

# The Major Yolk Protein in Sea Urchins Is a Transferrin-like, Iron Binding Protein

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The major yolk protein (MYP) in sea urchins has historically been classified as a vitellogenin based on its abundance in the yolk platelets. Curiously, it is found in both sexes of sea urchins where it is presumed to play a physiological role in gametogenesis, embryogenesis, or both. Here we present the primary structure of MYP as predicted from cDNAs of two sea urchins species, *Strongylocentrotus purpuratus* and *Lytechinus variegatus*. The sequence from these two species share identity to one another, but bear no resemblance to other known vitellogenins. Instead the sequence shares identity to members of the transferrin superfamily of proteins. *In vitro* iron binding assays, including both <sup>59</sup>Fe overlay assays of MYP enriched coelomic fluid and immunoprecipitation of native iron-bound MYP from coelomic fluid, support this classification. We suggest that one of MYP's transferrin-like properties is to shuttle iron to developing germ cells. © 2002 Elsevier Science (USA)

**Key Words:** oogenesis; major yolk protein (MYP); yolk platelet; vitellogenin; transferrin; iron.

## INTRODUCTION

Yolk platelets comprise nearly one-third of the volume of the sea urchin egg yet the precise function of its constitutive molecules is unclear. In other animals, yolk is known to provide a source of nutrients until the organism can feed on its own. The most abundant protein of both the sea urchin egg and the yolk platelet, is called the major yolk protein (MYP; Harrington and Easton, 1982; Kari and Rottman, 1985; Harrington and Ozaki, 1986; Ozaki *et al.*, 1986.) It is a 180 kDa glycoprotein and is characterized as a vitellogenin based on reported biochemical properties (Shyu *et al.*, 1986, 1987). However, the sea urchin MYP does not completely fit the profile of other known vitellogenins. For example, large amounts of MYP are produced in both males and females whereas vitellogenins are specific to females in other examined animals (nematode, insects, and vertebrates). In addition, the overall mass of MYP does not change during development up to the pluteus stage of embryogenesis (Kari and Rottmann, 1985; Armant *et al.*, 1986) negating the possibility that MYP serves as vitellogenin store of amino acids. So what is the function of this abundant protein?

MYP is present in the coelomic fluid of both sexes of sea

urchins, at a slightly higher molecular weight (195–200 kDa) than in the yolk platelets of eggs, and it has been estimated to comprise greater than 50% of the total protein content of this fluid (Harrington and Easton, 1982; Shyu *et al.*, 1986). Before gametogenesis the protein is stored in nutritive phagocytes (accessory cells) of both the testis and ovary (Ozaki *et al.*, 1986; Unuma *et al.*, 1998) although the major site of synthesis in both sexes appears to be the intestine (Shyu *et al.*, 1986). In males, MYP has been suggested to serve as a nutrient store for spermatogenesis as immunohistochemistry shows MYP depletion from degenerating nutritive phagocytes in the follicular lumen during spermatogenesis. The protein is, however, not detectable in stored spermatazoa (Unuma *et al.*, 1998). In females, MYP is retained following gametogenesis. It is transported during the vitellogenic phase of oogenesis from the nutritive phagocytes to the growing oocytes where it is packaged into yolk platelets that are stored in mature eggs (Ozaki *et al.*, 1986; Harrington and Ozaki, 1986; Unuma *et al.*, 1998). Its deposition there accounts for more than 10–15% of the total egg protein (Ichio *et al.*, 1978; Kari and Rottman, 1980; Ozaki, 1980; Harrington and Easton, 1982). During embryogenesis MYP is processed into characteristic lower molecular weight cleavage products that disappear by the pluteus stage (Kari and Rottman, 1985; Armant *et al.*, 1986; Scott and Lennarz, 1989; Lee *et al.*, 1989), although the significance of this processing is unclear.

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To elucidate the physiological function of MYP in sea urchins we set out to clone the full-length cDNA. Here we present the primary structure of MYP as predicted from cDNAs of two sea urchin species and find that instead of resembling a vitellogenin, MYP contains transferrin-like iron binding domains. We tested the functionality of these domains and demonstrate that MYP does bind iron. These results help us resolve conundrums of MYP utilization in development and its occurrence in the male gonad. We suggest that this protein transports iron to the ovary and testis to meet the proliferative demands for gametogenesis and embryogenesis.

## MATERIALS AND METHODS

All reagents were obtained from Sigma Co. (St. Louis, MO) unless otherwise noted.

### Animals

Adult *Strongylocentrotus purpuratus* were obtained from Charles Hollahan (Santa Barbara, CA) and kept at 15°C in artificial seawater (Coral Life, Carson, CA) until needed. Adult *Lytechinus variegatus* were obtained from Scotty Services and Susan Decker (Miami, FL) and kept at 20°C in artificial seawater until needed.

### Isolation of MYP Protein from Yolk Platelets of Whole Eggs

Isolation of yolk platelets was carried out according to the method of Yokota and Kato (1988) with modification. *S. purpuratus* gametes were obtained by intercoelomic injection of 0.5 M KCl, and the eggs were dejellied and washed twice in artificial sea water (McClay, 1986). To avoid contamination of the yolk preparation by the very abundant fertilization envelope and other cortical granule proteins we isolated the yolk protein following egg activation by one of two different methods. Initially, fertilization envelopes were removed by treating shed eggs with 1 mM 3-AT (3-Amino-1,2,4-triazole) before fertilization, to inhibit the cross linking of envelope proteins and thereby, aid in their solubilization for removal from the eggs (Showman and Foerder, 1979). Subsequently eggs were also treated with 10 mM dithiothreitol (DTT) to remove the vitelline layer before activation (Epel *et al.*, 1970) so the cortical granule proteins could be freely washed away. In both cases eggs were activated by the addition of the calcium ionophore A23187 (10 µg/ml). Eggs were kept on ice and washed twice with calcium-free sea water and then twice with KCl solution (0.55 M KCl; 1 mM EDTA, pH 7.0) and resuspended in 5 volumes of this solution. Eggs were homogenized in a Wheaton (Millville, NJ) 7 ml dounce tissue grinder by hand several times until near complete egg lysis was achieved. The egg homogenate was then centrifuged in a Sorvall RC-5B refrigerated Superspeed centrifuge with a SS-34 rotor for 4 min at 1900 rpm at 4°C, and the supernatant was collected and centrifuged for 10 min at 4500 rpm. The pelleted precipitate was resuspended in KCl solution and the above centrifugation procedure was repeated with the final precipitate used as the yolk platelet preparation. For immunoblot analysis and internal amino acid sequencing, a portion of this preparation was resuspended in 2× sample buffer (20% sucrose, 5 mM Tris-Cl pH 6.8, 2% SDS, 10 mM DTT), denatured for 5 min at 100°C, and electrophoresed on a

4–20% gradient polyacrylamide gel (OWL Separation Systems, Portsmouth, NH) run in Tris-SDS sample buffer. The gel was stained with Coomassie blue and a band at 180 kDa was excised and subjected to internal amino acid sequencing (University of Massachusetts Medical School, Shrewsbury, MA).

### cDNA Cloning

A complete cDNA sequence was obtained in several ways including: 5' RACE from total gut RNA, PCR screening of an ovary cDNA library, and expression screening of this same library using an MYP mAb. Initial cloning of MYP was carried out using the 5'-end amplification RACE (rapid amplification of cDNA ends) protocol described by Frohman *et al.* (1988). For first strand cDNA synthesis, *S. purpuratus* female total gut RNA was reversed transcribed with M-MLV reverse transcriptase (Gibco-BRL Gaithersburg, MD) using nested gene-specific primers designed to the *S. purpuratus* 980 bp 3' cDNA clone kindly provided by J. Villinsky and R. Raff (Indiana University) that consisted of an open reading frame of 240 bp followed by a stop codon (TAA) referenced in Shyu *et al.* (1987). A poly(A) tail was added to the primer extended products with dATP and Terminal Deoxynucleotide Transferase (Promega, Madison, WI). Second strand cDNA synthesis was carried out with the (dT)17-adaptor primer: 5'-GACTCGAGTC-GACATCGATTTTTTTTTTTTTTTTTTTT-3' with the *Xho*I, *Sal*I, and *Cl*aI restriction sites at the 5' end. These products were PCR amplified with *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD) using the adaptor primer and a nested gene-specific primer located upstream of the extension primer sequence. PCR products were ligated into the vector pGEMt-EZ (Promega Corp., Madison, WI). Plasmids with MYP inserts were identified by Southern Blot analysis and DNA sequencing was performed by the macromolecular sequencing facility at Brown University using an ABI 377 prism automated DNA sequencer.

To obtain full length cDNA encoding *S. purpuratus* MYP, internal primers were designed from these clones and subsequent rounds of RACE from total gut RNA were performed in conjunction with PCR screening of a *S. purpuratus* Lambda ZAP ovary cDNA library (Laidlaw and Wessel, 1994). Fortunately, we also isolated two overlapping MYP cDNA clones from an antibody expression screen of this same ovary cDNA library (V. Zaydfudim, personal communication). An MYP mAb was obtained as a contaminant from a vitelline layer preparation to which monoclonal antibodies had been made. By these three screening methods, over 10 separate but overlapping cDNA clones were isolated and sequenced. Less than 1% DNA sequencing variation between the clones was noted and this most likely reflects the polymorphism of the wild-type population.

Independently a partial *L. variegatus* MYP cDNA consensus sequence was obtained in our lab (personal communication A. Howell and J. Wong) from several separate but overlapping cDNAs obtained by an EST screen of two different *L. variegatus* Lambda Zap II ovary cDNA libraries (Stratagene, La Jolla, CA; Wessel *et al.*, 1998; and data not shown).

MYP sequence data were analyzed using the Sequencher program software (Gene Codes Corporation, Ann Arbor, MI). The consensus sequence of the cDNAs resulting from the above screens has been entered in the National Center for Biotechnology Information (NCBI, Bethesda, MD) database (accession numbers *Sp*:AY090112; *Lv*:AY090113).

## Immunological Approaches

For polyclonal antibody generation a *S. purpuratus* yolk preparation was run on a reducing 5% SDS-PAGE gel, and the MYP band at 180 kDa was excised, electrophoretically eluted, lyophilized, and resuspended in Freund's complete adjuvant. Polyclonal antisera were obtained by subcutaneous injection of a New Zealand white rabbit and boost injections were made every 3 weeks for 3 months. Plasma was collected from the ears 1 week after each boost (Harlow and Lane, 1988). Independently, monoclonal antibodies against MYP from *S. purpuratus* were generated using a DTT vitelline layer extract (Stem Cell Technology according to the manufacturer's protocol; V. Zaydfudim and J. Wong, personal communication). Although MYP is not a component of the vitelline layer it is not surprising that this monoclonal antibody was generated, since yolk proteins are very abundant and contaminate many egg protein preparations (Wessel *et al.*, 2000).

For immunoblot analysis, coelomic fluid and yolk platelets (1  $\mu$ g total protein) were subjected to SDS-PAGE and immunoblotting essentially as described (Towbin *et al.*, 1979). Samples for analysis were pelleted, resuspended in 2 $\times$  sample buffer, and denatured for 5 min at 100°C. The proteins were then resolved by SDS-PAGE and blotted. Blots were washed twice for 30 min in blotto (50 mM Tris-Cl, pH 7.5, 0.18 M NaCl, 0.05% Tween 20, 3% nonfat dry milk) and then incubated for 1 h in blotto containing anti-MYP diluted 1:50,000. The blots were then washed three more times over 1 h and incubated for 1 h with goat anti-rabbit antibodies conjugated with alkaline phosphatase diluted 1:30,000. Blots were washed in blotto three more times and then washed in blotto minus milk two times. Each of the above washes were 10 min in duration. Detection of immunolabel signals was carried out with BCIP/NBT development according to Harlow and Lane, 1988; Promega Corp. As a control blots were incubated with preimmune sera or secondary antibody alone and these experiments resulted in no detectable signal (data not shown).

## Preparation of Coelomic Fluid Protein and Iron Binding Procedures

Coelomic fluid was prepared essentially as described by Harrington and Easton (1982). Adult *S. purpuratus* male and females were opened by cutting the peristomial membrane around Aristotle's lantern. The perivisceral coelomic fluid was poured out and collected in a beaker on ice to allow the coelomocytes to clot. This clot was removed and the remaining supernatant was centrifuged in a Sorvall RC-5B refrigerated Superspeed centrifuge with a SS-34 rotor for 10 min at 10,000 rpm at 4°C. The supernatant was passed through a Corning disposable sterile syringe filter (Corning, NY) to remove any residual cellular material, dialyzed at 4°C overnight against several changes of deionized water, and vacuum dried. Total protein concentration was determined by a Bradford Assay. Samples were analyzed by Coomassie staining of SDS-Page gels and immunoblot analysis using MYP pAb sera and the presence of MYP was confirmed at over 50% of the total coelomic fluid protein content (representative shown in Fig. 4).

For slot blotting, all of the glass and plastic ware used were cleaned with RBS 35 detergent concentrate (Pierce Scientific, Rockford, IL) and all solutions were made with nanopure water to minimize contaminating metal ions. The slot blot was prepared by applying coelomic fluid preparations to a nitrocellulose membrane in a Hibri-Slot vacuum manifold from BRL (Bethesda Research Laboratories, Bethesda, MD). Partially iron-saturated human serum transferrin (10 mg/ml), BSA (10 mg/ml; EM Science, Gibbstown,

NJ) and gelatin (10 mg/ml) were used as controls. All slot blot incubations were performed at 23°C in a hybridization oven (VWR Scientific, Quincy, MA) with constant rotation. After applying the protein samples, the slot blot was removed from the manifold and washed twice for 30 min each in blotto. Iron was removed from protein bound samples by incubating the blots in an acidic stripping buffer (0.1 M EDTA, 0.1 M NaCl, pH 5.5) for 2 washes at 30 min each (Cochran *et al.*, 1984). During this incubation <sup>59</sup>Fe-nitrilotriacetic acid (NTA) was freshly prepared from <sup>59</sup>FeCl<sub>3</sub> (8.09 mCi/mg; 1 Ci = 37 GBq; NEN Life Sciences, Boston, MA) by adding <sup>59</sup>FeCl<sub>3</sub> to tenfold excess of NTA in HEPES buffer (50mM NTA in HEPES buffer pH 7.5). The iron stripped slot blot was then incubated overnight with an iron-loading buffer (50 mM HEPES, 150 mM NaCl, 25 mM sodium bicarbonate, pH 7.0) with a molar excess of <sup>59</sup>Fe-NTA (Gasdaska *et al.*, 1996). Excess, unbound iron was washed from the slot blot with 0.02 M Tris-HCl pH 8.0 twice at 30 min each room temperature (Bartfeld and Law, 1990). The slot blot was then air dried, placed on a phosphoimaging plate, and the iron signal was analyzed using a Fuji Film BAS-2500 phosphoimager (Fuji, Japan).

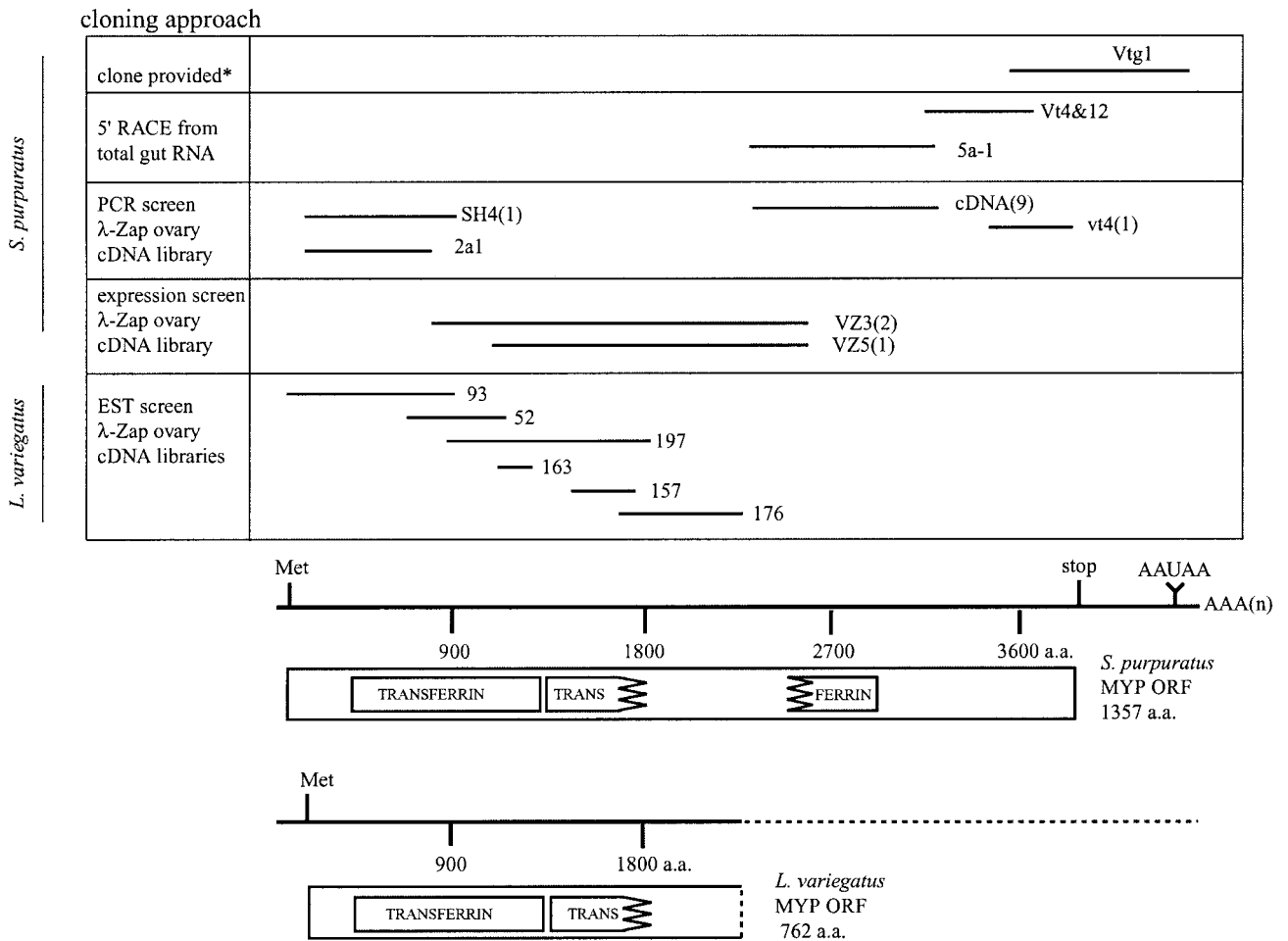
For immunoprecipitations following iron binding, all of the following were performed at 23°C. Radiolabeled coelomic fluid was prepared by first dialyzing overnight against several changes of acidic stripping buffer to remove any pre-existing bound iron. Two additional changes against stripping buffer without EDTA were then carried out to ensure that all of the EDTA was removed. The coelomic fluid was collected from the dialysis tubing and mixed with 10 $\times$  iron loading buffer to a final concentration of 1 $\times$ . <sup>59</sup>Fe-NTA was freshly prepared, added immediately to the coelomic fluid, and the mixture was incubated overnight in a hybridization oven to allow iron binding. Aliquots of coelomic fluid were distributed to Ependorf tubes and placed in a Labquake (Berkeley, CA) for constant mixing. An equal volume of TBS Tween 20 (0.15 M NaCl, 10 mM Tris pH 8.0, 0.1% Tween 20) and 2% BSA was added, and proteins were immunoprecipitated for 2 h using non-relevant pAbs to SFE 11 (Wessel *et al.*, 2000), preimmune sera to MYP, or pAbs to MYP. During this incubation, a slurry of protein-A Sepharose beads in a 10-fold excess of TBS Tween 20 and 2% BSA was prepared. A 20% mixture of these beads was added to the immunoprecipitation to a final concentration of 10% and the complexes were incubated for an additional 2 h. Complexes were allowed to settle by gravity sedimentation and were washed three times with TBS Tween 20 and then resuspended in Econo-safe counting cocktail (Research Products International Corporation, Mount Prospect, IL) for isotope analysis using a Beckman Instruments (Fullerton, CA) LS 6000SC scintillation counter.

## RESULTS

### cDNA Cloning of the Major Yolk Protein

Although the physiological function of the major yolk protein (MYP) in sea urchin has been analyzed for over two decades and its classification as a vitellogenin has yielded conflicting results, the complete sequence, and true function of this very abundant protein is not known. We set out to clone the full length MYP to help determine its role in this animal. First we isolated MYP from *S. purpuratus* yolk preparations prepared from whole eggs. After protein concentration and separation by SDS-PAGE electrophoresis, a prominent MYP band was visible by Coomassie staining at

## Clones used to generate the MYP open reading frames



**FIG. 1** Maps of the clones used to determine the full-length open reading frame for the *S. purpuratus* MYP and the partial open reading frame from the *L. variegatus* MYP. Included is a scale diagram of the putative protein encoded showing the predicted transferrin-like, iron binding domains (\*Shyu *et al.*, 1986).

180 kDa, consistent with the size reported (Harrington and Easton, 1982; Shyu *et al.*, 1986). This band was excised and subjected to amino acid analysis and internal amino acid sequencing. One tryptic peptide of 19 amino acids (NVELL-DDLVGVEGISDLVK) was obtained, with no strong similarities to database entries (NCBI database search), including vitellogenins.

Overlapping cDNAs encoding the full-length *S. purpuratus* MYP were isolated by several approaches including 5' RACE from total intestinal RNA, PCR screening, and expression screening of an ovary cDNA library (Fig. 1). We did not observe significant variations in sequence identity of the clones from the screens of intestinal RNA and the ovary cDNA library. Furthermore, cDNAs were confirmed to represent the same gene product by Northern blot

analysis using multiple cDNA probes spanning the MYP. A single RNA transcript of 5.1 Kb was detected in adult female and male gut and in ovaries and testis but not in eggs or embryos (data not shown), as reported previously (Shyu *et al.*, 1986). We also obtained a partial *L. variegatus* cDNA consensus as identified by several overlapping clones isolated in an independent EST screen of an ovary cDNA library (Fig. 1).

The complete *S. purpuratus* nucleotide and deduced amino acid sequence is shown in Fig. 2. The sequence contains 4,071 bp of open reading frame coding for 1357 amino acids beginning with an initiating methionine at position 30 followed by a 14 amino acid signal sequence and ending with a termination codon (TAA) at position 4101. We are confident that the sequence reported here is the sea

1	CATCAACTCAGCTCGGAGAAGTTGATCATGAGGGCTGCAATTCTCTTTGCGCTGGTGG	60	2101	ACAACCTCACCGGGCAGGAGTGGCTCTGTGGAAACACCCGCCACCTGGAACCTCTCTCA	2160
1	M R A A I L F C L V A	11	692	N F T G Q E W L L W N T P A T W N F L T	711
61	CCAGTTCATGGCCGTCGCAAGCGCTCGTTGGGCTCGAGGCGCGGAACCTGCCCACCTC	120	2161	CCTACAACAGGAAGGTTCTTACCGGTCTTGACATCAAGAAGCTGATTGAGCTGAAGAAGC	2220
12	S S M A V P S G S L G S R P G T C P P Q	130	712	Y N R K V S T G L D I K K L I E L K K Q	731
121	AAACCAGTATCAGTCAATGATTAAGCAACCGTGTCTTACGTGTATGGATTAACCT	180	2221	AGAACCTGACCTGCACATCTTCAACCAAAACCTCTCTCCCCCAGGAACCTGTAACCTC	2280
32	P S D Q V M I E A T R C S Y V Y G L T W	151	732	N L T S H I F N Q N L S S P R N V E L L	751
181	GGGACTGGAACCTGCAACAGCAAGCAAGAACTACAGTCTGCCAATATGAGAAGC	240	2281	TCGATGACCTCGTAGGGTTCGAGCAATCTCTGACCTGGTCAAGGGTATCCAGGACCCA	2340
52	D W N C S Q G G E N Y K C Q Y E N D	210	752	D D L V G V E G I S D L V K G V Q D T I	771
241	ACATCGAGATCTGTCTTCCAATCCAGCTGATGTGTAGAGGAGGTCGTGTGGAGC	300	2341	TCGGACCGGAGGCAAGCAGAGATGAACATCGTCCGTGACCGTCTGAGCAACTCTCTCC	2400
72	I R I C V P P I P A D V D E E V G V E Q	360	772	G P E G K Q K M N M L R D R L S N S F P	791
301	AACTTAGCCAGAGCGTGGACAGTCCCGCAGGCAATCCAGAAGACCCAGGACTTCATCC	420	2401	CCAACTTGAAGCGCTCGCACACTGTCGATTAAGGTCGATATCGTCAACAAAGTGAAG	2460
92	P S Q S V D Q V R Q A I Q K T Q D F I R	411	792	N F E A V R T L S D K V D I V N K M K D	811
361	GCAAGTGGGTCTGTACCCGCGCCCGCAGCGCGCGCAGCCCGCCAGCTCCCGACA	480	2461	ATGCTCGTCAAGAGGTTGAGAACAGGATCATCCATTTGGAAAGCTCAGGAGAA	2520
112	K V G L Y P A P D Q R R R T T P T P D T	480	812	A R Q Q R L Q N K D H P F G N V I Q E T	831
421	CAGTCAGATGGTGGCTCGAGCCGCTGCCAGATGACCAAGTCCAGAGGATGTCGACGG	540	2521	CATTCAGGGTCACTGTAGTGTGTGTTCAGCAAGCTCTCGAGCTCGTGTAGGAGCA	2580
132	V R W C Y S S R C Q M T K C Q D R M V S E	551	851	F Q G H L M V D V F S K L L E L R S D K	851
481	AGTTCACCTACAGCCCTAACATGTCCTCCAGGAACTGAAGTGCACCCAGCTACCTC	540	2582	AGATCAGCACCTTGGAGGATCATCTCTCACGTCAAGACCATTCATACCTCACAGACT	2640
152	F T Y S P N M V R R K Q W K C T Q A T S	540	852	I S T L E E I I S H V K T I P Y L T D F	871
541	CCAGGAGCAGTGTATGTCGGATTGAGCAAGATGGGCTGACATCATGACCAACAGCTG	600	2641	TCAGGATGTAGATCACCATCTCTCCAAACCCCTGCCATCATGAGTCACTGCGTCA	2700
172	O E O C M F W H I E O G W A D I M T T R E	600	872	K D V E I T T V L L K H P A I M S Y V E I	891
101	AGGACAGGTCCTACTCTGCAACACCACTTCAACCTGAAACCTATCGCTTATGAGCA	691	2701	TCCTACTCCCCCTCTCTCCAGACTTCTGTGCGAGCCCTTGATACCGTGAAGTCCGCT	2760
192	G Q V V Y S A N A N T T F N L K P I A X Y E T T	711	892	Y F P R L S Q T T V B E P F D N V E L R E	911
661	CCATCAATGATCAACAACANTGTCAGATCTCGAAGCATTACAGAAATGTGACCTTTG	720	2761	AGCCGGAATCAACCGCTCAACCACTCTCTGGCTTCTCCCAAGGTCACACCTACC	2820
212	I N D D Q P E I O I L K H Y Q N V T F A	721	912	R E F N R Y T N P L W L S P K V H T Y E	931
211	CCCTCAACTCATCCGCTCGTGAACCCACACTTCCCGAGTCCCGCAGCAAGACA	780	2821	ITGACCTGTCAAGATCACCAGACTGAGATCAACCAAGCTGCAACTCCAACTCCAC	2880
232	L K S S R I L V N E N T T F S E L R D K T E	251	951	D L V K N H Q T E I T K T C N S N L P L	951
252	C H A G S D M E D E B H P A S F A R P Y	271	2881	TCAACTTCAAGGGTATGAGGGCGCTCTTCGATGCTCAAGAGGGCGGTTCGGCAGCTGG	2940
841	TCGTCAATTCATCAAGAGGGCGTTATTCVCTACCCGAAACTGACATGAGAGCTTCT	290	892	N F K G Y E G A L R C L K S G V A D L A	971
272	C N L L K E G V I T G N Y I E S F S	900	2941	CTTCTGACAGGACCCCTCCGTGACAGGACCCCTCGTGTGACAGGACCTTCGAG	3000
901	CCGACTTTGTCAGGAGAGCTGTGTACAGGTGTCTCGAATGACCTTACAACAAGAGG	911	972	S S T S R P S V T R T L R D T D L S T G	991
292	D F V Q E S P F G V L N M T Y N K N G	960	1001	GGTGGTTCACCTCAAGAGCTTCGGCTTCTTTGCCCAACCGGAGGTGTGAGATCG	3060
961	GCACCTACCTCTTAGCCTGTACTCTGTGAGGATCAGCAGTACAAGTACTCGGGCA	1020	3012	W V H L Q R P P P S L T G P Q R V E I D	1011
312	F Y P L S L G T I C T G E D Q V Y K Y S G I	1080	3061	ACGTCAACATGACATCCCAAGTGTCAACTTTGGCAGGTCGAACACCTGTCTGG	3120
1021	TCAAGGAGCCCTCAGTTGCCCTGAGTCGGGCAAGGGAAGGTCACCTCTCGTCCGACAA	1140	1012	V N M D I A K V C N F G E V M N P V L V	1031
332	K G A L S C L E S G K G Q V T F P D K	351	3121	TTACCCGCTCAACACCTCAGGAAGTGGCGTGAACACTCAACAGGCTGTGATGAT	3180
1081	AGGTGATCAAGAAGATCATGAGTCCAAATGTGAGGGAACAATCCAGGTTGTGTGGC	371	1032	T A Y N T S G S V N R W N I T K A L M I A	1051
352	V I K K I M E D E N V R D N F Q V V Q R	371	3181	CCCACAGTCTGTGCGCCCTCCAGCCCTCTTTGGAGAGGACCGTCTATGSGCAAGS	3240
1141	GTGACGAGCAGGCGCTCTGACGAGGAAATCTTCCAGCAGCTCAGATGCCACGTGGCC	1200	1052	H Q S V A L F A L E F A L E G E T T V M H K D Y	1071
372	D E S R E L D E E L F E I D V I C H V G I H	391	3241	ATGACATGCTCTTCCATCGCCCTCTGAAACCACTGCTCAACCCCTCTCCGATCCA	3300
1201	ACACTGCTCGCCCACTTCTCATCAACAAGAACCAACCCAGCAGAGGAGATGATA	1260	1071	D M D L P L I A P L N Q V S Y Q P F L G S K	1091
392	T A R P T I F I N K N N T T Q Q E T D I	411	3301	AGCCACTCCGCTCCATGGGCGACTTCAAGGCTTCAATATATGATGCTGGTCAAGGAT	3360
1261	TTAAGACCTCTGCTGAAGATGATGAGCTTATGGCAACACTGACCCAGATGTCAACT	1320	1112	P L R S M E A I V K A S S Y D W F K D Q	1111
412	K T L L V Y K M H L E L V G N T L R D V N E	431	3361	AGACCCGATCTGCTACGGGCAAGCTTACCAACACTCTGTCAGCAGGCAACGAAACCT	3420
1321	TCAACATCTTGAATCTCGTCTGACAGTCTGGCAAGTGCAGATGACCCGCAAGCCGC	1380	1112	T G C I Y G E T Y T T N I V K Q R N B T C	1131
432	N I E D S S S V Y D C G G Q M T G S K P I I	451	3421	CCCAGCCGCTGCTAAGAGGAGTCAAGTGTGCTTCCACCCAGGATGAAGAGATCATG	3480
1381	TCAACAGAACCTGATCTTCTGGAAGAGTCCAACACCATGAAGATCGTCCAGCAGCA	1440	1132	Q A I V K D V T C V G T P R M K K I S V	1151
452	N K N L F L L E L E S T N K M V D D S K	471	3481	TCGGTCTGCTTCGGAGCCCAAGTCAAGATGATCAAGATGTCGAGCCCGCAAGCAAGT	3540
1441	AGGTCTAGCTGGCGAAGTCTAGTCCCTACAAGCTTCTCTCCAACTGGTGGCCAGC	1500	1152	G R F P A G A K Q F K M I K M C S R P S K F	1171
472	V Y Y A G R E Y Y A A Y N V C S K L V L R	491	3541	TTGTGGCAAGATGGCCGACTTCCAGTGGCAACTGGCTTCGGCTACCTCAACACAGTCA	3600
1501	CCCGTGGCAAGATCTGCGTCAACCAAGTTCACGAGTACGAGGCTGCGCCCGCTTCAAG	511	1172	V R K M A D D F Q C D N G F G Y L K P V I	1191
492	R A K I C V T N V T E Y E A C R R R F K G	511	3601	TCACTGCTGTGCTCGGAGTGTGCTATGTGAGGAGATGATCGAATAACAACCTCTT	3660
1561	GCATTGTGAGAACATCCAGAGGTCGAAGAAGTGTGCTGGGCTGTGCTTGGCAACT	1620	1192	T A V A C E C M L C E E M I E Y N T S F	1211
512	I A E N I P E P V T K K V A W G C V L A N S	1620	3661	TCACCGAGCAACATGTGGTCCGATGTCTCCACAAATACGTCTCAGCAGGAGAGCAGG	3720
1621	CCAGCATGAAATGATGCAAGCCCTCCACAACACCGCTGACCTCTTCAAGCCCAACC	1680	1212	T E D N M M S D V S N K Y V L T G E Q D	1231
532	S I E C M Q A V H N N T A D L F K A R P	1740	3721	ACATCTACAGAAATCCCTATCTGGGAAACCTCTACTTCTACGACATPACCTCGA	3780
1681	CAATGGAGACTTCATCGTGGCAAGGATTCCTGCTGACCCCTCTATGTCGCTCCACC	1740	1232	I Y R Q I P I W G N S Y F Y D H T L N	1251
552	M E T F I A G K E F L L D P L M S V H R	1740	3781	ACAAGAATTGAGCTGGAAACCTTCTATTCGTGTGAGCATCCAGACTGTTGTTG	3840
1741	GCAATGACACGCTAACCATGAACACCACTACACAGGACCTGGCAGTCAAGAGGT	1800	1252	K N F E L G N H S I I V E H V Q T V V	1271
572	N D S V T M N H T Y T R T L A V I K R S	1860	3841	TTGAAAGCCGAGCCGGAATTTGCTCAGGTCACCTCCGAAGTAGACCCCGAGGTCC	3900
1861	CTTCCCTGCCAAGTCCAGCCCTTCCAGCGTCTCGAAGGACCAACCAAGTACATCA	1860	1292	E R P S P G I I L S Q V N S E V D P E V Q	1291
592	S L A K F P G L L S V P E G Q P K Y I K	611	3901	AGGTCCAGATGGACTCCGAGCTCACCAAGACCTGTGAGAGGCTTGGAAAGCCGAG	3960
1861	AGGACCTCGAAGCTCAAGATCTGCTGTGCTCGAAGATTTCCAGCCTTCCACA	1920	1292	V Q M D S A S L T K T C E T V W N G Q S	1311
612	D L W K L K I C S A G L K N F S A F H S	631	3961	CGTGGCTCCGTGAGGTTCCAGGCTCAAGACGACCTGCTCCGCTGTGCTCCAGAG	4020
1921	GCCCACTGGTATCTCTCCCAAGCCAGCTCCACAGTATCGATCCGCTTTGAGT	1980	1312	W L P E R F G Y K T S G S C V V P E T	1331
632	P I G Y L L A N G T I P R I C S V F E S	651	4021	CCGAGCAAGCCCAAGTCTCGCTGGACCGCTCCCGCAGATCATGCAAGCAGCAAC	4080
1981	CTGTCAACAGGATCTCTCAGGCACTGGCTCCAGAAATCGAGCCGAGCTGGCGCT	2040	1332	G A N A K S R V D R P Q I M O R K Q Q	1351
652	V N R Y F Q A T C V P E I E P E T W R L	671	4081	AATTTGGTGCATCACCACAT*AAACCGCCCAAGATTAGAATTAACGGAAACCTACA	4140
2041	TGGACTGTATCTCTCCGCGGAGATGAACCTGGGATTTCTCCCTCCCAACATY	2100	1352	L V D H H H	1357
672	D S D L L L G R E M N W G F S S L N M Y	691	4141	TGACTTGTATATATGGAACCTTCTGGATCTTAAATCTTTGATTTATAATCAGAAAG	4200
			4201	CTTTGAGAATCTTATGAACTTTATCATTACGGTAGCTGAC	4244

**FIG. 2** Nucleotide sequence and amino acid translation of MYP cDNA from *S. purpuratus*. The signal sequence is italicized. The transferrin-like, iron binding domains are delineated by darker shading for the first complete domain (residues 132–489) and lighter shading for the second split domain (residues 494–662 and 945–1103). The sequence obtained by internal amino acid sequencing is in bold and underlined. The termination codon is marked with an asterisk.

urchin MYP based on several criteria. The amino acid sequence obtained from a tryptic fragment of our yolk platelet isolated MYP is encoded by the cDNA and verifies by biochemical means that the protein predicted from our cDNA sequence is in fact the major yolk protein. The overall amino acid composition predicted from this cDNA (Table 1) matches the profile of amino acids previously reported for the purified MYP (Kari and Rottman, 1985;

Scott and Lennarz, 1989). In addition, the theoretical pI from this cDNA is predicted to be 7.04 which is in the range of the previously measured pI of 7.0–7.8 for native MYP (Scott and Lennarz, 1989) and the predicted molecular weight from the cDNA is 154,019 kDa which is nearly identical to the MYP *in vitro* translation product of 155 kDa reported by Shyu *et al.* (1986). Furthermore, N-linked glycan sites on MYP characterized biochemically by Armant *et*

**TABLE 1**  
MYP Amino Acid Composition

Residue	Mol%	
	cognate cDNA sequence	Experimental*
Ala	4.4	5.2
Arg	4.6	6.2
Asx	11.4	13.0
Cys	3.0	0.5
Glx	10.9	14.1
Gly	5.0	3.5
His	1.7	1.5
Ile	5.5	5.5
Leu	7.3	8.0
Lys	6.3	7.0
Met	3.1	0.5
Phe	4.1	5.5
Pro	5.3	7.0
Ser	7.1	4.2
Thr	6.9	6.0
Trp	1.6	ND <sup>a</sup>
Tyr	3.3	3.5
Val	8.3	8.0
pI	7.04	7.0–7.8
MW	154 kDa	155 kDa

The amino acid composition of *S. purpuratus* MYP predicted from our cDNA recapitulates what was biochemically determined (\*Scott and Lennarz, 1989; reported error 6–8%).

<sup>a</sup> ND, not determined.

*al.* (1986) are found on our cDNA clone (data not shown) and the MYP tryptic digest for internal amino acid sequencing has HPLC peaks that suggest glycosylation (J. Leszyk; personal communication). Finally, both ends of our cDNA closely matched that reported from the MYP partial clones of Shyu *et al.* (1986, 1987). We also present a partial *L. variegatus* MYP cDNA consensus sequence consisting of 2485 bp and an ORF encoding 762 amino acids including an initiating methionine followed by a 14 amino acid signal sequence (NCBI accession no. AY090133).

### **The MYP Protein Is a Member of the Transferrin Superfamily**

When the full-length deduced amino acid sequence of *S. purpuratus* and the partial amino acid sequence of *L. variegatus* were aligned to one another (Lalign; Lipman and Pearson, 1985) 57.6% identity was shown in a 742 amino acid overlap (data not shown). When these sequences were compared with other proteins in the NCBI database surprisingly we found no similarity to reported vitellogenins. Instead, MYP contains two transferrin-like motifs (Fig. 1). Blast searches using the complete N-terminal transferrin-like domain from both species of sea urchin showed sequence identity between 24 and 28% to both vertebrate and

invertebrate transferrins. This level of identity in the transferrin superfamily is characteristic since the sequence between vertebrate and insect transferrins is only 25–30% identical (Baker *et al.*, 2001). Furthermore, we searched for conserved protein domains using Reverse Position Specific (RPS) Blast (NCBI; Altschul *et al.*, 1997) and each species of sea urchin MYP showed significant alignments (e-values; Sp  $2e^{-32}$ , Lv  $4e^{-35}$ ) to the Smart transferrin consensus sequence that was derived from 30 aligned transferrin domains (Shultz *et al.*, 1998). This consensus includes serum transferrin, melanotransferrin, ovotransferrin (from egg white), and lactoferrin (from milk, white blood cells, and other secretory fluids) from vertebrates and four transferrin domains from insects (conserved domain [CD] accession number: smart00094). The alignment for the complete N-terminal transferrin motif from both species of sea urchin was compared to the Smart consensus (Fig. 3). The sequence for *Blaberus discoidalis* (cockroach), an insect transferrin, is included for comparison and to show representative variability in the iron binding residues (see also Discussion for analysis of iron binding residues).

### **MYP Binds Iron**

To determine if MYP indeed binds iron, we first generated a monospecific antibody that binds MYP selectively and used coelomic fluid as the source of native intact MYP (Fig. 4). MYP occurs as a single species of 200 kDa in coelomic fluid that is processed into several distinct smaller fragments in yolk platelets once within the egg (Kari and Rottman, 1985; Armant *et al.*, 1986; Scott and Lennarz, 1989; Lee *et al.*, 1989). This processing is concomitant with the acidification of yolk platelets that occurs during embryogenesis and since many transferrins release iron at low pH, MYP processing may be linked to iron release from this protein. MYP abundance in coelomic fluid varies in accordance with the gravidity of the animal (data not shown) so we first used the MYP antibody to select for animals enriched for MYP. We then tested MYP iron-binding by using an iron overlay assay. Coelomic fluid proteins from both sexes of adult sea urchins were applied to a nitrocellulose membrane using a slot-blotting vacuum manifold. Additional protein samples for this experiment included a positive control of human transferrin and negative controls of gelatin, BSA, and blotto. Incubation of these blots in an acidic buffer (pH 5.5) containing EDTA strips and chelates pre-existing iron ions bound to proteins (Cochran *et al.*, 1984). Binding of radiolabeled iron to the stripped protein is then possible by transferring the blot to an iron binding buffer (pH 8.0) containing  $^{59}\text{Fe}$ -nitrilotriacetic acid (NTA) freshly prepared from  $^{59}\text{FeCl}_3$ . Iron binding is then detected using a phosphorimager and is shown in Fig. 5. The gelatin slots do not show detectable iron signal regardless of the protein concentration. Other negative control proteins, including BSA and blotto, showed similar results (data not shown). In sharp contrast, both the human serum transferrin (positive control) and coelomic fluid slots show

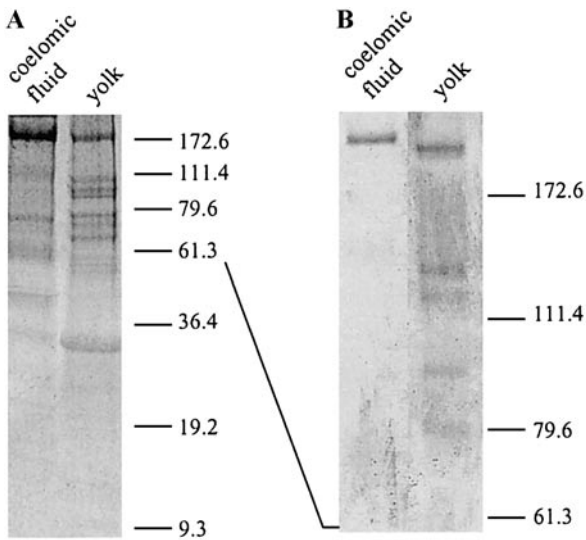
			*	*		*	*		▼																																								
Sp MYP	132	VRWCVSSRCQMTKQ	Q	R	M	V	S	E	F	T	Y	S	P	N	M	V	P	R	K	Q	W	K	C	T	Q	A	T	S	Q	E	C	M	F	W	I	E	Q	G	A	D	I	M	T	T	R	E	191		
Lv MYP	122	IRWCVSSPCQMKKQ	Q	R	M	V	S	E	F	T	Y	S	P	N	M	V	P	R	K	I	W	S	C	V	K	A	E	S	Q	E	C	M	F	W	I	E	R	G	A	D	I	M	T	T	R	G	181		
Bd trans.	29	YKVCVPEGALES-	C	H	R	M	S	Q	E	S	D	-----	L	H	M	T	C	V	A	A	R	D	R	I	E	C	L	D	K	I	K	H	R	E	A	D	F	A	P	V	D	P	79						
Smart con.	1	VRWCVVSNAEKSKC	D	R	W	S	V	N	S	R	G	-----	G	P	A	L	E	C	V	S	A	S	S	T	E	E	G	I	K	A	I	Q	K	G	E	A	D	A	V	T	L	D	G	55					
Sp MYP	192	QGVYSANTTFNLKPI	A	Y	E	T	-----	I	N	D	Q	-	Q	P	E	I	Q	I	L	K	H	Y	Q	N	V	T	F	A	L	K	S	S	R	L	V	N	P	N	T	F	S	E	L	246					
Lv MYP	182	EEVYVAN-TTFKLEPI	A	Y	E	K	T	I	K	P	Q	E	Q	E	-----	V	L	P	L	K	H	Y	Q	N	V	T	F	A	L	K	M	S	H	Q	I	A	P	M	T	W	S	E	I	236					
Bd trans.	80	EDMYVAAKIPQQDFI	I	F	K	E	I	-----	R	T	K	E	E	-----	P	D	E	E	F	R	Y	E	A	V	C	V	I	H	K	D	L	-----	D	I	T	S	I	H	G	L	129								
Smart con.	56	GDVYTAG-KPYNLVP	V	F	A	E	N	-----	Y	G	S	E	-----	E	D	P	T	C	Y	A	V	A	V	V	K	K	S	-----	A	I	F	T	W	N	Q	L	103												
Sp MYP	247	RDKTTCHAGIDMPDF	M	P	A	S	F	A	D	P	V	C	N	L	I	K	E	G	V	I	P	V	T	G	N	-----	Y	I	E	S	F	S	D	F	V	Q	E	S	V	P	G	V	L	303					
Lv MYP	237	RDKTTCHAGADFP	A	S	F	K	A	P	V	C	R	L	I	A	E	E	V	I	N	K	T	G	-	D	Y	A	E	S	-----	V	A	D	F	V	E	E	S	V	P	G	I	L	288						
Bd trans.	130	QGLKSCHTGVGR	N	V	G	Y	K	I	P	I	T	K	L	R	H	M	G	V	L	G	P	L	N	S	D	L	T	P	R	E	N	L	H	A	L	S	H	L	F	S	E	A	C	L	V	G	W	189	
Smart con.	104	KGKKSCHTGVGR	T	A	G	W	N	I	P	M	G	L	L	N	R	L	V	I	Q	T	P	N	C	D	F	D	T	A	-----	V	S	F	F	F	A	S	C	A	P	G	A	D	156						
Sp MYP	304	NMTYNKNG---TYPL	S	L	V	T	L	C	E	D	Q	Y	K	-----	Y	S	G	I	K	G	A	L	S	C	L	E	S	G	K	G	Q	V	T	F	V	D	Q	K	353										
Lv MYP	289	NKTYNKNMT---Y	P	M	N	L	V	S	L	C	E	K	D	Q	L	K	-----	Y	S	G	V	E	G	S	L	K	L	R	S	G	K	Q	V	T	F	V	D	H	K	I	337								
Bd trans.	190	APDPAQNQALKAK	Y	P	N	L	C	A	L	G	E	H	P	-----	E	I	C	D	Y	P	D	K	Y	S	G	Y	D	G	A	L	R	C	L	A	E	H	G	Q	V	A	W	T	K	V	Y	246			
Smart con.	157	KPDSNP-----	K	L	C	A	L	C	A	G	D	G	N	K	C	A	S	S	H	E	P	Y	Y	G	Y	T	G	A	F	R	C	L	A	E	G	A	G	D	V	A	F	V	K	H	S	207			
Sp MYP	354	IKKIMSDPNV-----	R	D	N	F	Q	V	V	-	C	R	D	E	S	R	P	L	D	E	E	I	F	T	D	V	I	C	H	V	G	H	T	A	R	P	T	I	F	I	N	400							
Lv MYP	338	VKKLMMDENERTT	T	W	L	S	A	R	-	A	R	A	S	-----	H	-	L	T	R	P	S	L	R	D	-	T	C	H	V	G	H	V	A	N	P	T	V	F	I	A	385								
Bd trans.	247	VKKHFGMAI--G	A	E	A	V	T	G	N	P	D	D	Y	A	L	C	P	D	A	-----	T	K	P	I	T	G	-	K	P	I	W	A	R	P	Q	Y	-	M	A	299									
Smart con.	208	VFENTDGKN--G	A	A	W	A	K	D	L	-	D	R	D	D	Y	E	L	L	C	P	D	N	-	G	T	R	K	P	V	T	E	Y	K	N	C	H	L	A	R	V	P	S	H	A	V	-	V	A	260
Sp MYP	401	KNNTQQ-KETDIK	T	L	V	V	K	M	M	E	L	Y	G	N	T	D	R	D	V	N	F	N	I	F	D	S	S	D	S	-----	G	K	C	Q	M	T	G	K	P	L	N	K	N	L	I	456			
Lv MYP	386	RNNTQV-KVEIK	D	L	L	R	K	I	L	S	-	L	Y	S	T	T	N	P	-----	E	I	E	L	N	I	F	D	S	S	V	Y	E	C	E	P	C	S	W	T	G	K	R	T	N	K	D	L	I	441
Bd trans.	300	N-----H	L	D	N	D	I	A	D	L	R	A	K	-----	I	S	L	A	D	T	I	G	E	T	E	-----	N	A	D	W	328																		
Smart con.	261	RS---DK-K	E	D	V	I	W	E	L	L	N	Q	Q	-	K	F	G	K	N	G	S	-	P	S	L	F	Q	L	F	G	S	P	T	G	-----	K	D	L	L	300									
Sp MYP	457	FLEESNTMKIV	D	S	K	V	Y	A	G	E	V	Y	A	A	N	V	C	S	K	L	V	P	-----	489																									
Lv MYP	442	FLEESTLKI	I	D	P	S	K	V	Y	A	E	K	F	F	Q	S	F	N	T	C	V	Q	-----	471																									
Bd trans.	329	LSKVL	D	L	N	N	K	T	I	P	I	D	N	Q	G	P	S	P	E	N	Y	L	N	K	A	N	Y	T	D	V	I	E	R	D	T	G	A	P	H	R	373								
Smart con.	301	FKDST	K	L	A	K	I	P	S	K	T	D	Y	E	L	Y	L	G	E	E	V	T	A	I	Q	N	L	R	K	S	R	E	T	S	-----	340													

**FIG. 3** The transferrin-like domains of MYP share sequence similarity with the iron binding domains of transferrin family protein members. The complete N-terminal transferrin domains of *S. purpuratus* and *L. variegatus* are compared to the transferrin consensus from the NCBI conserved domain database RPS-BLAST (Altschul *et al.*, 1997). The consensus is derived from Smart (simple modular architecture research tool) and represents 30 aligned transferrin domain sequences from vertebrates (26) including sero-, lacto-, melano-, and ovotransferrin, and four from arthropods. The sequence for *B. discoidalis* (cockroach), a representative insect transferrin is also included in the alignment. Asterisks and shading indicate the conserved cysteine residues while the putative vertebrate iron coordinating residues represented in the consensus (DYRYH) are shaded with their position marked by an arrowhead. Gaps were minimized since the entire alignment of 31 sequences has been compressed.

distinct iron binding with bands that increase in intensity in accordance with an increase in protein concentration. This observation suggests that coelomic fluid from both sexes of sea urchins contains proteins capable of binding iron. Because the predominant protein in this fluid is MYP (Fig. 4), and we report here that MYP sequence has iron binding domains, we hypothesized that MYP is responsible for the iron binding.

To determine specifically if MYP binds iron we used an immunoprecipitation approach. First, we determined by SDS-PAGE and Western blot analysis that the MYP pAb specifically immunoprecipitates MYP from coelomic fluid preparations when conjugated to protein A-Sepharose beads (data not shown). Coelomic fluid was

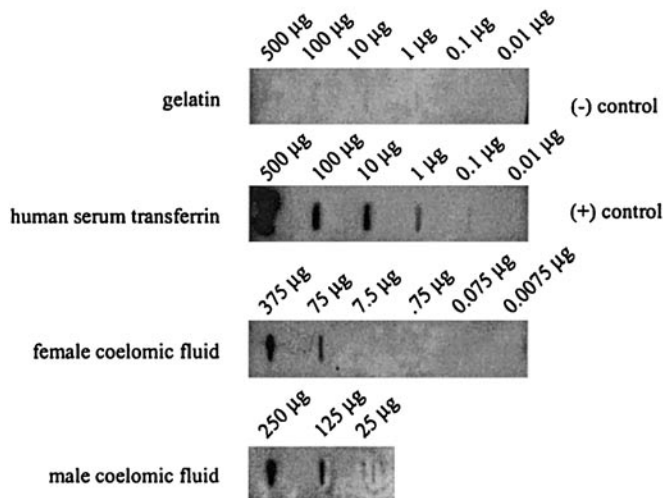
then stripped of iron by dialysis against iron stripping buffer, and this solution was transferred to the iron binding buffer, and fresh  $^{59}\text{Fe}$ -NTA was added. Antibodies were then added followed by protein-A Sepharose beads, and after extensive washes, iron binding was determined using a scintillation counter. As summarized in Fig. 6, radiolabeled iron was recovered almost exclusively by the MYP antibody. When MYP preimmune sera or a pAb was used to SFE 11, an oocyte-specific yolk protein absent from coelomic fluid and lacking iron binding domains (Wessel *et al.*, 2000), iron binding was negligible. These results were reproducible in three trials. We thus conclude that MYP binds iron and suggest that its function is to transport iron ions in coelomic fluid.



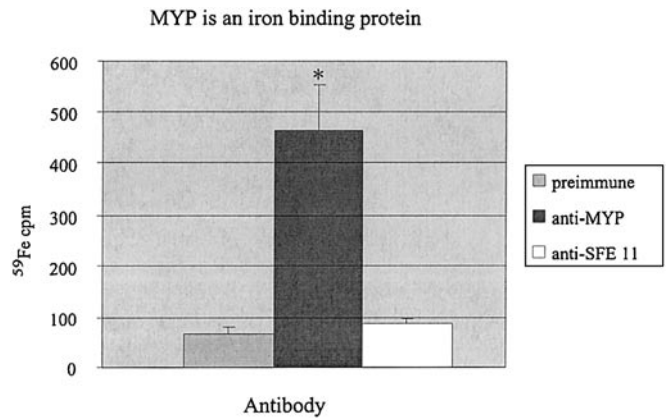
**FIG. 4** Analysis of protein preparations from *S. purpuratus* using the MYP pAb show that coelomic fluid is highly enriched with a 200 kDa species of MYP while yolk contains a 180 kDa species and the characteristic lower molecular weight cleavage products. (A) Coomassie stain of coelomic fluid and yolk preparations (10  $\mu$ g/lane) (B) immunoblot (1  $\mu$ g/lane) expanded above the 61.3 kDa marker to show the distinct MYP immunoreactive bands in this region.

## DISCUSSION

MYP is present in the adult sea urchin gut, coelomic fluid, testes, ovary, and yolk platelets of eggs and although



**FIG. 5** Coelomic fluid has iron binding capacity. These slot blots show that  $^{59}\text{Fe}$  binds to human serum transferrin and both female and male adult sea urchin coelomic fluid in a concentration dependent manner. It does not bind to gelatin (top) or other nonspecific proteins such as BSA and blotto (data not shown).



**FIG. 6.** Immunoprecipitations following  $^{59}\text{Fe}$  iron binding of coelomic fluid show that MYP binds iron. This binding is specific as shown by immunoprecipitations with both the MYP preimmune sera and an antibody to a SFE 11, a yolk protein absent from coelomic fluid and lacking iron binding domains (Wessel *et al.*, 2000). Each immunoprecipitation was conducted in triplicate and results were averaged. The MYP value indicated by the asterisk is significantly different ( $P < 0.001$ ) than MYP preimmune and SFE 11 by a two-sample t-test.

it is an abundant protein that has been extensively studied, its precise function is unknown. It has been suggested that MYP functions as a vitellogenin based on similarities of its physiological profile (Table 2). These aspects include its site of synthesis, secretion into circulation, delivery to the ovary, endocytosis by oocytes, and storage in yolk platelets. However, this characterization does not completely fit the attributes of other reported vitellogenins: MYP is not sex-specific, nor does it appear to provide a store of amino acids as a food supply for developing embryos since the mass of MYP is constant during embryogenesis. Here we find that

**TABLE 2**

Comparison of Properties of Vitellogenin-like and Transferrin-like Proteins

	Transferrin and Vitellogenin Similarities and Differences	
	Transferrin-like	Vitellogenin-like
Expression is sex-specific	no	yes, ♀ only
Expression can be regulated by hormones	yes	yes
Major site of synthesis:		
liver, fat body, gut	yes	yes
Secreted into circulation	yes	yes
Delivery to germ cells	♀ & ♂	♀ only
Function	transport & sequester Fe	nutrition

rather than being vitellogenin-like, MYP is a member of the transferrin superfamily of iron binding proteins, a classification that better fits its physiological attributes (Table 2). While we were initially surprised to find no similarity between MYP and vitellogenin sequences, in retrospect, this is not in conflict with any existing data. The assignment of MYP as a vitellogenin was previously based on its abundance in yolk platelets, coelomic fluid, and intestines, and not on any sequence data. In fact, the previous open reading frame sequence available (Shyu *et al.*, 1986, 1987) came from the 3' end of the open reading frame, and a small portion of the 5' end (Fig. 2). Combined, these reported amino acids represent only 13% of the MYP ORF and lie in regions that contain no sequence similarity to vitellogenin or transferrin.

Transferrins are glycoproteins characterized by their ability to bind iron reversibly. Vertebrate transferrins are monomeric glycoproteins (~80 Kd) that consist of two domains with similar amino acid sequence, each with a single iron-binding site. Crystallographic studies have shown that the iron binding sites are stabilized by many intrachain disulfides that coordinate iron binding (reviewed in Baker and Lindley, 1992). Transferrins from insects are similar in size, sequence, and function to vertebrate transferrins (Bartfeld and Law, 1990; Jamroz *et al.*, 1993; Kurama *et al.*, 1995; Yoshiga *et al.*, 1999) although most insect transferrins bind only one ferric iron because their C-terminal transferrin domains have lost iron-binding capacity due to amino acid substitutions at key residues (reviewed in Baker and Lindley, 1992). Interestingly, MYP may also only bind one iron molecule as its second transferrin domain is split by a 282 residue insertion predicted in both species of sea urchin (Fig. 1).

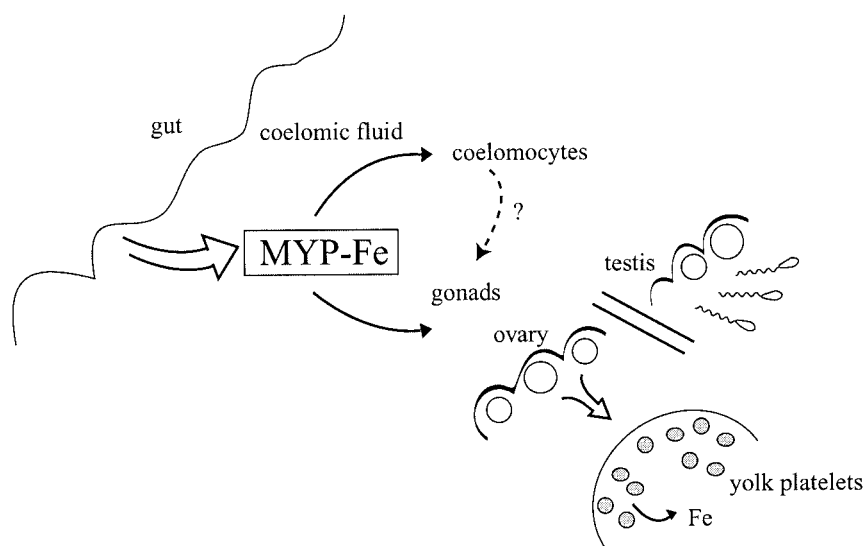
Disrupted iron binding domains are also seen in saxiphilin, a unique relative of the transferrin family from *Rana catesbeiana* (American bullfrog). This transferrin-like protein does not bind iron due to a 144 residue insertion that disrupts the first iron binding domain and numerous amino acid substitutions in the second. Curiously though, it binds saxitoxin, a potent paralytic neurotoxin produced by various dinoflagellates and cyanobacteria (Morabito and Moczydlowski, 1994). The sequence interrupting the second transferrin domain in MYP (Fig. 1) has no similarity to the insert in saxiphilin, or to sequences reported in the NCBI database.

Vertebrate transferrins have been crystallized (reviewed in Baker and Lindley, 1992; Hirose, 2000) and structural information shows that the diferric geometry relies on disulfide bridges that juxtapose four iron binding ligands, 1 Asp, 2 Tyr, and 1 His and an Arg (DYRYH) to coordinate a synergistic anion (usually bicarbonate) in each lobe of the protein (Fig. 3). In insects, transferrins have been shown to bind iron with high affinity even though there is a striking sequence variation including the His residue replacement, usually by Glu (DYRYQ; Fig. 3). Unfortunately, none of the insect transferrins have been crystallized to know how iron-binding pockets in transferrins have diverged in phy-

logeny. However, Baker *et al.* (2001) present a human H249Q mutant transferrin N-lobe as a model for iron binding in insect transferrins and find that the Gln ligand is coordinated to the iron atom. The sea urchin MYP has a high cysteinyl identity (Fig. 3) to the transferrin consensus. The residues involved in iron binding can be tentatively assigned based on the crystal structures of vertebrate transferrins (reviewed in Hirose, 2000). It should be noted that the SMART consensus is predominately representative of the vertebrate transferrins (26) with a small representation of the insect transferrins (4); therefore we included an insect transferrin to show variability and to compare to our MYP. For iron binding we postulate that MYP, like insect transferrins, shares four of the conserved vertebrate residues. Two of these residues share positional identity to the consensus (shaded residues marked by arrowheads) while two are in very close proximity (shaded residues). Similar positional differences have been suggested for insect transferrins (Bartfeld and Law, 1990). Only the Arg residue that serves as the bicarbonate synergistic anion-binding site is missing. This variation is interesting since in alternative iron-loaded crystal structures (Mizutani *et al.*, 1999; reviewed in Hirose, 2000), iron is stabilized at a nearby Thr rather than the Arg and in both sequences of MYP a Thr is nearby and may serve a similar function. Thus, MYP fits within the transferrin family, both in overall sequence, as well as postulated iron binding residues. Definitive specification of residues involved in iron binding for both sea urchin and a representative insect awaits crystallographic investigation, which would lead to a better understanding of the iron binding capabilities of these domains in general.

Iron is essential for a multitude of protein functions and physiological processes, yet careful control of iron transport and storage is critical for cell viability as free iron can generate oxidative radicals that damage lipids, proteins, and DNA (reviewed in Boltdt, 1999; reviewed in Nappi and Vass, 2000). In vertebrates, transferrins are the major vehicle of iron homeostasis. Mammals synthesize transferrin principally in the liver but important extrahepatic sites of expression have been discovered in both mouse and humans including the granulosa cells of the ovarian follicle (Briggs *et al.*, 1999) and the Sertoli cells of the testis (Skinner and Griswold, 1980; reviewed in Sylvester and Griswold, 1994). The localized synthesis of transferrin in granulosa cells has been implicated in follicle maturation (Briggs *et al.*, 1999). Furthermore, male mice lacking normal transferrin have abnormal spermatogenesis and a decreased number of germ cells (reviewed in Sylvester and Griswold, 1994). These observations recapitulate the pattern of expression of MYP in sea urchins since the majority of the protein is made in the gut but lower levels are also detected in both the ovary and testis. Thus, MYP may be the major regulator of iron homeostasis in the adult sea urchin.

In some insects, it is clear that iron ions are transported into oocytes during oogenesis by transferrin (Kuruma *et al.*, 1995; Hirai *et al.*, 2000). Kuruma *et al.* (1995) reported that transferrin purified from *Sarcophaga peregrina* (flesh fly)



**FIG. 7** Proposed model of MYP function. We suggest that MYP is an iron transporter in the coelomic fluid. Delivery of iron to the ovary and testis may support gametogenesis and the packaging of MYP into yolk granules may serve as a mechanism of iron delivery during embryogenesis. MYP packaging in coelomocytes could have a dual function of providing a bacteriostatic function in coelomic fluid as well as MYP-Fe delivery to the gonads.

hemolymph binds one iron and transports it to oocytes during the vitellogenic stage of oogenesis. This is similar to the pattern of MYP delivery in sea urchin oocytes. Deposition of iron into these cells could be important for incorporation into metalloproteins including the iron storage protein ferritin. In the eggs of sea urchins, iron metalloenzymes constitute an important group of iron containing molecules that provide energy, control and trigger egg activation, block polyspermy, and support early development. These iron containing proteins include the cytochromes of the many mitochondria, ovoperoxidase (a heme-dependent peroxidase that constitutes 0.5% of the egg protein and is responsible for the hardening of the fertilization envelope), plasma membrane bound NADPH oxidase, and nitric oxide synthase (reviewed in Wessel *et al.*, 2001).

A bacteriostatic function for MYP was also suggested by Cervello *et al.* 1994. They report that a subpopulation of coelomocytes circulating in the coelomic fluid release MYP under conditions of stress. In vertebrates it is well known that transferrin deprives pathogenic invaders of iron thereby preventing their growth (Kontoghiorghes and Weinberg, 1995). Transferrin has also been shown to be upregulated in cultured insect cells and adults animals (Yoshiga *et al.*, 1997, 1999) when challenged with bacteria. Together these results suggest that transferrin-like iron binding proteins may also be a general part of the insect and echinoderm immune response by sequestering iron from invading pathogens.

We present a model of MYP function in Fig. 7. MYP is present in the gut of adult sea urchins and is an abundant protein in the coelomic fluid where it functions to transport

iron ions to various tissues in adults including the nutritive phagocytes of the ovary and testis. During gametogenesis MYP is mobilized from these cells and in females it is selectively incorporated into the yolk platelets of developing oocytes where it delivers iron to cytoplasmic metalloproteins likely through a ferritin intermediate. MYP is processed into distinct smaller fragments in yolk platelets concomitant with their acidification that occurs during embryogenesis (Mallya *et al.*, 1992). This processing could be a timed mechanism for iron release as it has been shown that many transferrins release iron at low pH.

Placing our analysis into the context of previous studies, we have concluded that sea urchin MYP is a transferrin-like protein based on its amino acid sequence and its physiological profile. MYP binds iron and this role as a carrier protein is not unique since yolk proteins in general are important in regulating embryogenesis by sequestering diverse molecules for subsequent timed release. For example, in addition to serving as a reserve of amino acids, vitellogenins can be covalently modified with carbohydrates, phosphates, and sulfates and noncovalently bound lipids, hormones, vitamins, and metals (Lagueux *et al.*, 1981; Kunkel and Nordin, 1985; Byrne *et al.*, 1989; Dhadialla and Raikhel, 1990; Niimi *et al.*, 1993; Montorzi *et al.*, 1994; Mac Lachlan *et al.*, 1994). The yolk proteins of *Drosophila melanogaster* have been shown to bind ecdysteroids and it is proposed that their breakdown leads to timed release of this hormone (reviewed in Bownes *et al.*, 1988). It is of note here that the major yolk proteins of *Drosophila melanogaster* are also distinct from vitellogenin.

Currently for the sea urchin MYP we can only assign an

iron binding function, and formally, since native protein was used in these iron binding assays, we can not exclude the possibility that the iron is binding even in the nontransferrin domain. We can also not exclude the possibility that MYP serves as a carrier for other molecules. So, does the sea urchin contain a classical vitellogenin protein for nutrition during embryogenesis or is it using a yet to be identified alternate reserve?

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