IDENTIFICATION OF A PUTATIVE NA^+/H^+ EXCHANGER IN THE GILL OF THE ATLANTIC HAGFISH, *MYXINE GLUTINOSA*.

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Little is known about branchial ion exchange in hagfishes. In 1984, Evans (*J. Exp. Biol.* 113:465-469,1984) demonstrated a relationship between the extrusion of proton ions and the uptake of sodium ions in the Atlantic hagfish, (*Myxine glutinosa*). Although the exact mechanism for the ion transfer was unclear, he postulated that Na^+/H^+ exchange was occurring across the branchial epithelium. This theory was supported by McDonald *et al.*, (*J. Exp. Biol.* 161:201-215, 1991) who demonstrated that hagfishes were capable of regulating acid-base balance following acid infusion. Given the low urine flow rate of hagfishes, they suggested that the gill was the most likely site of ion exchange. Again, the exact mechanism of exchange remained unknown. The current study aims to identify the Na^+/H^+ exchanger in the gill of the hagfish (*M. glutinosa*) using molecular cloning and immunoblotting techniques.

Total RNA was isolated from *M. glutinosa* gills by the acid-phenol method (Chomczynski & Sacchi., *Analyt. Biochem.* 162:156-159 1987). Single-stranded cDNA was synthesised from total RNA using Superscript®II RNAse H- reverse transcriptase (Life Technologies). Degenerate primers based on highly conserved regions of known NHE sequences were initially designed to target a 673bp fragment (Towle *et al.*, *J. Exp. Biol.* 200:1003-1014, 1997). A 673bp product was ligated into a pCR2.1 plasmid vector (Invitrogen) and was transformed into competent *E.coli* INVαF′ cells. Positive clones were selected by blue/white screening, grown up overnight and plasmids isolated by alkaline lysis. Inserts were sequenced on an Applied Biosystems automated sequencer (Westmead Hospital, Australia). Homologous hagfish oligonucleotide primers based on the 673bp fragment were then designed and used in conjunction with the 3'Rapid Amplification of cDNA Ends kit (Life Technologies). Polymerase chain reaction was carried out following the protocol of Towle *et al.*, (*J. Exp. Biol.*, 200;1003-1014, 1997) the resulting 876bp 3'RACE product was subsequently cloned as described above and sequenced using an Applied biosystems 377 Primer DNA sequencer (Medical College of Georgia) (Fig 1).

Hagfish gill membrane proteins were prepared and blotted as previously described (Choe, K.P. *et al.*, *Bull. MDIBL* 37:38-39, 1998). A monoclonal antibody for mammalian NHE-1 (4E9) reacted with a 97 kDa band and a polyclonal antibody for mammalian NHE-3 (666) bound to a protein of approximately 88kDa (data not shown) indicating the expression of proteins antigenically similar to mammalian Na^+/H^+ exchangers. It is important to note that even though the molecular weights of the proteins detected in the present study approximately correspond to the mammalian transporters (McSwine, R. L. *et al.*, *Am. J. Physiol.* 275:C693-701, 1998; Cox, G. A. *et al.*, *Cell* 91:139-148, 1997), verification *via* antigen competition is required to rule out nonspecific antibody binding. Rabbit polyclonal serum 666 was graciously provided by Dr. Mark Musch at The University of Chicago School of Medicine and Mouse monoclonal antibody 4E9 was provided by Drs. Bliss Forbush and Daniel Biemesderfer at Yale University School of Medicine.

Fig 1. Nucleotide and predicted amino acid sequence of the gill NHE in *Myxine glutinosa.*

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N  D  A  V  T  V  V  L  Y  Q  L  L  S  V  L  A  D  L  P  S  V  P  A  S  S  V  L  L
aatgacgcggtgacggtggtgttgtaccagctgctgtcttgtctggccgatcttccttctgtgcctgcctcttccgtgctgttg
G  V  V  R  F  F  V  V  C  L  G  G  I  A  F  G  I  C  A  G  L  L  A  S  F  T  T  R
ggggtcgtccgcttcttcgttgtctcgctgggtggaattgcgtttggaatctgtgctggcttgcttgcttcatttaccaccagg
F  T  Y  P  P  L  E  P  I  L  I  L  L  T  C  Y  L  A  Y  L  I  T  E  M  L  H  L  S
ttcacatatccagcattggagcctatcctcatcctgctcacctgttacctcgcttarctaatcacagaaatgctccatctctct
G  I  M  A  L  I  S  A  A  L  T  M  R  S  Y  V  D  L  N  L  E  W  R  S  R  T  T  L
gggataatggcgcttatatcggcagctttaacgatgcgttcttacgtggatctgaatctggagtggcgttcccgtacaacccta
R  R  T  L  R  A  L  S  S  T  S  E  T  L  I  F  L  L  L  G  M  A  T  L  D  G  P  H
cgtcgcacacttcgagctctgtcatccaccagtgaaactctcatcttcttgcttttagggatggctacccttgatggaccccat
D  W  S  W  P  F  V  I  S  T  L  I  L  C  L  V  L  R  A  T  G  V  L  I  L  S  W  V
gactggagctggccatttgtcatctcgacattgattttgtgccttgtatggagagcaacaggtgtactgatactctcctgggta
A  N  R  V  R  L  V  P  I  S  Y  K  D  Q  F  I  I  A  Y  G  G  L  R  G  A  I  A  F
gccaaccgtgtgcgtttggtgcccatctcgtacaaagaccaatttattattgcatacggtggtttgaggggcgccatcgccttc
S  L  V  Y  L  I  P  K  V  F  H  H  R  A  L  F  T  T  A  T  I  T  T  L  L  F  T  V
tctctcgtttatctcattccaaaagttttccatcatcgagccctcttcaccactgccacaatcaccactcttctcttcacagtc
F  V  Q  G  M  T  I  P  S  T  G  *  S  L  G  S  E  K  E  M  R  D  *  A  N  S  N  *
tttgtgcagggaatgactataccgtccactggttgatctcttggaagtgaaaaagaaatgcgagactgagccaacagtaactga
R  N  K  H  S  D  I  *  S  S  S  R  W  Y  *  G  H  L  R  P  S  W  S  S  S  L  A  C
agaaataaacactcggatatttgatcatcttctcggtggtattgaggacatttgcggccatcttggtcatcatcactggcttgc
C  V  R  L  P  K  F  E  L  L  S  H  G  *  H  L  A  *  A  L  G  *  A  S  C  *  C  Y
Tgtgtacgtttacccaagtttgaacttctcagtcatggttgacatttggcatgagcattaggctaggcgtcttgctgatgctat
V  *  K  R  *  C  V  S  C  E  K  A  E  R  I  N  V  L  R  S
gtttaaaaaaggtaatgcgtaagctgcgagaaagctgaaagaataaacgtcttaaggagc
* denotes possible stop codon
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Fig 2. Clustal V multiple sequence alignment of *Myxine glutinosa* gill Na^+/H^+ exchanger.

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Hagfish                          NDAVTVVLYQLLSVLAD---LPSVPASSVL
ß-NHE                            NDAVTVVLYNLFEEFSK---VGTVTVLDVF
NHE1                             NDAVTVVLYHLFEEFAN---YDSIGISDIF
                                 ********* *    +      ++  + +

Hagfish   LGVVRFFVVCLGGIAFGICAGLLASFTTRFT--YPPLEPILILLTCYLAY
ß-NHE     LGVVCFFVVSLGGVLVGAIYGFLAAFTSRFTSHTRVIEPLFVFLYSYMAY
NHE1      LGFLSFFVVALGGVFVGVVYGVIAAFTSRFTSHIRVIEPLFVFLYSYMAY
          **++ **** ***+ +*  +* +*+***+***++    * *      * **

Hagfish   LITEMLHLSGIMALISAALTMRSYVDLNLEWRSRTTLRRTLRALSSTSET
ß-NHE     LSSEMFHLSGIMALIACGVVMRPYVEANISHKSYTTIKYFLKMWSSVSET
NHE1      LSAELFHLSGIMALIASGVVMRPYVEANISHKSHTTIKYFLKMWSSVSET
          *  *+ *********     ** **  *    * **    *   ** ***

Hagfish   LIFLLLGMATLDG-PHDWSWPFVISTLILCLVLRATGVLILSWVANRVRL
ß-NHE     LIFIFLGVSTVAG-PHAWNWTFVITTVILCLVSRVLGVIGLTFIINKFRI
NHE1      LIFIFLGVSTVAG-SHQWNWTFVISTLLFCLIARVLGVLVLTWFINKFRI
          ***  **  *  * +* * * ***+*+++**+ *  **+ * +  *  *

Hagfish   VPISIKDQFIIAYGGLRGAIAFSLVYLIPKV-FHHRALFTTATITTLLFT
ß-NHE     VKLTKKDQFIVAYGGLRGAIAFSLGYLLSNSH-QMRNLFLTAIITVIFFT
NHE1      VKLTPKDQFIIAYGGLRGAIAFSLGYLMDKKHFPMCDLFLTAIITVIFFT
          *    *****+************** **     +  + ** ** **   **

Hagfish   VFVQGMTIPSTGSLGSEKEMRDANSNRNKHSDI----------SSSRWY
ß-NHE     VFVQGMTIRPLVELLAVKKKKESKPSINEEIHTEFLDHLLTGVEGVCGHY
NHE1      VFVQGMTIRPLVDLLAVKKKQETKRSINEEIHTQFLDHLLTGIEDICGHY
          ********    *   *         *                    *

Hagfish   GHLRPSWSSSLA----CCVR--------------LPKF----------EL
ß-NHE     GHYH--WKEKLNRFNKTYVKRWLIAGENFK-EPELIAFYRKMELKQAIMM
NHE1      GHHH--WKDKLNRFNKKYVKKCLIAGERSK-EPQLIAFYHKMEMKQAIEL
          **    *   *                        *  *          ++

Hagfish   LSHGH----LAALGASCCYVK-------RCVSCEKA----------ER--
ß-NHE     VESGQ----LPSVLP--STISMQNI-QP------RAIPRVSKKREEEI--
NHE1      VESGG----MGKIPSAVSTVSMQNI-HPKSMASERILPALSKDKEEEI--
                +          +             + +          *

Hagfish   --------------------INVLRS----------------------
ß-NHE     --------------------RRILRANLQNNKQKMRSRSYSRHTLFDADE
NHE1      --------------------RKILRSNLQKTRQRLRS--YNRHTLVADPY
                               **+
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Related nucleotide sequences were identified by searching the Genebank database (National Center for Biotechnology Information). Analysis of the putative *M. glutinosa* gill NHE using the BLAST algorithm (Altschul *et al.*, *J. Mol. Biol.* 215:403-410, 1990) revealed an amino acid homology of 35% with both the trout β-NHE and mammalian NHE-1. Higher amino acid homology (54%) occurs within the regions highly conserved between different isoforms (Fig 2).

This study provides the first direct evidence of Na^+/H^+ exchangers in the gills of agnathan fishes. Further work is required to obtain the entire sequence of the gene and to verify the identity of the proteins that exhibited crossreactivity with the mammalian antibodies.

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