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Acheron, a novel member of the Lupus Antigen family, is induced during the programmed cell death of skeletal muscles in the moth *Manduca sexta*

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Abstract

In order to identify novel genes associated with the initiation of programmed cell death during development, we employed a differential screening protocol to isolate cDNAs that were induced when the intersegmental muscles (ISM) of the moth *Manduca sexta* become committed to die at the end of metamorphosis. In this report we provide the first description of Acheron (Achn), a novel protein that was isolated in this screen. Acheron contains three Lupus antigen (La) repeats, nuclear localization and export (NLS and NES) signals, and an RNA recognition motif. Achn defines a new subfamily of La proteins that appears to have branched from authentic La protein relatively late in metazoan evolution. Achn is widely expressed in various insect, mouse and human tissues. Consistent with its expression during ISM death, Achn has been shown in separate studies to control muscle differentiation and apoptosis in both mice and zebrafish. These data define Achn as a newly discovered regulatory molecule that presumably mediates a variety of developmental and homeostatic processes in animals.

Keywords

apoptosis; 20-hydroxyecdysone; development; metamorphosis; gene expression

1. Introduction

Virtually any cell can be killed by noxious treatment with any of a wide variety of compounds or environmental perturbations such as heat, salts, or detergents. These insults typically induce necrosis, a passive form of cell death that usually begins with plasma membrane disruption

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that allows salts and water to passively diffuse into the cell leading to swelling and subsequent lysis (Szabo, 2005). The resulting liberation of cellular debris serves as a potent inducer of immunological responses that then result in the recruitment of phagocytic and inflammatory cells.

In contrast, most programmed cell death (PCD) occurs via suicide. PCD is an ATP-dependent process that requires signaling cascades that lead to the activation of proteolytic systems. Apoptosis, the best-characterized form of PCD, can be triggered by a wide range of physiological stimuli, including: hormones, loss of growth factors, or engagement of membrane death receptors such as CD95/Fas (Green, 2005). These diverse signaling cascades lead to the activation of aspartate cleaving proteases known as caspases, which in turn cleave a large number of structural and regulatory proteins (Lettre and Hengartner, 2006). Apoptosis results in cellular shrinkage, genomic DNA degradation and the partitioning of the cell into small membrane-bound apoptotic bodies. *In vivo*, the apoptotic bodies are rapidly phagocytosed by neighboring cells or circulating macrophages.

Other cells undergo PCD via autophagy, a physiological process that involves cellular protein and organelle degradation by the ubiquitin-proteasome and lysosomal pathways (Yorimitsu and Klionsky, 2005). Autophagy can allow cells to sustain themselves for long periods in the absence of nutrients and can even confer resistance to apoptosis.

While the degradative machinery that mediates PCD has been described in exquisite detail, relatively little is known about the signal transduction pathways that allow this process to be controlled in a lineage-specific manner. Identification of these upstream regulatory molecules not only provides insights into the control of development and homeostasis, but also may identify potential targets for therapeutic interventions to regulate cell death during pathological processes.

To gain insight into the molecular mechanisms that mediate PCD, we exploited the death of the intersegmental muscles (ISMs) from the tobacco hawkmoth *Manduca sexta* (reviewed in Fahrbach et al., 2005). The ISMs are organized as sheets of syncitial skeletal muscle fibers that attach at the borders between abdominal segments in the larva and provide the motive force for crawling. When animals pupate, the ISMs in the first two and last two abdominal segments undergo PCD. The persisting ISMs are then used by the developing adult moth to emerge from the overlying pupal cuticle at the end of metamorphosis, after which they undergo PCD during the subsequent 30 hours (Finlayson 1956; Lockshin and Williams, 1965). The timing of ISM death is controlled by changes in the circulating titer of the insect molting hormone 20-hydroxyecdysone (Schwartz and Truman, 1982, 1983). The ISMs initiate cell death coincident with the emergence of the adult moth from the overlying pupal cuticle at the end of metamorphosis (eclosion). During the subsequent 30 hours the ISM degrade their contractile apparatus, involute their membranes, and undergo an autophagic cell death (Beaulaton and Lockshin, 1977; Fahrbach et al., 2005).

In previous studies we have demonstrated that ISM PCD requires *de novo* gene expression and have cloned a number of genes that are induced when the cells become committed to die (Schwartz et al., 1990a; Fahrbach et al., 2005). Some of these induced genes encode well-characterized proteins such as: 20S and 26S proteasome subunits (Dawson et al., 1995; Jones et al., 1995; Sun et al., 1996), polyubiquitin (Schwartz et al., 1990b), and apolipophorin III (Sun et al., 1995). Others encode novel proteins whose role in death and development are poorly understood, including: SCLP (Small Cytoplasmic Leucine Rich Repeat Protein) (Kuetlzer et al., 1999) and DALP (Death Associated LIM-Only Protein) (Hu et al., 1999).

In the present report we identify a previously unknown protein that is induced when the ISMs become committed to die, which we have termed Acheron (Achn) after the river that leads to

the realm of the dead in ancient Greek mythology. Sequence analysis revealed that Achn is structurally related to the La/SSB (Lupus antigen/Sjögren Syndrome) protein but defines a distinct subfamily that is highly conserved across phylogeny. In studies, we have found that Achn is a key regulatory protein that controls differentiative decisions in several lineages, most notably muscle. Loss of Achn in either mouse C_2C_{12} myoblasts *in vitro* or zebrafish embryos results in the failure to form multinucleated myotubes. These data suggest that Achn defines a new family of regulatory proteins that is highly conserved across phylogeny.

2. Materials and methods

2.1 Animals

The tobacco hawkmoth, *Manduca sexta* was reared as previously described (Schwartz and Truman, 1983). In some experiments, animals were injected on day 17 of pupal/adult development with 25 μg of 20-hydroxyecdysone (20-E) (Sigma) in 10% isopropanol to delay ISM death (Schwartz and Truman, 1983). In all experiments, animals were rapidly dissected under ice cold saline at the developmental stages indicated and tissues removed from all adhering material prior to flash freezing in liquid nitrogen.

2.2 Isolation of Achn cDNAs

A day18 ISM cDNA library in the λZAP II vector (Stratagene) was screened by plus/minus screening as previously described (Schwartz et al., 1995). Differentially expressed recombinants were plaque purified, and the cDNA clones recovered within pBluescript vector by *in vivo* excision. One of these recombinants, termed *Acheron* (*Achn*), was selected for further analysis.

To isolate the human *Achn* cDNA, an oligo-dT and random primed human hippocampus cDNA library in the λZAP II vector was screened with a hippocampal EST (Genbank accession no. ***M79107***) that encodes a protein with high sequence identity to *Manduca* Achn. The initial human recombinant was truncated at the 5′-end but additional library screening and inverse reverse transcriptase PCR were used to obtain the missing sequence.

The genomic human *Achn* sequence was isolated by screening a human PAC library (RPCI1) cloned in the pCYPAC2N vector with a ^{32}P-dCTP-labeled human *Achn* probe (996 bp: nts 459–1454). Two positive PACs (304E10 and 261E2) were recovered and shown by sequence analysis to contain the human *Achn* gene. The intron-exon boundaries were identified either by direct sequence analysis of PCR amplified fragments from the PAC clones or by using a GenomeWalker kit (Clontech) to analyze restriction enzyme digested fragments from these clones. Data were confirmed by comparison by BLAST analysis of the human genome project database (http://www.ncbi.nlm.nih.gov/projects/genome/seq/BlastGen/BlastGen.cgi?pid=9558).

2.3 Analysis of Achn expression

Total RNA was isolated from various *Manduca* and mouse tissues as previously described (Schwartz et al., 1990a). For *Manduca*, Northern blots were performed using 15 μg of total RNA denatured in formaldehyde and separated in a 1.5% agarose gel. RNA was transferred to Zeta-Probe membranes (Bio-Rad) and hybridized at high stringency to ^{32}P-labeled cDNA clones. Blots were stripped and reprobed with a cDNA clone encoding the constitutively expressed ubiquitin-fusion gene *18-2* (Bishoff and Schwartz, 1990) as a loading control. RNA from various mouse organs was used as a template for reverse transcriptase PCR (RT-PCR) with random hexamers and 25 cycles with 5′GCAAGATGCTCCTG GTCTATGATC (sense) and 5′AGACACCCTTG CGCT GTGC (antisense) primers. The resulting cDNA was analyzed by Southern blot using a ^{32}P-labeled mouse *Achn* probe. *Actin* was used as an internal control.

Human RD rhabdomyosarcoma cells were maintained at 37°C in Dulbecco's modified Eagle medium (Gibco) supplemented with 15% fetal bovine serum, 100 IU/ml penicillin G and 100 μg/ml streptomycin. Total RNA was isolated using the Ultraspec™ RNA reagent (Biotecx Laboratories, Inc.) and poly A^+ RNA isolated using oligo(dT)-cellulose columns (Pharmacia). Northern blots were generated using 10 μg of poly A^+ RNA as described above and probed with a human *Achn* cDNA.

To analyze Achn expression at the protein level in mouse and human tissues, an anti-human Achn polyclonal antiserum was generated in rabbits. A fragment of *Achn* cDNA corresponding to the coding region 97–1398 was amplified by PCR and subcloned into the pET-25b(+) expression vector (Novagen) to produce a 434 amino acid fragment of Achn that also contained both HSV tag and 6X-His epitope tags. The fusion protein was expressed in *E.coli* BL21(DE3-pLysS) and purified by affinity chromatography on Ni-NTA agarose beads (Qiagen) under native conditions according to the manufacturer's instructions. Polyclonal antisera were generated in rabbits by injection of approximately 100 μg of gel-purified fusion proteins in complete Freunds adjuvant. Boosting was carried out with subcutaneous injections every two weeks with ~100 μg of proteins in incomplete Freunds adjuvant. Serum was collected after the fifth boost and pre-immune serum was collected as control.

Total proteins were extracted in Laemli buffer, fractionated by size by 10% SDS-PAGE and transferred to Immobilon P membrane (Millipore). Membranes were blocked in 5% nonfat dry milk in PBS/0.05% Tween, reacted with the anti-Achn antiserum at 1:2,000 and binding was detected with a horseradish peroxidase-labeled mouse anti-rabbit antibody and the ECL chemoluminescence Western blotting system (Amersham Pharmacia) followed by film autoradiography.

A human *Achn* cDNA lacking the first 31 amino acids was cloned in-frame to the FLAG epitope in the pFLAG-CMV-2 vector (Sigma). RD cells were transfected with linearized plasmid using Lipofectamine (GIBCO/BRL), and antibiotic-resistant clones were selected with G418 (500 μg/ml). Protein extracts were collected for Western blot analysis with anti-Achn antiserum and the anti-FLAG mouse monoclonal antibody (Sigma).

2.4 Structural and phylogenetic analysis

Homology analysis with nucleic acid and protein databases (Genbank, EMBL and SwissProt) including human EST databases was performed using the BLAST algorithm from the National Center for Biotechnology Information (NCBI, National Library of Medicine). Amino acid analysis for putative post-translation modification sites was performed with PROSITE (Hofmann et al., 1999). Domains and motifs were identified by applying: BlockSort (Henikoff et al., 2000), MyHits (Falquet et al., 2002), NetNes (la Cour et al., 2004), Pfam (Bateman et al., 2004), and ProDom (Servant et al, 2002; Corpet et al., 2000). Multiple alignment analysis was carried out by using the MultAlign software (Corpet, 1988) and Clustal W (Thompson et al., 1994).

The La domains of *Drosophila melanogaster*, *Homo sapiens*, *Manduca sexta*, and *Mus musculus* were identified and used as a basis for a phylogenetic investigation. These domains were subjected to a BLAST search of NCBI databases and proteins with greater than 70% similarity were identified. These proteins were aligned using the CLUSTAL W algorithm against the search queries and putative La domains were identified. The La domain from the human La protein was identified in a similar manner and used as a basis for a second BLAST search. Again, sequences with greater than 70% similarity were aligned as previously described to identify the La domain among the La family members. Twelve La domains from the putative Achn family and 15 from the La families (Table I) were subjected to a parsimonious bootstrap analysis with 1000 replicates using PAUP and dendograms were generated (Fig. 5B). The

groupings were confirmed utilizing a pairwise analysis and generation of neighbor joining trees in the same program. Values presented in the figure represent branch lengths between nodes.

3. Results

3.1 Cloning and expression of moth Achn

A differential screening protocol was utilized to isolate new death-associated genes from the intersegmental muscles (ISMs) from the tobacco hawkmoth *Manduca sexta*. In a screen of 1,200 individual recombinants, four up-regulated sequences were cloned: *apolipophorin III* (Sun et al., 1995), *SCLP* (Kuelzer et al., 1999), *DALP* (Hu et al., 1999) and *Acheron* (*Achn*), the focus of this study.

Northern blot analysis of ISM RNA demonstrated that *Achn* transcripts are approximately 2.4 kb in size and are undetectable until day 17 of pupal/adult development, just prior to the commitment of the ISMs to die (Fig. 1A). By the next day, the expression of this transcript increased greater than 10 fold and continued to be expressed well into the death period.

Expression of *Achn*, like ISM death itself, is under the control of the circulating titer of 20-hydroxyecdysone (Schwartz and Truman, 1983). Injection of 20-E on day 17 prevented both ISM death and the increase in *Achn* mRNA that is normally observed on day 18 (Fig. 1A and B).

To ensure that elevations in *Achn* expression were correlated with the commitment of the ISMs to die rather than just changes in circulating hormones, we examined *Achn* mRNA in a variety of insect tissues including: flight muscle, male accessory gland, ovary, Malpighian tubules, and fat body. *Achn* mRNA was most abundantly expressed in the ISMs (Fig. 1C). The presence of a low abundance higher molecular weight *Achn* transcript in the ISMs may reflect unprocessed message or an alternatively splicing product. *Achn* mRNA was also detected in fat body and to a lesser extent flight muscle. No *Achn* mRNA was detected in Malpighian tubules, male accessory gland or ovary. Since the ovary is composed predominantly of unfertilized oocytes, this data suggest that moth *Achn* is not a maternal transcript.

The moth *Achn* cDNA clone contained 2247 nucleotides (Genbank accession no. ***AAN76709***). Open reading frame analysis suggests that it encodes an N-terminally truncated protein of 395 amino acids. Structural analysis identified several motifs within the Achn protein, the most notable of which, the La domain, shared 30% identity and 54% similarity over 134 amino acids with the La motifs in the human La protein (Fig. 2A). The moth Achn protein also contains several putative regulatory sequences including: a bipartite nuclear localization signal (NLS) within the La domain at (amino acids 118–135), a nuclear export signal (NES) (amino acids 208–223), an RNA binding domain (also known as RNA recognition motif) (amino acids 148–254), two putative glycosylation sites (amino acids 73 – 76 and 164 – 167), and an amidation site (amino acids 354–357). In addition, moth Achn has a number of putative phosphorylation sites, including those for: casein kinase II (starting at amino acids: 2, 15, 25, 37, 51, 63, 121, 212, 225, 226, 266, 268, 333 and 349); protein kinase C (staring at amino acids: 10, 93, 95, 99, 120 and 321); cAMP/cGMP- dependent protein kinase (starting at amino acids: 12, 118, 282, 302, 322, 341, 356 and 385); and tyrosine kinases (starting at amino acids: 255 and 385).

3.2 Cloning and sequence analysis of human Acheron cDNA

By searching the human EST database with the *Manduca Achn* cDNA as a query, we identified a human hippocampal EST (Genbank accession no. ***M79107***) that encodes an amino acid sequence with 59% identity and 68% similarity over 86 amino acids to the predicted *Manduca* Achn protein. Using this cDNA as a probe, we screened a human hippocampus cDNA

library and isolated several positive clones, the longest of which was 1895 nucleotides. This cDNA clone contains a 5′ end with high GC content, a 3′-UTR of 513 bp complete with a polyadenylation signal, a poly A^+ tail, and a 1360 bp open reading frame encoding an N-terminally truncated protein of 460 amino acids.

Alignment of this predicted amino acid sequence with the *Manduca* Achn protein displayed an overall 31% identity and 40% similarity. Based on these results we conclude that this sequence is the human Achn (hAchn) ortholog. We then isolated the missing 5′ end region via RT-PCR with RNA from human RD cells. The fully assembled *hAchn* cDNA has a total length of 2056 bp and a predicted open reading frame of 1987 nucleotides (Genbank accession no. ***AAN76711***). (The HUGO Gene Nomenclature Committee has since designated Acheron as La ribonucleoprotein domain family, member 6 (LARP6) (HGNC no. ***24012***). However since was originally deposited in Genbank by us as Acheron, we have retained this name). The cDNA encodes one putative ATG translation initiation codon within a Kozak consensus sequence (GCCATGG) at nucleotide position 1 and a termination signal (TAA) at nucleotide position 1474. This sequence encodes a protein of 491 amino acids with a predicted molecular mass of 55 KDa.

Structural analysis of hAchn amino acid sequence revealed that the protein, like *Manduca* Achn, contains a highly conserved N-terminal La domain (ProDom 004143) spanning a region of 71 amino acids between 99–171 (Fig. 2B). In addition, hAchn like moth Achn contains a putative: RNA recognition motif (amino acids 184–296), amidation site (amino acids 351–354), nuclear localization signal (PKKKPAK) (amino acids 297–303), and nuclear export signal (LLVYDLYL) (amino acids 186–193). In separate studies we have demonstrated that the NLS and NES sequences are functional and regulate Achn movement in and out of the nucleus (Shao, R., Glenn, H. and Schwartz, L.M., unpublished). Like moth Achn, human Achn has numerous sequences with a high probability for phosphorylation by a variety of kinases, including: casein kinase II (starting at amino acids: 4, 56, 58, 72, 338, 340, and 408); tyrosine kinases (starting at amino acids 41 and 322); protein kinase C (staring at amino acids: 128, 134, 194, 229, 247, 358, 393, and 455); and camp/cGMP-dependent kinase (starting at amino acids: 168 and 244). It also has a number of putative glycosylation sites (starting at amino acids: 315, 335, 372, and 403). Unlike the *Manduca* Achn, hAchn has three SP repeats (SPxxSPxxSPxx; amino acids 376–385) characteristic of transcription factors of the NF-AT family (Masuda et al., 1995). SP domains are thought to provide a β-turn in proteins that allows them to interact with DNA (Suzuki and Yagi, 1991).

Database analysis revealed that another Acheron cDNA containing an additional 591 bases of 5′ UTR, but encoding the same full-length protein, has also been deposited in Genbank (accession no. ***NM_018357***). Northern blot analysis with RNA from RD cells verifies that the *hAchn* mRNA is about 2.3 Kb in size (Fig. 3A). While hAchn has a predicted molecular mass of about 55 kDa, Western blot analysis with RD cells revealed a band of about 65 kDa (Fig. 3B). This discrepancy could have resulted from either expression from an alternative start site or post-translational modification. To help resolve this issue, we replaced the first 33 amino acids of hAchn with an N-terminal FLAG tag. This would allow us to resolve both the native protein and the smaller ectopically expressed version in the same cells. Following transfection and Western blot analysis, two predominant hAchn bands were observed: 65 kDa (wild-type) and 62 kDa (N-truncated) (Fig. 3B). These data suggest that the predicted ATG is used for initiation and that hAchn may be post-translationally modified in such a way as to add approximately 10 kDa.

To determine the distribution of *Achn* mRNA expression in mammalian tissues, we performed semi-quantitative RT-PCR followed by Southern blot analysis with mRNA isolated from various mouse organs (Fig. 4A). *Achn* mRNA was highest in brain, with strong expression in

heart, skeletal muscle and testis. Lower levels were observed in thymus, spleen and kidney. Comparable results were obtained with analysis of human mRNA (data not shown).

In agreement with this analysis, Western blots of mouse tissues revealed that Achn protein was highest in heart and brain, intermediate in kidney, skeletal muscle and testis, and lowest in spleen (Fig. 4B). There appears to be a non-specific antibody trapping by actin (43 kDa) in the skeletal muscle sample, an artifact that we have observed with a number of other unrelated antisera. Several low molecular weight proteins were also detected in some tissues, most notably spleen, but the identity of these cross-reactive species is unknown.

3.3 Genomic mapping and structure of human Achn gene

Based on our genomic and cDNA sequences, as well as sequences available in the public domain, we determined the genomic structure of the human *Achn* gene. It spans a region of approximately 22,600 bp and resides at 15q23 on chromosome 15 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=retrieve&dopt=full_report&list_uids=55323). This mapping agrees well with the radiation hybrid mapping we performed with the Genebridge 4 panel of 93 radiation rodent hybrid clones of the whole human genome which placed *hAchn* at q22.3–q23 region of chromosome 15 (data not shown). The *hAchn* gene has two introns, although EST database analysis revealed three additional putative exons between exons 1 and 2 consistent with the existence of alternatively spliced isoforms. One of these encodes a protein with a predicted size of 93 amino acids (**NP_932062**) although its expression in tissues has not been confirmed

3.4 Phylogenetic analysis of Achn proteins

Full length mouse (Genbank accession no. ***AK017372***) and *Drosophila* Achns (Genbank accession no. ***CG17386***) respectively demonstrate 94% and 46% similarity with human Achn. While this dissimilarity might argue against an evolutionary relationship between the proteins, their La domains display an extremely high level of conservation. Over the 13 amino acids spanning between the La-1 and La-2 motifs (residues 111–123; KDAFLLKHVRRNK), there is 100% sequence identity between insects and mammals. This conserved region is termed the “Acheron motif I” (Figures 2 and 5). Two additional highly conserved motifs within the RNA binding domain were identified and termed “Achn motif II ” ([V/I]-R-[V/I]-L-[K/R]-P-G) at position 230–236 and “Achn motif III ”(C-A-[I/L]-V-E-[F/Y]) at position 258–263.

Database and phylogenetic analysis also revealed extensive similarity within the La domain in other La family proteins outside of the Acheron family. This similarity was observed in multiple organisms ranging from yeast to mammals. Fifteen proteins were identified with greater than 70% similarity to the La domain of the human La protein (Table I). These were aligned with the La domains of the putative Achn family proteins (Fig. 5A). Based on this alignment, a dendrogram was generated displaying possible phylogenetic relationships among all proteins (Fig. 5B). Phylogenetic analysis using the fungus *Yarrowia lipolytica* La-like protein as an out group reveals a significant relationship between Achn and the remainder of the La family. Achn clusters to form an entirely independent family among the La proteins with a high degree of confidence (bootstrap values of 81 for the La grouping and 96 for Achn). Pairwise analysis of known Achn and La proteins confirms this with a division of the Achn and La proteins in 100% of all cases.

4. Discussion

We exploited the ISMs from the moth *Manduca* as a model system in order to gain insight into the molecular mechanisms that mediate programmed cell death during development. In previous studies we found that the commitment of the ISMs to die requires *de novo* gene

expression (Schwartz et al., 1990a). In some cases DNA sequencing and database analysis allowed these differentially expressed genes to be functionally identified, thus implicating specific biochemical pathways in the regulation of PCD. For example, ISM death is accompanied by exponential increases in the expression of ubiquitin-proteasome components, including: polyubiquitin (Schwartz et al., 1990b) and 20S and 26S proteasome subunits (Dawson et al., 1995; Jones et al., 1995;Sun et al., 1997). Several of the other genes isolated from the ISMs encode novel genes whose roles are undefined, such as *SCLP* (Kuelzer et al., 1999). Achn, the focus of this study, represents another novel death-associated gene from the ISMs.

Achn mRNA was essentially undetectable in the ISMs prior to their commitment to die late on day 17 of pupal-adult development. *Achn* expression, like ISM death itself, is under the control of the circulating ecdysteroid titer. Artificial elevation of 20-hydroxyecdysone on day 17 delayed both ISM death and the *de novo* expression of death-associated genes, including *Achn*.

While *Achn* expression is associated with the death of the ISMs, it is presumably not a component of the death machinery itself since it is constitutively expressed in several moth and mammalian tissues that are not undergoing PCD. Instead, Achn is presumably a signal transduction protein that can regulate a variety of different homeostatic and developmental processes, one of which may be death. In support of this hypothesis, we have found Achn regulates myogenesis in both C_2C_{12} myoblasts *in vitro* and zebrafish embryos *in vivo* (Wang, Z., Glenn, H., Liu, X.J., Seth, A., Karlstrom, R.O., and Schwartz, LM., unpublished). Achn is permissive for the expression of the myogenic differentiation factor MyoD but represses expression of the related basic helix-loop-helix transcription factor Myf5. In myoblasts, this facilitates both the differentiation of myotubes and the death of surplus satellite/reserve cells. Morpholino knock-down studies in zebrafish embryos have revealed that the loss of Achn is associated with defects in other lineages as well, thus supporting its role in diverse developmental pathways.

How Achn regulates cellular physiology is unknown. It has functional NLS and NES signals that allow it to translocate in and out of the nucleus (Shao, R., Glenn, H. and Schwartz, L.M., unpublished), although it is unknown in which compartment(s) it normally functions. Like other La family proteins, Achn also contains a putative RNA binding domain and three La-like motifs. Authentic La protein binds to UUU-OH sequences in the 3′ end of nascent RNA polymerase III transcripts where it plays a role in processing and stabilization of small RNAs (Copela et al., 2006; Huang et al., 2006; Wolin and Cedervall, 2002). Several lines of evidence suggest that La protein modulates mRNA translation by binding to the 5′ terminal oligopyrimidine (TOP) tracts on ribosomal and other mRNAs and directly facilitates their binding to polysomes (Cardinali et al., 2003; Crosio et al., 2000; Meyuhas, 2000). TOP mRNAs rapidly and reversibly bind to polysomes under the control of mitogens and thus may play a key role in the developmentally regulated protein synthesis (Caldarola et al., 2004; Loreni and Amaldi, 1996).

While La is a non-essential protein in yeast, it plays an early and essential role in mammalian embryogenesis (Wolin and Cederval, 2002; Park et al., 2006). Homozygous deletion of the La gene in mouse results in failure to form the inner cell mass in embryos (Park et al., 2006). The reason for this defect is unknown, but La does exert control over both the cell cycle and survival at several key regulatory points in animals. La protein binds to MDM2 mRNA and enhances translation, resulting in a subsequent decrease in the levels of the tumor suppressor protein p53 (Trotta et al., 2003). As well, La can modulate the stability and/or translation of other apoptosis-associated proteins such as Reaper (Vazquez-Pianzola et al., 2005) and the inhibitor of apoptosis protein (IAP) (Holcik and Korneluk, 2000). While most of the studies of La function

have focused on La itself, it is interesting to speculate that Achn may actually mediate some of the *in vivo* properties ascribed to La.

Phylogenetic analysis of 27 La domain-containing proteins found in GenBank demonstrated that the Achn proteins define a new sub-family of the La proteins (Fig. 5B). However, the patterns of divergence between La and Achn proteins do not match precisely, suggesting one of several possible evolutionary events. The grouping of *Bos taurus, Pan troglodytes, Homo sapiens, Rattus norvegicus, Mus musculus,* and *Canis familiaris* in the Achn family is one example of this differing evolutionary divergence. The equivalent grouping among the authentic La proteins lacks *Mus musculus* and *Rattus norvegicus*. A significant amount of evolutionary divergence exists between these two rodent species, which is denoted by the large branch that separates them. It is important to note however that these two species still group firmly within the La protein subfamily rather than in the Achn subfamily. One possible explanation is directional selection on rodents. Alternatively, there could be some sort of systematic sequencing error in the rodent Achn sequences present in the NCBI database. However, the available data do not allow us to discriminate between these two possibilities.

In addition to differences in species grouping, the La and Achn families display dramatically different patterns of evolutionary divergence. The Achn family presents a classical divergence pattern that suggests a relatively constant rate of mutations among family members over time. In contrast, the La family displays a star phylogeny, characterized by a large number of branches diverging from a single node. This is consistent with a rapid diversification of the La gene at a particular time in its history. Two different mechanisms could account for these distinct evolutionary patterns. The first is that La and Achn are sister families, diverging at the same time from an ancestral gene and then undergoing differential selective pressures. Those exerted on La presumably favored a more rapid diversification than took place for Achn. A second possibility is that the ancestral gene was more similar to Achn and at some point in evolution it duplicated and split to form the La protein, which then rapidly diversified. The observation that the yeast and nematode genomes encode orthologs for La but not Achn supports the former theory as the most likely evolutionary history of Achn. Thus Achn represents a new family of conserved regulatory proteins that may play diverse developmental and homeostatic role in higher eukaryotes.

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Abbreviations

20-E
: 20-hydroxyecdysone

Achn
: Acheron

cDNA
: complementary DNA

DALP
: Death Associated LIM-Only Protein

ISM
: intersegmental muscle

La
Lupus Antigen

PCR
polymerase chain reaction

PCD
programmed cell death

RNA
ribonucleic acid

RT-PCR
reverse transcriptase PCR

SCLP
Small Cytoplasmic Leucine Rich Repeat Protein

SDS-PAGE
sodium dodecyl sulfate polyacrylamide gel electrophoresis

UTR
untranslated region

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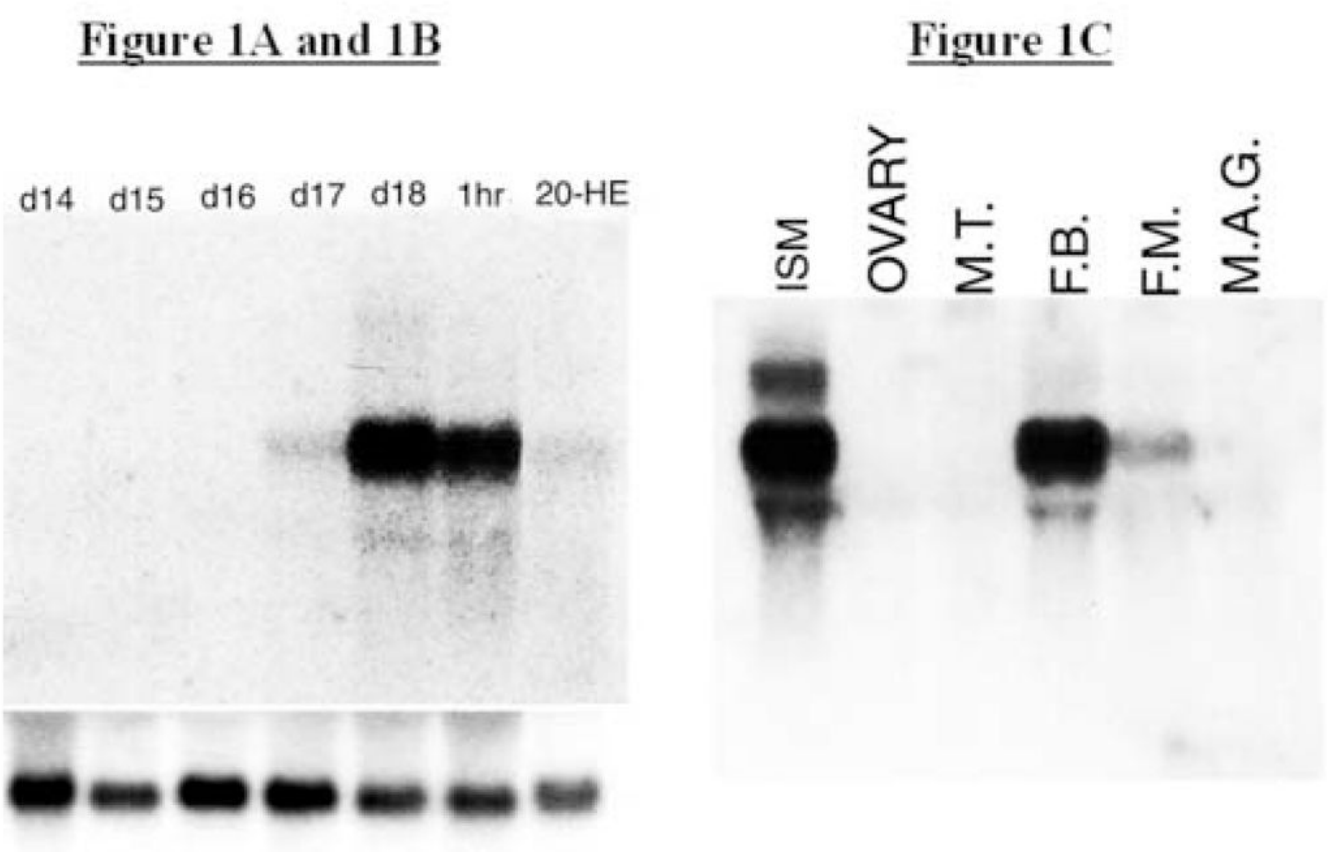

Figure 1. Expression of Achn mRNA in *Manduca*

(A) A developmental Northern blot of intersegmental muscle (ISM) RNA was hybridized with ^{32}P-labeled *Achn* cDNA (Genbank accession no. ***AAN76709***). ISMs become committed to die late on day 17 and begin to actively die during the hours following adult emergence late on day 18. RNA from the ISMs was collected on each day of development from day 15 until day 18 and after the onset of degeneration at 1 hr post-emergence on day 18. Treatment of day 17 animals with 25 μg of the steroid 20-hydroxyecdysone (20-E) delays death and the expression of death-associated transcripts when examined 1 hour post-eclosion (8). D = day of pupal-adult development; hrs = hours after adult emergence.

(B) The same blot was stripped and reprobed with the constitutively expressed ubiquitin-fusion gene, clone *18-2*, to verify that equivalent amounts of mRNA were loaded for each stage.

(C) RNA was extracted from several different moth tissues from animals on day 18 of pupal/adult development and used to generate a Northern blot. The membrane was probed with ^{32}P-labeled *Achn* cDNA. Abbreviations: ISM = intersegmental muscle; FM = flight muscle; FB = fat body; MT = Malpighian tubule; MAG = male accessory gland.

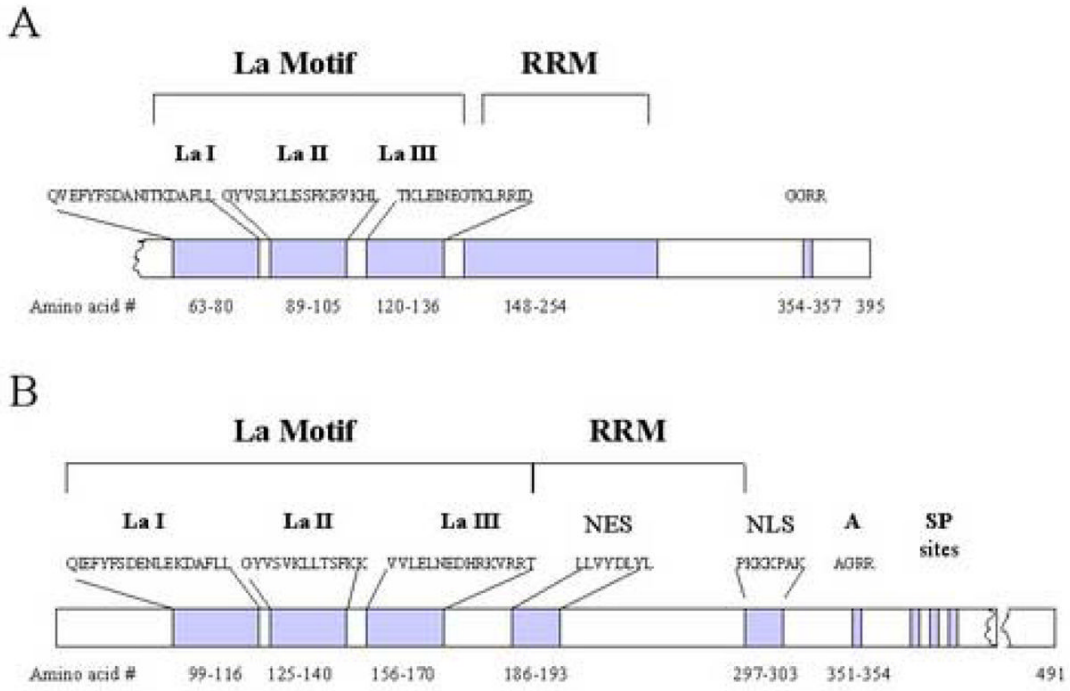

Figure 2. Protein Structure of Human Acheron

(A) Schematic representation of the N-terminally truncated *Manduca* Achn protein.

(B) Schematic representation of the full-length human Achn protein.

RRM = RNA recognition motif; NLS = nuclear localization signal; NES = nuclear export signal; A = amidation site; SP motifs. Note: distances in drawing are not to scale in order to accommodate inclusion of DNA sequences.

Fig. 3. Northern and Western blots of *hAchn* expression

(A) Northern blot of poly A^+ mRNA from RD rhabdomyosarcoma cells probed with the *hAchn* cDNA (Genbank accession no. ***AAN76711***). Mol. wt. markers are in kB.

(B) Western blot analysis of control and *hAchn* transfected RD cells. The first 33 amino acids of the *hAchn* open reading frame was replaced with a cassette encoding the FLAG epitope tag in-frame with the *hAchn*. Stable transfectants were selected and the proteins isolated for Western blot analysis with either anti-hAchn antisera (left) or a mouse monoclonal antibody directed against the FLAG epitope (right). Mol. wt. markers are in kDa.

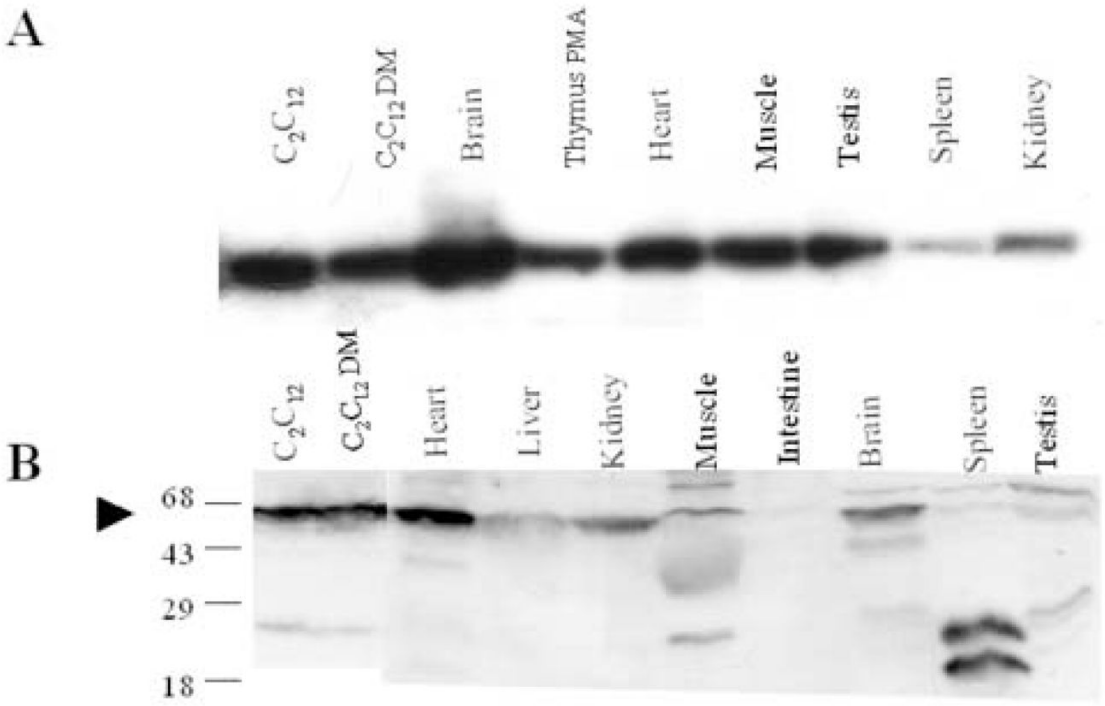

Figure 4. RNA and Protein expression of Achn in mouse

(A) Tissues were removed from adult mice and the RNA isolated. Semi-quantitative reverse transcriptase PCR was performed and the resulting products were fractionated in agarose, blotted to a nylon membrane and probed with a ^{32}P-labeled *Achn* cDNA.

(B) Tissues were removed from adult mice and the proteins isolated, fractionated by size in polyacrylamide, transferred to a nylon membrane and probed with an anti-hAchn antiserum. Achn is noted by the arrowhead. Mol. wt. markers are in kDa.

FIGURE 5A

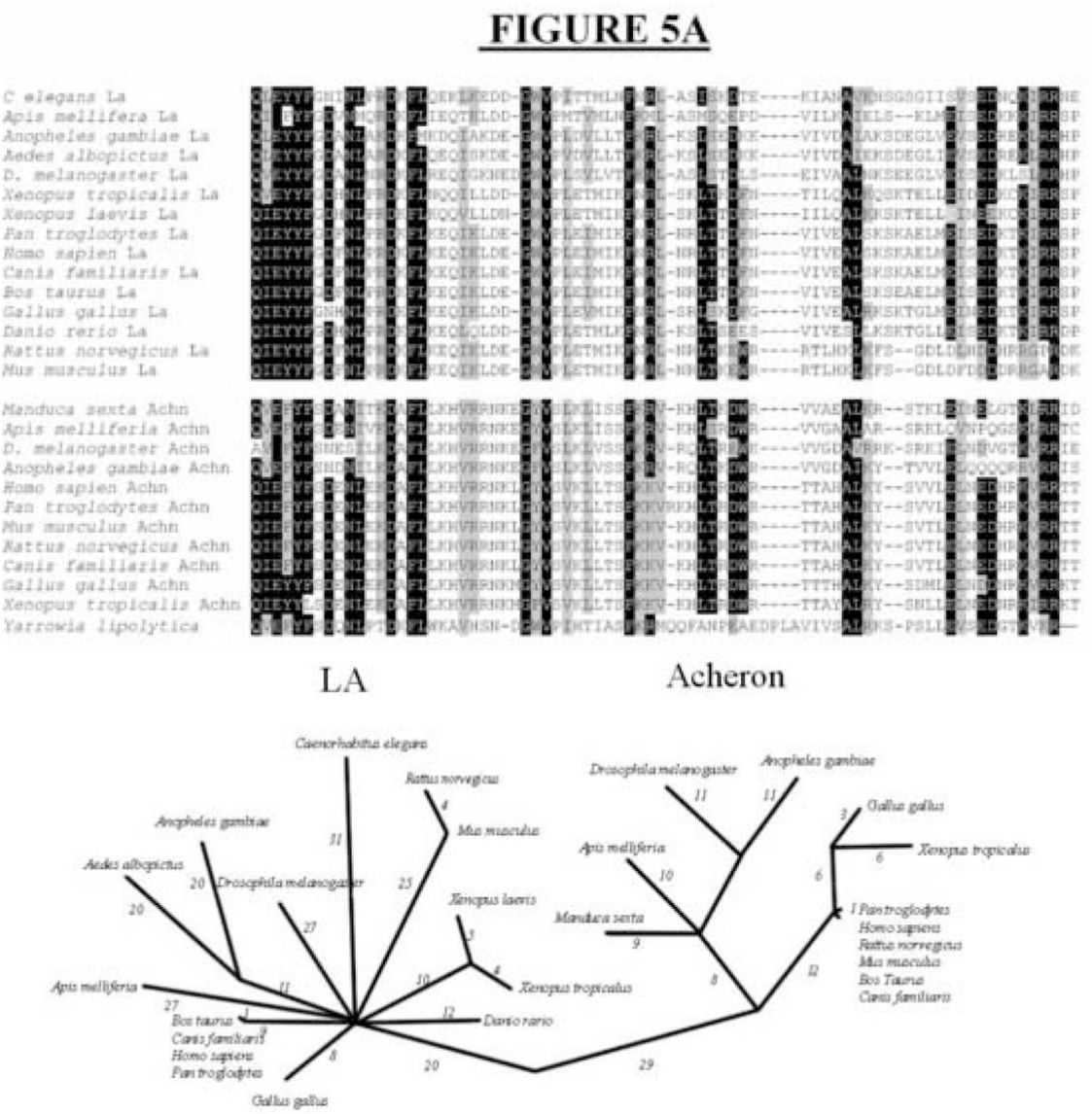

Figure 5. Alignment of Achn proteins from human, mouse, fly and moth

A. Comparison of the La domains from all known La-motif proteins.

Identical amino acid residues are highlighted in black box shading, whereas conservative amino acid replacements are presented in gray box shading. Gaps are introduced for optimal alignment. Accession numbers correspond to the proteins in the SwissProt-TrEMBL database (see Table I). Hum: *H. sapiens*, Mus: *M. musculus*, XEN: *X. laevis*, AEDAL: *A. albopictus*, Dro/DROME: *D. melanogaster*, mnd: *M. sexta*, Yeast: *S. cerevisiae*, SCHPO: *S. pombe*, EEE: *T. brucei*, EUP: *E. aediculatus*, ARA: *A. thaliana*, ORY: *O. sativa*, BRANA: *B. napus*, CAE: *C. elegans.*

(B) Phylogenetic analysis of the LA and Achn proteins

The tree was generated with the maximum parsimony criterion. The number near each node represents the branch length to the next nearest node. Note that Achn proteins represent an La protein subfamily distinct from the other La proteins.

TABLE I

ACHERON/LA PROTEINS

SPECIES	ACHN ACCESSION NUMBER	LA ACCESSION NUMBER
Apis mellifera	**XP_001120212**	**XP_395300**
Anopheles gambiae	**XP_315318**	**XP_319705**
Aedes albopictus	-	**Q26457**
Bos Taurus	**XP_611659**	**NP_788838**
Canis familiaris	**XP_544750**	**XP_535952**
Caenorhabdits elegans	-	**NP_491411**
Danio rerio	-	**AAH45392**
Drosophilia melanogaster	**AAX33496**	**P40796**
Gallus gallus	**XP_413777**	**NP_989911**
Homo sapiens	**AAH06082**	**NP_003133**
Manduca sexta	**AAN76709**	-
Mus musculus	**AK017372**	**NP_033304**
Pan troglodytes	**XP_510512**	**XP_001138021**
Rattus norvegicus	**XP_236338**	**NP_112381**
Xenopus laevis	-	**CAA48716**
Xenopus tropicalis	**XP_413777**	**NP_001001455**