intensity will be 1% smaller (because everything lasts 1% less time). Accordingly, the sum of the control of all process rates on maximum and final signal strength must be zero and the sum of their control on duration and on integral signal strength −1.

This statement, with respect to signal amplitude, dictates that the average control by the kinases (or, to be more precise, by the activating processes) should be equal (though opposite in sign) to the average control by the phosphatases (or, to be more precise, by all the inactivating processes). Accordingly, both kinases and phosphatases must control signal amplitude. Also, inescapably, in cascades where kinases exert stronger control on amplitude than in any reference cascade, the phosphatases must also exert stronger control than in that reference cascade. It is not possible that in one cascade control resides more with the kinases whereas, in a different cascade, the phosphatases are more in control.

With respect to duration and integral signal strength, this is different. By 'taking away' signal, the phosphatases accelerate the signal transduction dynamics. Also, the total control corresponds to this acceleration. In more precise terms, the total negative control by the phosphatases (or inactivating enzymes) on both duration and integral signal strength must always be 1 stronger than the total positive control exerted by the kinases (or activating enzymes). In cases where total control by the phosphatases is close to −1, there will be little control by the kinases. More generally, there is a tendency for the kinases to control duration and integral signal strength less than the phosphatases do. This then is the precise underpinning of the tendencies described in the earlier sections of this paper (Figs 3 and 4 and Tables 1 and 2).

Experimental validation

Kinases control amplitude rather than duration

We set out to test the predictions, based on the theoretical work here and in an earlier study [22], experimentally in a MAPK signaling network in living cells. We focused on the influence of the second kinase in the MAPK cascade, MEK, on the time profile of ERK (i.e. the third kinase) phosphorylation upon growth factor stimulation. The model predicted that MEK would mainly control the peak height and perhaps the final amplitude, and less so the peak duration (Figs 3A and 4A). To address this question, we arrested NRK fibroblasts in the G_0-phase using serum-starvation. The MAPK pathway was then stimulated with EGF, samples were taken after various incubation times and the ERK-PP concentration of cell lysates was measured by quantitative Western blotting. Stimulation with EGF was carried out in the presence of various concentrations of the noncompetitive MEK inhibitor PD98059 [26]. We observed a biphasic ERK-PP time profile, consisting of a rapid high peak followed by a low quasi-steady state (Fig. 5A). Increasing MEK inhibitor concentrations resulted in decreased peak heights. Added inhibitor had little affect the duration (width) of the peak. These results confirm the model predictions that kinase inhibition affects signaling amplitude much more than signaling duration.

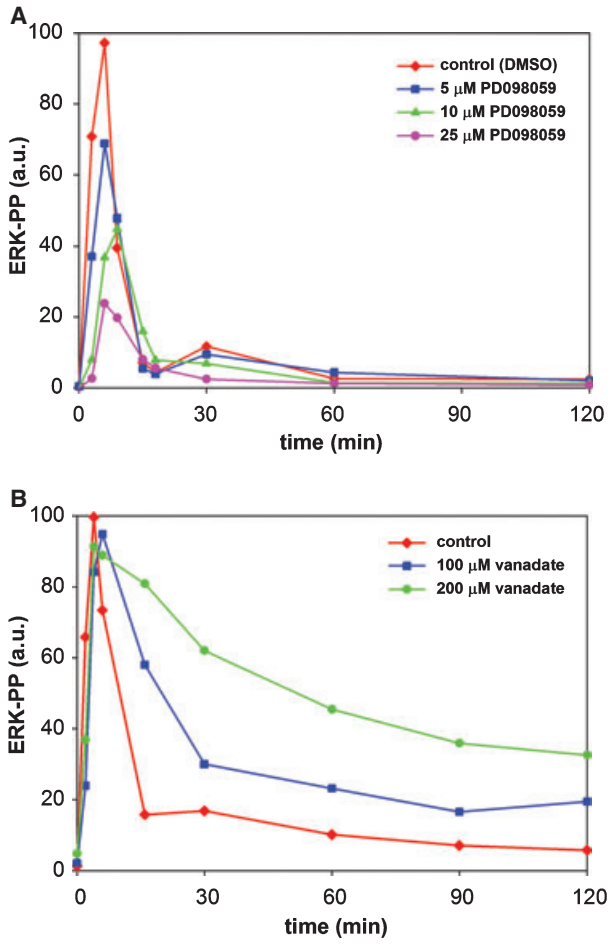


Fig. 5. Experimental validation of the theoretical results. (A) Inhibition of MEK, the second kinase in the MAPK pathway to ERK, using the noncompetitive inhibitor PD098059, led to a decrease in the peak ERK-PP concentration. (B) Protein tyrosine phosphatase inhibition, using orthovanadate, significantly increased the duration of the presence of ERK-PP.

Phosphatases control duration of signaling

In the model simulation, phosphatases controlled maximum signaling amplitude, signaling duration, final signal amplitude and integral signal strength. To test these predictions experimentally, we applied the protein tyrosine phosphatase (PTP) inhibitor, sodium orthovanadate, to arrested NRK fibroblasts that were stimulated subsequently with EGF. PTP inhibition resulted in a broader peak followed by a relatively high final quasi-steady ERK-PP concentration (Fig. 5B), both in consonant with the model predictions.

The peak height in the presence of the phosphatase inhibitor was no higher than that in control cells. This result corresponds closely to that simulated for the model of Figs 4B i.e. the case where the applied amount of EGF was close to saturating. Indeed, it has been previously shown that in NRK cells, this EGF concentration causes virtually all ERK to become doubly phosphorylated ([32], and our unpublished observation). We conclude that the experimental results obtained with a kinase inhibitor and with a phosphatase inhibitor were in complete correspondence with our modeling and mathematical results and those reported previously [22].

Discussion

Cellular behavior is brought about by the concerted action of many components. On one hand these components should be studied individually but on the other, cell physiology should address the functioning of the entire cell. What often remains unanalyzed is to what extent and how individual components contribute to (i.e. control) the functioning of cellular systems, such as the activity of signaling networks. Such analysis is of vital importance not only for understanding cellular systems but also for drug design, as it helps in the process of choosing potential drug targets

according to the magnitude of their control on cell pathology [11,33].

What do we know about the control of intracellular signaling? Its pathways, such as the MAPK cascades, are often composed of kinase and phosphatase pairs. But how important are the kinases and phosphatases relative to each other? Are all combinations of the control of signal transduction possible? Here, we combined the analysis of a kinetic model of a simple signaling pathway, with mathematics and with quantitative experimentation on MAPK signaling. We showed that there are general principles regarding the control of protein kinase signaling cascades, and also that these principles differ from the ones one might have guessed intuitively. The principles demonstrated here confirm and elaborate on previous predictions that were made on the basis of a theoretical analysis of a simple model of signal transduction [22]. Here, we also verify these predictions experimentally.

In a simplified mathematical model, we calculated the control distribution on four features of the activity of a signaling cascade: the amplitude of signaling; the final intensity of signaling; the duration of signaling and the integral signal intensity (which corresponds to the 'area-under-the-curve'). For MAPK and other signaling pathways, these features are important determinants for the biological response that is evoked. Amplitude should be important if a certain activation threshold must be exceeded to cause a downstream effect. For instance, such a form of MAPK activation is required for the proliferation of fibroblasts [25]. A paradigmatic example of the importance of duration in PC12 cells is that sustained MAPK activity leads to differentiation whereas transient MAPK signaling causes proliferation [7]. In rat hepatocytes, rapid, transient MAPK activation promotes progression through the G_1 phase of the cell cycle and entry into the S phase, whereas prolonged MAPK activation inhibits this process [8]. Furthermore, the repertoire of downstream genes that are expressed upon MAPK activation depends on the duration of signaling [9]. These data imply that critical cellular decisions are made at the level of the activation characteristics of signaling cascades, such as the MAPK pathway and that distinguishing between the early amplitude and late plateau, and duration and area-under-the-curve may be important for understanding differential control of downstream processes by the activities of kinases and phosphatases, and other (in-)activating processes.

In our analysis, we introduced new quantitative measures for the strength of control, akin to those used in metabolic control analysis, to calculate to what extent individual kinase and phosphatase reactions control these decisive signaling characteristics. For two examples of a signal transduction cascade we showed that control of the amplitudes, the duration and the integrated activation of signaling was distributed over all processes within the cascade and that the control exerted by the individual processes differ. This is in line with what is known about control distribution in metabolic pathways [29,31,34,35]. What is different from the control of metabolic pathways is the total control. For the main function of metabolic pathways, i.e. the rate of product formation (the 'flux'), control adds up to 1. Here, control of two characteristics of signal transduction adds up to 0 and of two other characteristics to −1. Although a summation rule for control coefficients of transient times also exists in control analysis when applied to metabolism [36], this further substantiates that control of signal transduction differs fundamentally from the control of metabolic flux [10].

These summation principles for signal transduction were then derived mathematically. This means that these principles are not accidental for the two examples of a linear signal transduction pathway analyzed here numerically but are general for any signal transduction pathway that fulfils the definition given here. This definition is quite general (Appendix). Signaling pathways are frequently regulated by (nonlinear) feedback and feed-forward circuitry [37,38]. The summation does not depend on a linear structure of the network.

Principles of general validity are also called 'laws': they could have been discovered experimentally, they require precise definition of conditions and properties, and they can be derived from underlying accepted principles by employing mathematics. Here the underlying principles include the usual types of deterministic kinetics and local stability of the system. It is not often that understanding of an aspect of cell biology can be achieved by using analytical mathematics.

The laws dictate that the control of all processes on the amplitude of signaling must equal 0 and that the total control on the duration (and integrated activity) of signaling must equal −1. This implies that (a) all kinases together are necessarily of exact equal importance for the amplitude (i.e. both the maximal and the steady state activity) as are all phosphatases together, and that (b) the total control on the signal duration and integrated strength by all phosphatases always exceeds the total control of all kinases. This statement should read 'all activating enzymes' for kinases and all 'inactivating enzymes' for the phosphatases, in case reactions other than kinases and phosphatases are involved in the cascade. Here, it should be noted that

this conclusion may depend on the structure of the signaling network. Non-linearity, caused by regulatory circuits, may yield unexpected control properties. For instance, if a kinase is involved in a nonlinear negative feedback loop, it is possible that its overall control on the activity of the network proves to be negative, rendering, e.g. a lower amplitude. However, total control on the amplitude must sum to 0, meaning that one or more of the other processes in the network will compensate for the negative control by the particular kinase.

Although many components of the MAPK pathway have been identified, not all processes that control its activity are known. Therefore, and due to the limitation of experimental possibilities, at present it is not feasible to determine exactly the control coefficients for all individual reactions in the MAPK pathway. Our experimental results, however, illustrate that the general principles we deduced in the theoretical work we report here and have reported previously [22] are in qualitative accordance with the experimental data for the functioning of a complex signaling network in living cells: a kinase (MEK) inhibitor affected the amplitude of signaling through MAPK, while leaving duration unaffected. A (protein tyrosine) phosphatase inhibitor influenced both duration of signaling to MAPK and its amplitude in the steady state. We did not find an effect of the phosphatase inhibitor on the first peak. This can be attributed to the fact that EGF stimulation causes virtually all ERK molecules to be doubly phosphorylated in the first peak ([32] and our unpublished observation). Phosphatase inhibition could therefore not further elevate the amplitude in the peak, and the experimental result was in line what we obtained by modeling for this case of maximum phosphorylation of ERK (Fig. 4B).

The summation laws have a number of implications for drug therapy, as well as for the understanding of oncogene function. For instance, for cell functions that depend on integrated concentration of phosphorylated ERK (such as total transcription of a target gene) the summation laws prescribes a constant total control of −1. The prescribed constancy of control implies that if the control exerted by one enzyme kinase (or phosphatase) is altered (which could be achieved by adding an inhibitor or by mutation of its gene), the control of at least one other enzyme (but most probably of many others) is altered as well. Application of such an inhibitor as a drug, or the occurrence of mutations affecting the control by one enzyme, will therefore almost always interfere with the regulation of the signal transduction pathway by all regulatory mechanisms, not just by the regulators that impinge on the step that is directly affected by the inhibitor or the mutation. This may well have implications for the application of signal transduction modulators in cancer treatment, such as tyrosine kinase inhibitors that have already been validated as promising clinical agents in targeted therapies [39,40]. A more positive note is that the effect of oncogenic mutations on the activity of a target molecule in tumor cells will affect cellular signaling, but in addition, the control that other kinases or phosphatases have on that signaling. Therefore, antitumor strategies need not only focus on the molecular target of the mutation, but could also be directed against other steps in the pathway, with largely similar results. Or, from a slightly different perspective, the oncogenic mutation should lead to redistribution of the control and hence to the emergence of additional new targets at other sites in the network. Network-based drug design, a systems biology approach, may help identify those targets [11] and enable rationalized combination therapies.

Another issue that may be more readily understood now, is that mutations in MAP kinases and phosphatases can differ in the extent to which they shift a cell between differentiation and proliferation. If kinases and phosphatases were considered to be precisely each others antipode, then less kinase activity should have the same effect as more phosphatase activity and a mutation in either should increase or decrease both differentiation and proliferation rather than cause a shift between them. Our demonstration that kinases and phosphatases affect the amplitude and duration of signaling differently, provides a possible explanation for such a shift: phosphatase inhibition should activate more the functions that depend on sustained transcription of a regulatory gene (such as differentiation), whereas kinase stimulation (or to be more precise stimulation of any of the activating processes, such as by Ras activation) should activate more the function depending on short-term transcription (perhaps proliferation).

Experimental procedures

Model description

We constructed a mathematical model of a simple linear signal transduction pathway that consists of a receptor and three consecutive kinase/phosphatase monocycles (Fig. 1). In the model the receptor (R) is activated instantaneously by added EGF. It is then inactivated over time (to become Ri). The inactive form of the receptor is re-circulated slowly to become active once again; the case where EGF remains present. The active form of the receptor phosphorylates and thereby activates the first kinase X1 (to become X1P). Through phosphorylation, this kinase can then activate the

second kinase (X2), which, in turn, can activate a third kinase (X3). All reaction steps follow Michaelis–Menten kinetic rate equations, with $V_{max} = 1.0$ and $K_m = 0.1$ for the activating (kinase) reactions and the receptor inactivation reaction (reaction **1**); $V_{max} = 0.3$ and $K_m = 1.0$ for the deactivating (phosphatase) reactions; and $V_{max} = 0.01$ and $K_m = 0.1$ for the receptor recirculation reaction. We consider explicit conservation relations for each of the three kinases, i.e. the concentrations of the active and inactive form, sum up to 1. The initial concentrations for Ri, X1P, X2P and X3P were set to 0 and the initial concentrations were set to 1 for X1, X2, X3 and to 0.5 for R. All concentrations are in mM. It must be stressed that the model was not constructed to accurately describe the MAPK pathway, but to derive general principles that apply to any signal transduction pathway of this type. The parameter values do not determine the general conclusions. The model is also available on our website (http://www.bio.vu.nl/hwconf/supplements/). The model was analyzed with GEPASI 3.30, a public domain software program specifically designed to simulate biochemical networks [41,42]. The kinase reactions were taken to depend proportionally on the concentration of their corresponding activators (i.e. the preceding phosphorylated kinase). To show that our conclusions do not depend on this specific set of model parameters (Fig. 4 and Table 2), the simulation was repeated with increased reaction rates for the kinases: the V_{max} of the kinase reactions were set to 1.2.

Cell culture

NRK fibroblasts were cultured in Dulbecco's modified Eagle's medium (Cambrex Bio Science Verviers SPRL, Verviers, Belgium), supplemented with 10% (w/v) fetal bovine serum (Gibco; Invitrogen Corporation, Carlsbad, CA, USA), 0.10 $g{\cdot}L^{-1}$ penicillin and 0.10 $g{\cdot}L^{-1}$ streptomycin in a humidified 5% (v/v) CO_2 incubator at 37 °C. For serum-starvation, cells were washed them with 1× Hank's buffered salt solution (Gibco) and then used the same medium, but with 0.5% (w/v) bovine serum albumin (AppliChem GmbH, Darmstadt, Germany) instead of serum.

Stimulation experiments

Cells were grown in culture dishes to subconfluency and then serum-starved for three days in order to be arrested in the G_0-phase of the cell cycle. Subsequently, cells were stimulated with 10 $ng{\cdot}mL^{-1}$ EGF (Becton Dickinson, Franklin Lakes, NJ, USA) for the indicated periods of time. Enzyme inhibitors were purchased from Calbiochem (San Diego, CA, USA). Where indicated MEK was inhibited by preincubation for 1 h with various concentrations of the noncompetitive inhibitor PD98059 [26]. We preincubated cells for 1 h with 0.20 mM sodium orthovanadate [43] to inhibit PTPs.

Western blot analysis

After stimulation, cells were washed twice with ice-cold phosphate-buffered saline (17 mM NaH_2PO_4, 38.5 mM Na_2HPO_4, 68 mM NaCl, pH 7.4) and incubated on ice with 'lysis buffer' [10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) SDS, 0.1% (v/v) Nonidet P40, 0.1% (w/v) sodium deoxycholate, 50 mM NaF, 1 mM sodium-orthovanadate, 1× Complete protease inhibitor mix (Roche, Basel, Switzerland)] for 20 min. Cell lysates were scraped using a cell scraper (25 cm per 1.8 cm, Costar, Dow Corning Europe, Seneffe, Belgium), collected, vortexed for 10 s, frozen in liquid nitrogen and stored at −80 °C. Protein contents in the cell lysates were determined with the BCA assay (Pierce, Rockford, IL, USA). Proteins were separated by SDS/PAGE, using a 12% resolving gel. Exactly 10 μg of total protein of each sample was loaded onto the gel in loading buffer [0.25 M Tris/HCl (pH 7.6), 8% (w/v) SDS, 40% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 20 mM dithiothreitol]. Proteins were electrotransferred to Immuno-Blot™ poly(vinylidene difluoride) membranes (Bio-Rad laboratories, Hercules, CA, USA) using a current of 0.40 A overnight at 4 °C. Membranes were washed in Tris-buffered saline [TBS: 20 mM Tris/HCl (pH 7.6), 150 mM NaCl] supplemented with 0.05% Tween 80 (TBS-T), preincubated for 1 h at room temperature with blocking buffer [5% skim-milk powder (Oxoid Ltd, Basingstoke, UK) in TBS-T], supplemented with 0.5 mM Na_3VO_4, and incubated overnight at 4 °C with a monoclonal anti-(phospho-p44/42 MAP kinase) Ig (Cell Signaling Technology Inc., Beverly, MA, USA) in blocking buffer (1 : 2000), supplemented with 0.5 mM Na_3VO_4. After washing, membranes were incubated for 1 h at room temperature with horse-radish peroxidase-conjugated goat anti-(mouse IgG) (Bio-Rad) in blocking buffer (1 : 3000). Membranes were washed again and then incubated for 5 min with Lumi-Light^{PLUS} Western Blotting Substrate (Roche). Signals were detected with a FluorS^{TM} MultiImager (Bio-Rad) and quantified using the MULTI-ANALIST software (Bio-Rad). All measurements were carried out in the linear range of the method. The standard error of the mean was 4.6% of the measured value.

Acknowledgements

We thank Boris N. Kholodenko for intensive discussions, and him and Mark Peletier for discussions on summation laws of this type.

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/EJB/EJB4404/EJB4404sm.htm

Appendix S1. A description of the model including kinetic equations and additional data.

Appendix

Summation law for the control of time dependent phenomena

We here discuss a dynamic reaction system with spatial homogeneity [23]. In this system, n reactions, numbered with the index i, take place, each at a rate $e_i.v_i$. Through v_i, the rates are functions of concentrations (x_j) of m reactive molecules in the system, and they are each further characterized by an activity e_i. In some systems these e_i values correspond with enzyme concentrations, such as the concentration of a protein kinase or a protein phosphatase. A chemical reaction v_i leads to a time-dependent increase in the concentration of substance x_j as given by [32] and Eqn (1):

$$\frac{dx_j}{dt} = \sum_{i=1}^{n} n_{ji} \cdot e_i \cdot v_i(\bar{x}). \quad (1)$$

Here, n_{ji} is a stoichiometric number, which is positive when x_j is a product of the reaction and negative when it is a substrate. The vector $\bar{x}$ contains the concentrations of all m reactive molecules. The equation assumes that during the time of observation, the environment of the system is constant i.e. any external change such as the addition of EGF should have occurred at or slightly before $t = 0$. The equation then defines the dynamics of the system in time. We shall assume that the above differential has a unique solution that is asymptotically stable in the sense of Lyapunoff [29].

Equation (1) and the concentrations at time zero, which we denote by $\bar{x}_0$, define the concentrations at any point in time:

$$x_j(e_i, t) = x_j(0) + \int_{\tau=0}^{\tau=t} \sum_{i=1}^{n} n_{ji} \cdot e_i \cdot v_i(\bar{x}) \cdot d\tau. \quad (2)$$

We shall now consider a transformation in which inspections are made at earlier times (i.e. at t' rather than t) or, at times expressed in seconds rather than in minutes. In addition, starting at $t = 0$, any process i is made to run faster by a factor λ_i (or its units are changed from min^{-1} into s^{-1}). Accordingly, the times are 'earlier' by a factor λ_t and activities have increased by the same factor λ_i:

$$t' \equiv t/\lambda_t \quad (3)$$

$$e_i' \equiv e_i \cdot \lambda_i \quad (4)$$

At time zero the untransformed and the transformed system have the same concentrations of all their components, i.e. $\bar{x}_0$. Substituting into Eqn (2), one obtains:

$$x_j'(e_i, t) = x_j(\lambda_i \cdot e_i, t/\lambda_t) = x_j(0) + \int_{\tau=0}^{\tau=t/\lambda_t} \sum_{i=1}^{n} n_{ji} \cdot \lambda_i \cdot e_i \cdot v_i(\bar{x}) \cdot d\tau$$

Writing z for $\tau \cdot \lambda_t$, this becomes:

$$x_j(\lambda_i \cdot e_i, t/\lambda_t) = x_j(0) + \int_{z=0}^{z=t} \sum_{i=1}^{n} n_{ji} \cdot \frac{\lambda_i}{\lambda_i} \cdot e_i \cdot v_i(\bar{x}) \cdot dz$$

Because z is just a dummy integration variable, this implies that:

$$x_j(\lambda_i \cdot e_i, t/\lambda_t) = x_j(0) + \frac{\lambda_i}{\lambda_i} \cdot \big(x_j(e_i, t) - x_j(0)\big)$$

We now consider the case where all these transformation factors are equal. They then drop out of the above equation, implying that:

$$\bar{x}(\lambda \cdot t, \lambda^{-1} \cdot e_i) = \lambda^0 \cdot \bar{x}(t, e_i).$$

This shows that the concentrations are homogeneous functions of order zero, of all process activities at order 1 and of time at order −1. Using Euler's theorem for homogeneous functions one then formulates the summation law for time-dependent concentration control coefficients [23]:

$$\sum_{i=1}^{n} C_i^x = C_t^x$$

where, the control coefficients have been defined by:

$$C_t^x = \frac{d \ln x}{d \ln t}$$

and

$$C_i^x = \frac{d \ln x}{d \ln e_i}.$$

It may be noted that these definitions generalize the control coefficients defined by metabolic control analysis for steady state properties to time dependent properties [44–46]. Where the control by time is zero, e.g. in steady state or at an extreme point in the transient dynamics, the law reduces in form to the traditional law derived from steady state equations [47]:

$$\sum_{i=1}^{n} C_i^{x_m} = 0.$$

We note however, that the traditional law was limited to steady states, whereas here, we have derived it so that it also applies to the maxima and minima in time dependencies in nonstationary phenomena. For the present discussion, the law applies both to the maximum level of ERK phosphorylation and to its steady state level. The integrated output ('area-under-the-curve') is defined as:

$$I(t) = \int_{t=0}^{t} x(\tau) \cdot d\tau.$$

We consider the same transformation as in Eqns (3) and (4) and note that:

$$I\left(\frac{e_i}{\lambda_i}, \lambda \cdot t\right) = \int_{\tau=0}^{\tau=\lambda \cdot t} x\left(\frac{e_i}{\lambda_i}, \lambda \cdot \tau\right) . d\tau = \int_{\tau=0}^{\tau=\lambda \cdot t} x(e_i, \tau) \cdot d\tau = \lambda \cdot \int_{\tau=0}^{z=t} x(e_i, \tau) \cdot dz = \lambda \cdot I(e_i, t).$$

Application of Euler's theorem gives:

$$\sum_{i=1}^{n} C_i^{I(t)} - C_t^{I(t)} = -1.$$

If the signal integral converges for long times, this yields for the total integral signal strength at infinite time:

$$\sum_{i=1}^{n} C_i^{I(\infty)} = -1.$$

In many cases the signal intensity does not drop to zero but attains a steady state level different from zero. Then the above integral does not converge; the area-under-the-curve continues to increase with time. In these cases, the area-under-the-curve should be evaluated up to the point where the curve drops below a certain value. If at that point in time the time derivative is small, then the simpler form of the law is retained. We now consider the time t it takes for the concentration to attain a certain value $\bar{y} + \bar{x}_0$. This time is given implicitly by the equation:

$$0 = \int_{\tau=0}^{\tau=t} \sum_{i=1}^{n} n_{ji} \cdot e_i \cdot v_i(\bar{x}) \cdot d\tau - y_j.$$

We now consider the transformation where all enzymes are activated. The time t' at which the concentration reaches the same magnitude is then found by solving the equation:

$$0 = \int_{\tau=0}^{\tau=t'} \sum_{i=1}^{n} n_{ji} \cdot e_i \cdot \lambda_i \cdot v_i(\bar{x}) \cdot d\tau - y_j.$$

Again, writing z for $\tau{\cdot}\lambda_i$ and taking all λ_i terms as equal, this becomes:

$$0 = \int_{t=0}^{z=\lambda\cdot t'} \sum_{i=1}^{n} n_{ji} \cdot e_i \cdot v_i(\bar{x}) \cdot dz - y_j$$

which proves that:

$$\lambda \cdot t' = \lambda \cdot t(\lambda \cdot e_i) = t(e_i)$$

i.e. the time at which a certain concentration is attained is a homogeneous function of time of the order −1 of the enzyme activities. Hence:

$$\sum_{i=1}^{n} C_i^{\tau} = -1$$

where τ is the time it takes for the signal to reach a certain magnitude given a certain local dynamic environment (this condition is added to accommodate the fact that the time needed to attain a certain signal magnitude may not be unique, e.g. because a signal increases and then decreases).