New class of blue animal pigments based on Frizzled and Kringle protein domains

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Running title: Frizzled/Kringle-related pigment
ABSTRACT

The nature of coloration in many marine animals remains poorly investigated. Here we studied blue pigment of a scyfid jellyfish *Rhizostoma pulmo* and determined it to be soluble extracellular 30 kDa chromoprotein with a complex absorption spectrum peaking at 420, 588 and 624 nm. Further we cloned corresponding cDNA and confirmed its identity by immunoblotting and mass spectrometry experiments. The chromoprotein, named rpulFKz1 consists of two domains, a Frizzled cystein-rich domain and a Kringle domain, inserted into one another. Generally, Frizzleds are members of a basic Wnt signal transduction pathway investigated intensely with regard to development and cancerogenesis. Kringles are autonomous structural domains, found throughout the blood clotting and fibrinolytic proteins. Neither Frizzled and Kringle domains association with any type of coloration nor Kringle intrusion into Frizzled sequence was ever observed. Thus, rpulFKz1 represents a new class of animal pigments, which chromogenic group remains undetermined. The striking homology between a chromoprotein and members of signal transduction pathway provides a novel node in the evolution track of growth factor-mediated morphogenesis compounds.

Keywords: coloration, chromoprotein, Rhizostomeae, Frizzled, Kringle, domain insertion
INTRODUCTION

Pigments in nature play important roles ranging from camouflage coloration and sunscreen to visual reception and participation in biochemical pathways (1). Considering the spectral diversity of pigment-based coloration in animals one can conclude that blue pigments occur relatively rare (as a rule blue coloration results from light diffraction or scattering, rather than the presence of a blue pigment). At least partially this fact is explained by an inevitably more complex structure of blue pigments comparing to yellow-reds. In order to appear blue a compound must contain an extended and usually highly polarized system of the conjugated $\pi$-electrons.

In many organisms blue coloration is achieved by combining a chromophore with a specific apoprotein, which organizes chromophore and provides red-shift upon binding. For example, carotenoprotein crustacyanin, a well-studied blue pigment from the lobster shell, contains orange carotenoid astaxanthin, which demonstrates strong bathochromic shift due to non-covalent binding to a protein of lipocalin family (2).

Several known types of protein-based animal pigments can be distinguished: carotenoproteins (including rhodopsins, where carotenoids feature an unusual covalent bond to apoprotein), hem-, flavin- or metal-containing proteins, pterins, melanins, ommochromes (1) and members of green fluorescent protein (GFP) family (3). Each type is characterized by specific chromogenic groups and protein sequences.

In the present work we studied blue pigment of the scyfoid jellyfish Rhisostoma pulmo. We anticipated to find either a carotenoprotein (being the most widely spread pigment class [1]) or GFP-like chromoprotein (as many fluorescent and colored GFP-like proteins were found earlier in Cnidaria [3]). However, this work revealed a new class of pigments based on a unique
combination of well-known Frizzled and Kringle protein domains that are tightly bound to a chromogenic group of an unknown structure.

EXPERIMENTAL PROCEDURES

Purification of blue pigment from Rhizostoma pulmo—Specimens of jellyfish Rhizostoma pulmo possessing intense blue coloration on bell margins were collected at the Russian shores of the Black Sea. Colored body pieces were excised and placed for extraction into sea water at 4°C for 4-7 days (the blue pigment is stable and retains coloration when stored as described for at least several months) so that significant amounts of the pigment was extracted into solution. The resulting solution was twice chloroform extracted. Aquatic fraction was subjected to ammonium sulfate extraction. First, ammonium sulfate was added to a final concentration of 60% saturation. Precipitated protein was removed by centrifugation while the blue pigment remained in solution. Addition of ammonium sulfate up to 100% saturation resulted in precipitation of the blue substance as floating flakes. These flakes were collected by centrifugation and dissolved with phosphate buffered saline (PBS) pH 7.5.

Standard protein denaturing discontinuous polyacrylamide gel-electrophoresis (SDS-PAGE) in 12% gel was used to analyze heated and unheated pigment samples.

Gel-filtration was performed using AKTAprome chromatograph (Amersham Pharmacia Biotech). Samples were loaded onto Sephadex S-200 and Sephadex S-400 columns (0.7 × 60 cm) and eluted with of 50mM Na - phosphate buffer (pH 7.0) and 100 mM NaCl. The columns were calibrated using MW-GF-200 standards from Sigma including Blue Dextran (molecular weight 2×10^6).
Absorption spectra were generated using Beckman DU520 spectrophotometer. Fluorescence analysis was performed by Varian Carry Eclipse fluorescence spectrophotometer.

RpullFKz1 gene cloning—Total RNA from small pieces of Rhizostoma pulmo bell margins was isolated using a NucleoSpin RNA II kit (Clontech). cDNA was synthesized and amplified with a SMART PCR cDNA Synthesis kit (Clontech).

The ammonium sulfate purified protein sample was applied to a 12% SDS-PAGE without boiling and a colored band of approximately 30 kDa was transferred onto Immobilon-P membrane and sequenced on an automatic protein sequencer. Twenty N-terminal amino acid residues were identified, reading SAVPAKMVQLPLEKLPLNIE. The following degenerated primers were designed corresponding to the amino acid sequences SAVPAK and SAVPAKMD: 5’-ATGTCTGCTGTTCCNGCNAA and 5’-TCTGCTGTTCCNGCNAA(A/G)ATGGT. Step-out PCR strategy (4) was used to amplify and clone 3’-fragment and then 5’-fragment of corresponding cDNA encoding the blue protein.

Antibodies generation—A part of rpulFKz1 Kringle domain, reading GECKMTTEGGDyrGVSQTFDGVKCQAWDTQEPHRHSVTAKTPND, was cloned into pQE31 (Qiagen) expression vector using BamHI and KpnI cloning sites. Soluble protein was purified using TALON affinity resin (Clontech) and injected into 2 rabbits 3 times in the course of 3 months 1 mg/injection. Anti-serum was used in Western-blotting procedures.

Western blotting—Western blots were performed by electrophoretic transfer of proteins resolved by SDS-PAGE to Hybond-C membrane. Upon electrophoretic transfer, membrane was blocked by 5% Bovine Serum Albumin in PBS buffer, incubated with polyclonal anti-Kringle antibodies at a 1:1000 dilution for 1 hr, washed in PBS 3 times 5 min each, incubated with HRP-
conjugated goat F(ab’)2 anti-rabbit IgG, washed in PBS 3 times 5 min each, followed by exposure to HRP (TMB kit, Vector).

Mass spectrometry—RpulFKz1 was purified with chloroform extraction and ammonium sulfate precipitation as for N-terminal sequencing, but instead of separating monomeric subunit by gel electrophoresis, we used gel-filtration to purify 1*10^8 Da fraction (multimeric form of the protein) on a Sephacryl S-400 column. The purity of the protein was confirmed by gel electrophoreses, which yielded a single band corresponding to a 28 kDa monomeric subunit of the RpulFKz1. The sample was handed to “Center of proteomic researches” (Moscow, Russia) for Matrix-Assisted Laser Desorption Ionization (MALDI) mass spectrometry of the trypsin digested protein.

RESULTS

In the summer of 2001 we collected several jellyfish specimens near the Russian shores of the Black Sea, readily identified as Rhisostoma pulmo (Cnidaria, Scyphozoa) (Fig. 1A). The specimens featured dark blue bell fringe. Primary characterization of the blue pigment was carried out on crude extract. Extraction was performed by placing medusae pieces into filtered sea water and storing for several days at 4°C. As a result, significant amounts of the pigment transferred from tissue into aqueous phase producing intensely colored solution.

To investigate the chemical nature of the pigment we performed several simple tests. First, phenol extraction of the colored samples was accomplished. This treatment resulted in a complete loss of the blue coloration. Since phenol denatures proteins we concluded that the blue pigment is protein-based. Then, we found that treatment with chloroform did not alter the color of the aqueous phase. It is well known that chloroform is not such a strong denaturing agent as
phenol and some proteins with firm tertiary structure remain stable in the presence of chloroform. In order to examine *Rhizostoma* blue pigment for carotenoproteins presence we used a common test, cold acetone extraction. Acetone did not extract any colored agents from the jellyfish tissues. We concluded that the blue pigment from *Rhizostoma* did not belong to the carotenoprotein family.

To purify the blue protein we used precipitation with ammonium sulfate. It was determined that in saturated (NH$_4$)$_2$SO$_4$ the pigment precipitated into floating blue flakes. Lower concentrations of ammonium sulfate did not significantly affect the pigment solubility allowing to remove most of the ballast protein.

When unboiled purified protein from the bell fringe was subject to standard gel-electrophoresis (SDS-PAGE), the colored bands were clearly visible after the run (not shown). The color of the pigment changed from blue to red. We attributed the color change of the pigment to SDS-dependent protein denaturing (see below), which could have disturbed the chromophore environment. The pigment migrated with approximate molecular weights of 30 kDa. The migration speed of the pigment changed upon the addition of β-mercaptoethanol to the loading buffer, resulting in a higher apparent molecular weight (approximately 32 kDa). This indicated that the colored protein possessed multiple sulfide bonds. Boiling did not affect the apparent molecular weight of the pigment. Surprisingly, gel-filtration experiments demonstrated the colored protein to be multimeric in the absence of SDS. When applied to chromatographic columns containing Sephacryl S-200 (fractionation range 5×10$^3$ - 2.5×10$^5$ Da), the blue protein mostly eluted in the void volume. However, Sephacryl S-400 gel-filtration, which allows separation of globular proteins of up to 8×10$^6$ Da, resulted in the elution of an individual chromatographic peak, tailing into low molecular weight zone. Summing up, we must consider a
multimeric protein-based pigment with the upper weight limit close to \(1 \times 10^7\) Da consisting of apoprotein monomers of less than 30 kDa.

Absorption spectrum of purified pigment was obtained peaking at 588 nm with a shoulder at 555 nm and smaller peaks at 420 and 624 nm (Fig. 2). Upon the addition of SDS the spectrum of rpulFKz1 became a single absorption peak with a maximum at 507 nm. Sample heating resulted in a strong blue shift of the absorption maximum up to 395 nm. No fluorescence in the visible part of the spectrum was detected from any spectral form.

As the colored band on SDS-PAGE of the ammonium sulfate purified sample was well separated from the neighboring protein bands, we decided to obtain the N-terminal sequence for the pigment. We have directly sequenced the first 20 amino acids and designed corresponding degenerated primers. Together with oligo-dT-containing primer they were used for the amplification of the specific cDNA 3' fragment. After cloning and sequencing of this fragment, we used 5'-RACE procedure (4) to obtain corresponding 5' flank of this cDNA. As a result, the sequence of the whole coding region for \textit{Rhizostoma} blue protein was obtained (GeneBank accession number AY507144). Standard BLAST analysis revealed two clearly detectable domains within the new protein: so called Frizzled cysteine rich domain and Kringle domain (Fig.1B). Notably, in \textit{Rhizostoma pulmo} pigment the Kringle domain is inserted into the primary structure of the Frizzled sequence. We named the pigment rpulFKz1 to emphasize the origin and domain architecture of the protein (from \textit{Rhizostoma pulmo Frizzled-Kringle-Frizzled}).

In order to confirm the identity of the obtained sequence and the jellyfish pigment we performed additional experiments. First, immunochemical approach was applied. We generated polyclonal antibodies against recombinant polypeptide corresponding to a part of rpulFKz1 Kringle domain. Protein samples from different \textit{Rhizostoma} body parts were probed with these antibodies. We confirmed that anti-Kringle antibodies stain the bands that appear colored on
acrylamide gels (not shown). Second, we utilized mass-spectroscopy on gel-filtration purified fragmented jellyfish pigment comparing theoretical and experimental data. One must emphasize, that unlike the initial protein sequencing experiments, when SDS-PAGE was used to obtain monomers of the apoprotein, this independent sample consisted of polymeric fraction with a mean molecular weight of $10^6$ Da. The sample was subjected to trypsin digestion followed by MALDI-TOF analysis. Alignment of experimental mass fingerprints against theoretical digests of RpuFKz1 covered 79% of the protein sequence, proving protein identity (Fig. 1B). The under-representation of 21% of the actual protein sequence by experimental peptide masses has the following reasons: (i) Fz domains are known to be glycosylated, and a potential N-glycosylation site is predicted in the sequence of RpuFKz1 in a peptide missing from the spectrogram (Fig. 1B); (ii) 16% of the protein length is represented by short fragments (molecular weight under 650 Da, 1-6 amino acid residues) and were not aimed to be detected (though 11 residues were detected as parts of longer peptides); (iii) The presence of covalently bound chromophoric group might change the expected peptide mass.

**DISCUSSION**

Blue proteinaceous pigments are known in a variety of plant and animal species. Upon observing the basic characteristics of RpuFKz1 we started wondering if similar pigments had ever been described. The unusual shape of the absorption spectrum and the color change in the presence of SDS and after rpulFKz1 heating closely resemble the spectral properties of the pigment named Cassio Blue from the related jellyfish *Cassiopea xamachanta* (5). Considering similarity of spectral and biochemical properties one can conclude that rpulFKz1 and Cassio Blue represent the same class of pigments. No primary structure was assigned to the *Cassiopea* pigment. The authors only inferred that the pigment is based on Cys-rich multimeric
glycoprotein consisting of about 30 kDa monomers that carries covalently bound chromophore which does not belong to any known pigment compounds. Cassio Blue is thought to provide photoprotection for the symbiotic algae in the jelly. As Cassio Blue was visually observed in mesoglea only and not inside cells, it was unclear whether jelly or algae cells are the source of the pigment. RpulFKz1 is also distributed throughout mesoglea, but *Rhizostoma pulmo* hosts no algae. Thus, it is very likely, that Cassio Blue pigment is of the jellyfish origin.

Study of Cassio Blue failed to reveal the chemical nature of its chromophore. Analogously, in case of rpulFKz1 we only can conclude that the chromogenic group is attached tightly (most probably covalently) to the protein chain and the chromophore properties are dissimilar to those of common classes of pigments. Some hints about the site of chromophore attachment in rpulFKz1 can be derived from mass spectrometry of its tryptic digest. Three stretches of the protein were not detected in the spectrogram (Fig. 1B). Among other explanation of these results there exists a strong possibility of the chromophore to be linked with one of the three stretches identified. Further studies are required to identify chromophore structure and its localization in rpulFKz1 and related pigments.

Domain content and structure of rpulFKz1 are of special interest. RpulFKz1 features two well-known domains - Frizzled and Kringle. Moreover, in rpulFKz1 the Kringle domain is inserted into the primary structure of the Frizzled domain. More often, these domains constitute separate proteins with very different functions. Frizzled CRDs are conserved in diverse proteins and organisms and all known family members serve as binding domains for Wnt growth factors. Wnts are known to be involved in diverse developmental processes such as cell differentiation, cell polarity and cell proliferation (6). Along with Frizzled-containing membrane receptors for Wnt, there is a class of soluble secreted Frizzled-related proteins that act as Wnt antagonists (7). Kringles are autonomous structural domains, found throughout the blood clotting and fibrinolytic
proteins (8). Kringle 5, sharing the strongest homology to rpulFKz1, can regulate cell proliferation (9). To date, the only protein family found to contain both Frizzled and Kringle domains is ROR-like receptor tyrosine kinases playing crucial role in developmental processes of invertebrates and vertebrates (10). To our best knowledge, neither Frizzled and Kringle domains association with any type of coloration nor Kringle intrusion into Frizzled sequence were ever observed.

Both Kringle and Frizzled domains are cysteine rich and positions of Cys residues are highly conserved. RpulFKz1 contains 14 out of 16 characteristic cysteine residues typically found in the CRD of Frizzleds and Kringles. Two cysteines, which rpulFKz1 lacks, belong to the Frizzled domain and would normally form a S-S bond. RpulFKz1 Kringle domain possesses majority of conserved amino acid residues (Fig. 1B), known to form ligand-binding cavity in the Kringle domains (11, 12). Probably, RpulFKz1 ligand specificity is similar to that of other Kringle 5 domains. Comparison of the rpulFKz1 Frizzled domain with other Frizzleds shows that the molecular surface region important for Wnt binding (13) is reasonably conserved and is not disrupted by the Kringle domain insertion. Thus, we must not rule out a possibility of the rpulFKz1-Wnt interaction in the jelly that should result in local Wnt inhibition and cell fate alteration. Thus body patterning variations may become visible due to the unique rpulFKz1 properties. Further investigation of Wnt pathway components interaction in Cnidaria may shed light upon the origin of growth factor-mediated morphogenesis.

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FIGURE LEGENDS

Fig. 1. A, Scyloid jellyfish *Rhizostoma pulmo* (photo by Miquel Pontes, www.marenostrum.org). White arrowheads indicate blue bell margins containing blue chromoprotein rpulFKz1. B, Amino acid sequence comparison of rpulFKz1 to Frizzled cysteine rich domain from rat Frizzled 2 precursor (blue boxes) and Kringle 5 domain from human plasminogen (yellow boxes). Signal peptide absent in the mature rpulFKz1 is outlined. Experimentally detected by MALTI-TOF rpulFKz1 tryptic digest fragments are underlined. Cysteine residues are shadowed in red. Residues important for Frizzled-Wnt interaction are marked by blue squares. Residues forming ligand-binding cavity in Kringle 5 are designated by yellow squares. Arrowheads show predicted sites for N-glycosylation.

Fig. 2. Absorption spectra for *Rhizostoma pulmo* purified blue pigment (solid line); pigment in 10% SDS (dashed line) and heated pigment (dotted line).
Figure 1
Figure 2
New class of blue animal pigments based on Frizzled and Kringle protein domains
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