FEBS Letters 533 (2003) 124–128 FEBS 26834

HmCRIP, a cysteine-rich intestinal protein, is expressed by an identified regenerating nerve cell

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Received 1 October 2002; revised 14 November 2002; accepted 19 November 2002

First published online 10 December 2002

Edited by Robert B. Russell

Abstract **A *Hirudo medicinalis* cDNA isolated from regenerating CNS tissue at 24 h post-axotomy was identified as a leech homologue of the mammalian cysteine-rich intestinal proteins (CRIPs) and named HmCRIP. HmCRIP is up-regulated within 6 h of axotomy, peaking at 24 h. This is the first demonstration of a CRIP homologue in regenerating CNS and in a serotonergic neurone. In rodents CRIP is an important factor in the regulation of the inflammatory immune response through control of Th1/Th2 differentiation. The role of HmCRIP in the regeneration competent environment of the annelid central nervous system is discussed.**

Key words: Leech; Central nervous system regeneration; Axotomy; LIM; Cysteine-rich intestinal protein

1. Introduction

Following trauma or experimental axotomy neurones in the mammalian central nervous system (CNS) do not regenerate, and their failure to reconnect to appropriate target cells leads to loss of function [1]. The devastating effects of traumatic spinal cord injury and brain damage have focussed attention on the mechanisms underlying the failure of CNS to repair. In contrast to CNS neurones, peripheral nervous system (PNS) neurones in mammals are able to regenerate. A comparison of the regenerative abilities of CNS and PNS neurones and the demonstration that CNS neurones will extend new processes when presented with a PNS graft [2,3] have led to the identification of factors in the glial microenvironment of damaged CNS neurones that are inhibitory for neurite outgrowth [4–7].

An alternative strategy for understanding nervous system repair is to study the molecular basis for successful repair in an animal whose CNS is regeneration competent. The leech *Hirudo medicinalis* has an impressive ability to repair damage to its CNS, and the ability of individual axotomised CNS neurones to survive injury, regrow processes and reform specific synaptic connections to restore function is well documented [8,9]. We have used identified neurones in this model organism to characterise the molecular events that follow nerve cell damage by isolating genes whose expression is altered following injury [10–12]. A serotonergic neurone cDNA library was screened using subtracted radiolabelled probes that we produced from regenerating and non-regenerating ganglia of leech CNS. This screen has demonstrated that a homologue of a human cysteine-rich intestinal protein (CRIP), HmCRIP (AY156993), is up-regulated following axotomy and is expressed in a serotonergic neurone. Quantitative analysis shows that HmCRIP is up-regulated by 6 h post-axotomy; its expression peaks at 24 h and remains at elevated levels up to 72 h post-axotomy. The CRIPs in mammals are involved with the modulation of the immune system. Their potential role in axotomy and regeneration is discussed.

2. Materials and methods

2.1. Subtracted probe production

Leeches (Ricarimpex, France) were anaesthetised in 0.2% chlorobutanol (Sigma), and the ninth ganglia axotomised as described previously [10,13]. Fifty ng of total RNA from ganglia at 0 and 24 h post-axotomy were used to produce cDNA using the SMART PCR cDNA synthesis kit (Clontech). Two rounds of forward and reverse subtractive hybridisation were conducted following manufacturer's protocols (cDNA Subtraction Kit, Clontech) to produce two cDNA probes, 'control' (transcripts enriched in non-axotomised ganglia) and '24 h' (transcripts enriched in regenerating ganglia).

2.2. Identification of differentially expressed genes

Twenty-five ng of each probe was radiolabelled with [α-^{32}P]dCTP to approximately equal specific activity using High Prime (Boehringer). A total of 1.25×10^5 pfu of an identified neurone (Retzius cell) library (Wang et al., in preparation) was screened following standard protocols [14]. Duplicate filter lifts of the library were hybridised with the subtracted probes at 65°C in Denhardts based buffer [14]. The filters were washed to 0.1×SSC, 0.1% SDS and exposed to X-ray film. Differentially expressed clones were isolated by comparing the hybridisation seen to each duplicate filter when probed with either control or 24 h cDNA. Clones of interest were excised as double stranded plasmids and sequenced using an ABI fluorescent sequencer.

2.3. Semi-quantitative analysis of gene expression following injury

Plasmids containing either HmCRIP or leech Elongation Factor 1-α (accession no. AAC03162) were PCR amplified using M13 and M13rev primers that flank the multiple cloning site of the vector. Amplicons were purified and diluted to a concentration of 100 ng μl^{-1}. One μl of this cDNA was spotted in duplicate on four individual nylon filters. The filters were then hybridised at 65°C with ^{32}P radiolabelled first strand cDNA probes produced as follows. The ninth segmental ganglia from five leeches was collected at each of the following time points: 0, 6, 24 and 72 h post-axotomy. RNA was isolated using the RNAqueous-4PCR extraction kit (Ambion, Austin, TX, USA). First strand cDNA was produced using SuperScript II reverse transcriptase whilst incorporating 1 μl of [^{32}P]dCTP (10 mCi ml^{-1}) to radiolabel the probes to high specificity. Probes were hybridised at 65°C overnight then washed to 0.1×SSC, 0.1% SDS. Hybrid-

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0014-5793/02/$22.00
doi:10.1016/S0014-5793(02)03741-9

isation intensity was quantified using the NIH IMAGE analysis package. Semi-quantitive data were obtained by comparison of the HmCRIP with an endogenous control gene leech Elongation Factor 1-α.

2.4. Sequence and phylogenetic analysis

The nucleotide sequence of HmCRIP was used to search the 'nr' database at NCBI using the BLASTx algorithm [15]. The conceptual ORF was obtained by translation of the nucleotide sequence in all six reading frames using the translate tool at Expasy (http://www.expasy.ch/) [16]. CRIP orthologues were detected using the tBLASTn option of the BLAST algorithm to search the EST_others database (non-human, non-mouse ESTs) at the NCBI. Conceptual translations of the ESTs were produced as above. The boundaries of individual LIM domain were determined using the SMART programme (http://www.smart.ox.ac.uk) [17] and the sequences trimmed to this region alone. The two LIM domains of human CSR2 (accession no. Q16527) [26], mouse CRP2 (accession NP_031818. 1) [27], the three LIM domains from *Schizosaccharomyces pombe* Leupaxin-like LIM domain protein (accession no. O74398) and two LIM domains from *S. pombe* GTPase-activator protein for Rho-like GTPase (accession no. O14014) [28] were also aligned. In each of these cases the numbering starts with the most N-terminal LIM domain. The LIM domain sequences were first aligned and boot strap values based on 1000 trials were calculated using Clustal-X [18]. Phylogenetic analysis was visualised using NJ-plot [19]. The Clustal-X alignments were coloured using the CHROMA programme [20].

3. Results

A clone of 360 bp identified as being up-regulated at 24 h post-axotomy was isolated from the Retzius library screen. The cDNA encoded a 78 amino acid glycine and lysine-rich protein. Using the BLASTx algorithm the highest scoring pair ($E = 7 \times 10^{-35}$) to the leech sequence was human cysteine-rich intestinal protein 1 (CRIP1, accession NP_001302) [21]. The leech clone was named *H. medicinalis* HmCRIP. Analysis of the HmCRIP protein using SMART showed that, like the human, rat and mouse CRIP's [22,23] the HmCRIP contains a single zinc-binding LIM domain. The translated HmCRIP amino acid sequence was used in a BLASTP search which isolated many proteins with high similarity to the LIM domain, but many were not true CRIP proteins. In order to identify potential HmCRIP orthologues other than the previously identified human, rat and mouse CRIPs, a search of the non-human, non-mouse, EST database at the NCBI was initiated. One hundred and eighty-three previously uncharacterised potential CRIP EST's from 28 different species were identified. Of these 28 species, 13 were from the Phylum Nematoda, one from the Arthropoda, one from the Annelida and 13 from the Chordata. Representative transcripts from each of the species were translated to amino acid sequence and the open reading frames aligned using Clustal-X. Representative mouse and human CRIP sequences were also included in the subsequent analysis. Fig. 1 shows a coloured alignment of potential CRIP orthologues. A high degree of similarity is seen across the different phyla. We note that the nematode sequences have two additional residues located between the fifth and sixth cysteines of the LIM domain, and also lack a conserved proline residue (Fig. 1). How this affects the binding of the zinc-finger domain is unknown. In addition to the high degree of similarity, the genes were considered HmCRIP orthologues because all conceptual open reading frames were of similar length (78–109 amino acids) and the C-terminal flanking region follows the consensus for the human CRIP glycine-rich region [23,24]. The previously characterised CRIP proteins are human, rat and mouse, although chicken, fish (*Lebistes reticularis*) and sea squirt (*Botryllus schosseri*) DNA has been shown to hybridise to a rat CRIP probe [22]. A *Caenorhabditis elegans* gene (accession no.

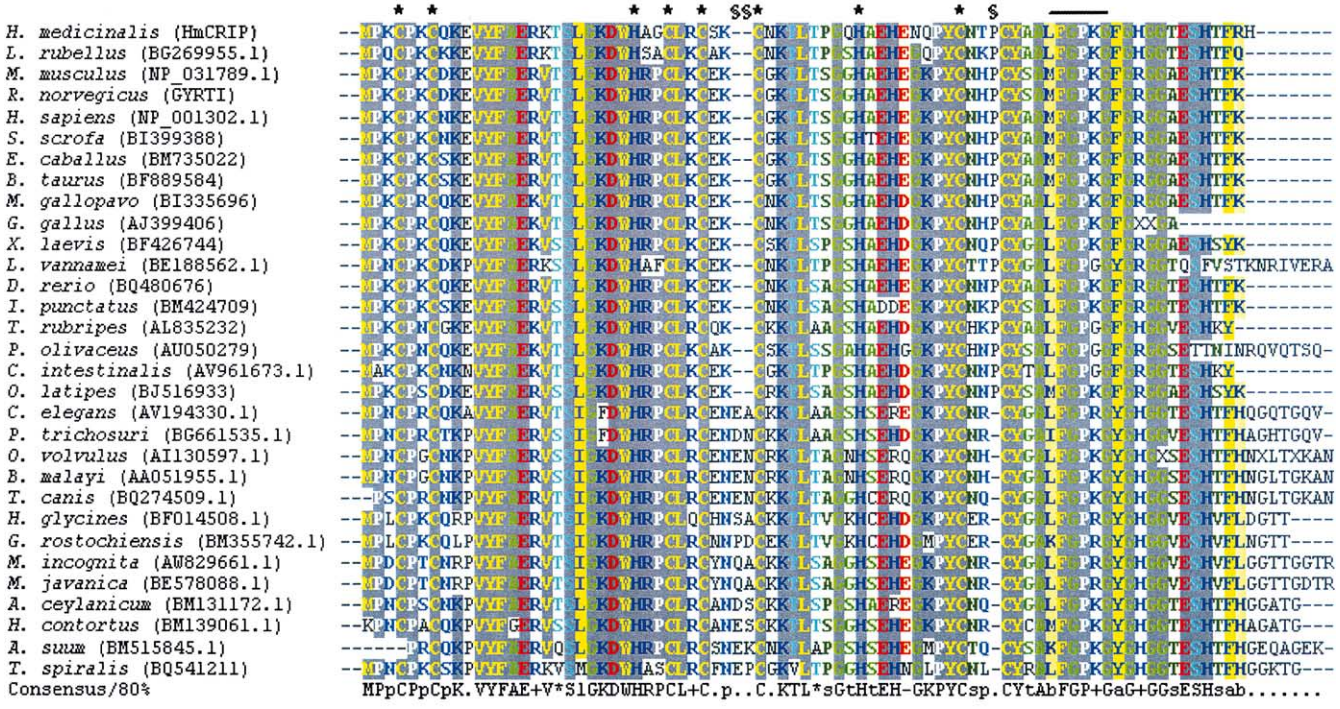

Fig. 1. Clustal-X alignment of the newly identified CRIP proteins. Conserved zinc-binding cysteine and histidine residues are highlighted with an asterix. The nematode specific differences are marked §. The conserved LIM domain sequence ((F/Y)GPKG) [23] is marked with a bar. Accession numbers for each of the sequences are given in parentheses. The alignment was coloured using the CHROMA programme [20].

AAA28023) has been annotated as a homologue of CRIP. Analysis using the SMART programme showed that this protein also contained Nebulin domains which bind actin [25] and a transmembrane domain. Thus AA28023 is a LIM domain containing protein, but not a CRIP orthologue. The newly discovered *C. elegans* EST (accession AV194330) may prove useful as an invertebrate model for study of the function of CRIP.

To investigate the evolutionary relationship of the new CRIP orthologues, the LIM domain from these 28 species plus human and mouse CRIP were aligned. The resulting phylogenetic tree shows that the proteins generally group according to taxonomic relationships, with the CRIP LIM domains separate from the non-CRIP LIM domain proteins (Fig. 2). This suggests that during the evolutionary history of the LIM domain, a domain duplication event produced the multiple LIM domain proteins from an ancestral protein with a single LIM domain, and that this ancestral form has subsequently been lost from certain phyla.

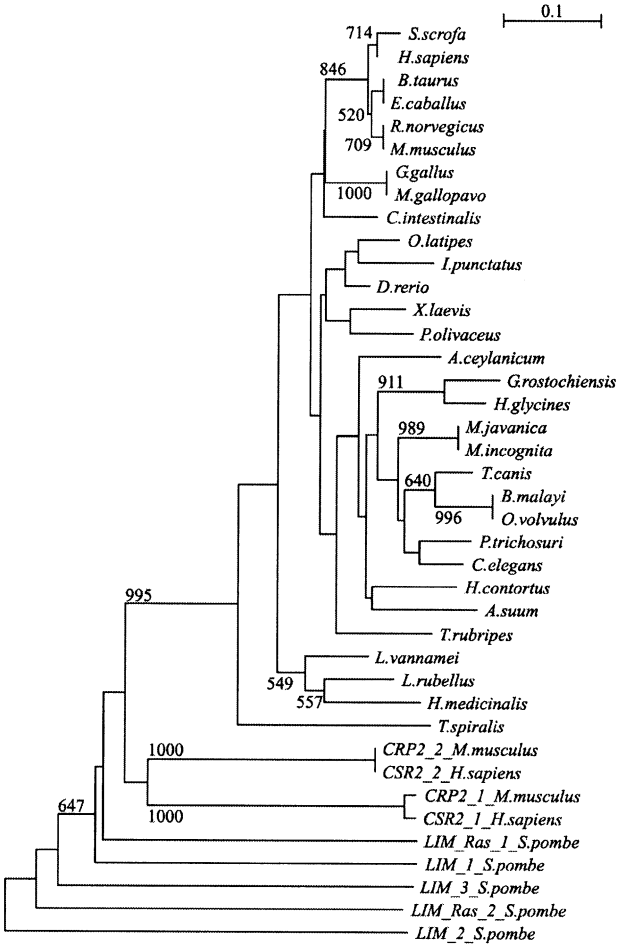

Fig. 2. Phylogenetic analysis of the LIM domains of identified CRIP proteins. Sequences were aligned using Clustal-X, bootstrap values are based on 1000 trials, bootstrap values greater than 50% are shown. The tree was produced using NJ-plot. LIM_RAs_n_S.pombe = *S. pombe* GTPAse activator protein (accession no. O14014), LIM_n_S.pombe = *S. pombe* Leupaxin protein (accession no. O74398), CRP2_M.musculus = *M. musculus* CRP2 (accession no. NP_031818. 1) and CSR2_H.spapiens = *H. sapiens* CSR2 protein (accession no. Q16527). n = LIM domain number as described in Section 2.

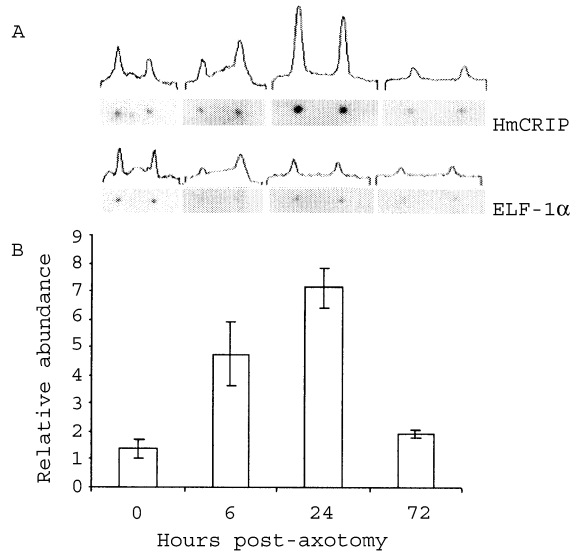

Fig. 3. Semi-quantitative gene expression dot blot. Nitrocellulose filters were dotted with duplicate spots of HmCRIP and leech Elongation Factor 1-α. The filters were probed with radiolabelled cDNA produced from five ganglia at appropriate time points. Activity was measured by densitomic analysis of exposed X-ray film (panel A). Relative abundance (panel B) was calculated as a ratio of HmCRIP expression to Elongation Factor 1-α (ELF-1α). Error bars represent one standard deviation.

The isolation of HmCRIP identified the clone as being up-regulated at 24 h post-axotomy. We subsequently analysed expression of the gene between 0 and 72 h post-axotomy. Semi-quantitative analysis of gene expression (Fig. 3) showed that up-regulation of HmCRIP could be detected at 6 h post-axotomy, when the level of expression was approximately three times greater than at time zero. The expression peaked at 24 h with levels approximately five times those at time zero, but dropped to nearly pre-axotomy levels by 72 h. As HmCRIP was identified in the serotonergic neurone (Retzius cell) library it would be interesting to determine if this specific neurone responds following injury. In in situ hybridisation experiments on leech ganglia, digoxigenin-labelled HmCRIP probes positively labelled many cells in addition to the Retzius cells (results not shown). Since digoxygenin-labelled probes do not give reliable quantitative data, future work will study the expression of HmCRIP by individual cells following injury using quantitative RT-PCR to determine whether different cells types differentially express HmCRIP in response to injury.

4. Discussion

The CRIPs are small zinc-binding proteins containing a single LIM domain [29]. The LIM domain comprises of a double zinc finger of the consensus sequence: C-X2-C-X16-23-(HCD)-X2-(CEH)-X2-C-X2-C-X16-21-C-X2-3-(CHD). It was first described in a set of three transcription factors Lin-11, Isl-1 and Mec-3, from which the name was derived [30–32]. Due to the plethora of proteins containing the LIM domain, schemes have been proposed to classify them into three groups [33]. Although the LIM homeodomain protein is abundant in developing CNS and is thought to confer motor neurone identity [34], as far as we are aware this is the first

demonstration that a LIM-only CRIP mRNA is expressed in regenerating CNS and in an identified nerve cell.

The CRIP1 protein was first described as an intestinal marker for the suckling-to-weaning transition in mice and rats [22]. However, our analysis has identified a much wider ranging taxonomic distribution than previously thought, encompassing the phyla Chordata, Arthropoda, Nematoda and Annelida. The LIM domain is wide spread in the Metazoa, Fungi and Viridiplantae kingdoms, but could not be detected in the Eubacteria or Archaea. The domain is therefore a relatively ancient structure that evolved after the divergence of the eukaryotes. Our phylogenetic analysis showed that the corresponding LIM domains of different proteins are more similar than two LIM domains within a single protein. This complements the findings of Tsui et al. [23], who compared the human cysteine-rich heart protein with rat CRIP.

Much work has focused on the role of the LIM domain as a mediator of protein–protein interaction [35–37], and on the LIM homeodomain proteins which are known to be important for cell fate specification and development [34,37]. The presence of the LIM domain led to CRIP being thought of as a protein that plays a role in the regulation of growth and differentiation of eukaryotic cells. The human form of CRIP is highly expressed in peripheral immune cells and over-expression of CRIP in transgenic mice modulates the expression of Th1/Th2 cytokines [38–40]. This immunological role for CRIP fits well with its high expression in the intestine [41], a well-established site of host protection and immunity [42].

How does a proposed immunological function fit with our findings of up-regulation of CRIP following nerve cell injury in the leech? The invertebrates are not thought to possess adaptive immune defence systems which rely on the possession of lymphocytes and antibodies [43]. However cytokine-like molecules have been characterised in the invertebrates [44,45]. A solution to this apparent anomaly has been proposed by Beschin et al. [46,47], who show that vertebrate and invertebrate cytokine-like molecules have evolved through convergent evolution, sharing similar structures, but are not homologous proteins, as is evident when analysed at the amino acid level. CRIP is different in this respect in that the amino acid sequences are highly conserved and HmCRIP and CRIP are certainly homologues.

As HmCRIP mRNA expression increases following CNS injury what cells could this signalling protein be affecting? Annelids possesses macrophage-like and natural-killer-like cells [48,49], and these cells become activated and show extensive migration following injury [50]. Could such cells be responding to damage response proteins such as HmCRIP? If pro-inflammatory signals are used to stimulate the activation and migration of these cell types, then one might predict that counter, anti-inflammatory control mechanisms also exist. It has been suggested that inflammatory and 'auto-immune'-like reactions following human injury lead to more damage than the original trauma [51]. The progression of conditions such as multiple sclerosis and experimental autoimmune encephalitis are thought to be moderated by the immune environment, such that pro-inflammatory Th1 type cytokines induce damaging autoimmune reactions, which can be reduced by the induction of a Th2 anti-inflammatory environment [52]. This Th1/Th2 modulatory role has been suggested for CRIP in rodents. The high degree of similarity between the leech and the human/mouse CRIPs would suggest that strong evolutionary forces have acted to maintain the function of the protein from annelids to mammals. HmCRIP is therefore a good candidate for a molecular switch controlling an invertebrate pro/anti-inflammatory response following injury.

A rapid expression of HmCRIP following injury (within 6 h) and a decline at 72 h post-axotomy would allow temporal regulation of the immune response following injury. If high levels of HmCRIP act to modulate the immune system as in rodents then an anti-inflammatory environment would be induced following axotomy, which may reduce cellular damage and promote regeneration.

The isolation of HmCRIP from the annelid *H. medicinalis* and the subsequent identification of orthologous genes in other invertebrate species, which are not thought to possess an adaptive immune system, is of interest from an evolutionary perspective. These findings suggest that either CRIP has an alternate role in the invertebrate species, or as we propose, that CRIP is an ancient gene expressed following cell damage which has been sequestered into the immune response repertoire of the chordates.

It would be of interest to follow the expression of CRIP following CNS injury in a mammalian species. This may shed light on the role of immune modulation in response to cellular insult, and how the control of the microenvironment affects capacity for CNS regeneration.

Acknowledgements: Supported by the Medical Research Council UK and The Human Frontiers Science Programme. We thank Chris Ponting for helpful advice and discussions.

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