Biochemical and Biophysical Research Communications 378 (2009) 376–382

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Receptor overexpression or inhibition alters cell surface dynamics of EGF–EGFR interaction: New insights from real-time single molecule analysis

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ARTICLE INFO

Article history:
Received 29 October 2008
Available online 17 November 2008

Keywords:
TIRF
EGF
Dimerization
Cancer cells
Receptor density
Anti-EGFR drugs
AG1478
EGFR inhibitor

ABSTRACT

Binding of Epidermal growth factor (EGF) to epidermal growth factor receptor (EGFR) in two types of cancer cells (HeLa; 5×10^4 EGFR/cell) and MDA-MB-468; 2×10^6 EGFR/cell) was studied using Total Internal Reflectance Fluorescence (TIRF) microscopy at single molecule precision. Mathematical modeling of the binding kinetics revealed that cells respond differently to the same concentration of EGF depending on the expression level of EGFR. Compared to Hela, MDA-MB-468 cells show; (a) higher number of pre-formed dimers, (b) improved EGF–EGFR interaction at lower ligand concentrations, and (c) shorter time-lapse between first and second EGF binding to the dimer. Treatment with a pharmacological inhibitor of EGFR, AG1478, produced strikingly different binding kinetics where the extent of pre-formed EGFR dimers increased substantially. Thus, single molecule approaches produce novel, quantitative information on signaling mechanisms of significant biological importance. Surface kinetics could also serve as surrogate markers to predict biological outcome of signaling pathways.

Epidermal growth factor receptor (EGFR) is a prototype of receptor tyrosine kinases that regulate fundamental biological processes such as cell proliferation, growth and development [1]. Binding of EGF to its receptor initiates many molecular and biochemical events. These include receptor dimerization/oligomerization, autophosphorylation of the receptor followed by interaction of the receptors with intracellular signaling molecules [2]. These molecular interactions activate specific signaling cascades such as Ras/Raf/mitogen-activated protein kinase [MAPK] pathway, PI-3K/Akt pathway and many others [3,4]. The signal ultimately culminates in regulation of cell proliferation, cell growth, survival, cytoskeleton remodeling and cell migration [5].

Defective EGFR signaling is one of the important molecular causes of cancer development. Constitutive activation of EGFR either by overexpression (e.g., due to gene amplification), mutation (e.g., truncated receptor with no ligand binding domain) or by autocrine or paracrine secretion of ligands (e.g., TGFα) causes oncogenesis [6]. Therapeutic importance of the members of EGFR family of receptors, particularly EGFR and EGFR-2 [HER2] is well documented [7,8]. Many promising new cancer drugs targeting the EGFR and/or HER2 are currently in various phases of clinical trial, while some are already in clinical use [7,9]. However, in some instances, development of unexpected resistance to such therapies highlight the need for better understanding of the dynamics at the molecular level [10,11].

Although abnormal activation of EGFR is undoubtedly an important event in oncogenesis, it is not clear whether EGF–EGFR signaling differs in cells expressing vastly different numbers of receptors either at the level of receptor–ligand interactions or at the level of temporal sequence of these events. Contrary to the existing notion that the receptors are induced to dimerize upon binding to EGF [12,13], a certain proportion of EGFRs is now shown to be present as dimers even before interaction with EGF [14]. It was also noted that only a small fraction (~1%) of EGFR needs to be occupied to induce EGF signaling [15]. These results question the prevalent dogma that EGFR amplification and/or sustained activation is a key molecular mechanism of enhanced proliferative/oncogenic signaling [6,16]. To address this, we analyze ligand–receptor interactions in two different types of cancer cells expressing vastly different numbers of EGFR. We investigate the effect of various concentrations of EGF and treatment with an EGFR-targeting drug AG1478

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doi:10.1016/j.bbrc.2008.11.018

[17] on EGF–EGFR association kinetics. We conclude that our approach is uniquely capable of distinguishing important, otherwise unmeasurable, alterations in the surface kinetics upon receptor overexpression or its pharmacological inhibition.

Materials and methods

Cell culture. HeLa cells were cultured in DMEM (Mediatech Inc., Herndon, VA) containing 10% FBS (Invitrogen, Carlsbad, CA) and MDA-MB-468 cells were cultured in MEM (Mediatech Inc.) containing 10% FBS. For single-molecule analysis, cells were cultured on No. 1.5 glass coverslips (Thermo Fisher Scientific Inc., Waltham, MA) and were serum-starved overnight in serum-free medium prior to EGF treatment.

Instrumentation settings. An inverted optical microscope (IX-71, Olympus, Tokyo, Japan) was used for an objective type total internal reflectance fluorescence microscopy utilizing a high numerical aperture objective (PlanApo 100X NA 1.4, Olympus). Samples were illuminated with a randomly polarized laser (543 nm, 2.0 mW, R-30972; Newport, Irvine, California) and images recorded on a dual intensified, cooled CCD camera (XR/TURBO-120Z ICCD, Stanford Photonics, Palo Alto, California).

Single-molecule measurement of EGF binding to cell surface EGFRs. Alexa 555-conjugated human EGF (Ax-EGF) was from Invitrogen (Carlsbad, CA). Ax-EGF was applied at three final concentrations (1, 5, and 10 ng/ml) and cells were observed under the microscope at room temperature. Cells were kept on ice before being mounted onto the microscope for measurements, to slow down EGFR internalization. In a previous work by Teramura et al. [18], an oblique illumination microscope was used to observe the apical surface of a living cell, in order to avoid the potential effect of the diffusion of EGF through the cavities between the basal surface of the cells and the coverslip becoming the determining factor of binding kinetics. However, the apical surface is ~μms away from the coverslip surface, at this distance the TIR field has already decayed to negligent levels; hence the advantage of superior resolution along the vertical direction provided by TIRF microscopy is lost. In fact, on the basal surface, EGF in the surrounding environment still has easy access to the outside ring (Fig. 1A), where diffusion is not the rate-determining factor. Therefore, we limited the analysis to the outside ring of the basal surface of the cells, in order to effectively utilize TIRF microscopy to study EGF–EGFR binding kinetics. Images of cells were recorded for several seconds at each of the indicated time-point after the application of Ax-EGF. For single-molecule tracking experiments, images were recorded continuously at 30 frames/s. Spots produced by random noise were removed through a noise-removal procedure in which all bright spots that only appeared in one frame were deleted before further analysis. Images of the whole cell surface were divided into sub-sections (2×2 μm) and fluorescence spots in each sub-section were tracked through the entire time-course. The number of bound Ax-EGF molecules on the whole surface of each cell was then re-constructed by adding in contributions from all sub-sections. Finally, the observed number of binding events was scaled to the total number of bound molecules per cell by scaling the observed area (area of the outside ring) with respect to the averaged surface area of the cell (4500 μ^2/cell, [15]). Data from multiple cells (N) were gathered for analysis ('N' is indicated in the figure legend for each experiment).

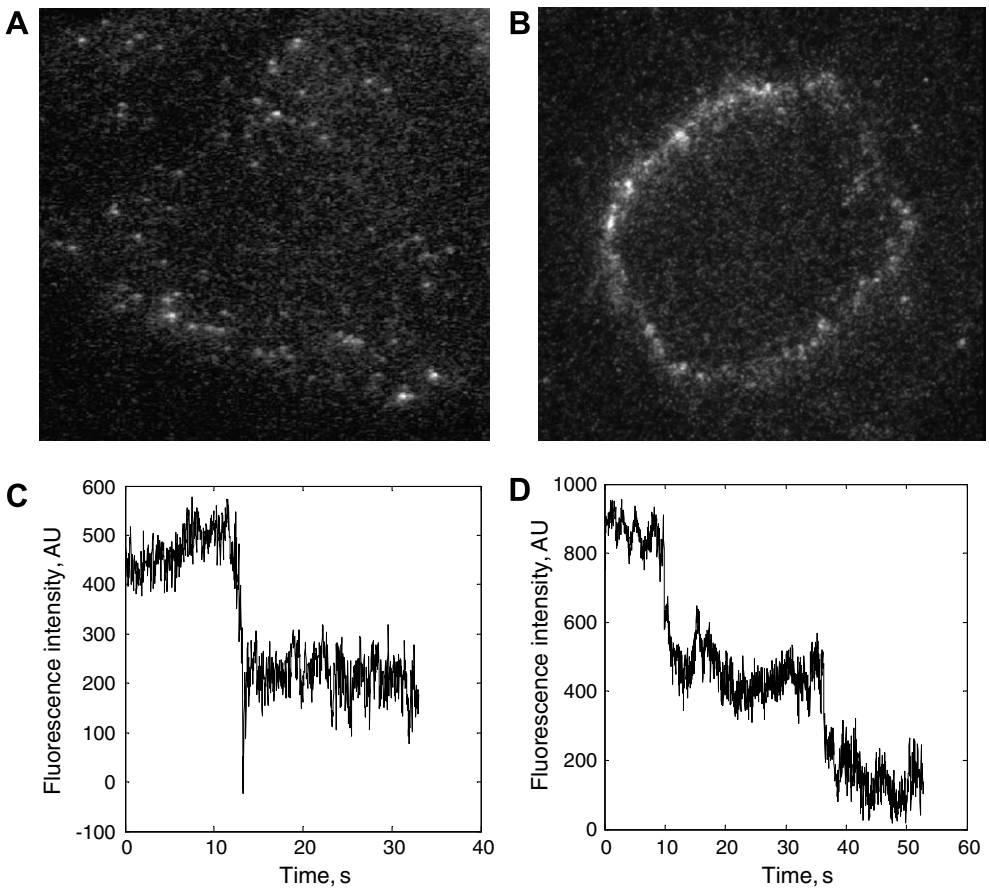

Fig. 1. (A and B) TIRF images of Ax-EGF binding to cell surface EGFRs acquired after 100 s of Ax-EGF application displaying the Ax-EGF binding to the outside rings on the basal surface of the cells; (A) HeLa, (B) MDA-MB-468. (C) One-step drop of fluorescence intensity observed due to photobleaching of Ax-EGF bound to EGFR monomer; (D) Two-step drop of fluorescence intensity observed due to photobleaching of Ax-EGFs bound to EGFR dimer.