Inhibition of Conditioned Stimulus Pathway Phosphoprotein 24 Expression Blocks the Development of Intermediate-Term Memory in *Hermissenda*

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Studies of memory consolidation have identified multiple phases or stages in the formation of memories. The multiple components of memory can be broadly divided into the three phases; short-term, intermediate-term, and long-term. Although molecular changes underlying short- and long-term memory have been examined extensively, the molecular mechanisms supporting the formation of intermediate-term memory are poorly understood. In several examples of cellular and synaptic plasticity, intermediate memory depends on translation but not transcription. One-trial conditioning in *Hermissenda* results in the development of intermediate memory that is associated with enhanced cellular excitability and the phosphorylation of a 24 kDa protein referred to as conditioned stimulus pathway phosphoprotein (Csp24). Using amino acid sequences derived from Csp24 peptide fragments, a full-length cDNA was cloned and shown to contain multiple β -thymosin-like domains. The expression of Csp24 and the development of enhanced excitability, a characteristic of intermediate memory, were blocked by antisense oligonucleotide-mediated downregulation of Csp24 without affecting the induction of immediate enhanced excitability, a characteristic of short-term memory. These results demonstrate that the synthesis of Csp24 is required for the development and maintenance of intermediate memory.

Key words: *Hermissenda*; Csp24; β -thymosin repeat protein; one-trial Pavlovian conditioning; actin-binding proteins; cytoskeleton

Introduction

Studies of memory formation have identified phases that can be differentiated on the basis of the relative contribution of signal transduction pathways, protein synthesis, and gene induction (Ng and Gibbs, 1991; Crow and Forrester, 1993; Rosenzweig et al., 1993; Yin and Tully, 1994; Nguyen et al., 1994; DeZazzo and Tully, 1995; Kane et al., 1997). In several examples of cellular and synaptic plasticity proposed as mechanisms of learning and memory, a distinct intermediate phase of plasticity has been identified that is dependent on translation but not transcription (Orani et al., 1989; Ghirardi et al., 1995; Crow et al., 1999; Sutton et al., 2001). However, specific proteins whose synthesis contribute to the expression of intermediate-term memory have not been identified.

One-trial conditioning of *Hermissenda* results in the phosphorylation of proteins identified from two-dimensional (2-D) gel analysis, the time-dependent development of enhanced cellular excitability in identified sensory neurons of the conditioned stimulus (CS) pathway, and the suppression of CS-elicited locomotion (Crow and Forrester, 1986; Crow and Siddiqi, 1997; Crow et al., 1997, 1999; Crow and Xue-Bian, 2000). An *in vitro* one-trial conditioning procedure produces similar biochemical and electrophysiological changes as obtained after one-trial *in vivo* conditioning (Crow et al., 1996, 1997, 1999; Crow and Siddiqi, 1997). A phosphoprotein regulated by one-trial condition-

ing and associated with the intermediate stage of memory is CS pathway phosphoprotein 24 (Csp24), a β -thymosin-like protein (Crow and Xue-Bian, 2000). The increased phosphorylation of Csp24 is observed after procedures that produce intermediate-term enhanced excitability (Crow et al., 1999), but not after procedures that only result in short-term changes in excitability (Crow and Xue-Bian, 2000).

Here we report the cloning of a full-length Csp24 cDNA and provide evidence that the Csp gene product is a *Hermissenda* homolog of the β -thymosin-like family of actin-binding proteins that contain multiple actin-binding domains (VanTroys et al., 1999; Boquet et al., 2000; Hertzog et al., 2002; Paunola et al., 2002). We show that incubation of isolated *Hermissenda* circumesophageal nervous systems with Csp antisense oligonucleotides (ON) decreases Csp24 synthesis. Treatment with antisense ON before one-trial conditioning blocked the development of enhanced excitability, a characteristic of intermediate-term memory, without affecting the induction of immediate enhanced excitability, a characteristic of short-term memory. In contrast, incubation of nervous systems with scrambled ON did not affect the development of either short- or intermediate-term enhanced excitability. Our results show that the synthesis of Csp24 is essential for the development and maintenance of intermediate-term memory produced by one-trial *in vitro* conditioning.

Materials and Methods

Cloning. The degenerate oligonucleotides Her 2F, 5'-CCNACNGCNGA-RGCNAT-3', and Her 1R, 5'-TCYTGNCDDATRTCTC-3', corresponding to the peptide sequences PTAEAI and DDIGQE, respectively, were used to amplify cDNA products from *Hermissenda* total RNA. The resulting products (251 and 137 bp) were cloned into pGEM-T, sequenced, and used in the design of additional primers to clone the 5' and

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3' ends from *Hermisenda* poly(A)+ mRNA using SMART rapid amplification of cDNA ends (RACE) (Clontech, Palo Alto, CA). The alignments in Figure 1 were performed using the Lasergene 99 software (DNASTAR, Madison, WI). Protein sequences, or conceptual translations of cDNA sequences, were retrieved from GenBank. Alignments for the phylogenetic analysis in Figure 2 were performed using the Clustal X program (Thompson et al., 1997), and an unrooted tree was generated by the neighbor-joining method (Saitou and Nei, 1987). For *in vitro* translation, full-length *Csp24* and *Csp29* cDNAs were amplified from total RNA using either Her exon 1a 5'-GACGGACTACGAAAGAGACC-3' or Her exon 1b 5'-GAGATACTGGTTATCAATTTGAC-3' paired with Her 10R 5'-AAAGTCCCATCTAAACATTGGA-3' and cloned into pCR-II Blunt TOPO (Invitrogen, Carlsbad, CA). One microgram of circular plasmid template was translated using TNT T7 Quick Coupled Transcription/Translation (Promega, Madison, WI) in the presence of 25 μ Ci [35 S]L-methionine (1000 Ci/mmol); 2 μ l of product was separated by SDS-PAGE (15% resolving) and transferred to polyvinylidene difluoride membrane. Blots were exposed for 12–18 hr to a phosphorimaging k-screen, and visualized with the Bio-Rad FX Molecular Imager and QuantityOne software. Immunodetection of the Csp24 and 29 proteins was performed with 1:7500 rabbit anti-Csp24 and 1:20,000 anti-rabbit IgG-HRP and visualized with Supersignal Pico (Pierce, Rockford, IL). Chemiluminescent images were captured with a CCD camera on the Gel Doc 2000 system (Bio-Rad, Hercules, CA).

Fluorescent labeling of Csp antisense ON. Nervous systems were incubated in biotinylated Csp antisense ON for 18–20 hr and fixed in 4% paraformaldehyde in 0.1 M PBS with 30% sucrose overnight at 4°C. Frozen nervous systems were sectioned at a nominal thickness of 16 μ m and incubated with 0.3% Triton X-100 in 0.1 M PBS followed by fluorescent labeling of the biotinylated Csp antisense ON with streptavidin Alexa Fluor 488 conjugate. Sections were viewed at 200 $\times$, and a serial stack of images was collected using a three-line, laser scanning confocal microscope (Bio-Rad Radiance 2000 system).

Conditioning procedures and statistical analysis. Adult *Hermisenda crassicornis* were maintained in an artificial seawater (ASW) aquaria at $14 \pm 1^\circ\text{C}$ on a 12 hr light/dark cycle. Isolated circumesophageal nervous systems were incubated for 18–20 hr in 5 μ M unmodified Csp antisense oligonucleotides 5'-GTGCAAGTCGACGGAAGGA-3' (ON), 5 μ M scrambled 5'-AGAGTGAGCACGGAGTCAG-3' Csp ON, or ASW. A BLAST search of the scrambled ON sequence showed no significant homology to known expressed gene sequences. Electrophysiological measurements of excitability and light-elicited activity of lateral type B photoreceptors were collected from the treated isolated circumesophageal nervous systems at different times after conditioning. The one-trial *in vitro* conditioning procedure consisted of a 5 min presentation of light; the CS (10^{-4} W/cm 2) paired with the application of serotonin (5-HT) (10^{-4} M final concentration) to the isolated nervous system. Unpaired control groups received the CS and 5-HT (10^{-4} M) separated by 5 min. For the unpaired control group, the 5-HT was applied in the dark and washed out after the 5 min exposure. Normal controls received the *in vitro* conditioning procedures after incubation in normal ASW. A two-way ANOVA was used to assess the effects of the various treatments on excitability after conditioning. After the assessment of overall significant differences, selected *post hoc* comparisons consisted of *t* tests for independent groups or correlated means.

Electrophysiology. Intracellular recordings were collected from identified lateral type B photoreceptors at 15 min intervals to 4 hr after conditioning. Animals were prepared for intracellular recording and stimulation with extrinsic current using previously published standard procedures (Crow and Siddiqi, 1997; Crow et al., 1999; Crow and Xue-Bian, 2000). The isolated circumesophageal nervous systems were maintained at $15 \pm 0.5^\circ\text{C}$ in ASW having the following composition (in mM): 460 NaCl, 10 KCl, 10 CaCl $_2$, 55 MgCl $_2$, buffered with 10 mM HEPES and brought to pH 7.6 with NaOH. Excitability was assessed with 2 sec 0.2 nA depolarizing current pulses from a holding potential of -60 mV. Spike frequency was determined by dividing the total number of action potentials by the duration of the extrinsic current pulse. Electrophysiological data were digitized and analyzed using spike 2 software (Cambridge Electronic Design).

1-D and 2-D gel electrophoresis. Protein synthesis after 18–20 hr incubation in 5 μ M Csp antisense ON or 5 μ M scrambled Csp ON was examined in preparations incubated for 2 hr in 175 μ l of oxygenated ASW containing 11 mM glucose and 0.25 mCi of [35 S]L-methionine. After the 2 hr incubation the samples were rinsed in an isotonic ice-cold wash solution containing (in mM): 460 NaCl, 10 KCl, 5 EDTA, and 100 Tris-HCl, pH 7.8, and lysed in a modified lysis solution containing 9.2 M urea, 2% Nonidet P-40, 5% β -mercaptoethanol, and 2% carrier ampholytes (1.6%, pH 5–8; 0.4%, pH 3.5–10), and stored frozen at -80°C . Samples were analyzed by 2-D PAGE using a first-dimension isoelectric focusing gel with an immobilized pH gradient (4–7) and a precast SDS polyacrylamide (8–18% linear gradient) second-dimension gel. Gels containing [35 S]methionine-labeled proteins were stained with SYPRO Ruby and exposed to storage phosphor screens for a period of 48 hr. SYPRO Ruby-stained gels and phosphor screens were computer scanned and analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA) for quantitative analysis. For some experiments, lysates of samples incubated in Csp antisense ON or scrambled ON were loaded for 1-D PAGE followed by protein band staining with SYPRO Ruby and phosphorimage analysis of [35 S]methionine-labeled protein bands. To compensate for potential difference in sample loading, [35 S]methionine-labeled bands from the Csp antisense ON group and scrambled ON group were normalized to actin levels for each sample.

Results

Isolation and characterization of Csp24 cDNA

Degenerate oligonucleotides based on Csp24 peptide sequences obtained in previous experiments (Crow and Xue-Bian, 2002) were used to amplify partial cDNA clones from *Hermisenda* total RNA. Putative full-length Csp24 cDNAs were subsequently obtained through 5' and 3' RACE cloning and characterized by *in vitro* translation and sequence analysis. The Csp gene consists of at least five exons that can undergo alternative splicing to generate at least four distinct mRNA species, resulting in two translated protein isoforms, Csp24 and Csp29 (Fig. 1A). Conceptual translations of the cDNA sequences detected two closely positioned putative start codons, with the upstream codon located within a stronger Kozak context (ATCgtT) than the downstream codon (CTCgtT) (Kozak, 1996). *In vitro* transcription/translation and bacterial expression experiments indicate that although both methionines can serve as translation start sites, there is a preference for initiation at the upstream codon. [35 S]L-methionine-labeled *in vitro* translation products of Csp24 and Csp29 cDNA clones comigrated with the endogenous Csp24 and a 29 kDa protein detected in circumesophageal protein samples with a Csp24 peptide-specific antibody (Fig. 1C), indicating that the cDNA clones contained full-length open reading frames (ORFs). These *in vitro* translation products could also be detected with the same antibody (data not shown). Interestingly, the proteins migrate higher on SDS-PAGE than their predicted molecular weights of 17.2 and 21.5 kDa, respectively (Fig. 1A, C).

Dot-plot analysis of the Csp24 amino acid sequence revealed a modular structure consisting of four internally repeated domains, whereas the 29 kDa isoform contained a fifth repeated domain. Within each of the repeated domains was an actin-binding motif similar to that found in the β -thymosins and related multi-repeat actin-binding proteins. Alignments of the multiple Csp β -thymosin homology domains (BTH1–5) with themselves, representative β -thymosins, and the repeated actin-binding domains of *Acanthamoeba* actobindin, *Drosophila* ciboulot, and *Caenorhabditis elegans* tetrathymosin β are shown in Figure 1, D and E. The Csp BTH domains exhibit 48.5–68.8% identity to β 10thymosin, and 34.4–51.2% identity to each other (Fig. 1E, Table 1). There is also strong homology between some of

Figure 1. Cloning and characterization of *Csp*. *A*, mRNA splice variants of *Csp*. The open box indicates 5' and 3' untranslated regions, the filled red box represents the predicted open reading frame, and the arrow indicates the putative start methionine. The black line indicates the position of the *Csp* antisense oligo; the blue line indicates the positions of the previously identified *Csp24* peptide fragments (Crow and Xue-Bian, 2000). *B*, Predicted amino acid sequence of the *Csp* ORF. Boxed sequences represent the core β -thymosin homology domains. Vertical arrowheads indicate the putative exon 2/3 and 3/4 boundaries. *C*, *In vitro* translation products. Lane 1, No template control. Lane 2, *Csp29* cDNA template translated in the presence of [35 S]methionine. Lane 3, Circumoesophageal protein extract probed with a *Csp24* peptide-specific antibody. Lane 4, *Csp24* cDNA template translated in the presence of [35 S]methionine. Filled arrowhead indicates the position of *Csp24*; open arrowhead indicates *Csp29*. *D*, Alignment versus β -thymosins. Alignment of the five *Csp* repeated domains with themselves and several representative members of the β -thymosin family. Identical amino acids are shaded in black; similar amino acids are shaded in blue. Boxed consensus represents the core β -thymosin homology sequence containing an actin-binding motif (accession numbers AAA36746, P20065, S21282, B59005, A54005, and AY12936). *E*, Alignment versus multidomain actin-binding proteins. Alignment of the five *Csp* domains with the four, three, and two repeated actin-binding domains of *C. elegans* tetrathymosin β , *Drosophila melanogaster* ciboulot, and *Acanthamoeba castellanii* actobindin, respectively. Asterisks denote the consensus actin-binding motif; filled circles denote highly conserved hydrophobic residues that may be involved in α -helix formation (accession numbers NP509430, NP525065, A36614, and AY129238).

the internally repeated domains, although they are less similar to β 10 thymosin: *Csp* β TH4, 5 and Cib D2, 3 (>50% identity), *Csp* β TH3, 5 and tetrathymosin β D1, 2, 3 (>40% identity). Figure 2 shows a phylogenetic analysis of representative actin-binding

proteins, clearly demonstrating that the *Csp* gene products belong to the β -thymosin superfamily of proteins. The clustering also suggests that the *Csp* gene products, in addition to ciboulot, tetrathymosin β , and actobindin, may constitute an

Table 1. Identity Matrix of β Thymosin Homology Domains

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
57.6	56.2	51.5	68.8	48.5	41.0	41.0	43.2	39.3	41.7	55.3	61.1	31.2	28.1	1	β 10Thymosin
	34.4	39.4	40.6	51.5	45.5	36.4	33.3	25.0	40.6	48.5	45.5	30.0	26.7	2	CSP β TH1
		37.5	46.9	43.8	46.9	53.1	53.1	33.3	43.8	50.0	46.9	27.6	27.6	3	CSP β TH3
			50.0	45.5	39.4	36.4	45.5	35.7	40.6	54.5	51.5	36.7	33.3	4	CSP β TH4
				40.6	50.0	40.6	53.1	22.2	43.8	56.2	62.5	27.6	24.1	5	CSP β TH5
					33.3	36.4	45.5	21.4	28.1	42.4	39.4	30.0	23.3	6	CSP β TH2
						33.3	45.9	25.0	41.7	42.1	47.2	37.5	40.6	7	Tetrathymosin β D1
							40.5	28.6	38.9	36.8	47.2	15.6	15.6	8	Tetrathymosin β D2
								32.1	41.2	47.2	48.6	28.1	31.2	9	Tetrathymosin β D3
									18.5	28.6	32.1	24.0	24.0	10	Tetrathymosin β D4
										41.7	45.7	31.0	34.5	11	Ciboulot D1
											59.5	32.3	29.0	12	Ciboulot D2
												33.3	30.0	13	Ciboulot D3
													67.7	14	Ac actobindin D1
														15	Ac actobindin D2

emerging subclass of actin-binding proteins containing multiple β -thymosin-like repeats.

Antisense ON selectively decreases Csp24 synthesis

To examine the role of Csp24 in intermediate memory, antisense oligonucleotides were used to downregulate its expression. Figure 3A shows an example of the uptake of fluorescent-labeled biotinylated Csp antisense in a section of a lateral B photoreceptor. To determine the efficacy and specificity of Csp antisense ON, isolated circumesophageal nervous systems were incubated in Csp antisense ON (5 μ M) or scrambled ON (5 μ M) for 18–20 hr followed by incubation in [³⁵S]L-methionine (0.25 mCi), and the lysates were subjected to 1-D and 2-D PAGE to separate labeled proteins. In addition to Csp24, the synthesis of three proteins previously implicated in conditioning of *Hermissenda* was analyzed in 1-D gels (Fig. 3B). The group data depicted in Figure 3C showed that Csp antisense ON treatment produced a significant reduction in Csp24 synthesis relative to the scrambled ON ($\bar{x}$ = 0.69 $\pm$ 0.06; t_4 = 11.7; p < 0.001), whereas the synthesis of the 21, 32, and 44 kDa protein bands was not significantly affected. Prints of 2-D gels from storage phosphor screens of [³⁵S]methionine-labeled proteins revealed that Csp antisense ON blocked the synthesis of Csp24 (Fig. 3E) as compared with nervous systems treated with scrambled ON (Fig. 3D). The analysis of the SYPRO-stained 2-D gels showed that total Csp24 was significantly reduced by the antisense ON treatment relative to controls treated with scrambled ON ($\bar{x}$ = 0.55 $\pm$ 0.08; t_4 = 5.2; p < 0.007). Summary data for the 2-D gels analysis depicted in Figure 3F showed that Csp antisense ON treatment resulted in a significant reduction in Csp24 synthesis relative to the scrambled ON treatment ($\bar{x}$ = 0.72 $\pm$ 0.09; t_4 = 6.9; p < 0.002).

Inhibition of Csp24 synthesis blocks the development of intermediate-term enhanced excitability after one-trial conditioning

To examine the role of Csp24 in the formation of intermediate-term memory, Csp24 protein synthesis was inhibited before conditioning. Isolated nervous systems were incubated in (5 μ M) Csp antisense ON, 5 μ M scrambled ON, or normal ASW for 18–20 hr followed by the presentation of the one-trial *in vitro* conditioning procedure or unpaired *in vitro* control procedure. After one-trial conditioning or control procedures, lateral type B excitability was examined every 15 min for 4 hr after conditioning. Group data depicting the mean spikes per second elicited by a 2 sec 0.2 nA extrinsic current pulse are shown in Figure 4A, and examples of current-evoked spikes at different times after conditioning associated with short- and intermediate-term memory are shown in Figure 4B–E for the different experimental treatments. The statistical analysis revealed overall significant differences between the experimental and control groups ($F_{(3,20)}$ = 22.6; p < 0.01) and significant differences in excitability between 15 min and 4 hr

Figure 2. Unrooted phylogenetic tree of selected actin-binding protein sequences. An unrooted phylogenetic tree was generated by the neighbor-joining method. Bootstrap analysis (1000 replicates) was performed to assess the reliability of the tree, and the resulting percentage values for the occurrence of each branch are shown. The β -thymosin superfamily branch is shown in red, with the cluster of related proteins containing multiple β -thymosin homology domains circled. Species abbreviations are as follows: Ac, *Acanthamoeba castellanii*; Ai, *Argemone irradicans*; At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Eh, *Entamoeba histolytica*; Hc, *Hermissenda crassicornis*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Om, *Oncorhynchus mykiss*; Rn, *Rattus norvegicus*; Sc, *Saccharomyces cerevisiae*. Accession numbers: A36614, B59005, NP179566, NP194664, NP194663, NP179567, AAB71308, NP525065, AAF53042, AAD29083, BAA07198, AB002757, AY129236, AAD31280, NP444252, D87459, AB026542, AB026543, AF115549, D88460, AF031588, NM009515, P20065, BAB24563, AAH02080, S21282, AJ303456, Q03048, NP014765.

after conditioning ($F_{(15,300)}$ = 3.4; p < 0.01). Significant differences in excitability between the conditioned groups and the unpaired control group were detected at a time associated with short-term memory (Crow and Xue-Bian, 2000) (45 min after conditioning) ($F_{(3,20)}$ = 6.7; p < 0.01). In addition, overall differences between groups were observed during intermediate-term memory (Crow et al., 1999) (90 min after conditioning) ($F_{(3,20)}$ = 10.7; p < 0.01). Paired comparisons of excitability at 90 min after conditioning revealed that the antisense ON group was significantly different from the scrambled ON group (t_{10} = 2.2; p < 0.05) and the conditioned group was significantly different from the unpaired controls (t_{10} = 3.8; p < 0.01). A similar outcome was detected 3 hr ($F_{(3,20)}$ = 10.5; p < 0.01) and 4 hr ($F_{(3,20)}$ = 49.4; p < 0.01) after conditioning. An analysis of changes in excitability over time for the different groups revealed that the conditioned antisense ON group expressed a decreased excitability ($F_{(15,300)}$ = 8.2; p < 0.01), whereas the scrambled ON group ($F_{(15,300)}$ = 1.7; NS) and conditioned group ($F_{(15,300)}$ = 1.6; NS) did not show a significant overall difference in excitability over the 4 hr time period (Fig. 4A). During intermediate-term memory (90 min), the conditioned antisense ON group exhibited a significant decrease in excitability (t_5 = 2.9; p < 0.03) that was stable at 4 hr after conditioning (t_5 = 12.9; p < 0.01) as compared with excitability assessed 15 min after conditioning. These results show that blocking Csp24 synthesis did not affect the induction or maintenance of short-term memory but was sufficient to block the

Figure 3. Antisense ON blocks the synthesis of Csp24. *A*, Confocal image of fluorescently labeled biotinylated Csp antisense ON, in a section of a lateral type B photoreceptor, using streptavidin Alexa Fluor 488 conjugate. *B*, Lysates of nervous systems ($n = 7$) incubated in [35 S]methionine and protein bands resolved with 1-D PAGE. Csp antisense ON ($5 \mu\text{M}$) blocked the synthesis of the 24 kDa protein band, but not the 44, 32, or 21 kDa protein bands. Incubation of nervous systems in scrambled ON ($5 \mu\text{M}$) did not block expression of [35 S]-labeled 24, 44, 32, or 21 kDa protein bands. *C*, Group data showing that Csp antisense ON blocked the synthesis of the Csp24 protein bands relative to the scrambled ON group. 21K, 21 kDa; 32K, 32 kDa; 44K, 44 kDa (apparent molecular weights). *D*, Print from a storage phosphor screen of a 2-D gel showing [35 S]methionine labeling of Csp24 (boxed) from nervous systems ($n = 7$) incubated in scrambled antisense ON ($5 \mu\text{M}$). *E*, Storage phosphor print of 2-D gel showing absence of [35 S]methionine labeling of Csp24 (boxed) in nervous systems incubated in Csp antisense ON ($5 \mu\text{M}$). *F*, Group data ($n = 5$) showing that Csp antisense ON significantly reduces the synthesis of Csp24 relative to scrambled ON controls.

formation of intermediate memory after one-trial *in vitro* conditioning.

We next examined the effect of blocking Csp24 synthesis on light-elicited activity examined during short- and intermediate-term memory (30 min and 2 hr after conditioning). Group data showing mean light-elicited spike activity are depicted in Figure 5A, and examples of light-evoked responses for the conditioned antisense ON and conditioned scrambled ON groups are shown in Figure 5, *B* and *C*. The number of light-elicited action potentials for the conditioned antisense ON group compared with the conditioned scrambled ON group 30 min after conditioning was not significantly different, but was significantly different 90 min ($t_5 = 3.2$; $p < 0.01$) and 2 hr after conditioning ($t_{10} = 4.3$; $p < 0.002$). Further analysis revealed that the conditioned antisense ON group exhibited a significant decrease in light-elicited spike activity at 2 hr as compared with 30 min ($t_5 = 4.7$; $p < 0.006$). In contrast, the conditioned scrambled ON group did not exhibit a change in light-elicited spike activity between 30 min and 2 hr ($t_5 = 0.3$; NS). Moreover, the finding that the conditioned antisense ON group expressed normal light-elicited generator potentials but reduced spike activity near the end of the recording session indicates that the decay in excitability and light-elicited spike activity were not attributable to a general rundown of the sensory neurons or reduced sensitivity to light.

Discussion

We have shown previously using both one-trial conditioning and one-trial *in vitro* conditioning procedures that translation and transcription exhibit different temporal domains for memory

consolidation (Crow et al., 1999). The protein synthesis inhibitor anisomycin, but not the mRNA synthesis inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole, significantly reduced phosphorylation of Csp24 2 hr after conditioning. Previously, inhibition of translation and transcription was shown to block long-term enhanced excitability, while leaving short-term enhanced excitability intact (Crow and Forrester, 1990; Crow et al., 1997). In addition, procedures that only produce short-term enhanced excitability did not result in Csp24 phosphorylation (Crow and Xue-Bian, 2000). These results suggested that Csp24 regulation via translation and phosphorylation may be involved in long-term enhanced excitability.

In this report we demonstrate that selective inhibition of Csp24 synthesis through the use of an unmodified antisense oligonucleotide blocks the expression of intermediate-term enhanced excitability while leaving short-term enhanced excitability intact. The change in excitability may be mediated by a local synthesis of Csp24 or generation of a new pool of available Csp24. Alternatively, the antisense knockdown experimental results could also be caused by a reduction of the Csp24 pool available to be phosphorylated. We have demonstrated previously that one-trial conditioning increases Csp24 phosphorylation (Crow et al., 1999). The regulation of Csp24 phosphorylation may be operating in part through protein kinase C (PKC), because pretreatment with GF109203X, an inhibitor of both Ca^{2+} -dependent and independent PKC isoforms, significantly reduced conditioning-dependent regulation of Csp24 phosphorylation (T. Crow, unpublished observations). Examination of the amino

Figure 4. Blocking Csp24 synthesis inhibits the development of enhanced excitability during intermediate memory but does not block short-term enhanced excitability. *A*, Group data depicting mean frequency (spikes per second) elicited by 2 sec 0.2 nA extrinsic current pulses for a conditioned group ($n = 6$) incubated in 5 μ M Csp antisense ON (18–20 hr), a conditioned group ($n = 6$) incubated in 5 μ M scrambled ON (18–20 hr), a conditioned group ($n = 6$) incubated in normal ASW (18–20 hr), and an unpaired control group ($n = 6$) incubated in ASW (18–20 hr). *B*, Examples of excitability assessed with extrinsic current pulses for the different experimental conditions at different times after conditioning. The example for the conditioned antisense ON group at 30 min exhibited short-term enhancement (*B1*) but not intermediate-term enhancement at 150 min (*B2*). In contrast, the scrambled ON group showed both short-term (*C1*) and intermediate-term (*C2*) enhancement similar to the conditioned group (*D1*, *D2*). The unpaired controls exhibited less short-term enhancement (*E1*) and no intermediate-term enhancement (*E2*). Cond, Conditioned.

acid sequence for Csp24 using the Prosite database revealed potential PKC phosphorylation sites at positions Serine⁸⁷, Serine⁹⁹, and Threonine¹⁵⁰ (Fig. 1*B*).

The contribution of different second messengers to stages of

Figure 5. Blocking the synthesis of Csp24 inhibits the development of intermediate-term light-elicited increase in spike activity, but not short-term light-elicited increase in spike activity. *A*, Group data depicting the mean frequency of light-elicited (2 sec) spike activity in a group ($n = 6$) incubated in 5 μ M Csp antisense ON (18–20 hr) before conditioning. *B*, Examples of light-elicited generator potentials and spikes for the conditioned Csp antisense ON group at 30 and 120 min and the conditioned scrambled ON group at 30 and 120 min. The antisense ON treatment reduced light-elicited spike activity at 2 hr after conditioning (intermediate memory) without affecting generator potential amplitude. Cond, Conditioned.

memory consolidation in *Hermisenda* is complex, depending on the conditioning paradigm and the cellular correlate associated with learning. Previous studies have shown that PKC is involved in the induction of enhanced excitability produced by conditioning procedures involving one or few conditioning trials (Matzel et al., 1990; Crow et al., 1991; Crow and Forrester, 1993) and 5-HT-dependent synaptic facilitation (Schuman and Clark, 1994). The expression of long-term enhanced excitability in type A and B photoreceptors produced by multi-trial Pavlovian conditioning can be reversed by PKC inhibitors, suggesting that enhanced excitability is influenced by a constitutively active kinase or a persistent kinase activator (Farley and Schuman, 1991; Fryszak and Crow, 1997). However, long-term synaptic facilitation produced by multi-trial Pavlovian conditioning was not reversed by injections of the PKC peptide inhibitor PKC (19–36) (Fryszak and Crow, 1997).

Additional second messengers and phosphoproteins have been implicated in studies of cellular and synaptic plasticity after Pavlovian conditioning in *Hermisenda*. The phosphorylation and activation of extracellular signal-regulated protein kinase (ERK) is observed after both one-trial and multi-trial Pavlovian conditioning (Crow et al., 1998). PKC has been shown to contribute to the 5-HT activation of the ERK pathway. The increased phosphorylation of ERK by 5-HT is reduced but not blocked by pretreatment with the calcium chelator BAPTA-AM or pretreatment with PKC inhibitors GF109203X or Gö6976 (Crow et al., 2001). The results showed that 5-HT-dependent ERK phosphorylation involves both a PKC-dependent and a PKC-independent pathway. Moreover, procedures that produce only short-term enhanced excitability did not result in increased ERK phosphorylation (Crow et al., 2001). A protein that has been identified previously with long-term memory in *Hermisenda* is calexitin/cp20, a low molecular weight GTP- and Ca^{2+} -binding protein (Ascoli et al., 1997) that is phosphorylated after multi-trial conditioning (Neary et al., 1981). However, the contribution of the ERK signaling pathway, and specifically calexitin, to intermediate memory formation has not been established. Taken collectively, it is likely that different stages of memory associated with one-trial and multi-trial Pavlovian conditioning may involve the interaction of distinct signaling pathways and the regulation of multiple phosphoproteins.

Sequence analysis revealed that Csp24 contains four putative

actin-binding domains. Actin coprecipitates with Csp24, and Csp24 is colocalized with presumed G-actin but not F-actin in the cytosol of photoreceptor cell bodies (Crow and Xue-Bian, 2002). In addition, our results show that Csp24 exhibits a strong sequence homology with domains of ciboulot found in *Drosophila* and tetrathymosin β in *C. elegans*. Recent studies of the biochemical properties and function of actobindin, ciboulot, and tetrathymosin β suggest that the contribution of this new family of actin-binding proteins to the control of actin filament dynamics may be different from β -thymosin (Hertzog et al., 2002; Paunola et al., 2002). All known vertebrate and invertebrate β -thymosins bind actin monomers (Nachmias, 1993; Safer and Chowrashi, 1997; Stoeva et al., 1997). Like β -thymosin, proteins of the β -thymosin repeat family can also sequester G-actin. However, unlike β -thymosin, these proteins participate in filament barbed-end assembly like the profilin-actin complex (Boquet et al., 2000; Hertzog et al., 2002). Therefore, it is likely that Csp24 may also contribute to the regulation of filament dynamics.

The assembly and disassembly of actin filaments induced by extracellular signals underlie a number of cellular processes. The actin cytoskeleton has been proposed to play a key role in cellular and synaptic plasticity (Fifkova and Morales, 1992; Kim and Lisman, 1999; Matus, 1999; Fischer et al., 2000; Halpain, 2000; Zhou et al., 2000). To perform functions supporting cellular plasticity, the organization of the actin cytoskeleton requires both temporal and spatial regulation. The activity of these proteins is in turn modulated by intracellular signals, resulting in the recruitment of actin nucleation and polymerization at specific cellular sites (for review, see Schmidt and Hall, 1998). The synthesis and regulation of Csp24 by phosphorylation is a likely mechanism that could influence actin dynamics.

A major aspect of the contribution of actin to cellular plasticity may also involve its role in anchoring ion channels to the plasma membrane, in addition to directly contributing to the regulation of K^+ channel activity (Ehrhardt et al., 1996; Maguire et al., 1998; Maruoka et al., 2000). Previous work has shown that the enhanced excitability of type B photoreceptors is associated with the 5-HT-dependent modulation of the sustained Ca^{2+} current ($I_{Ca,s}$), a hyperpolarization-activated inward rectifier current (I_h), and several K^+ conductances (I_A , $I_{K,Ca}$) (Acosta-Urquidí and Crow, 1993; Yamoah and Crow, 1995, 1996). Reductions in I_A and $I_{K,Ca}$ or an increase in I_h would be expected to enhance excitability. Therefore, the regulation of Csp24 by one-trial conditioning may contribute to changes in K^+ channel or I_h channel organization or density by influencing actin filament activity or affect channel gating by modifying actin filament dynamics in neurons of the CS pathway in the intermediate-term transition period between short- and long-term memory.

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