CXCL9 expression and purification: Identifying further structural and functional relationships with ligands

Eva Murphy, Dr. James Murphy
Advisor: Dr. Benjamin Alper
Sacred Heart University, Fairfield, CT
Sterling Hall of Medicine, Yale University, New Haven, CT

Abstract

Chemokines are proteins that induce tissue extravasation, promote differentiation, and induce chemotaxis. Because of these properties, the chemokine's role in antitumor immune response is of great interest to researchers. The CXCL9,10,11/CXCR3 axis is specific in that it regulates immune cell migration, differentiation, and activation, leading to tumor suppression. CXCL9 mainly mediates lymphatic infiltration to the focal sites and suppresses tumor growth. In this research, we expressed the novel CXCL9 protein within competent BL21 cells. Two variations of a pET22b plasmid were used, one with PelB (to cleave initial methionine on protein sequence) and one without. It was found that after induction, CXCL9 was expressed without the PelB leader sequence. From here we purified the CXCL9 protein and deduced that the overall expression of the protein was more than favorable. After isolating and concentrating the protein, a final concentration of 92 mg/ml was determined. With the purified protein, X-ray crystallographic studies will be used to determine the 3-D structure of the protein. This interface is important because it will impact the interaction with the receptor; therefore, altering the CXCL9/CXCR3 axis.

Introduction

Chemokines are small proteins, 8-15kD, which interact with G protein-coupled receptors in order to induce chemotaxis, tissue extravasation, and promote differentiation [1]. The CXCL9, 10, 11/CXCR3 axis is important because it regulates the differentiation of naive T cells to T helper 1 cells and leads to the migration of immune cells to specific tissues [5]. Due to these properties, the role of this specific axis in anti-tumor immune response is of great interest. The CXCL9, 10, 11/CXCR3 axis activates the paracrine axis, and inhibits the autocrine axis which makes it a favorable target for drug development [5]. It has been shown that agents who change the paracrine CXCL9,10,11 expression, and neutralize CXCR3 expression on cancer cells have shown anti-tumor activity in certain models [5]. Research has shown that plasmid borne CXCL9 plus cisplatin changed colon and lung cancer reduction and cytotoxic lymphocyte activation [7]. Additionally, intra- tumoral CXCL9 and interleukin 2 in renal cell carcinoma tumor model ended up reducing tumor growth and angiogenesis [3]. CXCL12, a similar small protein ligand, has been found to bind to heparin, and work with glycosaminoglycans to sequester the ligand to CXCR4 [2]. Identifying certain small molecules that bind the CXCL9 will be beneficial because the small molecules can alter the function of the ligand and alter the CXCL9/CXCR3 interaction and function, or will help describe how the ligand is sequestered and presented to the receptor.

The overall goal of the research is to purify CXCL9 and then use X-ray crystallography in order to determine the protein's structure, and possible structural relationships with ligands

Methodology

A transformation of three pET22b plasmids into competent Bl21(DE3) cells was performed. The three plasmids were wild type CXCL9, control, and CXCL9 with PelB following Agilent Technologies Bl21(DE3) competent cells protocol. An SDS-Page gel was run in order to determine if PelB was effective.

An LB+ ampicillin 15 mL culture from a fresh Bl21 colony transformed with pET22b was set in a shaker incubator overnight at 37*C. A subculture of 1:100 into LB liquid was created and placed in shaker incubator for 2 hours at 37*C. The culture was induced with 1.5mL 1M IPTG for 4 hours in a shaker incubator at 37*C. The cells were harvested and spun down at 8krpm. The pellets were then re-suspended in 30 mL 1XpBS+1%TX-100+1/5Protease tablet. The re-suspended cells were French Pressed twice. The cells were spun down again at 16 krpm for 25 minutes. Each pellet was re-suspended in 30 mL wash buffer and spun down again under the same conditions, this was repeated twice. The pellets were re-suspended in 6M Guanadine HCl to release the protein into the supernatant. An SDS page gel was ran for samples from the French press to the last wash. The supernatant was diluted 1:100 into refolding buffer and added dropwise, constantly stirring overnight at 4*C.

Once the protein of interest was isolated, it underwent purification procedures. The protein solution was run through an FPLC machine, the fractions were collected, and an SDS-page gel was ran to determine suitable fractions to pool. Next, the pooled fractions were run through an HPLC machine, and only the distinct peak containing the protein of interest was collected. The purified protein solution was condensed and then lyophilized overnight. The final purified product was re-suspended in 500uL of ddH2O, and an SDS-Page gel was ran of oxidized and reduced samples of CXCL9 to ensure the right protein was purified.

Crystallization protocols were followed. 12mg/mL of protein concentration was used, and then 88mg/mg of protein into pre-made wells from various distributors to maximize the possibility of crystallization using and NT8 Formulatrix Drop Setter.

Results

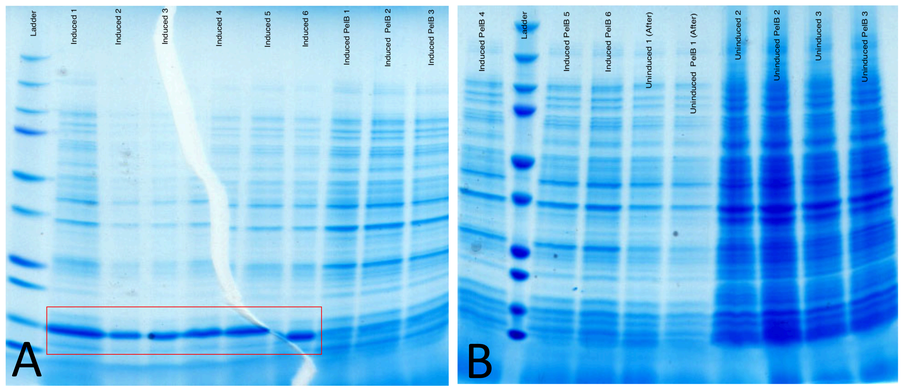

Figure 1: SDS-Page gel of induced and uninduced samples of WT and PelB CXCL9. (A) Induced samples 1-6, induced PelB samples 1-3. CXCL9 is expressed in the induced samples by a characteristic dark band (red outline) from the WT plasmids. The induced PelB 1-3 samples show no clear expression of the CXCL9 protein. **(B)** Induced PelB samples 4-6 show no clear expression of CXCL9. Uninduced samples were ran as a reference.

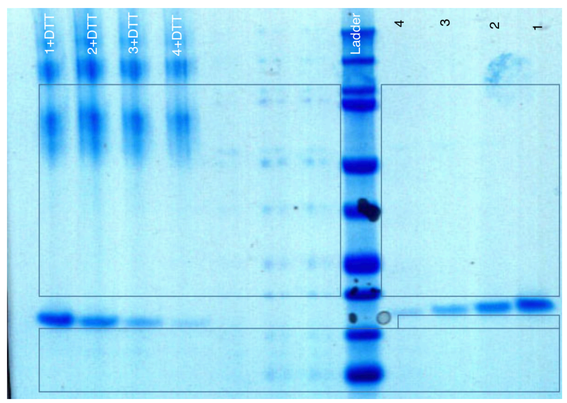

Figure 2: SDS-Page of final purified CXCL9 product, batch 1. The final concentration of 2 purification batches was determined to be 88mg/mL and 56mg/mL. A serial dilution was performed on the concentrated product, starting with a 1:50 dilution of protein to ddH2O, two times. The left side of the gel shows reduced CXCL9 treated with DTT. The reduced CXCL9 sample is shown to have traveled farther down the gel, which is expected as the disulfide bonds within the chemokine break. The right side of the gel shows the oxidized version of CXCL9. The oxidized chemokine should travel less distance through the gel when compared to the reduced version, as seen by the spacers under the protein band on the right. The density within each band series decreases as the dilution ratio increases.

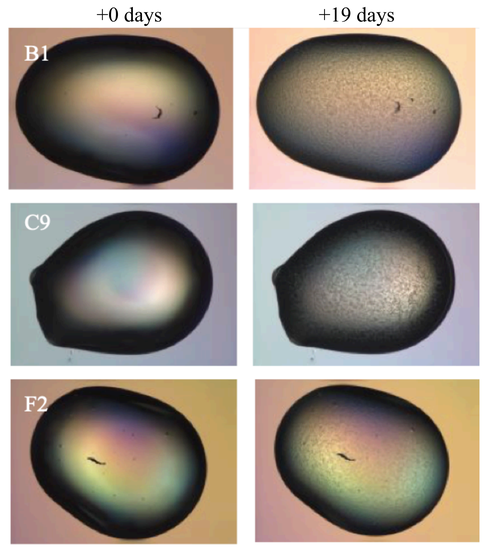

Figure 3: Three X-Ray Crystallography Hampton HT plates from 0 to 19 days with 0.4uL of an 88mg/mL CXCL9 concentration and 150uL well volume. Cells B1, C9 and F2 were determined to have some sort of crystalline structure. **(B1)** 0.2M Na3 Cit, 0.1M Tris 8.5 pH, 30 %v/v PEG 400. **(C9)** 4M Na form. **(F2)** 0.2M K Na Tart, 0.1M Na3 Cit 5.6 pH, 2M (NH4)2SO4.

Discussion and Implications

A concentration of 88mg/ml was determined for the first batch of CXCL9, and 56mg/ml for the second. These concentrations reflect a good expression of the CXCL9 protein. The proper conditions for CXCL9 to be knocked out of solution were tested. 12mg/mL was tested at first, this proved to be a weak concentration as no proteins crystallized. Even at an extremely high concentration of 88mg/mL, only three well out of 96 wells showed some sort of crystals. However, these crystals could also be inorganic salt precipitates. Since the protein is unable to be knocked out of solution, it could be too stable. The protein could also not be folded correctly and in solution like a big noodle; therefore, not allowing crystallization to happen. I would have liked to have a crystal structure by now, but due to the current climate that was not able to happen. Currently, I am working figuring out how to mutate the protein to decrease stability, if the protein is too stable. Furthermore, A2 and B22 virial coefficients might be helpful in terms of the fact that they can be manipulated for crystallography. A virial coefficient can be manipulated to provide correction to the ideal gas law and are characteristic of the interaction potential between particles and depend on the temperature [6]. This coefficient may be useful to manipulate within the well system in order to achieve proper crystallization.

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