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REGULATION OF RHODOPSIN PHOSPHORYLATION BY A FAMILY OF NEURONAL CALCIUM SENSORS

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SUMMARY: Recoverin is a calcium sensor that regulates rhodopsin phosphorylation in a calcium-dependent manner. Cloning experiments indicate the presence of a numerous gene family, called the NCS family, encoding recoverin-like proteins expressed predominantly in neurons. Here, we report the cloning of three novel NCS genes, and demonstrate that at least six distinct members of the NCS family (including recoverin, S-modulin, vilip1, NCS-1, *Ce*-NCS-1, and *Ce*-NCS-2) specifically inhibit rhodopsin phosphorylation. The presence of species homologues within the NCS family suggests that this function might be shared by at least 12 (out of 18) NCS proteins. Recent studies indicate that recoverin inhibits rhodopsin phosphorylation by directly regulating rhodopsin kinase, a G protein coupled receptor kinase (GRK). Since several NCS proteins are found in neurons throughout the entire nervous system, they may regulate other members of the GRK family. Together, our data suggest a general role for NCS proteins in the regulation of calcium-dependent phosphorylation in the nervous system.

Calcium sensors, such as calmodulin, are involved in the fine regulation of calcium-dependent specific pathways (1). In their calci-forms they are active as enzymes or as regulators of structural proteins, membrane ion channels and key enzymes including kinases, phosphatases, phospholipases, and proteases. The binding of Ca^{2+} to calcium sensors induces conformational changes that expose hydrophobic surfaces. Using whole cell recording, retinal-specific bovine recoverin and two homologs, visinin and Gecko p26, from chicken and Gecko retina respectively, were dialyzed into functionally intact Gecko rods, and shown to prolong the rising phase of the photoresponse without affecting the kinetics of response recovery (2). It was also demonstrated *in vitro* that bovine recoverin or its frog homolog S-modulin prolongs the lifetime and activity of frog rod cGMP-phosphodiesterase (3, 4). The S-modulin and recoverin effect is calcium-dependent and is mediated via the inhibition of rhodopsin phosphorylation at high

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physiological calcium (5, 6). Recent experiments indicate that Ca^{2+}-recoverin acts directly on rhodopsin kinase to decrease its catalytic activity (6, 7). Therefore, quenching of rhodopsin in the presence of retinal NCS occurs only at low physiological calcium concentration.

Eighteen amino acid sequences, related to recoverin and representing a novel subfamily of calcium binding proteins, are grouped and presented here (Fig. 1). This family was named the Neuronal Calcium Sensor (NCS) gene family as it represents neuron-specific proteins that bind Ca^{2+} and have 3-4 potential EF hands. Members of the NCS family have been isolated in several species such as drosophila, frog, chick, rat, mouse, bovine, and human. It includes recoverin (8, 9, 10), frequenin (11), S-modulin (3), neurocalcin (12), hippocalcin (13, 14), vilip1 (15, 16), vilip2 (17), vilip3 (17), visinin (18), and HLP2 (14), and NCS-1 (19).

In this work, we have identified three novel NCS genes and further characterized the in vitro function of several NCS proteins. The data show that the majority of the NCS family members can regulate the phosphorylation of rhodopsin.

MATERIALS AND METHODS

PCR, cDNA, and Genomic Cloning of NCS-1, *Ce*-NCS-1, and *Ce*-NCS-2. To isolate rodent and C. elegans NCS-1 homologous genes, we screened a rat brain cDNA library (Stratagene), a mouse genomic library, and a C. elegans cDNA library (20) at low stringency (50°C) with the chick NCS-1 probe (19). Several positive phages were characterized and their insert DNA subcloned in $pBSK^-$ (Stratagene), and sequenced using the dideoxy method of Sanger (21). *Ce*-NCS-2 from C. elegans was obtained by screening the cDNA library at low stringency with a C elegans NCS-1 probe.

Phylogenetic Tree Analysis. Progressive alignments of amino acid sequences were calculated with the program PileUp (Genetics Computer Group, Madison, Wisconsin, USA). The calculated branch lengths were obtained on an AXP/VMS system with the program TREE (R. F. Doolittle, UCSD, La Jolla, USA) modified by one of us (E. D. C.) to read input files directly from PileUp output formats. Distance scores between each pair of aligned sequences was determined by using the Minimum Mutation Matrix of Dayhoff. The angles used to draw the tree were arbitrarily chosen, in order to illustrate aesthetically the differences between the four branches.

Production and Purification of Recombinant NCS Proteins. Overproduction of NCS members in E. Coli was obtained with the system described in (22). The complete open reading frame (from ATG to the stop codon) of the cDNA clones RL25 (chick NCS-1, (19)), pTrec2 (bovine recoverin, (23)), 69h (chick vilip1, (16)), *Ce*-NCS-1 and *Ce*-NCS-2 were inserted in the expression vector pET3a and transformed in the strain BL21(DE3) in the presence of pLysS. After induction with IPTG, overexpressing bacteria (3 liters) were harvested by centrifugation, resuspended in 200 ml lysis buffer (50 mM Tris-HCl, pH 8. 0,2 mM EDTA; 0.1% (v/v) Triton X-100), frozen in liquid nitrogen, and sonicated for 3-5 min. After centrifugation, the supernatants were brought to ~40% saturation with ammonium sulfate and centrifuged. The pH of the supernatant was brought to ~4.3 with acetic acid, centrifuged, and the precipitate, which contained NCS proteins, was resuspended in 200 ml of 50 mM HEPES, pH 7.5, 2 mM $CaCl_2$, 0.1 M NaCl and charged on a 2 x 6 cm phenyl-Sepharose CL-4B column equilibrated in this buffer. The protein was eluted with the same buffer containing 10 mM EDTA instead of $CaCl_2$. Protein concentrations were determined from the UV absorption spectrum using a calculated molar extinction coefficient of ~22,000 $M^{-1}cm^{-1}$.

Rhodopsin Phosphorylation Assay. Rhodopsin phosphorylation was carried out as described in (5). For a typical phosphorylation assay, 15 µl of ROS membranes (containing ~10-15 µg rhodopsin) was added in the dark to 10 µl of a [γ^{32}P]ATP solution. The resultant reaction mixture consisted of (final concentration): rhodopsin (10-15 µM), ATP (0.1 mM); [γ^{32}P]ATP

(24 MBq/mmol of ATP); GTP (0.5 mM); cGMP (4 mM), exogenous NCS recombinant protein (4-5 μM). Thirty seconds later, a light flash bleaching 3.5 x 10^6 rhodopsin molecules per ROS was applied and two minutes later, the reaction was stopped by adding 180 μl of ice-cold 10% (w/w) trichloroacetic acid. The sample was centrifuged at 10'000 x g for 15 minutes, the precipitate washed once with 1 ml of K-gluconate buffer, centrifuged, and resuspended in a sample buffer for SDS-PAGE. The gel was stained with Coomassie and autoradiographed. The incorporation of ^{32}P into rhodopsin was obtained by counting ^{32}P activity of rhodopsin bands dissected from the SDS-PAGE gel. The Ca^{2+} concentration was buffered using a Ca^{2+} /EGTA buffering system as described (24).

RESULTS

Rodent NCS-1. By screening at moderate stringency a rat brain cDNA library with the chick NCS-1 probe (19), we were able to isolate several positive phages which had a cDNA insert encoding the rat NCS-1. A nucleic acid coding sequence comparison of chick and rat NCS-1 revealed 87% identity. However, surprisingly, the 191 amino acid open reading frames of both proteins are 100% identical (see Fig. 1).

NCS genes in Nematode. Nematodes possess a very simple nervous system (only 302 neurons in hermaphrodite worms) compared with mammalians. Therefore, C. elegans is an attractive

Figure 1. Amino acid sequence alignment of 18 members of the NCS family. Six members (three pairs) have an identical primary amino acid sequence: NCS-1 (chick/rat), vilip1 (chick/rat), hippocalcin(rat/human). X, Y, Z and -X, -Y, -Z represent the positions of amino acids binding to calcium ion in EF-hand. EF1 site is quite divergent from a consensus EF-hand. EF2, EF3, and EF4 are likely to bind calcium. For every vertical row, the most abundant amino acid is shown in upper case, whereas less abundant or unique amino acids are shown in lower case. The consensus line indicates the amino acid identical present in more than 15 sequences. The boxes underlined by arrows correspond to highly conserved regions from which sense and antisense primers for the original PCR reaction were derived. These sequence data are available from Genbank/EMBL/DDBJ under accession numbers: L27420 (Chick NCS-1), L27421 (rat NCS-1), L33680 (*Ce*-NCS-1), L33681 (*Ce*-NCS-2).

model to study neuronal functions during development and in fully developed adult animals (25). To determine whether calcium sensors exist in the nematode, we screened at low stringency cDNA and genomic DNA libraries of C. elegans with the rat full-length NCS-1 cDNA probe. We were able to isolate several positive phages that contained inserts encoding a novel calcium binding protein of the NCS family named *Ce*-NCS-1 (Fig. 1) because of its high homology with chick/rat NCS-1. Indeed, the *Ce*-NCS-1 deduced amino acid sequence has 75% identity with rodent or avian NCS-1. The high homology between rodent, avian and nematode NCS-1 amino acid sequences strongly suggests that we have characterized homologous genes from different species. To estimate the degree of gene diversity in the nematode NCS family, we screened at very low stringency both cDNA and genomic C. elegans librairies with the full-length *Ce*-NCS-1 probe. We isolated several positive phages which, after further characterization were shown to encode a single but novel neuronal calcium sensor named *Ce*-NCS-2 (Fig. 1). There is only 45% of amino acid identity between *Ce*-NCS-2 and either *Ce*-NCS1 or rodent/avian NCS-1. Northern blot analysis indicated that both *Ce*-NCS-1 and *Ce*-NCS-2 are expressed in adult C. elegans (data not shown). No additional NCS genes were found in C. elegans.

NCS-1, Ce-NCS-1, and Ce-NCS-2 Biophysical Properties. By analysis of the full-length amino acid sequence of NCS-1 from rat/chick, we predict a protein molecular weight (M_r) of 21'878 Daltons, an isoelectric point of 4.53, and three potential EF-hands (in Fig. 1: EF2,3,4), For the *Ce*-NCS-1 protein, we predict a M_r of 22'021 Daltons, a theoretical isolectric point of 4.95 and two potential EF-hands (EF2,3). For the *Ce*-NCS-2 protein, the calculated Mr is 21'985 Daltons and the theoretical isoelectric point is 4.92, and there are three potential EF-hands (EF2,3,4).

Phylogenetic Tree Analysis of the NCS Family. To date, a total of 18 sequences encoding NCS proteins have been isolated in several species including C. elegans, drosophila, chick, rat, mouse, bovine, frog, and man (Fig. 1). By using a phylogenetic analysis program (26), a tree representing the degree of homology between NCS proteins during evolution was calculated (Fig. 2), assuming that all sequences evolved from a common ancestor gene by successive duplications. Four main branches could be distinguished: 1) branch **I** is composed of S-modulin, three recoverins, and visinin, 2) branch **II** represents the three NCS-1, and frequenin, 3) branch **III** is composed of vilip1 to vilip3, hippocalcin, neurocalcin, and HLP2, 4) branch **IV** is only composed of C. elegans *Ce*-NCS-2. Species homologs of branch **I** are frog S-modulin, bovine, mouse, and human recoverins, and chick visinin; homologs of branch **II** are chick, rat, mouse NCS-1 and C. elegans *Ce*-NCS-1, and Drosophila frequenin; homologs of branch **III** are chick and rat vilip1, rat vilip3 and human HLP2, rat and human hippocalcin. It is not yet known if rat hippocalcin and bovine neurocalcin (87%) are species homologs.

Inhibition of Receptor Phosphorylation. Based on the recent function reported for retinal NCS such as native S-modulin (5), recoverin (2, 3) and Gecko p26 (2), we performed a functional *in vitro* assay with rod outer segment (ROS) membranes to measure the phosphorylation of rhodopsin in the presence or absence of recombinant NCS proteins. At high but physiological

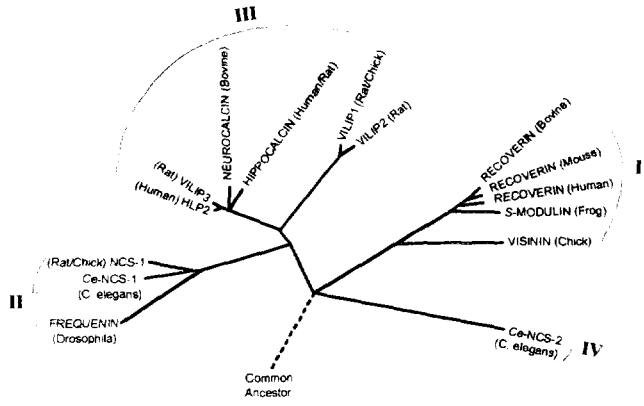

Figure 2. Phylogenetic tree of the NCS family. 4 main branches (**I** to **IV**) are observed. The relationship of a set of genetic sequences can be deduced from a tree analysis which gives more information than analysis based on percent identity or homology. The general topology of the tree gives information on how the sequences should be grouped, while evolutionary distances separating different sequences are shown by the branch lengths. Branch **I** represents retinal NCS. Branch **II** is composed of highly homologous NCS from distant species including rat, chick, drosophila, and C. elegans. Branch **III** represents another group of homologous NCS found in human, rat, chick, and bovine; hippocalcin is highly enriched in neurons of the hippocampus. Branch **IV** and its single member (C. elegans *Ce*-NCS-2) represents the most distant sequence among the family of NCS proteins identified to date.

calcium concentrations, recombinant NCS-1 inhibits rhodopsin phosphorylation (Fig. 3A). However, at low calcium, NCS-1 has no effect on receptor phosphorylation (Fig. 3A), suggesting a calcium-mediated effect. To determine whether it represents a shared property of all NCS family members, we performed the rhodopsin phosphorylation assay with several distinct NCS proteins including recoverin, vilip1, *Ce*-NCS-1, *Ce*-NCS-2, NCS-1 and S-modulin. All six NCS proteins inhibit rhodopsin phosphorylation in a calcium-dependent manner (Fig. 3B, 3C). A control experiment with a ubiquitous calcium sensor such as calmodulin, or with ovalbumin had only a marginal effect (~10%) on rhodopsin phosphorylation (data not shown). In this assay, we found no difference between native myristoylated NCS and recombinant non-myristoylated NCS.

DISCUSSION

NCS-1 Highly Related Proteins: Branch II of the NCS Family Tree. The NCS family seems to evolve from a common ancestor gene (Fig. 2). The most spectacular branch of the phylogenetic tree is branch **II** with frequenin from drosophila, NCS-1 from chick, rat, mouse, and *Ce*-NCS-1 from C. elegans, since it represents a group of highly conserved sequences and homologous genes from very distant species. To date, *Ce*-NCS-2 is the most divergent sequence of the NCS family since it possess only 37-49% of identity when compared to the other NCS members. However, the *in vitro* function of *Ce*-NCS-2 is similar to the one we observed with all the other NCS proteins tested, suggesting that the calcium-dependent inhibition of phosphorylation may represent a shared property of the entire NCS protein family.

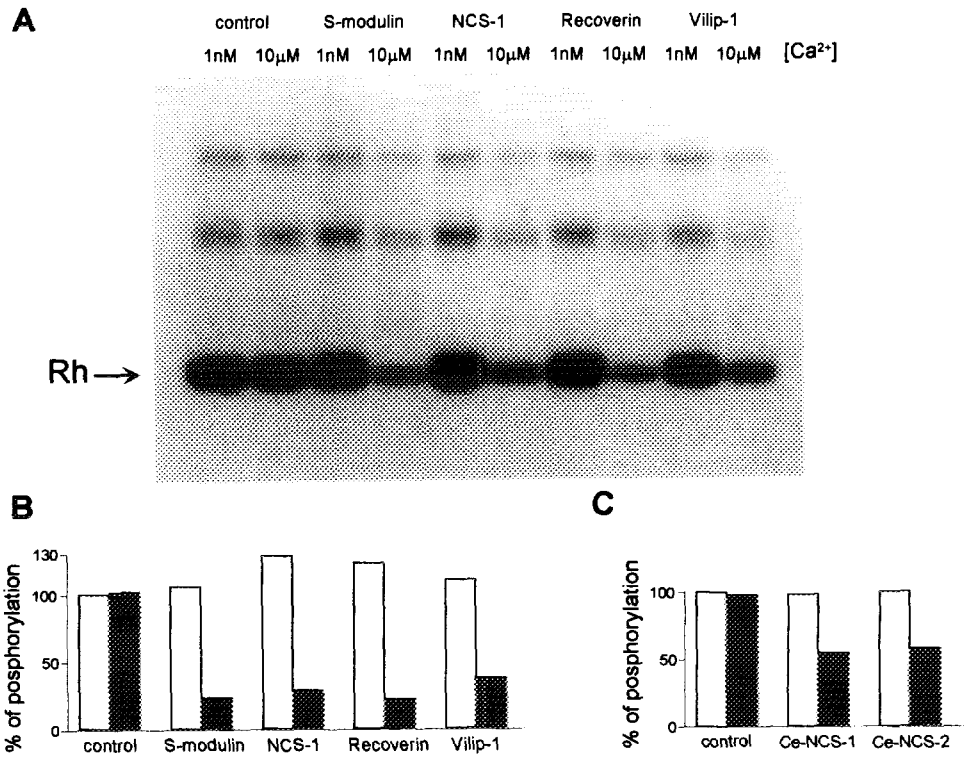

Figure 3. Ca^{2+}-dependent inhibition of rhodopsin phosphorylation by several members of the NCS family. A) ^{32}P-autoradiograph showing the inhibition of rhodopsin phosphorylation at high calcium (10 μM) with S-modulin, NCS-1, recoverin, and vilip1. Low calcium concentration was 1 nM. When present, recombinant NCS protein concentrations were 4-5 μM. No addition (control) means that no exogenous NCS protein was added. B) Graphical representation of the autoradiograph data shown in A. Values shown in B indicate percent of rhodopsin phosphorylation obtained with different NCS proteins at low (white boxes) or at high (black boxes) Ca^{2+} concentrations. C) Graphical representation of rhodopsin phosphorylation by *Ce*-NCS-1 and *Ce*-NCS-2 from C. elegans, or without NCS (control) at low or high Ca^{2+} concentrations (symbols as in B). Note that, in such an assay, calmodulin and ovalbumin have only a marginal effect (~10% of inhibition) on rhodopsin phosphorylation.

The NCS family: Inhibitors of Receptor Phosphorylation. Our *in vitro* assay reveals that, in addition to retinal-specific NCS, several distinct members of the NCS family expressed in neurons throughout the entire nervous system inhibit also rhodopsin phosphorylation *in vitro* in a calcium-dependent manner. Since hippocalcin has also been reported to have the same effect (27), there are 12 members out of 18 in the NCS family that share this property to date. Recent studies indicate that NCS may interact with members of the GRK family since: i) rhodopsin kinase, in vitro, is directly regulated by recoverin in a calcium-dependent manner (6, 7); ii) rhodopsin kinase is a member of the GRK family (28); iii) GRK5, a recently identified member of the GRK family, undergoes a rapid phospholipid-stimulated autophosphorylation (29). It is possible that the calcium-loaded form of NCS interacts with GRK5.

Conclusion. The principal finding reported here is the common functional characteristic of a family of calcium binding proteins present from human to nematode. The shared property of at least 12 NCS proteins, out of a total of 18 members, as *in vitro* inhibitors of rhodopsin phosphorylation suggests a potential role for NCS proteins in the calcium-sensitive phosphorylation of components of the signal transduction machinery.

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