Shc-dependent pathway is redundant but dominant in MAPK cascade activation by EGF receptors: a modeling inference

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Abstract In cell signaling cascades, one stimulus often leads to various physiological functions by multiple pathways. Perturbation of one pathway by blocking or overexpressing one of its components will result in changes in multiple pathways and multiple cell functions. Thus, it is important to reveal the relative contribution of each pathway to each function in order to assess the consequence of perturbations (e.g. drug delivery). By exploring an established mathematical model, the Shc-dependent pathway is found to be both redundant and dominant during activation of the mitogen-activated protein kinase cascade by epidermal growth factor receptor (EGFR). Its dominance results from the majority consumption of the common precursor ((EGF-EGFR*)2-GAP) by this pathway. The key steps for the dominance are the binding and phosphorylation of Shc. In conclusion, cells may prefer the long Shc-dependent pathway to the short Shc-independent pathway.

Key words: Epidermal growth factor receptor; Shc; Cell signal transduction; Biochemical simulation

1. Introduction

The epidermal growth factor receptor (EGFR) is one of the tyrosine kinase receptors and is involved in various physiological or pathological phenotypes including embryonic and postnatal development [1], progression of tumors [2], and transactivation processes [3]. Upon being bound by the ligand EGF at the extracellular domain, EGFR forms a dimer and phosphorylates itself at the intracellular domains. Phosphorylated EGFR then recruits a series of signaling molecules and induces activation of mitogen-activated protein kinase (MAPK) cascade [4]. The activated MAPK then regulates several cellular proteins and nuclear transcription factors [5]. There are two pathways used by EGFR to activate the MAPK cascade, Shc-dependent and Shc-independent [6,7]. Although Shc-mediated MAPK activation has been well established [8,9], dominant negative Shc or loss of Shc expression often results in only a partial loss of MAPK activation [10]. It remains unclear how much Shc contributes to MAPK activation. Recently a computational model for the dynamics of MAPK cascade activation by EGFR was proposed [11], which provided an insight into signal–response relationships between the ligand binding and activation of downstream proteins in the signaling cascade. However, the report did not address the issue of the relative contribution of Shc-dependent and -independent pathways. In this study, we further explore the model to quantitatively assess the relative contribution of the Shc-dependent and Shc-independent pathways to MAPK activation by EGFR. We report here that the Shc-dependent pathway is both dominant and redundant during activation of MAPK by phosphorylated EGFR. The redundancy is supported by several experimental results in the literature, and the key step responsible for the dominance is revealed in this study. Furthermore, the sub-pathways within the Shc-dependent pathway are dissected mathematically and their contributions to MAPK activation are discussed. As more and more signaling cascades are revealed to utilize multiple pathways (see the review by Liebmann [12]), the protocol demonstrated in this study for assessing their relative contributions will have a broad usage in simulating cell signal transduction and inferring the biological importance of alternative pathways.

2. Model and simulation

The model described by Schoeberl et al. [11] was used in this study. The scheme of the EGFR-induced MAPK cascade was modified so that each molecule species appears only once (Fig. 1). The Shc-dependent and Shc-independent pathways are separated graphically so that the components in each pathway are readily identified. The two pathways share the same events at the beginning, i.e. binding of EGF to EGFR, dimerization and phosphorylation of EGFR, and recruiting of GAP (not shown in Fig. 1). They diverge at the point of (EGF-EGFR*)2-GAP. Instead of Grb2, Shc is recruited in the Shc-dependent pathway. After phosphorylation of Shc, Grb2 is recruited to form (EGF-EGFR*)2-GAP-Shc*-Grb2, which is the equivalent of (EGF-EGFR*)2-GAP-Grb2 in the Shc-independent pathway (Fig. 1). The following common events are SOS binding and then Ras-GDP binding. The two pathways converge at the point of Ras-GTP production, and thereafter follow the same track to sequentially activate Raf, MEK and ERK (not shown in Fig. 1).

Simulation was conducted using the biochemical simulator GEPASI version 3.21 [13–15] The reactions, their kinetic parameters and the initial quantities of the components were input into GEPASI using its GUI (graphical user interface). Totally there were 125 reactions and 94 components, as described by Schoeberl et al. [11].

For assessing the relative contributions of the two or more pathways, the concentrations of the components are of limited values. Molecules may be produced and consumed at the same time. If both processes occur in the same strength, the concentration of a molecule remains constant. More importantly, the concentration is component (molecule species)-specific and does not reflect the contribution of a single reaction. Thus, it is more meaningful to observe production and consumption of each component dynamically via each

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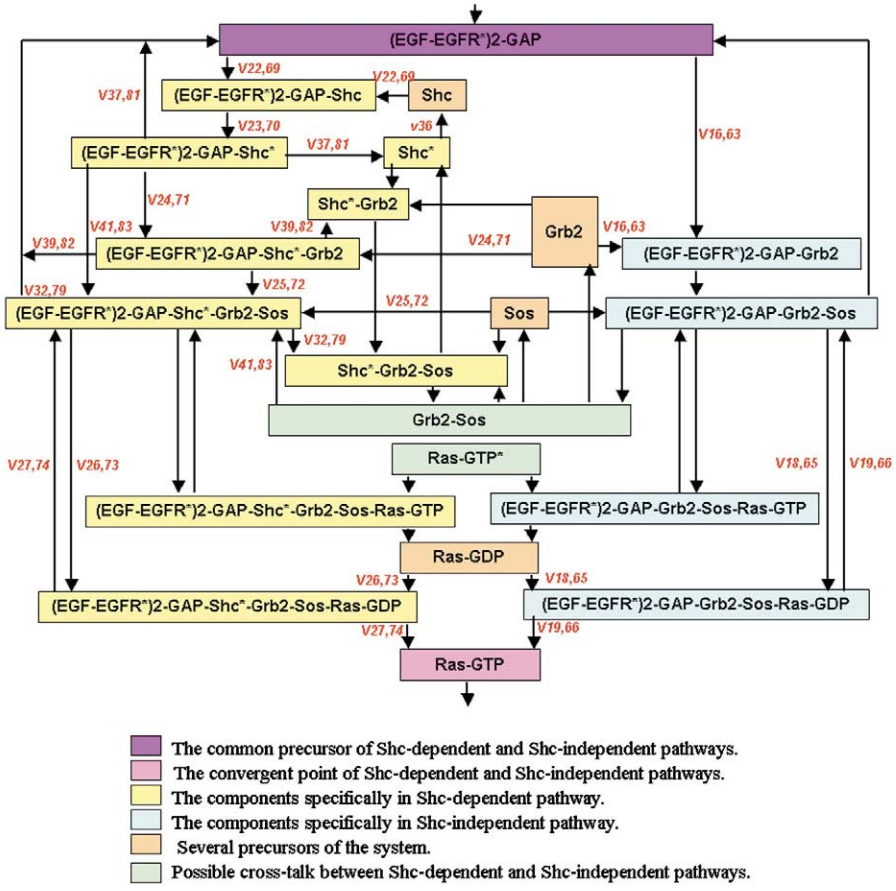

Fig. 1. Partial scheme of the EGFR-induced MAPK cascade. This figure aims to show the Shc-dependent and Shc-independent pathways. The Shc-dependent pathway is located on the left part, whereas the Shc-independent pathway is located on the right part. The common precursor of, convergent point of, possible cross-talks between the two pathways, and several initial components are located in the middle of the scheme. Each component label represents a molecular species at the cell surface, and internalized as well, if applicable. Arrows represent the reaction direction. In the case of a reversible reaction, the direction of the arrows determines the forward and reverse reaction parameters, which is not shown in the scheme. The IDs for the reactions begin with letter v and are close to the arrows representing the reaction. For simplification, only the IDs of the reactions being discussed are displayed. In the case of two IDs, the second one represents the reaction with the internalized component. (Modified from [11].)

reaction in which it is involved for the purpose of this study. To do so, the fluxes (velocity of the molecular flow, which is reaction-specific) of the reactions were traced. This function is one of the features of the software GEPASI. The amounts of molecular flow (which is reaction-specific, accordingly) were calculated with integration over the simulation time, using the following formula: molecular flow = $\Sigma(\Delta t \times F_t)$, where Δt is the small segment of time (typically 1 s in this study) and F_t is the flux at time t (in the unit of M/s). With molecular flows, it is easy to dissect the ratio of a component produced by different reactions, or the flows of a component into different reactions.

For reversible reaction steps, unidirectional arrows are used in the scheme for assignment of the forward and reverse reactions. The flux value can be positive or negative. When the value of the flux is positive, the flow of the molecule has the same direction as the arrow. If the value of the flux is negative, the molecular flow has a reverse direction to the arrow.

3. Results

It has been modeled previously that EGFR could be internalized before or after activation, and the internalized receptors transduce signal as well [11]. However, internalization of the receptors had very limited effects on production of Raf, MEK-PP and ERK-PP under the simulation condition used in this study (with 50 ng/ml EGF) (data not shown), which was in agreement with the published results [11]. Therefore, the effects produced by both cell surface and internalized receptors were combined for the simulation in this study.

The relative contribution of the Shc-dependent and Shc-independent pathways to MAPK activation was first assessed. The index of contribution was evaluated by the molecular flow to Ras-GTP from its direct precursors ((EGF-EGFR*)2-GAP-Shc*-Grb2-Sos-Ras-GDP and (EGF-EGFR*)2-GAP-Grb2-Sos-Ras-GDP for the Shc-dependent and Shc-independent pathways, respectively). The fluxes of the reactions were traced up to 60 min and production of Ras-GTP was calculated. As shown in Fig. 2, the Shc-dependent pathway was the dominant one used by EGFR to produce Ras-GTP, while only 2% of the total Ras-GTP was produced by the Shc-independent pathway, when the initial concentration of EGF was set at 50 ng/ml and the concentration of Shc at the reported value [11].

To further determine the extent to which the Shc-dependent pathway was dominant and also whether it was redundant as indicated by several experiments, the effects of initial Shc concentration on Ras-GTP production by the two pathways were tested, using the component concentration perturbation scanning, a function provided by GEPASI. The total production

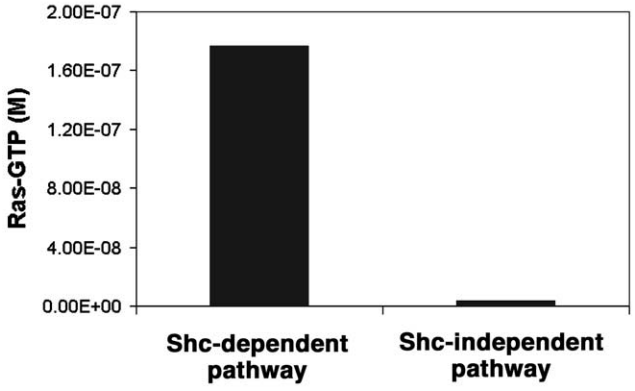

Fig. 2. Relative contributions of the Shc-dependent and Shc-independent pathways to production of Ras-GTP. The *y*-axis represents the molecular flow to Ras-GTP. The simulation was conducted using the same set of reaction parameters and initial concentrations as described [11]. EGF concentration was 50 ng/ml. The time course of fluxes for reactions v27(74) (in the Shc-dependent pathway) and v19(66) (in the Shc-independent pathway) were traced for 60 min with 1 s for each segment. The fluxes from reactions v27 and v74, and from reactions v19 and v66, were combined, respectively. Molecular flows were then calculated as the sum of the fluxes at each time point.

of Ras-GTP only had a small reduction (less than 5%) when the initial Shc concentration was set at 0. Under this condition, all of the Ras-GTP was produced via the Shc-independent pathway (Fig. 3). This result suggested that the Shc-dependent pathway was redundant in the MAPK activation by EGFR. Nevertheless, the existence of a small amount of Shc ($<12\%$ of the reported value) would restore Ras-GTP production almost to a normal level ($>99\%$). Furthermore, this small amount of Shc caused the Shc-dependent pathway to produce more than 83% of the total Ras-GTP. When the Shc level was half of the reported value, this ratio was as high as 95% (Fig. 3), indicating that a low level of Shc was enough to make this pathway dominate MAPK activation.

There are at least two possible scenarios for the dominance of the Shc-dependent pathway. One is the cross-talk between the pathways (see Fig. 1). The early components in the Shc-independent pathway may be produced in a considerable amount, but consumed by the cross-talk paths between the pathways, instead of leading to production of Ras-GTP via their own pathway. Alternatively, most of the common precursor of the two pathways ((EGF-EGFR*)2-GAP) is consumed by the Shc-dependent pathway in the presence of even a very small amount of Shc. To test these two possibilities, consumption of the common precursor ((EGF-EGFR*)2-GAP) by the two pathways was compared by tracing the fluxes from the common precursor and calculating the molecular flows. As shown in Fig. 4, a majority of the precursor (99.9%) flowed to the Shc-dependent pathway. To rule out the possibility of cross-talk, production of the intermediates equivalent in the two pathways ((EGF-EGFR*)2-GAP-Shc*-Grb2 vs. (EGF-EGFR*)2-GAP-Grb2, (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos vs. (EGF-EGFR*)2-GAP-Grb2-Sos, (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos-Ras-GTP vs. (EGF-EGFR*)2-GAP-Grb2-Sos-Ras-GTP, and (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos-Ras-GDP vs. (EGF-EGFR*)2-GAP-Grb2-Sos-Ras-GDP; see also Fig. 1) was also compared. There was no significant production of the intermediates in the Shc-independent pathway in comparison with their counterparts in the Shc-dependent pathway (data not shown). Thus it is concluded that the existence of Shc caused the common precursor ((EGF-EGFR*)2-GAP) flow to the Shc-dependent pathway, consequently leading to the dominance of the Shc-dependent pathway in Ras-GTP production.

A question remains as to which step(s) along the Shc-dependent pathway determines the majority flow of the common precursor to this pathway and subsequently production of most Ras-GTP. A systematic scanning of the parameters of all the reactions involved in the Shc-dependent pathway was conducted, taking advantage of the power of GEPASI for parameter perturbations. The results showed that changes in three of the parameters could significantly affect production of Ras-GTP by the Shc-dependent pathway. They were v22k_1 (forward rate constant of the reaction v22), v23k_1 and v26k_1 (Fig. 5A–C; also see Fig. 1 for their locations). Changes of parameters in all other forward reactions and all reverse re-

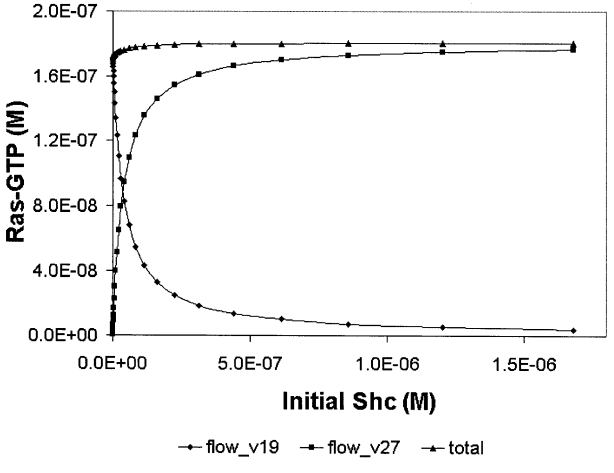

Fig. 3. Relative contributions of the Shc-dependent and the Shc-independent pathways to production of Ras-GTP at varied initial Shc concentrations. The *y*-axis represents the molecular flow to Ras-GTP. The simulation condition and calculation was the same as described in Fig. 2, except that the Shc concentration was scanned. The values of initial Shc concentrations were selected logarithmically, spanning from 0 to 1.68E-6 M.

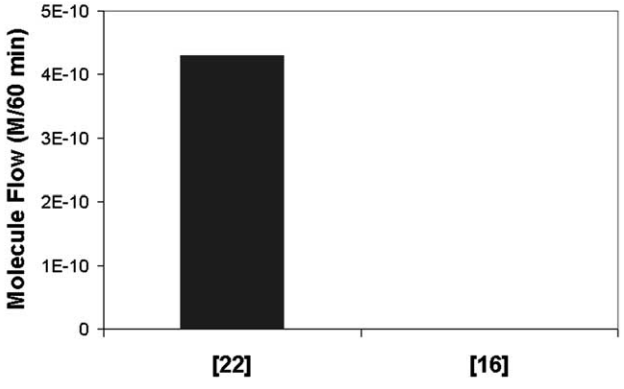

Fig. 4. Amount of the common precursor, (EGF-EGFR*)2-GAP, flowing to the Shc-dependent pathway via reactions v22 and v69 (combined and labeled as [22] in this figure), and to the Shc-independent pathway via reactions v16 and v63 (combined and labeled as [16] here). The simulation condition was the same as described in Fig. 2.

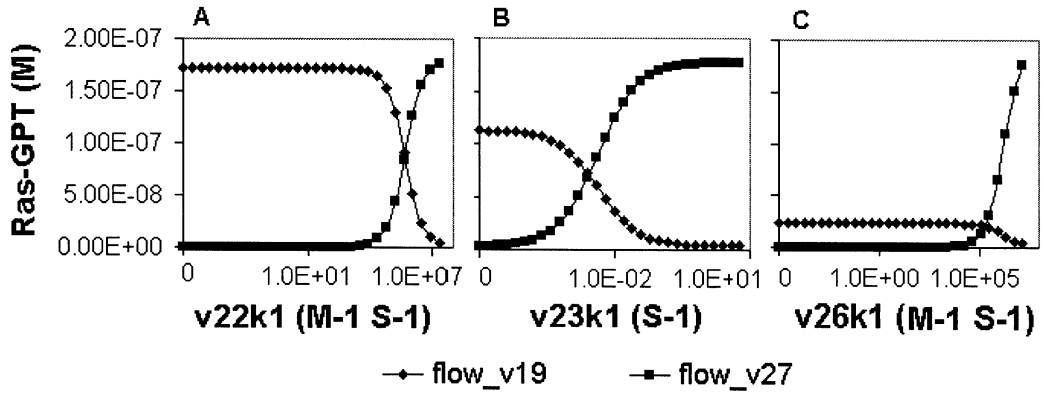

Fig. 5. Relative contributions of the Shc-dependent and Shc-independent pathways to production of Ras-GTP at varied values of rate constants. The *y*-axis represents the molecular flow to Ras-GTP. The simulation condition was the same as described in Fig. 3, except the rate constants were scanned instead of initial Shc concentration. Rate constant values were selected, and the *x*-axis values are displayed logarithmically. A: Rate constant k_1 of reaction v22 was scanned. B: Rate constant k_1 of reaction v23 was scanned. C: Rate constant k_1 of reaction v26 was scanned. flow_v19 and flow_v27 represent the contribution of the Shc-independent and Shc-dependent pathways, respectively. The effects of cell surface and internalized receptors were combined.

actions did not affect the differential production of Ras-GTP (data not shown). v22 is the first step from the common precursor in which Shc is bound, and v23 is the subsequent step in which Shc is phosphorylated. Both v22 and v23 are specific to the Shc-dependent pathway. v26 is the step in the Shc-dependent pathway in which the Ras-GTP precursor is produced and which has an equivalent in the Shc-independent pathway (v18). Both v26 and v18 have the same values for forward and reverse rate constants, suggesting that the key steps determining the flow of the common precursor are v22 and v23. Furthermore, as little as 0.05% of the reported v23k_1 value, or 2% of the reported v22k_1 value, resulted in the two pathways producing the same amount of Ras-GTP (Fig. 5A,B). Further, the effects of changes in v22k_1 and v23k_1 on the dynamics of the downstream ERK signal are also different. When v22k_1 or v23k_1 was set at 0, the total Ras-GTP production was almost unchanged (97.2%) or dropped to 63.3% of the full amount, respectively (Fig. 5A,B). Interestingly, when v26k_1 was set at 0, the total Ras-GTP production was reduced by 88% (Fig. 5C), suggesting that production of Ras-GTP by the Shc-independent pathway did not compensate the block of this step in the Shc-dependent pathway. It was hypothesized that in this situation most common precursor flowed to the Shc-dependent pathway and accumulated as the intermediates in this pathway during the blockage of reaction v26. The accumulation could be fulfilled via, for example, the loop: v22-v23-v37-v36-v22. This was confirmed by tracing each reaction in this loop, where their fluxes were significantly increased when v26k_1 was set at 0 (data not shown).

After quantitative dissection of the Shc-dependent and Shc-independent pathways, the sub-pathways in the Shc-dependent pathway were further dissected. In the Shc-dependent pathway, there are two sub-pathways that possibly lead to production of Ras-GTP. They are (also see Fig. 1):

1. (EGF-EGFR*)2-GAP → (EGF-EGFR*)2-GAP-Shc → (EGF-EGFR*)2-GAP-Shc* → (EGF-EGFR*)2-GAP-Shc*-Grb2 → (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos → (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos-Ras-GDP → Ras-GTP;
2. (EGF-EGFR*)2-GAP → (EGF-EGFR*)2-GAP-Shc → (EGF-EGFR*)2-GAP-Shc* → (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos → (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos-Ras-GDP → Ras-GTP.

Instead of reactions v24 and v25, (EGF-EGFR*)2-GAP-Shc*-Grb2-Sos is produced directly from (EGF-EGFR*)2-GAP-Shc* and Grb2-Sos via reaction v41 in the second sub-pathway. This is independent of the first sub-pathway, because Grb2-Sos is produced from pre-existing Grb2 and Sos. A flux-tracing experiment showed that, at first, both sub-pathways were used to produce Ras-GTP. Reactions v24 and v25 were reversed after 270 s and 126 s, respectively (Fig. 6). Thereafter, only the second sub-pathway was used (see the top curve in Fig. 7A). (EGF-EGFR*)2-GAP-Shc* is the common precursor of the two sub-pathways. Its flow to the two sub-pathways depends on the value of v41k_1 (Fig. 7). At low v41k_1, flow to the first sub-pathway was always prevalent. At considerable v41k_1, at first part of the common

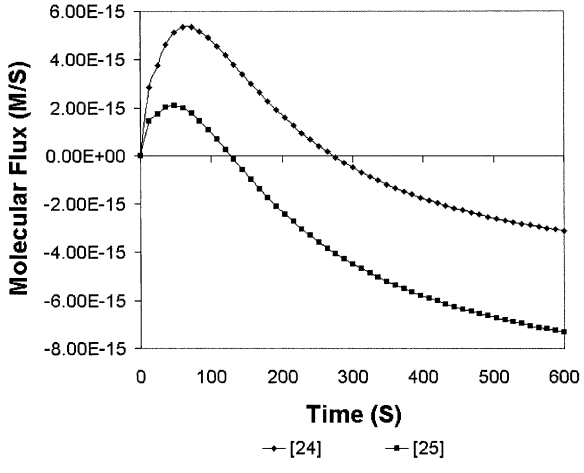

Fig. 6. Time courses of the fluxes of reaction v24 (including v71) and v25 (including v72). The simulation condition was the same as described in Fig. 2 except fluxes for reactions v24 (and v71) and v25 (and v72) were traced. At the beginning of the simulation, the molecular flows were towards Ras-GTP production. Then fluxes were reversed and these reactions negatively contributed to Ras-GTP production.

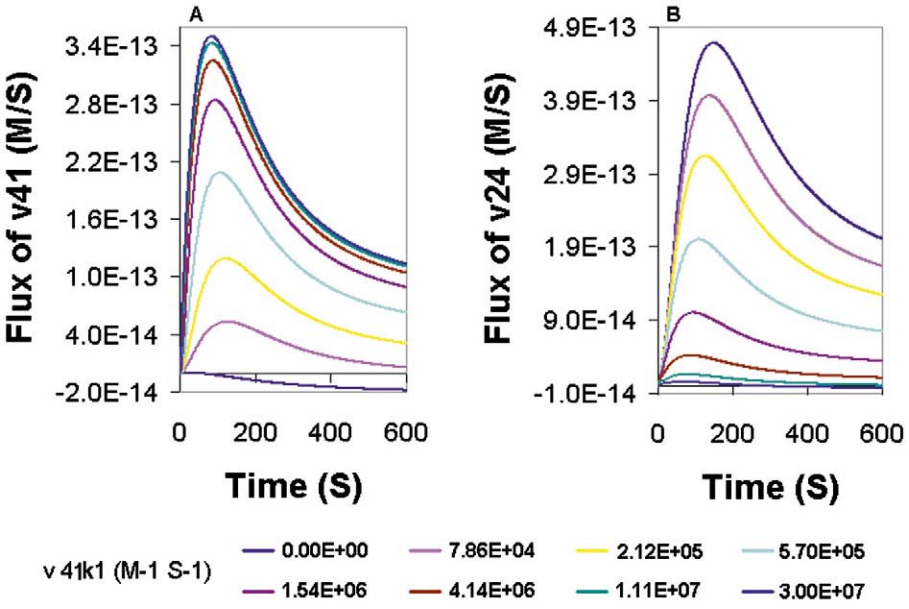

Fig. 7. The level of the rate constant v41k_1 determines the usage of two sub-pathways in the Shc-dependent pathway for producing Ras-GTP. Time course of fluxes of reaction v24 and v25 (not shown) and v41 in 10 min at different values for rate constant k_1 of reaction v41. Simulation condition was the same as described in Fig. 5, except v41k_1 was scanned and the fluxes of reactions v24 and v41 were traced. Note that at different v41k_1 values, different sub-pathways could be used in the Shc-dependent pathway.

precursor flowed to the first sub-pathway, then all the flow was through the second sub-pathway. At high v41k_1, flow to the second sub-pathway was always prevalent (Fig. 7). However, as previously demonstrated, change in a single kinetic parameter of reactions v24 or v25 or v41 did not affect the Ras-GTP production by the Shc-dependent pathway. Consequently, blockage of either of the sub-pathways would not affect the dynamics of the Ras-GTP production by EGFR.

4. Discussion

Shc is an adapter protein, its tyrosine is phosphorylated by several different types of receptors (growth factor receptors, antigen receptors, cytokine receptors, G protein-coupled receptors and hormone receptors, for review see [10]). The real role Shc plays in signal transduction is not clear. Two possibilities have been proposed: Shc provides a central and universal component of intracellular signaling, or the phosphorylation of Shc is a bystander effect [10]. In MAPK activation by EGFR, Shc provides an alternative pathway [6,7]. The existence of the Shc-dependent pathway increases the complexity of signal transduction networking. Although networking could contribute to genetic buffering [16], i.e. the robustness of the biochemical networking, the Shc-dependent pathway in this currently used model might not be aimed at this, based on the fact that this pathway shares all other signal molecules with the Shc-independent pathway.

In this study we revealed computationally the redundancy and dominance of the Shc-dependent pathway during MAPK activation by EGFR. The structural basis for the redundancy is the ability of phosphorylated EGFR to be bound by both Shc (via the Shc SH2 and PI domains) and Grb2 (via the Grb2 SH2 domain) as well as the binding of Grb2 to phosphorylated Shc (via the Grb2 SH2 domain) [6]. The redundancy of Shc in EGFR signaling has been observed in several types of cells, e.g. chicken DT40 B cell lines in which Shc was knocked out [17], and rat pheochromocytoma cell line PC12 where the Shc inhibitor TRK-Y490P was used [18]. Shc redundancy was also observed in several other situations. For example, when proliferation or viability signaling was activated by granulocyte/macrophage colony-stimulating factor, the tyrosine phosphorylation of Shc was not necessary [19]. In addition, Raf1 was activated by erythropoietin via the Shc-independent pathway [20] and 8-bromo-cAMP induced a proliferative response independent of Shc [21]. In contrast, Shc was non-redundant in thymic T cell development [22]. Also, mouse embryos with homozygous mutant at the ShcA locus died at day 11.5 with severe defects in heart development [9]. Obviously, the situations at the cellular or whole animal levels are much more complex than the model used in this study. The complexity comes, at least, from the multiple pathways Shc may be involved in. Besides MAPK activation, Shc is also related to the phosphatidylinositol 3-kinase/Akt pathway [23], c-Myc activation, [24], and the JNK signaling pathway [17]. Besides the EGF cascade, Shc was also reported to regulate cascades of other growth factors, cytokines or hormones such as estrogen [25], insulin-like growth factor-1 [26], and interleukin 2 [27]. While our results suggest that Shc is redundant in the EGF-EGFR stimulation of MAPK, they cannot easily be extrapolated to other systems. However, it is safe to say that Shc is redundant in the cases in which there exists alternative pathway(s) for the execution of the observed cell functions; and it is non-redundant in the cases in which there are no alternative pathway(s) for the cell to use for expressing specific phenotype(s). Perturbation or knockout experiments could provide clues on the existence of alternative pathways involved in the relevant cellular phenotypes.

The dominance of the Shc-dependent pathway during activation of MAPK was unexpected, considering that the Shc-dependent pathway is longer than the Shc-independent pathway. Also, this dominance, to our knowledge, has not yet been observed experimentally and its biological meaning re-

mains unknown. While one possibility is that activation of MAPK via the Shc-dependent pathway could be more efficient (i.e. resulting in larger amplification of the signal) than via the Shc-independent pathway, the result of Shc concentration scanning experiments (Fig. 3) does not support this. The real roles Shc plays in physiological and pathological processes can only be figured out with larger-scale and more detailed models. However, our hypothesis is that the importance of its dominance might not reside in EGFR activation of Ras-GTP itself. Rather, it might increase the competition of this activation with other receptor-induced pathways in which Shc is also involved. Besides, an interesting conclusion implied by this result is that, during MAPK activation by EGFR, the longer pathway (Shc-dependent), instead of the shorter pathway (Shc-independent), is preferentially used. Further experimental results are needed to confirm this computational inference. A possible experimental strategy is to measure the contents of the molecular species with newly developed techniques such as mass spectrometry-based proteomics. In a recent study, this methodology was applied to analyze the proteins from HeLa cells treated with EGF, and absorbed by immobilized Grb2 fusion protein [28]. Although increases in proteins absorbed by the Grb2 SH2 domain were observed, similar yet more quantitative (e.g. put the labeled proteins as inner references) experiments in a time series will be more informative.

As demonstrated, the key steps determining the dominance of the Shc-dependent pathway are Shc binding and phosphorylation by EGFR (rate constants $v22k_1$ and $v23k_1$). Three isoforms of Shc (46 kDa, 52 kDa and 66 kDa) could be expressed in the cells, and the major substrates for EGFR are 46 kDa and 52 kDa Shc (phosphorylated to similar extents), very limited 66 kDa Shc was phosphorylated by EGFR [29]. The parameters in the model used in this study were based on the kinetics of the 46 and 52 kDa Shc isoforms [11]. Thus, if the 66 kDa Shc is the predominant or exclusive isoform, utilization of the two pathways could be different from the modeling result, and it is possible for them to be utilized to a similar extent. However, more accurate modeling demands the differentiation of the Shc isoforms at the kinetic level.

While there might be more sub-pathways in the Shc-dependent pathway, via v37, v39 and v32, they are not considered important under the current modeling situation. First, all of them need phosphorylated Shc (Shc*) or complexes containing Shc* and thus they are dependent on v22 and v23. Second, flux-tracing results showed that they were all involved in the dissociation, instead of formation, of the intermediates of the Shc-dependent pathway (data not shown). Interestingly, in some tumor cells, Shc was constitutionally phosphorylated, because of the constitutionally activated tyrosine kinases [30]. Under those circumstances, the alternative sub-pathways might be used in Ras-GTP production. However, detailed modeling needs extra and/or renewed initial state variables.

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