

Rule-based modeling: a computational approach for studying biomolecular site dynamics in cell signaling systems

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Rule-based modeling was developed to address the limitations of traditional approaches for modeling chemical kinetics in cell signaling systems. These systems consist of multiple interacting biomolecules (e.g., proteins), which themselves consist of multiple parts (e.g., domains, linear motifs, and sites of phosphorylation). Consequently, biomolecules that mediate information processing generally have the potential to interact in multiple ways, with the number of possible complexes and posttranslational modification states tending to grow exponentially with the number of binary interactions considered. As a result, only large reaction networks capture all possible consequences of the molecular interactions that occur in a cell signaling system, which is problematic because traditional modeling approaches for chemical kinetics (e.g., ordinary differential equations) require explicit network specification. This problem is circumvented through representation of interactions in terms of local rules. With this approach, network specification is implicit and model specification is concise. Concise representation results in a coarse graining of chemical kinetics, which is introduced because all reactions implied by a rule inherit the rate law associated with that rule. Coarse graining can be appropriate if interactions are modular, and the coarseness of a model can be adjusted as needed. Rules can be specified using specialized model-specification languages, and recently developed tools designed for specification of rule-based models allow one to leverage powerful software engineering capabilities. A rule-based model comprises a set of rules, which can be processed by general-purpose simulation and analysis tools to achieve different objectives (e.g., to perform either a deterministic or stochastic simulation). © 2013 Wiley Periodicals, Inc.

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INTRODUCTION

How does the behavior of a cellular regulatory system emerge from the physical interactions of its constituent biomolecules? What information processing and decision-making functions are performed by these interactions? What are the design principles underlying these functions? The field of systems biology aims to address such questions using a suite of experimental and computational approaches.^{1–3} Among the computational approaches is rule-based modeling,^{4,5} which is particularly useful for studying cell signaling.^{6–26} A characteristic feature of this approach is the use of rules to represent interactions. As we will discuss, the use of rules streamlines specification and enables simulation of models that capture biomolecular site dynamics, the structurally resolved chemical kinetics of biomolecular interactions. Studies of site dynamics are needed to understand the behavior of cell signaling systems because (1) biomolecular interactions (e.g., protein–protein interactions) depend on specific structural interfaces^{27–31} and (2) these interactions are transient.^{27,32,33} In the first two sections that follow, we discuss aspects of biomolecular interaction networks and modeling concerns that motivate the rule-based modeling approach. After these sections, we provide an introduction to basic rule-based modeling concepts, which features discussion of an example model. We then discuss simulation techniques, including the advantages and disadvantages of various methods, and issues related to knowledge representation, including model visualization and annotation and new high-level approaches to model specification. We conclude with a brief discussion of the potential for model reuse and collaborative model development to contribute to our predictive understanding of cell signaling.

BIOMOLECULAR INTERACTION NETWORKS: COMPLEX, DYNAMIC, AND MODULAR

Complex networks of interacting biomolecules are ubiquitous in cells, and form the regulatory machinery controlling fundamental biological processes,³⁴ such as metabolism, gene expression, and signaling, which are interdependent.³⁵ We focus here on networks that mediate intracellular signaling, i.e., detection of environmental stimuli and processing of this information to yield a cellular response. These networks are overlapping and interconnected³⁶ partly because of promiscuity³⁷ and crosstalk, which can have functional consequences.³⁸ The most prominent biomolecules in signaling systems are proteins,^{39,40} but

lipids, nucleic acids, and small-molecule metabolites also play important roles. Proteins have been subject to a barrage of evolutionary pressures,^{41,42} which has led to emergence of modular protein elements: functional units that can operate somewhat independently and that offer adaptive benefits.⁴³ These functional units can be combined into diverse arrangements through mechanisms such as gene duplication and fusion,⁴³ or through deliberate engineering,⁴⁴ to generate new connections between pathways and new capabilities.⁴⁵

A protein with multiple functional components has the potential to interact with multiple binding partners simultaneously. A further layer of complexity arises because a protein may be subject to posttranslational modifications at multiple sites,^{46,47} which regulate interactions and modulate catalytic activities. Many different combinations of interactions and multisite modifications may be possible, with different combinations contributing to different signaling functions.³⁸ This hallmark feature of signaling systems has been termed combinatorial complexity.⁴⁸ The challenges posed by combinatorial complexity, discussed below, motivate the rule-based modeling approach.

In addition to being intricately connected, signaling networks are highly dynamic. Most interactions among proteins in such a network are noncovalent and transient,⁴⁹ and the outcomes of enzyme–substrate interactions within these networks, such as serine, threonine, and tyrosine phosphorylation, are known to be condition- and time-dependent.^{50,51} However, these networks are commonly depicted as static diagrams or graphs, in which nodes correspond to biomolecules and edges or arrows correspond to interactions and/or influences (e.g., activation and inhibition).⁵² Examples of such diagrams are shown in Figure 1, which illustrate proteins and interactions considered in various models for signaling by the epidermal growth factor (EGF) receptor (EGFR),^{15,53–60} a member of the ErbB family of receptor tyrosine kinases.⁶¹ The interactions considered in Figure 1 are further described in Table 1. Diagrams in Figure 1 have clear utility and summarize large amounts of qualitative knowledge in an intuitive and visual format. However, the logical consequences of the interactions depicted in the diagrams depend not only on qualitative factors (e.g., the connections and influences among the biomolecular components of a network) but also on quantitative factors (e.g., the copy number of a protein, which can modulate the abundance of a protein complex and/or the strength of a feedback loop).^{62,63} Whenever quantitative factors are important, intuition alone is limited, and reasoning

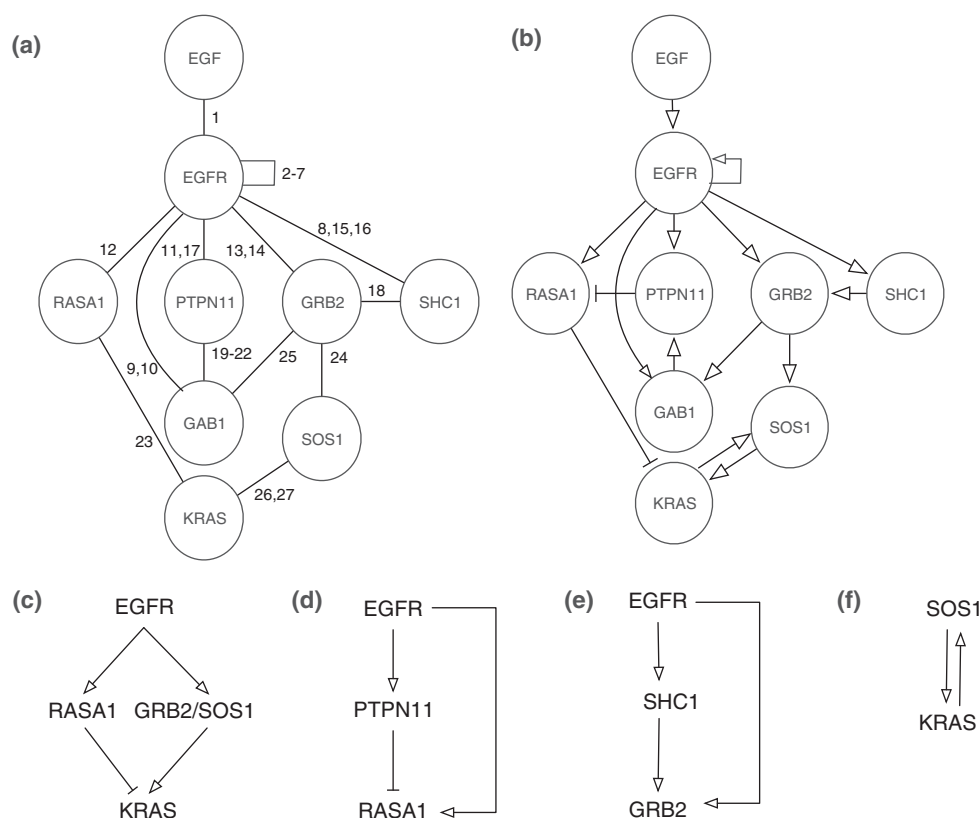


FIGURE 1 | Illustration of proteins and interactions involved in epidermal growth factor receptor (EGFR) signaling. (a) In this graph, nodes correspond to proteins and edges correspond to direct physical interactions. This type of graph is commonly used to visualize protein interaction networks.⁶⁴ Numbers next to edges refer to descriptions of interactions given in Table 1. (b) In this graph, arrows represent positive and negative influences. Note that there is a (negative) arrow connecting PTPN11 and RASA1 even though these proteins do not directly interact (cf. Panels a and b) because PTPN11 is responsible for dephosphorylation of a site in EGFR that interacts with RASA1 (Table 1). Interesting regulatory circuits embedded within the diagram of Panel b are highlighted in the panels at bottom. (c) EGFR generates competing positive and negative signals for KRAS activation. (d) An incoherent type 1 feed-forward loop (FFL) motif.⁶⁵ (e) A coherent type 1 FFL motif.⁶⁵ (f) A positive feedback loop.⁶⁶

aids, such as computational models, are needed to accurately predict the behavior of a system.

INVESTIGATING CELL SIGNALING WITH MODELS

Given the complexity of biomolecular interaction networks, researchers are increasingly using theoretical/computational methods to study biomolecular interactions.^{5,88} Among these methods is molecular dynamics (MD),^{89,90} which is routinely used to simulate the motions of atoms in biomolecules. However, the computational expense of this approach imposes limits on the timescales that can be simulated (typically nanoseconds to milliseconds), which are shorter than the timescale on which many signaling events occur (seconds to minutes to hours). Thus, although MD can be useful for studying isolated interactions,⁹¹ it is not useful for studying the integrated behavior of

a network of biomolecular interactions, except with coarse graining,⁹² which tends to be challenging and dependent on problem-specific details.⁹³ A menagerie of methods that overcome this limitation have been applied to characterize cell signaling systems, including Bayesian analysis,⁹⁴ regression analysis,⁹⁵ information theory,⁹⁶ constraint-based modeling,^{97,98} and Boolean/logical modeling.⁹⁹ However, the aforementioned approaches either ignore (without intentionally violating) physicochemical principles or are only loosely coupled to these principles. This disconnect can be beneficial (or irrelevant) for particular purposes. However, a model based on physicochemical principles has a number of highly desirable attributes. Such a model is grounded in causality (vs correlation) and its parameters (e.g., protein copy numbers and binding affinities) can be measured independently.^{74,100–102} Moreover, the interactions that underlie the structure of such a model can be (and have been) systematically elucidated

TABLE 1 | Description of Interactions Considered in Figures

Index	Description
1	EGF binds EGFR. ⁶⁷
2	EGF-induced dimerization of EGFR via EGFR-EGFR interaction. ⁶⁷
3	EGFR autophosphorylation at Y1016. ⁶⁸
4	EGFR autophosphorylation at Y1092. ⁶⁹
5	EGFR autophosphorylation at Y1110. ⁷⁰
6	EGFR autophosphorylation at Y1172. ⁶⁹
7	EGFR autophosphorylation at Y1197. ⁶⁹
8	EGFR-mediated phosphorylation of Y317 in SHC1 (isoform p52Shc). ⁷¹
9	EGFR-mediated phosphorylation of Y627 in GAB1. ⁷²
10	EGFR-mediated phosphorylation of Y659 in GAB1. ⁷²
11	PTPN11 binds pY1016 in EGFR. ⁷³
12	The SH2 domains in RASA1 mediate interaction with pY1016 in EGFR. ^{74,75}
13	The SH2 domain in GRB2 binds pY1092 in EGFR. ^{76–78}
14	The SH2 domain in GRB2 binds pY1110 in EGFR. ^{76–78}
15	The PTB domain in SHC1 binds pY1172 in EGFR. ^{78,79}
16	The PTB domain in SHC1 binds pY1197 in EGFR. ^{78,79}
17	PTPN11-mediated dephosphorylation of pY1016 in EGFR. ⁷⁵
18	The SH2 domain in GRB2 binds pY317 in SHC1 (isoform p52Shc). ⁷¹
19	The N-terminal SH2 domain in PTPN11 binds pY627 in GAB1. ⁸⁰
20	The C-terminal SH2 domain in PTPN11 binds pY659 in GAB1. ⁸⁰
21	PTPN11-mediated dephosphorylation of pY627 in GAB1. ⁸⁰
22	PTPN11-mediated dephosphorylation of pY659 in GAB1. ⁸⁰
23	The GAP domain in RASA1 binds KRAS causing an increase in GTPase activity that favors the GDP-loaded form of KRAS. ⁸¹
24	The N-terminal SH3 domain in GRB2 interacts with several C-terminal proline-rich sequences (PRS) in SOS1. ^{82,83}
25	The C-terminal SH3 domain in GRB2 interacts with two central proline-rich sequences (PRS) in GAB1. ⁸⁴
26	GTP-loaded KRAS binds the REM domain in SOS1, which increases the GEF activity of SOS1. ^{85,86}
27	The GEF domain in SOS1 binds KRAS causing a release of guanine nucleotide that favors the GTP-loaded form of KRAS. ⁸⁷

EGFR, epidermal growth factor receptor.

through a variety of both reductionist and high-throughput approaches^{29,103} (vs imperfectly reconstructed through statistical inference¹⁰⁴ on the basis of one or a few types of high-throughput data). Thus, there is a need for modeling approaches that are grounded in causality, constrained by physico-chemical principles (e.g., the law of mass action, the thermodynamic principle of detailed balance,^{105–107} and Fick's laws of diffusion^{108,109}), and applicable at the timescales relevant for studying cell signaling.

Addressing this need is the framework of chemical kinetics, which has been widely applied in studies of cell signaling.^{5,32,55,110–113} A traditional approach for modeling chemical kinetics involves

enumerating chemical species and reactions in a system to construct a reaction network, from which a system of coupled ordinary differential equations (ODEs) can be obtained.¹¹⁴ These equations characterize changes in concentrations of chemical species (or comparable state variables) with time. ODEs have been used to model chemical kinetics for approximately 150 years.^{115,116} The intense interest in this approach within the systems biology community is evidenced by the popularity of tools such as COPASI^{117,118} and the development and broad adoption of systems biology markup language (SBML),^{119,120} a standardized format for electronic storage and exchange of models that are defined

in terms of reaction networks. However, it can be difficult or even impossible to specify a complete reaction network when one is interested in structurally resolved chemical kinetics, meaning changes that occur at specific functional sites within biomolecules, because the modular functional components of these molecules can interact in myriad ways. These interactions have the potential to produce a vast number of chemical species.

If we aim to translate a protein interaction network into a chemical reaction network, we must (in a traditional modeling approach) enumerate the chemical species that can be populated and the connections and influences amongst these species (i.e., their reactions). To obtain an ODE model for this network, we then must write an equation for each chemical species that includes a term for each relevant reaction. This task can be daunting. As illustrated in Figure 2, the arrows in a typical pathway diagram imply many chemical species and hide a larger reaction network encompassing these species. As more biomolecular sites and interactions are considered, there is a combinatorial explosion and network size eventually becomes unmanageable. For example, Creamer et al.¹⁵ found that a subset of known posttranslational modifications and interactions of

an EGFR/ErbB3 heterodimer imply >10 distinct potentially populated chemical species. Clearly, not all of these species are relevant, but which can be safely ignored? Often, *ad hoc* assumptions are made to limit the chemical species included in a model to a manageable number. For example, different sites of phosphorylation in a protein may be lumped together as a single ‘virtual phosphorylation site’, as discussed by Birtwistle et al.⁵⁸ Such approaches are certainly serviceable for some purposes,⁵⁶ but most such assumptions are never evaluated and lumping of sites and other simplifying assumptions can limit the resolution of a model.^{56,121,122} Moreover, these assumptions may have unintended and undesirable consequences. For example, if the phosphosites of a receptor are lumped together and these sites interact with different adaptor proteins, then lumping may introduce a false competition amongst the adaptor proteins for binding to the receptor. In cases where (1) site-specific details are important, (2) these details entail significant combinatorial complexity, and (3) commonly used simplifications aimed at overcoming the barrier of combinatorial complexity have limited utility or require careful evaluation, what approach should one take to model biomolecular site dynamics?

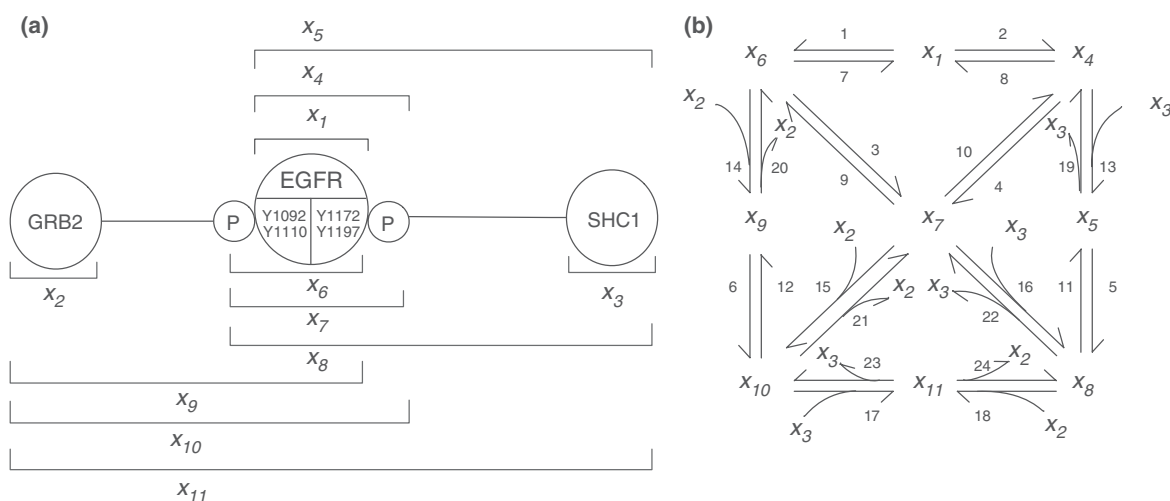


FIGURE 2 | The edges/arrows of a typical pathway diagram hide significant combinatorial complexity. (a) The interactions and phosphorylation states of the three proteins considered in this diagram [epidermal growth factor receptor (EGFR) and two adaptor proteins, GRB2 and SHC1] imply 11 distinct chemical species. The horizontal bars serve to label the 11 species and to delineate their compositions. Note that EGFR is taken to be phosphorylated at two sites of EGFR autophosphorylation: Y1092 (or both Y1092 and Y1110, which are important docking sites of GRB2) and Y1172 (or both Y1172 and Y1197, which are important docking sites of SHC1). GRB2 and SHC1 interact with EGFR via domains that recognize phosphotyrosines: the SH2 domain in GRB2 and the PTB domain in SHC1. The diagram shown here does not comprehensively depict the known interactions and phosphorylation states of these proteins; for example, EGFR-mediated phosphorylation of SHC1 and interaction between GRB2 and phosphorylated SHC1 are not considered. (b) The 11 chemical species are connected in a reaction network encompassing 24 unidirectional reactions. Reactions 1–6 are phosphorylation reactions, Reactions 7–12 are dephosphorylation reactions, Reactions 13–18 are bimolecular association reactions, and Reactions 19–24 are dissociation reactions. For simplicity, all reactions are represented as single-step transformations and catalysts are considered implicitly. For more information about the interactions considered here, see Table 1.

RULE-BASED MODELING

From Equations to Rules

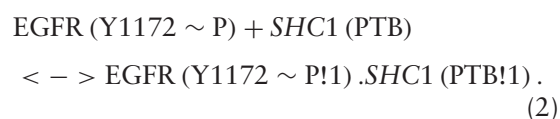
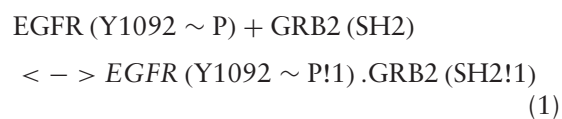
The key feature of rule-based modeling that makes this approach suitable for studying the site dynamics of biomolecular networks is the simplifying idea of representing biomolecular interactions in terms of local rules.⁴ Modeling approaches based on local rules, as in agent-based modeling,¹²³ are used in many fields, including physics, chemistry, and computer science.¹²¹

Local rules can be formulated in different ways and on the basis of different formalisms, such as that of process algebra,^{124–126} finite state machines,^{127,128} or graph rewriting.^{129–132} In one approach,^{129,130} which underlies a number of useful methods and software tools, such as BioNetGen,^{129,133,134} rules take the form of single-pushout graph transformations,¹³⁵ graph-rewriting rules with particular technical features, such as a mapping of vertices from the left-hand side (LHS) to the right-hand side (RHS) of a rule. (This mapping is usually implicit but can be made explicit, which is necessary for some purposes.¹³⁶) Rules are applied to graphs representing biomolecules and connected sets of graphs representing biomolecular complexes to determine the outcomes of interactions. These graphs are simple, colored, attributed graphs, in which vertices represent molecular components, undirected edges connecting vertices represent (noncovalent) bonds between components, and the vertices representing the components of a particular molecule type are all associated with a common ‘color’, the name of the molecule type. Moreover, vertices are optionally associated with attributes or internal states, which take the form of alphanumeric strings. Internal states are abstractions useful for representing local properties of sites such as conformation, phosphorylation status, and subcellular location. For example, a vertex representing a tyrosine residue in a protein that is a substrate of a protein tyrosine kinase and phosphatase can be associated with the attribute ‘P’ (or ‘0’) to indicate that the tyrosine is phosphorylated (or unphosphorylated). Accessible formal definitions of the graphs used in rule-based modeling are available.¹³⁷

Model-specification languages, such as the BioNetGen language (BNGL)¹³⁴ and Kappa,¹³⁸ which can be viewed as domain-specific programming languages, enable the graphs of a rule-based model, which represent biomolecules and complexes, to be encoded in a machine-readable plain-text format. In Figure 3, we illustrate BNGL-encoded representations of two proteins (EGF and EGFR) and a particular chemical species considered in a recently reported

rule-based model for EGF-induced oligomerization of EGFR.²² The structural resolution of these representations is intermediate between the finer resolution of an atomic model and the coarser resolution of a traditionally formulated model for chemical kinetics, in which each chemical species is represented simply by a unique name. As can be seen by comparing Figure 3(b) and (f), BNGL enables the connectivity (or topology) of a protein complex to be explicitly represented. This level of resolution is most common; however, geometrical aspects of complexes have been considered in some rule-based models.^{139–143}

BNGL¹³⁴ enables rules to be encoded in plain text. For example, interactions of the adaptor proteins GRB2 and SHC1 with phosphotyrosines in EGFR (Figure 2(a)) can be encoded as follows:



These rules indicate that the SH2 (PTB) domain in GRB2 (SHC1) can reversibly bind the phosphorylated form of Y1092 (Y1172) in EGFR.^{76,79} According to these rules, a free SH2 or PTB domain in an adaptor protein and the availability of a phosphorylated cognate binding site in EGFR are the only requirements for interaction. In general, rules define the necessary and sufficient conditions for interactions and transformations; the stringency of these conditions can be adjusted to match mechanistic understanding and assumptions. In accordance with the conventions of BNGL,¹³⁴ two components in direct physical interaction are identified (on the RHS of each of the above rules) through sharing of a bond name, which here is a number that is prefixed by a ‘!’ symbol. The ‘~’ symbol prefixes an internal state label. Here, the label ‘P’ is used to represent ‘phosphorylated’. In a complete model specification, the rules above would each be associated with rate laws for association and dissociation (which are specified by simply providing single-site forward and reverse rate constants if mass-action kinetics is assumed). BNGL-encoded rules are further discussed in Box 1. For readers interested in additional information about BNGL, a comprehensive description of BNGL is available,¹³⁴ as is a thorough tutorial.¹⁴⁹

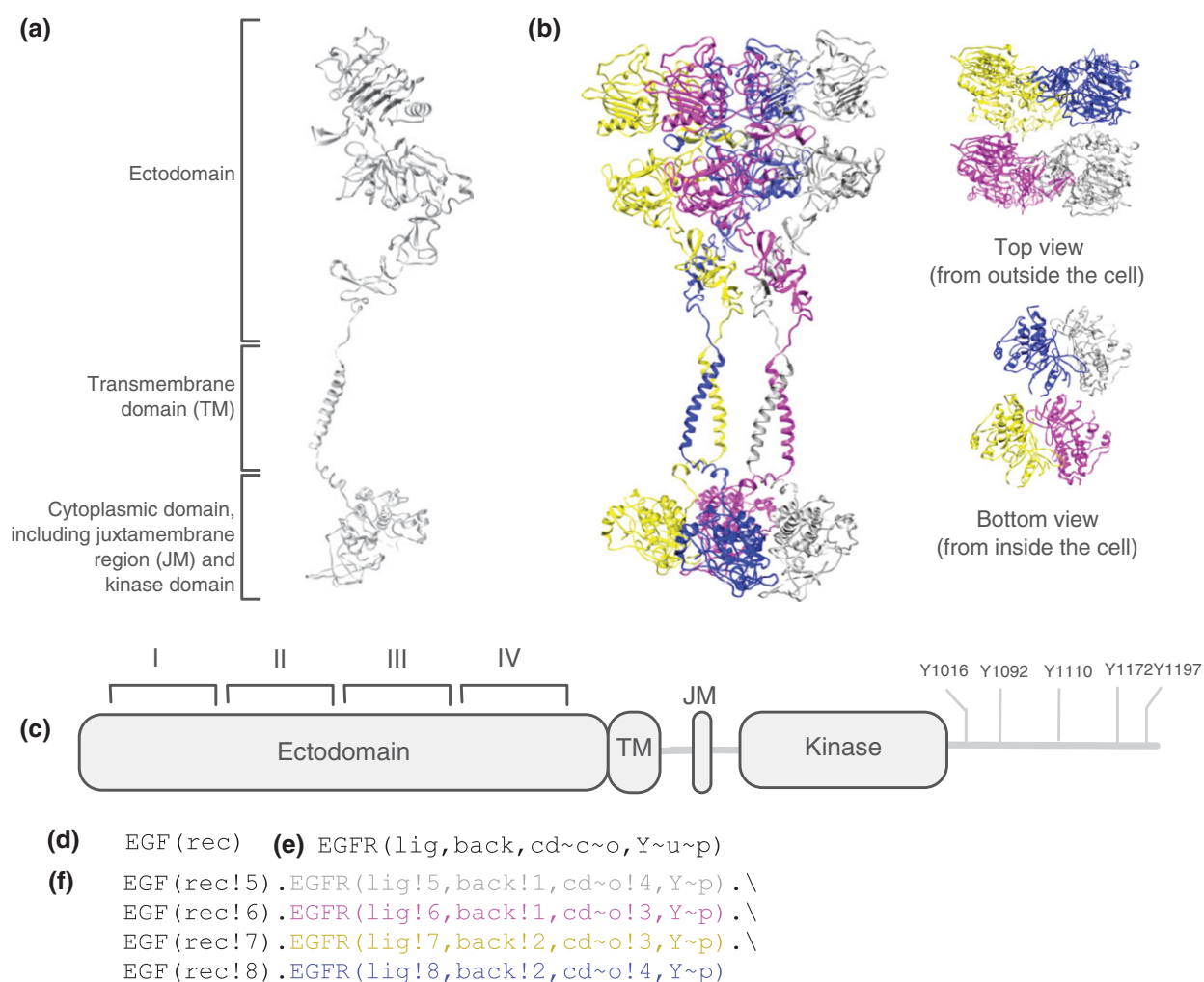


FIGURE 3 | A protein or protein complex can be represented at different levels of resolution. (a) An atomic model of epidermal growth factor receptor (EGFR) (amino acid residues 25–982). The model is based on experimentally determined structures of three domains of EGFR (PDB accession codes 3NJP, 2M20, and 2JIV).^{144–146} The model does not include the cytoplasmic tail of EGFR. (b) An atomic model of an EGF-induced oligomer of EGFR, a cyclic side-by-side dimer of dimers. EGF is not shown. The model was constructed with preservation of experimentally determined structural interfaces between domains and with preservation of the chemical integrity of individual domains, which were treated as rigid bodies. Consecutive domains are within the allowed lengths of the connecting loops. The top view highlights ectodomain–ectodomain interactions, and the bottom view highlights cytoplasmic domain–cytoplasmic domain interactions. (c) Domain architecture of EGFR. This diagram provides a schematic representation of EGFR and its component parts and includes a depiction of five sites of autophosphorylation (cf. Figures 2 and 5). (d) BioNetGen language (BNGL)-encoded representation of EGF. This encoding indicates that the molecule type EGF contains one functional component, *rec*. More formally, this line of code introduces a graph that has the color EGF and one vertex labeled *rec*. (e) BNGL-encoded representation of EGFR (cf. Panel a). This encoding indicates that the molecule type EGFR has four functional components: *lig*, *back*, *cd*, and *Y*. The *lig* component represents the ligand-binding site, which comprises domains I and III of the EGFR ectodomain.⁶⁷ The *back* component represents domain II of the EGFR ectodomain, which is responsible for a self-interaction.⁶⁷ The *cd* component, which is responsible for another self-interaction,¹⁴⁷ represents the cytoplasmic domain of EGFR and encompasses the juxtamembrane region and kinase domain. The *cd* component is taken to have two possible internal states (or more formally, vertex attributes): closed (*c*) and open (*o*). In the *o* state, the *cd* component is able to interact with a second copy of itself (also in the *o* state). The *Y* component represents an autophosphorylation site, which is taken to have two possible internal states. These states, *u* and *p*, represent different phosphorylation states, unphosphorylated, and phosphorylated. (f) BNGL-encoded representation of a cyclic EGFR tetramer (cf. Panel b). As indicated, this complex is held together through alternating back–back and cd–cd interactions, which are abstractions of the interactions illustrated at atomic resolution in Panel b. Atomic models were visualized using the UCSF Chimera package.¹⁴⁸ The model of Panel b represents only one plausible structure.

BOX 1

FORMAL ASPECTS OF BNGL-ENCODED RULES

In the formalism underlying BNGL,^{129,130} rules are strictly unidirectional. Thus, Eq. (1) or (2) is actually shorthand for two rules, one for association and one for dissociation. Rules are composed of pattern graphs representing molecular moieties.^{129,130} The set of pattern graphs on the LHS of a rule defines the necessary and sufficient conditions required of a set of sites for an interaction or transformation to occur. Pattern graphs identify reactants through 'matching'. A pattern graph matches a chemical-species graph (and identifies the corresponding chemical species as a potential reactant) if the two graphs are related by a subgraph isomorphism.¹³⁰ The set of pattern graphs on the RHS of a rule, together with a LHS-to-RHS mapping of vertices, defines the outcome of a transformation. The transformation, given implicitly by the difference between LHS and RHS, could be addition of an edge, removal of an edge, or a change of a vertex attribute. Rules can also represent processes that add or remove molecules or complexes. If a site in a biomolecule or a site's state does not affect an interaction, or if its influence is unknown and one wishes to make minimal assumptions, it is omitted from a rule. This practice is sometimes called the 'don't care, don't write' convention. Rules that include only sites (vertices) directly involved in an interaction are maximally permissive. Rules with additional sites are more stringent. The purpose of including nonreactive sites in rules is to impose contextual constraints on interactions.

Representation of an interaction in the form of a local rule is appropriate (and advantageous) if the interaction is modular, i.e., entirely independent of molecular context or only loosely coupled to nonreactive aspects of the environment in which the interaction occurs. An assumption of modularity can often be justified by Occam's razor and by the observation that proteins, as well as other biomolecules, tend to be composed of modular parts, as discussed earlier.

If the necessary and sufficient conditions for applicability of a rule are not highly stringent, then biomolecules in many distinct states are likely to satisfy the conditions and the rule will define multiple reactions. For example, the rules given above (Eqs. (1) and (2)) each corresponds to multiple

reactions in Figure 2(b): the forward transformation of the first rule (GRB2 association with EGFR) corresponds to Reactions 14, 15, and 18, and the forward transformation of the second rule (SHC1 association with EGFR) corresponds to Reactions 13, 16, and 17. It is this feature of rules that enables concise model specification. It should be noted that the benefit of concise representation comes with a cost. Because each (unidirectional) rule is associated with one rate law, all reactions implied by that rule are effectively assigned this rate law, which brings about a coarse graining of the chemical kinetics. However, the resolution/applicability of a rule can be adjusted as needed or desired. For example, if a nonlocal property of a site is a prerequisite for an interaction (e.g., through allostery, which is discussed below), this requirement can be specified in a rule to make the rule as specific as necessary. In general, the specification of a rule can be tuned to restrict or enlarge the set of species that qualify as reactants according to the rule. The most broadly applicable binding rule that could be specified would simply state that potentially reactive components must be free for binding to occur between them. At the other extreme, the finest rule would imply one and only one reaction, i.e., the rule would be equivalent to an individual reaction in the traditional sense. Thus, a set of rules can be viewed as a generalization of a reaction network.

Rule-based models are compositional, meaning that the rules that comprise these models can be specified somewhat independently. Often, adding consideration of an interaction to a model only requires adding a new rule for that interaction.¹²¹ Although in practice some additional modifications of the model may be necessary, the effort of making these modifications is usually modest in comparison to the effort required when adding an interaction to an equivalent ODE model.¹²¹ The ability to specify rules independently makes a rule analogous to a line of code; a set of related rules, defining for example the interactions involved in a Michaelis–Menten reaction mechanism, analogous to a software routine; and the complete set of rules of a model analogous to a program. Because model specification is separated from model simulation,¹³⁴ simulation is analogous to compilation. This analogy has been leveraged by recently developed software tools, most notably RuleBender^{150,151} and PySB.¹⁵² RuleBender, an Eclipse rich client platform (RCP) application, provides an integrated development environment (IDE) for specification, visualization, and simulation of rule-based models. PySB is a Python module that enables high-level abstractions and

treatment of rule-based models as Python-language programs, which has the potential to facilitate model reuse. PySB is further discussed below.

A Simple Example of a Rule-Based Model

As an example of a rule-based model, we consider the model of Kiselyov et al.¹⁵³ for insulin-like growth factor 1 (IGF1) binding to the IGF1 receptor (IGF1R) (Figure 4). IGF1R is a dimeric receptor containing a total of four extracellular binding sites that can interact with IGF1. As illustrated in Figure 4(a), a single ligand (IGF1) can bind two receptor sites simultaneously, thereby crosslinking the receptor subunits containing these sites. Formation of a crosslink coincides with a conformational change that causes sites distal from the crosslink to shift away from each other, preventing formation of a second crosslink (Figure 4(a)). Thus, ligand–receptor binding reactions depend on the states of sites that are capable of interacting directly (i.e., the states of the ligand and receptor sites), as well as nonlocal properties (e.g., the presence or absence of a crosslink). Another nonlocal property considered in this model is binding pocket occupancy; the model only allows one ligand per binding pocket (the space between opposing ligand-binding sites). The model of Kiselyov et al.¹⁵³ exemplifies the role of structurally resolved or site-specific details in modeling of biochemical kinetics. Two rules from this model are illustrated in Figure 4(b) (for ligand binding and crosslinking) using the graphical conventions of Faeder et al.¹⁵⁴ All rules of the model are shown in Figure 4(c) in BNGL,¹³⁴ along with definitions of molecule types (for IGF1 and IGF1R); seed species, which define an initial system state; and an observable, which is defined because the last rule of the model includes a local function. For this model, the definition of seed species is important beyond providing initial conditions, because this section of the model (together with the absence of rules for receptor dimer formation or dimer dissociation) indicates that receptors are static dimers. Local functions, a fairly new feature of BNGL, are discussed in Box 2.

BOX 2

LOCAL FUNCTIONS IN BNGL

In BNGL, ‘observables’ are user-specified outputs of simulation.¹³⁴ An observable is defined by a set of pattern graphs and a type. For an observable of the ‘Molecules’ type, its value, loosely speaking, is the number of times its

pattern graphs match a set of chemical-species graphs. Usually this set represents all species present in a system. However, observables can also be computed for smaller sets of chemical-species graphs. Observables with limited scope are called local observables. In BNGL, a graph can be tagged using the syntax ‘%label’ and the tag can be passed as an argument to an observable to limit its scope, as in the expression ‘Obs(label)’. BNGL also allows for the use of functions that refer to local observables in rate laws, which are called local functions. If the rate law of a rule includes such a function, the rate of an individual reaction implied by that rule will depend on the number of matches of the local observable’s pattern graph(s) to the reactant chemical-species graph(s). In Rule 5 of Figure 4(c), a local function is used to restrict crosslinking of receptor subunits by IGF1 to situations where a crosslink does not already exist. The local function of Rule 5 accomplishes this restriction by setting the forward rate constant for crosslinking (kcr) to zero if a chemical-species graph matching the LHS of the rule also matches the ‘Crosslink’ observable, i.e., if a crosslink is detected.

SIMULATION METHODS

Rule-based models can be simulated using a variety of approaches. These approaches fall into two broad categories: indirect and direct methods. Most available software tools for rule-based modeling, which are exhaustively listed in a recent review,¹²¹ implement indirect methods. In an indirect approach, a rule-based model is first converted to a model having a traditional model form, such as a system of ODEs, and then a simulation method/tool available for that model form (e.g., an ODE solver) is applied. In a direct approach, the simulation procedure is tailored to the native form of a rule-based model.¹⁵⁵ Currently available direct approaches are particle-based kinetic Monte Carlo (KMC) methods,¹⁵⁶ in which rules are used as event generators. The algorithmic differences between available direct approaches are compared in a recent review.¹²¹ A notable distinction between indirect and direct methods is the way in which system state is tracked. In an indirect method, system state is defined by traditional state variables, such as concentrations. In contrast, in a direct method, system state is defined by the collective states of individual sites or particles.¹⁵⁷ This aspect of direct methods is discussed further below.

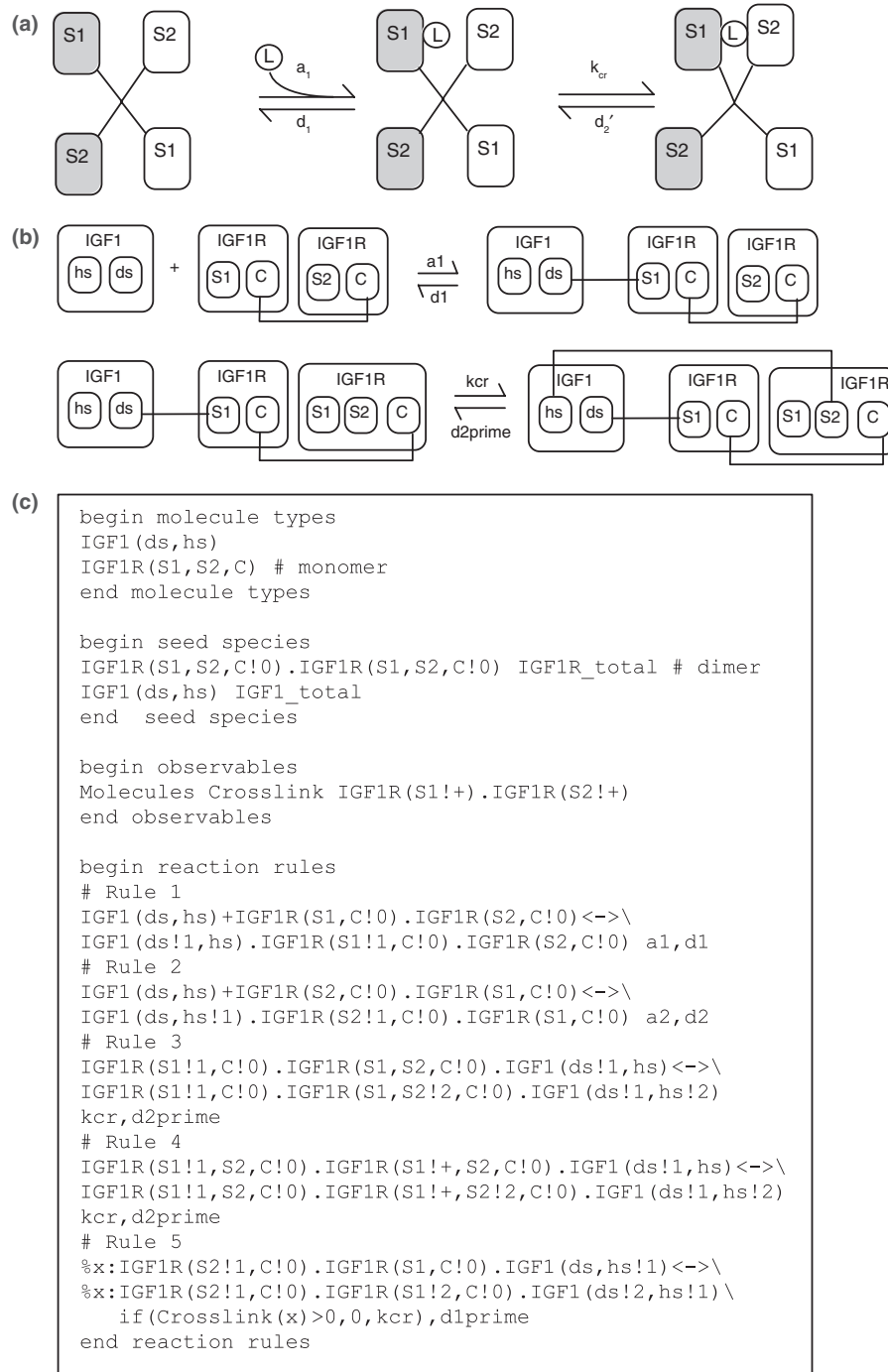


FIGURE 4 | A rule-based model for insulin-like growth factor 1 (IGF1) interaction with the receptor IGF1R. (a) Two reactions from the model of Kiselyov et al.¹⁵³ Reversible capture of a free ligand (IGF1) by an inactive, unbound receptor (IGF1R) is illustrated at left, and reversible ligand-mediated crosslinking of receptor subunits containing Sites 1 and 2 (or S1 and S2) is illustrated at right. A receptor is dimeric and each monomer contains two ligand-binding sites. Common shading indicates sites that are found in the same receptor monomer. (b) Two rules from the model of Kiselyov et al.¹⁵³ drawn according to the graphical conventions of Faeder et al.¹⁵⁴ The rule at top (bottom) implies the left (right) reaction in Panel a. (c) Excerpts from an executable encoding of the model of Kiselyov et al.¹⁵³ in BioNetGen language (BNGL)¹³⁴. In the listing shown here, definitions of graphs for IGF1 and IGF1R (i.e., molecule types) are given at top, definitions of seed species are given next, definition of a pattern used in a local function is given in the observables block, and definitions of rules are given at bottom. Rule 1 is the text encoding of the top rule in Panel b, and Rule 3 is the text encoding of the bottom rule in Panel b. Rule 2 is similar to Rule 1. Rules 3 and 4 together define crosslinking of Sites 1 and 2 when a ligand is bound at Site 1. Rule 5 defines crosslinking of Sites 1 and 2 when a ligand is bound at Site 2.

Indirect Methods

If either an indirect or direct method can be applied, it is usually the case that the indirect method is more efficient. However, indirect methods are not always feasible.⁴

Network Generation

The first step in an indirect simulation method is network generation,^{129,130,133} a process of enumerating the chemical species and individual reactions implied by a set of rules through iterative application of these rules to graphs representing seed species and their derivatives. In the first round of rule application, rules are applied to the graphs representing seed species, which are defined as part of a model specification. (A declaration of seed species is analogous to a declaration of an initial condition for an initial value problem.) In the second round of rule application, rules are applied to the graphs representing any novel products of reactions involving the seed species as reactants, which are generated in the first round of rule application. The novelty of a reaction product is detected through canonical labeling of chemical-species graphs.^{130,137} The process of network generation continues until no new products are generated or a specified stopping criterion is satisfied. In general, the process is not guaranteed to terminate. For example, network generation will not terminate, without specification of an arbitrary stopping criterion, if a model includes a rule defining a polymerization-like reaction.^{4,156} The size of a reaction network obtained via network generation can range from small (i.e., manageable) to large (i.e., unmanageable) depending on the details of the rules of a model and the initial data (i.e., the seed species). An important point that should be kept in mind is that the intrinsic complexity of a rule-based model is reflected by the number of rules of the model (which largely determines the number of parameters), not the number of chemical species or number of reactions implied by the rules. Analogously, the complexity of a model for protein dynamics is not defined by the number of molecular configurations that can potentially be sampled during an MD simulation. The reactions implied by the rules of a model are ancillary to the rules themselves, and these reactions would ordinarily only be derived for purposes such as simulation via an indirect method. A number of software tools provide network generation capabilities, including BioNetGen,¹³⁴ Simmune,^{158–161} and SSC.¹⁶² BioNetGen is the most used of these tools¹⁶³ and it has been extensively tested, which is important given the technical complexity of network generation.^{129,130,134,137}

Simulation of Well-Mixed Compartmental Models

Once a reaction network has been generated, simulations can be performed using the armamentarium of network-based deterministic and stochastic simulation methods for well-mixed reaction compartments. For example, a list of reactions and associated reaction propensities, which are determined by rate constants and system state, can serve as the input for a stochastic simulation algorithm.^{116,164} Alternatively, under the assumption of a continuum limit, an ODE model can be derived.^{114,116} BioNetGen¹³⁴ currently provides access to three built-in network simulators: (1) the stiff/non-stiff numerical ODE solver CVODE,¹⁶⁵ which offers multiple methods of numerical integration; (2) an implementation of an efficient version of Gillespie's method for stochastic simulations of chemical kinetics¹⁶⁴ with propensity sorting¹⁶⁶; and (3) a partitioned-leaping algorithm (PLA) for accelerated stochastic simulations.¹⁶⁷ Furthermore, stochastic simulations can be performed on-the-fly.^{129,168} Finally, BioNetGen is capable of exporting rule-derived reaction networks in SBML format,^{119,120} allowing for their simulation and analysis via SBML-compatible tools,¹⁶⁹ such as COPASI.^{117,118} It can also export a specification of the corresponding ODEs as a MATLAB M-file or MEX-file.

Simulation of Models with Coupled Reaction and Diffusion

Fully enumerated reaction networks can be simulated in a spatially resolved manner. The tools Simmune^{158–161} and SSC¹⁶² are designed to support spatial simulations of rule-based models, using subvolume-based continuum and stochastic simulators, respectively. Both tools enable native specification of rules. Smoldyn¹⁰⁹ includes native rule-based modeling features¹⁷⁰ and enables particle-based reaction-diffusion calculations, i.e., simulations based on Brownian dynamics (BD). A BioNetGen frontend utility is available for VCell,¹⁷¹ a web-based platform that provides access to continuum and particle-based spatial modeling capabilities. Particle-based simulations are performed through an interface with Smoldyn, and continuum simulations are performed on the basis of partial differential equations (PDEs) and PDE solvers.

Direct Methods

Indirect methods are not always tenable. Inclusion of a rule that defines a polymerization-like reaction will often result in an implied network bounded (without information about the parameters of rate

laws) only by the number of molecules in a system.^{4,156} Polymerization-like reactions arise, for example, in modeling of multivalent ligand–receptor binding.^{172,173} Even without rules that define polymerization-like reactions, the size of an implied reaction network can be effectively infinite,¹⁵ simply because of the combinatorial explosion in network size that comes with consideration of more and more interactions. In some cases, a truncated network (e.g., a network obtained from a limited number of rounds of rule application) can be useful. However, in some cases, it is impossible to find a truncated network of manageable size that captures all of the populated chemical species in a system.¹⁵⁶ Various methods are available for obtaining reduced-order models from rules.^{138,174–179} However, these methods are not guaranteed to yield a significant or meaningful reduction in the size of a model. In cases where a model in a traditional form cannot be obtained from a set of rules, currently, direct methods must be used.

Simulating rule-based models that correspond to large-scale reaction networks requires changing the system state representation from a population (concentration) perspective to a particle-based perspective. Intuitively, the idea is that if the number of particles (molecules/reactive sites) in a system is less than the total number of chemical species that are potentially populated, then it is computationally more efficient to operate on the system of particles than it is to enumerate all of the distinct populations in which these particles can potentially be found. A system of particles can be simulated using particle-based KMC methods,^{155–157,180–184} which are variants of standard stochastic simulation algorithms,¹²¹ such as Gillespie's method.¹⁶⁴ Because a reaction network is not enumerated, these methods are commonly referred to as network-free methods. Available network-free methods are similar to each other.¹²¹ The following steps are found in several algorithms: (1) rates (propensities) are calculated for the rules comprising a model by counting the number of molecular moieties, or reactive sites, that match the pattern graphs on the left-hand sides of rules; (2) the next rule to fire (i.e., to apply to the graphs representing the molecules in the system of interest) is chosen probabilistically based on the relative rates of rules; and (3) sites qualifying as reactive according to the rule chosen to fire are randomly selected and transformed in accordance with the rule.

A number of network-free simulators are available. The tools DYNSTOC,¹⁸² RuleMonkey,¹⁵⁷ and NFsim¹⁸³ are compatible with BNGL.¹³⁴ NFsim is conveniently distributed with RuleBender.^{150,151} KaSim¹⁸⁵ is compatible with Kappa.¹³⁸ Hybrid

methods, which combine aspects of direct and indirect simulation, are emerging.^{186,187} Although direct simulation methods allow one to perform new types of simulations and to ask new questions, such as questions about the composition of protein complexes at the proteome-wide scale,¹⁶ these methods have drawbacks. For example, because these methods update system state one reaction event at a time, they can be computationally expensive. Acceleration of stochastic simulation for traditionally formulated models is a topic that has received much attention.^{116,186,188} As noted above, a method for accelerated stochastic simulation, PLA,¹⁶⁷ is available with BioNetGen. However, there are many other useful methods in this class that have yet to become available in simulators for rule-based models. Another drawback of direct methods is the difficulty of identifying the parameters of a model that can only be simulated stochastically.^{186,189,190} In general, the various known drawbacks of stochastic simulation arise when using direct methods.

MODELS AS VEHICLES OF UNDERSTANDING

Visualization and Annotation

As it becomes possible to specify and simulate increasingly complex models, a new challenge arises in the question of how models can be communicated effectively. This challenge is twofold: concise representation of molecules and interactions captured in a model, and connection of model elements to relevant experimental data. To address these challenges, methods for visualization and annotation of rule-based models have been developed.¹⁹¹

Individual rules in a model are easily visualized (see Figure 4(b)), and more comprehensive model diagrams can be generated automatically by a number of software tools.^{150,151,192} A useful type of diagram is a contact map,¹⁹³ which can be generated automatically from a BNGL- or Kappa-encoded model specification. A contact map for the model of Figure 4 is shown in Figure 5. A contact map illustrates the molecules, components, component states, and binding interactions captured in a model. A limitation of a contact map is that it only captures information explicitly encoded in a model. Thus, a contact map will not illustrate aspects of a system that are implicitly captured in a model,¹⁹¹ such as an enzyme whose concentration is subsumed in an effective rate constant.

To encompass these details in visualization of a model, the concept of an extended contact

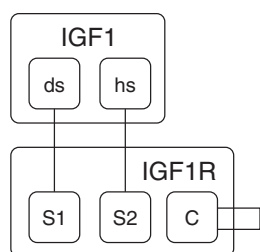


FIGURE 5 | Example of a contact map for the model of Kiselyov et al.¹⁵³ (Figure 4). The map shown here is essentially the same as the one that is generated automatically by RuleBender^{150,151} from the model specification that is partially shown in Figure 4(c).

map has been developed.¹⁹¹ Extended contact maps provide several types of information typically absent in an automatically generated contact map: enzyme–substrate relationships are depicted explicitly, hierarchical protein substructures¹³⁷ are illustrated through nesting of boxes representing the material constituents of a system, and the layout of a map is designed to reflect the flow of information during signaling. Construction of extended contact maps is aided by templates available online.¹⁹¹ A number of examples of extended contact maps have been published.^{14,15,21,26,194} Interactions and molecules illustrated in a map can be connected to formal model elements (e.g., rules) through a model guide, in which these elements are annotated with information from the primary literature or online resources.¹⁹¹ In this way, a visualized and annotated rule-based model can serve as an easily navigated archive of available knowledge about a signaling system. An extended contact map for the proteins and interactions considered in Figure 1 is shown in Figure 6. As can be seen, an extended contact map illustrates site-specific details, which are often omitted in pathway diagrams (cf. Figures 1 and 6). Because the elements of an extended contact map relate to the formal elements of a rule-based model,¹⁹¹ summary of available mechanistic knowledge in the form of an extended contact map can be valuable during development of a model and for communication of the finished product.

High-Level Modeling Languages

With development of larger and more complex models,^{59,195} the potential usefulness of collaboration among modelers has become increasingly apparent.¹⁹⁶ In collaborative model development, approaches used in computer programming are likely to prove useful because teams of programmers routinely write, maintain, and extend complex codes. Two products of recent efforts to bring software engineering concepts

to modeling in systems biology are PySB¹⁵² and LBS- κ ,¹⁹⁷ which are tools designed to ease the specification of models. A model specified using PySB takes the form of a Python-language program, which can be executed to obtain models in other formats and to direct simulations and analyses. PySB provides an embedded language for high-level abstractions and a capability to translate these abstractions into BNGL¹³⁴ and Kappa.¹³⁸ In contrast, LBS- κ is a domain-specific language that enables high-level abstractions. An earlier example of such a language is MetaKappa.¹⁹⁸

A PySB model may contain modules that represent sets of interactions. A module is useful for concisely representing a set of interactions that recurs many times within a single model or a set of interactions found in multiple models (e.g., the interactions of the reader/writer/eraser signaling motif¹⁹⁹), in part because a user can define different but related sets of rules by passing different arguments to the same module and by using loops and conditionals. Adding a module to a PySB model contextualizes the interactions represented by the module (e.g., through user-specified arguments). A benefit of using PySB-defined modules is that it becomes unnecessary to manually specify nearly identical sets of rules, such as sets of rules capturing a common enzymatic mechanism but for which the identity of the substrate varies (Figure 7). Another benefit is that modules can be assigned intuitive names and constructed through Python-native object-oriented composition so that a PySB model has a hierarchical structure that can be inspected at multiple levels of resolution.

PySB enables model reuse at several levels. At the simplest level, an existing PySB model can be duplicated and its elements can be modified or new elements can be added. A second type of model reuse involves independent manipulation of modules, allowing a modeler to swap out part of a model while other parts of the model are maintained. A third approach involves automatic generation of a set of models, each with a different combination of features of interest. To facilitate identification and annotation of formal model elements across different models, PySB provides the capability to tag model elements with terms compatible with the conventions of MIRIAM,²⁰⁰ a set of guidelines for model annotation. These capabilities combined with streamlined model specification and consistent annotation has the potential to make model development a more efficient and collaborative process than has previously been possible.

Because PySB provides an embedded language for high-level model specification (i.e., one that is used within a general-purpose programming language),

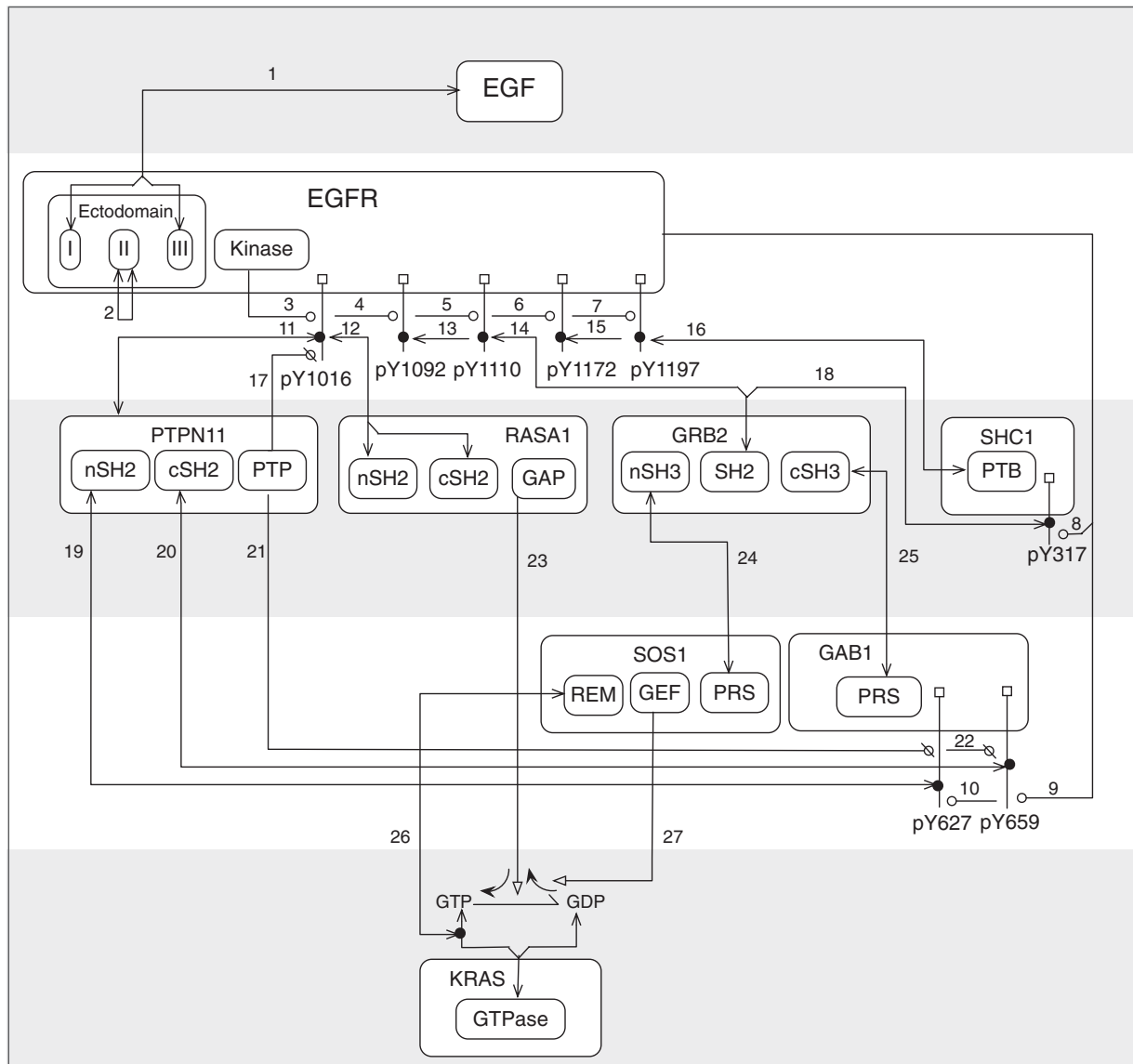


FIGURE 6 | Example of an extended contact map¹⁹¹ for selected proteins and protein–protein interactions involved in epidermal growth factor receptor (EGFR) signaling. The same proteins and interactions are considered in Figure 1. Boxes with rounded corners represent selected proteins, domains, and linear motifs. Small square boxes attached to vertical lines represent sites of phosphorylation. Lines that begin and end with an arrowhead represent noncovalent direct-binding interactions. Lines that begin at a box representing a catalytic subunit and end with a circle point to substrates of an enzyme (a kinase or phosphatase). An open circle indicates a posttranslational modification (phosphorylation); a circle with a line through it indicates reversal of a modification (dephosphorylation). See Table 1 for more information about the interactions represented in this map.

a modeler using PySB inherits the benefits of mature software development tools, such as those available for version control. In addition, all the features of a general-purpose programming language (e.g., conditionals, loops, and classes) are made available to a modeler. However, this feature can be a disadvantage if the flexibility available is abused, such that a model becomes difficult to interpret owing to absence of a standardized style for model specification. Moreover, the syntax of the host language (Python in

the case of PySB) must be retained, and this restriction is not always desirable, especially if a modeler is unfamiliar with the language. In comparison, a high-level domain-specific language, such as LBS- κ or MetaKappa, can be easier to learn and to use and can be tailored to meet specific needs of modelers, but such a language will generally not offer as much flexibility as an embedded language, which can be a disadvantage if an unanticipated model-specification problem arises.

```

(a) from pysb import *

Model()

# Declare molecule types
Monomer('IGF1', ['ds', 'hs'])
tyrosines = ['Y973', 'Y980', 'Y1161', 'Y1166', 'Y1280', 'Y1281', 'Y1346']
Monomer('IGF1R', ['S1', 'S2']+tyrosines, {i:['O', 'P'] for i in tyrosines})

# Create a dictionary of rate constants
kp = {}
for tyr in tyrosines:
    kp[tyr]=Parameter('kp'+tyr, 1.0) # all rate constants are set to 1

# Define phosphorylation rules for IGF1R tyrosines
for tyr in tyrosines:
    Rule("phos1"+tyr, IGF1(ds=1, hs=2) % IGF1R(S1=1) % IGF1R({'S2':2, tyr:'O'}) >> \
        IGF1(ds=1, hs=2) % IGF1R(S1=1) % IGF1R({'S2':2, tyr:'P'}), kp[tyr])
    Rule("phos2"+tyr, IGF1(ds=1, hs=2) % IGF1R({'S1':1, tyr:'O'}) % IGF1R(S2=2) >> \
        IGF1(ds=1, hs=2) % IGF1R({'S1':1, tyr:'P'}) % IGF1R(S2=2), kp[tyr])

(b) # Rule 1
IGF1(ds!1,hs!2).IGF1R(S1!1).IGF1R(S2!2,Y973~O)->\
IGF1(ds!1,hs!2).IGF1R(S1!1).IGF1R(S2!2,Y973~P) kpY973
# Rule 2
IGF1(ds!1,hs!2).IGF1R(S1!1,Y973~O).IGF1R(S2!2)->\
IGF1(ds!1,hs!2).IGF1R(S1!1,Y973~P).IGF1R(S2!2) kpY973
...
# Rule 13
IGF1(ds!1,hs!2).IGF1R(S1!1).IGF1R(S2!2,Y1346~O)->\
IGF1(ds!1,hs!2).IGF1R(S1!1).IGF1R(S2!2,Y1346~P) kpY1346
# Rule 14
IGF1(ds!1,hs!2).IGF1R(S1!1,Y1346~O).IGF1R(S2!2)->\
IGF1(ds!1,hs!2).IGF1R(S1!1,Y1346~P).IGF1R(S2!2) kpY1346

```

FIGURE 7 | Illustration of how PySB¹⁵² can simplify the specification of related but distinct rules. (a) PySB code that specifies two phosphorylation rules for each of seven sites of insulin-like growth factor 1 receptor (IGF1R) autophosphorylation. IGF1R is a receptor tyrosine kinase.⁶¹ The rules all have the same form, differing only with respect to substrate and symmetry of ligand binding. It is assumed that an IGF1-crosslinked receptor can mediate autophosphorylation of sites in both of its monomer subunits. (b) Examples of the 14 rules defined by the PySB code in Panel a, in BioNetGen language (BNGL) format.¹³⁴

CONCLUSION

Cellular regulatory systems compute through interactions of biomolecules,^{201–203} which generally comprise multiple functional components, or sites. These sites undergo continuous changes in state, as exemplified by the reader/writer/eraser signaling motif,¹⁹⁹ wherein a tyrosine residue is subject to the opposing activities of a kinase and phosphatase and serves as a docking site for SH2 domain-containing proteins when phosphorylated. The study of state changes at the functional sites of proteins and other biomolecules can be called biomolecular site dynamics. An appropriate theoretical framework for studying site dynamics, which evolve over seconds to minutes to hours, is that of chemical kinetics; however, traditional approaches for modeling chemical kinetics are difficult

to apply,⁴⁸ which has necessitated the development of the specialized method of rule-based modeling.⁴ This approach is based on the use of local rules to model biomolecular interactions and their consequences and appropriate data structures (viz. graphs) to capture the coarse structures (domain architectures) of biomolecules and the topological structures (connections) of biomolecular complexes. Nearly 30 software tools have been developed for rule-based modeling of cellular regulatory systems.¹²¹ These tools deliver capabilities to perform a variety of simulations, including simulations based on ODEs, PDEs, KMC, and BD algorithms. Coarse MD simulations are even possible.¹⁴² However, development of simulation and analysis capabilities is ongoing, and indeed, building the theoretical foundations of rule-based modeling

is an active area of research.¹⁹⁴ At present, the approach is mature enough to enable nontrivial modeling studies of intracellular signaling^{6–26} and other biological processes, such as metabolism^{204–206} and gene regulation.^{207–209}

Two promising directions for future research are model-based analyses of proteomic data and further development of capabilities and resources that are relevant for collaborative model development and model reuse, such as model repositories.^{210–212} Modeling and proteomics have not so far become intimately integrated, despite the ability to apply proteomic technologies to generate quantitative data in support of modeling efforts.²¹³ We expect that this situation will change because a rule-based model has the site-specific resolution needed to connect to high-throughput proteomic data,¹⁵ such as (time-resolved) measurements of site-specific phosphorylation^{50,214–216} and measurements of site-specific binding affinities.^{74,102} The approach can also

leverage our growing knowledge of protein–protein interfaces.^{28–31} Model reuse is an issue that must receive greater attention if we are to develop reliable comprehensive models for cellular regulatory systems that fully leverage our collective intelligence. Recent large-scale modeling efforts provide motivation for pursuing comprehensive models.^{217,218} As discussed here, rule-based models have features that should facilitate model reuse and community-driven model development. Efforts to model and analyze cellular regulatory systems in systems biology have tended to focus on small subsystems, such as the simple circuits illustrated in Figure 1(c)–(f).^{110,219} Increasing the scope of modeling efforts (without degrading model reliability) should enable deeper insights into cellular regulation and predictions of greater health significance. Rule-based modeling approaches, which are scalable with respect to both specification and simulation, may offer solutions to some of the problems posed by this challenge.²²⁰

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