Neuroscience 162 (2009) 1106–1119

THE RETINOIC ACID INDUCIBLE Cas-FAMILY SIGNALING PROTEIN Nedd9 REGULATES NEURAL CREST CELL MIGRATION BY MODULATING ADHESION AND ACTIN DYNAMICS

J. B. AQUINO,[a1,2] F. LALLEMEND,[a2] F. MARMIGÈRE,[a] I. I. ADAMEYKO,[a] E. A. GOLEMIS[b] AND P. ERNFORS[a]*

[a]*Unit of Molecular Neurobiology-MBB, Karolinska Institutet, Scheeles vag 1 A1:2, 171 77 Stockholm, Sweden*

[b]*Division of Basic Science, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA*

Abstract—Cell migration is essential for the development of numerous structures derived from embryonic neural crest cells (NCCs), however the underlying molecular mechanisms are incompletely understood. NCCs migrate long distances in the embryo and contribute to many different cell types, including peripheral neurons, glia and pigment cells. In the present work we report expression of Nedd9, a scaffolding protein within the integrin signaling pathway, in non-lineage-restricted neural crest progenitor cells. In particular, Nedd9 was found to be expressed in the dorsal neural tube at the time of neural crest delamination and in early migrating NCCs. To analyze the role of Nedd9 in neural crest development we performed loss- and gain-of-function experiments and examined the subsequent effects on delamination and migration *in vitro* and *in vivo*. Our results demonstrate that loss of Nedd9 activity in chick NCCs perturbs cell spreading and the density of focal complexes and actin filaments, properties known to depend on integrins. Moreover, a siRNA dose-dependent decrease in Nedd9 activity results in a graded reduction of NCC's migratory distance while forced overexpression increases it. Retinoic acid (RA) was found to regulate Nedd9 expression in NCCs. Our results demonstrate *in vivo* that Nedd9 promotes the migration of NCCs in a graded manner and suggest a role for RA in the control of Nedd9 expression levels.

Key words: HEF1, Cas-L, cytoskeleton, focal adhesion, multipotency, RA.

The emergence of vertebrates and their success can, in part, be attributed to the appearance of the neural crest which provides the embryological basis for the formation of vertebrate-specific features such as cranial skeleton, myelinated nerves, peripheral neurons and skin pigmentation (Baker and Bronner-Fraser, 1997; Glenn, 2005; Northcutt and Gans, 1983). The most prominent feature of neural crest cells (NCCs) is their high motility which makes them able to migrate extensively and populate tissues distant to their site of origin (Bronner-Fraser, 1995). While detaching from the dorsal neural tube—a process called delamination—NCCs adopt a mesenchymal morphology which allows them to invade the underlying mesoderm. Conversion of NCCs from a non-motile into a motile state requires interactions with various extracellular matrix (ECM) components and the acquisition of specific competences to properly sense these cues. While a number of factors regulating migratory properties of NCCs have been identified, the molecular framework that governs the movement of NCCs *in vivo* is just beginning to emerge.

Cell migration involves a concerted action of different mechanical forces within the cell. Cell adhesion to a substrate is necessary for cell spreading and motility and it requires the assembly of small focal contacts which link the actin cytoskeleton to the ECM (Huttenlocher et al., 1996; Small and Kaverina, 2003). Such focal adhesions and focal complexes are believed to provide the adhesive force necessary for traction at the leading edge of migrating cells. Integrin receptors are found clustered at focal adhesions and recruit a large complex of signaling molecules responsible for regulating actin dynamics and cell motility (Brunton et al., 2004). Numerous studies have demonstrated that the ECM functions as a scaffold onto which the NCCs migrate and that integrins play a prominent role in this process (Perris and Perissinotto, 2000). *In vitro* integrin signaling has been found to play a significant role in NCC spreading and migration onto a variety of ECM components (Desban and Duband, 1997; Erickson and Perris, 1993). Other studies have demonstrated that interfering with the function of integrins or their ligands, including laminin, is sufficient to perturb migration of NCCs (Bronner-Fraser, 1985; Lallier and Bronner-Fraser, 1993; Kil et al., 1996; Strachan and Condic, 2004, 2008; Coles et al., 2006). Nedd9/CasL/HEF1 (hereafter referred to as neural precursor cell expressed, developmentally downregulated 9, Nedd9) is a scaffolding protein of the Cas family (O'Neill et al., 2000). Nedd9 is a member of the β1-integrin signaling pathway found to be necessary for glioblastoma and Jurkat T cell motility (Natarajan et al., 2006; O'Neill et al., 2000; van Seventer et al., 2001). Investigations on other Cas family members suggest that this class of adaptor proteins might serve as key bridges for the assembly of actin–cytoskeleton signaling complexes (O'Neill et al., 2000). In the present work we investigated the mechanisms through which NCC migration is regulated. Nedd9 was identified by se-

[1] Present address: School of Medicine, Austral University, Av. Perón 1500, B1629AHJ Derqui-Pilar, Buenos Aires, CONICET, Argentina.
[2] These authors have equally contributed to this work.
*Corresponding author. Tel: +46-852487659; fax: +46-8341960.
E-mail address: Patrik.Ernfors@ki.se (P. Ernfors).
Abbreviations: bNCSC, boundary cap neural crest stem cells; DRG, dorsal root ganglion; ECM, extracellular matrix; EGFP, enhanced green fluorescent protein; HHst, Hamburger/Hamilton stage; ISH, *in situ* hybridization; NCC, neural crest cell; OD, optic density; PDL, poly-D-lysine; RA, retinoic acid; siRNA, small interfering RNA.

0306-4522/09 $ - see front matter
doi:10.1016/j.neuroscience.2009.05.035

quencing of mRNAs expressed in the early dorsal root ganglia (DRG) and their expression in migratory NCCs prompted further studies. Our results suggest that the motile capacity of multipotent NCCs is governed by an RA-controlled dynamic regulation of Nedd9 levels which influence cell adhesion and cytoskeleton properties.

EXPERIMENTAL PROCEDURES

All experimental procedures were approved by the Swedish National Board for Laboratory Animals (N17/2003, N404/2003 and N293/05). All experiments conformed to European guidelines on the ethical use of animals. The number of animals used as well as their suffering were minimized.

In situ hybridization (ISH)

Embryos were fixed in 4% paraformaldehyde/PBS and sectioned at 14-μm thickness. The mouse probe (498 bp) was amplified from mouse cDNA using the following primers: forward 5′-CCCAACAGCATCATGAACTCA-3′; reverse 5′-CTGAGCTGACGCAG CTGAA-3′, corresponding to nucleotide 1705 to 2203 in the mouse Nedd9 CDS. The chick probe was amplified from chick cDNA using the following primers, forward 5′-ATGAAGTACAAGAATCTTATGGCAA-3′; reverse 5′-GTCTTTTGTCCTGTGTAG TTGA-3′, corresponding to nucleotide 1–1191 in the chick Nedd9 CDS. Plasmids containing specific probes for mouse or chicken Nedd9 were used to synthesize digoxigenin-labeled antisense riboprobes according to the supplier's instructions (Roche Molecular Biochemicals, Mannheim, Germany). ISH was performed as previously described (Hjerling-Leffler et al., 2005).

Immunohistochemistry and immunocytochemistry

Cryoprotected tissue was sectioned at 14-μm thickness and mounted on slides. Sections were processed as previously described (Aquino et al., 2006). Primary antibodies specific to β-III-tubulin (TuJ-1, mouse, IgG, 1 μg/ml, Promega Biotech AB, Sweden), Brn3a (mouse, IgG, 1:50, Chemicon, Millipore AB, Solna, Sweden), HEF1 (Nedd9, 2G9, mouse, IgG, 1 μg/ml for mouse tissue and 2 μg/ml for chicken, Abcam, Cambridge, UK), Islet1 (39.4D5, mouse, IgG, 11 μg/ml, Developmental Studies Hybridoma Bank, DSHB, Iowa City, Iowa, USA), Sox2 (rabbit, IgG, 1:3000, from Dr. Thomas Edlund, Umeå University, Sweden), Sox10 (guinea pig, IgG, 1:1000, from Dr. Michael Wegner, University of Erlangen, Germany) and paxillin (mouse, IgG, 5.6 μg/ml, Upstate and mouse, IgG, 2.5 μg/ml, clone 349, BD Transduction Laboratories, Stockholm, Sweden) were used. The secondary antibodies were Cy3, Cy2 and Cy5-conjugated (Jackson ImmunoResearch, Suffolk, UK). Immunocytochemistry was performed following previously published protocols (Aquino et al., 2006). For phalloidin staining: cells were permeabilized with 0.1% Triton-X 100 (Merck, Whitehouse Station, NJ, USA; 10 min), washed and incubated with Alexa Fluor 546 or Alexa Fluor 647–phalloidin (Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA; 1:50 in PBS, 2 h, room temperature). Pictures were taken using a Zeiss LSM 510 confocal microscope. Measurements were obtained from pictures, taken with same settings from sections that were simultaneously processed for immunohistochemistry (IHC). For quantification purposes, 4.7 μm optical slice pictures (using an air 20× objective) were used. Only cells sectioned across their nuclei were included in the analyses. Optic density (OD) measurements were obtained using the ImageJ software (NIH) as follows: individual cells were carefully scanned, at least twice, by manually moving the cursor over the whole cell surface and maximum score obtained was registered for quantification. For *in vivo* cell counting, sections were first stained for Sox10 and Isl1 and the number of $EGFP^+$ cells was determined by counting the number of nuclei which were either $Sox10^+$ or $Isl1^+$. For this purpose, at least one slide with several serial sections (every 5th section taken) per embryo was used and only sections through the core of migrating cells/dorsal root ganglion (DRG) were considered for quantification. Criteria of inclusion of border DRG cells were as followed: $Sox10^+$ cells located within a two-cell-thick layer at the periphery of the DRG.

In ovo electroporation

For Nedd9 overexpression experiments, cDNAs encoding mouse Nedd9 (Law et al., 1998) and enhanced green fluorescent protein (EGFP) were separately sub-cloned into an expression vector driven by the chick actin promoter (pCA). Constructs were injected into the neural tube of either Hamburger/Hamilton stage (HHst) 10-11 or HHst13-14 chick embryos following published procedures (Marmigere et al., 2006).

For loss-of-function experiments, naked siRNA constructs (1.5 μg/μl) were electroporated in combination with the EGFP expression vector (0.5 μg/μl). The siRNA sequences were as follows: Nedd9 siRNA 1, 5′-UCAACCAACAGAAGAUCUAUCAAGUGC-3′ (MWG Biotech); and Nedd9 siRNA 2, 5′-AUUUAAGGGACUAAGCAUACCCUCC-3′ (Invitrogen); corresponding to Nedd9 coding sequence (from NCBI database, accession # AF104246.1) starting from nucleotides 261 and 332, respectively. As control experiments, either EGFP alone (0.5 μg/μl) or in combination with a control (scramble) siRNA (1.5 μg/μl; sequence: 5′-UCAACCAACAGAAGA UCUAUCAAGUGC-3′; Invitrogen) was used. E3.5: $n=5$ Nedd9 siRNA 1.5 μg/μl and control; $n=3$ Nedd9 siRNA 0.5 μg/μl. E4: $n=3$. E5: $n=4$. In Nedd9 siRNA plus mouse Nedd9 overexpression experiment, both the construct and siRNA (1.5 μg/μl each) together with the EGFP construct (0.5 μg/μl) were co-electroporated ($n=4$); as control condition pCA empty vector (1.5 μg/μl) was used instead of mouse Nedd9 full-length ($n=4$). For quantifications, only sections showing similar pattern and intensity of EGFP expression in the neural tube including the roof plate, were considered. Around 20 sections were analyzed for each experimental condition. Electroporation efficiency was 15% (measured in EGFP-electroporated E4 chicken as proportion of targeted cells within the DRG).

Chick neural tube explants

Chick E3 (24 h after electroporation) neural tubes between, but not including, forelimbs and hind limbs were dissected, cut into two to three somite long pieces and cleaned of somites. Neural tube explants were individually plated on 24-well plates coated with PDL and fibronectin (20 μg/ml, Sigma) in N2 medium supplemented with B27, BDNF (50 ng/ml), bFGF (40 ng/ml) and chicken embryo extract (0.5%, a generous gift from Dr. Hannu Sariola, University of Helsinky, Finland).

After 16–24 h, as indicated, cultures were either fixed or processed for NCC dissociation. After dissociation, NCCs were replated at 3000 cells/well, in 24-well plates on coverslips coated with either PDL and laminin (10 μg/ml, $n=3$ wells), PDL alone or heat-denatured laminin (10 μg/ml, 5′ at 99 °C, $n=3$ wells/condition) in a medium containing N2, B27 and all-trans retinoic acid (RA) (5 ng/ml). After 4 h, cultures were fixed and phalloidin or paxillin stained. A cell was considered to have spread when a flattened morphology and at least one process larger than its soma diameter was noted. The spreading area (or whole cell area) of EGFP co-transfected individual cells was measured using the EGFP signal: for this purpose the threshold signal was increased to cover the whole cell surface (ImageJ software). For value calculations, the average area score obtained from non-spread cells plated in PDL and denatured laminin was subtracted from individual cell area values.

Density measurements of phalloidin and paxillin stainings were calculated by obtaining from confocal images (using 60× objectives; phalloidin: stack projections from five 1 μm optic sections; paxillin: single 1.25 μm thick optic sections) the immunostained area value of individual cells and dividing it by total cell