Molecular cloning, functional expression and chromosomal localization of an amiloride-sensitive Na^+ channel from human small intestine

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Abstract Amiloride-sensitive Na^+ channels belonging to the recently discovered NaC/DEG family of genes have been found in several human tissues including epithelia and central and peripheral neurons. We describe here the molecular cloning of a cDNA encoding a novel human amiloride-sensitive Na^+ channel subunit that is principally expressed in the small intestine and has been called hINaC (human intestine Na^+ channel). This protein is similar to the recently identified rodent channel BLINaC and is relatively close to the acid sensing ion channels (ASICs) (79 and 29% amino acid identity, respectively). ASICs are activated by extracellular protons and probably participate in sensory neurons to nociception linked to tissue acidosis. hINaC is not activated by lowering the external pH but gain-of-function mutations can be introduced and reveal when expressed in *Xenopus* oocytes, an important Na^+ channel activity which is blocked by amiloride ($IC_{50} = 0.5$ μM). These results suggest the existence of a still unknown physiological activator for hINaC (e.g. an extracellular ligand). The presence of this new amiloride-sensitive Na^+ channel in human small intestine probably has interesting physiological as well as physiopathological implications that remain to be clarified. The large activation of this channel by point mutations may be associated with a degenerin-like behavior as previously observed for channels expressed in nematode mechanosensitive neurons. The hINaC gene has been mapped on the 4q31.3–q32 region of the human genome.

Key words: Na^+ channel; Amiloride; Degenerin; Small intestine; Human

1. Introduction

The amiloride-sensitive Na^+ channel and degenerin family (NaC/DEG) form a new family of cationic ion channels [1]. Members have been identified in many animal species ranging from the nematode *Caenorhabditis elegans* to human [2]. In *C. elegans*, neuronal and muscular degenerins are linked to mechanotransduction [3] and the intestinal FLR-1 protein controls the defecation rhythm [4]. The *Drosophila* dGNaC1 (also called ripped pocket) Na^+ channel may play a role in gametogenesis and early development [5,6] while the peptide FMRFamide ionotropic receptor FaNaC is presumably involved in neuronal modulations in the snail *Helix aspersa* [7,8]. The mammalian NaC/DEG channels already identified belong to two major groups. The first one includes the epithelial Na^+ channel ENaC associated with transepithelial Na^+ transport and Na^+ homeostasis, as well as taste perception [9,10]. The other group has been more recently discovered and comprises the neuronal acid sensing ion channel (ASICs) [1,11]. ASICs are external pH sensors present in brain and in sensory neurons where they are thought to participate in nociception associated with tissue acidosis, for instance in ischemic, damaged or inflamed tissue. Very recently, a novel related gene called BLINaC has been identified from rat and mouse [12]. It is present principally in liver, intestine and brain. BLINaC is only 30% identical to ASICs and is not activated by an extracellular acidification, although it is able to form an amiloride-sensitive Na^+-selective channel as revealed by the introduction of gain-of-function mutations.

The physiological importance of NaC/DEG channels was highlighted by the discovery of mutations in their genes that are associated to genetic disorders. In human, mutations in the α or β subunits of the epithelial Na^+ channel are involved in a hereditary form of human hypertension, the Liddle's syndrome [13,14], while a loss-of-function of ENaC caused by mutation on the α or β subunits are associated with pseudohypoaldosteronism type 1, a disease characterized by severe neonatal salt wasting with hyperkalaemic acidosis [15]. Mutations in certain *C. elegans* degenerins lead to degeneration of the expressing neurons (MEC-4 and DEG-1 [16,17]) or to muscle hypercontraction (UNC-105 [18]). A constitutive activation of mammalian channels of the NaC/DEG family by mutations equivalent to those occurring in *C. elegans* degenerins has been described [12,19] and leads to the death of the expressing cells [19]. However, no association of such mutations to genetic disorders has been described so far.

We describe here the molecular cloning, the functional expression and the chromosomal localization of a human amiloride-sensitive Na^+ channel that is essentially expressed in the small intestine. This channel is different from the already cloned human NaC/DEG channels. It is constitutively activated by gain-of-function mutations and can be considered as a novel human degenerin.

2. Materials and methods

2.1. Cloning of the hINaC gene

A human genomic library (2.5×10^6 clones; Clontech) was screened with a ^{32}P-labelled rat BLINaC cDNA probe corresponding to amino acids 1–214 of hINaC. One positive clone was obtained, digested with *Xho*I and subcloned into pBluescript SK^- vector (Stratagene). It contained a 10 kb DNA insert which includes two exons corresponding to amino acids 115–195 and 196–236 of hINaC. To identify the 3′ end of

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the hINaC coding sequence, total RNA from human duodenum was reverse-transcribed with superscript II reverse transcriptase (Gibco-BRL) using the SP6/KS-oligo-dT primer (5′-GATTTAGGTGACACTATAGAATCGAGGTCGACGGTATCCAGTCGAC$(T)_{18}$-3′). The RT sample was amplified by PCR (94°C for 0.5 min, 60°C for 0.5 min, 72°C for 3 min; 43 cycles) using either the primer 5′-GATTTAGGTGACACTATAGAA-3′ or 5′-TAGAATCGAGGTCGACGGTATC-3′, which are identical to parts of the SP6/KS-oligo-dT primer, and one sense primer complementary to the hINaC coding sequence (base position 510–531: 5′-ACTGATTTTGCTGCAAGTCACC-3′). To identify the 5′ end of the hINaC coding sequence, duodenum RNA was reverse-transcribed using oligo-$(dT)_{15}$ primer (Boehringer), poly G tailed with dGTP and terminal deoxynucleotidyl transferase (Promega), and used for PCR. For the PCR (94°C for 1 min, 45°C for 1.5 min, 72°C for 2.5 min; 10 cycles followed by 94°C for 1 min, 55°C for 1.5 min, 72°C for 2.5 min; 41 cycles), two primers (5′-CACTTGGAATTCGCGGCGTCA$(C)_{18}$-3′ and 5′-CACTTGGAATTCGCGGCGTCA-3′) complementary to the tail and a hINaC-specific antisense primer (base position 531–510: 5′-GGTGACTTGCAGCAAAATCAGT-3′) were simultaneously used. An additional PCR was performed (94°C for 1 min, 55°C for 1.5 min, 72°C for 2.5 min; 43 cycles) using one of the tail primers (5′-CACTTGGAATTCGCGGCGTCA-3′) and a second hINaC antisense primer (base position 287–265 5′-GATCTGCCATGTCACAAGTGAGA-3′). The PCR products were subcloned into pBluescript SK^- vector (Stratagene) and sequenced. These procedures allowed identification of the upstream of the first ATG codon and downstream of the stop codon. The full-length hINaC coding sequence was reconstructed from two overlapping PCR fragments located between a sense primer positioned just before the first ATG (nucleotide positions 36–58: 5′-ACGCTAGCCTGAAATCACAAATGGAGCAGAC-3′) and an anti-sense primer starting at nucleotide 1582 in the sequence (5′GTGGATCCGGTATCATGAAAAGGAAACTATT-3′). The cDNA was constructed in the *Xenopus* oocyte expression vector pBSK-SP6-globin. The hINaC A443 mutants were prepared by PCR using a modification of the method of gene splicing by overlap extension [20].

2.2. Northern blot and RT-PCR

Human multiple tissue Northern blots containing about 2 μg of poly A^+ RNA per lane were obtained from Clontech. The blots were hybridized with a ^{32}P-labeled hINaC 398 bp PCR fragment corresponding to bases 390–758 for 16 h at 65°C in the ExpressHyb hybridization solution (Clontech), washed in 0.5×SSC/0.1% SDS at 50°C and subsequently exposed to Kodak X-Omat AR film for 17 days at −70°C.

Two human multiple tissue cDNA (MTC) panels and a Human Digestive System Panel (Clontech) was utilized as described by the purchaser. Two primers (positions 442–464: 5′-GGCACATTGTATCCAAAGTCCTC-3′ and positions 708–730: 5′-CCTCTTCCAGAGACACTCACTTT-3′) were used in a 20 μl total reaction volume. Thirty high cycles of amplification (30 s at 95°C, 1 min at 60°C and 1 min at 72°C) for the MTC panel or 32 cycles of amplification (30 s at 95°C, 1 min at 55°C and 1 min at 72°C) for the human digestive system panel were performed using *Taq* DNA polymerase (Promega), except for GAPDH for which only 24 cycles were done. One fifth of each PCR reaction was loaded on a 2% agarose gel and than transferred onto nylon membrane (Hybond N^+, Amersham). The membrane was hybridized with a ^{32}P-labeled hINaC cDNA probe, washed in 0.2×SSC/0.1% SDS at 60°C and then exposed to Kodak X-Omat AR film at −70°C.

2.3. Expression and electrophysiology in Xenopus oocytes

Isolation, maintenance and injection of stage V and VI *Xenopus* oocytes with WT or mutated hINaC were performed as described previously [21]. cRNA was synthesized from the *Not*I-digested pBSK-SP6-globin hINaC vector using a kit from Stratagene. *Xenopus* oocytes were injected with 0.25–10 ng of cRNA, and microelectrode voltage-clamp assays were performed 1–3 days after injection. The ND96 bathing solution contained (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, pH 7.4 (with NaOH). In some experiments, NaCl was replaced by KCl.

2.4. Fluorescent in situ hybridization (FISH) on the human genome

Metaphase spreads were prepared from phytohemagglutinin-stimulated human lymphocytes cultured at 37°C for 72 h. 5-Bromodeoxyuridine was added for the final 7 h of culture (60 μg/ml of medium) to ensure a chromosomal R-banding of good quality. The plasmid containing the 10 kb genomic hINaC *Xho*I insert (see above) was biotinylated by nick translation with biotin-16-dUTP, as outlined by the Boehringer Mannheim protocol. Hybridization to chromosome spreads was performed with a standard protocol [22]. For each slide, 200 ng of biotinylated DNA was used. Before hybridization, the labelled probe was annealed with a 150-fold excess amount of Cot-1 human DNA (Gibco-BRL) for 45 min at 37°C to compete with the non-specific repetitive sequences. The hybridized probe was detected by means of fluorescence isothiocyanate-conjugated avidin (Vector laboratories). Chromosomes were counterstained and R-banded with propidium iodide diluted in antifade solution (pH 11) as described in Lemieux et al. [23].

3. Results

Screening of a human genomic library with a probe corresponding to the rat BLINaC Na^+ channel cDNA led to the isolation of a partial genomic clone of 10 kb. Sequence comparison with the rat BLINaC cDNA suggested the presence of two putative exons in this clone. The sequence of these putative exons was used to clone a full length cDNA from human duodenum by 3′ and 5′ RACE-PCR. The cDNA is 1692 bp long and contains an open reading frame preceded by one stop codon and encodes a protein of 505 amino acids (Fig. 1A). This protein is 79% identical to rat and mouse BLINaC (subsequently referred as rBLINaC and mBLINaC) (Fig. 1B). The same identity (79%) was found at the nucleotide level. The structure of the human protein is similar to that of rodent BLINaC except for a small 10 amino acid extension at the C-terminus. Assuming that the topology of the new protein is similar to the proposed structure of related proteins of the NaC/DEG family [24–26], the N-terminal intracellular region contains three putative protein kinase C phosphorylation sites and one potential casein kinase II phosphorylation site (Fig. 1A). The large extracellular loop contains eight putative *N*-glycosylation sites.

A band of 2.1 kb was only detected by Northern blot on human small intestine poly A^+ RNA (Fig. 2A). No signal can be detected on brain or on liver that has been shown to be the major site of BLINaC expression in mouse [12]. The distribution of hINaC mRNA along the human digestive tract determined by RT-PCR confirmed its predominant expression in the small intestine (duodenum and jejunum), a small signal being detected in stomach, ileum and rectum (Fig. 2B). This pattern is close to that found for rBLINaC or mBLINaC except that a low and variable expression was also found in rodent colon (not shown). A more sensitive detection of hINaC mRNA by RT-PCR showed, in addition to a signal in the small intestine, a weaker signal in testis while no signal was detectable in the brain or in the liver even in PCR conditions described to allow detection of rate transcripts (Fig. 2C).

The functional properties of hINaC have been analyzed after expression of its complementary RNA into *Xenopus* oocytes. A current of low amplitude (several tens of nA) that does not discriminate between Na^+ and K^+ was recorded in hINaC injected oocytes (not shown). This current is only partially inhibited by 1 mM amiloride (not shown). A similar type of current was also observed in rBLINaC injected oocytes [12]. It is not clear at present if this current is related to hINaC itself or to the non-specific activation of some endogenous ion channel. However, it has been possible to obtain

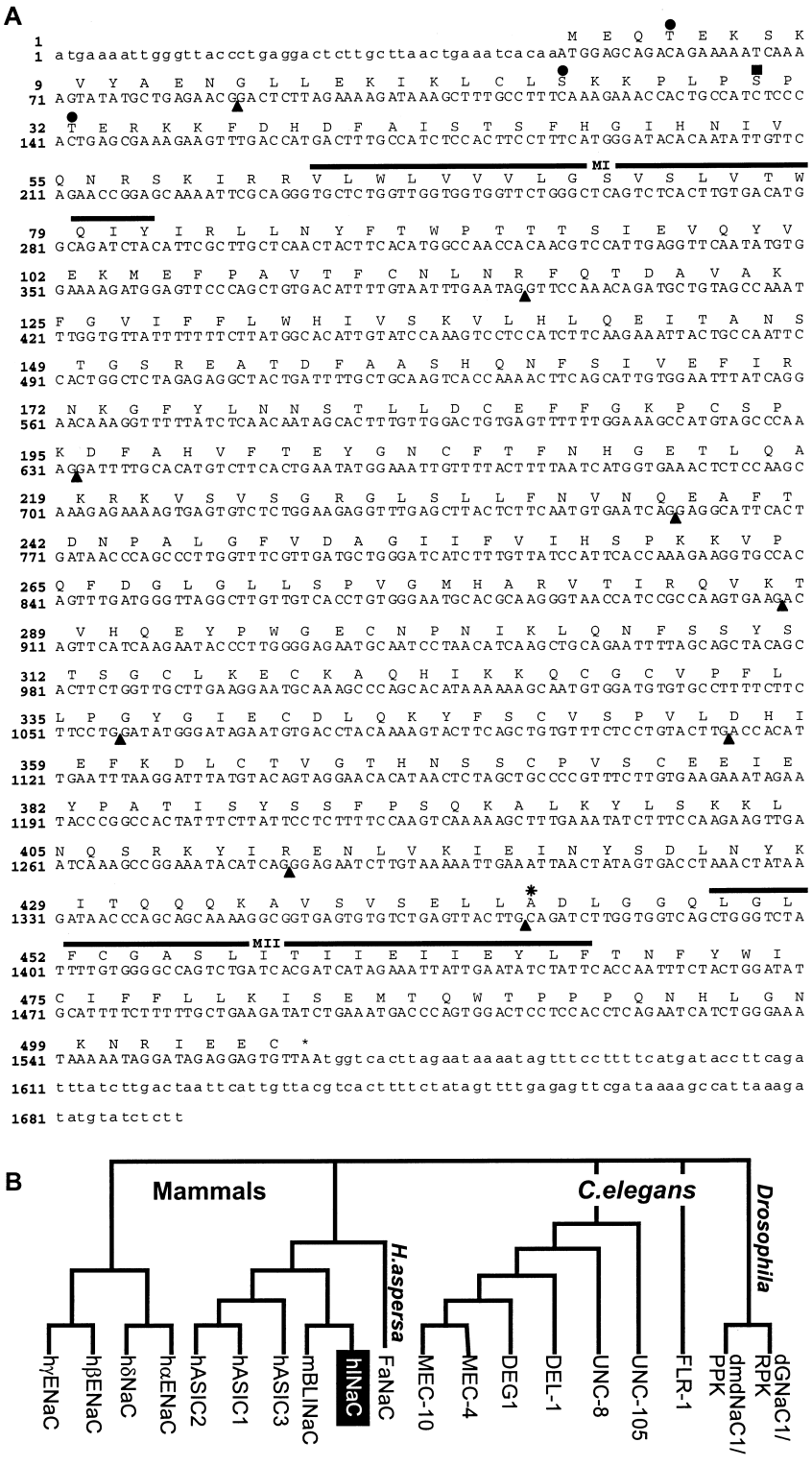

Fig. 1. Sequence of hINaC. A: The nucleotide sequence and the predicted protein sequence (505 amino acids) are shown. MI and MII correspond to the two putative transmembrane domains, the filled circles represent the putative protein kinase C phosphorylation sites, the filled square represents the putative casein kinase II phosphorylation site. Alanine 443 that when mutated leads to gain-of-function is marked by an asterisk. The intron positions in the hINaC gene are marked with filled triangles. The accession number for hINaC is AJ 252011. B: Phylogenetic analysis of several NaC/DEG family members isolated from *Drosophila* (dGNaC1, also named ripped pocket, and dmdNaC1, also named pickpocket), *C. elegans* (including the degenerins MEC-10, MEC-4, DEG-1, UNC-8, and UNC-105), *H. aspersa* (the FMRFamide-activated Na^+ channel FaNaC) and mammals (including the human epithelial Na^+ channel ENaC and the human ASICs). The hINaC protein is 79% identical to mouse BLINaC and 29% identical to human ASICs. The tree was obtained using the GCG Distances program with Kimura substitution followed by the GCG Growtree program with the UPGMA option, from an alignment generated with the GCG Pileup program.

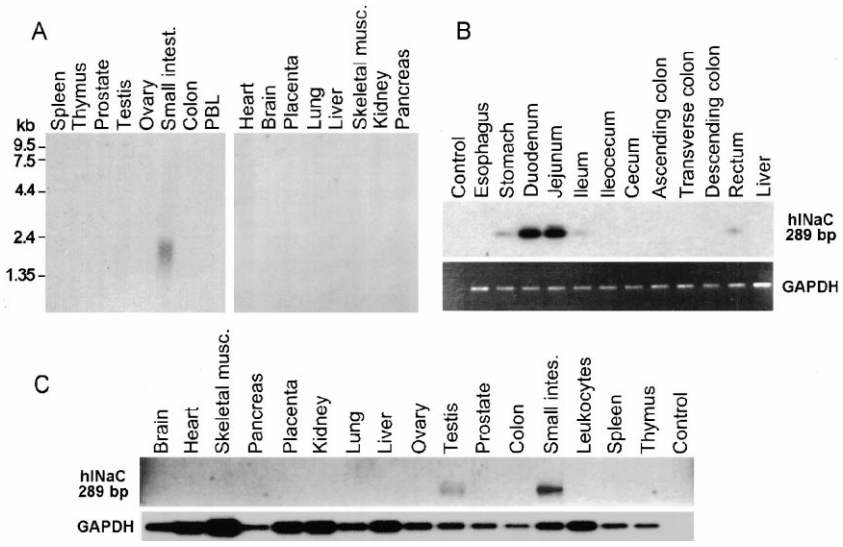

Fig. 2. Tissue distribution of hINaC. A: Northern blot analysis. A broad band of 2.1 kb was detected only on human small intestine poly A^+ RNA. B: The localization along the intestinal tract was assessed by RT-PCR using hINaC specific primers (upper panel, Southern blot analysis of the product of 32 cycles of amplification). A strong signal is detected in duodenum and jejunum and a weaker signal in stomach, ileum and rectum, confirming the predominant expression of hINaC in the small intestine. Unlike in rat and mouse, no signal was detected in liver. C: A more sensitive localization of hINaC mRNA in human tissues was obtained by RT-PCR (upper panel, negative image of an ethidium bromide staining of the product of 38 cycles of amplification). The significant expression of hINaC in the small intestine was confirmed and a weak signal was also detected in testis, while no signal was detected in brain and in liver. The lower panel in B and C corresponds to a GAPDH amplification control (ethidium bromide staining for B and Southern blot analysis for C).

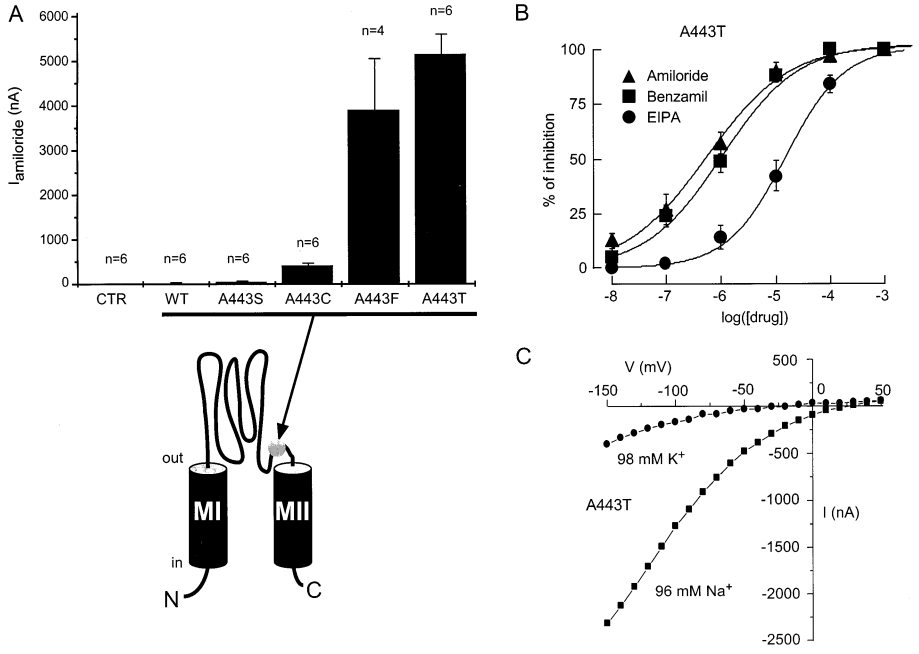

Fig. 3. Expression of hINaC in *Xenopus* oocytes. A: Wild-type hINaC or A443 mutant cRNAs have been injected into *Xenopus* oocytes (10 ng per oocyte) and the amiloride-sensitive current (1 mM amiloride) was recorded at a holding potential of −70 mV 2 days later using the two-microelectrode voltage clamp. Expression of WT hINaC led to the appearance of a current of very low amplitude that is weakly sensitive to 10^{-3} M amiloride and discriminates poorly between Na^+ and K^+ (not shown). The A443S mutant did not modify the amplitude of the current while the A443C and particularly the A443F and the A443T mutants are associated to very large amiloride-sensitive currents of different properties. The number of oocytes tested in each condition is given above the histograms. The control oocytes have been water-injected. A schematic representation of the hINaC protein including the position of the gain-of-function mutations is shown below the histograms. Extracellular and intracellular domains are referred to as out and in, respectively, and the two putative transmembrane domains as MI and MII. B: Dose–response curves of the inhibition of the A443T gain-of-function mutant current recorded at −70 mV by amiloride and its derivatives benzamil and EIPA. Each point represents the mean of 4–6 oocytes. The corresponding IC_{50} are 0.5 μM, 0.8 μM and 18 μM for amiloride, benzamil and EIPA, respectively. C: Typical current–voltage relationship of the amiloride (1 mM)-sensitive current recorded from a representative oocyte expressing the A443T mutant in sodium-rich (ND96, 96 mM of external Na^+) or in potassium-rich (98 mM of external K^+) medium. The reversal potential in ND96 medium is 35 mV.)

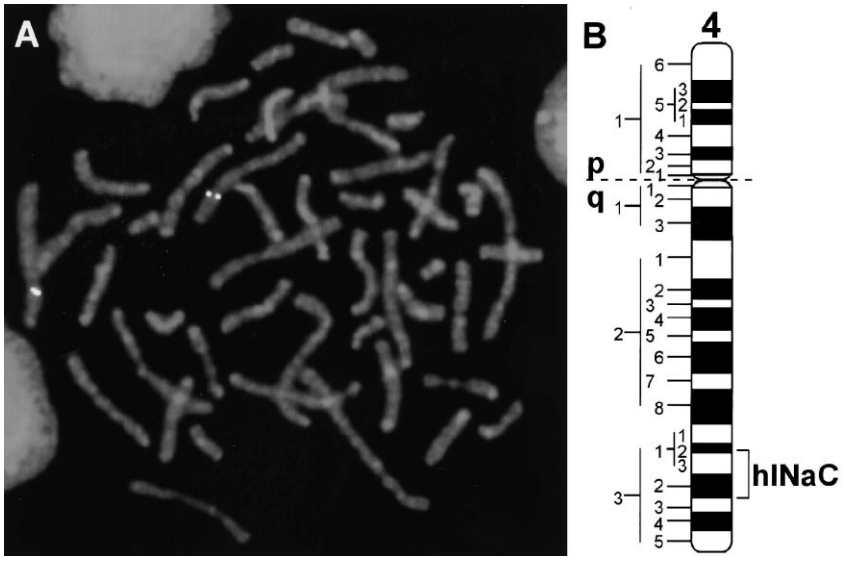

Fig. 4. FISH mapping of the hINaC probe to human chromosome 4q31.3–q32. A: Metaphases observed with the large band FITC Zeiss filter. Chromosomes are stained with propidium iodide and the FITC fluorescent signal is shown on the figure as white dots. B: Ideogram of the human G-banded chromosome 4 with the site of hybridization at 4q31–q32.

very large activation of hINaC by mutating a particular residue situated just before the second transmembrane domain (TM2). Similar mutations are responsible for a gain-of-function when present in some *C. elegans* degenerins [16,17] and in some mammalian NaC/DEG channels [12,19]. These mutations consisted of the replacement of the alanine 443 of hINaC by amino acids with larger side chains, such as cysteine, phenylalanine or threonine. They led to a gain-of-function associated to the appearance of large constitutive currents (Fig. 3A). The current amplitude did not notably change for the A443S mutation compared to the WT channel. However, it significantly increases for the A443C mutant and raised to several μA for the A443F and A443T mutants (Fig. 3A). The properties of the A443T mutant have been analyzed in more detail. The large decrease of the current after replacement of external Na^+ by K^+ and the positive reversal potential in Na^+ rich medium (25 ± 6 mV, $n = 5$, Fig. 3C) provide evidence that the gain-of-function mutant channel is highly selective for Na^+ ions. The current is reversibly blocked by the diuretic amiloride ($IC_{50} = 0.5$ μM) and its derivatives benzamil ($IC_{50} = 0.8$ μM) and EIPA ($IC_{50} = 18$ μM) (Fig. 3B).

The chromosomal localization of hINaC has been examined by FISH (Fig. 4). A total of 30 metaphase cells was analyzed and 90% of the cells showed specific fluorescent spots on the 4q31.3–q32 region of the human genome (Fig. 4A,B). In addition, the intron/exon organization of the hINaC gene has been deduced from the sequence of the partial genomic clone initially isolated and from a human genomic sequence released by the Whitehead Institute/MIT Center for Genome Research (accession AC021433). The hINaC gene comprises at least 10 exons (Fig. 1A).

4. Discussion

A homology screening allowed the cloning in human of a cDNA encoding a member of the amiloride-sensitive Na^+ channel and degenerin superfamily of ion channels. This subunit is highly similar to a novel amiloride-sensitive Na^+ channel that has been recently cloned from rodent [12]. hINaC shares a lot of the properties previously described for rodent BLINaC. Its sequence is close (79% identity) to that of rodent BLINaC both at the amino acid and at the nucleotide level. The hINaC gene is localized on chromosome 4 that is syntenic to the mouse chromosome 3 where the mBLINaC gene is located [12]. Both hINaC and BLINaC mRNAs are significantly expressed in the small intestine. Both channels are activated by gain-of-function mutations and the mutated channels have nearly identical electrophysiological and pharmacological properties. hINaC is thus probably the ortholog of the rodent BLINaC. However, some differences exist. In particular, no expression of hINaC has been detected in the brain and in the liver, even by RT-PCR, while the liver is the major site of mouse BLINaC expression.

The very large activity associated to the hINaC gain-of-function mutants and the properties of these mutants (Na^+ selectivity, amiloride-sensitivity) suggest that this protein forms a channel on its own that needs some activator to open. The sequence and the structure of hINaC are close to those of the ASICs (Fig. 1B) but hINaC is not a novel ASIC since it is not activated by extracellular protons (not shown). Phylogenetic analyses link hINaC to the group of ligand-gated channels of the NaC/DEG family comprising ASICs and FaNaC (Fig. 1B). An extracellular ligand, possibly a peptide, is thus a possible candidate to activate hINaC. All the putative ligand tested (e.g. FMRFamide and related mammalian peptides) were without effect on this channel (not shown). The discovery of such a ligand, if it exists, will be a challenging task. A second essential task to elucidate the hINaC function is to determine the exact nature of the expressing cells. This will require the development of dedicated tools like specific antibodies directed against hINaC. There is no physiological description in the small intestine of ion channels with properties similar to those of hINaC. The small intestine has been shown to display an amiloride-sensitive Na^+ transport in certain conditions like after proctocolectomy [27] but the channel involved in that process appears to be the epithelial

Na^+ channel ENaC. However, the properties of the native hINaC are still unknown and can be significantly different from those of the gain-of-function mutants described here, even if the data obtained for the proton-gated channel ASIC2a suggests that the properties of gain-of-function mutants of ASIC2a are not very different from those of the pH-activated channel [28].

Gain-of-function mutations in ion channels are often associated to human pathologies [29]. The constitutive activation of a Na^+-selective channel will undoubtedly lead to serious damage in the expressing cells as previously described in vitro [19]. This situation occurs in the nematode *C. elegans* where mutations in the degenerins are responsible for neurodegeneration or muscle hypercontraction. The localization of hINaC to the 4q31.3–q32 region of the human genome has not yet revealed links to putative channelopathies but the specific expression of this novel channel subunit in the human small intestine will certainly gain the interest of pathologists working on intestinal diseases.

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