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Expression analysis of members of the neuronal calcium sensor protein family: combining bioinformatics and Western blot analysis

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Abstract

We have used in silico mining of public databases (NCBI UniGene and NCI SAGE Anatomic Viewer) as a tool to obtain the tissue distribution pattern of three members of the neuronal calcium sensor protein family, namely VILIP-1, hippocalcin, and NCS-1 in humans. The theoretical human mRNA expression profile of the calcium sensor proteins derived from expressed sequence tag (EST) and serial analysis of gene expression (SAGE) data was compared with expression data from human tissues obtained by Western blot analysis. Since the EST databank searches do not yet give comparable results for rat which is often used as model animal, we have also analyzed the protein expression in rat tissues. Similar to the human expression profile in rat tissues calcium sensor proteins are mainly detected in the nervous system, but the data consistently implicated the additional expression in peripheral tissues with remarkable differences between the calcium sensor proteins.

Keywords: Bioinformatics; Calcium-binding protein; EST; Immunocytochemistry; NCS proteins; Peripheral expression; SAGE; Western blot

Functional characterization of the genes in the human genome remains a major challenge in the postgenomic era. Draft sequences of the human [1,2] and many other genomes have been completed. The availability of a large number of genomic sequences and expressed sequence tags from EST databases of mammalian genomes allows one to systematically search for gene expression. A major source of available information is the National Center for Biotechnology Information database of expressed sequence tags (dbEST, http://www.ncbi.nlm.nih.gov/dbEST/) which contains a total of nearly 18 million sequences with over 5 million human, 4 million mouse, and 0.5 million rat EST data (*Homo sapiens* 5,654,825, *Mus musculus* + domesticus 4,235,142, *Rattus* sp. 636,658 in July 2004) which represent almost the entire genome of human and mouse. However, before genomic information can be put to practical use, it must be converted into biologically useful information, e.g., such as the tissue distribution of a given gene or a family of genes. A fast way to obtain an overview on the tissue expression of a protein is to perform an in silico mining of EST databases as a tool to obtain the requested tissue distribution pattern at least on the transcript level. The UniGene collection (UniGene, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene) comprises more than 106,934 human clusters of preselected EST sequences which allow one to generate a transcript map of genes of interest. In

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addition, the National Cancer Institute with its Cancer Genome Anatomy project offers tools such as the SAGE Anatomic Viewer (http://cgap.nci.nih.gov/SAGE/AnatomicViewer). The SAGE database uses a method of computational analysis of large numbers of mRNA transcripts by sequencing short, usually 10 bp, sequence tags which allows one to visualize human gene expression in tissues in the form of virtual Northern blots. Thus, various computational methods using comparative genomic approaches are available and are powerful tools to receive novel information about proteins which might be useful for the design of new experimental questions [3].

We are interested in neuronal calcium sensor (NCS) proteins which have been described as a nervous system specific gene family with expression outside the nervous system in some cases [4]. An exception from the rule has been, e.g., the family member VILIP-3 also known as REM-1 which had been described in cells of the haematopoietic system and in the gut [5] but also in kidney, spleen, and testis [6]. With the description of new NCS family members, the Kv channel-interacting proteins (KChIPs) [7], and the observation that KChIP2 which is strongly expressed in the heart acts as a regulatory subunit of the cardiac potassium channel complex [8], attention has raised for a possible peripheral expression and function of these proteins. Similarly, NCS-1 was shown to be expressed in the heart and to modulate potassium channels in the mammalian myocardium [9]. Thus, besides their role in influencing neuronal signalling NCS proteins may also play important roles in physiology of other organs. We were interested as to what extent these primarily neuronal proteins are expressed in the periphery. Therefore, in this study we have used the UniGene database to screen for EST sequences of members of the NCS protein family including VILIP-1, Hippocalcin, and NCS-1 in order to obtain novel information on their peripheral tissue distribution. In addition, we have used the SAGE Anatomic Viewer to obtain a virtual mRNA distribution of the proteins derived from EST and SAGE data. To validate the in silico results we have compared the mRNA expression of the NCS proteins obtained by virtual Northern blots with the protein expression pattern obtained by Western blot analysis from human and rat tissues.

Materials and methods

Bioinformatics. The UniGene gene clusters [3] for the human NCS proteins VILIP-1 (Hs.2288), Hippocalcin (Hs.288654), and NCS-1 (Hs.301760) were analyzed (NCBI, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). For graphical presentation of tissue distribution the sequences with a pathological context or other undefined sequences (e.g., pooled sequences from different organs) were subtracted from the total amount of sequences. The remaining number of sequences was set to 100% and the tissue-specific sequences were calculated in percent of total sequences (see Table 1). A virtual Northern blot for the three genes is derived from EST and SAGE data (http://cgap.nci.nih.gov/SAGE/AnatomicViewer). In the anatomic viewer in each tissue the expression is computed by dividing the number of ESTs or SAGE tags representing the NCS protein by the total number of ESTs or SAGE tags in all libraries used with the given tissue. This ratio is then multiplied by 200,000, giving the number of ESTs or SAGE tags per 200,000. The value is visualized as band intensity on a logarithmic basis. These spot images represent the virtual mRNA expression level of the genes in the different tissues.

Antibodies. Rabbit polyclonal antibodies against VILIP-1 and hippocalcin were raised against recombinant His-tagged VILIP-1 or hippocalcin fusion proteins and were affinity-purified as previously described [10,11]. Chicken polyclonal antibody against NCS-1 was purchased from Calbiochem (Temecula, CA, USA).

Western blotting and immunodetection. A human multiple tissue blot (Oncogene Research Products, San Diego, CA) with 75 μg protein per sample which had been homogenized in the presence of protease inhibitors, separated on a 4–20% SDS–PAGE and blotted onto PVDF membrane, was used for Western blot analysis. For rat tissue homogenates were lysed with 1% SDS, 1% Triton X-100 in phosphate-buffered saline (PBS, pH 7.6) including a protease inhibitor cocktail (Boehringer–Mannheim, Germany). Protein concentrations were determined using a BCA kit (Pierce, Rockfort, IL, USA). For all examined peripheral tissues 50 μg protein (except for testis

Table 1
Tissue-specific expression of NCS proteins as deduced from human UniGene database

	VILIP-1	Hippocalcin	NCS-1
UniGene cluster	Hs.2288	Hs.288654	Hs.301760
Total No. EST sequences	196	96	299
Pathological	23	26	121
Other (pooled, unknown)	15	3	19
No. EST sequences counted	158 (100%)	67 (100%)	161 (100%)
Tissue			
Brain	117 (74%)	55 (82%)	76 (47%)
Nerves	14 (9%)	1 (1.5%)	10 (6%)
Eye/optic nerve	6 (4%)	9 (13.4%)	18 (11%)
Heart	1 (0.64%)		6 (3.7%)
Liver	2 (1.3%)		
Spleen			
Lung	3 (1.9%)		2 (1.2%)
Kidney	4 (2.5%)		7 (4.3%)
Stomach	1 (0.64%)		1 (0.6%)
Colon	1 (0.64%)		4 (2.5%)
Skin			6 (3.7%)
Testis	2 (1.3%)	1 (1.5%)	1 (0.6%)
Ovary			1 (0.6%)
Placenta/uterus	2 (1.3%)		5 (3%)
Glands, adrenal gland	1 (1.3%)		9 (5.6%)
Pancreas			5 (3%)
Prostate	4 (2.5%)	1 (1.5%)	4 (2.5%)
Breast			6 (3.7%)

UniGene cluster numbers which were used to analyze a transcript map for VILIP-1, NCS-1, and Hippocalcin using the UniGene databank from the National Center for Biotechnology Information database (Unigene, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene) are indicated. The sequences with pathological context or undefined sequences, such as unknown or pooled sequences from different organs, were subtracted from the total amount of sequences. The number and percentage of sequences in nervous tissues and most peripheral organs is shown.

10 μg) and as control 20 μg of brain tissue (hippocampus) were separated on a 5–20% gradient SDS–polyacrylamide gel using the Laemmli buffer system [12] and blotted onto PVDF. After blocking of unspecific binding sites for 2 h with blocking buffer (5% low-fat milk powder, 0.1% Tween 20 in TBS) the membranes were incubated overnight at 4 °C with rabbit polyclonal anti-VILIP-1, Hippocalcin antibodies or chicken polyclonal anti-NCS-1 antibody. The immunoreactivity was visualized using HRP-coupled goat anti-rabbit or anti-chicken antibodies (Dianova, Hamburg, Germany) and the ECL chemiluminescence detection system (Amersham Biosciences, Freiburg, Germany).

Results

Tissue-specific expression of NCS proteins as deduced from human UniGene database

The NCBI UniGene database and thus, the complete human expressed sequence tag database (dbEST) was analyzed for the human NCS proteins VILIP-1 (Hs.2288), Hippocalcin (Hs288654), and NCS-1 (Hs.301760). After elimination of disease related sequences and some sequences of undefined origin, such as pooled tissue from different organs, an estimate of the pattern and levels of mRNA transcript expression for the NCS proteins in a variety of adult and fetal tissues could be obtained (see Table 1).

VILIP-1 EST clones and thus, expression of VILIP-1 mRNA was found primarily in nervous tissues (approximately 87% of the analyzed EST sequences) such as fetal brain, hippocampus, hypothalamus, and amygdala, but it was also found in eye, retina, and optic nerve or nerves in general. In peripheral tissues VILIP-1 EST sequences appeared in a variety of tissues (only human EST sequences are shown in decreasing abundance of EST sequences). Prostate, kidney, and lung had highest EST numbers whereas in tissues such as liver, testis, placenta, heart, adrenal gland, stomach, and colon only 1–2 EST sequences per organ were available (Fig. 1A). Hippocalcin EST clones showed up mainly in nervous tissues (approximately 98%) with only low sequence numbers in peripheral tissues when compared to VILIP-1. Only one EST sequence for Hippocalcin was found in prostate and one in testis in the human EST database (Fig. 1B). For NCS-1 only about 63% of the analyzed clones were found in nervous tissues and expression in most other organs was observed (Fig. 1C). Major expression in the periphery occured in glands, kidney, skin, heart, breast, uterus, pancreas, colon, and prostate with up to 4–5% of EST clones per organ. The EST sequences for NCS-1 had weaker abundance in lung, stomach, testis, and ovary with only one to two sequences per organ.

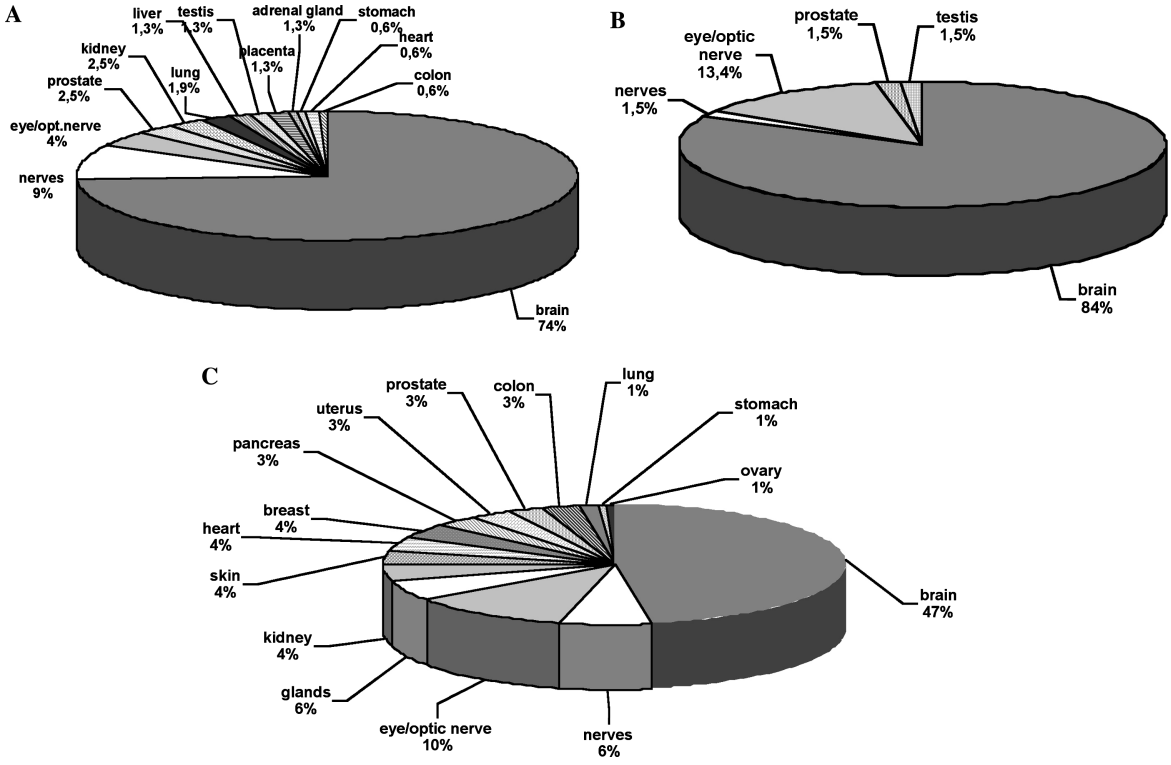

Fig. 1. Relative expression of NCS proteins VILIP-1, Hippocalcin, and NCS-1 in brain and peripheral tissues according to GenBank EST sequences. Tissue expression of VILIP-1 (A), Hippocalcin (B), and NCS-1 (C) in percent of total number of EST sequences found in UniGene cluster for the different NCS proteins.

Virtual Northern blot analysis of the expression of NCS proteins in peripheral tissues based on EST and SAGE data

Virtual Northern blot data (Fig. 2) for the tissues of interest for the three genes were taken from EST and SAGE databases as supplied by (http://cgap.nci.nih.gov/SAGE/AnatomicViewer). In the anatomic viewer the expression level was computed for each tissue and the value was then visualized as spot intensity. Similar to the manual dbEST data analysis (Fig. 1) the highest level of mRNA expression based on EST data for the three NCS proteins was found in the brain (Fig. 2A), with additional expression in heart, liver, lung, kidney, and testis for VILIP-1, and heart, lung, kidney, ovary, colon, and skin for NCS-1, but no additional peripheral expression for hippocalcin. Interestingly, the virtual mRNA distribution based on SAGE data (Fig. 2B) showed some differences in tissue distribution. Again all three proteins showed highest expression levels in the brain with Hippocalcin being completely brain specific. VILIP-1 showed expression in heart, kidney, pancreas, and colon, whereas NCS-1 was additionally expressed in kidney, ovary, and pancreas.

Western blot analysis of the expression of NCS proteins in peripheral tissues

The search in the human EST database and virtual Northern blot data from EST and SAGE databases indicated expression of VILIP-1, Hippocalcin, and NCS-1 in several peripheral tissues. In order to confirm these in silico results on an experimental level for human tissues we performed Western blot analysis with a human multiple tissue blot (Fig. 3A). The blot was probed with affinity-purified VILIP-1 or Hippocalcin antiserum or a chicken NCS-1 antiserum. In brain tissue the antibodies reacted strongly with a protein band in the region of 22 kDa in the case of VILIP-1 and Hippocalcin and 25 kDa in the case of NCS-1 (Fig. 3A, brain). Compared to brain tissue VILIP-1 showed a weaker but still strong reactivity in heart, lung, liver, and testis and a weaker reactivity in ovary, kidney, spleen, and pancreas. Besides brain NCS-1 showed strongest expression in kidney, testis, ovary, and pancreas and weaker expression in heart, liver, and lung. Similar to the previous mRNA data Hippocalcin was brain specific. In addition, we examined the protein expression pattern in rat tissues (Figs. 3B and C). Therefore, equal amounts of protein from embryonic (E19) and adult (8 weeks) rat tissues were subjected to Western blot analysis and probed with the different antibodies. Again all three proteins showed strongest expression in brain (20 μg/lane) with Hippocalcin being brain specific. Strongest expression for VILIP-1 was seen in the embryonic tissue (Fig. 3B) in heart, lung, and testis, the latter tissue with only 10 μg protein/lane. Additionally, we found strong expression in stomach and skin (not present on the human blot, compare Figs. 3A and B). A weaker but still detectable reactivity was observed in liver, spleen, kidney,

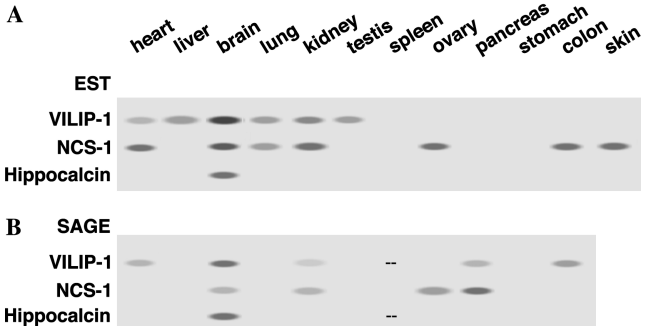

Fig. 2. Virtual Northern blot analysis of the expression of NCS proteins in brain and peripheral tissues of human. Tissue expression of VILIP-1, Hippocalcin, and NCS-1 mRNA for different peripheral tissues such as heart, liver, brain, lung, kidney, testis, spleen, ovary, pancreas, stomach, colon, and skin as calculated from EST (A) and SAGE (B) data. The intensity of the virtual mRNA bands and therefore the expression strength was visualized on a logarithmic basis following calculation of the ratio of the number of ESTs/SAGE tags in a given tissue and the total number of ESTs/SAGE tags in this tissue. The notation '–' means that no libraries for the given tissue were available.

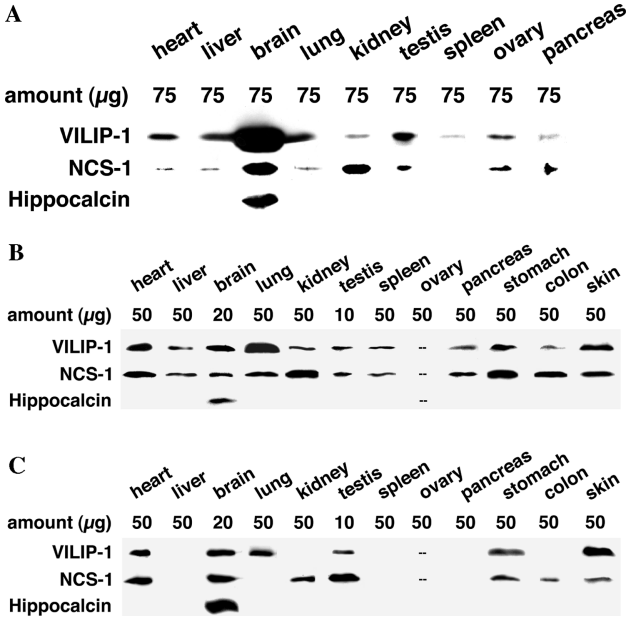

Fig. 3. Western blot analysis of the expression of NCS proteins in brain and peripheral tissues of human and rat. Tissue expression of VILIP-1, Hippocalcin, and NCS-1 in Western blots of homogenates of different peripheral tissues such as heart, liver, brain, lung, kidney, testis, spleen, ovary, and pancreas from human (A) and heart, liver, brain, lung, kidney, testis, spleen, ovary, pancreas stomach, colon, and skin from rat embryonic (B) and rat adult (C) tissues. Western blots were probed with affinity-purified polyclonal antibodies against VILIP-1, Hippocalcin or polyclonal chicken antibodies against NCS-1. The amount of protein applied per lane is indicated.

pancreas, and colon. In adult tissues a similar expression pattern was observed (Fig. 3C), however in comparison to the embryonic tissue much less or no immunoreactivity appeared in liver, spleen, kidney, colon, and pancreas. Hippocalcin showed highest expression levels in brain and virtually no expression could be detected in peripheral tissues in embryonic and adult tissues (Figs. 3B and C). NCS-1 showed besides expression in the brain quite some expression in most organs analyzed (Figs. 3B and C). However, highest expression levels occured in heart, kidney, testis, and lesser expression in lung in embryonic and adult tissues. Similar to VILIP-1 in adult tissues expression was reduced in some organs in particular in stomach, colon, and skin or lost completely in liver, lung, and pancreas (Fig. 3C). Therefore, the expression level became more comparable to the distribution in human tissue (compare Figs. 3A and C). In summary, except for the case of Hippocalcin which showed almost no peripheral distribution the expression patterns for VILIP-1 and NCS-1 suggested by virtual Northern blots were mainly confirmed by Western blot analysis.

Discussion

In this study novel information on the peripheral tissue distribution of members of the NCS protein family including VILIP-1, Hippocalcin, and NCS-1 was obtained by mining public DNA databases and confirming these data experimentally by Western blot analysis. All NCS proteins showed major expression in the nervous system. However to varying degrees different members showed an additional differential expression in peripheral tissues. For VILIP-1 we have detected major expression in heart, liver, lung, and testis in human and rat but also stomach and skin in rat. On the mRNA level some expression of VILIP-1 in heart and lung had already been implied by Northern blot data [13] and could now be confirmed on the protein level. Furthermore, the presence of VILIP-1 in heart and colon was recently shown by RT-PCR [14], the presence in skin was shown by immunohistochemistry [15], thus, reconfirming our results. Therefore, from these data it can be concluded that VILIP-1 is mainly a neuronal protein, however significant expression levels can also be found in some peripheral tissues in a developmentally regulated manner. In contrast to the other NCS proteins, Hippocalcin showed a very restricted protein expression pattern exclusively in brain tissues. Although EST sequences were found in a few additional peripheral tissues no protein expression could be detected. For NCS-1 protein a wide distribution was observed in most peripheral organs of human and rat which is in line with previous findings for heart and the gastrointestinal system [8,14] and with the EST and SAGE sequence data. In the human blot the strength of NCS-1 expression was lower than expected from the EST and SAGE data which indicated strong and wide distribution. Moreover, differences in the degree of expression were observed for VILIP-1 and NCS-1 especially in heart, liver, lung, and kidney in human and lung, kidney, testis, and skin in rat. Whereas in lung and skin VILIP-1 showed high and NCS-1 low expression levels it was vice versa in kidney, possibly indicating organ specific functions of the two NCS proteins. The peripheral distribution of NCS proteins in relation to brain expression was much higher in embryonic tissue compared to adult rat tissues, indicating a developmental restriction of NCS protein expression. The developmental changes in the strength of NCS protein expression occurred as a decrease in expression in liver, lung, kidney, spleen, pancreas, and colon and may additionally hint to a specific function of defined NCS proteins during development of organs. Finally, EST clones containing NCS protein sequences were also found in quite a few disease-related tissues (see Table 1) especially in cancer tissue such as nervous tumor, hepatocellular carcinoma, adenocarcinoma, oligodendroglioma, lung squamous cell carcinoma, and head/neck tumors. Thus, it is likely that NCS proteins not only play a role in the development and physiology of peripheral organs but also under certain pathophysiological conditions. This has recently been shown for VILIP-1 in the form of an involvement in growth and invasiveness of head/neck tumors [15].

In summary, on the basis of data mining from the human EST and SAGE databases, a rough estimation of the expression pattern of a gene seems to be feasible. Detailed organ specific examination of protein expression has to substantiate the preliminary in silico data. The strength of the in silico data comes also from additional information for pathological conditions which may be useful for the design of new experimental approaches in the future. For the NCS proteins the experimental data indicate that NCS-1 shows the widest and Hippocalcin the least distribution in peripheral tissues which is in line with the in silico results. It seems that a change in expression pattern from a more general expression as observed for NCS-1 to a pattern more restricted to the brain in the case of VILIP-1 and even more obvious for Hippocalcin occurs. From an evolutionary point of view this makes sense if one looks at the evolutionary tree of the NCS protein family [4,16]. Whereas NCS-1 is a protein which is already present in organisms such as yeast, the VILIPs and especially Hippocalcin have branched off only much later in development. Hippocalcin seems to have developed at last and therefore seems to have a much higher brain specificity and shows a much more restricted expression pattern in the periphery. Hippocalcin may have adapted to specific brain functions in the hippocampus, whereas VILIP-1 and NCS-1 still fulfill some peripheral organ specific functions.

The terminology neuronal calcium sensor proteins for this family of EF-hand calcium binding proteins which is now commonly used [4,16,17] seems to be still valid in regard to the major expression of the proteins in the nervous system.

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