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Handling Complex Rule-Based Models of Mitogenic Cell Signaling (on the Example of ERK Activation upon EGF Stimulation)

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***Abstract*—To continue our research on systems biology of mitogenesis, we have developed and fitted according to the experimental data a highly-branched network model of ERK activation in response to EGF stimulation. To produce a network with full number of possible complexes and reactions that may emerge during signal propagation, we used the rule-based software tool in systems biology, BioNetGen 2. Although our network model contains more than 650 complexes and 5500 reactions, we showed the ability the handle this complexity, even using the manual parameter fitting. Analyzing the results of model fitting, we discuss possible details of protein-protein interaction, such as preferable sites/domains for binding one another, sequestration of active enzymes via binding to huge protein complexes etc. Plans for experimental validation of modeling results are also considered.**

Keywords-systems biology, mitogenic cell signaling, rule-based network modeling, model fitting, protein-protein interaction

I. INTRODUCTION

Complex molecular machinery that determine malignization of a cell, has been systematically investigated for more that a decade [1]. Receptor tyrosine kinase (RTK)-initiated signaling networks that determine growth factor, apoptotic, survival, cytokine-induced etc. response of a cell involve a plethora of types of proteins, which, in their own turn, possess multiple binding sites/domains. As a result, pure experimental research methods may mot always give an exhaustive answer to the question of molecular etiology for a certain cancer case, since it may require detailed measurements of interactions between several dozens of proteins that transfer the mitogenic signal.

On the other hand, much valuable information on the details of protein-protein interaction may be obtained using *in silico* analysis of chemical kinetics for signal transduction [2]. Such researches can be useful to understand what protein activity should be either down-regulated or enhanced for the prevention of carcinogenesis or tumor suppression and destruction. Computational approach in molecular oncology has been also extensively applied for more than a decade [3-6]. This approach means that the researcher constructs the kinetic model of protein-protein interaction, performs the ODE integration for concentrations of various chemical species (i.e., protein complexes in certain states), adjusts the unknown kinetic constants to fit the experimental data of activation kinetics and, finally, makes predictions about the different details and conditions of signal propagation.

Although aggregation of protein molecules into sophisticated complexes leads to the combinatorial explosion of the number of chemical species that emerge during signal propagation [7-9], we have previously developed and tested several models that take into account the combinatorial complexity. These models include those that describe activation of one the most important mitogenic effectors, extracellular regulatory kinase (ERK), as well as the serine/threonine kinase AKT that is important for anti-apoptotic response, upon the stimulation of the HEK293 cells with epidermal growth factor (EGF) [10], as well as with insulin [11].

We have shown experimentally that in HEK293 cell line insulin does not activate ERK much, although it considerably amplifies the ERK response upon EGF stimulus [11]. Using mathematical modeling, we have hypothesized [11] the essential role of the adapter protein Grb2-associated binder 1 (GAB1) that plays the key role in signal propagation upstream of the small GTPase Ras [10], whereas the major mechanism of GAB1-depdndent signal amplification is recruitment of GAB1 (and other adapter proteins) to the plasma membrane via phosphatidylinositol-3,4,5-triphosphate (PIP_3) [10-11]. Our hypotheses were checked and confirmed during the series of experiments, such as Western blotting, inhibition of keystone enzymes in signaling pathways, as well as siRNA-based depletion of important proteins in signal transduction process [10-11].

Nevertheless, some details of GAB1 interaction with its partners remain unclear. This protein molecule is known to posses multiple docking sites that specifically bind numerous partners such as phosphatidylinositol-3 kinase (PI3K) [12], GTPase activation protein RasGAP [13], tyrosine phosphatase SHP2 [11], tyrosine kinases of the Src family [14] etc. Moreover, one of major GAB1 partners, growth factor receptor binder 2 (Grb2), has been reported to bind to GAB1 according to multiple ways. First, the association of Grb2 and GAB1 can be performed via on phosphorylated tyrosine residues at GAB1 and SH2 domain at Grb2 [15-16]. Second, the above-mentioned binding may be performed via

the C-terminal Src homology 3 (SH3) domain of Grb2 and the proline-rich domain (PRD) of GAB1 [17-18]. Our previous *in silico* studies [10-11] have not favored any of these possible scenarios due to the limited description of combinatorial complexity in these models. To obtain several hints and insights on the details of protein-protein interaction within the mitogenic signaling network, we decided to build and investigate a full-scale combinatorially complex network model.

II. Materials and Methods

Since our goal was to distinguish different scenarios of protein-protein interaction using computational methods, we decided to build as detailed model as possible. That is why the model was developed using the software BioNetGen 2 [19], which, along with StochSim [20-21], Kappa [22-23], Moleculizer [24] and *Lillte b* [25], describes highly-branched kinetic networks using the rule-based approach. This approach means that all possible chemical complexes (species) that emerge during the signal propagation are generated algorithmically according to the user-specified reactions rules that describe certain events on certain sites on certain protein molecules. Along with the entire graph of chemical transformations of molecules and their complexes, the rule-based systems biology software builds the corresponding system of ordinary differential equations (ODE) for concentration of each species. Like most code packages for rule-based description of molecule interactions, BioNetGen 2 allows ODE integration using both deterministic Runge-Kutta and stochastic Monte Carlo (Gillespie) [26] method.

III. Results and Discussion

A. Protein-protein interaction in the model

We have developed a highly-branched model for activation of mitogenic (Ras/ERK) and survival (AKT) targets upon EGF stimulation. Our model is a further step in computational research of mitogenic signaling pathways that we have conducted during the past decade [10-11]. Our previous descriptions showed the ability to describe considerable amount of measured data and to make experimentally verifiable predictions on the details of cell signaling propagation. However, they both were "manually" developed: all species and reactions in the network were specified by the model developers.

Although the network structure of the current model differs significantly from our previous models, most proteins and their principal relationships are quite similar to what we have described previously (see Fig. 1, which is pretty similar to the corresponding figures in [10] or [11]). Signal propagation starts with activation of the cell-surface receptor [3]. Upon EGF binding, the receptor dimerizes and undergoes transphosphorylation at tyrosine residues in the cytoplasmic tail [3]. These residues can bind Shc, Grb2 (followed by binding of the guanine exchange factor SOS), p85 subunit of PI3K and GTPase RasGAP [3-5]. All the species that contain phosphorylated EGFR may be endocytosed and degraded, releasing the binding partners of EGFR. Primary activation of the tyrosine kinases of the Src family (SFK) is also done by EGFR [5].

The major role in the amplification of the Ras/MEK/ERK signaling is played by the adapter protein GAB1. GAB1 is recruited to the plasma membrane through PIP_3 via the plekstrin homology (PH) domain [27]. When bound to the membrane, either via PIP_3 or via Grb2-(Shc)-EGFR, GAB1 may be phosphorylated by EGFR or SFK, which is followed by binding Grb2 [15-16], PI3K [12], RasGAP [13] (for the sake of simplicity, we assume competitive binding of these partners to GAB1) or SHP2 [12]. When bound to GAB1, SHP2 exhibits phosphatase activity against the phosphorylation sites on GAB1, as well as on EGFR [12].

Likewise, Grb2 also possesses scaffolding properties. We took into account even in [10] that it has two SH3 domains (the N-terminal domain specifically binds SOS, while the C-terminal one binds GAB1 [17-18]. In addition, the SH2 domain at Grb2 binds phosphotyrosine residues, both on EGFR and GAB1 [15-16].

To make distinction between two modes of GAB1-Grb2 binding, we created two variants of signaling models that are symbolically called A and B (see Fig. 2). The only difference between protein-protein interaction in models A and B is the sites/domains for Grb2-GAB1 binding. Whereas the model A assumes that these proteins bind each other via the proline-rich domain (PRD) of GAB1 and C-terminal SH3 domain of Grb2, in the model B these proteins associate via one of the numerous tyrosine residues at GAB1 and SH2 domain at Grb2.

PIP_3 (which is produced by the membrane-recruited PI3K) recruits to the plasma membrane adapter protein GAB1 and a serine/threonine kinase PDK1. Membrane-recruited PDK1 causes Akt phosphorylation at Thr308 residue [28-29].

Membrane-recruited SOS produces transformation of Ras-GDP into Ras-GTP complex [3, 30]; the reverse process is catalyzed by membrane-recruited RasGAP. Ras-GTP causes primary activation of Raf [31], however, for full activation of Raf, active SFK is needed [32]. Active Raf causes MEK activation by dual phosphorylation of MEK activation loop [33]. Active MEK causes ordered phosphorylation of ERK [34].

As a serine/threonine kinase, active ERK may impose negative feedbacks via phosphorylation of serine/theronine inhibitory sites at SOS and GAB1 [35-37].

B. Model size in comparison with our previous results

Our first large-scale model of signaling networks [10] assumed independent binding of multiple partners to the scaffolding proteins like GAB1 and Grb2. Since it was constructed manually, we needed certain model reduction methods [7-9, 38] that replace highly branched networks that describe transitions between the states of a scaffold protein with more compact pathways that involve several virtual ("macroscopic") proteins that possess fewer sites than a real scaffold. Contrary, for the sake of simplicity, during modeling of coupled insulin-EGF signaling [11], we used the assumption of competitive binding of partners to the scaffold proteins, which reduces the number of chemical species and reactions, however, makes impossible any predictions on the

details of protein-proteins interaction (identification of binding sites/domains etc).

In contrast to our previous work, the current model exploits the ability of BioNetGen 2 to generate all possible complexes and reactions that may arise during the signal propagation according to the user-specified reaction rules. In our example, BioNetGen 2 generated produced more than 2000 chemical species and 12000 reactions for the model A and more than 650 species and 5500 reactions for the models B, which makes our model one of the biggest in current systems biology of mitogenesis (at least, it leaves our previous attempts to describe mitogenic signaling networks quantitatively far beyond in terms of model size). Table I presents the comparison of size and overall topology of signaling network models that we have developed during the past decade.

Despite rather large model size, total computation time (including signaling network generation, equilibration of species concentration prior the stimulation followed by calculation of signaling kinetics after adding EGF) was 15.8 min for the variant A and only 1.43 min for the variant B at a personal computer with CPU frequency of 1.82 GHz and RAM capacity of 1 Gb.

C. Fitting kinetic parameters and model predictions

Methods for network signaling model handling and verification that we used in the current work were similar to what we applied in previously published works [10-11]. During the "training"/"fitting" process, one must be cautious not to exceed the boundaries for kinetics parameters that are imposed by experimental hints for the similar processes. In addition, any reaction should not be faster than it is prohibited by the diffusion limit.

TABLE I. OVERVIEW OF OUR SIGNALING NETWORK MODELS

	Ref. [10]	**Ref. [11]**	**Current work**
Initiating ligands	EGF	EGF + insulin	EGF
Number of species	~200	78	2022 (model A) 665 (model B)
Number of reactions	~500	111	12148 (model A) 5733 (model B)
Combinatorial complexity	Independent binding with "manual" model construction	Competitive binding with "manual" model construction	Independent binding with automated model construction

To make the model more "robust", after completion of parameter fitting, the researcher should make experimentally verifiable predictions, which include, for example, some model perturbations, such as inhibition of certain enzymes or increases/decrease of certain protein abundances via protein overexpression or, perhaps, siRNA-assisted depletion.

Fig. 3 shows the results of our model "training" on the example of ERK (left column) and Akt (right column) activation patterns. Models A (upper row) and B (middle row) were "trained" for three values of EGF dose (20 nM, 1 nM and 0.2 nM), as well as for action of PI3K inhibitor wortmannin (WT), according to the experimental (Western blotting) data that we published previously for HEK293 cells (lower row, taken form [11]). Although the activation curve shapes for models A and B are not always strictly similar, it was impossible to make clear preferences for the variant A or B at this "training"/"fitting" step. The same conclusion may be made for the GAB1, Ras and MEK activation curves (data not shown).

Previously, we have demonstrated [10-11] the crucial role of GAB1 as an enhancer of mitogenic signaling upon EGF simulation. When GAB1 is recruited to the plasma membrane via PIP_3, it may bind PI3K, which produces more PIP3, thus increasing the concentration of the membrane-recruited GAB1 and closing the positive feedback loop. The effects of feedback loop disruption by GAB1 depletion, which we computationally predicted in our earlier models, have been experimentally verified using the siRNA method [10-11].

Interestingly, our calculations (see Fig. 4) show that only model B (middle panel) was capable to reproduce experimental results (right panel) for the influence of GAB1 depletion on the ERK response to EGF stimulation of HEK293 cells. Contrary, the model A (left panel) did not show the decrease of ERK signal even for the total removal of GAB1 for the cell. This surprising effect is caused by the specific "sequestration" of Grb2 by GAB1 in the model A. If a large GAB1-containing complex binds Grb2-EGFR or Grb2-Shc-EGFR complex via the C-terminal SH3 domain of Grb2, the resulting reaction product may exceed the critical number of protein molecules in the complex (we assumed than any complex cannot contain more than five molecules), thus preventing SOS binding to Grb2 via the N-terminal SH3 domain. However, in the model B, GAB1 and EGFR cannot bind to Grb2 simultaneously (see lower right panel in Fig. 2), so that GAB1 cannot "sequester" the membrane-recruited (via EGFR) Grb2 from the pool of molecules that are capable to recruit SOS to the membrane.

D. Plans for experimental validation of modeling results

Although our results showed feasibility and traceability of large-scaled combinatorially complex signaling network models that are automatically generated using the rule-based software for systems biology, our finding that model B may be more adequate than model A, needs further verification.

It should be notified that the experimental validation, which may favor scenario A or B, may introduce several experimental errors or uncertainties. Direct measurements may lead to triple immunoblotting (detection of certain phosphotyrosine residue in the simultaneous Grb2-GAB1 precipitate), which has very low registration efficiency, and, consequently, large relative errors.

Experiments with mutant proteins (for example, substitution of GAB1 tyrosine residue that binds SH2 domain for Grb2 with phenylalanine) inevitably involve animals (e. g., mice), and the use of animals may introduce extra uncertainties due to the human-murine differences in the genome and proteome.

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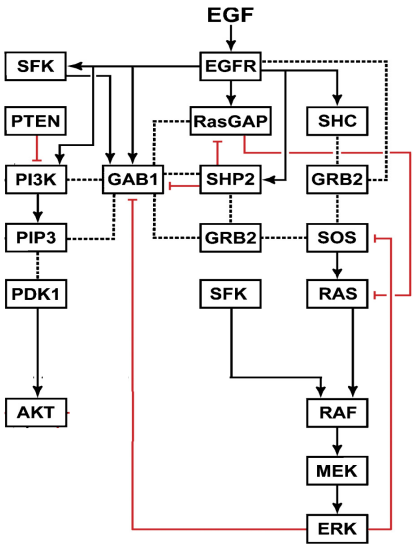

Figure 1. Flow chart of signal propagation through the EGFR signaling network (pretty similar to our previously published models [11]). Solid lines with arrows show the activation or tyrosine phosphorylation of proteins and lipids. Dotted lines represent direct protein–protein and protein–lipid interactions. Red lines with blunt ends show inhibition.

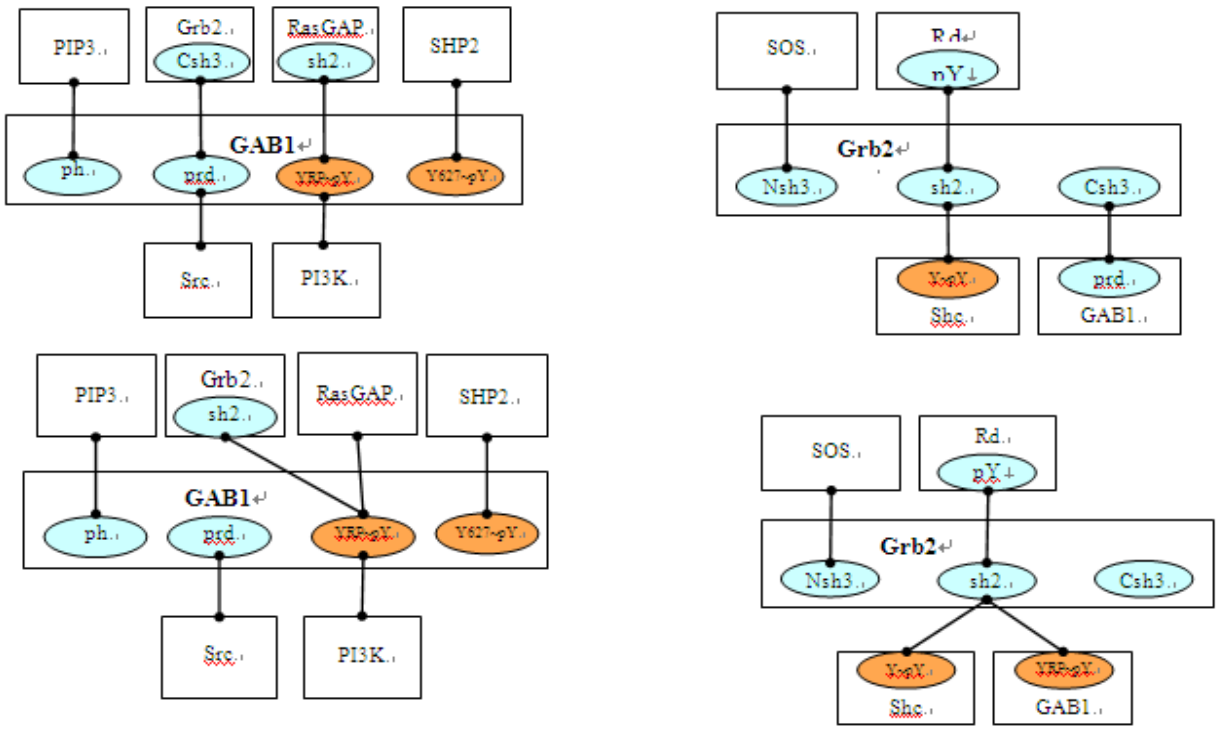

Figure 2. Domain/site structure and binding partners of two major scaffold proteins, GAB1 and Grb2, according to the variants A (upper row) and B (lower row) of the signaling network model. Rectangles represent protein or lipid molecules, blue ovals – protein domains, orange ovals – tyrosine residues.

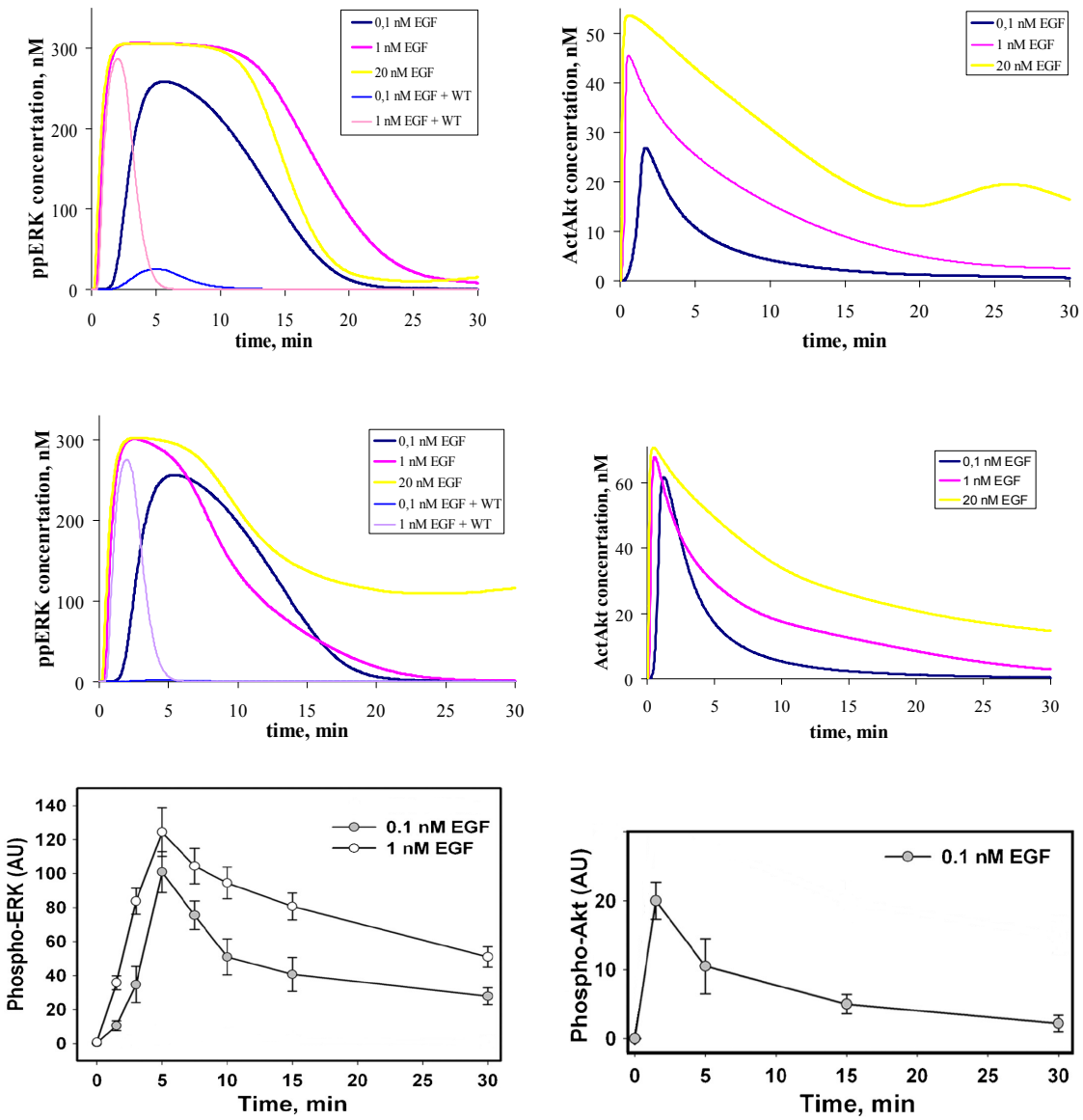

Figure 3. Model "training/fitting" according to the experimental data on ERK (left column) and Akt (right column) activation. Upper row: results of model A fitting for different EGF doses and application of wortmannin (WT), a PI3K inhibitor. Middle row: the same results for model B fitting. Lower row: experimental western blotting data, which we obtained previously on HEK293 cells [11]

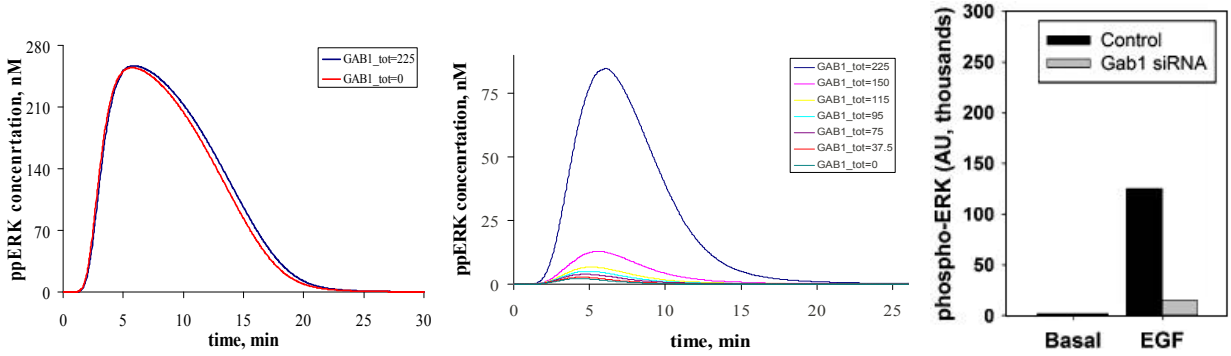

Figure 4. Model predictions on effects of siRNA-induced GAB1 depletion for EGF concentration of 0.1 nM. Left panel: model A. Middle panel: model B. Right panel: experimental (western blotting) validation [11] for the influence of siRNA-induced GAB1 depletion on ERK activation at 1.5 min after EGF stimulation of HEK293 cells.