New and Notable

Pinning Down the EGF Receptor

Thomas M. Jovin[1,*]
[1]Laboratory of Cellular Dynamics, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany

According to leading investigators in the field of cellular signal transduction, the epidermal growth factor (EGF) receptor (EGFR, ErbB1, HER1), ubiquitously encountered in signaling mechanisms and thus in human tumors, is the best studied yet least prototypic of receptor tyrosine kinases in general (1). This perception arises primarily from the fact that activation of the EGFR is thought to be conformational/allosteric, i.e., not requiring covalent modifications at or near the active site (2). However, a number of outstanding, perplexing questions exist that might still place the EGFR in the well known but poorly understood category. Delineating these issues requires an outline of the current dogma.

The EGFR monomer is a glycosylated transmembrane protein, comprising an ectodomain with the capacity of adopting a ligand (growth factor)-binding conformation; a single-pass transmembrane domain with distinct bilateral juxtamembrane segments; and a cytoplasmic domain containing a latent tyrosine kinase core and a C-terminus with nine tyrosine residues serving as phosphorylation targets. In response to ligand (e.g., EGF) binding, although also in its absence under certain conditions, the EGFR forms homodimers and heterodimers with the three other members of the HER family. As a consequence, the kinase domain(s) auto- and trans-phosphorylate the C-terminal tail(s). The pY products constitute specific recognition elements funneling directly or indirectly via adaptor proteins into downstream intracellular signaling pathways, including Ras/Raf/MEK/ERK1/2, phosphatidylinositol 3-kinase (PI3K)/Akt, and phospholipase C (PLCγ). In most cases, membrane translocation and recycling of one or more components is involved.

Let us regard the same system from a biophysical perspective, posing certain questions (Q1–Q9, listed below) for which answers are as yet incomplete. I propose defining the EGFR as a "multifaceted signal transducer". Signaling is bidirectional with respect to the plasma (or endosomal) membrane, mediated by interactions with the classical peptidic growth factors as well as with other regulatory molecules (proteins, individual lipids, lipid microdomains, carbohydrates). Concerted (Q1) reactions couple ligand binding (Q2) to conformational transitions (Q3) leading to formation (or reconfiguration) of a dimer (Q4) stabilized by the extension and intertwining of dimerization arms; the interactions can be homotypic and heterotypic. The ectodomain—and as a consequence, the kinase subdomains—adopt active configurations (Q5). The primary targets of phosphorylation are the C-terminus of the same and/or partner EGFR monomer (Q6) but other cellular proteins are also phosphorylated (Q7). EGFR signaling is abrogated by phosphatases (Q8) before and after clathrin-dependent cellular uptake.

In view of these considerations, I deem it expedient to invoke four distinct activated states or entities of the EGFR: i), ectodomain configuration(s); ii), the phosphorylated carboxy-terminal tail; iii), the activated kinase(s); and iv), monomeric or oligomeric derivatives of the primary activation dimer. The complex interplay of thermodynamic states and complexes, and the corresponding kinetic parameters at steady state or full equilibrium are of primary importance. For example, the external "clasps" and internal (juxtamembranar) "latches" presumably stabilize dimers for only a finite time, as evidenced in single-molecule tracking experiments (3). This aspect of the EGFR system is further complicated by the identification, already in 1993, of numerous factors leading to aggregation and thus activation of the EGFR by non-canonical mechanisms (Q9). Mass action (molecular crowding) suffices to induce EGFR kinase activity, even if the interactions are unspecific and/or polymorphic. Thus, EGFR activation can be achieved or enhanced by protein modification, high expression levels, focal ligand exposure (functionalized microbeads and nanoparticles), coaggregation with protein and peptidic kinase substrates, interaction with polyaminoacids and polyamines, and the targeted application of physical forces. The influences of the lipid environment (4) are undoubtedly key but as yet not fully elucidated, for example in relation to feedback and feedforward regulation (5).

Q1: Is a rigorous biophysical definition in terms of distinct states (conformational, complexation, association) possible at this time?
Q2: How many molecules? Is there negative and/or positive binding cooperativity? Role of nonspecific (physical) factors?
Q3: Involving which distinct states of the tripartite molecule?
Q4: Role of preformed dimer? Symmetric versus asymmetric?; 1 or 2 ligands?
Q5: Mechanism of transmembrane crosstalk? Does reciprocal conformational inhibition and activation of the ecto- and endodomains occur? What are the influences of ligand identity and EGFR subcellular compartmentalization (plasma membrane, endosomes, filopodia, nucleus)?

Submitted October 17, 2014, and accepted for publication October 24, 2014.

*Correspondence: tjovin@gwdg.de

Editor: David Piston.

0006-3495/14/12/2486/3 $2.00

http://dx.doi.org/10.1016/j.bpj.2014.10.037

Q6: Are one or both monomers activated in the dimer? What determines a (the) particular pattern of tyrosine modification? Random?

Q7: How many? How can (large) protein substrates be accommodated stereochemically by a dimer?

Q8: How long do the other activated states persist before and after? For example, is a dissociated, C-terminally phosphorylated monomer active in signal transduction?

Q9: What is the relative influence of ligand-dependent and ligand-independent processes on the distribution of EGFR and its downstream signaling partners?

A number of these issues are addressed in an intriguing new study of the output interface(s) of the EGFR from the Baird-Holowka group at Cornell University (6). The authors inverted the usual ligand-to-EGFR experimental paradigm by generating an ordered micron-sized array of EGF on silicon surfaces, using techniques honed in numerous long-standing studies of FcεRI. A parylene-patterned silicon substrate with 1.5-4 μm features was functionalized with Alexa568-streptavidin and incubated with EGF-biotin. Removal of the parylene yielded defined patterns of bound EGF, which were readily recognized by NIH-3T3 cells overexpressing the EGFR deposited on the surface (unfortunately, neither the number of receptors per cell nor the density of immobilized EGF was specified). The images presented indicate that there were $<$10 EGF domains per overlying cell. The activation of EGFR was determined immunocytochemically as EGFR-pY. Multiprotein EGFR signaling complexes formed at the plasma membrane in response to the micropatterned EGF and the images were subjected to correlation and distribution analyses.

Immobilized EGF has been used previously to study the influences of ligand density and diffusion barriers on the degree of activation of EGFR ((7) and references therein; (8)). Stabley et al. (8) demonstrated that clustering of the EGFR required phosphorylation to a degree that was inversely related to cluster size, as well as interaction with one or more forms of F-actin. Singhai et al. (6) exploited the micropatterned EGF to obtain much more detailed information about the correlated spatiotemporal distribution of activated EGFR, downstream effectors, and certain molecules involved in cell-cell and cell-extracellular matrix interactions. The initial step of receptor recruitment to the EGF patches occurred in 10–40 min at 37°C; the authors attribute this very long time course primarily to the accommodation of the cell to the surface and secondarily to ligand density and accessibility. However, inasmuch as the association of EGF to the streptavidin surface-coupling agent is reversible in principle, one can ask whether the EGF molecules can (must) detach and redistribute to fully populate EGFR dimers in such an experimental system (Q2); this process would be very slow. Phosphorylation (pY-1068) accompanied cellular attachment and EGFR clustering, and was inhibited by the kinase inhibitor Iressa. However, Iressa did not inhibit the clustering per se, a clear demonstration that ligand binding and many of its consequences can be uncoupled from kinase function (to be distinguished from kinase activation; Q2–Q5). Paxillin, a component of integrin-mediated signaling at focal adhesions, was also recruited to the clusters of activated EGFR and underwent phosphorylation, although not by EGFR but probably by Src, which modulates EGFR function via phosphorylation at Tyr-845. Is compartmentalization required for this process (Q5)? F-actin and integrin $\alpha5\beta1$ were likewise corecruited, in the latter case preferentially to sites at the cell periphery. Could unliganded mobile phosphorylated monomers be involved (Q8)? In the case of the Erk signaling pathway, EGFP-labeled H-Ras and N-Ras concentrated at the EGF patches, as did the downstream effectors MEK and pErk. The phenomena were physiological in that the latter molecules underwent subsequent translocation to the nucleus. Other upstream members of this signaling cascade (Grb2, Shc, SOS, Raf), were not monitored. Their relative stoichiometry in the EGFR-overexpressing cells would be important, inasmuch as their physical recruitment would have to antecede that of MEK and pErk.

An additional finding was the inhibition by cytochalasin D of the recruitment of paxillin and pErk, but not of the GTPase dynamin 2, a mediator of EGFR endocytosis. Does the involvement of F-actin imply the need for sequestration of scaffold and 14-3-3 proteins, which regulate the kinetics, strength and position of MEK/ERK signaling? Interestingly, PLCγ1, an enzyme responsible for hydrolysis of PI(4,5)P2, was also recruited to the patterned EGF, and inhibition of PI(4,5)P2 biosynthesis suppressed the recruitment of F-actin and pErk. The demonstration that phosphoinositides are involved in the stabilization of signaling complexes by F-actin is novel and important (4), as is the evidence of a differential distribution of focal adhesion components and F-actin, with implications for downstream signaling involved in cell migration.

The conclusion one can derive from the impressive study from the Baird-Holowka lab is that the spatial distribution of signaling entities is orchestrated by a hierarchy of local and external factors, starting with the input and output interfaces mediating activation of the EGFR (Q9) and other members of the HER family. One can conceive of biophysical extensions to address Q2–Q5. Assessing recruitment and kinase activation as a function of the density of immobilized EGF would be very instructive. Photocleavable attachment of the EGF to the micropatterned surfaces would enable assessment of diffusion-dependent and dissociation processes. Optogenetic control of gene expression and molecular states could greatly enhance the scope of the imaging based analyses.

Spatially controlled photobleaching would permit isolation of individual stages in sequential responses. Single-molecule techniques (3), including those based on fluorescence resonance energy transfer, lifetime, and hyperspectral signatures, should also be feasible and instructive. From a cell biological standpoint, one would wish to study other cell types and assess the processes of EGFR endocytosis and recycling.

The bottom line? After 31 years, the EGFR (and Q1) remain elusive…but within reach.

REFERENCES

1. Schlessinger, J., and M. A. Lemmon, editors. 2014. Signaling by Receptor Tyrosine Kinases. *Cold Spring Harb. Perspect. Biol*., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1–478.
2. Endres, N. F., T. Barros, …, J. Kuriyan. 2014. Emerging concepts in the regulation of the EGF receptor and other receptor tyrosine kinases. *Trends Biochem. Sci.* 39:437–446.
3. Pryor, M. M., S. T. Low-Nam, …, J. S. Edwards. 2013. Dynamic transition states of ErbB1 phosphorylation predicted by spatial stochastic modeling. *Biophys. J.* 105:1533–1543.
4. Coskun, Ü., M. Grzybek, …, K. Simons. 2011. Regulation of human EGF receptor by lipids. *Proc. Natl. Acad. Sci. USA.* 108:9044–9048.
5. Avraham, R., and Y. Yarden. 2011. Feedback regulation of EGFR signalling: decision making by early and delayed loops. *Nat. Rev. Mol. Cell Biol.* 12:104–117.
6. Singhai, A., D. L. Wakefield, …, B. Baird. 2014. Spatially defined EGF receptor activation reveals an F-actin-dependent phospho-Erk signaling complex. *Biophys. J.* 107:2639–2651.
7. Gonçalves, R., M. C. Martins, …, M. A. Barbosa. 2010. Bioactivity of immobilized EGF on self-assembled monolayers: optimization of the immobilization process. *J. Biomed. Mater. Res. A.* 94:576–585.
8. Stabley, D., S. Retterer, …, K. Salaita. 2013. Manipulating the lateral diffusion of surface-anchored EGF demonstrates that receptor clustering modulates phosphorylation levels. *Integr. Biol. (Camb).* 5:659–668.