Characterization of a Recently-Discovered Mutant Fetal Hemoglobin

Arindam Sarkar and Dr. John S. Olson

Abstract

Last summer, Dr. Mitchell Weiss and his colleagues at Children's Hospital of Philadelphia discovered a new hemoglobinopathy in a baby from Toms River, New Jersey, who was born cyanotic and with enlarged spleen and liver tissues. Sequencing of the baby's hemoglobin alleles revealed a missense mutation in a segment of DNA that codes for the gamma chains of fetal hemoglobin (HbF), the oxygen-carrying protein in red blood cells of human fetuses. The objective of our work is to use recombinant DNA technology to construct the Hb Toms River mutation, γ Valine 67 (E11) Methionine, in plasmid DNA which can then be used to express and purify mutant protein using E. coli. We plan to characterize the mutant HbF in order to understand its clinical manifestations and, perhaps, to develop treatments options. This paper provides an overview of HbF developmental biology, our initial hypothesis of how the Hb Toms River mutation might lead to cyanosis, and our strategy for expressing and characterizing the γ Val67 to Met mutation in recombinant HbF.

Introduction

During a consultation with pediatricians at Children's Hospital of Philadelphia, Dr. Mitchell Weiss discovered a new blood disorder in a child who was born cyanotic and with an enlarged spleen and liver. These symptoms resolved roughly two months after her birth, and she was normal and healthy by six months. Based on their initial clinical observations, Dr. Weiss and the treating physicians suspected that a mutant fetal hemoglobin might be the cause of the baby's symptoms, so they drew small amounts blood samples for analysis from the baby several days after birth. They discovered that her condition appears to fall into a class of hematological disorders known as hemoglobinopathies, which are genetic defects in the DNA sequences that produce hemoglobin. Hemoglobin is the primary oxygen transport protein in humans, and when the baby's DNA was analyzed, it was discovered that a Val67 to Met (V67M) mutation was present in one of the child's γ chain alleles. This mutation occurs in a region of DNA that gives rise to the eleventh amino acid along the E helix of the γ globin chain, which is called Val (E11) for its spatial location in the three dimensional structure of hemoglobin subunits (Figure 1).

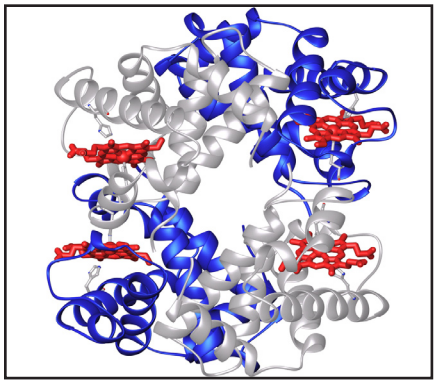

Figure 1. (PDB: 1fdh). The HbF tetramer illustrating the two alpha (α) subunits (silver) and the two gamma (γ) subunits (blue). The red molecules are heme. (Image courtesy of Dr. Jayashree Soman, Department of Biochemistry & Cell Biology, Rice University.)

The original mutant fetal protein could not be studied directly because of physical and clinical limitations which prevent the withdrawal of significant amounts blood from an anemic infant. Another problem was that fetal hemoglobin production switches to adult hemoglobin production shortly after birth as part of normal developmental processes. Thus, resolution of the cyanotic condition occurred when the γ gene (characteristic of HbF) was silenced, and only normal adult hemoglobin was present in the baby's red blood cells, which occurred 6 to 8 weeks after birth. To obtain enough starting materials for study, we chose to produce mutant HbF in our laboratory using recombinant technology. The objective of our work is to use structural biology to characterize the γ V67M mutation in HbF, examine the role of the E11 position in O2 binding in γ chains, and then understand why the mutation caused cyanosis and spleen enlargement.

Hemoglobin Development

Hemoglobin is a complex iron-containing protein in the blood that picks up oxygen from the lungs and carries it to respiring cells; at the same time, it assists in transporting carbon dioxide away from the peripheral tissues. Mammalian red cell hemoglobins are tetramers consisting of four polypeptide chains and four planar prosthetic groups known as heme molecules [2, 3, 9]. Each red blood cell contains about 280 million hemoglobin molecules [7].

Different kinds of hemoglobins are commonly identified by the specific combination of polypeptide chains or subunits within each tetramer. During development and birth, three main types of hemoglobin are expressed (Figure 2). The first type is known as embryonic hemoglobin, which consists of two α and two γ globin chains. The low oxygen conditions of the uterine wall demand a higher oxygen affinity than either HbF or adult hemoglobin confer, but embryonic hemoglobin functions well in this environment. After 10 to 12 weeks of development, the primary form of hemoglobin switches from embryonic hemoglobin to fetal hemoglobin HbF ($\alpha_2\gamma_2$). At this point, the fetus's red blood cells have access to the oxygen passing though the placenta and umbilical cord. Like embryonic hemoglobin, HbF has a higher oxygen affinity than

adult hemoglobin, thus allowing the fetus to extract oxygen from the mother's blood in the placenta.

When a baby is born and begins to breathe air, γ chain production ceases and β chains are produced which result in adult hemoglobin HbA ($\alpha_2\beta_2$) production. At birth, HbF comprises 50-95% of the child's hemoglobin. These levels decline to almost zero after six months as adult hemoglobin synthesis is completely activated. Hemoglobin genes exist on Chromosomes 11 and 16 (Figure 3).

Genetic abnormalities can suppress the switch to adult hemoglobin synthesis, resulting in a condition known as hereditary persistence of fetal hemoglobin [6]. In adults, HbF production can be rekindled pharmacologically, which is one of the main treatment options for sickle-cell disease [5]. The mechanisms by which erythroid cells switched from the synthesis of HbF to that of HbA during the neonatal period appeared normal for the patient with HbF Toms River. As a result, this genetic disorder did not persist as a threat to the child.

Analysis of the V67M Mutation

A necessary stage for understanding the Hb Toms River disorder will be acquiring large amounts of the cyanotic child's mutated fetal hemoglobin. In this case, our only choice was to construct the mutation in vitro with recombinant DNA techniques and then express the mutant γ chain with wild-type α chains in bacteria. The intestinal bacterium, Escherichia coli, is an excellent choice for expressing recombinant proteins because of its high tolerance for synthesizing large amounts of heterologous proteins and the ease of performing site-directed mutagenesis on plasmids that can be taken up by this bacterium. The plasmid system pHE2 was originally developed by Chien Ho's group at Carnegie Mellon University (Shen et al., 1993) to produce adult hemoglobin, and we obtained the pHE2 expression system for HbF from Professor Kazuhiko Adachi at Children's Hospital of Pennsylvania (Adachi, 2002). This vector contains one wild-type α gene, along with one wild-type γ^G gene from human HbF. We created the single-site V67M mutation using the Stratagene QuikChange Site-Directed Mutagenesis Kit. The plasmids for expression of hemoglobins were transformed into E. coli JM109 cells. E. coli cells were grown in 2x YT medium. Expression was induced by adding isopropyl-β-thiogalactopyranoside (IPTG) at 0.1 mM at 37oC and then supplemented with hemin (30 μg/ml). The harvested cell lysate was passed through a Zn2+ binding column, Fast Flow Q-Sepharose column, and finally a Fast Flow S-Sepharose column using an FPLC.

We are currently evaluating the purity and authenticity of our HbF mutant by performing gel electrophoresis and protein sequencing reactions from aliquots of the purified protein. Our long-term goal is to characterize HbF Toms River in terms of its relative stability and O2 affinity with the hope that recombinant technology can help us understand the clinical symptoms of the hemoglobinopathy and perhaps suggest a treatment.

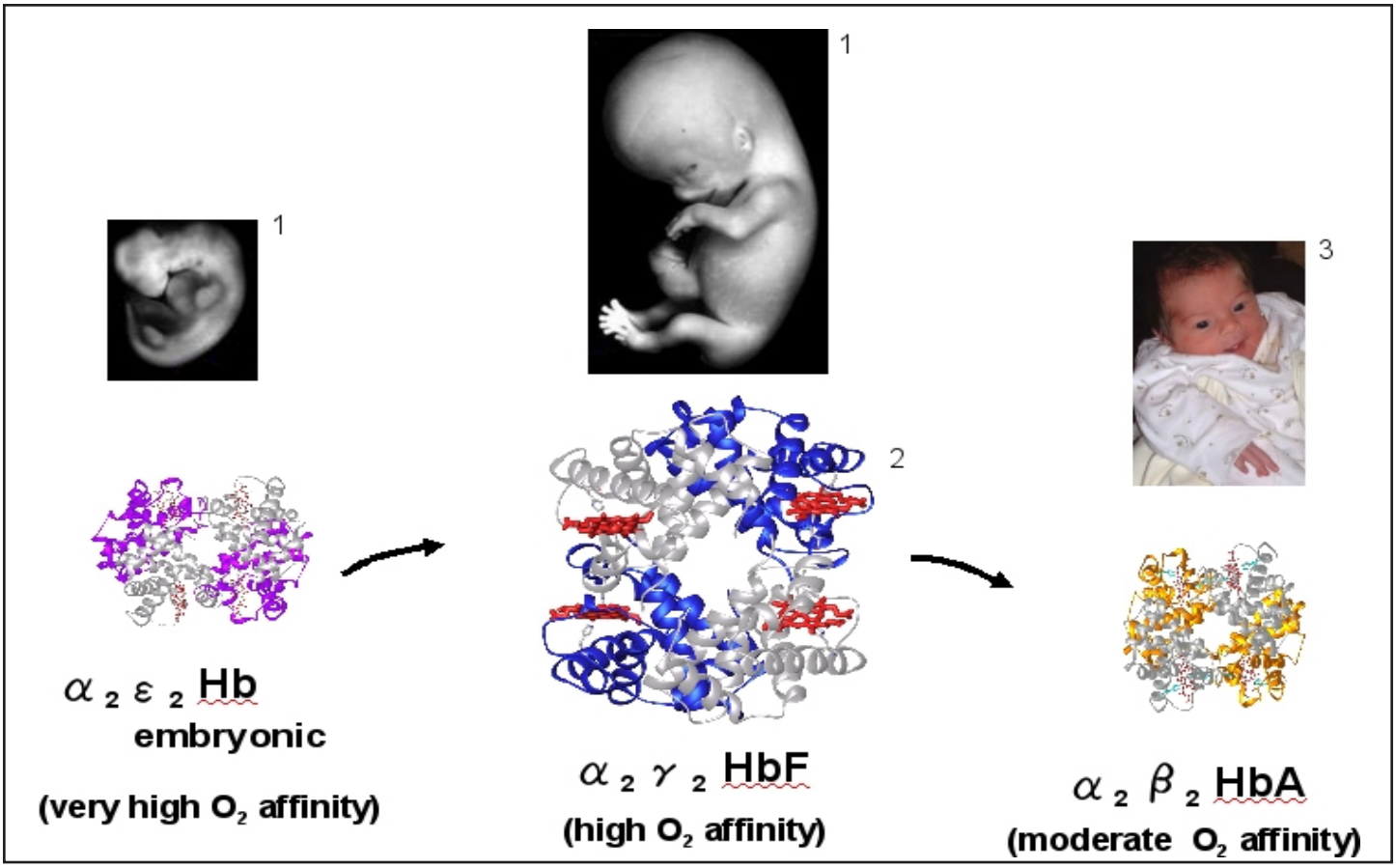

Figure 2. Developmental Biology of Human Hemoglobin. After 10-12 weeks hemoglobin switches from embryonic to fetal. At birth 98.5% converts to HbA. 1. Image from M.A. Hill, UNSW Embryology <http://embryology.med.unsw.edu.au/wwwhuman/Stages> Accessed: Feb. 12, 2009 2. Image provided by Dr. Jayashree Soman. 3. Image courtesy of J.S. Olson. [PDB: HbE 1fdh; HbF 1A9W; HbA 1A00]

For the past twenty years Dr. John Olson's laboratory in the Department of Biochemistry & Cell biology at Rice has been examining O_2 binding to mutants of mammalian myoglobin and the α and β subunits of HbA. Much of this work has focused on amino acid substitutions within the oxygen binding pocket, including at the valine E11 position. In 1995, an undergraduate honors research student in Dr. Olson's laboratory, Joshua Warren (Rice BA 1996; Yale PhD 2002), used sperm whale myoglobin (Mb) as a model system to examine the effects of valine E11 to methionine, phenylalanine, tyrosine, and tryptophan mutations on oxygen binding. All four of these amino acids have much larger side chains which fill up the interior portion of the pocket which captures diatomic gases, including O_2, CO, and NO. Warren observed dramatic decreases in the rates of O_2 uptake and release due to the valine E11 to methionine replacement. Similar marked decreases compared to wild-type Mb were observed for the Phe, Tyr, and Trp mutations, which also decrease the size of the binding pocket [4].

The mechanism of O_2 binding to either Mb or a Hb subunit is analogous to catching a baseball in a fielder's glove. As the thumb opens, by upward and outward movement of the histidine E7 side chain (Figure 4), incoming oxygen can be "caught" in the pocket of the glove. If the available space of the glove is made too small by limiting it with a large amino acid like methionine, the ball or O_2 will "bounce" back out of the globin requiring that multiple tries be made until it is finally captured and bound to the iron atom. Thus, we expect the valine E11 to methionine mutation to appreciably slow O_2 binding.

At the moment, a structure of the γ valine E11 to methionine mutant has only been simulated (Figure 4) and recombinant HbF Toms River not been characterized. However, based on Warren's results with Mb, we predict that O_2 binding may be slowed so much that red blood cells containing the HbF mutant cannot uptake oxygen quickly enough during passage through the placenta or new born lungs. Consequently, the blood will only be partially saturated with O_2 and appear a purplish or cyan color associated with cyanosis. We are currently setting up the HbF expression system and, as a control, show that wild-type γ subunits have kinetic and stability parameters very similar to those of HbA β subunits.

With luck this system will allow complete characterization of the HbF Toms River mutation, and recombinant DNA technology will allow us to study the clinical disorder associated with this hemoglobinopathy without having to obtain the infant's blood. This in vitro approach represents an important advance in characterizing genetic defects in hemoglobins and will provide a general approach for determining the underlying mechanisms for the phenotype

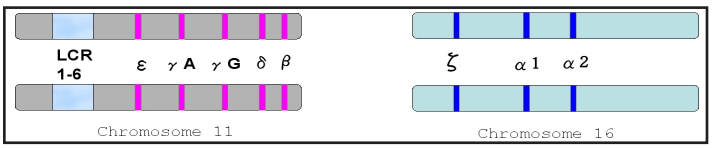

Figure 3. The Locus Coding Region (LCR) is responsible for silencing the different allele loci at different stages of the development. First, ε gene expression is down regulated by trans acting factors such as GATA-1 and EKLF. The "switching," continues to suppress the γ gene after birth. The ζ gene is expressed during the very initial stages of embryonic development.

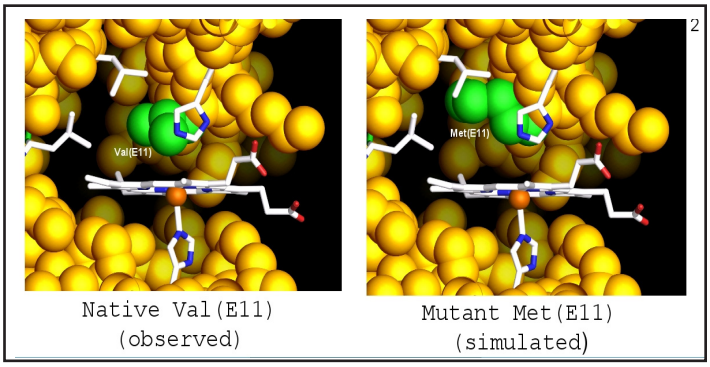

Figure 4. Currently, the mutant structure is hypothetical and the mutant recombinant HbFγ subunit has not been characterized. Using visual induction, one can predict the larger residue implicating increased steric hindrance for ligand binding. (Image courtesy of Dr. Jayashree Soman)

associated with the hemoglobin mutations and possible treatments to restore normal physiological function.

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