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REVIEW ARTICLE

The neuronal calcium sensor family of Ca^{2+}-binding proteins

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Ca^{2+} plays a central role in the function of neurons as the trigger for neurotransmitter release, and many aspects of neuronal activity, from rapid modulation to changes in gene expression, are controlled by Ca^{2+}. These actions of Ca^{2+} must be mediated by Ca^{2+}-binding proteins, including calmodulin, which is involved in Ca^{2+} regulation, not only in neurons, but in most other cell types. A large number of other EF-hand-containing Ca^{2+}-binding proteins are known. One family of these, the neuronal calcium sensor (NCS) proteins, has a restricted expression in retinal photoreceptors or neurons and neuroendocrine cells, suggesting that they have specialized roles in these cell types. Two members of the family (recoverin and guanylate cyclase-activating protein) have established roles in the regulation of phototransduction. Despite close sequence similarities, the NCS proteins have distinct neuronal distributions, suggesting that they have different functions. Recent work has begun to demonstrate the physiological roles of members of this protein family. These include roles in the modulation of neurotransmitter release, control of cyclic nucleotide metabolism, biosynthesis of polyphosphoinositides, regulation of gene expression and in the direct regulation of ion channels. In the present review we describe the known sequences and structures of the NCS proteins, information on their interactions with target proteins and current knowledge about their cellular and physiological functions.

Key words: channels, frequenin, NCS-1, neurocalcin, phototransduction phosphoinositide, recoverin.

INTRODUCTION

Increases in intracellular Ca^{2+} concentration play a central role in the function of neurons by acting as the trigger for neurotransmitter release [1,2]. In addition, many aspects of neuronal activity, ranging from rapid modulations of channel function within a millisecond timescale [3] to long-term switches in gene expression, are controlled by changes in the cytosolic concentration of Ca^{2+} [4]. These various actions of Ca^{2+} must be mediated by Ca^{2+}-binding proteins which, in turn, interact with target proteins. It is well known that the ubiquitous Ca^{2+}-binding protein calmodulin [5] is involved in many aspects of Ca^{2+} regulation in many cell types, including neurons. However, many other EF-hand-containing Ca^{2+}-binding proteins have been identified. One subfamily of these, the neuronal calcium sensor (NCS) proteins, are expressed predominantly or solely in retinal photoreceptors or neurons [6], suggesting that they have specialized roles in these cell types. Some abundantly expressed neuronal Ca^{2+}-binding proteins are likely to act only as Ca^{2+} buffers, since they bind Ca^{2+} with high affinity but do not undergo any significant conformational change on binding of Ca^{2+} [7,8]. The NCS proteins, by contrast, bind Ca^{2+} with an affinity above resting free Ca^{2+} concentration and undergo substantial conformational changes on Ca^{2+} binding, consistent with activity as Ca^{2+} sensors and switches. Recent work has demonstrated the roles of the NCS protein family in phototransduction, regulation of neurotransmitter release, the control of cyclic nucleotide metabolism, control of gene expression, regulation of ion channels and in phosphoinositide metabolism. They are also likely to have other, as yet unknown, functions. Our aim in the present review is to focus on more recent findings on the biochemistry and function of these proteins. For the earlier literature and detailed information on their cellular distribution, we advise readers to consult a previously published review [6].

GENERAL ASPECTS OF THE NCS PROTEIN FAMILY

NCS proteins from many different species have been identified and sequenced, and a wide array of terminologies has been used to describe them. Unfortunately, in several cases the same protein has been cloned, sequenced and renamed several times, leading to considerable confusion, both in the literature and in sequence databases. It will be preferable in future, to avoid confusion, for the particular proteins under study in any publication to be identified by their accession number, which will allow unequivocal identification of the protein described. The availability of an almost complete human genome sequence allows the definition

Table 1 Members of the NCS protein family and classification into subfamilies

Class	Name of subfamily	No. of human genes	First appearance*
A	Frequenin (NCS-1)	1	Yeast
B	VILIPS†	4	Nematodes
C	Recoverins	1	Amphibians
D	GCAPs	3	Amphibians
E	KChIPs	3	Mammals

* Based on currently available sequences.
† Neurocalcins and hippocalcin.

Abbreviations used: CaMK, calmodulin-dependent protein kinase; DREAM, downstream-regulatory-element-antagonist modulator; GC, guanylate cyclase; GCAP, GC-activating protein; GRK, G-protein-coupled receptor kinase; KChIP, Kv-channel interacting protein; NCS, neuronal calcium sensor; NMDA, *N*-methyl-D-aspartate; PC, pheochromocytoma; VILIP, visinin-like protein; PI-4-K, phosphatidylinositol 4-hydroxykinase.

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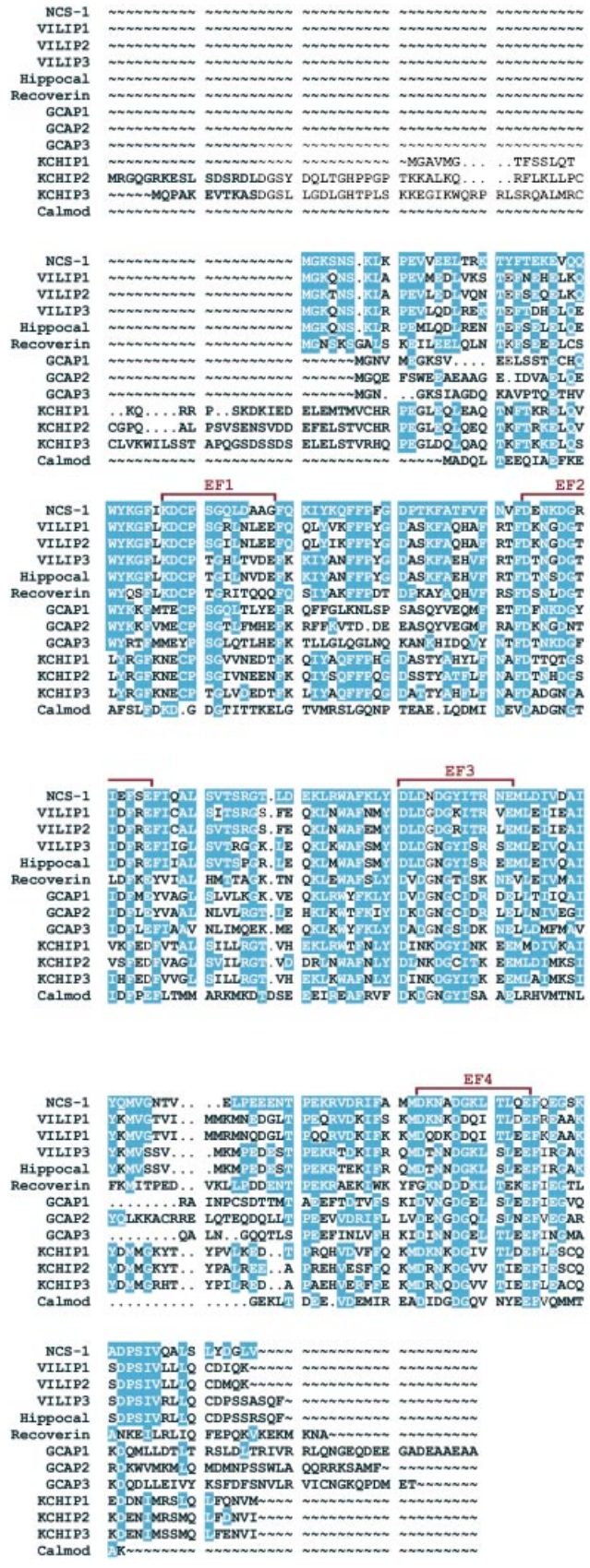

Figure 1 Alignment of the sequences of the human NCS proteins

The database accession numbers for the sequences are as follows: NCS-1, X84048; VILIP-1 (hpl3), AB001104; VILIP-2 (hpl4), AB001105; VILIP-3 (BDP-1), D16227; hippocalcin (BDP-2), D16593; recoverin, S43855; GCAP1, L36859; GCAP2, AF173229; GCAP3 AF110001; KChIP1, AF199597; KChIP2, AF199598; KChIP3, AF199599; calmodulin, J04046. Amino acids identical with those in NCS-1 are highlighted in blue. Two sequences for human NCS-1 in the GenBank® database have a predicted proline residue at position 178. This is not present in various human expressed-sequence-tag sequences, suggesting that it may result from a sequencing error. The positions of the EF-hand loops are indicated in red above the sequences.

of all NCS genes in humans by sequence. We have, therefore, classified the proteins primarily by reference to the human proteins and avoided names used in other species unless essential. The various names given for the NCS proteins in other species have been reviewed previously [6]. The members of the NCS family expressed in mammalian species and present in the human genome are listed in Table 1 in the order of their evolutionary appearance based on currently available sequences. Their sequences are shown in Figure 1, their phylogenetic relationships in Figure 2 and their degree of sequence identity in Table 2. The various members of the NCS family show 25–35 % identity with calmodulin. The primordial member of the NCS family appears to be frequenin. Despite these proteins having a predominant expression in the nervous system [6] and neuroendocrine cells [9] in higher organisms, a member of the family, recognizable as a

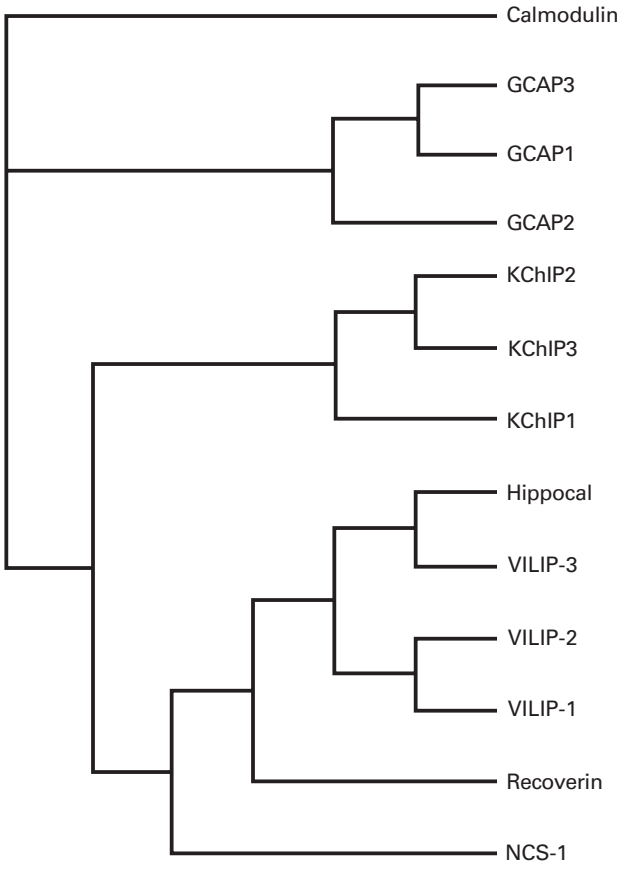

Figure 2 Evolutionary relatedness of the human NCS proteins

A consensus maximum parsimony tree based on the human NCS family members in Figure 1 is shown that was generated using the University of Wisconsin Genetics Computer Group version of the program PAUP [121]. The strict case of 100 maximum parsimony trees is depicted. The consistency index for each of the individual most parsimonious tree used to construct the consensus tree was 0.8.

Table 2 Comparison of the human NCS proteins

The Table shows the percentage of identical residues in comparisons of all of the human NCS proteins shown in Figure 1.

	VILIP-1	VILIP-2	VILIP-3	Hippocalcin	Recoverin	GCAP1	GCAP2	GCAP3	KChIP1	KChIP2	KChIP3
NCS-1	58.5	57.4	60.1	59.6	46.5	41.2	46.2	35.8	45.6	45.6	43.3
VILIP-1	–	88.5	67.7	67.7	46.0	41.7	42.3	36.9	46.3	43.4	41.1
VILIP-2	–	–	67.2	69.4	46.1	41.0	44.6	37.3	44.0	44.6	40.6
VILIP-3	–	–	–	91.7	50.5	46.5	47.8	39.5	43.2	42.6	42.0
Hippocalcin	–	–	–	–	53.5	45.6	45.8	38.2	43.8	43.8	40.9
Recoverin	–	–	–	–	–	37.2	34.5	32.0	35.6	36.2	36.4
GCAP1	–	–	–	–	–	–	49.7	50.3	38.0	36.9	33.5
GCAP2	–	–	–	–	–	–	–	38.8	36.2	35.2	33.3
GCAP3	–	–	–	–	–	–	–	–	31.3	33.1	31.3
KChIP1	–	–	–	–	–	–	–	–	–	70.7	69.9
KChIP2	–	–	–	–	–	–	–	–	–	–	68.6
KChIP3	–	–	–	–	–	–	–	–	–	–	–

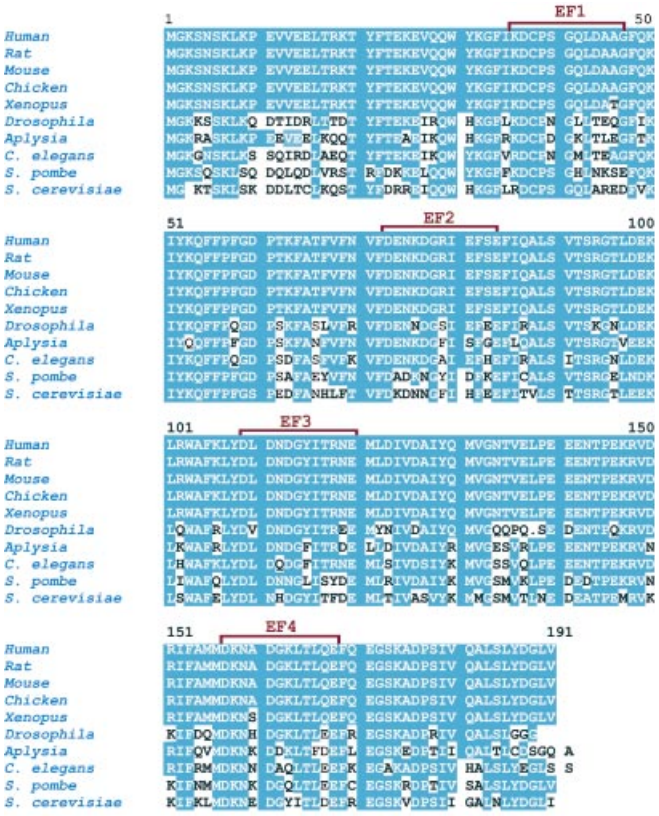

Figure 3 Sequence comparison of the frequenin/NCS-1 proteins from yeast to humans

The sequences of frequenins from human, rat, mouse, chicken, the frog *Xenopus*, the fruitfly *Drosophila*, the marine snail *Aplysia*, the nematode *C. elegans* and the yeasts *S. pombe* and *S. cerevisiae* were aligned. The database accession numbers for the sequences are as follows: human, X84048; rat, L27421; mouse, AF020184; chicken, L27420; *Xenopus* U27274; *Drosophila*, L08064; *Aplysia* U61222; *C. elegans*, L33680; *S. pombe*, CAA90589; *S. cerevisiae*, AAB64809. Amino acids identical with those in the human NCS-1 are highlighted in blue. The positions of the EF-hand loops are indicated in red above the sequences.

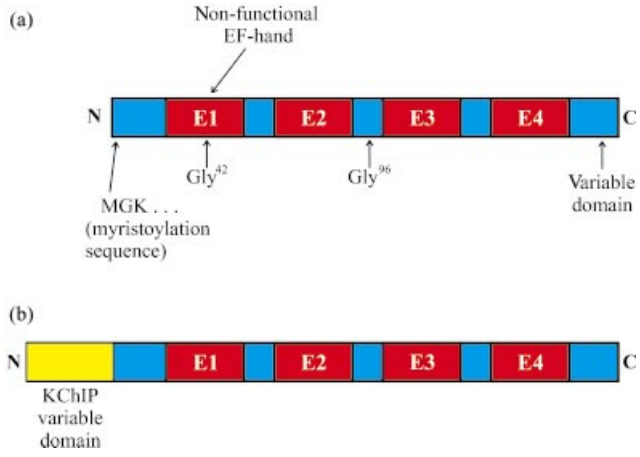

Figure 4 Schematic diagram of the domain organization of the NCS proteins

All members possess four EF-hands (red), of which EF-1 is non-functional. The KChIPs 2 and 3(**b**) have a variable N-terminal extension (yellow), while the other members of the family have an N-terminal myristoylation consensus sequence (**a**). Gly^{42} and Gly^{96}, which are conserved in all members of the family, are the sites of rotation following Ca^{2+} binding in recoverin.

frequenin [10], is expressed in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Frequenins (also known as NCS-1) from yeast to man show a high degree of sequence conservation, with the *S. cerevisiae* frequenin being 59% identical with the human protein (Figure 3). Frequenin and a single visinin-like protein (VILIP), neurocalcin, which is most similar to VILIP-3 (neurocalcin δ), are present in the *Caenorhabditis elegans* (a nematode worm) and *Drosophila* (fruitfly) genomes. In birds and mammals, four VILIPs are known (VILIP-1, -2 and -3 hippocalcin). In addition, other members of the family appeared later in evolution, including the recoverins and guanylate cyclase (GC)-activating proteins (GCAPs), which are expressed only in retinal photoreceptor cells and whose appearance coincided with the evolution of the vertebrate eye. Finally, a more diverse subfamily, the Kv-channel-interacting proteins (KChIPs), function in controlling a subclass of K^+ channels (the A-type K^+ channels), and are known so far only in mammalian species [11]. All members of the family possess four EF-hand domains (Figure 4). The EF-hand motif consists of a 12-amino-acid loop within which Ca^{2+} is co-ordinated with α-helices on either side [12]. The consensus sequence is defined in PROSITE (PDOC00018). In the NCS proteins only two or three of these motifs are able to

bind Ca^{2+} depending on the particular member of the family, as described below.

A considerable literature exists on the cellular localization of the neuronal NCS proteins, frequenin, hippocalin and VILIPs based on Northern blotting and immunohistochemistry. Use of antibodies generated against NCS proteins in localization studies is questionable unless care has been taken to establish a lack of cross-reactivity with other family members. In addition, comparison between studies has been difficult, owing to the differences in techniques used. A recent study, involving a parallel study using *in situ* hybridization to assess mRNA expression in the rat brain [13], has now determined which cells express the Class A and B NCS proteins. This has established that certain neuronal cell types express several or all (e.g. hippocampal pyramidal neurons) of these NCS proteins, but that the overall pattern of expression is unique for each protein. This suggests that, despite their high sequence similarity, the NCS proteins are likely to perform distinct functions specific for particular neuronal cell types.

THE IMPORTANCE OF NEURONAL CALCIUM AND THE ROLE OF NCS PROTEINS

Calmodulin is expressed ubiquitously in all the cells and tissues of eukaryotic organisms and, in addition, plays key roles in several aspects of neuronal Ca^{2+} signalling and regulation. Ca^{2+} entry through voltage-gated Ca^{2+} channels triggers exocytosis of synaptic vesicles and neurotransmitter release in a process that, in certain synapses, requires high (100–200 μM) free Ca^{2+} concentrations. These high concentrations will only be found close to Ca^{2+} channels at the sites of exocytosis [2,14,15]. It is noteworthy, however, that this might not apply to all synapses [16,17]. Changes in intracellular Ca^{2+} due to entry through channels or release from intracellular stores in neurons also mediates rapid modulation of ion-channel function [3], short-term changes in synaptic efficacy [18] and long-term changes in gene expression. The essential Ca^{2+} receptor responsible for vesicle fusion is not calmodulin, and currently the most likely candidate is the low-Ca^{2+}-affinity, C_2-domain-containing protein synaptotagmin [19,20]. Calmodulin is involved, however, in controlling synaptic-vesicle recruitment via activation of Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) I and II and the phosphorylation of the synapsin proteins on the synaptic vesicle. This phosphorylation leads to dissociation of synapsins from the vesicle, release of synaptic vesicles from a cytoskeletal attachment and vesicle movement to the presynaptic plasma membrane [21–23]. Calmodulin is important in many other aspects of Ca^{2+}-dependent modulation of neuronal function. These include post-synaptic changes during synaptic plasticity [24–26], alterations of gene expression [27], Ca^{2+}-dependent inactivation of voltage-gated Ca^{2+} channels [28–31], activation of Ca^{2+}-dependent K^+ channels [32–34] and modulation of glutamate receptor {*N*-methyl-D-aspartate (NMDA) [35] and metabotropic [36]} function. Some of these aspects are likely to be regulated by Ca^{2+} entry, but perhaps also by local Ca^{2+} changes of a lower magnitude following release from intracellular Ca^{2+} stores [37–39]. These effects of Ca^{2+} are often due to actions via CaMK II [22] and CaMK IV [27]. In the case of the regulation of Ca^{2+} and Ca^{2+}-activated K^+ channels and glutamate receptors, there is a direct interaction of calmodulin with the channel subunits [3].

The diverse actions of calmodulin raise the question as to why additional neuronal-specific EF-hand Ca^{2+}-binding proteins are required. Clearly the NCS proteins may have evolved to play specific roles that apply only for neuronal function, but it should be taken into account that a frequenin exists in yeast [10], suggesting some more generalized function. The answer may lie in the differences in properties of the NCS proteins and calmodulin. With the exception of KChIP2 and KChIP3, all the NCS proteins have N-terminal myristoylation consensus sequences. Several have been shown to be substrates in tissues and/or in *Escherichia coli* for *N*-myristoyltransferase, including recoverin [40,41], VILIP-1 [42,43], VILIP-3 (neurocalcin δ) [44–46], hippocalcin [47], NCS-1 [48] and GCAP-2 [49,50]. Myristoylation may be responsible for membrane targeting of the NCS proteins, which, in the case of recoverin, has been shown to be due to exposure of the myristoyl group following Ca^{2+} binding [51] (see below). Other NCS proteins can be membrane-associated, however, even at low Ca^{2+} concentration [48,49,52]. The second, perhaps more relevant, aspect is the high affinity of Ca^{2+} binding shown by all the NCS proteins so far characterized (Table 3). The affinity of Ca^{2+} binding to the KChIPs is, at present, unknown [11], but the majority of the family members show high-affinity Ca^{2+} binding *in vitro*. Binding occurs not far above basal Ca^{2+} concentrations (100 nM) and is half-maximal below 1 μM free Ca^{2+}. Ca^{2+} binding is also usually co-operative, with Hill coefficients of about 2 [9,40,44,47,49, 50,53–56]. One possible exception is VILIP-1 (neurocalcin α), which showed a lower Ca^{2+} affinity than NCS-1 in a direct comparison under the same conditions. VILIP-1 also did not show positive co-operativity of Ca^{2+} binding, although it still showed

Table 3 Ca^{2+} affinities of NCS proteins

The data are derived from a variety of assays as indicated.

Protein	Assay	Ca^{2+} affinity (μM)	Co-operativity (Hill coefficient)	Reference
NCS-1	Ca^{2+}-binding	0.3	Yes (2)	[53]
NCS-1*	Ca^{2+}-dependent increase in tryptophan fluorescence	<0.1	?	[48]
NCS-1	Phosphodiesterase activation	0.1	?	[54]
VILIP-1 (neurocalcin α)	Ca^{2+}-binding	1	No	[53]
VILIP-3 (neurocalcin δ)*	Ca^{2+}-binding	≈ 0.6	Yes (>2)	[44]
	Ca^{2+}-dependent increase in tryptophan fluorescence	1.3–1.7	Yes (1.8)	[44]
	Ca^{2+}-dependent membrane binding	0.75	Yes (2.3)	[44]
Hippocalcin*	Ca^{2+}-dependent membrane binding	0.6	?	[47]
Recoverin*	Ca^{2+}-dependent membrane binding	2.1	Yes (2.38)	[40]
GCAP1*	GC activity	0.26	Yes (?)	[55,56]
GCAP2*	GC activity	0.25	Yes (1.7–1.9)	[50]
GCAP3*	GC activity	0.25	Yes (?)	[55]

* Data from myristoylated protein.

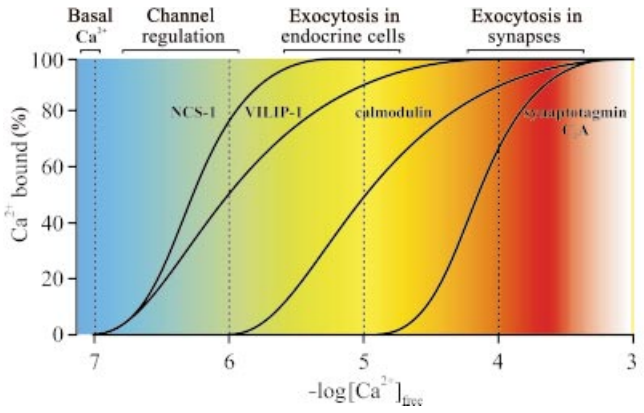

Figure 5 Comparison of the Ca^{2+}-binding affinities for various Ca^{2+}-binding proteins expressed in neurons

The data for NCS-1 and VILIP-1 (neurocalcin α) are derived from equilibrium-dialysis measurement of Ca^{2+} binding [53], as are the data for calmodulin [57]. In each case the binding was assayed in the presence of 1 mM $MgCl_2$. The data for the C_2A domain of synaptotagmin I were derived from fluorescence charge on Ca^{2+} binding in solution. In the presence of lipid, Ca^{2+} binding to the synaptotagmin I C_2A domain is shifted to higher affinity [122]. To put these affinities into context, the Ca^{2+} concentrations required for certain key neuronal functions are indicated.

half-maximal Ca^{2+}-binding at 1 μM free Ca^{2+} [53]. Assays using identical techniques for calmodulin showed it to have about a 10-fold lower affinity for Ca^{2+} [57,58]. It is known that calmodulin shows higher Ca^{2+} affinities when bound to target proteins and controls phenomena *in vivo* in the submicromolar range. Nevertheless, these *in vitro* data show a basic biochemical difference in potential Ca^{2+} affinities of these proteins. The significance of these Ca^{2+}-affinity values is that they indicate that NCS proteins could be close to being fully activated at intracellular Ca^{2+} concentrations that would produce activation of only a small fraction of calmodulin [54]. The presence of multiple Ca^{2+}-binding proteins in the same neuron with different Ca^{2+} affinities will, therefore, increase the dynamic range over which Ca^{2+} can regulate neuronal activities. This will allow responses to small changes over the resting Ca^{2+} concentration up to increases into the mid- to high-micromolar range (Figure 5). In addition, if certain NCS proteins are already membrane-targeted or associated with target proteins at resting Ca^{2+} concentrations, they will be poised to rapidly transduce small changes in Ca^{2+} concentration. These will be detected even if they are localized to submembrane domains close to Ca^{2+} channels on the plasma membrane or on intracellular Ca^{2+}-releasing organelles.

STRUCTURE OF THE NCS PROTEINS

Figure 1 shows a sequence alignment of the 12 NCS proteins present in the human genome. From this analysis we propose a classification into five subfamilies. A previous classification [6] has an additional class based on a single protein, NCS-2, expressed in *C. elegans*. NCS-2 has around 45–50 % identity with NCS proteins of other classes. There do not appear to be any orthologues of this divergent protein in mammalian or other species, suggesting that it is possibly a unique *C. elegans* protein. As full genomic data on several species are now available, we have taken an evolutionary standpoint and listed the subfamilies in order of their first appearance during evolution. On the basis of current information, frequenin (NCS-1) is the first member of the NCS family (see above). NCS proteins of classes A–D are all of a similar size, possess at least 50 % identity with each other and possess an N-terminal myristoylation sequence. In contrast the KChIPs (class E), found only in mammals, are more divergent and show a high level of sequence similarity over the core four EF-hand domains, but possess distinct N-terminal extensions. In two of them this replaces the myristoylation site. In all of the proteins, the first EF-hand is predicted to be unable to bind Ca^{2+} owing to the presence of a conserved Cys-Pro substitution in the Ca^{2+}-binding loop. This has been confirmed from structural analysis, which has shown that EF-1 is involved instead in interaction with the myristoyl group in the Ca^{2+}-free forms of the proteins (see below). In some proteins (class C) the fourth EF-hand is also unable to bind Ca^{2+}.

The three-dimensional structure of the NCS proteins and the conformational changes that occur on Ca^{2+} binding have been extensively characterized from a study of the bovine photoreceptor protein recoverin – the first NCS protein to be identified (Figure 6). Recoverin has only two functional EF-hands (EF-2 and EF-3), owing to the formation of an internal salt bridge in EF-4 [51]. Initially, the crystal structure of unmyristoylated recoverin with a single Ca^{2+} bound in EF-3 was solved [59]. Subsequently NMR spectroscopy was used to solve the solution structure of the myristoylated form in both a Ca^{2+}-free state [60] and also with two bound Ca^{2+} ions [51]. The Ca^{2+}-free form has a compact structure with the myristoyl group buried within a hydrophobic pocket formed from residues within EF-1 and hydrophobic residues contributed by other helices. It was also apparent that recoverin possesses two distinct domains with a bent linker between the N-terminal (EF-1 and EF-2) and C-terminal (EF-3 and EF-4) domains. Comparison of the Ca^{2+}-free form with the Ca^{2+}-bound form of recoverin [51] demonstrated extensive conformational changes due to Ca^{2+} binding. In the Ca^{2+}-bound form the myristoyl group has 'flipped out' into the aqueous solution, leaving an exposed hydrophobic surface (Figure 6) potentially able to interact with target proteins. In addition, the N-terminal domain is rotated by 45° relative to the C-terminal domain in the Ca^{2+}-bound form. The C-terminal domain is relatively unaffected, apart from small changes to accommodate the bound Ca^{2+} in EF-3.

The major conformational changes in Ca^{2+}-bound recoverin occur as a result of rotation of the backbone at residues Gly^{42} and Gly^{96}. These two residues are conserved in all of the NCS proteins (Figures 1 and 3), as are the hydrophobic residues that cradle the myristoyl group. It was suggested, therefore, that similar conformational changes should occur in other members of the family following Ca^{2+} binding [51]. In support of this idea, the crystal structure of bovine VILIP-3 (neurocalcin δ) [61] and the NMR-solved solution structure of GCAP2 [62] in the Ca^{2+}-bound forms are very similar to that of Ca^{2+}-bound recoverin. VILIP-3 (neurocalcin δ) and GCAP2 differ from recoverin in having three bound Ca^{2+} ions in EF-2, EF-3 and EF-4. The only structures currently available for these other NCS proteins are for the unmyristoylated proteins.

THE FUNCTIONAL SIGNIFICANCE OF N-TERMINAL MYRISTOYLATION OF THE NCS PROTEINS

The structural studies on recoverin [51], combined with data from biochemical assays [40,63], led to the concept of the so-called 'Ca^{2+}-myristoyl switch'. The idea is that Ca^{2+} binding would allow extrusion of the myristoyl group, which would, in turn, allow membrane association of recoverin as the myristoyl group inserted into the lipid bilayer. A key aspect of activation, therefore, would be Ca^{2+}-driven translocation to the plasma

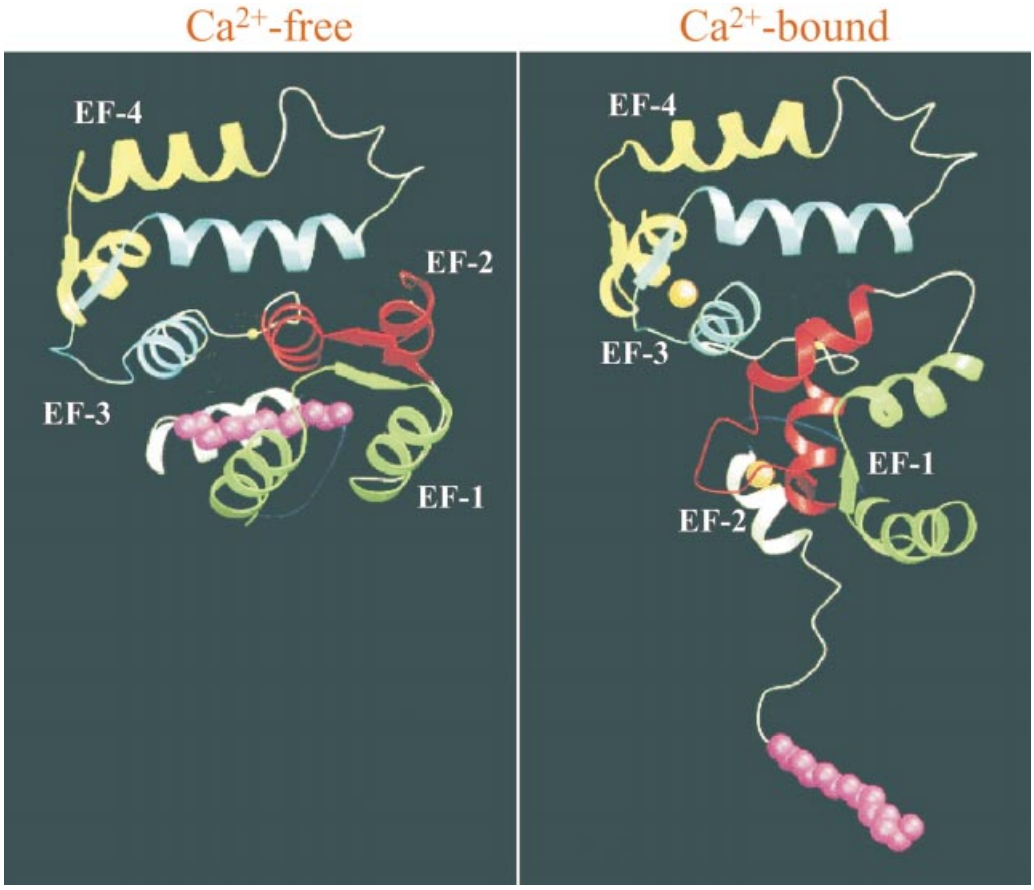

Figure 6 Structure of myristoylated recoverin in the Ca^{2+}-free and Ca^{2+}-bound forms

In the Ca^{2+}-bound form the myristoyl group is 'flipped out', and a major conformational change in the N-terminus of recoverin is obvious from a comparison of the two structures. Animated versions of these structures and those of other members of the NCS family that have been determined can be accessed at http://www.BiochemJ.org/bj/353/bj3530001add.htm

membrane. This interpretation fits data showing that recoverin only binds to membranes in a Ca^{2+}-dependent manner [40]. Other NCS proteins, such as VILIP-3 (neurocalcin δ) [44,46] and hippocalcin [47], show similar properties. This model may not, however, be applicable to all NCS proteins and, in fact, myristoylation of recoverin does not seem to be essential for its ability to inhibit rhodopsin kinase [64]. VILIP-1 translocates to membranes in a Ca^{2+}- and myristoyl-dependent manner, but significant amounts remain membrane-associated, even in the absence of Ca^{2+} [43,46,52]. Myristoylated NCS-1 is able to bind to membranes in the absence of Ca^{2+} [48], and an even more marked difference is shown by GCAP2, which is membrane-bound at low Ca^{2+} and dissociates as Ca^{2+} is elevated [49]. It will be interesting to know whether GCAP2 also extrudes its myristoyl group on Ca^{2+} binding, as would be predicted from its similarity to recoverin, and is exposed to the aqueous medium, as suggested by NMR studies [65]. If it does so, it will then pose a mechanistic riddle as to how extrusion of myristate in one protein leads to membrane association and to dissociation in another protein. One possible explanation is that the myristoyl group does not interact with the lipid bilayer but instead with membrane-associated proteins that behave differently in the case of recoverin and GCAP2. Another possibility put forward is that the behaviour of GCAP2 could be related to dimer formation [62]. Both recoverin and VILIP-3 (neurocalcin δ) are present as dimers in the crystal structures. Dimer formation could shield exposed myristate groups and allow dissociation of GCAP2. It is not clear, however, whether or not dimers form under physiological conditions. One study has shown that membrane-association or activation of GC by GCAP2 does not require its myristoylation [49]. In contrast, another study claims that myristoylation of GCAP1 is essential [66]. The exact role of myristoylation in the function of the GCAP proteins is, therefore, still something of a mystery. The KChIP2 and KChIP3 proteins (in contrast with KChIP1) do not possess a myristoylation consensus sequence [11], but may be membrane-associated owing to a direct Ca^{2+}-independent interaction with K^+ channels.

CELLULAR FUNCTIONS OF THE NCS PROTEINS

Regulation of retinal phototransduction

Two classes of NCS proteins, the recoverins and the GCAPs, are expressed only in photoreceptor cells of the retina in vertebrates and have key roles in phototransduction (Figure 7). In the well-studied pathway in rod photoreceptors, light activates rhodopsin that is coupled to the G-protein transducin [67,68]. The activated α-subunit of transducin stimulates phosphodiesterase, leading to the hydrolysis of cGMP. The fall in cGMP levels allows closure of the cyclic-nucleotide-gated cation channels and consequent hyperpolarization of the cells to reduce neurotransmitter release. This phototransduction pathway can be significantly modulated

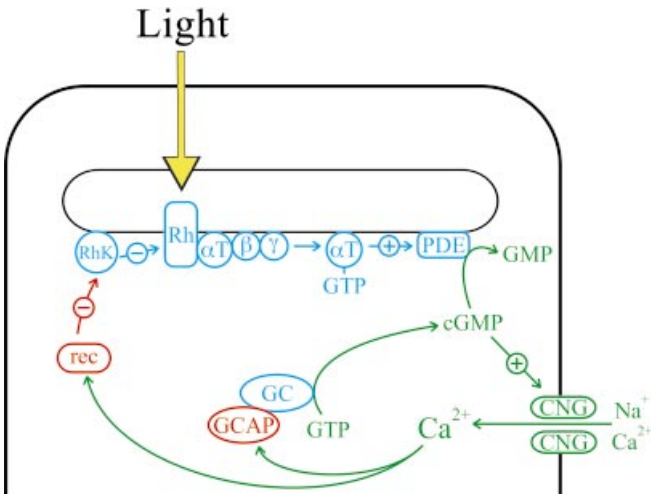

Figure 7 Regulatory roles of recoverin and GCAPs in retinal phototransduction

Light activates rhodopsin (Rh), which leads to the conversion of the α-subunit of transducin (αT) into the GTP-bound active form. αT stimulates phosphodiesterase (PDE), leading to a reduction in cGMP levels and closure of the cyclic nucleotide-gated (CNG) cation channels in the plasma membrane. At high Ca^{2+} levels in the dark, recoverin (rec) inhibits rhodopsin kinase (Rhk). Activation of GC by GCAPs occurs, by contrast, in the light, when Ca^{2+} is low, so that the increased cGMP concentration opens the CNG channels.

by Ca^{2+} in the process of adaptation, allowing phototransduction to be attuned to a wide range of illumination intensities. The search for the Ca^{2+}-binding proteins involved in adaptation resulted first in the discovery of the recoverins and then in that of the GCAP proteins.

Recoverins (known as S-modulin or visinin in certain species) were discovered in a search for activators of retinal GC [69–71]. As noted above, recoverin has been the model protein leading to the proposed Ca^{2+}-myristoyl switch mechanism. It is now clear that the function of recoverin is to regulate cGMP levels, not by a direct effect on GC, but instead by inhibiting rhodopsin kinase [72–76] by a direct Ca^{2+}-dependent interaction [64]. By using competing synthetic peptides and site-directed mutagenesis it has been established that a series of residues in the N-terminal region of the frog recoverin, S-modulin, are required for interaction with rhodopsin kinase [76]. These residues (Phe^{22}, Glu^{26}, Phe^{55} and Thr^{92}) are normally buried in the Ca^{2+}-free form of recoverin and are exposed on Ca^{2+} binding. Phosphorylation of rhodopsin by rhodopsin kinase is an inactivating mechanism, and so, at high cytosolic Ca^{2+} concentrations (as found in the dark), recoverin would prevent rhodopsin inactivation and thereby increase the lifetime of activated phosphodiesterase. Recoverin could act, therefore, to regulate light-sensitivity, but this has not yet been established as a physiological mechanism [77]. Several other proteins, including NCS-1, VILIP-1 [78] and *Drosophila* neurocalcin [45], have been shown to inhibit rhodopsin kinase *in vitro*, but this is unlikely to be of physiological relevance, as these are neuronally expressed proteins and rhodopsin kinase is retina-specific. It was suggested [79] that NCS proteins could be general regulators of G-protein-coupled receptor kinases (GRKs), despite the absence of any data apart from that for rhodopsin kinase (GRK1). VILIP-1 and -3 have been shown, however, to partially inhibit (by 30%) phosphorylation of the M_2 muscarinic receptor by GRK2 [80], but it still remains to be established whether the GRKs are genuine targets for NCS proteins.

The *bona fide* GC activators are the GCAPs. The interaction of GCAP proteins with the retinal GC isoforms GC1 and GC2 has been investigated in some detail over the past few years [49,50,55,56,65,81–85]. The GCAPs show unusual properties in that they activate GC when in their Ca^{2+}-free form, and, at higher Ca^{2+} concentrations, GCAP2 and GCAP3 (but not GCAP1), instead became inhibitors of GC activity [49,50,55]. This switch occurs over the range of free Ca^{2+} concentrations between 0.1 and 1.0 μM. GCAP activity would lead to an increase in GC activation in the light when cytosolic Ca^{2+} is low. Myristoylation of GCAP2 does not appear to be necessary for either stimulation of GC or membrane association [49]. In addition, unlike recoverin, GCAP2 dissociates from membranes when Ca^{2+} is elevated [49].

The importance of the GCAPs is that stimulation of GC after bleaching, when Ca^{2+} is low, is required to increase cGMP levels to inhibit the nucleotide-gated cation channel. The functional role of GCAPs in adaption of the phototransduction pathway has been discussed in a recent review [67] and will not be addressed in detail here. Evidence for the importance of GCAPs has come from analysis of mutations in humans that lead to retinal degeneration. In one such disease, in which the degeneration is cone-specific, this has been attributed to a single mutation of Tyr^{99} to Cys in GCAP1 [86]. The effect of this mutation is to modify the switch to the inhibitory function at high Ca^{2+}, so that GC remains activated at elevated Ca^{2+}. Binding of Ca^{2+} to the EF-3-hand domain appears to cause a significant conformational change in the region of Tyr^{99} [56] and this must therefore be important for the inhibition of GC.

The significance of the GCAPs as regulators of retinal GC has been clearly established [67]. It has been suggested recently that VILIP-3 (neurocalcin δ) may have a similar function [87]. The effect of VILIP-3 (neurocalcin δ) on retinal GC seems unlikely to be physiologically important, however, as it only activated at very high Ca^{2+} concentrations, with half-maximal activation at 20 μM free Ca^{2+}.

One aspect of the function of NCS proteins that has in general been little studied, with the exception of recoverin, is the nature of the residues and domains required for interaction with target proteins. The lack of information is mainly due to an absence of clear insight into specific targets for several of the NCS proteins. In the case of the GCAPs, domain-swapping or mutagenesis studies have been carried out to determine the requirements for GC interaction [84,85]. In addition, synthetic peptides have been used as competitors of the interaction [88]. These studies have not yet provided a clear picture. Both approaches have demonstrated the importance of the N-terminal 20 amino acids of GCAP1, but the significance of other domains has still not been resolved. Surprisingly, analysis of GCAP2 [85] showed that the extreme N-terminus was not an important specific region for the interaction, as it could be replaced by the N-terminus of recoverin or VILIP-3 (neurocalcin δ). The difference between GCAP1 and GCAP2 may not be surprising, as they show less than 50% identity and the conserved residues are mainly within the EF-hands. This suggests, therefore, that the molecular basis of the specific interaction with GC may differ for GCAP1 and GCAP2, although common regions close to EF-1 and in the C-terminal domain were required for GC activation by both proteins [84,85].

Frequenin and the modulation of neurotransmitter release

Frequenin was identified during the characterization of the molecular basis of neuronal hyperexcitability in a *Drosophila* mutant known as the V7 mutant [89,90]. This was studied in

parallel with mutants that had similar phenotypes and which were found to be due to the absence of certain functional K^+ channels such as in the *Shaker* mutants. In contrast, the V7 phenotype was found to be due to the fact that these mutant flies overexpressed the NCS protein frequenin. The V7 mutants or, alternatively, flies engineered to overexpress frequenin under the control of the heat-shock promoter, showed an impairment of neuronal branching [91,92]. From electrophysiological analysis, the striking consequence of frequenin overexpression was a marked increase in neurotransmission during a series of repetitive depolarizations [93]. An explanation for this would be that, as Ca^{2+} concentration built up during a train of depolarizations, frequenin would be activated and result in a facilitation of neurotransmitter release. The underlying molecular mechanisms for this action of frequenin in *Drosophila* have not been clarified. It has been suggested that it is due to changes in K^+ channel function [94] or $Na^+ \leftrightarrow Ca^{2+}$ exchange [95], but it is unclear if this is how frequenin acts.

Frequenins were subsequently identified in several other species [96–100]. Frequenin from the frog *Xenopus* was also shown to increase evoked neurotransmitter release at the neuromuscular junction following injection of recombinant protein into blastomeres [97]. Following identification of frequenin (also known as NCS-1) in birds [100] and mammals [98], it was also shown to regulate Ca^{2+}-dependent exocytosis of dense-core granules in the PC12 rat adrenal tumour cell line (PC is pheochromocytoma) [9]. This was based on an increase in evoked release in PC12 cells transfected to overexpress NCS-1. This effect was seen, however, only in intact cells stimulated by agonist, but not in permeabilized cells, where exocytosis was directly stimulated by Ca^{2+} addition [9,48]. These results suggest that frequenin/NCS-1 does not directly regulate the exocytotic machinery, but instead exerts its effects on the stimulus–secretion-coupling pathway, via changes in Ca^{2+} signalling or via modulation of plasma-membrane channels. Consistent with a role for frequenin in modulating neurotransmission, it has been found that, following the induction of long-term potentiation, a phenomenon involved in long-term increases in neurotransmission efficiency, the level of the message for frequenin increased [101]. NCS-1 is unlikely, however, to have a purely presynaptic function, as it is expressed in all neuronal compartments in the central nervous system and is not restricted to synaptic terminals [98,102].

One possibility that has been examined, as the basis of the effect of overexpression on neurotransmission, is that frequenin increased the activity of voltage-gated Ca^{2+} channels. This does not seem to be the case, however, as overexpression of wild-type NCS-1 in adrenal chromaffin cells had little effect on Ca^{2+} channel currents. In contrast, expression of an EF-hand mutant with a glutamate-to-glutamine mutation at position 120 in EF-3 [NCS-1(E120Q)], designed as a potential dominant inhibitor, increased the overall magnitude of Ca^{2+} channel currents [103]. From that study it appears that NCS-1 acts in a pathway required for an inhibitory regulation of Ca^{2+} channels in chromaffin cells. This is mediated by an autocrine feedback loop due to secretion of ATP and opioids that act on their respective G-protein-coupled receptors. NCS-1 appears to be required for this inhibitory pathway to function. In another study, overexpression of wild-type NCS-1 in neuroblastoma cells [104] reduced high-voltage-activated Ca^{2+} currents. This finding is consistent with the data from chromaffin cells, but overexpression also increased the magnitude of low-voltage-activated Ca^{2+} currents, indicating differential regulation by NCS-1 of different forms of voltage-gated Ca^{2+} channels.

Three NCS proteins have been described in *C. elegans* [78,105,106]. Two of these (Ce-NCS-1 and Ce-NCS-3) are homologues of frequenin, although Ce-NCS-1 shows the highest sequence identity with *Drosophila* frequenin (70% and 55% identity for Ce-NCS-1 and Ce-NCS-3 respectively). Null mutants of both Ce-NCS-1 and Ce-NCS-3 have been generated. The Ce-NCS-3 mutant had no detectable phenotype and this was attributed to redundancy [106]. The phenotype of the Ce-NCS-1 null mutant [105] has not yet been described in detail, but will hopefully be more informative about the physiological role of frequenin/NCS-1.

Frequenin as an activator of phosphatidylinositol 4-hydroxykinase (PI-4-K)

NCS-1 has been found to interact *in vitro* with multiple potential target proteins [48] and can activate purified calcineurin and 3′,5′-cyclic nucleotide phosphodiesterase on its own, and nitric oxide synthase in conjunction with calmodulin [54]. The physiological significance of these interactions is unknown, but another interaction has been confirmed to be essential *in vivo*. A single member of the NCS-1 family is expressed in yeast that is clearly an orthologue of frequenin. This gene was found to be essential for survival, owing to the requirement for frequenin as an activator of PiK1, one of the two non-interchangeable PI-4-Ks in *S. cerevisiae* [10]. This is a key enzyme in the pathway for synthesis of polyphosphoinositides and, therefore, for the maintenance of phosphatidylinositol bisphosphate levels. The physical interaction of yeast frequenin with PiK1 was Ca^{2+}-independent, but no data were provided on the Ca^{2+}-dependency of the activation of PiK1. The importance of frequenin for PI-4-K activation in other species is unknown, but frequenin has been shown to co-immunoprecipitate with the mammalian PiK1 homologue PI-4-Kβ from Madin–Darby canine kidney cells overexpressing rat frequenin [107]. It will be important to determine if this interaction is specific and whether or not other NCS proteins can interact with PI-4-Kβ. In addition, it remains to be determined whether PI-4-Kβ is activated by frequenin and if it is the major target for frequenin action in mammalian cells as it is in yeast. Some doubt has been cast on the importance of this interaction, due to a failure to demonstrate a direct interaction in nervous tissue [108], and so further work will be required to resolve this issue.

KChIPS and K^+ channel function

The A-type voltage-gated K^+ channels are expressed in brain and heart, where they are functionally important in controlling excitability. In an attempt to discover accessory subunits of the Kv4 A-type channel required for full function, the cytoplasmic domain of the channel was used in a yeast two-hybrid screen [11]. Three interacting proteins were identified and termed KChIP1, KChIP2 and KChIP3. These turned out to be members of the NCS family. The KChIPs had marked effects on A-type K^+ channel magnitude and inactivation kinetics and were found to be constitutive subunits of the channel required for normal activity. Their binding to the Kv4 proteins was not Ca^{2+}-dependent, but modulation of channel function was lost if EF-hands EF-2, EF-3 and EF-4 were mutated. The KChIP2 and KChIP3 proteins are unusual within the NCS family in not possessing an N-terminal myristoylation site and all three KChIPs have an extended and variable N-terminal domain. Surprisingly, deletion of this N-terminal domain from KChIP1 or KChIP2 did not prevent the modulation of the Kv4 channels, and so the functional significance of this domain is unknown. A further surprise is that KChIP3 is almost identical, at the nucleotide level, with the previously characterized protein DREAM

(**d**ownstream-**r**egulatory-**e**lement-**a**ntagonist **m**odulator) that functions as a Ca^{2+}-dependent repressor of transcription [109]. It will be important, therefore, to determine if this protein does indeed have two completely distinct physiological functions. In addition, KChIP3 is identical with the protein calsenilin identified as a presenilin-interacting protein [110]. Mutations in presenilin have been shown to result in familial Alzheimer's disease. The significance of the interaction with calsenilin is unknown, although it has been shown that calsenilin can prevent a potentiation of Ca^{2+} signals in *Xenopus* oocytes due to presenilin 1 [111].

NCS protein interactions with nucleic acids

Two NCS proteins have been found to interact in a Ca^{2+}-dependent manner with nucleic acids. One, DREAM, with DNA and the other, VILIP-1, with double-stranded RNA, indicating that NCS proteins may have roles more diverse than those mediated by protein–protein interactions.

A number of genes are regulated by a downstream-regulator element which acts in the repression of gene transcription. The NCS protein DREAM, which is related to KChIP3, binds to this regulatory element in the absence of Ca^{2+} and dissociates at elevated Ca^{2+} [109]. Its dissociation relieves the repression and allows transcription to occur. DREAM can act, therefore, as an activity-dependent regulator of gene expression. One important issue that remains to be resolved is the exact relationship between DREAM, calsenilin and KChIP3. The predicted protein sequences of calsenilin [110] and KChIP3 [11] are identical, but DREAM was predicted to have an alternative start codon with 20 additional N-terminal amino acids [109]. It is not clear, at present, whether this is due to sequencing error or alternative splicing, whether both of these proteins are expressed in cells, or whether the same protein has quite distinct functions.

Specific mRNA species are found in the dendrites of certain classes of neurons. In an attempt to find proteins that may determine this subcellular localization, VILIP-1 was discovered to be a double-stranded-RNA-binding protein that can recognize structured regions of mRNAs [112]. VILIP-1 binding was Ca^{2+}-dependent and was not detectable in the absence of Ca^{2+}. The ability of VILIP-1 to associate also with the cytoskeleton [52] resulted in the suggestion that this might provide a mechanism for the physical association of mRNAs with the cytoskeleton in an activity-dependent manner to modify mRNA localization [112]. It would be interesting to know whether other VILIPs can interact with double-stranded RNA or if this is a VILIP-1-specific function.

NCS proteins in the control of cyclic nucleotide levels

As noted above, the GCAP proteins control the levels of cGMP in photoreceptor cells directly by activation of GC. Recoverin does so indirectly by inhibition of rhodopsin kinase. The possibility that other NCS proteins have a general role in regulation of cyclic nucleotide metabolism has been discussed previously [6]. Certain non-retinal NCS proteins can modify cGMP levels in photoreceptor preparations [93], but it is not clear if this is physiologically relevant. Other studies have suggested, however, possible roles for NCS proteins in controlling both cAMP and cGMP concentrations. NCS-1 has been shown to stimulate 3′:5′-cyclic nucleotide phosphodiesterase [54], and stable expression of VILIP-1 in glioma cells produced an enhancement of cAMP production in response to β-adrenergic-receptor stimulation [42]. The effect of VILIP-1 was dependent upon myristoylation of the protein and appeared to be downstream of the receptors, as increased cAMP concentration was also seen when the adenylate cyclase was stimulated directly with forskolin. More recently it has been shown that VILIP-1 directly interacts with GC [113]. It is possible, therefore, that VILIP-1 may modulate cyclic nucleotide levels through direct interaction with both adenylate cyclase and GC. This interpretation is complicated by the opposing finding that VILIP-1 inhibits adenylate cyclase activity in membranes from olfactory neurons [114]. In contrast with these effects of VILIP-1, no effects of myristoylated NCS-1 were seen on cAMP or cGMP levels following its introduction into permeabilized adrenal chromaffin cells [48].

Hippocalcin and apoptosis

Hippocalcin shows very high identity with VILIP-3 (neurocalcin δ), being 91 % identical [115]. It appears that, in contrast with VILIP-3, it has a restricted distribution, being expressed at high levels in, for example, hippocampal pyramidal neurons [13,115,116]. This cell-specific expression is due to specific promotor elements in the 5′-untranslated region of the gene [117,118]. Recently hippocalcin was detected in a yeast two-hybrid screen for proteins that interact with the neuronal-inhibitor-of-apoptosis protein [119]. Expression of hippocalcin was found to potentiate the protection from apoptosis due to neuronal-inhibitor-of-apoptosis protein. It remains to be determined if this is the sole function of hippocalcin or if it has other specialized functions relating to the physiology of hippocampal pyramidal cells and the other neuronal cell types in which it is expressed. A yeast two-hybrid approach also detected an interaction of hippocalcin with the mitogen-activated protein kinase kinase kinase MLK2 [120]. The significance of this interaction was not established.

PERSPECTIVES

The first member of the NCS family (recoverin) was discovered 10 years ago, but, with the exception of recoverin and the GCAPs, their functions have only begun to emerge in the past couple of years. It seems that the NCS proteins as a group have diverse roles in regulating different aspects of neuronal function. Considerable additional work will be required to determine their full range of physiological activities. Even in the case of recoverin, its exact physiological role in light adaptation in retinal photoreceptors is not established. For other members only a single function has been discovered so far. For example, the KChIPs were discovered as specific regulators of A-type K^+ channels. This may not be their only function, however, as the closely related DREAM protein appears to be a regulator of transcription. Other members such as frequenin and the VILIPs have been shown to have particular physiological functions. Various data have shown, however, their ability to interact with multiple target proteins. In addition, some NCS proteins also interact with nucleic acid (with RNA in the case of VILIP-1 [112] and with DNA in the case of DREAM [109]). These findings suggest that the NCS proteins are likely to have multiple regulatory roles. Identification of the range of target proteins with which NCS proteins interact will be essential for further progress in the understanding of their regulatory functions.

Genetic evidence in support of proposed functions of NCS proteins is available only for frequenin and GCAP1. The most clearly established role for frequenin, therefore, is in the activation of PI-4-K in *S. cerevisiae*, and GCAP1 is clearly important in retinal phototransduction on the basis of its mutation in a human genetic disorder. The V7 mutant, in which frequenin is overexpressed, established this NCS protein as

playing some physiological role in the modulation of neurotransmitter release. Further genetic manipulation of NCS proteins by targeted gene disruption, or by expression of dominant-negative mutants, will be important in the future to allow precise determination of their respective physiological roles. The extent to which NCS proteins can functionally replace one another is unknown. Redundancy in gene knockouts may be a problem if the NCS proteins have overlapping functions, but a dominant-negative approach will provide information on the extent of any overlap. Since the NCS proteins are expressed by different subsets of neurons, effects on the function of specific identified neurons should be informative. In addition, clear information should be generated by genetic manipulations in simple organisms (e.g. *C. elegans* and *Drosophila*) that express only a limited subset of NCS proteins [105].

In 1999, the NCS proteins were described as "... proteins in search of a function ..." [6]. As we have outlined here, progress since then has resulted in a number of functions being revealed. It is clear, however, that NCS proteins could play multiple roles in many different regulatory pathways in neurons that are yet to be discovered. We expect that considerably more insight into their function is sure to emerge in the next few years.

Note added in proof (received 21 November 2000)

The structure of the *S. cerevisiae* frequenin has recently been determined using NMR [123]. The myristoyl group appeared to be solvent-exposed, even in the Ca^{2+}-free form, suggesting the absence of a Ca^{2+}–myristoyl switch mechanism for this protein and consistent with the ability of the mammalian frequenin to associate with membranes in the absence of Ca^{2+} [48].

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