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Comparative phosphoproteomic analysis of intestinal phosphorylated proteins in active versus aestivating sea cucumbers

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ABSTRACT

The sea cucumber *Apostichopus japonicus* is becoming an excellent model marine invertebrate for studies of environmentally-induced aestivation. Reversible protein phosphorylation as a regulatory mechanism in aestivation is known for some terrestrial aestivators but has never before been documented in sea cucumbers. The present study provides a global quantitative analysis of the role of reversible phosphorylation in sea cucumber aestivation by using tandem mass tag (TMT) labeling followed by an IMAC enrichment strategy to map aestivation-responsive changes in the phosphoproteome of sea cucumber intestine. We identified 2295 unique phosphosites derived from 1283 phosphoproteins and, of these, 211 hyperphosphorylated and 65 hypophosphorylated phosphoproteins were identified in intestine during deep aestivation compared with the active state based on the following criterion: quantitative ratios over 1.5 or less than 0.67 with corrected p-value <0.05. Six major functional classes of proteins exhibited changes in their phosphorylation status during aestivation: (1) protein synthesis, (2) transcriptional regulators, (3) kinases, (4) signaling, (5) transporter, (6) DNA binding. These data on the global involvement of phosphorylation in sea cucumber aestivation significantly improve our understanding of the regulatory mechanisms involved in metabolic arrest when marine invertebrates face environmental stress and provide substantial candidate phosphorylated proteins that could be important for identifying functionally adaptive variation in marine invertebrates.

Significance: Sea cucumber *Apostichopus japonicus* is an excellent model organism for studies of environmentally-induced aestivation by a marine invertebrate. The present study provides the first quantitative phosphoproteomic analysis of sea cucumber aestivation using isobaric tag based TMT labeling followed by an IMAC enrichment strategy. These data on the global involvement of phosphorylation in sea cucumber aestivation significantly improve our understanding of the regulatory mechanism involved in metabolic arrest when marine invertebrates face environmental stress and provide substantial candidate phosphorylated proteins that could be important for identifying functionally adaptive variation in marine invertebrates. This study also demonstrates the usefulness of the TMT-based quantitative phosphoproteomics approach to explore the survival responses of a non-model marine invertebrate species to seasonal changes in its environment.

1. Introduction

Aestivation, a state of aerobic dormancy, is used as a survival strategy by many animals, both terrestrial and aquatic, typically as a response to environmental stresses including nutrient limitation, high temperatures, or aridity [1–3]. Key features of long-term aestivation involve strong metabolic rate depression and descent into a hypometabolic state where both cellular (transcription, translation, cell cycle, etc.) and physiological processes (e.g. feeding and locomotion, heart and breathing rates, etc.) are suppressed. By reprioritizing energy use and reorganizing cellular metabolism a new balance is achieved between ATP-producing and ATP-consuming processes in cells that allows long term survival based on stored fuel reserves built up in the body before aestivation began [4]. This has been well reported for various fish, amphibians, and reptiles, as well as invertebrates such as land snails. However, only limited research has been directed toward the aestivation phenomenon in aquatic environments [1].

The sea cucumber, *Apostichopus japonicus*, first attracted attention from researchers because of its phylogenetic position (an invertebrate deuterostome) and its special regeneration characteristics but more recently has become a good model organism for studies of environmentally-induced aestivation by a marine invertebrate [5,6]. Unlike other aestivators, high water temperature (a rise above 25 °C) is the only trigger inducing this species to enter aestivation [7]. Complete cessation of feeding and locomotion are observed during a four month natural aestivation period of this species in the shallow seas around the Chinese coast. When sea water cool again to about 15 °C,

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sea cucumbers arouse back to normal function [8]. Hypometabolism in aestivating sea cucumbers is evidenced by strong decreases in the rates of oxygen consumption and ammonia nitrogen excretion, and degeneration of the intestine into a very thin filament [8]. During the aestivation period, the sea cucumber does not lose the intestine and once aestivation is complete, the intestine regenerates in a very short time.

Multiple studies have shown that a primary regulatory mechanism involved in metabolic arrest is reversible protein phosphorylation (RPP) [3,9,10]. The addition or removal of phosphate groups can result in major changes to the biochemical characteristics of a protein including on-off control of activity as well as conformational changes affecting parameters such as substrate affinity, susceptibility to allosteric activators or inhibitors, binding to other proteins, etc. [11,12]. For example, in the land snail, *Otala lactea*, RPP regulation of several enzymes is involved in suppressing carbohydrate catabolism during aestivation including glycogen phosphorylase, phosphofructokinase, pyruvate kinase and pyruvate dehydrogenase [13]. Similarly, glucose-6-phosphate dehydrogenase (that produces NADPH for biosynthesis and antioxidant defense) and glutamate dehydrogenase (which links carbohydrate and amino acid metabolism) are regulated by RPP during snail aestivation. The transmembrane ion channels, sodium-potassium ATPase and sarco(endo)plasmic reticulum Ca-ATPase, are also regulated by RPP in *O. lactea* as are the eukaryotic initiation factor 2 (eIF2) and eukaryotic elongation factor 2 (eEF2) which are crucial regulators of protein synthesis [13]. Some subunits of the 20S proteasome involved in protein degradation were also shown to be phosphorylated and this accompanied reduced rates of protein degradation during *O. lactea* aestivation [14].

Although previous studies in other species have revealed important unifying regulation of metabolism by RPP, only a limited range of proteins (representing just a few cell functions) have been examined to date; these include various proteins with roles in glycolytic metabolism, antioxidant defense, transmembrane ion transport, protein synthesis and protein degradation. Furthermore, RPP as a regulatory mechanism in aestivation has only been examined in a few species and has never been documented in aestivating sea cucumbers. In recent years, protein mass spectrometry has emerged as a key technology for screening protein posttranslational modifications including phosphorylation [15–17]. It allows simultaneous phosphorylation site mapping and quantification in a single experiment. The 6-plex tandem mass tag (TMT) labeling technology enables the comparison of six different samples in one mass spectrometry-based experiment as well as the capacity to assess large numbers of cellular proteins simultaneously.

The aim of the present study was to profile global phosphorylation patterns in aestivating versus active sea cucumbers. By applying a TMT-based quantitative phosphoproteomics strategy, we identified a group of differentially phosphorylated proteins serving as targets for regulation of sea cucumber aestivation.

2. Materials and methods

2.1. Animals

Adult sea cucumbers (two years old, body weight 70–108 g) were collected from culture ponds in Jiaozhou Bay of the Yellow Sea in China. Animals sampled in April (sea water temperature about 15 °C) had gone through the aestivation period, regenerated their tissue and returned to active status; these were used as non-aestivating controls. The deep aestivating animals were collected in mid-August (sea water temperature above 25 °C); the animals had stopped feeding and locomotion and the intestine had degenerated into a very tiny string (about 2–3 mm), usually after 15 days of continuous torpor [18]. To ensure adequate coverage of phosphosites, 3 biological replicates (total of 6 samples) including 3 non-aestivating and 3 deep-aestivating animals were collected. The intestine of each animal was dissected without contents and immediately frozen in liquid nitrogen, then kept at −80 °C until subsequent analysis. No permission was needed for sea cucumber collection. The sea cucumber (*A. japonicus*) is not an endangered or protected species.

2.2. Protein extraction

Intestine samples (~200 mg each) were ground to a powder under liquid nitrogen and homogenized using a Polytron in lysis buffer (8 M urea, 1% Triton-100, 65 mM dithiothreitol [DTT], with 0.1% Phosphatase Inhibitor Cocktail Set II and Protease Inhibitor Cocktail Set IV added [Calbiochem]). The homogenates were transferred to 50 mL centrifuge tubes and subjected to mild sonication three times on ice using a high intensity ultrasonic processor (Scientz, Ningbo Scientz Biotechnology Co., Ltd.). The samples were centrifuged at 4 °C and 20,000 g for 10 min. Proteins were precipitated from supernatants using cold 15% w:v trichloroacetic acid (TCA) for 2 h at −20 °C. Precipitated proteins were pelleted by centrifugation (4 °C, 5000 g for 10 min), the supernatant was removed, and the pellets were washed with cold acetone three times. Protein was resuspended in rehydration buffer (8 M urea, 100 mM triethylammonium bicarbonate (TEAB) buffer, pH 8.0) and the protein concentration was determined using the 2D Quant kit (GE Healthcare, Piscataway NJ) according to the manufacturer's instructions.

2.3. Trypsin digestion

Trypsin (Promega) was added into extracts at 1:50 w:w ratio of trypsin to protein and digestion was allowed to proceed at 37 °C for 16 h. DTT was then added to a final concentration of 5 mM followed by incubation at 50 °C for 30 min. After that, 20 mM iodoacetamide was added to alkylate proteins followed by incubation at room temperature (RT) in the dark for 45 min. The alkylation reaction was quenched by addition of 30 mM cysteine (final concentration) at room temperature for 30 min. Trypsin was then added again at a 1:100 w:w ratio for digestion at 37 °C for 4 h to complete the digestion cycle.

2.4. TMT labeling

Tandem mass tag (TMT) labeling was carried out as per manufacturer instructions (Thermo Scientific) for 6-plex with minor modifications. Briefly, one unit of TMT reagent (defined as the amount of reagent required to label 300 μg of protein) was thawed and reconstituted in 24 μl acetonitrile (ACN). The peptide digest from each condition was reconstituted in 0.5 M TEAB, mixed with the TMT reagent (labeling each sample with a different tag) and incubated at RT for 1 h. After the labeling, peptide mixtures were pooled and incubated for 2 h at RT, desalted using a Strata X C18 SPE column (Phenomenex) and dried by vacuum centrifugation.

2.5. HPLC fractionation and IMAC enrichment

Each dried peptide sample was fractionated by high pH reverse-phase HPLC coupled with an Agilent 300 Extend C18 column (5 μm particles, 4.6 mm ID, 250 mm length). Briefly, peptides were first separated with a gradient from 2% to 60% acetonitrile in 10 mM ammonium bicarbonate (pH = 8) over 80 min into 80 fractions. Then, the peptides were combined into 6 fractions and dried by vacuum centrifugation. Phosphopeptide enrichment was performed as described previously [19] with some modifications. Briefly, IMAC (immobilized metal affinity chromatography) was applied by re-suspending the dried peptide samples in 4 ml of 40% ACN and 0.1% trifluoroacetic acid (TFA). After incubation with IMAC beads (Dalian Institute of Chemical Physics, Chinese Academy of Sciences) under vibration for 60 min, the IMAC beads with enriched phosphopeptides were collected by centrifugation, and the supernatant was removed. To remove non-specific adsorbed peptides, the IMAC beads were washed sequentially with 50% ACN + 6% TFA followed by 30% ACN + 0.1% TFA. Bound phosphopeptides were