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Thioester function is conserved in SpC3, the sea urchin homologue of the complement component C3

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Abstract

The amino acid sequence of the thioester site in the α chain of SpC3, the sea urchin homologue of C3, is conserved. This implies a conserved function of covalent bond formation with amine or hydroxyl groups on target molecules. When coelomic fluid (CF) was incubated with ^{14}C-methylamine, a classic assay for thioester binding function, the α chain became labeled. When CF was treated to induce autolysis, peptide bond cleavage occurred at the thioester site. Autolysis could be blocked or reduced by pre-treating CF with either methylamine or yeast, both of which are known to bind to thioester sites C3 proteins from other organisms. The data suggest that SpC3 can bind to target cell surfaces, constituting indirect evidence that it can covalently bind to pathogen surfaces and function as an opsonin in vivo. This activity may be an important aspect of host defense in the sea urchin. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Sea urchin; Coelomic fluid; Complement; Autolysis; Thioester; Echinoderm; Opsonization; Evolution

1. Introduction

Current understanding of the activities and functions of the innate immune system in higher vertebrates indicates that the complement system is one of the major subsystems involved in host protection and is important for activating the adaptive immune system [1–6]. Based primarily on the close association between the classical pathway of complement and activation by antigen/antibody complexes, the complement system was historically believed to be present only in higher vertebrates. However, homologues of complement components have been identified in lower deuterostomes that do not exhibit adaptive immune capabilities. These include C3 homologues in the hagfish, *Eptatretus stouti* [7–9], the lamprey, *Lampetra japonica* [10,11], the tunicate, *Halocynthia roretzi* [12] and the sea urchin, *Strongylocentrotus purpuratus* [13,14]. In mammals, C3 has been characterized as a member of the acute phase reactants, showing a moderate increase in serum concentration as a result of hepatic production [15], with significant localized increases of C3 in tissues as a result of responses by macrophages to infection or injury [16,17]. In sea urchins, SpC3 is expressed in two subpopulations of macrophage-like coelomocytes called phagocytes [18], and the level of

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Abbreviations: LPS, Lipopolysaccharide; α2M, alpha 2 macroglobulin; TEP, thioester-containing proteins; CF, coelomic fluid; SDS, sodium dodecyl sulfate; PBS, phosphate buffered saline; TBS, Tris buffered saline; α-SpC3, rabbit serum to SpC3; anti-α′pep, rabbit antiserum to the deduced α′ peptide from SpC3; GαRIg-HRP, goat antiserum to rabbit immunoglobulins labeled with horseradish peroxidase

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SpC3 in the coelomic fluid (CF) of immunoquiescent sea urchins increases rapidly after challenge with lipopolysaccharide (LPS) [19,20]. Together, these results suggest that SpC3 may be an invertebrate acute phase reactant and be important in host defense.

Although the biochemical activities of the complement system in sea urchins, and SpC3 in particular, have only been predicted based on sequence analysis of the cDNA [14], preliminary data on phagocyte function suggest that SpC3 acts as an opsonin and can augment phagocytosis of opsonized particles [21,22]. The thioester site is the functionally defining region of the thioester family of proteins which include the complement components C3, C4, C5 (although C5 has an altered thioester site and cannot form covalent bonds) (reviewed in Ref. [23]), alpha 2 macroglobulin (α2M) (reviewed in Ref. [24]), and thioester-containing proteins (TEPs) which have been characterized in Drosophila [25] and Mosquito [26]. Covalent binding activity is based on an internal thioester bond that forms between the side chains of a cysteine and a nearby glutamine. When C3 or C4 become activated by proteolytic cleavage of the α chain, the thioester, which is normally buried within the protein, becomes exposed to the medium. When this happens, the glutamine side chain can form either an ester or amide bond with a target molecule [23,27,28]. Similarly, when a protease cleaves the 'bait' region of α2M, the thioester site is activated and binds covalently to the protease. Simultaneously, a conformational change occurs in α2M that encages the protease and sequesters it from further activity [24]. The insect TEPs appear to have activities that are similar to those for both α2M and C3/C4. Mosquito aTEP-1 may be activated by proteases, perhaps secreted by microbial invaders, which cleave and cause a conformational change in the TEP molecule. This activates the thioester which can bind the foreign molecule like α2M, which then functions as an opsonin like C3 [26].

In early studies that established thioester function in human α2M and complement proteins, investigators used two classic assays. The first was to show that small nucleophiles such as methylamine or hydroxylamine would covalently bind to proteins containing thioester sites. This activity has been well characterized and is generally accepted as an assay for thioester activity in vertebrates. Recently, this assay has been used to demonstrate the thioester activity for the C3 homologue in a tunicate [12] and for Mosquito aTEP-1 [26]. Another classic assay used to demonstrate thioester function is autolysis. Under denaturing conditions at high pH and high temperature, the peptide bond between the glutamic acid and glutamine within the thioester is cleaved (for review, see Ref. [29]). Although autolysis is a non-biological reaction that occurs in the test tube, the chemistry is directly related to the normal activities of an active thioester site and its ability to form covalent bonds with target molecules.

Because SpC3 has been established as a member of the thioester containing protein family and as a homologue of C3 based on sequence analysis [14], the functional activities of this protein must be established. The data presented here demonstrate that SpC3 binds methylamine, and under the proper treatment conditions, undergoes autolysis. Furthermore, when the thioester is engaged with either methylamine or yeast, autolysis can be reduced or blocked. These data confirms our preliminary results [22,30] and indicate that the thioester activities in SpC3 are conserved. The results presented here, in addition to other data [21] predict that SpC3 plays an important role in host protection, functioning as an opsonin to augment particle uptake by phagocytic coelomocytes in the sea urchin.

2. Materials and methods

2.1. Animals

Sea urchins were obtained from the Southern California Sea Urchin Co. (Corona del Mar CA), housed in 100 gal marine aquaria equipped with several filtering systems, and fed rehydrated Chinese kelp as previously described [14,18,20,31].

2.2. Coelomic fluid

Samples of whole CF, composed of coelomocytes plus coelomic fluid, were collected as previously described [19,20,32]. Briefly, a 23-gauge needle was inserted through the peristomial membrane into the coelomic cavity and whole CF was withdrawn into a 1 ml syringe. Coelomic fluid was transferred to a 0.5 ml microfuge tube and allowed to clot for up to

15 min at room temperature. Whole cells and cellular debris were pelleted, and the resulting cell free CF was used immediately.

2.3. Coelomic fluid treatments

Autolysis. Samples of cell free CF (20 μl) were mixed with 14.8 μl of autolysis buffer (final concentration after mixing with CF: 4 M urea, 0.42% sodium dodecyl sulfate (SDS), 0.42 M Tris, pH 10, 0.42 M dithiothreitol (DTT); according to Ref. [33]), and heated to 95 °C for 5 min. Control CF samples (20 μl) were mixed with 14.8 μl non-autolysis buffer (final concentration: 0.42% SDS, 0.42 M Tris pH 6.8, 0.42 M DTT) and heated to 37 °C for 15–30 min. Samples were spun briefly and analyzed by SDS–PAGE (4.5% stacking gel, 8% running gel) [34].

Incubation with yeast. Dehydrated baker's yeast (100 mg) (Sigma Chemical, St Louis MO) was resuspended in 2.0 ml phosphate buffered saline (0.15 M $NaPO_4$, 0.9 M NaCl), washed six times, and heat killed at 95 °C for 20 min. Yeast particles were counted on a hemocytometer, pelleted, resuspended in 80 μl CF (2×10^{10} ml^{-1}), and incubated in a rotator at room temperature. Samples (20 μl) of the CF–yeast mixture were removed after varying periods of time, the yeast were pelleted and the CF was treated to induce autolysis followed by analysis by SDS–PAGE and Western blot. Yeast were omitted from incubations of control CF prior to inducing autolysis.

^{14}C-Methylamine incorporation. Coelomic fluid was incubated in 10 mM ^{14}C-methylamine (33 μCi, Amersham Biosciences, Piscataway NJ) at 37 °C for 1 h and then cold methylamine was added to 100 mM for an additional 30 min at 37 °C. The treated CF was split into two subsamples and one received heat killed brewer's yeast (4.0×10^8 ml^{-1}). Samples were incubated at room temperature, and aliquots were taken at 1 and 4 h. Yeast were removed by centrifugation and supernatants were treated to induce autolysis prior to analysis by SDS–PAGE. Gels were either stained with Coomassie Brilliant Blue and dried for autoradiography or proteins were transferred to nitrocellulose for Western blot analysis to localize the SpC3 bands (see below). After Western blot results were obtained, filters were dried and autoradiographed (X-OMAT AR; Eastman Kodak Co, Rochester NY) for 19 days to identify the bands that had incorporated ^{14}C.

2.4. Western Blots

Proteins were electroblotted from gels to nitrocellulose filters with a Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules CA) with blotting buffer (20% methanol, 20 mM Tris pH 8.8, 0.15 M glycine, 0.05% SDS) at 8 °C in a chilled water bath (Neslab Inst. Co. Portsmouth NH). Filters were stained with 0.1% Ponceau S (Sigma) in 5% acetic acid, de-stained in 5% acetic acid, trimmed, and the edges of the gel and the positions of the lanes and standard bands were marked with needle holes. Filters were washed briefly in Tris buffered saline (TBS: 0.2 M Tris pH 7.4, 138 mM NaCl) with 0.1% Tween 20 (TBS-Tween) and blocked by rocking over-night at room temperature in blotto (5% dehydrated milk in TBS-Tween). Filters were rocked for 3 h at room temperature in a mixture of two primary antisera (see below). The filters were washed twice in TBS-Tween followed by two washes in TBS, and incubated in the secondary antiserum (goat-anti-rabbit-immunoglobulins labeled with horseradish peroxidase; GαRIg-HRP [Pierce, Rockford IL], 1:80,000 in blotto). Filters were washed as before and incubated in luminol (Super Signal West Pico; Pierce) and exposed to film (X-OMAT AR; Eastman Kodak Co., Rochester NY).

2.5. Antisera

Rabbit antiserum to SpC3-6His (αSpC3-6H; used at a dilution of 1:15,000 in blotto) was raised to a fusion protein expressed in bacteria that consisted of a 50 kDa fragment of the N-terminal region of the SpC3 α chain to which had been added a histidine tag (Qiagen, Valencia CA) for affinity isolation [20]. The second rabbit antiserum was raised to a synthetic peptide (anti-α′pep; used at a dilution of 1:1000 in blotto) that corresponded to the deduced N-terminal sequence of the SpC3 α′ chain (SGGDGGEQ-NAAVKVRDDFRETWFFDC) which was kindly provided by J. Lambris (for detailed methods, see Refs. [14,18]). Both antisera were characterized by analyzing their binding patterns against bacterial lysates, coelomic fluid and coelomocyte lysates on Western blots. Because the rabbits were only sensitized to the bacterially produced fusion protein or the synthetic peptide, no other sea urchin proteins were identified by these antisera. The prebleed controls

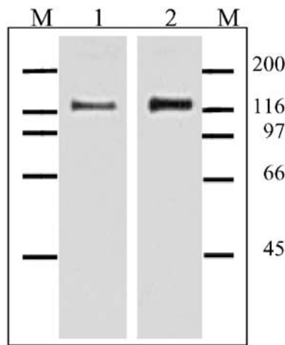

Fig. 1. Anti-SpC3 antisera both recognize the α chain of SpC3. Aliquots of CF proteins were separated by SDS–PAGE, electroblotted onto nitrocellulose and incubated with two different antisera raised to SpC3 that were used together throughout this study. Lane 1, α-SpC3-6His (1:15,000 in blotto). Lane 2, anti-α′pep (1:1000 in blotto).

were consistently negative on Western blots. The anti-α′pep antibody bound to the N-terminal end of the α′ fragment, while the α-SpC3-6H bound to the N-terminal region of the intact α chain. Both antisera identified the α chain (Fig. 1), but because each recognized slightly different regions of the α chain, the two antisera were used together to detect as much of the α chain as possible.

3. Results

3.1. Sequence conservation of the thioester site in SpC3

The conserved thioester site in SpC3 has the identical amino acid sequence at the thioester, GCGEQ, that is found in other members of the thioester containing family of proteins (Fig. 2, and see Ref. [14]). In addition to the complement proteins and α2M, a number of other vertebrate proteins have thioester sites including mammalian pregnancy zone protein, mouse sex linked protein, murinoglobulin, and some protease inhibitors (see legend to Fig. 2). Non-deuterostome members of this protein family include five TEPs from Drosophila [25], one from the Mosquito [26] and two unknown gene products with conserved thioester sites from Drosophila that were identified in GenBank. An alignment of the thioester region from these proteins is shown in Fig. 2. The strict conservation of the thioester site in SpC3 suggested that this echinoderm protein might have activities that are similar to at least some of the activities that have been characterized in detail for human C3. As an initial approach to demonstrate the functional homology between SpC3 and other thioester containing proteins, the ability of SpC3 to bind methylamine and to undergo autolysis was determined.

3.2. SpC3 binds ^{14}C-methylamine

The CF from five sea urchins (CF from one animal was repeated three times) were incubated with ^{14}C-methylamine for 1–4 h. Results for one experiment are shown in Fig. 3, which includes the Coomassie-stained protein gel (Fig. 3(A)). A Western blot of a gel run simultaneously as that shown in Fig. 3(A) with aliquots of the same samples, revealed the position of the SpC3 α chain (130 kDa, Fig. 3(B)) which corresponded to the second to the largest band on the protein gel (Fig. 3(A)). Autoradiography of the dried filter (or the dried gel, not shown) indicated that 130 kDa α chain identified by the antisera, also acquired the ^{14}C label (Fig. 3(C)). These results indicated that SpC3 is a major constituent of the fraction of large CF proteins that stained with Coomassie, and demonstrated that SpC3 can covalently bind small nucleophiles. This suggested that the thioester site in SpC3 functions through a conserved mechanism, a

Fig. 2. Alignment of a region of the α chain from the family of proteins containing thioester sites. The alignment was done with Clustal W [60] and the region surrounding the thioester site is shown. The thioester bond is formed between the cysteine and the glutamine, which is indicated above the alignment as a connection between C and Q. The horizontal lines separate different groups of thioester proteins. The top group includes the C3 and C4 proteins. The middle group includes the insect members of the family including the TEPs. The bottom group includes the alpha 2 macroglobulins and other proteins with conserved thioester sites. Jfounder = Japanese flounder, DrosGeneProd = unknown Drosophila gene product, TEP = thioester-containing protein, A2M = alpha 2 macroglobulin, A1M = alpha 1 macroglobulin, A1INH3 = alpha 1 inhibitor 3, A2INH3 = alpha 2 inhibitor 3, GHamster = Golden hamster, Gpig = Guinea Pig, Murinoglob = murinoglobulin, PZP = pregnancy zone protein, Celegans = *Caenorhabditis elegans*. * = identical among all proteins; : = similar amino acids; – = gap.

Species protein	thioester site	accession number
SeaUrchin SpC3	GLDHLVRQPR**GCGEQ**TMIYLAPTLFVYQYLIAVGSDTAEQEAR	T14074
Branchiostoma C3	GLGTLLRLPT**GCGEQ**TMIKLAPNVYVLSYLHCTDQITKDVEEK	AB050668
Tunicate HrC3	-LQNLINSPG**GCGEQ**NMIRIAPVVYIHAYRSNLEAFTVTDAQR	BAA75069
Hagfish C3-4	KISNLLRLPR**GCGEQ**NMMYTSITVMVARYLNRSDQWNKMGDPQ	P98094
Jflounder C	SMGTLIYQPS**GCGEQ**NMIHMTLPVIAATYLDKTNQWETVGFQK	BAA88901
Medaka C3-1	SMGTLIYQPS**GCGEQ**NMIHMTLPVIATIYLDKTNQWEAVGFQK	BAA92285
Trout C3-1	SLGSLIVQPV**GCGEQ**NMIYMTLPVIATHYLDNTKKWEDIGLDK	P98093
Trout C3-3	PLGDLLRQPG**GCGEQ**NMIGMTLPLSPHITWTRPNTWDKVGLER	AAC60015
Carp C3-H1	FMGRLIIQPN**GCGEQ**IMIGMTLPVIATHYLDSTSQWETVGMER	BAA36619
Carp C3-H2	FMGRLIVQPS**GCGEQ**IMIGMTLPVIATHYLDSTSQWETVGFER	BAA36620
Carp C3-Q1	FMGSLIVQPS**GCGEQ**NMIGMTLPLIATHYLDSTNQWDTIGTER	BAA36622
Carp C3-Q2	FMGRLIVQPS**GSGEQ**NMMLMTLPLIATHYLDSTSQWDTVGMER	T30517
Carp C3-S	FMGRLIVQPS**GCGEQ**NMIGMTLPVIATHYLDNTNQWEAVGIQR	BAA36621
Xenopus C3	NMNHLIVVPA**GCGEQ**NMISTTPSVIATRYLDASGQWERVGVNR	AAB60608
Cobra C3	KLNHLIITPS**GCGEQ**NMITMTPSVIATYYLDATGQWENLGVDR	Q01833
Chicken C3	KLKHLIVTPS**GCGEQ**NMIGMTPTVIAVHYLDSTMQWETFGINR	I50711
Mouse C3	RLKHLIVTPA**GCGEQ**NMIGMTPTVIAVHYLDQTEQWEKFGIEK	AAC42013
Rat C3	RLKHLIVTPS**GCGEQ**NMIGMTPTVIAVHYLDQTEQWEKFGLEK	NP_058690
GPig C3	RLKHLIITPS**GCGEQ**NMIGMTPTVIAVHYLDQTEQWEKFGLEK	P12387
Human C3	RLKHLIVTPS**GCGEQ**NMIGMTPTVIAVHYLDETEQWEKFGLEK	NP_000055
Carp C4A	GINKLINLPT**GCAEQ**TMVKMSPAIHAMRYLDATNQWISLKAER	BAB03284
Medaka C4	-VSNLIHLYK**GCLEQ**TMSTFAPTTLAMRYLDLSQQWFTLEPGA	BAA92287
Chicken C4	IGDSLLRSPR**GCGEQ**SLMSMAPTAAALRFLDESEGWGQLPPGH	T28153
Mouse C4	GVASLLRLPQ**GCAEQ**TMIYLAPTLTASNYLDRTEQWSKLSPET	X05314
Human C4A	GVASLLRLPR**GCGEQ**TMIYLAPTLAASRYLDKTEQWSTLPPET	CAB89302
Human C4B	GVASLLRLPR**GCGEQ**TMIYLAPTLAASRYLDKTEQWSTLPPET	NP_009224
Mosqito aTEP-1	NLDNLLAVPT**GCGEQ**NMVKFVPNILVLDYLYATGSKEQHLIDK	AAG00600
Dros TepI	HLDDLVHLPC**GCGEQ**NMFNFVPSILALSYLKAKNRQDQEIENK	AAF53490
Dros TEP1	HLDDLVHLPC**GCGEQ**NMFNFVPSILALSYLKAKNRQDQEIENK	CAB87807
Dros TEP2	NLDNLVRMPY**GCGEQ**NMVNFVPNILVLKYLEVTGRKLPSVESK	CAB87808
Dros TEP3	NLDSLILLPT**GCGEQ**TMVNFVPNLIVLRYLGRLRQLTPEVELR	CAB87809
Dros TEPIV	NLENLLRLPS**GCGEQ**TMSKLVPNYLVRDYLKSIKKLTPALDTR	AAF53826
Dros GeneProd1	NLDNLVRMPY**GCGEQ**NMVNFVPNILVLKYLEVTGRKLPSVESK	AAF52540
Dros GeneProd2	NLDNLVRMPY**GCGEQ**NMVNFVPNILVLKYLEVTGRKLPSVESK	AAF52539
Celegans A2M	NAHKLVQMPY**GCGEQ**NMLNLVPNILVVKYLRATNRNESQLETK	CAB05006
Limulus A2M	NLDHLVRLPT**GCGEQ**NMVKFVPNIFVLDYLTATGSITDSIKEK	T18544
Lamprey A2M	NLDKLLTLPT**GCGEQ**NMVKFAPNIYIQEYLQNSGQLTDAVRDK	T43166
Carp A2M1	NLHGLLQMPY**GCGEQ**NMAILSPNIYILQYLENTEQLTSAIRER	BAA85038
Carp A2M3	NLHGLLRMPY**GCGEQ**NMAILSPNIYILQYLEKTEQLTSVIRER	BAA85040
GPig A2M	NIQNLLQMPY**GCGEQ**NMVLFAPNIYVLDYLNETQQLTPDIKSK	JC5143
Mouse A2M	NLQNLLQMPY**GCGEQ**NMVLFVPNIYVLNYLNETQQLTEAIKSK	NP_031402
Human A2M	NTQNLLQMPY**GCGEQ**NMVLFAPNIYVLDYLNETQQLTPEVKSK	NP_000005
Rat A1M	NLQNLLQMPY**GCGEQ**NMVLFVPNIYVLEYLNETQQLTEAIKSK	A42210
Rat A1INH3	NTQNLIQMPY**GCGEQ**NMVLFAPNIYVLKYLNETQQLTEKIKSK	A29953
Rat A2INH3	NTQNLIQMPY**GCGEQ**NMVLFAPNIYVLKYLNETQQLTEKIKSK	P14046
GHamster A1Inh3	NTQNLLHMPF**GCGEQ**NMVLFAPNIYVLKYLNETQQLTQNIKSK	A41081
Mouse Murinoglob	NTQNPLHMPY**GCGEQ**NMVLFAPNIYVLKYLNETQQLTQKIKTK	AAA73048
Mouse Murinoglob2	NTQNLLHMPY**GCGEQ**NMVLFAPNIYVLKYLDKTQQLTQKIKTK	P28666
GPig Murinoglob	NTQNLLQMPY**GCGEQ**NMVLFAPNIYVLKYLNETDQLTQEIKSK	JC5114
Human PZP	NIQNLLQMPY**GCGEQ**NMVLFAPNIYVLNYLNETQQLTQEIKAK	NP_002855
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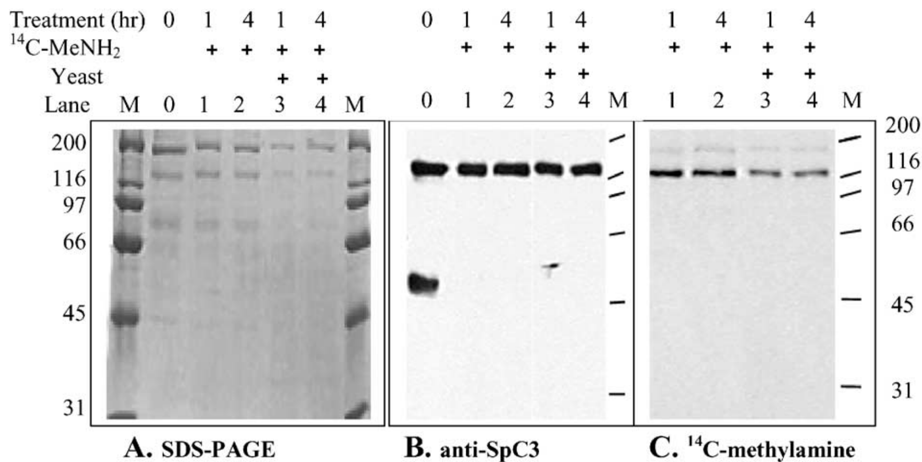

Fig. 3. Methylamine is incorporated into the α chain of SpC3. Coelomic fluid was collected and not treated (lanes 0), pretreated with 10 mM ^{14}C-methylamine followed by post incubation in the presence or absence of yeast for 1 or 4 h. (A) CF proteins were separated by SDS–PAGE and stained with Coomassie Brilliant blue. (B) A gel run simultaneously with that shown in panel A, using aliquots of the same samples, was electroblotted onto nitrocellulose. The filter was analyzed with anti-SpC3 antisera which recognized the 130 kDa α chain of SpC3. Bands (50 kDa) in lanes 0 and 3 result from autolysis of active α chain that was induced prior to loading. The apparent difference in size of the 50 kDa bands in lanes 0 and 3 is due to differences in the amount of fragment generated as reflected in band thickness. (C) After the Western blot was completed, the filter in panel B was dried and autoradiographed for 19 days to reveal the ^{14}C-labeled bands. ^{14}C-methylamine was incorporated into the 130 kDa α chain of SpC3 and into a larger band of about 185 kDa. Neither the antisera nor methylamine labeled the β chain of SpC3, which is about 80 kDa [14]. M = broad range protein marker (BioRad). Sizes of the marker bands are indicated at the sides of the figure.

result predicted from the conserved amino acid sequence in that region of the protein.

Autoradiography of the dried filter and the dried gel (not shown) also revealed a second band that had acquired ^{14}C-methylamine labeling in addition to the 130 kDa α chain (Fig. 3(C)). This band was about 185 kDa, did not cross react with the antisera (no 185 kDa band is present in Fig. 3(B)), and corresponded to the largest and most prominent band on the protein gel (Fig. 3(A)). Although there are no reports in the literature of an echinoderm homologue of α2M, the presence of a band of this size that is not the SpC3 α chain and becomes labeled with ^{14}C-methylamine suggests that it may be present in the sea urchin.

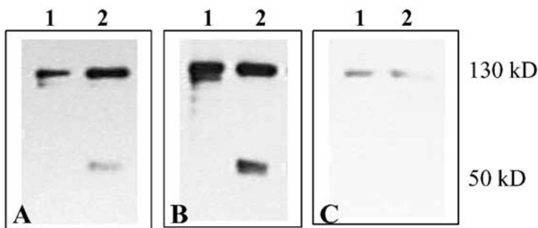

Fig. 4. SpC3 undergoes autolysis. Coelomic fluid from three sea urchins was processed so that autolysis was not induced (lanes 1; non-denaturing conditions, pH = 7.4, 37 °C) or treated to induce autolysis (lanes 2; denaturing conditions, 4 M urea, pH = 10, 95 °C) and analyzed by Western blot. (A) and (B) Two examples of CF containing SpC3. Additional examples are shown in Figs. 5 and 6. (C). An example of one CF sample with a decreased amount of SpC3 and that did not undergo autolysis.

3.3. SpC3 autolysis

Because ^{14}C-methylamine labeled the α chain of SpC3, which includes the thioester site, it was useful to employ an alternative assay to demonstrate that it was the thioester that was involved in binding methylamine. Therefore, CF was treated to determine whether SpC3 would undergo autolysis. After heating to 95 °C under denaturing conditions at pH 10, most samples of CF showed the appearance of a 50 kDa fragment on Western blots in addition to the full-length 130 kDa α chain (lane 2 in Fig. 4(A) and (B); see also lanes 0 and 3 in Fig. 3(B)). When the CF was processed for SDS–PAGE at 37 °C under non-denaturing conditions at pH 7.4, the 50 kDa band did not appear (lane 1 in Fig. 4(A)–(C)). The 50 kDa band

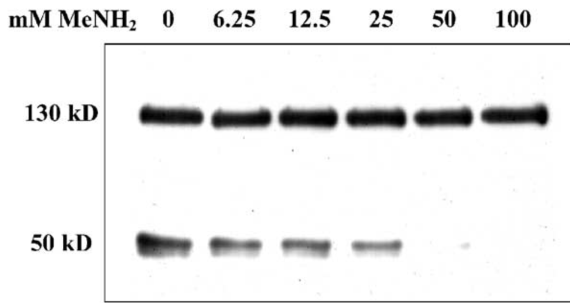

Fig. 5. Methylamine blocks SpC3 autolysis. Coelomic fluid was incubated for 30 min at 37 °C with varying concentrations of methylamine and analyzed for autolysis by Western blot using α-SpC3 antisera. The 130 kDa band is the full-length α chain and the 50 kDa band is the N-terminal autolytic fragment of the α chain. The 75 kDa fragment from the C-terminus of the α chain is not detected by the antisera.

corresponded in size to the N-terminal fragment of the α chain that had been cleaved at the thioester site. Expected sizes were deduced from the sequence of SpC3 in addition to estimations of the N-linked glycosylations at the four conserved sites in the α chain [14]. This interpretation of the fragment sizes was also based on the region to which the antisera were known to bind, i.e. the N-terminal portion [18–20]. The 75 kDa C-terminal fragment that was also generated by autolysis of the α chain was not detected by the antisera which had been raised against determinants in the N-terminus.

Under the conditions employed in this assay, only some of the α chain underwent autolysis, leaving some 130 kDa, full-length α chain in each sample (Fig. 4). A similar result has been noted in studies of autolysis for human C3 and α2M, where only those proteins that are in the active conformation undergo autolysis [33,36,37]. Variations in the amount of SpC3 in CF and the level of autolysis were noted when CF was obtained from 46 different sea urchins. Representative results of autolysis assays from three sea urchins are shown in Fig. 4. The animals were randomly chosen from the same aquarium and CF samples were collected at the same time, yet varying amounts of SpC3 were present in equal volumes of CF loaded onto the gels. Furthermore, different levels of autolysis were identified as noted by differences in intensities of the 50 kDa bands shown on the blots in Fig. 4(A) and (B). In total, 89.2% of the autolysis assays performed on CF from different sea urchins showed weak to strong autolytic bands while 10.8% of the CF samples did not undergo autolysis (Fig. 4(C)). These results suggested that the thioester site in SpC3 can undergo autolysis in a conserved mechanism, similar to autolysis of human C3, C4 and α2M. Furthermore, because some of the full-length α chain was still detected on the blots after autolysis was induced, this indicated that only a fraction of the SpC3 molecules were in an active conformation and capable of undergoing autolysis. This is in agreement with data from thioester containing complement proteins from higher vertebrates [29,33].

3.4. Autolysis can be blocked by engaging the thioester site

When the thioester site of human C3 is engaged either with methylamine or hydroxylamine, autolysis can be blocked [33]. Since methylamine was shown to bind to the α chain of SpC3 (Fig. 3) and since SpC3 undergoes autolysis, it was useful to know whether methylamine treatment of CF could block autolysis of SpC3. When CF from eight different sea urchins was incubated with methylamine followed by induction of autolysis, Western blot analysis showed that with increasing concentrations of methylamine, reduced amounts of the 50 kDa autolytic band were observed (representative results are shown in Fig. 5). Furthermore, by increasing incubation time with a constant concentration of methylamine, the amount of the 50 kDa autolytic fragment decreased (data not shown). These results suggested that methylamine was bound by the thioester site in SpC3 and that when the thioester was engaged, autolysis was blocked.

Because methylamine is small enough to gain access to the thioester site in proteins that are in the non-activated conformation, methylamine binding by SpC3 only serves as indirect evidence that SpC3 might function as an opsonin by binding to larger target molecules. To test the opsonization functions of SpC3, CF was incubated in the presence or absence of heat-killed brewer's yeast for varying time periods followed by induction of autolysis. Results showed that in the presence of yeast there was a decrease in the appearance of the 50 kDa autolytic fragment with longer incubation times (Fig. 6, see also Fig. 3(B). In addition, the intensity of the full-length α chain

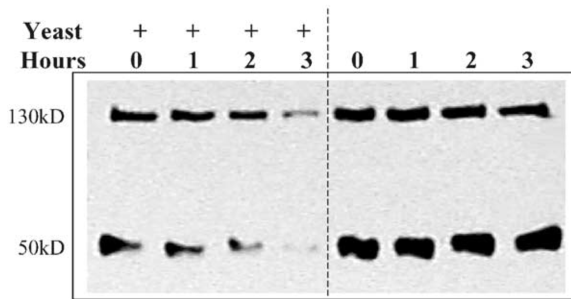

Fig. 6. Yeast blocks SpC3 autolysis. Coelomic fluid was incubated in the presence or absence of brewers' yeast for 0, 1, 2 or 3 h at room temperature. Samples were centrifuged to remove the yeast, treated to induce autolysis and analyzed for autolysis by Western blot with antisera to SpC3. The 130 kDa band is the full-length α chain of SpC3, and the 50 kDa band is the N-terminal autolytic fragment from the α chain.

band also decreased after increased incubation with yeast cells. When yeast were omitted, controls showed that autolysis could be induced at all time points, and that the intensity of the 130 kDa band did not decrease with time. The decrease in the intensity of the 130 kDa band in the presence of yeast (Fig. 6) was presumed to be because SpC3 bound to the yeast and was removed from the aqueous phase of the reaction when the yeast were pelleted prior to loading the supernatant onto the protein gel. The results indicated that the thioester site was involved in binding to the yeast cells, and when bound, could not undergo autolysis.

If the thioester was involved in opsonizing yeast, prior incubation with methylamine should block SpC3 binding to yeast and its subsequent removal from the sample, as shown in Fig. 6. Therefore, CF was pretreated with ^{14}C-methylamine followed by incubation with yeast for 1 and 4 h. Results from both Western blot (lanes 3 and 4 in Fig. 3(B)) and autoradiography (lanes 3 and 4 in Fig. 3(C)) showed that the intensity of the full-length α chain did not change after contact with yeast in the presence of methylamine. These results indicate that methylamine can block thioester-mediated binding to yeast, which predicts that SpC3 functions as an opsonin in vivo.

4. Discussion

When a thioester site is activated and exposed under normal conditions, the result is the formation of either an ester or amide bond with a target molecule, or the hydrolysis and deactivation of the thioester upon reaction with water. The covalent bonds that form are a result of a nucleophilic attack by hydroxyl or amine groups on the target upon the carbonyl carbon involved in the thioester bond [23,27]. The classic assay for this activity is detected by binding small radiolabeled nucleophiles to the thioester site, typically methylamine or hydroxylamine, followed by autoradiography. This assay has been used extensively to analyze complement components and other mammalian proteins with thioester sites (reviewed in Ref. [33]), and recently it has been employed to analyze proteins with thioester sites in the hagfish [7], and invertebrates including tunicates [12], insects [26], and now shown here for the sea urchin. Another classic assay of thioester function, the autolytic reaction, is artificial because the nucleophilic attack is internal, originating from within the protein itself, and is induced by non-biological conditions created in the laboratory. The internal nucleophile has been shown in human proteins to be the nitrogen atom in the peptide bond between the glutamic acid and glutamine, and that it attacks the carbonyl carbon of the thioester bond formed between the side chains of the glutamine and the cysteine [29]. As a result of this interaction, the thioester site passes through a number of transient intermediate structures and culminates in cleaving the peptide bond between glutamic acid and glutamine.

Autolysis was initially observed for isolated human C3 when the samples were boiled in lysis buffer prior to analysis by SDS–PAGE. Two bands of 46 and 74 kDa appeared on the gels in addition to the expected bands for the α chain (116 kDa) and the β chain (70 kDa) [33]. The additional fragments did not correspond to normal degradation products resulting from the activities of factor I and factor H on C3 and were not a result of contaminating proteases in the sample that were isolated in parallel with C3. Furthermore, it was noted that these fragments did not appear when the thioester site was occupied, inactivated or when C3 had been cleaved at the C3-convertase site to form C3b. Similar reactions could be demonstrated for C4 and α2M but not for C5 which does not have a functional thioester [23]. These results suggested that an active thioester bond was required for this

reaction to occur. Subsequent analysis of the reaction and the fragments that were formed demonstrated that under denaturing conditions to expose the site from its protected pocket within the protein using high pH and high temperature, the protein was induced to undergo an internal peptide bond cleavage at the thioester site [33,35–42]. Consequently, autolytic reactions have been employed to demonstrate the presence of a typical thioester bond within a given protein and has recently been used to demonstrate thioester activity in Mosquito aTEP1 [26] and tunicate C3 [12]. Here we have demonstrated this reactivity for SpC3.

In higher vertebrates, C3 in its active form is known to be very short lived. It is quickly inactivated through a number of mechanisms including hydrolysis by water. Therefore, at any time, only a fraction of the C3 molecules in serum are in an active conformation that can support covalent bond formation. Autolysis has been employed as a simple assay to characterize the relative amount of active C3 that is present in the serum at any point in time [33]. Similarly, autolytic assays of sea urchin CF show that only some of the SpC3 molecules undergo autolysis, and in fact, the level of active SpC3 molecules in different CF samples can vary from approximately half to none (based on visual inspection of the intensity of the 130 kDa band compared to the 50 kDa band in Fig. 4). This indicates that only some of the SpC3 molecules in the CF of any given sea urchin at any given time are capable of forming covalent bonds with target molecules or cell surfaces, a result that has also been noted for human C3 [33]. Although the basis for differences in SpC3 activity between sea urchins is not clear, there may be a correlation with the level of immunoquiescence in animals that have been housed for long periods of time in our aquarium systems (for a discussion of immunoquiescent sea urchins, see Ref. [19]). Essentially, we have found that when sea urchins have been kept for more than about eight months, their immune systems become down-regulated showing decreased amounts of SpC3 in the CF [20]. A reduced amount of SpC3 may be correlated with reduced levels or no molecules in an active conformation.

Two bands were detected by autoradiography of ^{14}C-methylamine-treated CF. The smaller band corresponded with SpC3 as recognized on Western blots using anti-SpC3 antisera, however, the larger band has not been previously identified. Its size of 185 kDa is within reason for a homologue of a2M. The presence of two bands identified by ^{14}C-methylamine on gels of separated CF proteins, albeit of vastly different sizes, also raises the possibility that there may be more than one copy of genes encoding SpC3 homologues in the sea urchin. All vertebrates have numerous members of the thioester family of proteins including complement proteins (C3, C4 and C5) and other thioester containing proteins, some of which are included in Fig. 2. In addition, the teleosts have multiple copies of C3 ([43–45]; reviewed in Refs. [6,46–48]); and multiple copies of a2M [48]. If two or more copies of SpC3 genes are present in the sea urchin genome encoding proteins of very similar size, the proteins may not be separated by one-dimensional gels. Although this possibility cannot be ruled out, there are there are a number of reasons why SpC3 gene duplication may not have occurred in the echinoderms. Based on the locations of mammalian C3, C4 and C5 on different chromosomes and within paralogous copies of the MHC, this has been used, in part, as evidence for two rounds of genome wide duplications that are thought to have occurred prior to the appearance of the elasmobranchs (reviewed in Ref. [49]). Furthermore, additional duplications are thought to have occurred in the teleost lineage [50,51] resulting in additional copies of complement proteins, as has been noted in several fish species [43,44,52]. Since the genome duplications are thought to have occurred well after the divergence of echinoderms and chordates, multiple copies of complement proteins located on different chromosomes in the sea urchin are not expected. Furthermore, no evidence has accumulated for SpC3 gene duplication based on genome blots [14] or through EST analysis. Recent BLASTn searches of EST databases using the SpC3 cDNA sequence has identified 22 matches, all of which show perfect sequence alignments (given a low level of random mismatches due to sequencing errors). Matches to sea urchin coelomocyte EST sequences [13] indicate that the 3′ end of the SpC3 message was identified eight times (EST117 [accession number R61971]; EST385 [R62116]; EST083 [R61951]; EST119 [R61973]; EST113 [R61967]; EST375 [R62107]; EST354 [R62094]; EST179 [R62008]). In the original publication, these ESTs were included in the unknown category because none included open reading frame sequences and therefore

BLASTx searches failed. Furthermore, in two EST studies of sea urchin embryos, the SpC3 message was found once in cleavage stage embryos (GenBank accession number AF122324; [53]) and 12 times in secondary mesenchyme cells (GenBank accession numbers: BG781881, BG781758, BG785784, BG784497, BG781085, BG783152, BG785352, BG780359, BG785194, BG786755 BG786433, BG780257; [54]). None of these ESTs included open reading frame sequences and were listed as unknowns. For these embryonic messages, all showed very low probability of random matches to SpC3 (BLAST E value $= 7 \times 10^{-4}$ or less) with a clear distinction between significant matches to the known sequence of SpC3 and random matches (BLAST E value = 0.003). This shows that there were no matches between the known SpC3 and ESTs with a level of mismatch that would be expected between duplicated genes with similar sequences. These results indicate that duplicate copies of the SpC3 gene may not be present in the sea urchin genome.

If SpC3 gene duplication has occurred in the sea urchin producing putatively linked SpC3 genes, the protein encoded the second gene cannot be identified by the analyses presented. If present, the protein would either have to be dissimilar enough not to be recognized by the antisera raised to the known SpC3, or of identical size to appear as a single band with the known SpC3 on Western blots. In addition, if present and of a different size, it would not have a functional thioester site because a band of similar size to the known SpC3 α chain does not appear by autoradiography of ^{14}C-methylamine labeled CF. In fish, duplicated copies of C3 show differences in size and antisera can be generated to differentiate between them [43]. Furthermore, thioester activity in the fish C3 proteins vary in their abilities to form covalent bonds with target molecules. Based on the data presented, if a duplicate copy of SpC3 is present in the genome and expressed in coelomocytes, it would either have to be of identical size to the known SpC3 and be masked on Western blots, be antigenically dissimilar enough not to be recognized by the available antisera to SpC3 or have a non-functional thioester site that would not bind ^{14}C-methylamine and therefore not appear by autoradiography.

Opsonization of foreign particles and pathogens by C3 resulting in augmented phagocytosis is generally recognized as an important function of the mammalian complement system [55,56]. Alpha 2 macroglobulin also opsonizes foreign endopeptidases and promotes their destruction after trapping them to control their activity (reviewed in Ref. [29]). The TEP molecules of the insects have somewhat similar opsonization functions [26]. The complement systems in lower deuterostomes including tunicates [12,57,58], hagfish [7,59] and lamprey [10,11], which function (apparently) without a terminal pathway for cell lysis, probably employ complement-mediated opsonization as an important mechanism for host protection. Data presented here indicate that the sea urchin complement system may function similarly. Because autolysis of SpC3 can be blocked by first engaging the thioester with either methylamine or yeast and that binding to yeast can be blocked with methylamine, this suggests that the sea urchin complement system also functions as an opsonization system leading to augmented phagocytosis in vivo. Preliminary data indicate that opsonization of yeast and bacteria results in elevated phagocytosis by coelomocytes [21,22]. This aspect of host protection in the sea urchin may be the most important function of this simpler complement system and this activity appears to be mediated by the thioester in SpC3. Data directly addressing this point constitutes a publication to be published elsewhere [21]. Because the sea urchin complement system represents an estimation of the ancient complement system in the deuterostome ancestor, it serves to identify the original functions of the alternative pathway and underscores the importance of the family of proteins containing thioester sites in host defense in modern vertebrates.

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