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Ligand depletion negatively controls the mitogenic activity of epidermal growth factor

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Abstract

EGF activates the ErbB1 receptor, but there appears only a limited correlation between its receptor binding affinity and mitogenic activity. This is indicated by our present observation that in cells with high ErbB1 expression, including SUM102 breast tumor cells, low affinity EGF/Notch chimeras have similarly high mitogenic activity as EGF, in spite of the fact that EGF is superior in inducing receptor tyrosine phosphorylation and p42/p44 MAP-kinase activity. However, as a result of receptor-mediated internalisation high-affinity ligands such as EGF are depleted much more rapidly from the extracellular medium than low-affinity EGF/Notch chimeras. As a consequence, the mitogenic activity of EGF on ErbB1 overexpressing cells is limited by substantial degradation of internalised ligand in the period before cells enter S-phase, a phenomenon that is not observed for low affinity mutant ligands. The mitogenic activity of EGF on ErbB1 overexpressing cells does therefore not only depend on the applied concentration but also on the total amount of ligand added, and is strongly underestimated when tested in a limited assay volume. No such dependence on the incubation volume was observed for EGF activity on cells with low ErbB1 expression levels and on cells for which EGF is growth inhibitory.

Keywords: Epidermal growth factor mutant; Receptor internalisation; Ligand degradation; Mitogenic stimulation; ErbB1 overexpression

Introduction

The ErbB signalling network is currently one of the main targets for the development of anti-cancer drugs [1,2]. It consists of four related ErbB receptors and a dozen soluble ligands with distinct receptor specificity. The best characterised of these ligands are epidermal growth factor (EGF) and transforming growth factor-alpha (TGFα), which both interact specifically with ErbB1 [3]. Recent crystallographic data have provided a wealth of information on the structure of the ErbB1/EGF and ErbB1/TGFα complex, and on the mechanism by which ErbB receptors dimerise upon ligand binding (reviewed in Ref. [4]). Much less is known, however, how receptor activation is translated into a mitogenic response. For example, cells with low ErbB1 levels can respond much stronger to growth stimulation by EGF than cells with intermediate receptor levels, while for cells that strongly overexpress ErbB1 EGF can become growth inhibitory and even induce apoptosis [5,6].

Binding of EGF to ErbB1 results in the rapid activation of the receptor intrinsic tyrosine phosphokinase activity and the subsequent generation of a large variety of intracellular second messenger molecules [7]. However, this process is attenuated by the subsequent internalisation and degradation of the ErbB1/EGF complex in the lysosomes [8,9]. Although ligand-induced receptor downregulation is generally considered as a negative feed-back mechanism in growth factor signalling, recent evidence indicates that also after internal-

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; MAP, mitogen-activated protein; NCS, newborn calf serum; PBS, phosphate-buffered saline; TGFα, transforming growth factor-alpha.

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isation, the activated ErbB1 receptor is able to generate intracellular second messengers [10–12], and can contribute to cell proliferation [12]. In contrast to EGF, TGFα dissociates from internalised ErbB1 in the acidic endocytotic vesicles, resulting in recycling of ErbB1 to the plasma membrane, concomitant with degradation of TGFα in an unknown cellular compartment [8]. In spite of these differences in inducing receptor recycling, the mitogenic activities of EGF and TGFα are very similar in most biological systems tested, with only minor differences in their receptor activation kinetics [13].

It is well established that induction of DNA synthesis into quiescent cells requires the continuous presence of growth factors for at least 8 h which corresponds to the time cells need to reach the G1/S-phase boundary [14]. The sequence of events during this relatively long period of time is still poorly understood, but recent studies indicate that two short pulses of platelet-derived growth factor (PDGF) or EGF are already sufficient to induce the signalling required for mitogenesis [15,16]. Activation of the second messengers MAP-kinase and c-Myc seems particularly important for triggering quiescent cells to enter the G1-phase, while PI3-kinase appears essential for inducing cells to make the G1/S-phase transition [15,16]. These results imply that 8 h after addition of a single dose of growth factor, active receptor–ligand complexes must still exist to direct cells into the S-phase.

From theoretical considerations, a direct relationship has been proposed between EGF receptor occupancy and mitogenic response [17]. On the other hand, many mutant forms of EGF have been characterised that have strongly reduced binding affinity for ErbB1, but still show similar dose-dependence for mitogenic activation as wild-type EGF [18–21]. The molecular basis for the high mitogenic activity of these low affinity ErbB1 ligands is still poorly understood, and has been attributed to enhanced resistance to proteolytic degradation [18], reduced cellular uptake [19], more efficient induction of receptor dimers [20] and increased generation of intracellular second messengers [21].

In order to understand the relation between ErbB1 binding and mitogenic activity in more detail, we have analysed in this study a set of EGF-Notch chimeras, which show a gradual decrease in ErbB1 binding affinity with increasing Notch content but maintain a high mitogenic activity [22]. Here, we show that the mitogenic activity of these low affinity EGF mutants strongly depends on the ErbB1 density of the cell. Low-affinity ligands are much less rapidly internalised and degraded than wild-type EGF, and as a result, they show similar receptor occupancy at the time cells are triggered to enter the S-phase. Our results furthermore indicate that the mitogenic activity of EGF in vitro is controlled by the rate of ligand depletion, and is therefore a complex function of the cellular ErbB1 receptor density, the applied ligand concentration, and the total amount of ligand molecules present in the assay.

Materials and methods

Cell lines

HER-14 cells, NIH-3T3 cells transfected with the human (h) EGF receptor, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS; Hyclone) in gelatinised flasks. These cells, which express 4.0×10^5 hEGF receptors/cell [23], were a generous gift from Dr. J. Schlessinger (Yale University, New Haven CT). A431 human epidermoid carcinoma cells containing 2.0×10^6 hEGF receptors/cell and mouse Swiss 3T3 cells fibroblasts, clone C7C2 (5) expressing 6×10^3 murine EGF receptors/cell were cultured similarly. SUM102 cells, derived from a microinvasive primary human breast tumor, were a generous gift from Dr. S. P. Ethier (University of Michigan, Ann Arbor MI) and Dr. M. Schutte (Erasmus Medical Center, Rotterdam, The Netherlands). These cells, which contain an estimated 1×10^5 hEGF receptors per cell, were cultured in Ham's F12 medium containing 0.2% bovine serum albumin (BSA), 1 μg/ml hydrocortison, 10 μg/ml insulin and 10 ng/ml EGF, basically as described [24], with an additional supplement of 5% growth factor-inactivated fetal calf serum [25].

Growth factors

Chimeras between hEGF and EGF repeat 13 of *Drosophila* Notch were generated by exchanging domains bordered by the shared cysteine residues as described previously [22]. E3N4E is a chimera composed of EGF in which the B-loop (amino acids 21–30) has been exchanged for the corresponding 8 amino acids HDKINGFK sequence from Notch. In Notch E3N4E/MY, this B-loop region has been exchanged for MYKINGFK, in E3N4E/MA for MDKINGFA, and in E3N4E/MYA for MYKINGFA. Recombinant wild-type hEGF and the various EGF/Notch chimeras were expressed in *Escherichia coli* initially linked at the N-terminus to Protein A [26]. Fusion proteins were isolated from the periplasm by affinity purification on IgG-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) followed by removal of the Protein A tag by Factor X_a and an additional run on IgG-Sepharose. Recombinant ligands were finally purified by reverse phase high-performance liquid chromatography and quantified by their absorption at 229 nm [26]. Recombinant hTGFα and the EGF/TGFα chimera E4T [27] were expressed in a similar manner.

Cell proliferation assays

HER-14 cells were seeded in gelatinised 24-wells plates at a density of 6.0×10^4 cells/1.8 cm^2. After 24 h of incubation, the medium was replaced by 0.9 ml of serum-free medium containing 0.5% BSA, as described [26]. After an additional 48 h of incubation, growth factors to be tested were added. In experiments in which the incubation volume