Long-Term Sensitization Training in *Aplysia* Leads to an Increase in the Expression of BiP, the Major Protein Chaperon of the ER

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Abstract. Long-term memory for sensitization of the gill- and siphon-withdrawal reflexes in *Aplysia californica* requires RNA and protein synthesis. These long-term behavioral changes are accompanied by long-term facilitation of the synaptic connections between the gill and siphon sensory and motor neurons, which are similarly dependent on transcription and translation. In addition to showing an increase in overall protein synthesis, long-term facilitation is associated with changes in the expression of specific early, intermediate, and late proteins, and with the growth of new synaptic connections between the sensory and motor neurons of the reflex. We previously focused on early proteins and have identified four proteins as members of the immunoglobulin family of cell adhesion molecules related to NCAM and fasciclin II. We have now cloned the cDNA corresponding to one of the late proteins, and identified it as the *Aplysia* homolog of BiP, an ER resident protein involved in the folding and assembly of secretory and membrane proteins. Behavioral training increases the steady-state level of BiP mRNA in the sensory neurons. The increase in the synthesis of BiP protein is first detected 3 h after the onset of facilitation, when the increase in overall protein synthesis reaches its peak and the formation of new synaptic terminals becomes apparent. These findings suggest that the chaperon function of BiP might serve to fold proteins and assemble protein complexes necessary for the structural changes characteristic of long-term memory.

Sensitization, an elementary form of learning, gives rise to both short-term and long-term memory. In the marine snail *Aplysia californica*, a sensitizing stimulus applied to the tail of the animal activates facilitatory neurons that synapse onto the presynaptic terminals of the gill and siphon sensory neurons. The facilitatory neurons release the modulatory neurotransmitter serotonin (5-HT) (Glanzman et al., 1989). The action of 5-HT on the sensory neuron leads to an increase in the strength of the synaptic connections between the sensory neurons and their target cells, the interneurons and motor neurons of the reflex, and contributes to the increase in the strength and duration of the gill- and siphon-withdrawal reflexes (Castellucci et al., 1970; Castellucci and Kandel, 1976).

A single noxious stimulus to the animal or a single application of 5-HT to sensory and motor neurons grown in dissociated cell culture produces short-term changes in behavior and synaptic strength (Frost et al., 1985; Montarolo et al., 1986). The same sensory neurons that respond to a single pulse of 5-HT to produce a short-term change respond to repeated pulses of 5-HT giving rise to a long-term change (Montarolo et al., 1986). Similarly, in the intact animal, long-term sensitization of the gill withdrawal reflex can be produced by presenting four or more noxious stimuli at regular intervals (Frost et al., 1985). Although the synaptic facilitation underlying long-term memory in *Aplysia* resembles that for short-term memory, long-term memory can be distinguished from short-term memory at the structural and the molecular level. The long-term alterations in synaptic strength require RNA and protein synthesis (Montarolo et al., 1986) and are accompanied by the addition of new synaptic structures after training (Bailey and Chen, 1983, 1988*a*,*b*, 1989; Glanzman et al., 1990).

These findings provided a rationale for analyzing the role of protein and RNA synthesis in long-term sensitization and facilitation. Using analytical two-dimensional (2-D) gel electrophoresis, we next identified changes in protein synthesis after behavioral training or prolonged exposure to 5-HT (Barzilai et al., 1989; Castellucci et al., 1988). Specifically, we found that exposure of sensory neurons to 5-HT induces at least three waves of specific protein synthesis: early, intermediate, and late proteins.

Here, we report the identification and molecular cloning of one of the late proteins. The cDNA encodes a protein of 667 amino acids homologous to BiP/GRP78, hereafter referred to as BiP. BiP is localized in the lumen of the ER and assists the folding and assembly of newly synthesized secretory and transmembrane proteins (Bole et al., 1986; Gething

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et al., 1986; Munro and Pelham, 1986; Pelham, 1988; Normington et al., 1989; Rose et al., 1989; Rothman, 1989). The synthesis of *Aplysia* BiP protein increases 3 h after serotonin treatment, the time at which an increase in overall protein synthesis peaks and when the formation of new synaptic terminals first becomes apparent. After behavioral training there is a similar increase in the steady-state level of *Aplysia* BiP mRNA. Levels of BiP mRNA are induced in cells that specifically participate in long-term sensitization but remain unchanged in cells not involved in the memory process.

Materials and Methods

Oligonucleotides

Oligonucleotides were synthesized using an Applied Biosystems Inc. (Foster City, CA) DNA Synthesizer at the Howard Hughes Protein and DNA Core Facility (Columbia University). Oligonucleotides used as S1 probes were gel purified. Desalting was carried out using an OPC cartridge (Applied Biosystems Inc.). Oligonucleotide sequences are as follows (X = A + G; Y = T + C: Z= A + G + T):

Primers used in PCR
DAQ 1 NotI sense, AAGCGGCCGCTAYTTYAAYGAYGCYCA
NPDE 1 EcoRI antisense, AAGAATTCGCYTCXTCYGGXTTZAT

Probes used in S1 nuclease analysis, the 15 or 20 nucleotides of 3'-nonmatching sequences were derived from the rabbit β-globin promoter (Dierks et al., 1983) and are indicated in lower case:

Aplysia Cam probe
GGTTGTGATGGTGCCATCTCCATCTTTATCGAAAAGACTGAATGC-cagcagctgccctgc (sequence of *Aplysia* calmodulin was provided by James H. Schwartz).

Aplysia HSC70 probe
CACCAGGACAATTTCATGGACTTGAGACTTGTCCATTTTGGCATC-cagcagctgccctgc

Aplysia BiP probe
CACCAACCAACACAATTTCATCAATGTCATCAGTCTTCAAGTCAGCAT-CTTCCAACACCTGTTTCACTGGCTTCATGcagcagctgccctgctctgc

Protein Analysis

One- and two-dimensional electrophoretic analysis was conducted as described (Barzilai et al., 1989; Castellucci et al., 1988). For protein microsequencing, *Aplysia* BiP protein was isolated directly from Coomassie blue-stained preparative gels of central nervous system lysate and digested with V8 protease; the resulting proteolytic peptides were electrophoretically resolved and blotted onto Immobilon (Millipore Corp., Bedford, MA) as described (Kennedy et al., 1988). Protein sequence analysis was carried out at the Howard Hughes Protein and DNA Core Facility (Columbia University) by automated sequential Edman degradation using an Applied Biosystems 470A automated gas phase sequencer. The amino acid sequence obtained was IVLVGGSTXIXKXXQ (see Figs. 1 and 2).

cDNA Synthesis

First strand cDNA synthesis was generated from 1 μg of glucose-starved *Aplysia* total nervous system RNA (see glucose starvation protocol below). Oligo dT primers (Pharmacia Fine Chemicals, Piscataway, NJ) were hybridized to mRNA, adjusted to standard reverse transcriptase conditions (Sambrook et al., 1989) in 50 μl, and incubated with M-MuLV reverse transcriptase (BRL, Gaithersburg, MD) for 90 min at 37°C. The RNA was degraded by alkaline treatment, and the cDNA was extracted with phenol and recovered by ethanol precipitation and resuspended in 100 μl of water.

Polymerase Chain Reaction Conditions and Cloning of Polymerase Chain Reaction Products

Amplification reactions were in 100 μl with 5 μl of first strand cDNA, 1 μg of each degenerate oligonucleotide pool, 2 mM $MgCl_2$, 0.2 mM dNTPs, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 μg/ml gelatin, and 2.5 U of Ampli Taq polymerase (Cetus Corp., Berkeley, CA). The samples were overlaid with 100 μl light mineral oil and incubated in a Perkin Elmer Cetus Thermocycler (Perkin-Elmer Cetus Instruments, Norwalk, CT). Cycling times for denaturation, annealing, and extension were, respectively, 2, 1, and 4 min for 40 repetitive cycles. Denaturation was at 92°C, annealing at 50°C, and extension at 72°C. The degenerate oligonucleotides successfully used for polymerase chain reaction (PCR)[1] were oligo DAQ 1, which includes the codons for the amino acids YFNDA and the first two nucleotides encoding for Q as well as an NotI linker in the sense strand, and oligo NPDE 1, amino acids INPDE and the first two nucleotides encoding for A with an EcoRI linker in the antisense strand (see Fig. 2, and under oligonucleotides in the beginning of this section, single letter amino acid code is used). The resulting 660-bp PCR products were cloned into pKSM13- (Stratagene Inc., La Jolla, CA). Plasmid DNA from individual colonies was isolated by standard methods (Sambrook et al., 1989).

Library Screening

Recombinant plasmids from two amplified *Aplysia* nervous system libraries (Pfaffinger et al., 1990; Mayford et al., 1992) were plated at a density of 2×10^4 colonies per 15-cm petri dish. Colony lifts were prepared and probed on duplicate filters as described (Sambrook et al., 1989). The probe was a random primed 660-bp fragment of the *Aplysia* BiP PCR-clone. Positive colonies were isolated and their plasmid DNA extracted by standard methods (Sambrook et al., 1989).

Nucleotide Sequencing and Sequence Analysis

Nucleotide sequencing was carried out with Sequenase II kits (United States Biochemical Corp., Cleveland, OH) using double-stranded DNA templates purified with Quiagen plasmid kits (Quiagen). Multiple synthetic oligonucleotides (17 mers) were used to sequence both DNA strands of two independent clones for *Aplysia* BiP from the library screen, and both strands of the 660-bp PCR-clone for *Aplysia* HSP70a, *Aplysia* HSP70b, *Aplysia* HSC70, and *Aplysia* BiP. DNA and protein sequence analysis was performed using DNASTAR Inc. (Madison, WI) software.

Glucose Starvation

Aplysia (50–70 g, Miami) were anesthetized with approximately one-half their body weight of isotonic $MgCl_2$ and the total central nervous system was dissected. Tissue was then allowed to recover in complete culture medium at room temperature for 2 h. The medium was then changed for glucose starvation medium lacking all sugars and other supplements (460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, 10 mM Hepes, 2.5 mM $NaHCO_3$, 50 U Pen/Streptomycin, pH 7.6). For the isolation of RNA, tissue was starved for 3, 7, and 18 h, at which time it was quick frozen in EtOH chilled on dry ice. Control tissue was treated in the same way except that it was incubated in complete culture medium. Extraction of total central nervous system RNA and S1 nuclease analysis of specific messages was carried out as described below. For analysis of protein, clusters of pleural sensory neurons were dissected and glucose starved as described above. Labeling with [^{35}S]methionine was for 2 h at the indicated time points (see Fig. 3). The sensory cells were homogenized and analyzed by 2-D gel electrophoresis and fluorography as described (Barzilai et al., 1989).

Long-Term Sensitization Training

Pretraining, training, and posttesting were carried out essentially as described (Castellucci et al., 1988). Animals (100–150 g, Miami) were rested for 5 d from their time of arrival in individual cages and the duration of siphon withdrawal was determined for all animals 1 d before the beginning of training. Experimental animals were then given four trains of four shocks to the tail and also to each side of the body wall to facilitate both the abdominal and the pleural sensory neurons. 24 h after the last training trial, the duration of siphon withdrawal was again determined for all animals. Training significantly increased the duration of siphon withdrawal. Control animals that were not trained showed no significant change in the duration of the siphon withdrawal (data not shown). After training, animals were anesthetized, the total central nervous system dissected out and immediately frozen in a mixture of 2 M NaCl and propylene glycol (1:1) on dry ice. Microdissection of the nervous system was similarly carried out in 2 M NaCl/propylene glycol (1:1) on a bed of dry ice.

1. *Abbreviation used in this paper*: PCR, polymerase chain reaction.

RNA Preparation and S1 Mapping

Standard RNAs for *Aplysia* HSC70 and *Aplysia* BiP were in vitro transcribed (Stratagene RNA transcription kit, Stratagene Inc.). In vitro-transcribed standards were quantified by incorporating 1 dpm of ^{32}P-UTP into each 300 pg of transcript synthesized. Total RNA isolation from glucose-starved tissue was as described (Auffrey and Rougeon, 1980). Total RNA from trained animals was isolated as described (Brunet et al., 1991). For each part of the central nervous system examined, material from five animals was pooled. ~5 μg of total RNA was used per S1 nuclease digest. Each digest was performed on material pooled from five animals; however, each animal was assayed only once per probe per tissue and therefore each assay represents a completely independent measurement. Quantitative S1 nuclease analysis was performed essentially as described (Kuhl et al., 1987; Westin et al., 1987). Single-stranded DNA probes were end labeled with ^{32}P using T4 kinase (BRL). S1 nuclease digestion was with 400 U S1 at 30°C for 2 h. The sequences of the 97-mer probe complimentary to *Aplysia* BiP mRNA and the 60-mer probes, complimentary to *Aplysia* HSC70, and *Aplysia* Cam mRNA are given in the beginning of this section. 20 and 15 bp, respectively, were derived from a 5′-untranscribed sequence from the rabbit β-globin promoter (Dierks et al., 1983), to differentiate digested from undigested probe.

For quantification of RNA messages after glucose starvation, radioactivity recovered after S1 mapping in the *Aplysia* BiP band, *Aplysia* calmodulin band, and blank areas were determined by Cherenkov counting. Steady-state levels of *Aplysia* BiP mRNA as measured by S1 analysis 24 h after training were quantified as follows. Multiple autoradiographic exposures were scanned with a Molecular Dynamics 100-A laser densitometer (Molecular Dynamics, Sunnyvale, CA) and analyzed with the Quantity-One software package commercially available from Protein Databases, Inc. Values are expressed as the percent change in the steady-state level of *Aplysia* BiP mRNA with respect to the steady-state level of the internal reference *Aplysia* HSC70 mRNA. The optical density of the *Aplysia* HSC70 signal was adjusted to a value of 100 optical density units. A proportionally normalized value for the signal probe was calculated with respect to this adjustment. The optical density of each measurement was determined to be within the linear range of the autoradiograph by comparison of known standards exposed in parallel. P values were calculated using a one tailed *t* test.

Results

Protein 1603 Is the Aplysia Homolog of BiP

Protein 1603 was first identified on analytical 2-D gels in studies designed to identify changes in protein synthesis in the sensory neurons of *Aplysia* after either behavioral training (Castellucci et al., 1988) or repeated or prolonged exposure to 5-HT (Barzilai et al., 1989), a modulatory transmitter released during behavioral training. Exposure to 5-HT caused an increase in the rate of synthesis of protein 1603 within 3 h, which was maintained for at least 24 h (Barzilai et al., 1989). A similar increase was detected with behavioral training (Castellucci et al., 1988). We isolated protein 1603 from preparative 2-D gels, digested the protein in situ, and sequenced the resulting peptides. The partial amino acid sequence obtained for protein 1603 showed significant similarity to proteins of the HSP70 family, being most similar to BiP (Fig. 1). Furthermore, its induction by glucose starvation (see below), pI of 5.1 and MW of 78, are consistent with the suggestion that protein 1603 is the *Aplysia* homolog of BiP.

Cloning of the Aplysia BiP Gene

On the basis of this assumption we cloned the corresponding cDNA. Complementary DNA and genomic sequences encoding BiP have been cloned from a variety of species (for example see Munro and Pelham, 1986; Ting and Lee, 1988; Heschl and Baillie, 1989; Normington et al., 1989; Rose et al., 1989). BiP is a member of the heat shock protein 70

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                                                         MW
APLYSIA 1603                      IVLVGGSTXIXKXXQ        78

HUMAN BiP                   SDIDE IVLVGGSTRIPKIQQ LV     78
                               360^      370^

HUMAN HSP70                 AQIHD LVLVGGSTRIPKVQK LL     70
                          330^        340^       350^

HUMAN HSC70                 SQVHD IVLVGGSTRIPKVQK LL     71
                          330^        340^       350^

C. elegans BiP              DDVHE IVLVGGSTRIPKVQQ LI
                          360^        370^       380^

C. elegans HSP70            SQVHD IVLVGGSTRIPKVQK LL     70
                          330^        340^       350^
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Figure 1. Aplysia protein 1603 is a member of the HSP70 family. Comparison of the partial amino acid sequence obtained for *Aplysia* protein 1603 with various members of the HSP70 family. Amino acids are given in single letter code. Numbers below the sequences refer to the respective position in the corresponding protein. Identical amino acids are shaded. The amino acid sequence obtained from protein 1603 is identical to corresponding residues in human (Ting and Lee, 1988) and *C. elegans* BiP (Heschel and Baille, 1989), and is homologous to human HSP70 (Hunt and Morimoto, 1985), human HSC70 (Dwornizcak and Mirault, 1987), and *C. elegans* HSP70 (Snutch et al., 1988).

(HSP70) family, which in most species comprises >10 closely related genes (Munro and Pelham, 1986). A unique DNA sequence encoding a portion of the *Aplysia* BiP gene was obtained by mixed oligonucleotide primed amplification of cDNA (MOPAC) from glucose-starved *Aplysia* total nervous tissue (Lee et al., 1988). Cloning and subsequent sequence analysis of the 660-bp amplification product identified four distinct HSP70 related genes. Three clones deviated from the amino acid sequence obtained from protein 1603 in one or more residues. The remaining clone encoded a sequence that matched this sequence at every position and was most similar to mammalian BiP. The partial *Aplysia* BiP clone was used to identify clones containing full-length cDNA from cDNA libraries prepared from *Aplysia* total nervous system mRNA. The isolated full-length cDNA clones contain 5′ and 3′ nontranslated sequence and encode a protein of 667 amino acids. Fig. 2 shows the extensive stretches of identity between the predicted amino acid sequence of *Aplysia* and human BiP proteins. Overall, 80% of the residues are identical. In contrast, the predicted amino acid sequence of this clone shared <65% amino acid sequence identity with each of the three other identified *Aplysia* HSP70 proteins, which are >80% identical to each other (Fig. 2). This degree of identity is very similar to that found between mammalian BiP and HSP70 proteins (Munro and Pelham, 1986). Notably, *Aplysia* BiP differs from the three other *Aplysia* HSP70 genes in lacking the consensus for N-linked glycosylation (N-X-S/T). These are absent in yeast and other eukaryotic BiP proteins but a common feature of cytoplasmic heat shock proteins (Munro and Pelham, 1986; Normington et al., 1989; Rose et al., 1989). As is the case with BiP from other species, *Aplysia* BiP contains a long span of hydrophobic residues at the NH_2 terminus that constitutes the presumed leader peptide directing the synthesis of BiP into the ER. The carboxy terminus ends with the sequence KDEL, the ER retention signal tetrapeptide (Munro and Pelham, 1987).

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Human BiP              MKLSLVAAMLLLLSAARAEEE--DKKEDVGTVVGIDLGTTYS
Aplysia BiP      MDRFTPFFLLVILFSSNLLVRADGDEEDEGDKKKSEVGTVIGIDLGTTYS

Human BiP        CVGVFKNGRVEIIANDQGNRITPSYVAFTPEGERLIGDAAKNQLTSNPEN
Aplysia BiP      CVGVFKNGRVDIIANDQGNRITPSYVAFTADGERLIGDAAKNQLTSNPEN

Human BiP        TVFDAKRLIGRTWNDPSVQQDIKFLPFKVVEKKTKPYIQVDIGGGQTKTF
Aplysia BiP      TIFDVKRLIGRTFDDKSVQHDIKFYPFKVTNANNKPHIQAATGEGD-RSF

Human BiP        APEEISAMVLTKMKETAEAYLGKKVTHAVVTVPAYFNDAQRQATKDAGTI
Aplysia BiP      APEEISAMVLSKMRDIAEEYLGKKITNAVVTVPAYFNDAQRQATKDAGTI
Aplysia HSP70b                                             RQATKDAGAI
Aplysia HSC70                                              RQATKDAGTI
Aplysia HSP70a                                             RQATKDAGAI

Human BiP        AGLNVMRIINEPTAAAIAYGLDKR-EGEKNILVFDLGGGTFDVSLLTIDN
Aplysia BiP      AGLNVMRIINEPTAAAIAYGLDKK-EGEKNILVFDLGGGTFDVSLLTIDN
Aplysia HSP70b   TGLNVLRMINEPTAATLRYGLDKGQSGEKNVLIFDLGGGTFDVSVLTIDE
Aplysia HSC70    SGLNVLRIINEPTAAAIAYGLDKKVGTERNVLIFDLGGGTFDVSILTIED
Aplysia HSP70a   AGLNVLRIINEPTAAALAYGLDKGQKGEKHVLIFDLGGGTFDVSVLAIDE

Human BiP        G-VFEVVATNGDTHLGGEDFDQRVMEHFIKLYKKKTGKDVRKDNRAVQKL
Aplysia BiP      G-VFEVVSTNGDTHLGGEDFDQRVMEHFIKLYKKKKGKDIRKDNRAVQKL
Aplysia HSP70b   GSMFEVKATAGDTHLGGEDFDNRLVSHFLQEFKRKHNKDISKNARATRRL
Aplysia HSC70    G-IFEVKSTAGDTHLGGEDFDNRMVNHFIQEFKRKHKKDIADNKRAVRRL
Aplysia HSP70a   GSIFEVKATAGDTHLGGEDFDNRLVAHFIQEFKRKHNKDISKNARAIRRL

Human BiP        RREVEKAK-ALSSQHQARIEIESFYEGEDFSETLTRAKFEELNMDLFRST
Aplysia BiP      RREVEKAKRALSSAHQVRLEIESFFDGEDFSESLTRAKFEELNMDLFRST
Aplysia HSP70b   RTACERAKRTLSSSSEASIEIDSLFEGMDYYTKISRARFEELCADLFRST
Aplysia HSC70    RTACERAKRTLSSSTQANIEIDSLFEGIDFYTTITRARFEELNADLFRGT
Aplysia HSP70a   RTACERAKRTLSSSSEASIEIDSLFEGIDYYTKVSRARFEELCGDLFRNT

Human BiP        MKPVQKVLEDSDLKKSDIDEIVLVGGSTRIPKIQQLVKEFFNGKEPSRGI
Aplysia BiP      MKPVKQVLEDADLKTDDIDEIVLVGGSTRIPKVQQLVKEYFNGKEPSRGI
Aplysia HSP70b   LEPVESALRDAKLDKGKIDEVVLVGGSTRIPKIQKLLSNFFNRKELNKS
Aplysia HSC70    LEPVEKSMRDAKMDKSQVHEIVLVGGSTRIPKIQKLLQDFFNGKELNKS
Aplysia HSP70a   LEPVETALRDAKLDKGKIDEVVLVGGSTRIPKIQKLLTDFFNGKELNKS

Human BiP        NPDEAVAYGAAVQAGVLSGDQDTGDLVLLHVCPLTLGIETVGGVMTKLIP
Aplysia BiP      NPDEAVAYGAAVQAGVLSGEEDTGDLVLLDVNPLTMGIETVGGVMTKLIP

Human BiP        SNTVVPTKNSQIFSTASDNQPTVTIKVYEGERPLTKDNHLLGTFDLTGIP
Aplysia BiP      RNTVIPTKKSQIFSTAADNQPTVTIQVYEGERSMTKDNHLLGKFDLTGIP

Human BiP        PAPRGVPQIEVTFEIDVNGILRVTAEDKGTGNKNKITITNDQNRLTPEEI
Aplysia BiP      PAPRGVPQIEVTFEIDVNGILKVTAEDKGTGSKNQIVIQNDQNRLSPEDI

Human BiP        ERMVNDAEKFAEEDKKLKERIDTRNELESYAYSLKNQIGDKEKLGGKLSS
Aplysia BiP      ERMINDAEKYADEDKKVKEKVDAKNELESYAYSLKNQIGDKEKLGAKLSD

Human BiP        EDKETMEKAVEEKIEWLESHQDADIEDFKAKKKELEEIVQPIISKLY---
Aplysia BiP      EDKEKITEAVDEAIKWLESNAEAESEAFNEKKTELEGIVQPIMTKLYEQS

Human BiP        GSAGPPPTGEEDT--AEKDEL
Aplysia BiP      GGA-PPPSGEEESEEAEKDEL
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Figure 2. Alignment of the predicted amino acid sequence of *Aplysia* BiP with other members of the *Aplysia* HSP70 family and human BiP. Alignment of the protein sequences of Human BiP (Ting and Lee, 1988), *Aplysia* BiP, *Aplysia* HSP70a, *Aplysia* HSP70b, and *Aplysia* HSC70 are shown with gaps introduced to optimize matches. Identical amino acids are shaded. The region of *Aplysia* BiP from which partial amino acid sequence was obtained and amino acids encoded by the degenerate PCR primers used in this study are underlined. *Aplysia* HSC70 shares the highest amino acid sequence identity with heat shock cognate protein 70, HSC70: 90% of the 209 amino acids encoded in the amplified fragment are identical to the human HSC70 protein (Dworniczak and Mirault, 1987). Its corresponding mRNA was not induced by heat shock or glucose starvation of *Aplysia* central nervous system (data not shown), consistent with the constitutive expression of HSC70 gene in other species. The mRNA corresponding to *Aplysia* HSP70a and HSP70b were both strongly induced after heat shock (data not shown). Both clones are ~80% identical to human HSP70 (Leung et al., 1990) at the amino acid level and are 90% identical to each other. The sequence data for *Aplysia* BiP, *Aplysia* HSP70a, *Aplysia* HSP70b, and *Aplysia* HSC70 are available from EMBL/GenBank/DDBJ under accession numbers Z15041, Z15037, Z15038, and Z15039, respectively.

Expression of Aplysia BiP Protein and RNA is Induced during Glucose Starvation

BiP was originally identified in fibroblasts as one of two proteins whose rate of synthesis is increased when cells are starved of glucose (Shiu et al., 1977). To further test our assumption that protein 1603 and the identified cDNA clone are BiP we dissected the bilaterally paired pleural sensory clusters of *Aplysia* and exposed one of the pair to glucose starvation, while the other was used as a control. Various time points were examined by labeling with [^{35}S]methionine followed by 2-D gel analysis. Fig. 3 shows an example of the response of *Aplysia* BiP protein expression to glucose starvation. The electrophoretic profile of BiP protein, induced by glucose starvation, was indistinguishable from that found previously for protein 1603. Using single-stranded DNA probes and S1 nuclease analysis we also measured the steady-state level of mRNA transcripts encoding BiP and calmodulin. Fig. 4 shows that glucose starvation leads to the induction of BiP mRNA, as was previously reported for BiP in other species (Lee, 1987; Munro and Pelham, 1986; Normington et al., 1989; Rose et al., 1989). In contrast, the mRNA level of *Aplysia* calmodulin was not significantly altered (Fig. 4). A time course experiment of BiP mRNA expression after glucose starvation is summarized in Fig. 5.

Aplysia BiP mRNA Levels Are Induced After Long-Term Sensitization Training

Earlier work in our laboratory has shown that *Aplysia* protein 1603, which we here have identified as BiP, shows an increased rate of protein synthesis after long-term facilitation and sensitization (Barzilai et al., 1989; Castellucci et al., 1988). To determine whether the increase in BiP protein reflected an elevation in the steady-state level of its mRNA, we carried out S1 protection assays 1 d after completion of behavioral training. We trained animals for 4 d and tested the strength of the reflex 24 h after the last training trial (see Castellucci et al., 1988). Training significantly increased the duration of siphon withdrawal. Control animals which were not trained showed no significant change in the duration of siphon withdrawal (data not shown). After training the pleural ganglion sensory neurons, the remainder of the pleural ganglion, the abdominal ganglion sensory wedge, and the abdominal ganglion neuroendocrine bag cells were microdissected and RNA isolated, and subjected to quantitative S1 analysis. In the S1 analysis uninduced and induced BiP transcript levels were normalized relative to the transcript levels of the corresponding comapped *Aplysia* HSC70 reference gene. This allowed us to eliminate errors resulting from differences in recoveries and S1 mapping (Kuhl et al., 1987). *Aplysia* HSC70 is particularly well suited as reference for two reasons. First, its transcript levels remain unchanged under various induction conditions. They are not significantly altered with heat shock or glucose starvation (data not shown) or a cocktail containing IBMX, cAMP analog, forskolin, and 5-HT, which induces early and intermediate genes in *Aplysia* (Kaoru Inokuchi, unpublished results). Second, its level of transcription is comparable with that of BiP thus allowing comparison and quantification on the same exposure. Fig. 6 *A* shows that the steady-state level of BiP messenger RNA is increased after behavioral training in the sensory neurons of the pleural ganglia, in the remainder of the pleural ganglion (Fig. 6 *B*), and in the abdominal wedge (Fig. 6 *C*), but remains unchanged in bag cells not involved in the memory process for long-term sensitization (Fig. 6 *D*). These results are summarized and quantified in Fig. 7.

Discussion

Duration of memory for behavioral sensitization varies as a

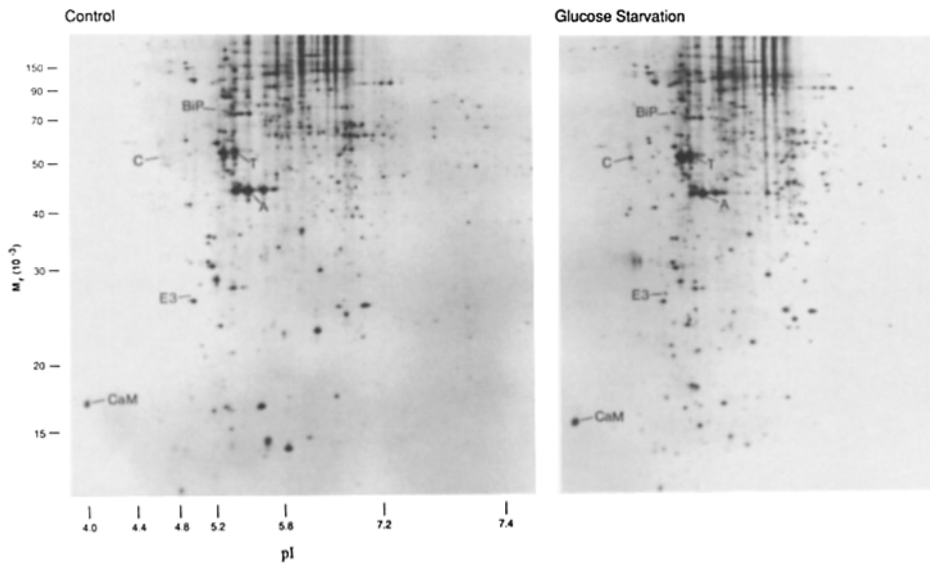

Figure 3. Glucose starvation induces proteins in *Aplysia* pleural ganglion sensory neurons. Autoradiograph of 2-D gel analysis of *Aplysia* pleural ganglion sensory neuron proteins following glucose starvation. *Aplysia* pleural ganglion sensory neuron clusters were labeled with [^{35}S]methionine between 22 and 24 h after being incubated in glucose starvation medium. The incorporation of label into the proteins BiP, calreticulin (*C*), and *E3*, a protein of unknown identity, increases during glucose starvation. Similar changes in expression were detected by labeling between 18 and 20 h or 32 and 34 h after the onset of glucose starvation. No change was detected between 0 and 2 h after the onset of glucose starvation (data not shown). Incorporation of label into tubulin (*T*), actin (*A*), and calmodulin (*CaM*), remains unchanged after glucose starvation as also indicated.

function of the number of training trials. The short-term memory produced by a single shock to the tail or a single pulse of 5-HT lasts minutes to hours and involves covalent modification of preexisting proteins. Long-term memory produced by four or more training trials or by repeated pulses of 5-HT lasts days to weeks and requires new protein and RNA synthesis (Castellucci et al., 1989; Montarolo et al., 1986). This requirement for RNA synthesis has made it attractive to think of mechanisms for the induction of long-term memory which require changes in transcriptional state, much as seen in differentiating cells in response to growth factors or hormones (Goelet et al., 1986). To determine whether the increase in BiP protein reflected an elevation in the steady-state level of its mRNA, we carried out S1 protection assays and found a significant increase in the level of BiP mRNA 1 d after the completion of behavioral training.

This increase in BiP, together with the increase in calreticulin, a calcium-binding protein (Kennedy et al., 1992), provide the first direct demonstrations that behavioral training produces changes in the level of mRNAs encoding specific late proteins. Moreover, these changes in mRNA occur in

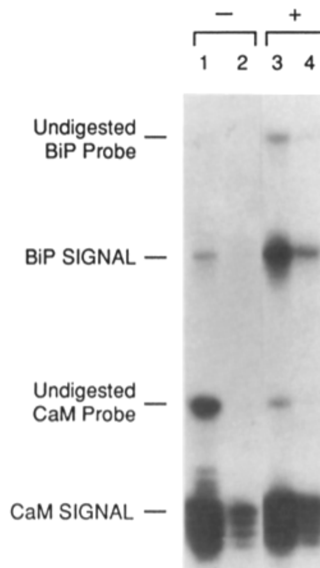

Figure 4. Glucose starvation induces *Aplysia* BiP mRNA. Autoradiograph of S1 nuclease analysis. Analysis was carried out with BiP and calmodulin probes. Positions of undigested probes and S1 protected signals are indicated. Lanes *1* and *2* contained 25 and 5 μg, respectively, of RNA isolated from control *Aplysia* central nervous system. Lanes *3* and *4* contained 25 and 5 μg, respectively, of RNA isolated from *Aplysia* central nervous system glucose starved for 18 h. Results for the 3- and 7-h time points are shown in Fig. 5.

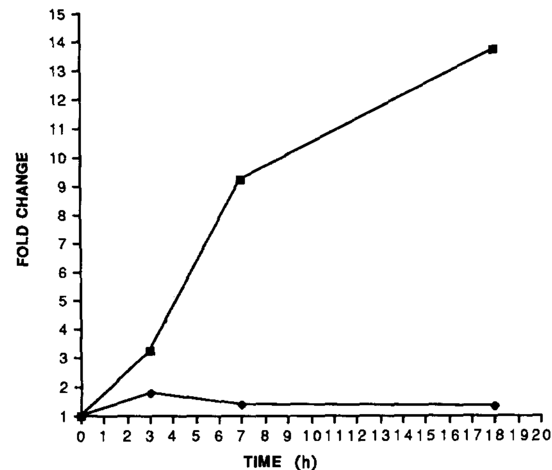

Figure 5. Time course of induction of *Aplysia* BiP mRNA after glucose starvation. Results of S1 nuclease analysis at 3, 7, and 18 h after glucose starvation are plotted as the average cpm determined for the protected S1 BiP signal in glucose-starved central nervous system over the average cpm determined for the protected S1 BiP signal in control central nervous system. The corresponding values for calmodulin are included for reference. Numbers for BiP at 3 h are: 3.2-fold increase, control 7.4 $\pm$ 7.4, $n = 2$, glucose starved 23.6 $\pm$ 11.4, $n = 2$, units are cpm $\pm$ SEM; At 7 h: 9.2-fold increase, control 19.6 $\pm$ 1.9, $n = 6$, glucose starved 180.4 $\pm$ 6.8, $n = 5$; at 18 h: 13.7-fold increase, control 7.4 $\pm$ 7.4, $n = 2$, glucose starved 101.1 $\pm$ 2.0, $n = 2$. Numbers for calmodulin are at 3 h: 1.8-fold increase, control 470.7 $\pm$ 29.5, $n = 8$, glucose starved 822.8 $\pm$ 31.5, $n = 4$; at 7 h: 1.4-fold increase, control 313.1 $\pm$ 21.7, $n = 4$, glucose starved 446.5 $\pm$ 75.1, $n = 5$; at 18 h: 1.4-fold increase, control 470.7 $\pm$ 29.5, $n = 8$, glucose starved 638.8 $\pm$ 31.2, $n = 4$. ●, CAM; ■, BiP.

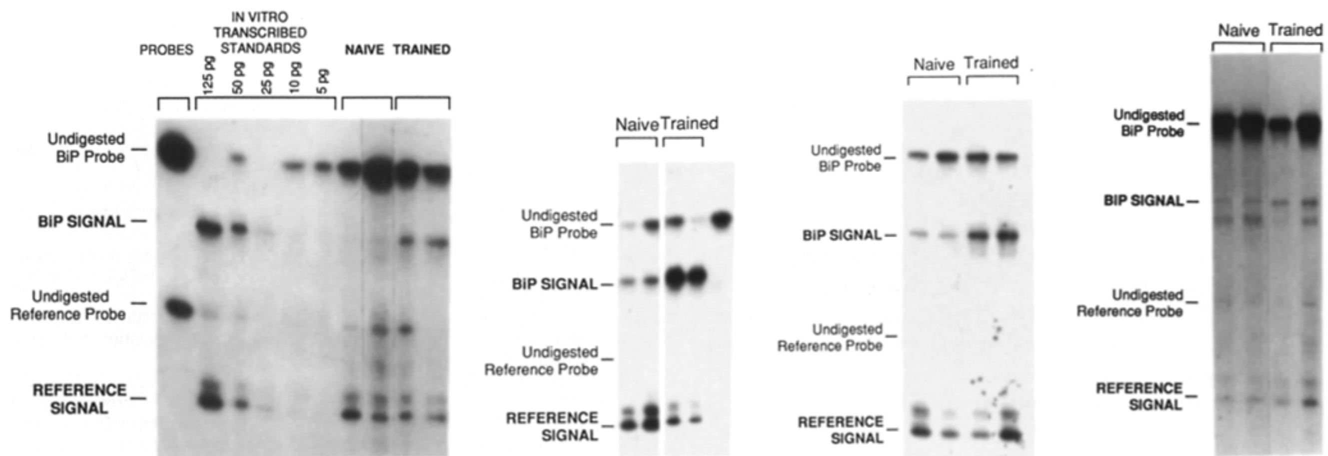

Figure 6. Aplysia BiP mRNA is induced in specific neurons after long-term sensitization training. (*A*) Autoradiograph of representative S1 nuclease analysis of RNA extracted from the pleural sensory clusters of naive and long-term sensitized *Aplysia.* 5 μg of total RNA was used per analysis. Each lane, naive and trained, contains RNA pooled from five animals; however, each animal was assayed only once and therefore each lane represents a completely independent experiment. Known quantities of in vitro–transcribed RNAs corresponding to *Aplysia* BiP and *Aplysia* HSC70 were analyzed in parallel indicating that the S1 nuclease assay was quantitative and sensitive enough to detect 5 pg of cognate transcript. Analysis was carried out with *Aplysia* BiP and *Aplysia* HSC70 probe, whose protected signal served as reference. Positions of undigested probe and protected signal are indicated. The lane labeled as probes at the top of the figure contains only undigested *Aplysia* BiP and *Aplysia* HSC70 S1 probes and no RNA. Lanes with in vitro–transcribed standards are as indicated at the top. The labels naive and trained at the top of the figure signify lanes with RNA of naive and trained animals, respectively. (*B*) Autoradiograph of representative S1 nuclease analysis of RNA extracted from the pleural ganglion after removal of the sensory clusters of naive and long-term sensitized animals. RNA amounts, probes, and abbreviations are as in Fig. 6 *A*. Last lane shows S1 nuclease analysis of 50 μg of yeast carrier RNA; no protected signal for *Aplysia* BiP or *Aplysia* HSC70 was observed. (*C*) Autoradiograph of representative S1 analysis of RNA extracted from the abdominal ganglion sensory wedge of naive and long-term sensitized animals. RNA amounts, probes, and abbreviations are as in Fig. 6 *A*. (*D*) Autoradiograph of representative S1 nuclease analysis of RNA extracted from the abdominal ganglion bag cell neurons of naive and long-term sensitized animals. RNA amounts, probes, and abbreviations are as in Fig. 6 *A*.

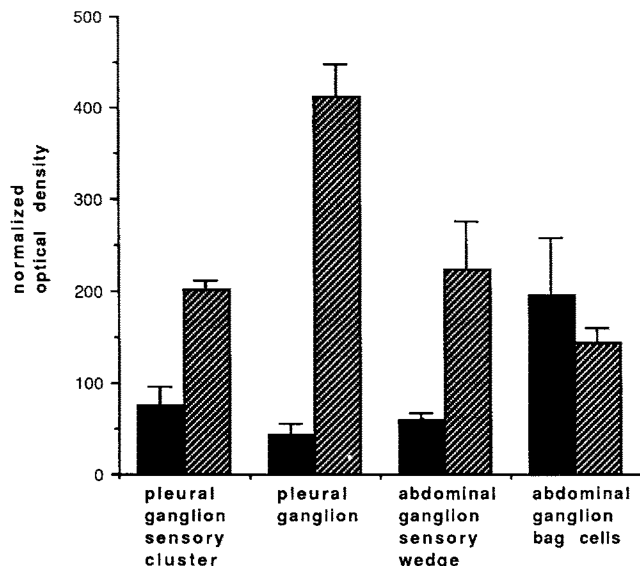

Figure 7. Quantification of S1 nuclease analysis after training. Multiple exposures of autoradiographs were scanned (see Materials and Methods) and results are plotted. After training the steady-state level of BiP mRNA was significantly increased in pleural ganglion sensory neurons (2.7-fold increase, control 75 ± 21, $n = 4$, trained 202 ± 10, $n = 4$, $P < 0.01$, units are normalized OD ± SEM), in the remainder of the pleural ganglion (9.3-fold increase, control 44.5 ± 10.5, $n = 4$, trained 412.2 ± 35.8, $n = 4$, $P < 0.01$), and the abdominal ganglion sensory wedge (3.8-fold increase, control 59.0 ± 10, $n = 5$, trained 244 ± 51, $n = 5$, $P < 0.01$) but remained unchanged in neuroendocrine bag cells, which are not involved in the reflex (0.7-fold decrease, control 197 ± 61, $n = 4$, trained 143 ± 16, $n = 5$, not significant at $P < 0.05$). ■, control; ▨, trained.

cells that specifically participate in a long-term memory process. They are evident in the sensory neurons of the pleural ganglia, which mediate the tail-withdrawal reflex, and in the abdominal ganglion sensory wedge, which contains the sensory and motor neuron cell bodies of the gill-withdrawal reflex. We also detect a large increase in the remainder of the pleural ganglion after removal of the sensory neuron cluster, suggesting that plastic changes induced during long-term sensitization training are not limited to the sensory neurons. Indeed, electrophysiological changes in both interneurons and motor neurons were found during short-term memory for sensitization (Frost et al., 1988). In contrast, we did not observe any significant change in the level of BiP transcripts in neuroendocrine bag cells of the abdominal ganglion, which are not involved in the reflex. Similarly, we did not detect an increase in any other identified member of the HSP70 family of proteins besides BiP during long-term sensitization or facilitation in our 2-D gel analysis (Barzilai et al., 1989; Castellucci et al., 1988). Although HSP70 proteins are related by sequence, their functional role in the cell differs, and their genes are differentially regulated (data not shown; see also Watowich and Morimoto, 1988). The induction of BiP in the absence of the induction of cytoplasmic HSP70 family members may reflect the specific demands imposed on neurons by the synthesis of exocytic proteins during long-term sensitization.

BiP's role as a molecular chaperon is thought to be essential for growth. In yeast, disruption of the BiP gene results in a recessive lethal mutation, demonstrating that BiP is essential for cell viability (Normington et al., 1989; Rose et al., 1989). BiP is synthesized constitutively under normal growth conditions in mammalian cells but is induced by a va-

riety of stimuli (see for example, Brostrom et al., 1990; Drummond et al., 1987; reviewed by Lee, 1987). The signal for induction is thought to be the accumulation of nascent proteins in the ER (Kozutsumi et al., 1988; Normington et al., 1989; Rose et al., 1989).

After long-term sensitization training in *Aplysia*, the sensory neurons grow new presynaptic terminals. In addition, there is an increase in the number and size of active zone release sites within the terminals as well as an increase in the number of vesicles loaded into these release sites (Bailey and Chen, 1983, 1988*a*,*b*, 1989). Similar changes are produced by 5-HT or cAMP in dissociated sensory and motor neurons in primary cell culture (Glanzman et al., 1990; P. Montarolo, personal communication), and by injection of cAMP into pleural sensory neurons (Nazif et al., 1991), consistent with the finding that the promoter region of the BiP gene contains a cAMP-response element (Alexandre et al., 1991). The structural changes first become apparent 3 h after the onset of the 5-HT application (S. Schacher and E. R. Kandel, personal communication), when the increase in BiP is first detected, and at a time when the increase in overall protein synthesis reaches its peak (Barzilai et al., 1989). Thus, it seems likely that BiP contributes to protein processing essential for the growth of neurites and the formation of synaptic connections involved in the establishment of long-term memory. The increase in BiP expression may be a response to the posttranslational challenge of a general increase in protein synthesis. The consequent increase in BiP expression may in turn generate conditions which are permissive for the growth of new synapses.

Alternatively, at least part of the general increase in protein synthesis may represent decreased degradation resulting from an enhancement in protein assembly consequent to the increase in BiP. The individual subunits of the acetylcholine receptor and perhaps other multimeric proteins are thought to be synthesized constitutively and are quickly degraded in the ER when one subunit is limiting (Blount and Merlie, 1990; Claudio et al., 1989). Thus, the finding that BiP increases with the onset of new synapse formation suggests the possibility that BiP itself may be limiting. According to this view, BiP may perform regulatory function and its level of expression might control the availability of protein essential for laying down synaptic structures. It should be possible to distinguish between these two possibilities by selectively overexpressing BiP in the sensory neuron (see Kaang et al., 1992). In the extreme case, if BiP is limiting, simply overexpressing this one protein might induce synaptic growth.

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