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Cbl-mediated Ubiquitination and Downregulation of the EGF Receptor

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Upon ligand-stimulated epidermal growth factor receptor (EGFR) activation, the receptor dimer is transphosphorylated on specific tyrosines by its intrinsic tyrosine kinase activity. Phosphorylated Tyr1045 recruits c-Cbl, a ubiquitin ligase responsible for EGFR ubiquitination, which targets the receptor for lysosomal degradation. Tyr1045 is followed by two serine residues that are known to be phosphorylated in vivo. It has been previously suggested that these serine residues play an important role in the endocytosis and downregulation of the EGFR. In contrast to EGFR, ErbB2 has low affinity binding of Cbl, and is poorly downregulated. ErbB2 contains a Tyr residue at position 1091 and a Ser residue at position 1092, corresponding to Tyr1045 and Ser1046 of EGFR. However, ErbB2 contains a Glu at position 1093 instead of the Ser1047 present in EGFR, which could be the reason for the poor binding of Cbl to ErbB2. We hypothesized that phosphorylation of Ser1046 and Ser1047 plays an important role in Cbl binding to and subsequent ubiquitination of the EGFR. In order to test this hypothesis, EGFR S1046A, S1047A, S1046/47A, S1046E, S1047E (introduces the putative ErbB2 Cbl binding sequence into EGFR), S1046/47E and Y1045F mutants were transiently transfected in NIH3T3 cells. After EGF stimulation, all EGFR mutants were tyrosine phosphorylated, and all mutants except Y1045F were ubiquitinated and co-immunoprecipitated Cbl. Immunofluorescence studies showed that the EGFR S1046/47A and S1046/47E mutants were present on the surface of transfected NIH3T3 cells and upon EGF-mediated stimulation were internalized in a similar fashion as EGFR wt. We conclude that: (I) introduction of the putative Cbl binding sequence of ErbB2 into EGFR does not reduce activation, ubiquitination and binding of Cbl to the receptor, (II) phosphorylation of Ser 1046 and 1047 is not required for efficient receptor activation, internalization and ubiquitination.

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Direct Association of EGF Receptor with Hsc70 and α-Actinin 4

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Our previous work established that the dileucine motif (679-LL) in the juxtamembrane domain of the human EGF receptor is essential for delivery of ligand-receptor complexes to lysosomes for degradation. To identify the cellular components that interact with this motif, we used EGFR juxtamembrane domain sequences fused to GST to isolate cytosolic binding partners that were then identified by mass spectrometry. This enabled the discovery of 3 interactive proteins: 70-kDa heat shock cognate protein (Hsc70), the uncoating ATPase of clathrin-coated vesicles; and its mitochondrial and ER homologues, Grp75 and Grp78, respectively. Two of these proteins, Hsc70 and Grp75, were co-immunoprecipitated with EGFR from cell lysates. The association between Hsc70 and EGFR was not affected by ligand stimulation, but was ATP and temperature dependent. Both Hsc70 and Grp75 also specifically interacted with ErbB2 in lysates from SK-BR3 breast cancer cells where ErbB2 is over-expressed. Although ErbB2 lacks a dileucine motif corresponding to 679-LL in EGFR, NMR analysis revealed that juxtamembrane domains in both receptors forms similar amphipathic helices, suggesting that secondary structure is important for Hsc70 association rather than the 679-LL motif per se. Since molecular chaperones such as Hsc70 interact with numerous proteins, we analyzed Hsc70 immunoprecipitates by mass spectrometry to identify potential protein binding partners that might also associate with EGFR. Thus α-actinin 4, an actin-binding protein involved in membrane ruffling in chemotactic cells, was identified as an Hsc70 interactive partner that was co-immunoprecipitated with EGFR independent of EGF stimulation. These data suggest that Hsc70 functions as an adaptor protein linking EGFR and/or ErbB2 with cellular proteins involved in multiple cellular functions including receptor trafficking. Supported by NIH grants GM063243 and DK54178.

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Temporo-Spatial Control of Signaling Responses: Tenascin-C EGF-like Repeats Stimulate EGFR-Mediated Fibroblast Migration

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During wound healing both migration and proliferation of various cell lineages must be controlled in an exacting manner. EGF family members are expressed during all stages of healing, acting as strong motogenic and mitogenic stimuli for keratinocytes and fibroblasts. Tenascin cytotactin (TenC) is an extracellular matrix (ECM) glycoprotein that is found preferentially upregulated at the edges of healing wounds. Select EGF like (EGFL) repeats of TenC, including the 14th repeat (Ten14), can signal through the EGF receptor (EGFR), but bind only transiently at ultralow affinities. We hypothesize that this leads to plasma membrane-proximal signaling and preferentially those responses controlled in that space, such as motility. We used NR6 fibroblasts over-expressing human EGFR to demonstrate a partitioning of cell signaling responses, with sustained migration over 2D surfaces at levels of Ten14 that failed to stimulate proliferation. At higher Ten14 concentrations there were comparable levels of migration and proliferation. For a classic soluble ligand, EGF, the migration and proliferation responses were equivalent across all concentrations. Maximal activation of PLCγ1 and calpain, membrane proximal biochemical responses, occurs at 15 minutes (mins) post treatment with ligand for all concentration of Ten14, concomitant with the activation of phosphoEGFR. The activation of phosphoERK remains low for 5, 15 and 60 mins of treatment with Ten14 compared to EGF. Elk1 transcriptional activation was similarly less robust for Ten14 as compared to EGF. Our data suggest that Ten14 preferentially stimulates migration due to strong motility-associated signaling, PLCγ1 and calpain, which is located in the peri-plasma membrane space, while the cytosolic and nuclear signals related to proliferation, Elk1 and p90rsk, are only weakly activated at subsaturating levels of ligand. Thus, the temporospatial nature of signaling through growth factor receptors dictates the cell response.

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Antitumor Activity of Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors in Human Hepatocellular Carcinoma Cell Lines

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Hepatocellular Carcinoma (HCC) is one of the most common malignancies and is responsible for more than one million deaths worldwide. Epidermal growth factor receptor (EGFR) with tyrosine kinase activity is highly expressed in many human tumors including HCC. Therefore, inhibition of EGF receptors could be a potential target for anticancer therapy in HCC. In this study, we investigated the effects of two EGFR tyrosine kinase inhibitors, PD153035 and its analogue 4-[[3-Chloro-4-fluorophenyl]amino]-6,7-dimethoxyquinazoline hydrochloride, on cell proliferation of three human HCC cell lines expressing EGFR by MTT proliferation assay. Effect of EGFR inhibitors on EGFR autophosphorylation and downstream signaling were determined by western blot. The effect of EGFR inhibitors on HCC cell cycle distribution was evaluated by flow cytometry. Our results demonstrated that both EGFR inhibitors inhibited tumor cell growth in a dose-dependent manner. These two specific inhibitors not only blocked EGF-stimulated EGFR autophosphorylation but also targeted EGFR signaling including ERK and Akt pathways in human HCC cell lines. Decreased phospho-Erk and phospho-Akt were observed after EGFR inhibitors treatment, whereas the expression of inactive form of Erk and Akt remained unchanged. Furthermore, EGFR inhibitors treatment induced a delay in cell cycle progression and a G_1 arrest together with a partial G_2/M block. In conclusion,this study demonstrated that PD153035 and its analogue 4-[[3-Chloro-4-fluorophenyl]amino]-6,7-dimethoxyquinazoline hydrochloride are potent inhibitors of tumor growth in HCC. Our data suggest that blockage of EGF receptors may provide an effective therapeutic approach for human HCC.

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Heterodimerization and Activation of EGF Receptors

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The epidermal growth factor receptor (EGFR) family, a member of protein tyrosine kinases, has frequently been implicated in cancer cell proliferation and metastasis. Therefore, an understanding of the receptor activation mechanism is an important step towards design of novel anti-cancer drugs. It is widely believed that EGF receptors are activated through ligand-induced dimerization of the receptor (dimerization model). There are, however, observations showing that EGFR homodimers exist on the cell surface in the absence of bound ligand, and we have previously proposed a 'rotation mode' in which ligand induce the rotation or twist of the receptor's transmembrane domains and dissociate the inactive homo-dimeric kinase domains for its activation. Here, we have investigated heterodimerization between the EGFR family members. 32D and B82 cells, which do not express the family receptors, were co-transfected with EGFR and HER2, or HER3 and HER4, respectively. Stably cotransfected cell lines were confirmed by Western blotting and flow cytometry. Chemical crosslink and sucrose density-gradient centrifugation were carried out to observe heterodimeric receptors without bound ligand. These results indicate that heterodimers between the EGFR family members exist prior to ligand binding, and are consistent with the rotation model, but not with the dimerization model.

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FRET Based Detection of Preformed EGFR Homo- Dimers on the Cell Surface

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The epidermal growth factor receptor (EGFR) is one of the four ErbB family of receptor tyrosine kinases. EGFR regulates cellular proliferation, differentiation and survival. Aberrant activation of the receptor has frequently been responsible for cancers. Formation of a homo-dimer is a prerequisite for phosphorylation of the carboxyl terminal regulatory domain by the tyrosine kinase, leading to intracellular signaling. Two models have been proposed to describe the activation of EGFR. The ligand EGF induces dimerisation of the receptor molecules in which the cytoplasmic kinases trans-autophosphorylate (dimerisation model), or rotates the transmembrane domains of preformed dimers, and dissociates the dimeric cytoplasmic kinase for activation (rotation model). We have previously shown by chemical and cysteine cross-linking and sucrose density-gradient centrifugation that in the absence of bound ligand the