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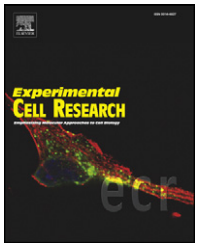

Research Article

Eps8 involvement in neuregulin1-ErbB4 mediated migration in the neuronal progenitor cell line ST14A

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ABSTRACT

Stable expression of the tyrosine kinase receptor ErbB4 confers increased migratory behavior to the neuronal progenitor cell line ST14A, in response to neuregulin 1 (NRG1) stimulation. We used gene expression profiling analysis to identify transcriptional changes associated with higher migratory activity caused by the activation of a specific ErbB4 isoform, and found constitutive up-regulation of the epidermal growth factor receptor pathway substrate 8 (Eps8), a multimodular regulator of actin dynamics.

We confirmed the increase of Eps8, both at the mRNA and at the protein level, in stable clones expressing two different ErbB4 isoforms, both characterized by high migratory activity.

Using Transwell assays and experimental manipulation of Eps8 expression level, we demonstrated that Eps8 synergizes with ErbB4 to increase both basal and ligand induced cell migration, whereas siRNA mediated Eps8 silencing strongly impairs cell motility and NRG1 induced actin cytoskeleton remodeling.

By transient knockdown of Eps8 through *in vivo* siRNA electroporation, followed by explant primary cultures, we demonstrated that Eps8 down-regulation affects migration of normal neuronal precursors.

In conclusion, our data demonstrate that Eps8 is a key regulator of motility of neuronal progenitor cells expressing ErbB4, both in basal conditions and in response to external motogenic cues.

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Abbreviations: ADAM 17, a disintegrin and metalloproteinase; EGFR, epidermal growth factor receptor; Eps8, epidermal growth factor receptor pathway substrate 8; GO, gene ontology; HGF/SF, hepatocyte growth factor/scatter factor; NRG1, neuregulin 1; OB, olfactory bulb; PI3K, phosphatidylinositol 3-kinase; qRT-PCR, quantitative real time RT-PCR; RMS, rostral migratory stream; SVZ, subventricular zone; TACE, tumor-necrosis-factor-α-converting enzyme

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Introduction

ErbB4 is a tyrosine kinase receptor belonging to the epidermal growth factor receptor (EGFR/ErbB1) family, involved in regulating neuronal migration [1–3]. This protein, like the other members of the EGFR family, can form homo- and hetero-dimers and presents an extracellular domain for interaction with the ligands, a single transmembrane region and a cytoplasmic domain that possesses tyrosine kinase activity [4]. The ErbB4 gene is characterized by two alternative splicing, producing four different isoforms. One isoform pair is characterized by alternative splicing of exons located in the extracellular juxtamembrane region, conferring susceptibility (JMa) or not (JMb) to proteolytic cleavage [5] by the metalloproteinase TACE ("tumor-necrosis-factor-α-converting enzyme") also known as ADAM 17 ("a disintegrin and metalloproteinase"). ErbB4 ectodomain shedding triggers a second presenilin-dependent γ-secretase cleavage, which releases from the membrane a cytoplasmic ErbB4 domain, that translocates into the nucleus and regulates transcription [6–13]. The other ErbB4 isoform pair is characterized by the presence (cyt1) or absence (cyt2) of a cytoplasmic exon containing a docking site for phosphatidylinositol 3-kinase (PI3K) [14,15] and for WW-domain containing ubiquitin ligases involved in ubiquitination and degradation of the receptor [16–19].

Two of the four ErbB4 isoforms (JMa-cyt2 and JMb-cyt1) confer, upon neuregulin 1 (NRG1) stimulation, a high migratory activity to the neuronal progenitor cell line ST14A [20,21].

We show here that in stable ST14A clones expressing these two ErbB4 isoforms, Eps8 ("EGFR pathway substrate 8") mRNA and protein are up-regulated. Eps8 has been identified among proteins phosphorylated on tyrosine following EGFR activation [22]; Eps8 exists in two isoforms, of 97- and 68-kDa [22], and is involved in regulation of actin dynamics. The actin cytoskeleton participates in many cellular processes including regulation of cell shape, motility and adhesion. Remodeling of the actin cytoskeleton is dependent on actin binding proteins, which organize actin filaments into specific structures that allow them to perform specialized functions. Eps8 is involved in actin regulation in at least three different ways: 1) forming a complex with Abi1, p85 (the regulatory subunit of phosphatidylinositol-3-kinase, PI3K) and the guanine exchange factor Sos-1, promoting the activation of Rac [23]; 2) capping the barbed-end of actin [24]; and 3) bundling actin filaments [25].

In this study we analyzed the involvement of Eps8 in the ErbB4 mediated migration in a cellular model of neuronal progenitors.

Materials and methods

Cell cultures and reagents

All chemicals and reagents were purchased from Sigma (St. Louis, MO), unless otherwise stated. The ST14A cell line (kindly provided by Prof. Elena Cattaneo) was derived from primary cells dissociated from embryonic day 14 rat striatal primordia as previously described [20]. Cells were cultured on dishes (BD Biosciences, San Jose, CA, USA) in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 1 mM sodium pyruvate, 2 mM L-glutamine and supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin and 10% fetal bovine serum (FBS, Gibco®, Carlsbad, CA, USA) inactivated for 30 min at 56 °C (or 2% FBS or serum free medium for starvation before assays, as described below). Stable transfectants were grown in medium containing 5 μg/ml puromycin. Cells were grown as monolayers at the permissive temperature of 33 °C in a 5% CO_2 atmosphere saturated with H_2O. Cells were allowed to grow to near-confluence, and adherent cells were harvested by the trypsin/EDTA method. For ligand stimulation experiments, 12.5 ng/ml hepatocyte growth factor/scatter factor (HGF/SF, Sigma) or 5 nM recombinant NRG1β1 (henceforth called NRG1) were used; the EGF-like domain of mouse NRG1 has been produced in the laboratory as a His-tag fusion protein in *E. coli* [26]. For experiments with PI3 kinase or γ-secretase inhibitors, stable clones B1.15 and A2.1 were grown in 10% FBS DMEM until 60% confluence. Then the serum was lowered to 2% and cells were treated for 24, 48, 72, and 96 h with 10 μM LY294002 (Calbiochem), or with 10 μM DAPT (γ-secretase inhibitor compound IX, Calbiochem). As a mock control, cells were treated with an equal volume of solvent (DMSO). The medium containing inhibitors or DMSO was replaced every 48 h.

RNA extraction from ST14A cells

Total RNA was prepared using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Microarray

We first optimized the conditions for a migratory response: starting from the standard Transwell assay conditions (2% serum, 5 nM NRG1, 18 h) we performed time course experiments to determine the optimal stimulation time and growth conditions. We could detect a specific migratory response to NRG1 already after 12 h of stimulation and we hypothesized that transcriptional regulation of effectors involved in the regulation of cell migration would be detectable after this time interval. RNA was extracted from NRG1 stimulated stable clone A2.12 and mock ST14A cells. Total RNA was amplified and labeled using Ambion Message Amp I. Cy3- and Cy5-labeled RNAs were hybridized with a dye-swap replication scheme on Whole Rat Genome Microarray (Agilent Technologies, Santa Clara, CA, USA) containing 41,000 unique probes that represent 21,337 well-known rat genes and transcripts, including homologues to human and mouse genes. Arrays were scanned with the Agilent B scanner and image files were loaded into Feature Extraction software version 7.6 (Agilent Technologies) to obtain raw data. Raw data files were then loaded onto the Resolver SE System (Rosetta Biosoftware, Seattle, WA, USA) to perform data processing and normalization using the platform-specific error model. The two replicated ratio profiles were combined, associating each gene to an expression fold-change and a p-value to assess the statistical significance of its modulation in the A2.12 ST14A sample compared with the mock one, both following stimulation with 5 nM NRG1 for 12 h. RNA quality was checked by Agilent 2100 Bioanalyzer (Agilent Technologies). Concentrations and labeling were also checked by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Microarray data analysis

A gene ontology (GO) enrichment analysis with Database for Annotation, Visualization and Integrated Discovery software (DAVID, http://david.abcc.ncifcrf.gov/) was performed to identify enrichment clusters in which highly related genes are bunched in functional