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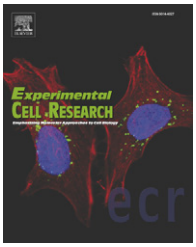

Research Article

NHS-A isoform of the *NHS* gene is a novel interactor of ZO-1

Shiwani Sharma[a,*], *Katrina SY Koh*[b], *Caitlin Collin*[b], *Alpana Dave*[a], *Amy McMellon*[a], *Yuki Sugiyama*[c], *John W. McAvoy*[c], *Anne K. Voss*[b], *Jozef Gécz*[d,e], *Jamie E. Craig*[a]

[a]*Department of Ophthalmology, Flinders University, Bedford Park, SA 5042, Australia*
[b]*Department of Neurobiology, Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia*
[c]*Save Sight Institute, Sydney Eye Hospital, University of Sydney, Sydney 2001, Australia*
[d]*SA Pathology, Women's and Children's Hospital, North Adelaide, SA 5006, Australia*
[e]*School of Paediatrics and Reproductive Health, University of Adelaide, SA 5005, Australia*

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ABSTRACT

Mutations in the *NHS* (*N*ance-*H*oran *S*yndrome) gene lead to severe congenital cataracts, dental defects and sometimes mental retardation. *NHS* encodes two protein isoforms, NHS-A and -1A that display cell-type dependent differential expression and localization. Here we demonstrate that of these two isoforms, the NHS-A isoform associates with the cell membrane in the presence of intercellular contacts and it immunoprecipitates with the tight junction protein ZO-1 in MDCK (Madin Darby Canine Kidney) epithelial cells and in neonatal rat lens. The NHS-1A isoform however is a cytoplasmic protein. Both Nhs isoforms are expressed during mouse development. Immunolabelling of developing mouse with the anti-NHS antibody that detects both isoforms revealed the protein in the developing head including the eye and brain. It was primarily expressed in epithelium including neural epithelium and certain vascular endothelium but only weakly expressed in mesenchymal cells. In the epithelium and vascular endothelium the protein associated with the cell membrane and co-localized with ZO-1, which indirectly indicates expression of the Nhs-A isoform in these structures. Membrane localization of the protein in the lens vesicle similarly supports Nhs-A expression. In conclusion, the NHS-A isoform of *NHS* is a novel interactor of ZO-1 and may have a role at tight junctions. This isoform is important in mammalian development especially of the organs in the head.

Introduction

Mutations in the *NHS* (*N*ance-*H*oran *S*yndrome) gene lead to an X-linked genetic disorder, namely the Nance-Horan syndrome [1]. Characteristic features of the syndrome include severe bilateral congenital cataracts, dental anomalies, dysmorphic facial features and in some cases mental retardation and behavioral disturbance [2–4]. Microphthalmia and microcornea have been reported in some families [5–7]. Cataracts in affected males cause profound vision loss requiring surgery early in life. Heterozygous females display a similar albeit less severe phenotype than affected males [7,8]. Frameshift and nonsense mutations in the *NHS* gene lead to premature truncation of the protein in all affected individuals reported to date [1,9–12].

The *NHS* gene is highly conserved among vertebrates. It is expressed in a spatially and temporally regulated manner during mouse development in organs affected in Nance–Horan syndrome [1]. *In situ* hybridisation revealed its expression in the lens vesicle

* *Corresponding author.* Fax: +61 08 8277 0899.
E-mail address: shiwani.sharma@flinders.edu.au (S. Sharma).

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and in the developing ocular lens suggesting importance of this gene in lens development [13]. The mammalian *NHS* gene encodes two major isoforms, *NHS-A* (named *Nhs1* in mouse) and *NHS-1A* (named *Nhs_v1* in mouse) by using alternate transcription start sites respectively in exon 1 and 1A of the gene [13,14]. The resulting protein isoforms have unique N-termini but identical distal sequence. Neither isoform has any known functional domain or significant homology to a protein with known function. *Ex vivo*, the two isoforms are differentially expressed in a mutually exclusive and cell-type dependent manner [13]. The *NHS-A* isoform is expressed in epithelial and neuronal cells whereas *NHS-1A* expression is specific to fibroblast cells. The *NHS-A* isoform is expressed in the adult human lens, retina and both fetal and adult brain. The NHS-A protein associates with the cell membrane in cultured epithelium as well as in the mammalian lens epithelium. *Ex vivo* in polarized epithelium it co-localizes with the tight junction protein ZO (zonula occludens)-1 at the uppermost portion of the lateral cell membrane [13]. Expression of the *Nhs-1A*/*Nhs_v1* isoform has been reported in the mouse embryonic mouth, paw and eye [14]. The protein localizes in the cytoplasm in cultured fibroblast cells, but its *in vivo* localization is as yet unknown.

An insertion mutation in intron 1 of the *Nhs* gene knocks out the Nhs1/Nhs-A isoform in the *Xcat* mouse and leads to cataract formation [14]. While male *Xcat* mice develop severe bilateral congenital cataracts, the cataract severity in heterozygous females varies from totally clear to totally opaque lens [15]. Hence the *Xcat* mouse is considered a model for Nance–Horan syndrome, although it has not been reported to display any other features of the syndrome observed in humans. As deficiency of the Nhs1/Nhs-A isoform alone is sufficient to cause cataracts in mice, and mutations in the first exon of the *NHS* gene, encoded only in the NHS-A isoform, lead to typical syndromic features in humans [1,10], we hypothesize that this isoform is important in mammalian development particularly in development of the ocular lens.

Here we investigated the function of the NHS-A isoform to gain an insight into its role during development. The presented data show that the ability to associate with the cell membrane is exclusively exhibited by the NHS-A isoform of *NHS*, and is reliant upon the presence of intercellular contacts. Biochemical interaction of this isoform with ZO-1 in epithelial cells and in the lens suggests a role at tight junctions. Membrane-associated distribution of the NHS protein during embryogenesis in epithelial and neuroepithelial cells is indicative of NHS-A expression and is supported by the co-localization data. The present findings implicate a role for NHS-A at tight junctions during development.

Materials and methods

Mammalian cell lines and cell culture

The MDCK (Madin Darby Canine Kidney) cells were kindly provided by Dr. Stephen A Wood, Women's and Children's Health Research Institute, North Adelaide, South Australia, Australia. The HEK (Human Embryonic Kidney) 293A cells were from Qbiogene and rat PC12 cells from the American Type Culture Collection, Virginia, USA.

MDCK and HEK 293A cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin. PC12 cells were grown in DMEM supplemented with 10% horse serum, 5% fetal bovine serum and penicillin/streptomycin. All cell cultures were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

Generation of GFP-fusion constructs

The generation of GFP (green fluorescent protein)-NHS-A fusion construct was described elsewhere [13]. To generate the GFP-NHS-1A fusion construct, the 1.32 kb EcoRI/EcoRV cDNA fragment encoding residues 2–442 of NHS-A was replaced with the 0.79 kb EcoRI/EcoRV fragment encoding residues 2–278 of NHS-1A in the GFP-NHS-A fusion construct.

Western blot analysis of GFP-fusions

3×10^5 HEK 293A cells seeded in 6-well plates were transfected on the following day with GFP-fusion or control plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Approximately 48 h post-transfection the cells were harvested and proteins extracted in lysis buffer (phosphate buffered saline [PBS], 1% Triton X-100, 0.1% SDS, 1 mM EDTA, Protease Inhibitor Cocktail (Roche Diagnostics), phenylmethyl sulfonyl fluoride [PMSF] and 0.1% β-mercaptoethanol). Total soluble proteins were size fractionated by SDS-PAGE and transferred onto Hybond-C Extra (GE Healthcare). Western blot was probed with anti-GFP mouse monoclonal antibody (Roche Diagnostics) (1:500) and sheep anti-mouse Ig-horseradish peroxidase secondary antibody (Chemicon) (1:1000). Antibody binding was detected with ECL Western Blotting System (GE Healthcare).

Localization of GFP-fusions in mammalian cells

3×10^5 MDCK or HEK 293A cells were seeded onto glass coverslips in 6-well plates. Cells were transfected on the following day with GFP-fusion or control plasmid using Lipofectamine 2000 according to the manufacturer's protocol. Approximately 48 and 72 h post-transfection respectively, HEK 293A and MDCK cells were fixed in 4% paraformaldehyde/PBS and mounted on slides in buffered glycerol. Confocal microscopy was performed on an Olympus AX70 microscope attached to a Bio-Rad 1024 MRC scanning confocal system equipped with an Argon Ion and a Helium Neon laser using LaserSharp 2000 software. GFP was excited with 488 nm laser line and detected at 522 nm.

Expression analysis

Total RNA from PC12 cells was extracted using the RNeasy mini kit (Qiagen) following the manufacturer's protocol. E12.5 mouse RNA was prepared by phenol–chloroform extraction. First strand cDNA synthesis from PC12 and E12.5 RNA was performed with Superscript III (Invitrogen) using random hexamers. RT-PCR on PC12 cell cDNA was performed with *Nhs-A* specific primers 5′-TGCAGCCTCTTCCAGGAGCTCGAGA (forward) and 5′-ATTG-GGTTTTTCGGCCTCTGCCCTA (reverse) and HotStar Taq (Qiagen) at 95 °C—15 min; 94 °C—30 s, 60 °C—30 s, 72 °C—30 s for 42 cycles. RT-PCR on E12.5 mouse cDNA was performed with the *Nhs-A* specific primers mentioned above at 95 °C—3 min; 95 °C—30 s, 58 °C—30 s, 68 °C—30 s for 45 cycles; 68 °C—7 min, and with *Nhs-1A* specific primers 5′-TACCGGAATTCTGCTCTGGCCTGCTGCATGCCCAAGAATG