Biochemical and Biophysical Research Communications 398 (2010) 79–85

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Extracellular Nm23H1 stimulates neurite outgrowth from dorsal root ganglia neurons *in vitro* independently of nerve growth factor supplementation or its nucleoside diphosphate kinase activity

K.T. Wright [a], R. Seabright [b], A. Logan [b], A.J. Lilly [c], F. Khanim [c], C.M. Bunce [c], W.E.B. Johnson [d,*]

[a] Keele University at the RJAH Orthopaedic Hospital, Oswestry, Shropshire, UK
[b] Neuropharmacology and Neurobiology, School of Clinical and Experimental Medicine, Birmingham University, Birmingham, UK
[c] Biosciences, Birmingham University, Birmingham, UK
[d] Life and Health Sciences, Aston University, Birmingham, UK

ARTICLE INFO

*Article history:*
Received 7 June 2010
Available online 15 June 2010

*Keywords:*
Nm23H1
Nerve growth
Nucleoside diphosphate kinase
Extracellular activity

ABSTRACT

The nucleoside diphosphate (NDP) kinase, *Nm23H1*, is a highly expressed during neuronal development, whilst induced over-expression in neuronal cells results in increased neurite outgrowth. Extracellular Nm23H1 affects the survival, proliferation and differentiation of non-neuronal cells. Therefore, this study has examined whether extracellular Nm23H1 regulates nerve growth. We have immobilised recombinant Nm23H1 proteins to defined locations of culture plates, which were then seeded with explants of embryonic chick dorsal root ganglia (DRG) or dissociated adult rat DRG neurons. The substratum-bound extracellular Nm23H1 was stimulatory for neurite outgrowth from chick DRG explants in a concentration-dependent manner. On high concentrations of Nm23H1, chick DRG neurite outgrowth was extensive and effectively limited to the location of the Nm23H1, i.e. neuronal growth cones turned away from adjacent collagen-coated substrata. Nm23H1-coated substrata also significantly enhanced rat DRG neuronal cell adhesion and neurite outgrowth in comparison to collagen-coated substrata. These effects were independent of NGF supplementation. Recombinant Nm23H1 (H118F), which does not possess NDP kinase activity, exhibited the same activity as the wild-type protein. Hence, a novel neuro-stimulatory activity for extracellular Nm23H1 has been identified *in vitro*, which may function in developing neuronal systems.



## 1. Introduction

Nm23H1 belongs to a family of multifunctional, nucleoside diphosphate (NDP) kinases with roles relating to signal transduction, gene expression, embryonic development, tumour progression and cell migration [1]. Early studies demonstrating an inverse correlation of *Nm23H1* expression with the aggression of a variety of tumours, e.g. breast cancer and malignant melanoma [2,3], together with inhibited tumour cell migration and metastasis following exogenous *Nm23H1* over-expression [4,5], account for its naming as Nm (non metastatic) 23H1 [6]. However, evidence suggests that Nm23H1 also plays a physiological role in the regulation of nerve growth. During mouse embryogenesis, *Nm23M1* (the murine orthologue) is expressed at high levels in central and peripheral neural tissues, including the brain, spinal cord and spinal and cranial ganglia [7]. In addition, *Nm23M1* transfection of PC12 cells, an established model of neuronal differentiation, was associated with increased neurite outgrowth, whilst anti-sense *Nm23M1* transfection inhibited neurite outgrowth [8]. Transfection of murine N1E-115 neuroblastoma cells with human *DR-Nm23*, another member of the NDP kinase family with 70% homology to *Nm23H1*, similarly enhanced neurite outgrowth [9].

Extracellular Nm23H1 is present in body fluids, where its protein levels in blood were correlated with poor prognosis in acute myelogenous leukemia (AML), malignant lymphoma and neuroblastoma [10–12]. The presence of Nm23H1 in blood may relate to lysis of effete erythrocytes [13] or of tumour cells themselves [14], but may also arise through secretion as Nm23 proteins have been detected in conditioned culture medium from established, viable tumour cell lines [15,16]. Furthermore, supplementing culture medium with recombinant Nm23H1 alters the growth and survival of normal hematopoietic cells, AML cells and embryonic stem cells [14,17–19]. Therefore, evidence suggests that extracellular Nm23H1 may regulate cell activity. Here, we have used established *in vitro* protocols to examine the effects of extracellular recombinant Nm23H1 on nerve growth.

* Corresponding author. Address: Life and Health Sciences, Aston University, Birmingham B4 7ET, UK. Fax: +44 (0)121 204 3696.
*E-mail address:* w.e.johnson@aston.ac.uk (W.E.B. Johnson).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.06.039

## 2. Materials and methods

### 2.1. Explant cultures of embryonic chick dorsal root ganglia (DRG)

Embryonic day 10 (E10) chick DRG explants were prepared according to previously described methods [20] and seeded into culture plates pre-coated with recombinant Nm23H1 or collagen type I (prepared as described below). The DRG explants were cultured in DMEM/F12 culture medium, supplemented with insulin, transferrin, and selenium (Invitrogen, Paisley, UK), non essential amino acids (Invitrogen) and with or without 50 ng/ml nerve growth factor (NGF, Sigma, Poole, UK).

### 2.2. Dissociated adult rat DRG cultures

Dissociated DRG neurons were prepared from adult male Sprague Dawley rats, as described previously [21]. About $2 \times 10^3$ cells per well were seeded into culture plates pre-coated with recombinant Nm23H1 and collagen type I and maintained in Neurobasal A medium (Invitrogen Life Technologies) supplemented with or without 50 ng/ml NGF (Sigma).

### 2.3. Preparation of recombinant wild type (WT) Nm23H1 and mutant Nm23H1 (H118F)

The Nm23H1 his-tagged clone was kindly provided by Dr. Patricia Steeg (Bethesda, MD). The clone was transformed into *E. coli* BL21 (DE3) cells (Novagen, CN Biosciences UK Ltd., Nottingham, UK) according to the manufacturer's instructions. Bacterial cultures for induction were prepared by inoculation of L-Broth media, containing 100 μg/mL Ampicillin (Sigma), grown to an $OD_{600}$ of 0.6. Recombinant Nm23H1 protein expression was induced with 1 mM IPTG (Sigma) and prepared by the Bugbuster Ni–NTA His Bind Purification Kit (Novagen). To generate the mutant form of Nm23H1 (H118F), which is NDP kinase inactive [22], complementary oligonucleotides containing the histidine to phenylalanine mutation at position 118 were synthesised such that:

3′-TTGGCAGGAACATTATACATGGCAGTGATTCTGTGGAGAGTGC-5′

3′-TTGGCAGGAATTTTATACATGGCAGTGATTCTGTGGAGAGTGC-5′

These oligonucleotides were used with the Qiagen Quikchange XL site-directed mutagenesis kit (Qiagen, Crawley, UK). The integrity of the mutant cDNAs was verified by base sequencing. The mutated plasmid was transformed into XL10-Gold ultra competent cells (Strategene, Leicester, UK) and the recombinant mutant protein produced as for the WT Nm23H1. The Kinase-Glo® Luminescent Kinase Assay (Promega, Madison, WI, USA) with substrates of ATP and UDP was used to confirm the NDP kinase activity and inactivity of recombinant WT Nm23H1 and Nm23H1 (H118), respectively (data not shown).

### 2.4. Coating of culture plates with recombinant Nm23H1

A choice assay of culture substrata has been used extensively to model the interaction of neurons with immobilised ECM molecules present within developing and injured neural systems [21,23,24]. This assay was adapted to examine the response of neurons to immobilised extracellular recombinant Nm23H1 proteins as follows: culture plates were pre-coated with a thin layer of protein-binding nitrocellulose (BA85, Schleicher and Schuell, Dassel, Germany), which was then blotted with strips of filter paper presoaked in recombinant WT Nm23H1 or Nm23H1 (H118F) proteins at various concentrations (10–1000 μg/ml). After the filter strips had dried to completion, they were removed and the plates were coated with a solution of 100 μg/ml of collagen type I (in phosphate buffered saline, PBS; both Sigma) for 5 min, and washed in PBS prior to seeding with chick DRG explants or rat DRG neurons. The immobilisation of the recombinant Nm23H1 on the culture plates was routinely visualized by inclusion of 10% (v/v) rhodamine B (Sigma) in the Nm23H1 solutions and confirmed in separate plates by Nm23H1 immunoblotting (data not shown). In separate experiments, nitrocellulose-coated culture plates were uniformly coated with solutions of laminin (40 μg/ml, Sigma) or Nm23H1 (800 μg/ml) prior to seeding with chick DRG explants in NGF-supplemented culture medium. After 48 h, the culture medium was then further supplemented with the JG22 monoclonal antibody (1/200 final concentration, Developmental Studies Hybridoma Bank, University of Iowa, USA) or with an irrelevant negative control antibody. Treatment of chick DRG neurons with the JG22 antibody has been demonstrated to specifically block neuronal integrins binding to the Asp-Gly-Asp (RGD) motif of laminin [25,26]. The extent of continued outgrowth of DRG neurites on these uniform substrata in the presence of the JG22 function blocking antibody versus control was then monitored for a further 24 h.

### 2.5. Treatment of chick DRG explants with soluble Nm23H1

E10 chick DRG explants were seeded in culture plates uniformly coated with 100 μg/ml collagen type I and then treated with 20–200 μg/ml of soluble WT Nm23H1 in serum free culture medium without NGF supplementation. Control cultures were set identically in the absence of WT Nm23H1. After 48 h, the DRG neurite outgrowth was quantitated using previously described methods [24].

### 2.6. Beta-III tubulin immunocytochemistry

Rat DRG neurons were 10% formalin-fixed, washed repeatedly in PBS, incubated with a blocking buffer, and then incubated with rabbit anti-rat beta-III tubulin antibodies (Sigma). Control immunolocalisation was performed on parallel cultures omitting the primary antisera only. Immunoreactivity was revealed with a secondary anti-rabbit Ig-Alexa 488 conjugated antibody (Invitrogen). No immunoreactivity was observed when the primary antibodies were omitted (data not shown).

### 2.7. Microscopy, image capture and analysis

Cultures were viewed using phase contrast and fluorescence microscopy (Nikon, Kingston-upon-Thames, UK). Digitized images were captured with a Hamamatsu digital camera (Hamamatsu photonics, Welwyn Garden City, UK) and analysed using IPLab software (Becton Dickinson). Adapting methods previously described [23,24], the digitised images were used to quantitate (i) the number of beta-III tubulin-immunopositive rat DRG neuronal bodies present on substrata of recombinant Nm23H1 proteins versus an equivalent area of adjacent collagen; (ii) the proportion of those DRG neuronal bodies that extended neurites, i.e. processes greater than 10 μ in length. For time-lapse microscopy, digitized images were captured using a digital video camera (JVC, London, UK) and converted into video files using Media Studio Video Editor (Ulead Systems, Karst, Germany).

### 2.8. Statistical analysis

Experiments were performed independently at least three times. Data derived from images of rat DRG neurons were pooled and analysed using the non-parametric Mann Whitney *U* test. Data on chick DRG neurite length in the presence of soluble Nm23H1 versus control were pooled and analysed using the Mann Whitney *U* test. The effects of supplementing culture medium with Nm23H1 in solution were examined using a non-parametric analysis of variance, the Kruskal–Wallis test. In all figures, levels of significance