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Molecular cloning and biophysical characterization of CXCL3 chemokine

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ABSTRACT

CXCL3 is a neutrophil activating chemokine that belongs to GRO subfamily of CXC chemokines. GRO chemokine family comprises of three chemokines GRO α (CXCL1), GROβ (CXCL2), and GRO γ (CXCL3), which arose as a result of gene duplication events during the course of chemokine evolution. Although primary sequences of GRO chemokines are highly similar, they performs several protein specific functions in addition to their common property of neutrophil trafficking. However, the molecular basis for their differential functions has not well understood. Although structural details are available for CXCL1 and CXCL2, no such information regarding CXCL3 is available till date. In the present study, we have successfully cloned, expressed, and purified the recombinant CXCL3. Around 15 mg/L of pure recombinant CXCL3 protein was obtained. Further, we investigated its functional divergence and biophysical characteristics such as oligomerization, thermal stability and heparin binding etc., and compared all these features with its closest paralog CXCL2. Our studies revealed that, although overall structural and oligomerization features of CXCL3 and CXCL2 are similar, prominent differences were observed in their surface characteristics, thus implicating for a functional divergence.

1. Introduction

Chemokines play an essential role in numerous pathophysiological functions including wound healing, inflammation, angiogenesis, and cancer metastasis etc. Chemokines regulate the biological events by interacting with their cellular partners such as glycosaminoglycans (GAGs) and G-protein coupled receptors (GPCRs) [1–3]. Chemokines are subdivided into different groups namely, CXC, CC, CX3C, and C chemokines, based on their arrangement of Cys residues at the N-terminal [4]. ELR-CXC chemokines, also known as neutrophil activating chemokines (NACs) are a subgroup of CXC chemokine family comprising of seven proteins namely CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8 [4].

Three NACs comprising of CXCL1 (GRO α), CXCL2 (GRO β), and CXCL3 (GRO γ) are called as "GRO chemokines" (Growth related oncogene chemokines) [5]. GRO family arose as a result of two rounds of gene duplication during the course of evolution [6–8]. GRO chemokines are closely related to each other, and are involved in growth and progression of melanoma tumors [9]. Moreover, biological studies reported the differential expression and regulation patterns of all these three GRO genes in tissue and signal specific manner [10–12]. Comparative CXCR2 chemotactic activity studies of GRO genes evidenced highest efficacy for CXCL1 and intermediate efficacies for CXCL2 and CXCL3 [11]. Further, structural studies suggested that the oligomerization features of these chemokines vary remarkably, and they do form homo and heterodimers [13].

CXCL3, also known as CINC-2 alpha (Cytokine-induced neutrophil Chemoattractant), exerts its functions through a number of signaling pathways including p38 MAPK, ERK1/2 MAPK and JAK2/STAT3 etc., by activating CXCR2 receptor [10,12,14,15]. It accomplishes specific functions by following distinctive molecular mechanisms in comparison with other two GRO chemokines [14,15]. CXCL3 is highly expressed during the number of tumorous conditions including melanoma, prostate, colorectal, aggressive breast cancer tumors, hepatocellular carcinoma (HCC) and also during hepatic injury and inflammation [9,16,17]. Furthermore, CXCL3 is also associated with vascular invasion and tumor capsule formation, thus can potentially serve as a prognostic biomarker and therapeutic target for HCC [12].

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Albeit, structural details and receptor/GAG binding interactions are elucidated for both CXCL1 and CXCL2 chemokines, no such information is available for CXCL3 [18,19]. Therefore, in order to throw light on the molecular interactions of CXCL3 chemokine, production of recombinant CXCL3 is essential. In the present report, we have produced the recombinant murine CXCL3 and investigated its divergent structural and biophysical characteristics. The molecular features of CXCL3 were compared with its closest paralog CXCL2, to unravel their differential structural/stability features.

2. Materials and methods

2.1. Cloning, expression, purification, and in-vitro refolding of CXCL3

Full length CXCL3 gene (NCBI Ref seq: NM_203320.2) was purchased from Sino-biologicals, China in pMD18-T cloning vector (Cat. No. MG50258-M). The gene encoding CXCL3 protein was amplified using appropriate forward and reverse primers, and subcloned into a pET32 bacterial expression vector between the Kpn1 and Nco1 restriction sites.

The cloned CXCL3 expression plasmid (pET32-CXCL3) was transformed in *E. coli* BL21 (DE3) cells, and the protein was expressed at both 37 °C and 20 °C by inducing with IPTG. Large scale expression of CXCL3 protein was carried out by transferring the overnight seed cultures (10 ml) into 1 l of either LB, or M9 minimal medium containing isotopically labeled $^{15}NH_4Cl$. The protein was expressed by growing the post induced cells at 20 °C, 220 rpm for 18 h.

CXCL3 present in inclusion bodies fraction (CXCL3_RF) was solubilized in lysis buffer (20 mM Tris and 500 mM NaCl, pH 8) containing 8 M urea at room temperature using cell homogenizer. Lysate was then centrifuged at 14000 rpm for 20 min, and the supernatant containing the denatured protein was separated from the cell debris. Denatured protein was refolded by dialyzing it twice (8 h for each cycle of dialysis) against the lysis buffer at room temperature. The refolded CXCL3 fusion protein (CXCL3_RF) was obtained by centrifugation. Both the supernatant fraction containing the CXCL3_RF, and the natively folded CXCL3 protein (CXCL3_NF) from the cytosolic fraction were purified using a series of chromatography techniques. The detailed protocol related to cloning, expression, and purification of CXCL3 is presented in supplementary materials and methods.

2.2. Size exclusion chromatography (SEC)

SEC was performed using HiLoad 16/60 Superdex 75 prep grade column on GE healthcare AKTA prime FPLC system equipped with zinc lamp. Natively folded (NF), and refolded (RF) CXCL3 proteins were loaded on to the pre-equilibrated column (50 mM sodium phosphate, 100 mM NaCl, and 1% Glycerol at pH 6.0) at a flow rate of 1 ml/min. To assess the oligomerization characteristics of CXCL3; CXCL2 and other standard reference proteins (pepsin, chymotrypsin, cytochrome C and aprotinin) with known molecular weight were loaded (1 ml each, 1 mg/ml) onto the column under similar experimental conditions.

2.3. Heparin binding assay

Heparin binding assay was performed using 1 ml HiTrap heparin high performance column from GE, which was pre-equilibrated with Tris-NaCl buffer (20 mM Tris and 50 mM NaCl at pH 7). GRO proteins at fixed concentration of 0.2 mg/ml were injected onto the column, and were eluted using a linear gradient of 0 M to 2 M NaCl (flow rate – 1 ml/min) by monitoring the absorbance at 215 nm.

2.4. NMR spectroscopy

^{1}H-^{15}N HSQC spectra for CXCL3_NF and CXCL3_RF were acquired at 25 °C on a Bruker 500 MHz spectrometer equipped with TXI probe. ^{15}N labeled CXCL3 samples (~1mM) were prepared in 50 mM sodium phosphate buffer (pH 6.0) with 10% D_2O. ^{1}H-^{15}N HSQC spectra were recorded with 32 scans and 256 increments in the indirect dimension. A spectral width of 16 ppm and 28 ppm was used for ^{1}H and ^{15}N dimensions, and their carrier frequencies were set to 4.7 ppm and 120.5 ppm respectively. 2D-DOSY experiments were carried out on Bruker 800 MHz spectrometer as described elsewhere [20]. All the DOSY measurements were carried out in 100% D_2O solvent. The proteins CXCL2, hen egg lysozyme (HEL) and chicken SH3 domain were used as standards to assess the molecular weight of CXCL3.

2.5. Optical spectroscopy

CXCL2 and CXCL3 samples with a concentration of 50 μM (20 mM Tris and 50 mM NaCl, at pH 7) were used for all optical spectroscopy studies.

2.5.1. Circular dichroism (CD) measurements

Far UV-CD experiments were carried out on a Jasco J-1500 CD spectrophotometer using 1 mm path length quartz cuvette in the range of 190–250 nm. Thermal denaturation profiles of CXCL2 and CXCL3 were monitored in the temperature ranges 20 °C to 90 °C by recording the CD spectra at regular intervals of 10 °C with gradual increase in temperature (1 °C/min), and an incubation time of 5 min at each resting temperature. To analyze the reversibility of structural transitions, proteins were steadily cooled back to 20 °C from 90 °C, and the spectra were recorded again at 20 °C.

2.5.2. Fluorescence spectroscopy

All the fluorescence experiments were recorded on a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon Spex®) containing xenon lamp. The spectra were measured at 25 °C using a 4 mm path length quartz cuvette with an excitation and emission slit width of 2.5 nm. To monitor the ANS (8-Anilinonaphthalene-1-sulfonic acid) binding to CXCL3 and CXCL2, samples were excited at 380 nm and the emission spectra were scanned in the range of 400–600 nm. A fixed concentration of 1:5 (protein: ANS) was used to record the spectra.

2.6. In-silico functional and structural analysis

Coding sequences for 13 rodent GRO genes and 27 primate GRO genes were collected from the NCBI sequence database (http://www.ncbi.nlm.nih.gov/genbank/) (**Table S1**, Supplementary material). Sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic trees based on protein sequences were constructed separately for rodents and primates using MEGA 6.0 software using neighbor–joining (NJ) method [21]. The reliability of the trees was assessed using 1000 bootstrap replications. Functional divergence between paralogous clusters of GRO chemokines were analyzed using the "functional distance analysis" module of DIVERGE 3.0 [22]. Amino acid conservation in GRO chemokine sequences was analyzed using 'weblogo' (http://weblogo.berkeley.edu/logo.cgi).

CXCL3 structural model was generated through homology modeling using Swiss model server [23]. Structure was obtained by employing crystal structure of CXCL2 as a template (PDB ID: 3N52). The quality of the obtained structure was validated using PROCHECK. Structural differences among GRO chemokines were obtained by calculating the RMSD values using PyMOL. Comparative contact maps depicting Cα contacts among GRO chemokines

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