Molecular characterization of a novel human PDZ domain protein with homology to INAD from *Drosophila melanogaster*

Stephan Philipp, Veit Flockerzi*

Pharmakologisches Institut der Universität Heidelberg, Im Neuenheimer Feld 366, D-69120 Heidelberg, Germany

Received 6 June 1997; revised version received 27 June 1997

Abstract PDZ domains are thought to act as protein-binding modules mediating the clustering of membrane and membrane-associated proteins. The INAD protein has been shown to interact via a PDZ domain with the calcium channel TRP which contributes to capacitative calcium entry into *Drosophila* photoreceptor cells. We have cloned the cDNA of a human INAD-Like protein (hINADL) of 1524 amino acids in length containing at least five PDZ domains. Additionally, two truncated versions hINADL$_{\Delta 304}$ and hINADL$_{\Delta 853}$ were identified. *hInadl* transcripts of differing size are expressed in various tissues including brain, where transcripts are abundant in the cerebellum.

Key words: PDZ domain; INAD; GLGF repeat; Capacitative calcium entry; Membrane protein cluster; DHR domain

1. Introduction

PDZ domains have been identified in a rapidly expanding number of intracellular proteins. These approximately 100 amino acid domains were originally found in a family of proteins comprising the **P**ostsynaptic density protein (PSD-95, [1]/SAP90, [2]), the **D**isc-large tumor supressor (dlg, [3]) and the **Z**onula occludens protein (ZO-1, [4]) which are located at synaptic, septate or tight junctions, respectively. These **PDZ** proteins are characterized by the presence of several PDZ domains, an additional Src homology domain (SH3) and a modified guanylate kinase domain (GUK) and are combined as a subclass of the membrane-associated guanylate kinases (MAGUK, reviewed in [5]). The PDZ amino acid sequence motif could be detected in further proteins of yet unknown function, in the dystrophin-associated syntrophins [6] and in the family of protein tyrosine phosphatases (PTP, [7,8]), which possess an additional phosphatase domain and a band 4.1-like region.

PDZ domains are also known as **D**iscs-large **H**omology **R**epeats (DHR) or GLGF repeats, according to the presence of a GLGF amino acid motif. They represent protein binding domains which mediate the interaction to their binding partners by at least two mechanisms. First, they bind to specific amino acid motifs located at the very C-terminal end of the target proteins. As shown for example for the interaction of the second PDZ domain of PSD-95 and the ionotropic NMDA receptor [9] or the shaker type potassium channel [10] this C-terminal amino acid motif consists of a serine or a threonine residue followed by any amino acid and a valine residue (consensus S/TXV). Second, different proteins are able to interact via their PDZ domains and build heterodimers. Accordingly, the second PDZ domain of PSD-95 bind directly to the PDZ domain of the neuronal nitric oxide synthase [11]. Thus, the same the PDZ domain can bind target proteins in two different ways.

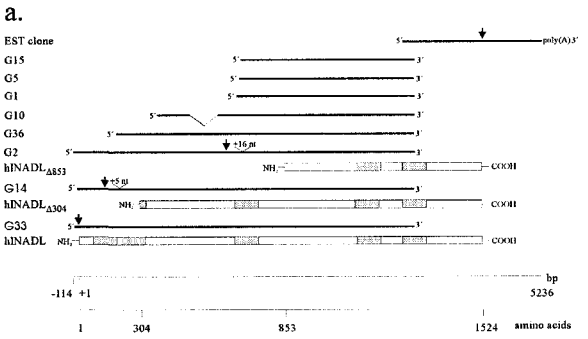
Interaction of PDZ proteins to their target molecules play a role in clustering proteins to specific locations at the plasma membrane. In this model PDZ proteins mediate cytoskeletal anchoring of membrane or membrane-associated proteins of which the proper function depend on the specific location and/or the vicinity of other regulatory components. Thus, PDZ proteins contribute to the formation of macromolecular complexes but may have also indirect or direct regulatory influence on the function of their target proteins.

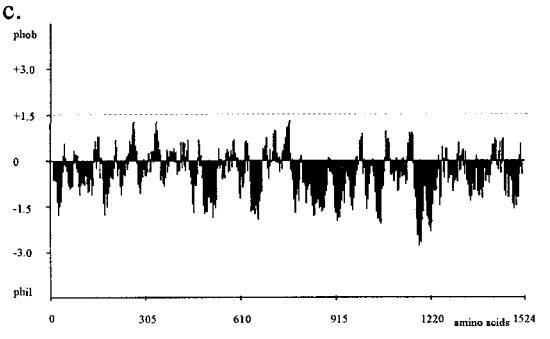
The inactivation no after-potential D (*InaD*) mutation in *Drosophila melanogaster* affect the visual transduction in the compound eye of the fly. As reported by Shieh and Niemeyer [12] the *Drosophila InaD* gene (*dInaD*) encode a hydrophilic protein with two amino acid repeats that share sequence similarity to the PDZ domains of the MAGUK family. Recently, it could be shown that a PDZ domain of dINAD bind to the C-terminally located STV amino acid residues of the TRP calcium channel [13], which is thought to be involved in capacitative calcium entry (CCE). The association of dINAD and TRP is prevented by a point mutation in the *InaD* mutant that converts a methionine residue located in the second PDZ domain to a lysine. These findings have led to the suggestion that dINAD is a regulatory subunit of the TRP channel. Similarly, the INAD protein of *Calliphora vicina* (cINAD) interacts with *Calliphora* TRP and additionally with phospholipase C, the key enzyme of the inositol(1,4,5)trisphosphate ($InsP_3$)-cascade [14]. These three proteins and an eye specific protein kinase C seem to built a multimeric signalling complex. A structural significance of dINAD is obvious by the fact that INAD deficient *Drosophila* mutants undergo retinal degradation probably due to the lack of the TRP-INAD interaction [15].

Similar to INAD the TRP protein was identified as a mutation affecting the phototransduction in *Drosophila*. The lack of TRP led to a transient receptor potential due to the defect in light-induced Ca^{2+} entry across the membrane. Functional analysis of TRP showed that it is a Ca^{2+} channel which is activated following Ca^{2+} release from internal stores upon stimulation of the $InsP_3$ cascade [16–18]). We have cloned the cDNA of two capacitative calcium entry channels CCE1 [19] and CCE2 [20] from mammals. Both mammalian proteins possess significant amino acid identity to TRP [21] and also

*Present address: Institut für Pharmakologie und Toxikologie der Universität des Saarlandes, D-66421 Homburg, Germany.
Tel.: (49) 6841-166400; Fax: (49) 6841-166402.
E-mail: stephan.philipp@med-rz.uni-sb.de or veit.flockerzi@med-rz.uni-sb.de

PII S0014-5793(97)00877-6


a.
EST clone
poly(A)3'
G15
G5
G1
G10
G36
G2
+16 pt
hINADL$_{\Delta 853}$
NH$_2$
COOH
G14
+5 pt
hINADL$_{\Delta 304}$
G33
hINADL
bp
-114 +1
5236
1
304
853
1524
amino acids


c.
phob
+3.0
+1.5
0
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-3.0
phil
0
305
610
915
1220
amino acids 1524

b.

PDZ 1
PDZ 2
PDZ 3
PDZ 4
PDZ 5

Fig. 1. Cloning strategy (a), nucleotide and deduced amino acid sequence (b) and hydrophobicity plot (c) of hINADL. Nucleotide sequences are indicated by black lines, the deduced amino acid sequences by white bars. PDZ domains are highlighted by hatched bars. Insertions of 16 and 5 nucleotides (nt) in clone G2 and G14, respectively, are indicated. The first ATG triplet in frame downstream of a stop codon (arrow) was taken as translation start site leading to three protein isoforms comprising 1524 (hINADL), 1221 (hINADL$_{\Delta 304}$) and 672 (hINADL$_{\Delta 853}$) amino acids, respectively. (b) Nucleotide and amino acid residues are numbered on the right beginning with the first residue of the ATG triplet encoding the initiating methionine and the initiating methionine, respectively. Stop codons in frame are signed by asterisks. PDZ domains are highlighted by grey bars and are indicated on the right. Nucleotide insertions of 5 nt (clone G2) and 16 nt (clone G15) leading to a frame shift and alternative translation start sites at methionine 304 and methionine 835 (highlighted in black), respectively, are indicated by small letters. An in frame deletion (nt 1981–2112) present in clone G10 is boxed. Base substitutions in hINADL$_{\Delta 304}$ and hINADL$_{\Delta 835}$ are indicated by small letters, one leading to an amino acid exchange (R744C). A predicted ATP/GTP-binding site (P-loop) is underlined by a black bar. Predicted phosphorylation sites for cAMP- and cGMP-dependent protein kinase are marked with a black square, for tyrosine kinase by a black circle. Numerous predicted phosphorylation sites for protein kinase C and for casein kinase II are not indicated. The sequence of the *hInadl* cDNA has been deposited in GenBank. (c) The hydropathy profile was obtained according to the method of Kyte and Doolittle [30] using a window of 19 amino acids.
←

share structural and functional properties with the *Drosophila* protein [16–20,22].

In search for putative subunits of mammalian CCE channels we have cloned the cDNA of a new human PDZ protein with significant homology to dINAD, which we call human INAD-Like (hINADL). Here we report the structure and expression of hINADL and variants of it, which in future experiments may help to further elucidate the molecular basis of capacitative calcium entry.

2. Materials and methods

2.1. Isolation and sequencing of cDNA clones

A cDNA library was constructed by reverse transcription of the primer 5′-CCC ACC ACA AAT ATG CTG CAT-3′ (nucleotides 3803–3784, Fig. 1b) with poly(A)$^+$ RNA from human Girardi cells as template. An oligodesoxyribonucleotide 5′-ATG GAC TTG - GAC TCA GCC TTG CTG GTA ATA-3′ (nucleotides 3740–3769, Fig. 1b) was phosphorylated using T4 polynucleotide kinase and γ[^{32}P]ATP and served as a probe to screen the library using standard protocols [23]. Hybridization was carried out at 50°C overnight and the filters were washed at 55°C with 15 mM NaCl/1.5 mM sodium citrate, pH 7.0/0.1% sodium dodecylsulfate. DNA inserts of all isolated clones were sequenced on both strands by cycle sequencing using infrared dye labelled primers and an automated sequencer (Licor).

2.2. Preparation of poly(A)$^+$ RNA

L(tk-) cells were grown in Dulbecco's modified Eagles medium supplemented with 10% foetal calf serum. Girardi cells which were shown to be identical to HeLa [24] were cultivated in the same medium containing glutamine (2 mM). For the isolation of total RNA, rabbit or bovine tissues (10 g each), or 10^9 cell culture cells sedimented and washed with cold phosphate-buffered saline were homogenized in 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% Na-*N*-lauroylsarcosine, 1% β-mercaptoethanol and 3% antifoam A (Sigma) and sedimented by centrifugation on a CsCl gradient [23]. The RNA was washed 3 times with 70% ethanol and solved in 600 μl 10 mM Tris-HCl/1 mM Na-EDTA, pH 7.5, followed by chloroform/butanol (4:1) extraction and ethanol precipitation. Poly(A)$^+$ RNA was prepared using oligo(dT) cellulose columns.

2.3. Northern blot analysis

Unless otherwise stated 10 μg of poly(A)$^+$ RNA was applied to a formaldehyde gel, electrophoresed as described [19] and transferred to nylon membranes (Amersham). After a short rinse in 0.3 M NaCl/30 mM sodium citrate, pH 7.0, the RNA was UV-crosslinked to the membrane. For analysis of hINADL in human brain a multiple tissue Northern blot (Clontech) was used. A cDNA fragment corresponding to nucleotides 3730–4528 (Fig. 1b) was labelled by random priming using α[^{32}P]dCTP and used as a probe for hybridization. The filters were exposed to X-ray films with intensifying screens at -80°C for the desired period of time. As a control of the integrity and the amount of the transferred RNA all filters were stripped and rehybridized to a 239 bp PCR-fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

2.4. Data base analysis and sequence alignments

A human partial sequence (dbEST Id 615332, GenBank acc. AA005420) homologous to INAD of *Drosophila melanogaster* was identified in the EST database. A search for hINADL homologous proteins was performed with the BLASTP (Basic Local Alignment Search Tool [25]) and BEAUTY (BLAST Enhanced Alignment Utility, [26]) program (Human Genome Center, Baylor College of Medicine, Houston, TX) at the National Center for Biotechnology Information (NCBI). Specific sequence patterns in hINADL were identified by eye or using the prosite motif library [27]. Protein sequences were aligned with the clustal [28] and the bestfit [29] programs using standard parameters (HUSAR sequence analysis program package at the German Cancer Research Center (DKFZ), Heidelberg, Germany).

3. Results

3.1. Identification of a novel PDZ domain protein

In search for putative homologs of dINAD in mammals, a partial 439 bp sequence was identified in the dbEST database showing considerable homology to INAD of *Calliphora vicina* (cINAD) and to INAD of *D. melanogaster* (dINAD). Analysis of this EST clone revealed a sequence of 1506 bp followed by a poly(A) tail (Fig. 1a). As shown in Fig. 2 the deduced amino acid sequence of the EST clone shares significant sim-

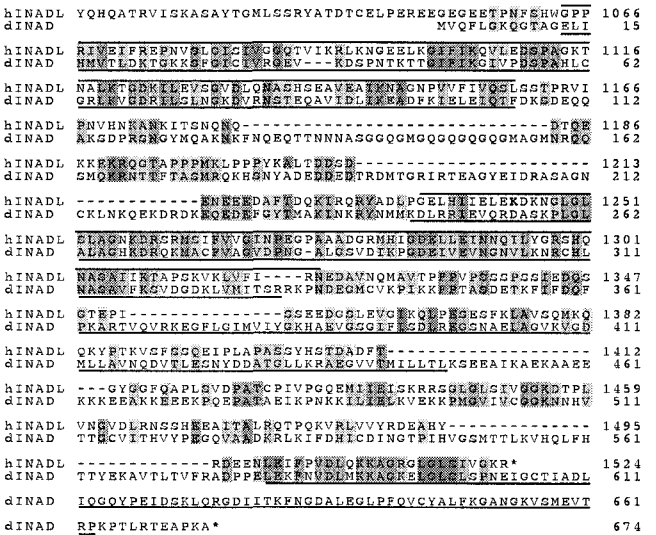

Fig. 2. Alignment of hINADL to INAD of *D. melanogaster*. The N-terminal amino acid residue K_{1244} of hINADL corresponding to the 5′-end of the EST-cDNA clone is indicated by a bold letter. Identical amino acids are highlighted in deep grey, conserved in pale grey. The 1016 N-terminal amino acid residues of hINADL are not shown. PDZ domains are indicated by black bars, stop codons by asterisks.

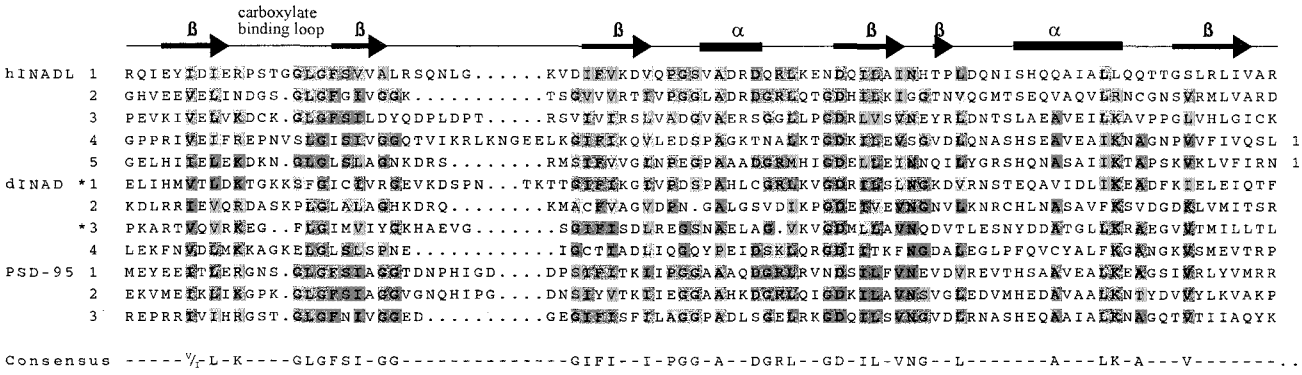

Fig. 3. Alignment of PDZ domains of hINADL, dINAD and PSD95. Amino acid residues are numbered on the right. A consensus sequence is given below for residues with a minimal frequency of 50% which are highlighted in deep grey. Conserved amino acids corresponding to the consensus sequence are shown in pale grey. The PDZ domains in dINAD originally identified by Shieh and Niemeyer [13] are signed by asterisks. The secondary structure elements predicted for the third PDZ domain of PSD-95 [31] are shown by arrows (β sheet), bars (α helix) and lines (connecting loops).

ilarity with the dINAD protein sequence. The first amino acid residue encoded by the 5′-cDNA sequence of the EST clone corresponds to D_{255} of dINAD indicating that the EST protein is homologous to the C-terminal region of dINAD but lacks the N-terminus. To get the full-length cDNA sequence, we constructed a primer extension cDNA library using a specific primer derived from the 5′-region of the EST sequence and poly(A)$^+$ RNA from human Girardi cells as template for the first-strand synthesis. 8×10^6 transformants were probed and 8 of more than 100 positive signals were analysed (Fig. 1a). All clones isolated contained the 5′-region of the EST clone comprising 74 bp. Three of the clones obtained, G2, G14 and G33, contained an in frame ATG triplet that appears downstream of a stop codon.

The 5350 nt cDNA sequence derived from clone G33 and the EST clone contained an open reading frame encoding a sequence of 1524 amino acids with a calculated molecular mass of 167 358. Sequence analysis revealed that this protein which is called human INAD-like (hINADL), contains at least five PDZ domains assigned as PDZ1 to PDZ5 (Fig. 1). Two clones, G2 and G14, which are of similar length as clone G33, possess insertions of 5 and 16 nucleotides, respectively (Fig. 1a) leading to shifts in the reading frame and the appearance of other in frame stop codons upstream of ATG triplets. As a consequence, Met^{304} and Met^{853} are assigned as the translation initiation sites in G2 and G14, respectively. The resulting proteins hINADL$_{\Delta304}$ and hINADL$_{\Delta853}$ comprise 1221 and 672 amino acids (Fig. 1). The truncation of the N-terminus leads to the absence of two (hINADL$_{\Delta304}$) or three (hINADL$_{\Delta853}$) PDZ domains, respectively. HINADL appears to be the most abundant isoform expressed in Girardi cells because all other clones which were analysed correspond to G33 and do not contain the insertions detected in G2 and G14. A hydropathy analysis [30] of the amino acid sequence of hINADL (Fig. 1c) reveals the absence of a typical membrane spanning region. Additionally, the N-terminal sequence does not resemble a hydrophobic signal sequence. These properties are consistent with the assumption that hINADL is a hydrophilic protein which is not an integral part of the cell membrane but might be associated with intracellular domains of membrane proteins.

3.2. Sequence and structural similarities

A close inspection of the hINADL sequence reveals the presence of five PDZ domains with a length between 84 and 96 amino acids (Fig. 1b) covering amino acids 130-219, 244-

Table 1
Amino acid sequence similarity and identity of different members of the PDZ family

									Similarity (%)	
	hINADL	dINAD	PSD-95	SAP97	SAP102	DLG	ZO-1	SNT B1	Chapsyn	PTP1E
hINADL		52.3	50.7	46.4	50.1	45.2	40.7	43.7	47.4	46.7
dINAD	27.1		43.9	41.4	40.2	41.7	44.4	40.1	43.8	45.5
PSD-95	28.2	21.1		86.3	82.2	76.2	55.0	45.3	84.7	48.2
SAP97	21.6	18.8	74.2		78.5	75.7	46.5	44.6	74.8	48.3
SAP102	24.6	19.6	67.6	66.4		75.7	46.3	45.3	82.2	49.0
DLG	21.7	18.4	58.3	60.9	59.8		46.9	43.6	72.5	43.4
ZO-1	19.6	20.8	31.1	25.1	25.0	26.2		43.1	50.8	41.5
SNT B1	20.6	18.4	22.0	24.1	23.2	21.4	18.9		44.6	41.0
chapsyn	24.8	19.1	70.7	86.2	69.4	57.0	28.0	20.5		44.7
PTP1E	23.6	22.5	24.6	24.8	25.6	22.7	19.8	20.8	22.6	

The amino acid sequences were aligned using the BestFit program and the local homology algorithm of Smith and Waterman [31]. GenBank accession numbers: dINAD (drosophila), U15803; PSD-95 (rat), M96853; SAP97 (rat), U14950; SAP102 (rat), U50147; DLG (drosophila), M73529; ZO01 (human), L14837; beta1-syntrophin (SNT B1, human), L31529; PTP1E (human), U12128.

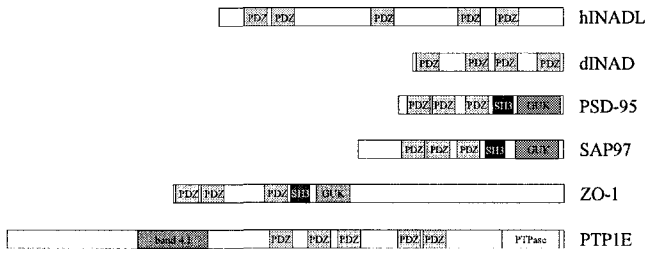

Fig. 4. Schematic diagram of the structural domains within different PDZ proteins. Domains indicated in the figure are: PDZ, PDZ domain; GUK, guanylate kinase domain, SH3, Src homology domain; band 4.1, band 4.1-like domain; PTPase, catalytic protein tyrosine phosphatase domain.

327, 682-771, 1064-1159 and 1235-1321. The alignment of these amino acids to the well-characterized PDZ domains of PSD-95 and PDZ domains present in dINAD (Fig. 3) indicates that both hINADL and dINAD belong to the PDZ family of proteins. Originally, it has been reported that dINAD possess two PDZ domains [12,13,15] but after sequence analysis it appears that at least two additional repeats are present (Fig. 2). A hINADL peptide sequence comprising amino acid residues 1433–1523 might represent a sixth PDZ domain within hINADL. The secondary structure revealed by an analysis of the crystal structure of the third PDZ domain of PSD-95 [31] locates the binding site for the C-terminus of the target proteins between two β-sheets. This carboxylate binding site as well as other structural elements of the proteins are highly conserved as it is in hINADL (Fig. 3). So, hINADL possess the molecular features of a PDZ protein and may function as a membrane protein clustering module.

The deduced amino acid sequence of hINADL is considerably longer than the sequence of dINAD. Nevertheless, the sequence of the dipterian protein possess significant sequence similarity to hINADL with only a few gaps and it is obvious that the similarity is not restricted to the PDZ domains (Fig. 2). Using the deduced amino acid sequence of hINADL as a query to perform a BLASTP+BEAUTY search in the protein database only proteins of the PDZ family were found. A separate search of hINADL sequence stretches outside the PDZ domains revealed no further significant similarities to other proteins. Although *D. melanogaster* is phylogenetically distant to mammals, a comparison of different PDZ proteins of varying length revealed highest sequence identity and similarity of hINADL to INAD of *Drosophila* (Table 1). Vice versa dINAD is most similar to hINADL. There is also an obvious similarity of hINADL to other members of the PDZ family as for example to PSD-95. To get deeper insight into the relationship of the PDZ family and to find out the closest relative of hINADL we compared the functional domains of several PDZ proteins. Fig. 4 shows a schematic diagram of the domain structure of hINADL and related proteins. MAGUK proteins are characterized by additional protein domains like the guanylate kinase (GUK) and a Src homology (SH3) domain and the protein tyrosine phosphatases (PTP) possess a phosphatase activity (PTPase) and a band 4.1-like region. No such additional domains could be detected in hINADL and dINAD indicating the close relationship of these proteins which apparently constitute a subfamily of PDZ proteins.

3.3. *Tissue distribution of hInadl mRNA*

Northern blot analysis shows that transcripts of about 9.0, 5.4, 4.1 and 1.5 kb are abundant in adrenal gland, kidney, ureter (Fig. 4a), heart and epithelial Girardi cells (Fig. 4b). Lower amounts of the transcripts are also present in other tissues including testis, lung (Fig. 4a), medulla and spinal cord (Fig. 4c). The 5.4 kb transcript, most prominent in human mRNA from cerebellum and Girardi cells correspond to the size of the cloned hINADL cDNA (5350 bp). The 9.0 kb transcripts and the less abundant and smaller mRNAs may differ in the 5′-untranslated sequence as a result of alternative mRNA processing. Alternatively, these signals may represent

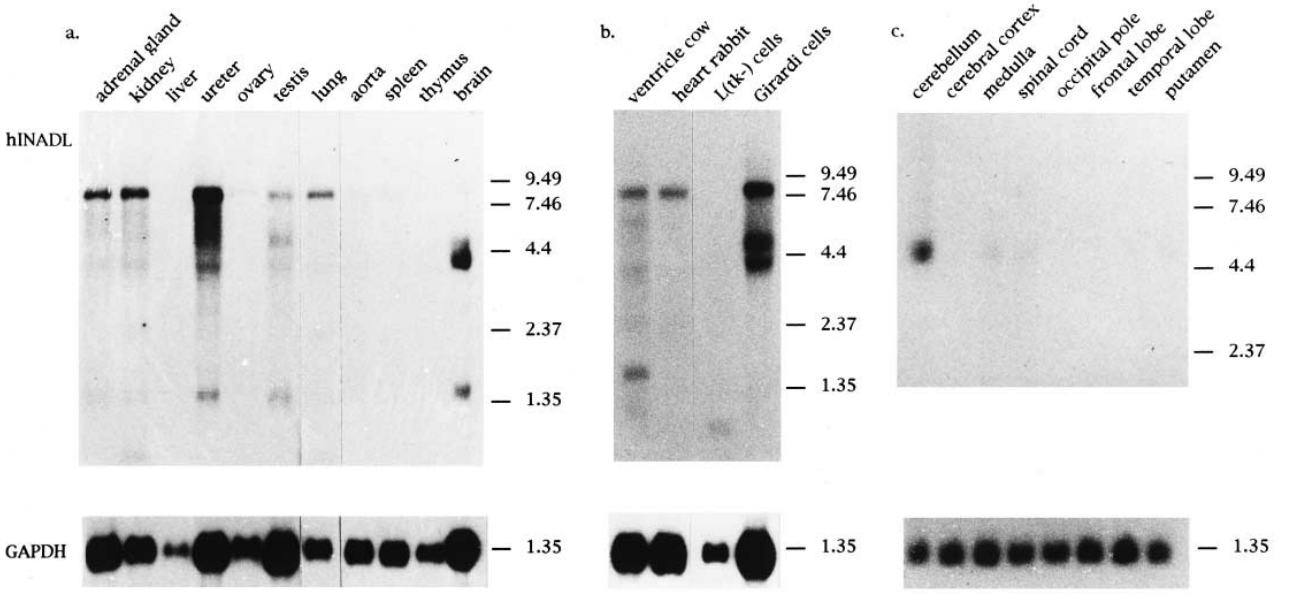

Fig. 5. Northern blot analysis of *hInadl* gene expression in rabbit tissues (a), in bovine ventricle, rabbit heart and cell lines (b) and in human brain (c). The upper panel shows the result using the *hInadl* probe after 10 days exposure of the filters. Lower panels are result of the same filters hybridized with the human glyceraldehyde-3-phosphate dehydrogenase probe.

rabbit, bovine and human mRNAs which are similar but not identical to hINADL (Fig. 5a,b).

4. Discussion

This study describes the molecular characterization of a novel human protein, hINADL, which contains at least five amino acid repeats which could be characterized as PDZ domains. Like other PDZ proteins including dipterian INAD, hINADL is hydrophilic and might contribute to clustering of membrane proteins. Sequence comparison of hINADL reveals significant similarity to INAD of *Drosophila* suggesting that hINADL represents the human homolog of dINAD. Although similarities to other PDZ proteins are obvious, hINADL as well as dINAD lack significant structural features of these proteins like guanylate kinase or protein tyrosine phosphatase domains. A major difference of the two proteins is their length. HINADL comprises 1524 amino acid residues whereas the dipterian INAD proteins are considerably smaller (674 and 665 amino acids) and in this respect resemble the N-terminally truncated isoforms $hINADL_{\Delta 853}$ and $hINADL_{\Delta 304}$. As shown for other PDZ proteins, hINADL may form multimeric complexes and interact with the carboxyl terminus of unrelated membrane proteins via its PDZ domains and thereby contribute to macromolecular complex formation within the cell membrane. Ongoing expression experiments will show whether — in analogy to the INAD/TRP interaction in the *Drosophila* compound eye — mammalian capacitative calcium entry channels like CCE1, CCE2 and other mammalian TRP homologs could be the molecular targets of hINADL. This is supported by the similarity of the tissue specific expression pattern of hINADL and CCE1/CCE2 [19,20]. Apparently, the C-terminal five amino acid residues in CCE1 and CCE2 might contribute to the interaction to hINADL. They comprise the amino acid motif TXL resembling the known interaction site of other PDZ domains S/TXV.

Acknowledgements: We thank Ute Soltek for technical support. This work was supported in part by the Deutsche Forschungsgemeinschaft, the Thyssen Stiftung and the Fond der Chemischen Industrie.

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