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Neuregulin-1 only induces *trans*-phosphorylation between ErbB receptor heterodimer partners

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Abstract

ErbB2, ErbB3 and ErbB4 are members of the Epidermal Growth Factor Receptor (EGFR) sub-family of Receptor Tyrosine Kinases (RTKs). Neuregulin-1 (NRG-1) is a ligand of ErbB3 and ErbB4 receptors. NRG-1-induced ErbB2/ErbB3 or ErbB2/ErbB4 heterodimerization, followed by receptor phosphorylation, plays multiple biological roles. To precisely determine the phosphorylation status of each ErbB receptor in ErbB2/ErbB3 and ErbB2/ErbB4 heterodimers, an immunoprecipitation–recapture of the ErbB receptors was performed to exclude any co-immunoprecipitated heterodimer partners from cells with co-expression of ErbB2/ErbB3, ErbB2/ErbB4, or ErbB2/ErbB4D843N, a kinase-inactive ErbB4 mutant, in which the aspartic acid at 843 (D843) was replaced by an asparagine (N). Here, we provide direct biochemical evidence that ErbB2 was only *trans*-phosphorylated by ErbB4, but not by ErbB3 or ErbB4D843N. By contrast, ErbB3, ErbB4 and ErbB4D843N were *trans*-phosphorylated by ErbB2 in the co-transfected cells. Therefore, we conclude that *trans*-phosphorylation, but not *cis*-phosphorylation occurred between ErbB2/ErbB3 and ErbB2/ErbB4 heterodimer partners by NRG-1 stimulation.
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Keywords: Neuregulin-1; ErbB receptors; Heterodimerization; *trans*-phosphorylation

1. Introduction

ErbB receptors belong to subclass I of the receptor tyrosine kinases (RTKs) super-family. ErbB-mediated cell signaling plays a critical role in the embryo development and adult organ functions [1]. ErbB receptors mediate signaling pathways for cell proliferation, differentiation, migration and structure reorganization [2,3]. There are four members in the ErbB family: epidermal growth factor (EGF) receptor (EGFR, also termed ErbB1); ErbB2/Neu; ErbB3 and ErbB4. Neuregulin (NRG) binds to the extracellular domain of ErbB3 or ErbB4 receptors leading to dimerization. To date, no ligand has been found to bind to ErbB2. However, ErbB2 serves as a preferred heterodimer partner for ErbB3 or ErbB4, and plays important roles in activation of ErbB receptor signaling [1].

In vivo studies with gene-targeting experiments indicate that developmental defects resulted from inactivation of *ErbB2* are similar to those observed in *neuregulin-1* (*NRG-1*)-inactivated animals [4,5]. Both animals showed defects in the development of neural crania ganglia and heart trabeculae. Furthermore, *ErbB3* or *ErbB4* gene-inactivated mice showed similar or overlapping phenotypes with *ErbB2* knockout mice in nervous and cardiac development [6–8]. NRG-1 signaling via an ErbB2/ErbB4 heterodimer also contributes to the neuroendocrine control of mammalian sexual development [9]. These findings strongly suggest that ErbB2 participates in NRG-1-activated ErbB3 or ErbB4 signaling pathways *in vivo*. In breast cancer cells, ErbB2 over-expressing is associated with a high level expression of ErbB3 [10,11]. Elevated expressions of ErbB2

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and ErbB3 are involved in the induction of mammary tumor cell growth in transgenic mice, suggesting a role of ErbB3 in association with ErbB2 in breast cancer [12]. The ErbB2/ErbB3 heterodimer was found to be a functional unit, in which ErbB2 requires ErbB3 to stimulate breast tumor cell proliferation [13]. These data suggest that ErbB2 plays essential roles in development and functions as the heterodimer partner of ErbB3 or ErbB4.

ErbB2 can act as a heterodimer partner for all other ErbB receptors *in vitro* [14,15]. Recently, a pre-activated structure of ErbB2 was revealed by a crystallization study, showing ErbB2 in a pre-activated form for homodimer or heterodimer without ligand stimulation [16]. ErbB2/ErbB3 or ErbB2/ErbB4 heterodimers show higher affinity for NRG-1β (<1 nM) than that of ErbB3 or ErbB4 homodimers (1–100 nM) [17]. Thus, the administration of NRG-1 to cells expressing ErbB3 or ErbB4 and ErbB2 would result the formation of ErbB2/ErbB3 or ErbB2/ErbB4 heterodimers [3]. Following heterodimerization, phosphorylation of ErbB receptors was markedly increased by ligand stimulations. However, it is not fully defined whether *cis*-phosphorylation or *Trans*-phosphorylation occurs between ErbB2/ErbB3 or ErbB2/ErbB4 heterodimer partners. *trans*-phosphorylation occurs between EGFR/ErbB2 dimer partners after stimulation with EGF [18], and between ErbB2 homodimer partners [19]. Due to the lack of kinase activity of ErbB3, the phosphorylation of ErbB3 in human cancer cells can be up-regulated by ligands, indicating that *trans*-phosphorylation occurred [20]. *Cis*-phosphorylation was thought to be occurring in ErbB2, ErbB3 co-expression cells too [21]. It is still not fully clear whether or not ErbB2 can be *cis*-phosphorylated in heterodimer of ErbB2/ErbB3 or ErbB2/ErbB4. Several observations with cultured cells suggest that ErbB2/ErbB3 and ErbB2/ErbB4 play different biological roles [22–24]. Thus, the phosphorylation status of ErbB2/ErbB3 and ErbB2/ErbB4 could be a critical factor to initiate distinct signaling pathways.

Here, we provide direct evidence that NRG-1 only induced *trans*-phosphorylation in ErbB2/ErbB3 and ErbB2/ErbB4 heterodimers. By co-immunoprecipitation, ErbB3 or ErbB4 was detected in ErbB2 precipitants from co-transfected NIH3T3 cells, indicating that heterodimers were formed in the co-transfected NIH3T3 cells. Using immunoprecipitation–recapture, each ErbB receptor was purified from heterodimers to examine its phosphorylation status. After NRG-1 stimulation, the phosphorylation of ErbB3, but not ErbB2, was up-regulated in ErbB2/ErbB3 transfectants. In addition, an intracellular domain-truncated form of ErbB3 suppressed the phosphorylation of ErbB2. The phosphorylations of ErbB2 and ErbB4 were both up-regulated by NRG-1 in ErbB2/ErbB4 co-transfected NIH3T3 cells. However, if a kinase-impaired ErbB4 mutant (ErbB4D 843N) was used in the co-transfection experiment, ErbB4D 843N, but not ErbB2, was *trans*-phosphorylated. Therefore, these results suggest the existence of a *trans*-phosphorylation mechanism involved in the activation of ErbB2/ErbB3 or ErbB2/ErbB4 heterodimers. Unlike in ErbB2/ErbB3, in which NRG-1 does not result in ErbB2 phosphorylation, *trans*-phosphorylation of ErbB2 in ErbB2/ErbB4 heterodimers may thus lead to distinct signal pathways.

2. Materials and methods

2.1. Materials

NIH3T3 cell line was purchased from American Type Culture Collection. LipofectAMINE™ transfection reagent was obtained from Life Technologies, Inc. NRG-1 was purchased from Neo Markers. The Cross-linking reagent BS^3 was obtained from PIERCE Chemical Company. The ErbB2 expression plasmid pRC/CMV-ErbB2 encoding a full length human ErbB2 cDNA and ErbB3 and 4 expression plasmid pCMVneo-ErbB3 and 4 encoding a full-length human ErbB3 and 4 cDNAs were kindly provided by Drs Rodney Fiddes and Roger Daly (The Garvan Institute of Medical Research, Darlinghurst, NSW 2010, Australia). ErbB3 C-terminal deletion mutants (ErbB3-640 and ErbB3-676) were constructed by PCR using above ErbB-3 cDNA plasmid as templates with a T7 promoter primer from vector sequence (5′-TAATACGACTCACTATAGGGAGA), thus a XbaI site was included from the polylinker sequence for subsequent cloning, the primer ErbB3-640 for the transmembrane and intracellular domain-deleted mutant (5′-CGAT***CTCGAG***GTTTTGCCGATCAGC) and ErbB3-676 for the intracellular domain-deleted mutant (5′-CGATCTCGAGCCCTTTTATTCTG.). The amplified gene fragments were sequenced and cloned into pCMVneo at XbaI and XhoI for expression. Primers for ErbB4 mutant (ErbB4*) used were 5′-TCGTT CATCGG*A*ATTTGGCAGCCCGTAATG and 5′-CATTACGGGCTGCC AAAT*T*CCGATGAACGA for the mutant of gene (G2560A) or protein (D843N). The site-directed mutagenesis followed the instruction manual of STRATAGENE. Antibodies recognizing ErbB2 (NCL-CB11 and NCL-PC11) were purchased from Novocastra Laboratories, Ltd. Anti-ErbB3 was purchased from Santa Cruz Biotechnology. Anti-phosphotyrosine (Recombinant RC20: HRPO) was purchased from Transduction Laboratories. Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) reagents were purchased from Amersham Bioscience.

2.2. Cell culture and transient transfections

NIH3T3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, GIBCOBRL) supplemented with 10% fetal bovine serum (FBS, GIBCOBRL) and the selective antibiotics at 37 °C in a 5% CO_2 atmosphere. NIH3T3 cells were transfected with the ErbB2, ErbB3, ErbB4 and ErbB4D843N cDNA either individually or in combination using the LipofectAMINE™ reagent according to manufacturer's instructions (GIBCOBRL).

2.3. Immunoprecipitation and immunoblot analysis

For analysis of ErbB receptors, transfected cells ($1–2\times10^6$) were washed with cold PBS and solubilized in 1 mL of lysis buffer (*i.e.*50 mM Tris, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Boehringer Roche) on ice. The lysates were incubated with protein A (Sigma) or protein G (Amersham Pharmacia Biotech.) sepharose complexes at 4 °C for 1 h on an orbital rotor before being clarified by centrifugation at 12,000 rpm for 15 min. For immunoprecipitations, cell lysates were incubated with specific antibodies and protein A (or protein G) for 3 h on an orbital rotor at 4 °C. The immunocomplexes were spun at 4000 rpm for 3 min and washed four times with ice-cold lysis buffer. Cell lysates or immunoprecipitated proteins were solubilized by boiling in SDS-PAGE sample buffer for 5 min and were subjected to SDS-PAGE. The proteins were transferred to PVDF membranes (Millipore, Bedford, NY). After blocking with 5% skim milk in PBS with 0.02% Tween 20 at 4 °C for 1 h or overnight, the membranes were probed with primary antibodies followed by secondary antibodies, each for 1 h at room temperature. The proteins were visualized by the ECL detection system. For reprobing, the blotted membranes were stripped with 1% SDS, 0.2 M Tris (pH 8.0) by shaking at RT for 2 h, and then were reprobed with respective antibodies. The immunoprecipitation–recapture followed the protocol in *Current Protocols in Molecular Biology* (1999).

2.4. Chemical cross-linking assay

Before cross-linking and immunoprecipitation assays, the transfected cells were starved overnight in serum-free DMEM. The following day, they were

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