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Proteomic Analysis of Post-Translational Modifications in Conditioned *Hermisenda*

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Abstract

Post-translational modifications of proteins are a major determinant of biological function. Phosphorylation of proteins involved in signal transduction contributes to the induction and maintenance of several examples of cellular and synaptic plasticity. In this study we have identified phosphoproteins regulated by Pavlovian conditioning in lysates of *Hermisenda* nervous systems using 2DE in conjunction with ³²P labeling, fluorescence based phosphoprotein in-gel staining, and mass spectrometry. Modification of protein phosphorylation regulated by conditioning was first assessed by densitometric analysis of ³²P labeled proteins resolved by 2DE from lysates of conditioned and pseudorandom control nervous systems. An independent assessment of phosphorylation regulated by conditioning was obtained from an examination of 2D gels stained with Pro-Q Diamond phosphoprotein dye. Mass spectrometric analysis of protein digests from phosphoprotein stained analytical gels or Coomassie blue stained preparative gels provided for the identification of phosphoproteins that exhibited statistically significant increased phosphorylation in conditioned groups as compared to pseudorandom controls. A previously identified cytoskeletal related protein, Csp24, involved in intermediate-term memory exhibited significantly increased phosphorylation detected 24 hrs post-conditioning. Our results show that proteins involved in diverse cellular functions such as transcriptional regulation, cell signaling, cytoskeletal regulation, metabolic activity, and protein degradation contribute to long-term post-translational modifications associated with Pavlovian conditioning.

Keywords

β-thymosin repeat protein; *Hermisenda*; cytoskeletal proteins; Pavlovian conditioning; post-translational modification

Post-translational modifications that change the properties of proteins are of major importance for biological function. For example, a number of biological events such as signal transduction, transcriptional regulation, control of enzyme activity, and cell division are regulated by the phosphorylation of proteins (for reviews see Hunter and Karin, 1992; Johnson and Vaillancourt, 1994; Hunter, 1995; Karin and Hunter, 1995; Johnston and O'Reilly, 1996; Mann and Jensen, 2003). In addition, phosphorylation is now recognized as important in the induction

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and maintenance of cellular and synaptic plasticity (for example see Neary et al., 1981; Sweatt and Kandel, 1989; Crow et al., 1996; Kondo et al., 2005; Wang et al., 2005; Baratier et al., 2006; Moulton et al., 2006; Sun et al., 2006; Sunyer et al., 2008). The onset and duration of protein phosphorylation varies over time following stimulation (Mooney and Anderson, 1989; Wahl et al., 1989; Ahn et al., 1990; Donaldson et al., 1991; Hagiwara et al., 1992; Moreland et al., 1992; Sato et al., 1999), and may be transient or long-term, depending upon the duration of the experimental treatment (Sweatt and Kandel, 1989; Homayouni et al., 1995, 1997). However, protein phosphorylation may also be detected within minutes-to-hours following a single experimental treatment (Crow et al., 1996).

In the marine mollusc *Hermissenda* the regulation of several phosphoproteins has been examined following multi-trial and one-trial Pavlovian conditioning (Neary et al., 1981; Alkon and Nelson, 1990; Crow et al., 1996; Nelson et al., 1996). Two phosphoproteins that have been identified and characterized in *Hermissenda* sensory neurons (photoreceptors) are calexcitin (CE), a GTP- and Ca^{2+} binding protein (Kuzirian et al., 2001), and Csp24, a protein containing repeated β -thymosin homology domains (Crow and Xue-Bian, 2000, 2003; Crow et al., 2003). The phosphorylation of Csp24 is regulated by one-trial *in vitro* conditioning. Previous studies of *Hermissenda* isolated nervous systems showed that one-trial *in vitro* conditioning resulted in a time-dependent increase in the phosphorylation of several proteins (Crow et al., 1996, 1999; Crow and Xue-Bian, 2003). However, with the exception of Csp24, additional phosphoproteins regulated by one-trial conditioning have not been identified or characterized. In addition, it is not known if multi-trial Pavlovian conditioning regulates the same phosphoproteins that are modified following one-trial *in vitro* conditioning procedures. Here we report that multi-trial Pavlovian conditioning results in a significant increase in the phosphorylation of 12 of 20 proteins from the sample selected for analysis, as compared to control groups that received pseudorandom presentations of the conditioned stimulus (CS) and unconditioned stimulus (US). Tryptic digests of the selected spots from 2D gels of conditioned and control lysates followed by mass spectrometric sequencing resulted in peptide sequences that were assigned by homology to proteins with diverse cellular functions.

EXPERIMENTAL PROCEDURES

Pavlovian conditioning

A total of 170 animals were used in the experiments. Adult *Hermissenda crassicornis* obtained from Sea Life Supply, Sand City, CA were maintained in artificial sea water (ASW) aquaria at $14 \pm 1^\circ\text{C}$ on a 12 hr light/dark cycle. The details of the conditioning procedure have been described in previously published papers (Crow and Alkon, 1978; Crow and Offenbach, 1983; Crow, 1985). Briefly, adult *Hermissenda* were conditioned using light as a conditioned stimulus (CS) and orbital rotation as the unconditioned stimulus (US). Animals received 100 trials of the 10 sec CS paired with a 9 sec US. Controls received 100 trials of the CS and US programmed on independent random schedules with the restriction that the CS and US not overlap in time e.g. pseudorandom. Behavior was tested 24 hours following conditioning using automated procedures to determine start latencies in response to the CS, the measure of inhibition of light-evoked locomotion (Crow and Offenbach, 1983; Crow, 1985). Start latencies were examined before conditioning to establish a baseline for all animals. The animals were placed into glass tubes filled with ASW and confined to one end of the tube by a small foam plug used as a starting gate. Tubes were placed into spring clips on a modified turntable housed inside an incubator to maintain temperature at 15°C . Animals were dark-adapted for 12 minutes prior to baseline testing of behavior. A light spot (10^{-4} w/cm^2 , white light) provided by a fiber optic bundle was projected onto the center of the turntable, illuminating a circular area 15-16 cm in diameter. Following the period of dark adaptation the foam plugs were removed, and the elapsed time to initiate locomotion in the presence of the test light was recorded when an

animal moved between an infrared emitter and a phototransistor at the starting end of each glass tube. When the infrared beam was interrupted, a free-running digital clock was automatically turned off and the time recorded for later data analysis. The conditioned groups were compared to pseudorandom controls that had received the same number of CS and US presentations as conditioned animals.

2D gel electrophoresis and protein phosphorylation

Following behavioral testing, modification of protein phosphorylation was examined in lysates of isolated circumesophageal nervous systems from conditioned and pseudorandom control preparations. Proteins in lysate samples were resolved by 2DE using a first-dimension isoelectric focusing (IEF) gel with an immobilized pH gradient (4-7) and a precast SDS polyacrylamide (8-18% linear gradient) second-dimension gel. In the first stage of the experiments protein phosphorylation in conditioned and control preparations were conducted with radiolabeling of proteins. The isolated nervous systems were incubated for 2 hr in 200 μ l of oxygenated ASW containing 11 mM glucose and 0.125 mCi of $^{32}\text{PO}_4$ (carrier-free; PerkinElmer Sciences, Waltham, MA). To minimize potential animal-to-animal variability in ^{32}P uptake, nervous systems from five animals were used from conditioned and control procedures in five independent replications (total number of animals = 50). After the 2 hr incubation the samples were rinsed in an isotonic ice-cold wash solution (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), 100 mM NaF, 1 mM sodium orthovanadate, 0.1 mM okadaic acid and stored frozen at -80°C . Proteins in the samples were resolved by 2DE as described above. Each precast gel provided the opportunity to run a side-by-side sample from a conditioned group and a pseudorandom control, thus providing paired comparisons of proteins resolved under the same conditions. Gels containing ^{32}P labeled proteins were stained in SYPRO Ruby protein stain for 90 min, destained for 30 min and air dried overnight followed by exposure to storage phosphor screens for 24 hr. Phosphor screens were scanned and analyzed using ImageQuant software (GE Healthcare, Piscataway, NJ) for quantitative analysis of ^{32}P labeling. Densitometric analysis of matched spots from SYPRO Ruby protein stained gels was conducted to determine total protein levels for conditioned groups and pseudorandom controls in order to control for potential differences in protein loading, and provided for normalization of ^{32}P levels of phosphoproteins relative to total protein.

In the second stage of the experiments phosphorylation was examined in conditioned and control samples using a fluorescence-based technique for the detection of phosphoproteins (ProQ Diamond phosphoprotein gel stain, Molecular Probes, Eugene, OR). Proteins separated by 2DE from 10 circumesophageal nervous systems of conditioned and control animals in six independent replications (total number of animals = 120) were stained using the phosphoprotein dye. The fluorescent dye staining was used in conjunction with SYPRO Ruby stain or Coomassie blue to provide for visualization from the same gel of phosphoproteins and total protein levels. Gels were fixed in 100 ml fixation solution (50% methanol, 10% acetic acid) for 30 min, followed by a change in fresh fixation solution for overnight fixation. The gels were washed with distilled H_2O and incubated in 100 ml Pro-Q Diamond stain in the dark for 90 min, and destained (20% 1,2-propanedial, 50 mM sodium acetate, pH 4.0, three changes of 30 min and the 5 min wash period in distilled H_2O). Images of the stained gels were acquired using a Molecular Dynamics Typhoon 9400 imager (GE Healthcare, Piscataway, NJ) with a 532 nm laser excitation and 580 nm bandpass emission filter. Following the scans of phosphoproteins the gels were stained in SYPRO Ruby protein stain for 90 min, destained in 10% methanol 17% acetic acid for 30 min, and 10% glycerol for 20 min. Gels were air dried overnight in the dark and scanned by a STORM 860 imager (GE Healthcare, Piscataway, NJ). For some experiments phosphoprotein stained gels were post-stained with Coomassie blue.

Spot selection from 2D gels

Only readily visible ^{32}P labeled spots that could be easily and reliably identified from all 2D gels were included in the sample. With regards to the MS identification of proteins, protein abundance based upon the matching of the ^{32}P labeled spots to corresponding Coomassie blue stained preparative gels was a criterion for selection. Previously analyzed, but unidentified phosphoproteins from studies of one-trial *in vitro* conditioning were also included in the final sample. The inclusion of all ^{32}P labeled phosphoproteins that could be detected from 2D gels was not attempted.

Following densitometric analysis, proteins of interest were excised by hand from Coomassie blue stained 2D gels or gels treated with the phosphoprotein stain and prepared for MS analysis. In the first experimental stage involving radiolabeling, ^{32}P labeled proteins were matched to multiple Coomassie blue stained preparative gels. Spot matching from multiple gels was carried-out by measurements of the selected spots relative to several abundant evenly spaced proteins used as reference points. For some experiments the reliability of the procedure for localizing spots from multiple Coomassie stained gels was validated by an independent comparison of the results of *de novo* sequencing from a second group of samples. Overall validation was based upon identical MS results from the analysis of phosphoprotein stained analytical gels and preparative gels matched to ^{32}P labeled samples.

Protein identification by Mass Spectrometry

Protein Digestion—Gel spots were dehydrated in 50% acetonitrile in 0.1M NH_4HCO_3 , dried in a SpeedVac and dissolved in 0.05 M NH_4HCO_3 containing 10ng/ μl modified trypsin (Promega) and digested for 20hr at 37° C. The supernatant was removed and fragments extracted with aqueous 50% acetonitrile, 1% formic acid for 30 min. Samples were then evaporated to ~10 μl , acidified with formic acid to ~pH 3 and desalted on a C_{18} ZipTip (Millipore). Peptides were eluted from the ZipTip with 3-5 μl of an aqueous solution of 50% acetonitrile containing 2% formic acid for analysis by mass spectrometry.

MALDI-TOF and Nanospray MS/MS Analysis of Protein Digests—Protein digests (1 μl) were spotted on a MALDI target plate, with matrix (HCCA, alpha-cyano-4-hydroxycinnamic acid), dried and analysis performed in reflector mode on an ABI/SCIEX 4700 Proteomics Analyzer TOF/TOF mass spectrometer with internal calibration using trypsin autodigestion peptides. Detected monoisotopic peptide masses were analyzed using MS-Fit (Protein Prospector, University of California, San Francisco) or ProFound (PROWL, Rockefeller University) for protein database searches and protein identification by peptide mass fingerprinting (PMF). For most of the samples there were no good matches or identifications by PMF since the *Hermisenda* genome has not been sequenced. The remainder of the digests were then analyzed on an ABI 4000 Q TRAP, a hybrid triple quadrupole linear ion trap mass spectrometer. Samples were directly introduced into the mass spectrometer via a nano-electrospray capillary tube (Protana, Odense, Denmark). MS/MS fragmentation spectra were collected on selected peptide ions in the positive ion mode and sequences were determined by visual inspection. The NCBI database was searched using the MS-Pattern search program from Protein Prospector, allowing for several mismatches in the sequence that was entered. A protein was identified as the target protein if it contained at least 2 peptide sequences that were homologous to those determined from the MS/MS peptide sequence that was entered.

RESULTS

Conditioning

The experiments were conducted in two stages. In the first, 25 animals were in the conditioned group and 25 animals were assigned to the pseudorandom control group. Protein

phosphorylation after conditioning was assessed by ^{32}P labeling of isolated nervous systems. In the second stage, 60 animals were conditioned and 60 animals were in the pseudorandom control group. Differences in protein phosphorylation between groups was assessed by phosphoprotein staining of 2D gels in the second stage. The conditioned groups received 100 conditioning trials and control animals received 100 pseudorandom presentations of the CS and US. Inhibition of light-evoked locomotion, the conditioned response, was tested 24 hr following conditioning by comparing the post-conditioning start latencies elicited in light to baseline start latencies elicited in light before conditioning. Assessment of the effectiveness of conditioning was determined by computing suppression ratios ($A/A+B$) that compared post-conditioned behavior (B) with pre-conditioned measurements (A). The mean suppression ratios for the conditioned group and pseudorandom controls were 0.35 ± 0.04 and 0.52 ± 0.03 respectively. The statistical analysis revealed that 100 conditioning trials produced significant inhibition of light-elicited locomotion as compared with the group that received 100 pseudorandom presentations of the CS and US ($t_{168}=2.7$; $p < .005$).

Post-conditioning ^{32}P labeling of proteins

Changes in protein phosphorylation were examined by 2DE separation of proteins in lysates from the nervous systems of conditioned and control animals incubated in $^{32}\text{PO}_4$ after behavioral testing. To minimize animal-to-animal variability in ^{32}P uptake, the circumesophageal nervous systems from five conditioned and five pseudorandom controls were used for each experimental replication. To reduce the effects of gel-to-gel variability, each experimental replication ($n=5$) consisted of lysates from conditioned and pseudorandom controls run side-by-side on the same 2D gel. Therefore densitometric comparisons were between the conditioned group and the corresponding pseudorandom control on each 2D gel. Densitometric analyses of the 2D gels revealed that multi-trial Pavlovian conditioning resulted in an increase in ^{32}P incorporation into proteins as compared to the lysate samples from pseudorandom controls. Representative images from scans of SYPRO Ruby stained proteins in conditioned and control samples are shown in Fig. 1A1-B1. A sample of twenty phosphoproteins were selected for analysis from ^{32}P labeled proteins and spots extracted from phosphoprotein stained gels based upon the criteria listed in the Methods section. Densitometric analysis of the selected proteins on SYPRO stained 2D gels was conducted to verify approximately equal loading of proteins after the experimental and control procedures, and to provide for normalization of phosphorylation relative to total protein to compensate for potential differences in protein abundance between samples. Figure 1A2-B2 shows representative examples of images from a storage phosphor screen of 2D gels with ^{32}P labeling of proteins from nervous systems of conditioned and pseudorandom control animals from the same gels stained with SYPRO Ruby shown in Fig. 1A1-B1. The phosphoproteins selected for statistical analysis are enclosed by white rectangles shown in Fig. 1A2-B2. Adjacent spot comparisons between examples of phosphoproteins from conditioned and pseudorandom controls are shown in Fig. 1. The spots indicated by the arrows in Figure 2 correspond to the different regions of interest shown in Fig. 1A2-2B2. The densitometric analysis of the phosphoimaging scans of ^{32}P labeled proteins shown in Fig. 1A2-B2 and Fig. 2 revealed that conditioning resulted in an increase in ^{32}P incorporation in proteins from conditioned samples relative to the samples from pseudorandom controls. The statistical analysis (ANOVA) of the densitometric measurements of sample phosphoproteins indicated significant overall differences between the conditioned group and pseudorandom controls ($F_{(1,72)}=5.4$; $p < 0.02$). Differences in ^{32}P incorporation in phosphoproteins from both the conditioned group and controls also showed an overall significant difference ($F_{(8,72)}=5.5$; $p < .001$). Since each side-by-side 2D gel contained proteins from conditioned preparations and controls separated under the same conditions, experimental/control ratios were computed for the statistical analysis of paired comparisons of individual proteins. For the statistical analysis of the densitometric measures, ^{32}P incorporation into each protein was normalized to the corresponding

densitometric analysis of protein levels generated from scans of the SYPRO stained 2D gels. Densitometric analyses also were conducted on the ratios of phosphoprotein dye stained spots from conditioned and pseudorandom control samples (see below). The results of the statistical analysis of the ratios of conditioned to pseudorandom controls for each of the proteins selected for analysis revealed significant differences in ^{32}P incorporation or phosphoprotein staining for 12 of 20 phosphoproteins in the sample (see Table 1).

Phosphoprotein staining

To provide for the identification of phosphoproteins independent of radiolabeling experiments, we examined proteins in lysate samples from conditioned and pseudorandom control circumesophageal nervous systems separated by 2DE, stained with Pro-Q Diamond phosphoprotein fluorescent dye and SYPRO Ruby or Coomassie blue stain, followed by MS analysis. The compatibility of the phosphoprotein stain with MS provided for the opportunity to obtain sequence data from each analytical gel. This provided an independent replication of protein identification obtained initially from the Coomassie stained preparative gels. Six independent replications consisting of nervous systems from 10 conditioned and 10 pseudorandom controls were used in the analysis of phosphoprotein stained gels. Figure 3A shows a representative image from a scan of a 2D gel from conditioned preparations stained with Pro-Q Diamond phosphoprotein dye and Fig. 3B is an example of an image from a scan of a 2-D gel from the corresponding pseudorandom controls stained with the phosphoprotein dye. The phosphoproteins enclosed by the white boxes were selected for MS analysis. Consistent with the results of the radiolabeling experiments, statistically significant changes in the density of phosphoprotein staining of 2D gels from conditioned and control samples were detected following multi-trial Pavlovian conditioning. The statistical analysis (ANOVA) of the densitometric measurements of the spots revealed a significant overall difference between the conditioned group and pseudorandom controls ($F_{(1,150)}=8.77; p < .01$).

MS/MS identification of proteins

Proteins in lysate samples from nervous systems of conditioned animals were separated by 2DE, and stained with Coomassie blue or phosphoprotein stain. Spots were excised from preparative and analytical 2D gels followed by tryptic digests, the generation of fragmentation data and *de novo* peptide sequencing. Since no molluscan genome has been sequenced, protein assignment was based upon database searches for homologous proteins exhibiting conserved peptides. The database homology search engine using the amino acid sequences of peptides derived from MS/MS fragmentation spectra resulted in the assignment of 19 phosphoproteins in the sample not previously examined in conditioned *Hermisenda*. The amino acid sequences of peptides generated from the MS/MS analysis are shown in Table 2. The spot numbers of identified proteins in Table 2 correspond to the spots enclosed by the numbered white boxes in Fig. 3. Five of the 20 MS analyzed spots were not identified, and are listed with their peptide sequences in Table 2 as unknown. Assignment based upon the MS/MS analysis resulted in the classification of proteins with diverse functions and interactions potentially contributing to the establishment of long-term memory. The functions of proteins in the sample involved cell signaling, cytoskeletal changes, transcriptional and translational control, protein degradation, and metabolic changes. In addition to previously identified Csp24, a number of proteins in the sample identified by conserved peptide sequences were cytoskeletal-related. Intermediate filament protein A, retrograde protein 51, actin, tubulin folding co-factor B, tropomyosin, and Csp24 exhibited significant regulation of phosphorylation in conditioned animals. Interestingly, actin did not exhibit changes in ^{32}P incorporation with conditioning. However phosphorylation regulated by conditioning was detected from phosphoprotein stained gels. Proteins that were visible with the phosphoprotein stain but not observed on the phosphor image may represent phosphorylated proteins that have lower turnovers. Three additional phosphoproteins were identified from the phosphoprotein stained 2D gels that were not initially selected for analysis

from Coomassie stained preparative gels. Neurocalcin, pirin, and ribosomal protein PO were identified from the MS/MS analysis of phosphoprotein stained analytical gels. Pirin ($t_5=2.64$; $p<.025$) and ribosomal protein PO ($t_5=2.34$; $p<.05$) exhibited statistically significant increased phosphorylation with conditioning. Neurocalcin showed an increase in phosphorylation in conditioned animals (mean= $1.52\pm.4$) that was not statistically significant ($t_5=1.29$; NS). Phosphoprotein Csp24 that was originally identified from ^{32}P labeling, also exhibited increased phosphorylation with conditioning using the Pro-Q Diamond stain. These results provide the first evidence for long-term post-translational modification of Csp24 by multi-trial Pavlovian conditioning.

DISCUSSION

Pavlovian conditioning in *Hermissenda* produces modifications in synaptic efficacy and intrinsic excitability in identified neurons in the CS pathway (for a recent review see Crow, 2004). Associated with the electrophysiological and biophysical modifications produced by conditioning are post-translational modifications of proteins in the circumesophageal nervous system and in sensory neurons of the CS pathway (Neary et al., 1981; Crow et al., 1996; Crow and Xue-Bian, 2000; Kuzirian et al., 2001). One identified protein, CE is a GTP- and Ca^{2+} -binding protein that is activated by Ca^{2+} influx and binds to ryanodine receptors to increase cytosolic Ca^{2+} (Nelson et al., 1996; Ascoli et al., 1997). CE is phosphorylated by PKC which results in its translocation to membranes. Conditioning increases CE levels in the sensory neurons (type B photoreceptors) and contributes to enhanced excitability by K^+ -channel inactivation (Kuzirian et al., 2001). A second protein that has been characterized, and contributes to enhanced excitability produced by one-trial conditioning is Csp24. Csp24 is a β -thymosin-like protein that is homologous to members of the family of β -thymosin repeat proteins that contain multiple actin binding domains (Crow and Xue-Bian, 2000; Crow et al., 2003). Actin co-precipitates with Csp24, and is localized with Csp24 in the cytosol of type B photoreceptor cell bodies (Crow and Xue-Bian, 2003). Protein kinase C influenced co-precipitation of Csp24 with actin is regulated by one-trial *in vitro* conditioning and recombinant Csp24 sequesters G-actin *in vitro* (Redell et al., 2007). The phosphorylation of Ser-122, but not Ser-49 of Csp24 is produced by conditioning procedures that result in intermediate-term and long-term intrinsic enhanced excitability, but not after procedures that result in short-term changes in excitability of sensory neurons (Crow et al., 1999; Crow and Xue-Bian, 2000, 2007). Moreover, treatment with a *Csp* antisense oligonucleotide (ON) before one-trial conditioning blocks intermediate-term enhanced excitability, without affecting the induction of short-term enhanced excitability (Crow et al., 2003). One-trial conditioning in isolated type B-photoreceptors produces a depolarized shift in the steady-state activation curve of the A-type transient K^+ current (I_A) without altering the inactivation curve (Yamoah et al., 2005). The conditioning-dependent reduction in I_A is blocked by pre-incubation of isolated B-photoreceptors with *Csp* antisense ON. We now show that Csp24 phosphorylation is also regulated by multi-trial Pavlovian conditioning, and the increased phosphorylation of Csp24 persists for at least 24 hrs following conditioning and behavioral testing. Taken collectively, studies of post-translational modification of Csp24 suggest that it may be an important link in the regulation of K^+ conductances underlying conditioning-dependent intrinsic enhanced excitability.

In addition to a role supporting cellular plasticity, the interaction between the cytoskeleton and β -thymosin, including β -thymosin repeat proteins, has been implicated in structural remodeling underlying development, growth and regeneration (Bouquet et al., 2000; Hertzog et al., 2004; Van Troys et al., 2004; Colby et al., 2005; Hermann et al., 2005; Van Troys et al., 2007). The loss of the function of the β -thymosin repeat protein *Cib* results in axonal growth defects in the central nervous system of *Drosophila* (Bouquet et al., 2000). Following nerve axotomy in *Aplysia*, β -thymosin and β -thymosin mRNA were detected at growth cones and in

regenerating axons *in vivo* (Colby et al., 2005). In *Hydra*, Pedin, a β -thymosin-like repeat protein contributes to bud outgrowth (Hermann et al., 2005). Tetrathymosin β identified in *Caenorhabditis elegans* has four thymosin β_4 -like repeats and contains both G- and F-actin interaction sites. The activity of tetrathymosin β may be of critical importance at specific developmental stages requiring actin polymerization (Van Troys et al., 2004). Recently identified β -thymosin^{His} and β -thymosin^{Glu} found in hemolymph and whole ganglia releasates of *Aplysia* support the anchoring of neurons in culture and increase neurite sprouting and total neurite outgrowth in culture (Romanova et al., 2006).

In addition to Csp24, other proteins identified in this study whose phosphorylation is regulated by Pavlovian conditioning have been implicated in growth, regeneration and plasticity. The ubiquitin-proteasome pathway plays a major role in the regulation of long-term synaptic plasticity (for reviews see DiAntonio and Hicke, 2004; Hegde, 2004). Proteasomes are continually assembled and disassembled and their subunits are subjected to a variety of post-translational modifications (Glickman and Raveh, 2005). Proteasome activity in the synaptic terminals is higher compared to activity in the nucleus (Wood et al., 2005; Upadhyaya et al., 2006) and ubiquitin c-terminal hydroxylase is involved in spatial learning and working memory (Wood et al., 2005). Intermediate filament protein A has been reported to contribute to 5-HT-dependent synaptic facilitation in *Aplysia* (Homayouni et al., 1997). In *Lymnaea*, inhibition of the intermediate filament protein RGP51 by RNA interference inhibits regenerative outgrowth of adult neurons in culture (Perlson et al., 2004). In addition, tropomyosin is a major regulator of F-actin functional specialization in migrating cells and is important for regionally defining the properties of the actin cytoskeleton in mediating changes in cell morphology (Gupton et al., 2005). Our results also showed that conditioning produces a significant increase in the phosphorylation of tubulin cofactor B. Previous work has shown that cytoskeletal reorganization is regulated by microtubule dynamics induced by p21-activated kinase 1 (Pak1) phosphorylation of tubulin cofactor B (Vadlamudi et al., 2005).

The post-translational modification of cytoskeletal-related proteins by conditioning may contribute to actin-filament dynamics underlying structural remodeling. The activity of proteins is modulated by intracellular signals, resulting in the recruitment of actin nucleatin and polymerization at specific cellular sites. Structural proteins characterized in this study could play a pivotal role in influencing actin dynamics supporting the development of long-term memory.

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Abbreviations

ASW	artificial sea water
CS	conditioned stimulus
US	unconditioned stimulus
2DE	two-dimensional electrophoresis
MS	mass spectrometry
IEF	isoelectric focusing
SDS	sodium dodecyl sulfate

Csp24	24kDa conditioned stimulus pathway phosphoprotein (accession number AAN08024)
CE	calexcitin
I _A	A-type transient K ⁺ current

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Fig. 1. Pavlovian conditioning results in an increase in ^{32}P incorporation into phosphoproteins. (A1) Representative print of a scan of SYPRO Ruby labeled proteins separated by 2DE from lysates of nervous systems (n=5) from conditioned animals. (A2) Print from a representative phosphorimaging scan of the same gel showing ^{32}P labeling of proteins from the conditioned group. (B1) Representative print of a scan of SYPRO Ruby labeled proteins separated by 2DE from lysates of nervous systems (n=5) from pseudorandom control animals. (B2) Print from a representative phosphorimaging scan of the same gel showing ^{32}P labeling of proteins from the pseudorandom control groups. The white rectangles numbered 1-4 enclose the areas of interest containing phosphoproteins that were included in the sample subjected to densitometric analysis.

Fig. 2.

Enlargements of the four areas indicated by the white rectangles in Figure 1. For each pair of rectangles the upper enlargement is from the conditioned group and lower is from the corresponding pseudorandom controls. The white arrows in each upper rectangle indicate proteins exhibiting statistically significant increased ^{32}P incorporation following Pavlovian conditioning as compared to the pseudorandom control groups.

Fig. 3. Phosphoprotein stained proteins from lysates of conditioned and pseudorandom control circumesophageal nervous systems separated by 2DE. (A) Print of a 2D gel stained with Pro-Q Diamond phosphoprotein dye from conditioned nervous systems (n=10). The 15 phosphoproteins enclosed by the white boxes were extracted from the analytical gels for identification by MS analysis. Identified proteins are indicated by the numbers corresponding to the assignments listed in Table 2. (B) Print of a 2D gel from pseudorandom control nervous systems (n=10) stained with phosphoprotein dye.

Table1

Sample of identified phosphoproteins regulated by conditioning

Name	Mean ratio (Cond./Pseudo.) $\pm$ SEM		Computed statistic		Significance Level	
	(a)	(b)	(a)	(b)	(a)	(b)
Intermediate Filament Protein A	1.6 $\pm$.2	2.8 $\pm$.6	$t_4=3.2$	$t_5=2.7$	$p<.025$	$p<.025$
Retrograde Protein 51	2.04 $\pm$.4	2.23 $\pm$.4	$t_4=2.4$	$t_5=3.03$	$p<.05$	$p<.025$
Enolase I	1.43 $\pm$.16	1.31 $\pm$.1	$t_4=2.6$	$t_5=3.02$	$p<.05$	$p<.025$
Actin	----	1.51 $\pm$.2	----	$t_5=2.2$	----	$p<.05$
Tubulin Folding Co-factor B	1.74 $\pm$.2	1.6 $\pm$.2	$t_4=3.09$	$t_5=2.6$	$p<.025$	$p<.025$
Tropomyosin (A)	1.7 $\pm$.3	1.34 $\pm$.14	$t_4=2.42$	$t_5=2.34$	$p<.05$	$p<.05$
Tropomyosin (B)	2.09 $\pm$.4	1.62 $\pm$.22	$t_4=2.51$	$t_5=2.75$	$p<.05$	$p<.025$
20S Proteasome subunit	1.82 $\pm$.2	1.50 $\pm$.21	$t_4=3.43$	$t_5=2.35$	$p<.025$	$p<.05$
Pirrin	----	1.59 $\pm$.2	----	$t_5=2.64$	----	$p<.025$
Ribosomal Protein PO	----	1.33 $\pm$.1	----	$t_5=2.34$	----	$p<.05$
Csp24	3.92 $\pm$.5	5.43 $\pm$.9	$t_4=4.8$	$t_5=4.53$	$p<.005$	$p<.005$
Hypothetical Protein	2.73 $\pm$.7	1.49 $\pm$.17	$t_4=2.7$	$t_5=2.9$	$p<.05$	$p<.025$

a) ^{32}P labeling

b) phosphoprotein stain

Table2

Peptide sequences used for protein assignments

Spot ^(a)	Name	Accession Number	Peptide Sequences ^(b)	%ID ^(c)	% coverage ^(d)
1	Intermediate Filament Protein A	CAA42839	YENELAQAR LVGLQDEISQLR KIDSLGNQIGIOEYEGELHTLR	84	9
2	Retrograde Protein 51	AAT01542	LAGLQDEIGSLR IDSLGNQLGEF EGELQSLR	78	7
3	Enolase I	NP-O11770	GNPTVEVELTTEK SIVPSGASTG VHEALEMER IGSEVYHNLK	100	13
4	Actin	AAS02073	VAPEEHPVLLTEAPLNPK	100	5
5	Tubulin-Folding Cofactor B	AAI06703	FEISEDHYDKR TDFKPGYWVGK	78	9
6	Tropomyosin (A)	AAA27817	IQLEEEDLE TLSIQNDQASQ	95	8
7	Tropomyosin (B)	BAB01765	TTENNFDVANEQLQEANK LQLEEEDFE LAFTEVDLER	76	14
8	20 S Proteasome α subunit	AH55520	HITFSPSGR AINQGGLTSVAVR	96	9
9	Pirin	XP001380970	NLDPFLVLDEFK TPTNYLDFK	86	7
10	Ribosomal Protein PO	ABY87386	ITALFDEYPK TGVVAPLD VRVPAQ(U)LTALGPEK	75	12
11	Hypothetical Protein	XP001204192	KSGHI(U)LNISSDSGR KFFVEGVTQVLR	73	11
12	Short-Chain Alcohol Dehydrogenase	EAA11743	VAVVTGASSGIGA VTSVSPGAVKT	75	10
13	C _{sp} 24	AAN08024	NPLPTAEISQER TVLPSIDDIGGEKK NSLPPQEA VETEK	100	26
14	Hypothetical Protein	EEA40671	TNIALPAGGFEFR MFYFDVGSNR DI(U)LGEYAEK	75	14
15	Neurocalcin	Q16982	FAEHVFR LSIEEFEGAK	95	10
16	Unknown		YES(FM)(U/L)NA(U/L)DDHK (FM) VATDVNPAC(FM)(U/L) E		

Spot ^(a)	Name	Accession Number	Peptide Sequences ^(b)	%ID ^(c)	% coverage ^(d)
17	Unknown		(I/L)VE (I/L)DGR (R/K)ESPED(I/L) P(I/L)ESNP (R/K)A[F/oxM]SN[F/oxM](I/L)K (R/K)(I/L) T[F/oxM] DENEK		
18	Unknown		VGELLWEK VVT(I/L)P(K/Q) VNAA(D/N)		
19	Unknown		NDVVHD(I/L)PH E(ND)GVNVYY(I/L)EE V(FM)SNAEMN(FM)		
20	Unknown		(R/K)(I/L)N[F/oxM]AE[F/oxN]MAD[F/oxM]YSR (R/K)(I/L)SDE(I/L)END (R/K)(I/L)S[F/oxM]DE[F/oxM]YSA[F/oxM]NK		

^{a)} Spot# 1-15 correspond to those shown in Figure 3.

^{b)} Ambiguous residues represented within parentheses. Modifications to specific residues designated by lower case abbreviation.

^{c)} % ID refers to the degree of identity between published peptide sequences and peptide sequences from *Hermisenda*

^{d)} % sequence coverage was calculated by dividing the number of sequenced amino acid residues by the total number of amino acid residues of the protein