

A Protein from the Salivary Glands of the Giant Amazon Leech with High Sequence Homology to a Nicotinic Acetylcholine Receptor Subunit[§]

Sancha Salgueiro^{a,*}, Susan Potts^a, E. Ann McIlgorm^a, Keith H. Ansell^a,
Jürgen Hemberger^b and Detlef Güssow^{a,2}

^a E. Merck Laboratory, MRC Collaborative Centre 1–3, Burtonhole Lane, Mill Hill,
London NW7 1AD, U. K. Fax (081) 906 1395.

^b Merck K GaA, BIOMED Fo Gbt, Frankfurterstr. 250, D-64271 Darmstadt, Germany

* Author for correspondence and reprint requests

Z. Naturforsch. **54c**, 963–971 (1999); received January 8/May 19, 1999

Haementeria ghilianii, RACE-PCR, Nicotinic Acetylcholine Receptor

A gene coding for a soluble protein with homology to the β subunit of the nicotinic acetylcholine receptor from goldfish was isolated from a cDNA library of *Haementeria ghilianii* salivary glands. Comparison of the leech protein sequence with the database showed that the N terminus has high homology with the extracellular portion of acetylcholine receptor β subunits, whilst the C terminus, highly charged, has homology to proteins which may be involved in chelating divalent cations. The leech protein was expressed in mammalian cells and the product compared to the native protein. Both proteins are glycosylated and form polymers which are disrupted by heat but not by reducing agents. A role for this protein in salivary gland secretion is suggested.

Introduction

The salivary glands of the giant Amazon leech *Haementeria ghilianii* have been used as a model of glandular secretion to investigate the relationship between electrical impulses and secretory events (Jones *et al.*, 1985; Wuttke *et al.*, 1989). The salivary system of *H. ghilianii* consists of two pairs of glands which lie at the base of the proboscis (Sawyer *et al.*, 1982; 1991). The cells from the anterior gland are not coupled, *i.e.*, the cells are not connected by gap junctions, nor do they connect in a common ductule (Jones *et al.*, 1985). The mechanism of release of secretions from the salivary gland cells is not known; secretions from specific cells seem to be discharged at discrete sites in the proboscis tip (Wuttke *et al.*, 1989). Electrophysiology studies indicated that the anterior salivary gland cells of *H. ghilianii* are electrically excitable and produce Ca^{2+} -dependent action potentials (Marshall and Lent, 1984; Wuttke and

Berry, 1988). Placing intact salivary glands in a Ca^{2+} -free solution causes the production of copious amounts of secretion from the proboscis (Wuttke *et al.*, 1989) but so far it was not possible to correlate the activity of a membrane protein with secretion. The secretions in the salivary system of *H. ghilianii* have been shown to consist of complex mixtures of proteins, some with anticoagulant activity, presumed to have a role in the feeding mechanism (Budzynski *et al.*, 1981; Sawyer *et al.*, 1991).

Although the mechanisms of secretion in the salivary system of this leech have been well studied from an electrophysiology point of view and there has been a volume of work on the biochemical characterisation of the secretion components, very little is known about the cellular mechanisms underlying the process of secretion. A suramin-insensitive membrane receptor activated by ATP has been shown to be involved in the regulation of intracellular Ca^{2+} (Wuttke *et al.*, 1996). It is likely that protein receptors will be involved in different aspects of the secretion mechanisms. While purifying a protein with anticoagulant activity from the Amazon leech salivary glands, a contaminant protein was detected in the final steps of purification. This protein was shown to have homology to the extracellular domain of one of the

¹ Present address: Biochemistry and Physiology Department, IACR Rothamsted, Harpenden, AL5 2JQ, UK

² Present address: Merck K GaA, BIOMED Fo Gbt, Frankfurter Str. 250, D-64271 Darmstadt, Germany

[§] The sequence presented in this paper has been submitted to the Genebank database and was given the Accession No. AF104404.



subunits of the nicotinic acetylcholine receptor (nAChR) from goldfish and its biochemical properties lead us to suggest that it may have a role in the secretion mechanism in *H. ghilianii* salivary glands.

Materials and Methods

Protein purification

Specimens of the giant Amazon leech, *Haementeria ghilianii* were obtained from Biopharm UK. Salivary glands homogenates were subjected to ultracentrifugation, ion-exchange and gel filtration in the E Merck laboratories (Darmstadt, Germany; method unpublished). Two proteins copurified in the same fractions: one was a protein with anticoagulant activity, the other was a protein with unknown function. This latter protein was analysed by matrix-assisted laser desorption ionisation-time-of-flight (MALDI-TOF) and shown to have a molecular weight (M. W.) of 26.5 kDa. The protein was digested with two different proteases and the peptides obtained were subjected to automated Edman degradation.

RNA preparation and RT-PCR (reverse-transcriptase-polymerase chain reaction) RNA was prepared from salivary glands using the method of Chomczynski and Sacchi (1987): freshly dissected salivary glands (including both anterior and posterior glands and the proboscis) were ground in liquid nitrogen and the powder mixed with 1 ml denaturing solution per 10 salivary gland pairs. Approximately 40 µg of total RNA was used as template for cDNA synthesis for RT-PCR reactions, as follows. First strand cDNA was synthesised with oligo d(T) as primer and Superscript II (Gibco-BRL), following the manufacturer's instructions. In some cases, messenger RNA (mRNA) was purified using a Poly(A)tract kit (Promega) and cDNA synthesised with random hexanucleotides (Pharmacia). This cDNA was used as template in PCR reactions with different primers, as described below.

Oligonucleotides 1 and 2 (Fig. 1, positions and orientation indicated by the arrows) were used at concentrations of 400 nM in PCR reactions, with 2.5 U of Taq polymerase (Perkin-Elmer). Each reaction contained 200 µM dNTPs (deoxy-nucleotide triphosphates), 10 mM Tris/HCl pH 8.0; 50 mM

KCl; 2 mM MgCl₂; 0.05% Nonidet P-40 (Sigma). The reactions were cycled 30 times at 95 °C for 30 seconds, 40 °C for 45 seconds and 72 °C for 2 minutes. After cycling, the entire reaction volume was spin dialysed through Sepharose CL6B (Sigma) and 1/20th of the volume was used as template for a second PCR reaction with the same conditions. Reactions were analysed by agarose gel electrophoresis and bands of the expected size were gel-purified using the Qiaex (Qiagen) gel purification kit and ligated into pCR II (Invitrogen) following the instructions of the manufacturer.

Oligonucleotides 3 and 4 (Fig. 1) were used in a 5'-RACE (rapid amplification of cDNA ends) PCR to clone the 5' region of the gene, using total salivary gland RNA as template for the cDNA reaction and the Amplifinder kit (Clontech), following the instructions of the manufacturer. Poly(A)tract-purified mRNA was used as template in a first strand cDNA reaction primed with an anchor-oligo d(T)18 (anchor is CCTCTGAAGGTTCCAGAATCGATAG). The cDNA was spin dialysed and used as template for a PCR reaction with oligonucleotide 5 (Fig. 1) and the anchor oligonucleotide to amplify the 3' region of the mRNA (3'-RACE). Oligonucleotides 6 (5') and 7 (3') (sequence underlined on Fig. 2) were used to amplify a 900 base-pair (bp) DNA product which was cloned into pCR II (Invitrogen). Two clones were sequenced in both directions using Sequenase (USB).

cDNA library

Salivary glands mRNA was purified using the Oligotex system (QIAGEN). cDNA was synthesised using the Great Lengths kit (Clontech), following the instructions of the manufacturer and ligated to CIP-*Eco*RI-cut λZAP arms (Stratagene). Ligated phage were packaged with Gold Giga Pack (Stratagene) according to the instructions of the manufacturer. 16 000 plaques of non-amplified library were screened using a riboprobe made from the 900 bp PCR clone representing the complete leech gene. The riboprobe, in antisense orientation, was made using the Promega transcription *in vitro* kit, and labeled with DIG-11-UTP (Boehringer). Duplicate GeneScreen (DuPont) filters were taken from each plate, treated with alkali and dried, as described in the manufac-

turer's instructions and pre-hybridized for 1 hour in 0.25 M Na₂HPO₄, 1 mM EDTA, 20% SDS at 60 °C. The probe (0.1 mg/ml solution) was added to the pre-hybridization solution and filters were allowed to hybridise overnight at 60 °C. Filters were washed three times for 20 min in 20 mM Na₂HPO₄, 1 mM EDTA, 1% SDS at 60 °C and developed as described in Engler-Blum *et al.* (1993). After three rounds of screening, 11 putative positive clones were purified to homogeneity. The plasmid was excised from the phage and digested with EcoRI. Digests were analysed by agarose gel electrophoresis and Southern blot, using positively charged nylon membranes (Amersham) and following the manufacturer's instructions. The blot was probed with the same probe used to screen the library. Eight of the excised plasmids had inserts which cross-hybridised with the probe. All clones were sequenced using the dideoxy termination sequencing kit from Amersham, following the manufacturer's instructions. Sequences were analysed using the DNASTAR package of programs for the Apple Macintosh.

Expression in COS cells

The insert containing the complete leech open reading frame (ORF), was cloned into a vector which had the human citomegalovirus (CMV) early promoter region, the Kozak consensus of initiation, a strong poly-adenylation signal (Enriquez-Harris *et al.*, 1991) and the C2 terminator (Ashfield *et al.*, 1991). Ten mg of recombinant vector was electroporated into 0.8x10⁷ COS7 subconfluent cells (V = 1.9 kV; C = 25 mF). Cell culture supernatants were filtered 72 hours post electroporation and analysed by polyacrylamide gel electrophoresis (PAGE). Transformed COS cells were washed with PBS and concentrated by centrifugation. The cell pellets were lysed by resuspending the cells in RIPA (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS) buffer with the "cOmplete"™ protease inhibitor cocktail from Boehringer (10 million cells/ml RIPA) and incubating 60 min at 4 °C with rotation. The lysis reactions were centrifuged for 5 min at 10000×g to clear the lysate from cell debris. Cell lysates were analysed by PAGE.

Polyacrylamide gel electrophoresis (PAGE) and Western blots

H. ghilianii salivary glands were homogenised in 20 mM HEPES, 10 mM Calcium buffer, pH 7.5 and sonicated. The homogenate was centrifuged for 15 min at 12000×g and the supernatant was collected. The pellet was extracted once more with the same buffer, the two supernatants combined and centrifuged at 100000×g for 60 min (Malinconico *et al.*, 1984). The supernatant of this centrifugation was analysed by PAGE using Laemmli's discontinuous gel system and loading buffer (Laemmli, 1970). Gels were blotted onto Immobilon membranes (Millipore). Membranes were blocked with PBS-T (phosphate buffer saline) (T = 0.1% Tween 20) containing 5% dried powdered milk for one hour at room temperature or overnight at 4 °C. Blots were incubated in PBS-T with primary rabbit antisera raised against the leech protein for 1 hour at room temperature. Following three washes of 10 min each with PBS-T, blots were incubated with anti-rabbit HRP (horseradish peroxidase) conjugate (Sigma) diluted according to the manufacturer's instructions in PBS-T and 0.5% BSA (bovine serum albumin). Blots were exposed to film after detection of enzymatic activity using the ECL system (Amersham).

Results and Discussion

Cloning of the leech gene

Products from two consecutive PCR reactions using cDNA to leech salivary gland RNA and the degenerate oligonucleotides 1 and 2 (Fig. 1) were analysed by agarose electrophoresis and a band of 80 bp was identified as having the expected size for the region coding for the N terminal peptide (Fig. 1). This product was ligated into pCRII and several clones were sequenced. A length of 43 bp of non-degenerate sequence was thus determined, which was used in further PCR reactions. Oligonucleotides 3 and 4 (Fig. 1), used in a 5' RACE PCR reaction amplified a 300 bp DNA region, which was cloned and sequenced. This sequence was found to contain the 5' non translated (NT) region of the message and the beginning of the ORF.

The 3' region of the mRNA was also amplified by PCR, using oligonucleotide 5 and an anchor oligonucleotide. These oligonucleotides amplified

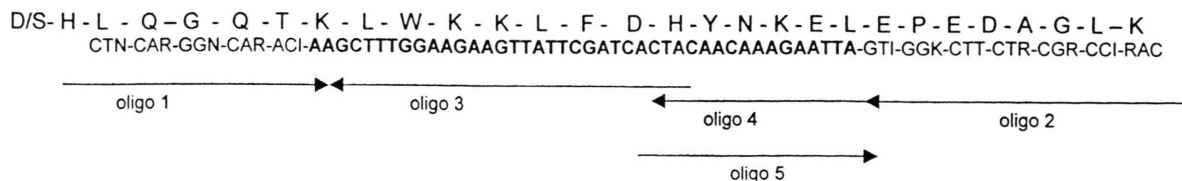


Fig. 1. Amino acid sequence of the N terminal peptide of the leech protein (first line). The second line represents the back-translated nucleotide sequence. The degenerate sequence of the oligonucleotides used for the initial PCR reactions is shown, as well as the non-degenerate sequence of the first clone (bold characters).

I = inosine. Arrows indicate the orientation and position of the oligonucleotides used in the PCR reactions (see text).

a 800 bp region which was cloned and sequenced. Using the sequence information of the clones described above, a PCR reaction was designed to amplify the complete gene. This reaction yielded a 900 bp DNA band which was cloned and sequenced. These clones contained an uninterrupted ORF (Fig. 2). The 900 bp PCR clone was used to synthesise a riboprobe to screen a λ ZAP cDNA library from salivary glands. Three of the selected clones were sequenced and shown to match the PCR clones.

The complete leech cDNA clone includes a 114 bp long 5' NT region and an ORF coding for a protein of M. W. approximately 27 kDa, which terminates with a TAA stop codon and is followed by 265 nucleotides of non-translated 3' region, including a poly-adenylation signal (Fig. 2). The first 19 amino acids in the ORF are likely to represent the signal peptide because they are not present in the mature protein sequence. This region has the characteristics of a signal peptide: it constitutes a very hydrophobic region with a small amino acid at position -1 and an isoleucine at position -3, all characteristics of signal peptides (von Heijne, 1986). The 27 kDa protein is probably processed and secreted as a 24 kDa protein.

The leech protein has homology to a subunit from an acetylcholine receptor

Comparison of the translated sequence of the leech gene with the Swiss and PIR and EMBL translated database showed that the cloned protein has homology (34.9%) to the β subunit of the nicotinic acetylcholine receptor from goldfish (Fig. 3A). The region of highest homology was between the extracellular region of the goldfish β subunit and the N terminal portion of the cloned leech protein. The position of most prolines and

tryptophan residues, which are important in determining protein folding, is conserved in the two protein sequences. The leech protein also showed homology to a consensus of the extracellular portion of β subunits of nAChRs from *Drosophila*, *Xenopus* and electric ray (Fig. 3A). These homologies seem to indicate that the leech protein has similarities in spatial conformation to the extracellular portion of the β subunits of nAChRs. It is possible, thus, that this protein is part of a receptor in the leech salivary glands. The β subunit of acetylcholine receptors is thought to contribute to the binding of some agonists (Luetje and Patrick, 1991) and it is possible that the leech protein, as part of a membrane receptor, could have a role in ligand binding.

The leech protein terminates in a highly repetitive and charged sequence of amino acids (histidines and aspartic acids). This type of repetitive sequence is not present in nAChRs β subunits but homologies were found between the leech protein C terminus and the C termini of two eggshell proteins of the blood fluke (*Schistosoma mansoni*) (Chen *et al.*, 1992, Johnson *et al.*, 1987). This region of the leech protein has also homology to the C terminus of the codworm haemoglobin gene (Dixon *et al.*, 1981) (Fig. 3b).

The function of such a highly charged region in these proteins is not fully understood: in the eggshell proteins of *Schistosoma*, the high histidine content is thought to be involved in regulation of polymerisation of the components of the eggshell from the "emulsion" state inside vesicles to the polymerised form on the egg (Wells and Cordingley, 1991). It is possible that this histidine-rich sequence could be involved in binding of divalent cations. This region includes the pattern found for Cu^{2+} binding in haemocyanins from arthropods

```

1  tggttcggcccacctctgaaggttccagaatcgatagtaaatatcaaatcggttcaattgc 60
61  gaagaacttgctgtctcctcaggatctcctctactcatctcatcttgcaagcatcatgaa 120
    M K
121  gttgttcttgggttgaggttttgggtggtctgtctgtcgagaacatattctgttctcactt 180
    L F L V A V L V A L S V E N I F C S H L
181  gcaaggccaaactaagctttggaagaagttattcgatcactacaacaagaattagaacc 240
    Q G Q T K L W K K L F D H Y N K E L E P
241  cgaagatgcaggtcttaagttcgggtctggtcttcggtcgtgctcattatgagaacaatc 300
    E D A G L K F G L A F V R A H Y E K Q S
301  tcatgaagtgcagtgtccatgtctgggaaagatactcctggaccgacaaacgtctcagctg 360
    H E V H V H V W E R Y S W T D K R L S W
361  ggagccatccgagttcgatgggtgtgaccagtttgagagtttctcacgatcatttgtggct 420
    E P S E F D G V T S L R V S H D H L W L
421  tcctgactttcaactgtaccatgggttcgaagagagtagagaccaagctctcctcac 480
    P D F Q L Y H G F E E R V E T K A L L T
481  ccacgaaggtcacttgggtctacgtgccagctgttctgtacaaaacccactgtgaaaaggg 540
    H E G H L V Y V P A V L Y K T H C E K G
541  ggccaaccattctctccactgccacttcaagggtcgatcatgggggtgctgatgcagtgc 600
    A N H S L H C H F K V G S W G V D A V T
601  ccttcctttggccaaatgggacacaggagccaccctggactactaccagacaaaacctc 660
    L P L A K W D T G A T L D Y Y Q T K T S
661  tccccttgaggctcacttgggtgaagtgaactcgtcaccaagagctattccgccttcca 720
    P L E A H L G E V E L V T K S Y S A F Q
721  gaaccttccatactctgccctcgaagtttcagttgatttccaccataaggacagtcatga 780
    N L P Y S A L E V S V D F H H K D S H D
781  tgatgatcatcatgataacgatcatcatgatcattaattccttcatcatcatcggtgttg 840
    D D H H D N D H H D H *
841  tgaatgaaaatcggagtagtaacgataataatattaacattaatagcaataatacgaaaa 900
901  aaaataacctacaaataacatttgttgactcggttaattattataattgattgggttatctg 960
961  tttgcttgattgctcgaatgggtgattgattttggttgattggttgattgacaacaatca 1020
1021  tttcgcaatatattatgatttcttgtgaagaatttattcaataaaaataaatctcaaattct 1080
1081  cgaaaaaaaaaaaaaaaaaaaaa 1101

```

Fig. 2. Complete nucleotide sequence and amino acid translation of the gene coding for the leech protein. The signal peptide is boxed and the poly-adenylation signal is indicated by a bar above the sequence. The sequence of the two oligonucleotides used to amplify the complete ORF is underlined. The star (*) indicates the position of the stop codon.

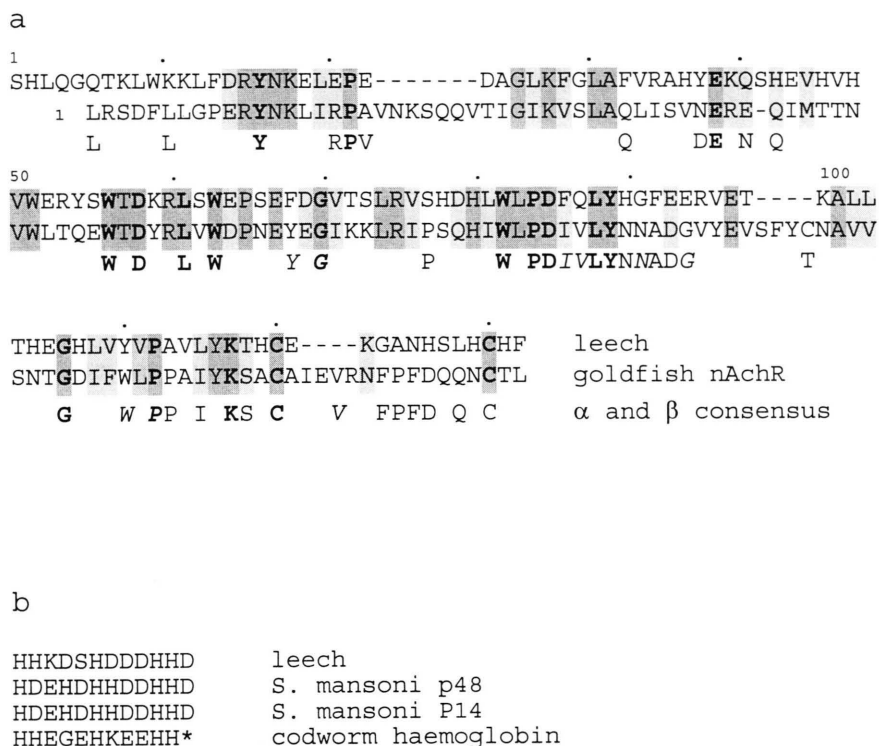


Fig. 3a. Alignment of the N terminal region of the leech protein with the extracellular portion of the β subunit of the goldfish nAChR and the consensus for the extracellular portion of α subunits (Elgoyhen *et al.*, 1994, italics) and β subunits from nAChRs of non mammals. Consensus for the β subunits was derived by aligning the extracellular region from amino acid sequences of nAChRs β subunits from *Drosophila* (accession Nos.P04755 and P25162), *Xenopus* (accession V04618), goldfish (Accessions P19370 and P18257) and electric ray (Accessions A93294, 94250, A03171) from the Swiss and PIR and translated EMBL+Genebank databases. Residues identical in leech and goldfish sequences are indicated by shaded background, residues with same chemical properties at conserved positions are marked by lighter shading. Residues identical in both sequences and in the α and β subunit consensus are in bold format.

Fig. 3b. Alignment of the histidine and aspartate-rich C terminus of the leech protein with corresponding regions of two *S.mansoni* eggshell proteins and codworm haemoglobin. Star indicates the position of the stop codon in each of the proteins.

(HN₃HN₈H) (Volbeda and Hol, 1989). There are indications that this leech protein can form polymers (see below) and that polymerisation was dependent or involved the presence of divalent cations because it can be disrupted by EDTA (results not shown). The histidine-rich tails of the leech protein monomers might be able to coordinate divalent cations in polymers.

The recombinant and native proteins have similar biochemical properties

The recombinant protein is larger than the native protein, as it can be observed in PAGE fol-

lowed by Western blotting (Fig. 4). On these blots, probed with an anti-leech protein-specific antiserum, the native protein, in reducing Laemmli loading buffer, boiled, appears as several bands: a group of M. W. from 24 kDa to 28 kDa, and other bands of M. W. 30 kDa and between 47.5 kDa and 70 kDa (lane 2). The recombinant protein treated in the same conditions, has a similar pattern but the major group of bands is between 28 kDa and 29 kDa, a band of M. W. 52 kDa and fainter bands of M. W. 48 kDa and 65 kDa and 24 kDa (lane 3). One possibility is that the larger M. W. proteins represent multimers of the smaller protein. The differences in apparent M. W. between the native

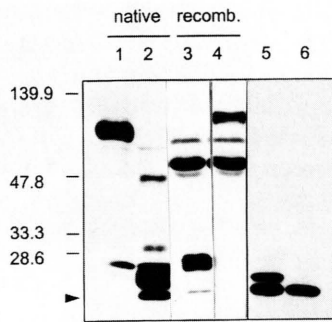


Fig. 4. Western blots of PAGE of natural and recombinant leech protein probed with antiserum against the leech protein.

Samples of native protein (lanes 1 and 2) in reducing (Laemmli) buffer, not boiled (lane 1) or boiled (lane 2); transformed COS cells' supernatant (lanes 3 and 4) in reducing buffer, not boiled (lane 4) or boiled (lane 3). Arrowhead indicates the small 24 kDa protein. Lysates of transformed COS cells grown in the absence (lane 5) or presence (lane 6) of tunicamycin in the culture medium. Numbers on the left indicate the position of the M. W. markers.

and recombinant proteins could result from different glycosylation patterns in the recombinant protein, as compared to the native glycosylation pattern of the protein in the leech. The smaller protein (approximately 24 kDa) has the M. W. expected for the product of the gene and probably represents the non-glycosylated form of the protein (arrowhead on Fig. 4). To test this hypothesis, transformed COS cells were treated with tunicamycin, which inhibits glycosylation. Lysates of tunicamycin-treated COS cells, analysed on PAGE and blotted onto membranes, contained only one protein which was detected by the anti-leech protein antiserum and co-migrated with the smaller protein from untreated COS cells (Fig. 4, lanes 5 and 6). None of the larger M. W. forms was detected in these blots, which seems to indicate that the multimers are only formed in the supernatants, once the protein is secreted from the cells, or that they were disrupted completely under the conditions used to lyse the cells. The difference between the M. W. of this small 24 kDa protein, which matched that expected for a product of this gene, and the M. W. determined by MALDI-TOF (26.5 kDa), could be explained by the presence of sugar residues in the purified leech protein.

The native protein formed aggregates spontaneously, the major form being of M. W. approxi-

mately 100 kDa. The multimers were partially disrupted by boiling but not by reducing agents alone (Fig. 4, lane 1). This indicated that the interaction between the monomers was not mediated by S-S bridges. The recombinant protein formed equally polymers, the larger with apparent M. W. 110 kDa (consistent with the greater M. W. of the monomer) which was also partially disrupted by boiling but not by reducing agents alone (Fig. 4, lane 4). Although the larger protein forms were disrupted by boiling, some small multimeric forms remain. It is possible that a more prolonged treatment at high temperature would be necessary to completely disrupt these multimeric forms or that they are maintained by other stronger interactions. The M. W. of these forms indicated that they could be polymers of four monomer units. In a previous study of salivary gland proteins by Budzynski and collaborators (1981), proteins extracted from the salivary glands of *H. ghilianii* which had been boiled in Laemmli reducing loading buffer or simply loaded in buffer without reducing agents had been analysed by PAGE and Coomassie stained. In these gels, a protein species of M. W. approximately 75 kDa seemed affected by boiling in the presence of reducing agents. It is possible that this protein corresponds to the species of similar M. W. detected by our antibody in crude salivary gland extracts. Preliminary results indicated that cations are involved in maintaining the multimers, as treatment with EDTA disrupted the multimers, in the absence of a temperature treatment. The interaction with a cation would be responsible for maintaining some degree of multimerisation, resistant to high temperature treatment, resulting in the higher M. W. forms of the protein observed in the samples boiled for 5 min (Fig. 4, lanes 2 and 3).

Conclusions

We have cloned and expressed in mammalian cells a protein from the salivary glands of the giant Amazon leech with homology to the extracellular portion of the β subunit of the nAChR from goldfish and, to a smaller extent, to other nAChR subunits from other species. Significantly, several amino acids important for the definition of the secondary structure and conserved in all α and β subunits of acetylcholine receptors sequenced so far are conserved in the leech protein (Fig. 3a). The

homology comparisons of the C terminus of the leech protein with the database showed that it included the consensus sequence for coordination of divalent cations, leading us to speculate that this leech protein may have a function involving the binding of cations, possibly in regulating the Ca^{2+} balance in the salivary glands. This protein may constitute a subunit of a membrane protein complex involved in Ca^{2+} transport or recognition. It is important to remember that the protein in question is a soluble protein, not a membrane protein. Recently, evidence has been found for the involvement of a $\alpha 9$ -type nicotinic acetylcholine receptor in the control of membrane polarisation in the serotonergic Retzius neurons of the medicinal leech midbody ganglia (Szuzupack *et al.*, 1998). It is pos-

sible that a similar receptor is to be found in the control of nervous impulse and secretion in the salivary glands of the Amazon leech.

Western blot analyses provided evidence that the native protein forms polymers and that the interaction between the monomers is not disrupted by reducing agents.

Acknowledgements

We would like to thank Dr R. Sawyer from Biopharm, UK for supplying the leeches and for useful discussions on leech physiology and feeding behaviour, Drs. S. Finney and L. Seate and Mr K. Peters from Biopharm, UK, for the dissection of the salivary glands. This work was funded by E Merck (Germany).

- Ashfield R., Enriques-Harris P. and Proudfoot N. J. (1991), Transcriptional termination between the closely linked human complement genes C2 and Factor B: common termination factor for C2 and c-myc? *EMBO J.* **10**, 4197–4207.
- Budzynski A. Z., Olexa S. A. and Sawyer R. T. (1981), Composition of salivary gland extracts from the leech *Haementeria ghilianii*. *Proc. Soc. Exp. Biol. Med.* **168**, 259–265.
- Chen L., Rekosh D. M. and LoVerde P. L. (1992), *Schistosoma mansoni* p48 eggshell protein gene characterization, developmentally regulated expression and comparison to the p14 eggshell protein gene. *Mol. Biochem. Parasitol.* **52**, 39–52.
- Chomczynski P. and Sacchi N. (1987), Single-step method of RNA isolations by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.
- Dixon B., Walker B., Kimmins W. and Pohajdak B. (1981), Isolation and sequencing of a cDNA for an unusual haemoglobin from the parasitic nematode *Pseudoterranova decipiens*. *Proc. Nat. Acad. Sci. USA* **88**, 5655–5659.
- Elgoyhen A. B., Johnson D. S., Boulter J., Vetter D. E. and Heineman S. (1994), $\alpha 9$: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell* **79**, 705–715.
- Engler-Blum G., Meier M., Frank J. and Muller G. A. (1993), Reduction of background problems in non-radioactive Northern and Southern blot analyses enables higher sensitivity than ^{32}P -based hybridisations. *Anal. Biochem.* **210**, 235–244.
- Enriquez-Harris P., Levitt N., Briggs D. and Proudfoot N. J. (1991), A pause site for RNA polymerase II is associated with termination of transcription. *EMBO J.* **10**, 1833–1842.
- Johnson K. S., Taylor D. W. and Cordingley J. S. (1987), Possible eggshell protein gene from *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* **22**, 89–100.
- Jones P. G., Sawyer R. T. and Berry M. S. (1985), Novel preparation for studying excitation-secretion coupling in the isolated single cell. *Nature* **315**, 679–680.
- Laemmli U. K. (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Luetje C. W. and Patrick J. (1991), Both α - and β -subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptor. *J. Neurosci.* **11**, 837–845.
- Malinconico S. M., Katz J. B. and Budzynski A. Z. (1984), Hementin: anticoagulant protease from the salivary gland of the leech *Haementeria ghilianii*. *J. lab. Clin. Med.* **103**, 44–58.

- Marshall C. G. and Lent C. M. (1984), Calcium-dependent action potentials in leech giant salivary cells. *J. exp. Biol.* **113**, 367–380.
- Sawyer R. T., Powell-Jones C. and Munro R. (1991), The biological function of hementin in the proboscis of the leech *Haementeria ghilianii*. *Blood Coagulation and Fibrinolysis* **2**, 153–159.
- Sawyer R. T., Damas D. and Tomic M. T. (1982), Anatomy and histochemistry of the salivary complex of the giant leech *Haementeria ghilianii* (Hirudinea – Rhynchobdellida). *Arch. Zool. exp. gen.* **122**, 411–425.
- Szuzupak L., Peralta E. J. and Kristan W. B. Jr. (1988), Long-lasting depolarization of leech neurons mediated by receptors with a nicotine binding site. *J. exp. Biol.* **201**, 1895–1906.
- Volbeda A. and Hol W. G. J. (1989), Pseudo 2-fold symmetry in the copper-binding domain of arthropodan haemocyanins. Possible implication for the evolution of the oxygen transport protein. *J. Mol. Biol.* **206**, 531–546.
- von Heijne G. (1986), A new method for predicting signal sequences cleavage sites. *Nucleic Ac. Res.* **14**, 4683–4689.
- Wells K. E. and Cordingley J. S. (1991), *Schistosoma mansoni*: eggshell formation is regulated by pH and calcium. *Exp. Parasitol.* **73**, 295–310.
- Wuttke W. A. and Berry M. S. (1988), Calcium dependent action potentials in giant salivary gland cells of the leech *Haementeria ghilianii*: calcium removal induces dependence on sodium. *J. exp. Biol.* **138**, 431–453.
- Wuttke W. A., Sawyer R. T. and Berry M. S. (1989), Extraglandular innervation of the salivary cells of the leech *Haementeria ghilianii*: neuronal stimulation elicits gland-cell action potentials and secretion. *J. exp. Biol.* **143**, 389–410.
- Wuttke W. A., Munsch T. and Deitmer J. W. (1996), Inhibition of voltage-dependent Ca^{2+} influx by extracellular ATP in salivary cells of the leech *Haementeria ghilianii*. *J. exp. Biol.* **199**, 1335–1341.