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Simulation of the regulation of EGFR endocytosis and EGFR-ERK signaling by endophilin-mediated RhoA-EGFR crosstalk

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Abstract Deregulations of EGFR endocytosis in EGFR-ERK signaling are known to cause cancers and developmental disorders. Mutations that impaired c-Cbl–EGFR association delay EGFR endocytosis and produce higher mitogenic signals in lung cancer. ROCK, an effector of small GTPase RhoA was shown to negatively regulate EGFR endocytosis via endophilin A1. A mathematical model was developed to study how RhoA and ROCK regulate EGFR endocytosis. Our study suggested that over-expressing RhoA as well as ROCK prolonged ERK activation partly by reducing EGFR endocytosis. Overall, our study hypothesized an alternative role of RhoA in tumorigenesis in addition to its regulation of cytoskeleton and cell motility.

Keywords: ERK activation; Pathway simulation; EGFR endocytosis; RhoA; ROCK

1. Introduction

Enhanced EGFR-ERK signaling is related to various cellular processes [1], cancers [2], and developmental disorders [3]. Apart from over-expression and gain of function mutations [4,5], enhanced signaling can be facilitated by increased EGFR stability via reduction of its internalization and subsequent degradation [6–12]. The dynamics of EGFR endocytosis significantly affects the magnitude and timing of EGFR-ERK signaling. For instance, EGFR endocytosis has been found to partly contribute to the transient activation of ERK signal in PC12 cells [13]. The dynamics of ERK activation has profound functional implications in affecting cell fate (proliferation or differentiation) [14,15] and in promoting tumorigenesis [16], cardiovascular disease [17,18], and urinary bladder dysfunction [19]. Therefore, understanding how the dynamics of EGFR endocytosis affects EGFR-ERK signaling is an important issue to address.

Wild-type EGFR undergoes rapid endocytosis from the plasma membrane followed by lysosomal degradation. However, Shtiegman et al showed that mutations such as L858R and L858R/T790M at EGFR that impaired c-Cbl–EGFR association undergo significantly slow endocytosis than wild type EGFR. Thus, these EGFR mutants resist ligand-induced degradation and their phosphorylated forms are detectable even several hours after stimulation with EGF [8]. As a result, the relatively slow clearance of mutant EGFR molecules enables persistent signaling leading to stronger mitogenic and antiapoptotic signals that may leads to cancers.

In addition to specific mutations on EGFR that impaired c-Cbl–EGFR association, there is alternative mechanism that causes delayed EGFR endocytosis. The small GTPase RhoA has been identified as a negative regulator of EGFR endocytosis via its effector Rho kinase (ROCK) to endophilin A1-mediated crosstalk in specific cell types [12]. Activated EGFR recruits Grb2 to mediate EGFR binding to c-Cbl, which subsequently initiate EGFR internalization and degradation process [20]. An important step is the binding of adaptor proteins CIN85 and Eps-15 to the EGFR and c-Cbl complex, which subsequently recruits endophilin, dynamin-2 as well as clathrin interacting proteins such as BAD2 and AP2 to drive clathrin assembly and budding that leads to EGFR endocytosis. However, activation of the RhoA effector ROCK phosphorylates endophilin A1 at Thr-14 that hinders the recruitment of endophilin A1 to the EGFR–c-Cbl–CIN85 complex, thereby reducing the level of EGFR endocytosis [12].

Apart from its regulatory role in EGFR endocytosis, RhoA as a small GTPase like Ras, acts as a molecular switch cycling between inactive GDP-bound and active GTP-bound forms and play important roles in regulating cytoskeleton formation and cell migration [21] as well as ERK activation via other mechanisms [22]. For instance, RhoA is required for the sustained ERK signal by repressing the Rac/Cdc42 pathway allowing expression of cyclin D1 in mid-G1 phase of NIH-3T3 cells [22]. These effects are expected to collectively contribute to the observed facilitating actions of active RhoA on

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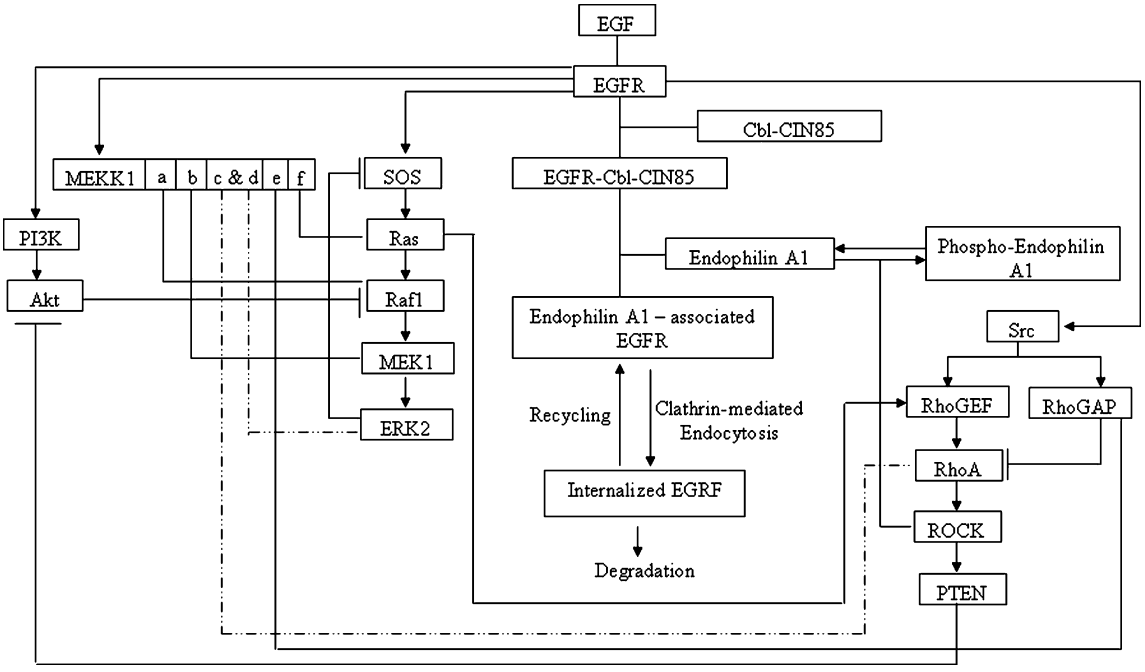

Fig. 1. Pathway model used in this study.

glomerular epithelial cells survival [23]. Given the important roles of RhoA in enhanced EGFR-ERK signaling [12] and the observations that it is over-expressed in several cancer cell lines possibly involved in cancer cell invasion and metastasis [24,25], it is important to study the effects of RhoA on the dynamics of EGFR endocytosis in regulating EGFR-ERK signaling via endophilin A1-mediated RhoA-EGFR crosstalk.

Simulation models of EGFR-ERK pathway have been extensively developed and analyzed by several groups to study important biological events associated with ERK activation such as proliferation and differentiation [26–29]. Kholodenko et al. have used ODE-based approach to develop a EGFR signaling network model to investigate the short-term pattern of cellular responses to EGF in rat hepatocytes [29]. This model consists of 25 reactions involving 23 different reacting species that include crosstalk to phospholipase C gamma (PLCγ). The kinetic parameters in this model are based on the scientific literature or derived from basic physicochemical quantities. Similarly, Brightman and Fell have developed an ODE-based mathematical model of the EGF signal-transduction pathway in PC12 cells to investigate the factors influencing the kinetics of ERK cascade activation [28]. There are 30 reactions involving 29 reacting species containing modules of the activation and internalization of EGFR. From these established mathematical models of EGFR-ERK pathway, Schoeberl et al. [27] have developed a more detailed ODE-based mathematical model (125 reactions involving 94 reacting species) describing the dynamics of the EGFR-ERK signal-transduction pathway to investigate the effects of receptor internalization on the ERK cascade [27]. They investigated the signal to response relationship between the binding of EGF to its receptor at the cell surface as well as the activation of downstream proteins in the signaling cascade.

To the authors' knowledge, mathematical model of endophilin-mediated EGFR endocytosis regulated by RhoA and its effector ROCK has not been developed. Given that deregulation of endophilin-mediated EGFR endocytosis can result in excessive ERK signaling and cancers, it is important to develop a mathematical model to understand its regulation at systems level. In this work, by extending the published ODE-based mathematical models of EGFR-ERK pathway [27–32], we developed a generic EGFR-ERK pathway simulation model that include endophilin A1-mediated RhoA-EGFR crosstalk (Fig. 1) to specifically study the relevant effects in those cell types that contain this crosstalk. As shown in Fig. 1 we also included scaffold protein MEKK1 that cross-talks RhoA to Raf-MEK-ERK cascade to capture the overall effects of RhoA on ERK activation. Detailed molecular interactions and the corresponding kinetics data were obtained from those used in the published simulation models and further search of literatures. Our simulation model was validated by evaluating whether the simulated results are in agreement with published experimental and computational findings. The validated model was then used to study the possible effects of the over-expression of RhoA and its effector ROCK on the duration and degree of ERK activation as malfunction of RhoA and ROCK had been observed in several types of cancers such as breast and colon cancers [33,34].

2. Results and discussion

2.1. Model validation against observed effects of impaired c-Cbl–EGFR association by EGFR mutations on EGFR endocytosis and signaling in lung cancer.

High levels of EGFR leads to prolonged ERK signal that is known to be associated with cancers. Recent study by Shtiegman et al. showed that EGFR association with c-Cbl is impaired by specific mutations in EGFR such as L858R/T790M. These mutations hindered EGFR ubiquitination and endocytosis leading to prolonged EGFR signaling in lung cancer [8]. These known mutational effects were used to validate

our model developed in this study. We first examined the effects of the amount of c-Cbl in relation to internalized EGFR. We found that at normal state of c-Cbl–EGFR binding, increasing the amount of c-Cbl promotes EGFR endocytosis as showed in increased amount of internalized endophilin A1-associated EGFR (Fig. 2a). Higher amount of internalized EGFR subsequently reduced the duration of ERK activation (Fig. 2b). Hence, the trends of our simulation results are consistent with the observation made by Shtiegman et al. that c-Cbl attenuated ERK signals via enhanced receptor endocytosis [8].

We next examine the effects of impaired c-Cbl–EGFR binding in our model. To mimic the effects of specific EGFR mutations that impair c-Cbl–EGFR association the K_f value of respective equation was reduced by 100- and 1000-folds (Eq. (114) in the Supplementary Table S1). The simulation results in Fig. 2c showed that reducing the binding strength between c-Cbl and EGFR by 100- and 1000-folds (0.005 and 0.0005 $\mu M^{-1}\ s^{-1}$, respectively) almost diminished endocytosis of EGFR. As shown in Fig. 2d the reduced amount of internalized receptor caused by this impaired c-Cbl–EGFR association prolonged ERK activation. This prolonged ERK activation causes stronger mitogenic signal that eventually leads to uncontrolled cell proliferation in cancers.

2.2. Model validation against reported experimental and simulation studies of EGFR-ERK signaling

We also validated our model with other published experimental and simulation studies on EGFR-ERK signaling. The validated results are shown in Fig. 3a–d in which simulated trends of time-dependent protein concentration and activity profiles of ERK, PP2A, MKP3, and RhoA are in reasonable agreement with experimentally determined profiles. For instance, at 50 ng/ml of EGF, the simulated ERK activation peaks at ~5 min and decayed within 50 min (Fig. 3a). The simulated result is consistent with experimental finding that treatment of 50 ng/ml and 100 ng/ml EGF in PC12 cells transiently activates ERK which peaks within 5 min and decays within 30–60 min [31].

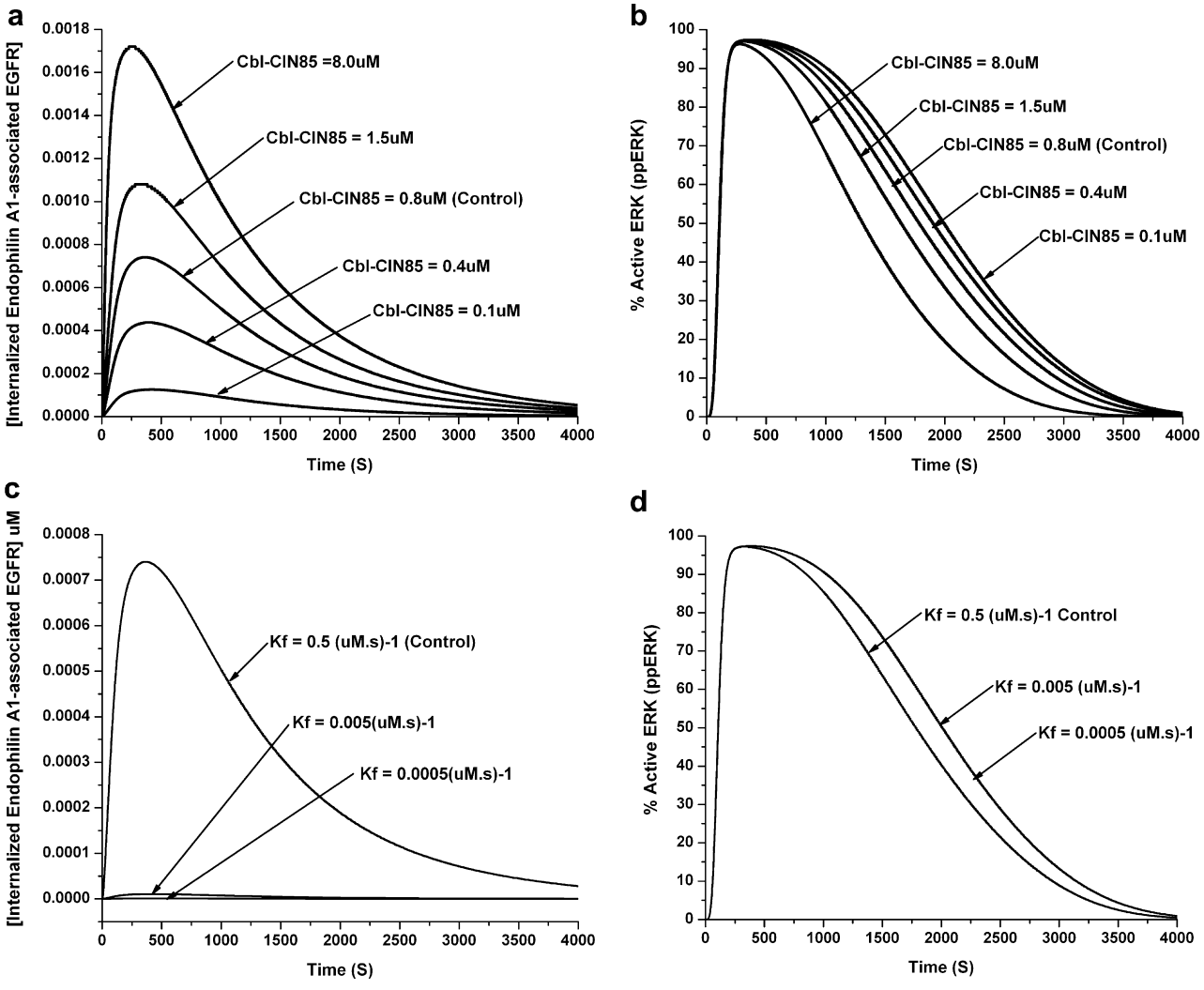

Fig. 2. (a) Profile of internalized endophilin A1-associated EGFR concentration at different Cbl-CIN85 concentrations. (b) Profile of active ERK concentration (in units of percentage of initial ERK concentration) at different Cbl-CIN85 concentrations. (c) Profile of internalized endophilin A1-associated EGFR concentration at different phoaphatases K_f value (forward kinetic value for Eq. (114) in the Supplementary Table S1). (d) Profile of active ERK concentration (in units of percentage of initial ERK concentration) at different phoaphatases K_f value (forward kinetic value for Eq. (114) in the Supplementary Table S1).

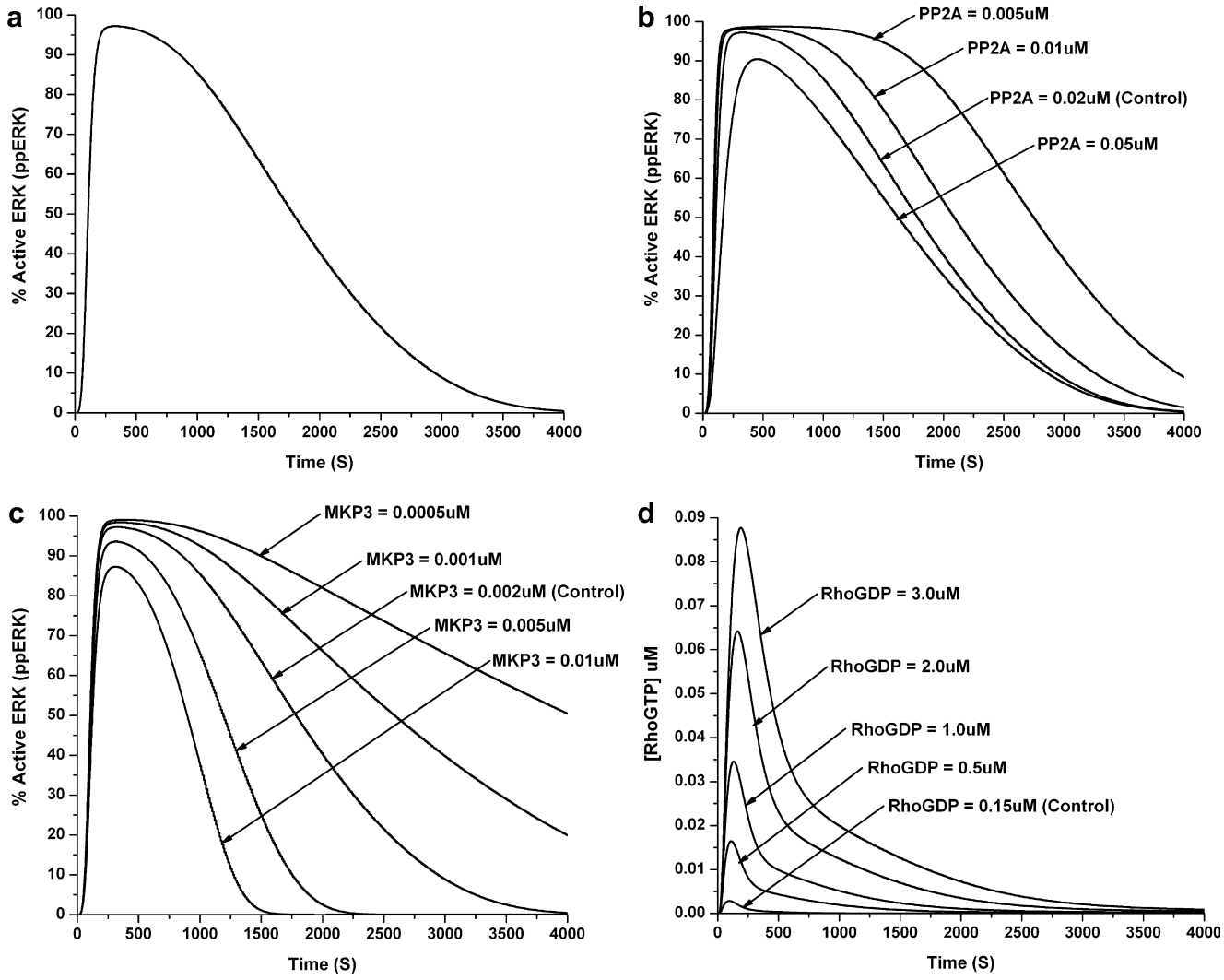

Fig. 3. (a) Profile of active ERK concentration (in units of percentage of initial ERK concentration) upon activation by EGF. (b) Profile of active ERK concentration (in units of percentage of initial ERK concentration) at different phoaphatases PP2A concentrations. (c) Profile of active ERK concentration (in units of percentage of initial ERK concentration) at different phoaphatases MKP3 concentrations. (d) Profile of active RhoA (RhoGTP) concentration at different RhoA (RhoGDP) concentrations.

The validity of our simulation model was further evaluated with two additional published simulation results and experimental data. As shown in Fig. 3b, low levels of PP2A (from 0.005 to 0.01 μM) that differ by 2-folds show little effect on the change of maximal amount of active ERK but substantially affect the duration of ERK activation (Fig. 3b). On the other hand, low levels of MKP3 (from 0.0005 to 0.001 μM) show little effect on the change of maximal amount of active ERK but significantly affect the duration of ERK activation, as the period of ERK activity within these low levels is significantly extended (Fig. 3c). However, high levels of MKP3 (from 0.005 to 0.01 μM) significantly decrease the duration of ERK activation more than the change of maximal amount of active ERK. These simulated results are consistent with the results of a reported simulation study [35]. It is noted that, at higher levels of PP2A and MKP3, the degree and duration of active ERK decreases. In addition, for RhoA activation profile increasing initial concentrations of GDP-bound RhoA increases the level of active GTP-bound RhoA (Fig. 3d). This showed that at normal cycle of RhoA the activity or amount of active GTP-bound RhoA is directly correlated with the amount of inactive GDP-bound RhoA.

2.3. Effects of RhoA and ROCK on EGFR endocytosis via endophilin A1-mediated crosstalk

From the above validated results, we next explored the regulation of RhoA and ROCK on EGFR endocytosis. Active GTP-bound RhoA activates its effector ROCK. The activated ROCK subsequently phosphorylates endophilin A1 at Thr-14 thereby preventing its recruitment to the EGFR-c-Cbl-CIN85 complex leading to delayed endocytosis of EGF-bound EGFR to the lysosomal pathway [12,36]. As the extent and duration of ERK activation is dependent on the amount of active EGFR in cell membrane, increased amount of RhoA or ROCK is expected to prolong ERK activation. We first showed that amount of internalized endophilin A1-associated EGFR is dependent on amount of active ROCK. Simulation results shown in Fig. 4 indicated that increased amount of ROCK reduces the amount of internalized endophilin A1-associated EGFR, which is consistent with experimental obser-

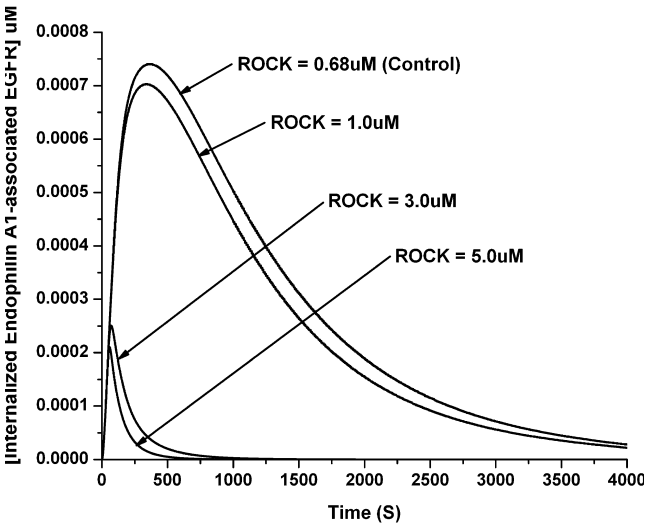

Fig. 4. Profile of internalized endophilin A1-associated EGFR concentration at different ROK concentrations.

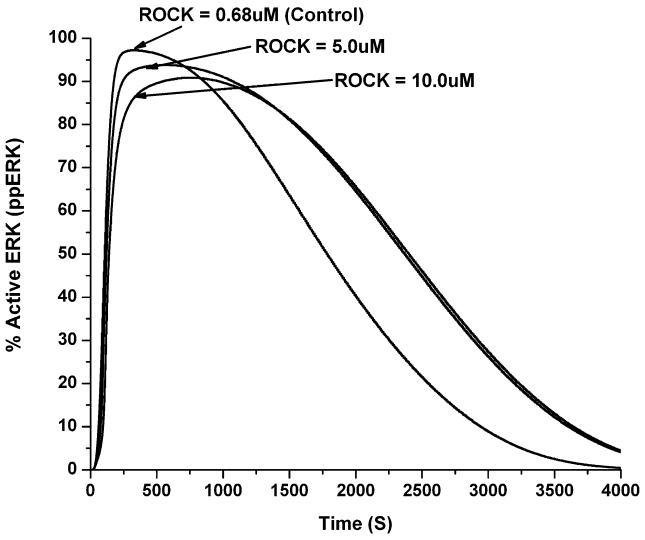

Fig. 6. Profile of active ERK concentration (in units of percentage of initial ERK concentration) at different increased ROCK concentrations.

vations [12,36]. In particular, when the amount of ROCK is >3.0 μM, both the amount and duration of internalized endophilin A1-associated EGFRs are significantly decreased. We next showed that the increased amount of active ROCK is dependent on activation of RhoA. As shown above increasing initial concentrations of GDP-bound RhoA increases the level of active GTP-bound RhoA (Fig. 3d), simulation results shown in Fig. 5 indicated that increasing the initial concentrations of GDP-bound RhoA significantly enhances the activation of ROCK. The simulation results suggested that amount of active ROCK is indeed dependent on amount of active RhoA that is consistent with experimental facts that ROCK is the effector of RhoA [37,38]. Consequently, increased amount of RhoA has a significant impact in preventing the association of endophilin A1 to the EGFR-c-Cbl-CIN85 complex via its effector ROCK.

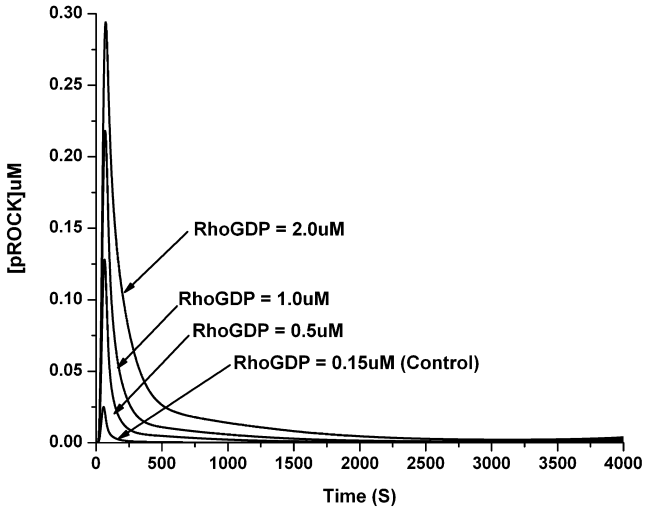

Fig. 5. Profile of active ROCK concentration at different RhoA (RhoGDP) concentrations.

2.4. Effects of RhoA and ROCK on ERK activation

We next examined the overall effects of RhoA to the activation of ERK in our model. As activation of ERK is dependent on the amount of active EGF-bound EGFR and RhoA negatively regulates EGFR endocytosis via ROCK and activates kinase activity of scaffold MEKK1, as a whole RhoA plays a positive regulator of ERK activation in cells containing this RhoA-EGFR crosstalk. Our simulation results showed that, when the amount of ROCK is increased from 0.68 μM to 15.0 μM the duration of ERK signaling is slightly prolonged and its maximal level is slightly decreased (Fig. 6). As indicated previously in Fig. 4, increased amount of ROCK reduced the amount of internalized endophilin A1-associated EGFR. Hence, prolonged duration of ERK activation by increased ROCK is due to reduced EGFR endocytosis. Our next simulation results indicated that over-expressing RhoA also prolonged the duration of ERK activation but with decreased maximal level of active ERK (Fig. 7).

We next examined the contribution of RhoA in ERK activation via inhibition of endocytosis of endophilin A1-bound EGFR by ROCK and RhoA-MEKK1 crosstalk. We "turned off" the interaction of RhoA to the scaffold protein MEKK1 by setting K_f values of corresponding equations to zero (Eqs. (178)–(181) in the Supplementary Table S1). In this condition, increasing the amount of RhoA to the activation of ERK is only due to delayed EGFR endocytosis mediated by ROCK. However, turning off the interaction between RhoA and MEKK1 does not affect the amount of internalized EGFR. Over-expressing ROCK in this condition produced similar results as shown in Fig. 6 and slightly prolonged ERK activation due to reduced amount of internalized EGFR (data not shown). Although there is no competitive binding between RhoA and ERK to MEKK1, increasing amount of GDP-bound RhoA prolonged ERK activation but to a lesser extend than that shown in Fig. 7 and the maximal levels of active ERK were reduced to a slightly larger extend than that shown in Fig. 6 (data not shown). This is due to the reason that over-expression of RhoA significantly activate ROCK to further reduce EGFR endocytosis (as shown in Fig. 5) that enhanced

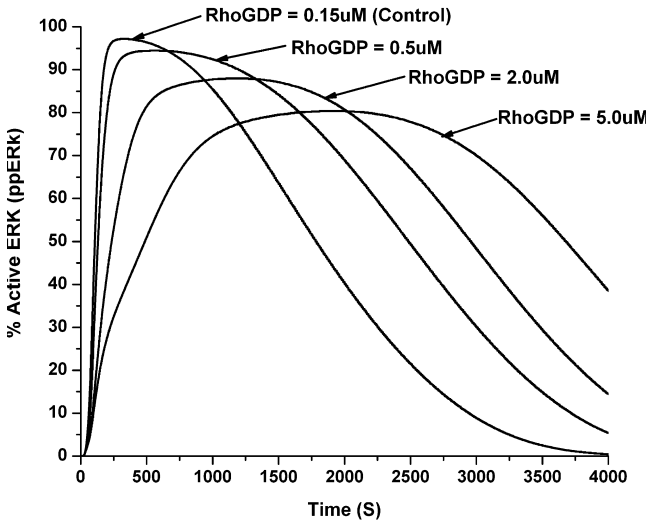

Fig. 7. Profile of active ERK concentration (in units of percentage of initial ERK concentration) at different decreased RhoA (RhoGDP) concentrations.

ERK activation. However, active ERK served as a negative feedback loop to active SOS. Hence, over-expression of RhoA causes prolonged ERK activation by reducing EGFR endocytosis but with slight reduced level of active ERK due to this negative feedback.

Next we "turned off" the interaction of RhoA to ROCK by setting Kf values of corresponding equations to zero (Eqs. (106) and (107) in the Supplementary Table S1). In this condition, increasing the amount of RhoA to the activation of ERK is only due to the crosstalk of RhoA to MEKK1. MEKK1 is a scaffold protein that binds ERK2 cascade components such as Ras, Raf1, MEK1, and ERK2 [39]. The unique feature of MEKK1 from other scaffold proteins is that it possesses a kinase domain that can further activate ERK2 cascade besides its scaffolding role. Binding of Raf1 to MEKK1 activates its kinase domain. The activated kinase domain of MEKK1 subsequently phosphorylates and hence activates bound MEK1 [40]. Activated MEK1 subsequently phosphorylates ERK2 once it binds to MEKK1. RhoA is capable to bind to MEKK1 and activates its kinase activity to further activate ERK2 [41]. Hence, our simulation results (data not shown) showed that over-expressing RhoA alone prolonged duration of active ERK via activating kinase activity of MEKK1. On the other hand, reduced maximal amount of active ERK with increased RhoA is partly due to the competitive binding mode of RhoA and ERK to the overlapping binding sites on the scaffold protein MEKK1 (see Section 4 for detail description of scaffold modeling). These results suggested that RhoA plays independent role in regulating the activity of ERK via ROCK-mediated inhibition of EGFR endocytosis and MEKK1-mediated activation of ERK.

Our simulation results from current mathematical model suggested that RhoA prolongs the duration of active ERK via inhibition of endophilin A1-mediated EGFR endocytosis by ROCK and MEKK1-mediated ERK activation. Overall, our current mathematical model hypothesizes alternative role of RhoA in promoting mitogenic signals in tumorigenesis by prolonging ERK activation in addition to its regulation of cytoskeleton and cell motility.

3. Concluding remarks

Our simulation model was developed to incorporate the effects of RhoA in regulating EGFR endocytosis via endophilin A1. Our simulation results suggested that RhoA and its effector ROCK are negative regulators of EGFR endocytosis via the crosstalks. In other words, RhoA positively regulates ERK activation partly via ROCK to the endophilin A1-mediated crosstalk. Elevation of RhoA substantially prolongs the duration of ERK activation but with reduced maximal amount of active ERK. It is noted that the effects of collective regulations by other key regulators such as Ras, SOS, PI3K, and SHP2 [42,43] via other cascades or cross-talks are not covered in our mathematical model. Further investigation of this and other relevant cascades and cross-talks is required [44,45]. A more comprehensive understanding of the primary and secondary signal transduction roles of RhoA and other key players in different cell and tissue types [46], the mechanism of their involvement in the collective promotion of cell proliferation and invasion in carcinoma cells [47], and the mechanism and effects of anti-proliferative agents targeting these proteins and pathways [48] await for future studies.

4. Materials and methods

4.1. Model development and collection of kinetics parameters

One of the most commonly used approaches to model biological pathway systems is that of ordinary differential equations (ODEs). In general, a differential equation can be used to describe the chemical reaction rate that depends on the change of participating species over time. The temporal dynamic behavior of molecular species in the biological signaling pathway network can be captured by a set of coupled ODEs [49]. The overall pathway internal links and architecture of our mathematical model is shown in Fig. 1. The information for the topology of the crosstalk was collected from various published works [27–32]. All equations for molecular interactions in this study were derived based on laws of Mass Action. The kinetic parameters for forming protein–protein interaction complexes in forward (K_f) and complex dissociation in backward reactions (K_b) were used. For enzymatic and irreversible reactions, the reaction constant (K) and turn over rates (K_{cats}) were used instead of the Michaelis–Menten constants (K_m) that are primarily applicable to steady-state models.

Kinetic parameters used in this study are mostly obtained from published experimental data. For those kinetic parameters that are unavailable from experimental data, similarity-based strategies were used to derive putative kinetic constants. Ranges of kinetic parameters are constrained based on the literature data and in vivo measurements of signaling kinetics [27,29–32,50]. A set of coupled ODEs was used to describe the reaction network. Our model contains 205 equations and interactions with 194 distinct molecular species, characterized by 313 kinetic parameters and 38 initial molecular concentrations. These ODEs were then solved using the Ode45 solver of MatLab [51]. The systems biology markup language (SBML) of our model is also provided in the Supplementary Material (the Supporting Information can be found on the FEBS Letters web site).

4.2. Modeling MEKK1 as scaffold for crosstalk of RhoA and ERK

The scaffold protein considered in this model is MEKK1. The MEKK1 is represented as MEKK1abcdef in the chemical equations where a, b, c, d, e, and f are binding sites for Raf1, MEK1, ERK2, RhoA, p115 RhoGAP, and Ras, respectively [39,41,52–55]. Site "a" corresponds to residues 221-370 which is a proline-rich region, site "c" corresponds to residues 370–559 which constitute PHD domain, site "d" corresponds to residues 437–456 which constitute PHD domain, and site "e" corresponds to residues 565–636. Sites "b" and "f" are binding sites of MEK1 and Ras that are corresponding to N-terminal and C-terminal of MEKK1, respectively. For instance, binding of MEK1 and Ras to MEKK1 can be simplified as: MEK1 + MEKK1abcdef = MEKK1abMEK1cdef and Ras-

GTP + MEKK1abcdef = MEKK1abcdefRasGTP, respectively. Since site "d" is within site "c" that are correspond to the binding of RhoA and ERK2, we model the binding of these proteins to MEKK1 as competitive manner where only one component binds at one time.

The kinetics parameters for MEKK1-mediated protein–protein interactions were obtained from yeast scaffold analog Ste5 [40] and were constrained according to experimental observations. Due to multiple binding sites on the scaffold protein by multiple signaling molecules, the possible combinations for scaffold-signaling molecule complexes are enormous. In this study, only scaffold complexes that involved in main processes were considered. The full list of scaffold-mediated reactions and corresponding kinetics parameters are given in the Supplementary Table S1.

4.3. Model optimization and validation

Mathematical models developed at systems levels are generally unable to reproduce exact quantitative values in all systems but are capable to produce known behavior or trend that agreed well of those systems under investigating. For instance, mathematical model developed for a biological pathway from parameters obtained experimentally from one cell type can behave slightly different in another cell types. The different of the behavior of the model in these cell types can be due to the present or absent of a crosstalk (i.e. the topology and hence the boundary of the mathematical model) and variation in values of kinetic parameters used. Hence, in this study we developed a generic model of EGFR-ERK signaling pathway with endophilin-mediated RhoA-EGFR crosstalk to investigate the role of RhoA in regulating endophilin-mediated EGFR endocytosis.

The simulated results are represented in curves of concentrations of a chemical species over time that are validated against available experimental data. If the trend or dynamics of the curves of a particular reactant or product behave as the experimental data suggest, then the model is said to be optimized and can be used to analyze and predict unknown biological phenomena within the boundary of the model. If the simulation results were not fair in well agreement with known experimental facts, then the definition as well as the boundary of the model has to be revisited to examine possible errors such as incorrect interaction kinetics or values of kinetics parameters.

Optimized parameters obtained from previous mathematical models are not necessarily optimized in current study as the boundaries of these models are different. As a biological network is robust and binding affinity of protein–protein interactions for proteins in similar family that mediate similar types of biochemical reactions (such as Ras and RhoA) differ within 10-fold range hence the values of kinetics parameters obtained from previous models are optimized within these ranges. Likewise, for parameters that are not available from previous models are obtained from proteins in similar family. For instance, the parameters of RhoA activation cycle are obtained from Ras activation cycle and are further optimized in 10-fold ranges. The cycle of optimization and validation are repeated in order to obtain simulated results that agreed well with known experimental trends.

Conflict of interest statement

We declare that we have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.05.026.

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