Cloning, Expression, Purification and Crystallization of NHR3 Domain from Acute Myelogenous Leukemia-related Protein AML1-ETO

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Abstract   The t(8;21) translocation is one of the most frequent chromosome abnormalities in acute myeloid leukemia. This translocation creates a fusion between the acute myelogenous leukemia 1 (AML1, a transcription factor) gene on chromosome 21 and the eight-twenty-one (ETO, a zinc finger nuclear protein) gene on chromosome 8, leading to the repression of certain AML1 target genes. We cloned NHR3 domain coding fragment into vector pGEX-6p-1 using PCR and obtained recombinant plasmid pGEX-6p-1-NHR3, which can be induced to stably overexpress fusion protein in *E. coli*. Through the purification on GST affinity chromatography column and PreScission protease cleavage, a large amount of NHR3 protein with high purity was obtained. In order to avoid possible interference of some strong negative charged molecules, NHR3 protein was further purified by Mono Q anion exchange chromatography. The NHR3 crystals were grown with hanging drop/vapor diffusion method and the first crystals appeared after four weeks at 18 °C in 0.2 M Tris-sodium citrate dihydrate, 0.1 M sodium cacodylate, pH 6.5, and 30% iso-propanol (*V/V*). ESI mass spectrum showed that the molecular weight of this domain was in agreement with its primary structure sequence prediction, and circular dichroism spectral data (190–250 nm) showed that NHR3 had a well-defined secondary structure of 25.9% α-helix, 23.2% random coil and 50.9% turn, which was consistent with GOV4 software prediction.

Key words   AML1-ETO; NHR3; crystallization; ESI mass spectrum; circular dichroism spectrum; glutathione S-transferase (GST)

Nearly 40% of acute myeloid leukemia (AML) M2 cases are associated with the t(8;21)(q22;q22) chromosome translocation [1]. This translocation creates a fusion between the AML1 gene on chromosome 21 and the eight-twenty-one (ETO) gene on chromosome 8. The resulting chimeric protein contains the DNA-binding domain of AML1 and nearly the full length of ETO [2–5]. AML1 is a hematopoietic cell-specific transcription factor and is essential for definitive hematopoietic development

[6–8]. Recent studies show that chimeric protein AML1-ETO mainly interferes with function of transcription factor AML1 by combination of N-CoR/SMRT corepressor, mSin3 nuclear co-repressor complex and further recruitment of histone deacetylase (HDAC) through ETO moiety [9]. ETO moiety from AML1-ETO is homologous to the *Drosophila melanogaster* in four regions (NHR 1–4). NHR4 domain contains two zinc finger motifs, which yields key function in integration of reciprocal actions between ETO and N-CoR/SMRT, and NHR2 domain mediates the dimerization and strongly interacts with mSin3A [10–13]. Generally, NHR3 was considered as a domain of unknown function [14], but recent research demonstrated that interaction between ETO and N-CoR required both NHR4 and NHR3 domains [15].

To explore the role of NHR3 domain in recruiting nuclear co-repressor complex and involvement of transcription repression via structure solution, the corresponding coding region was cloned into pGEX-6p-1, and the domain was expressed, purified, and preliminarily crystallized. These experiments paved the way for NHR3 structure solution and co-crystallization with some vital motifs of nuclear co-repressor complexes.

Materials and Methods

Materials

pMD18-T vector was purchased from TaKaRa; pGEX-6p-1 was from Pharmacia; *E. coli* DH5α strain was from Invitrogen. Restriction enzymes, T4 DNA ligase, Ex *Taq* DNA polymerase, IPTG and glass milk kit were purchased from TaKaRa. PreScission protease was from Pharmacia Biotech. IZIT (crystal indicator) was from Hampton Research. General reagents were of analytical grade. Forward primer is 5′-CG**GGATCC**GCACTAGAC-GCGCATCGG-3′ (*Bam*HI site in bold) and reverse primer is 5′-CCG**CTCGAG**TCACTCCTGCTGATTGATAAC-3′ (*Xho*I site in bold).

PCR amplification and construction of the expression vector

The NHR3 domain was amplified by the special primers from the template pQE30-ETO. The PCR product was isolated and purified by glass milk kit. NHR3 domain was first cloned into pMD18-T vector and then into pGEX6p-1 expression vector using *Bam*HI and *Xho*I restriction sites. The clone was named pGEX6p-1-NHR3.

Expression and purification

pGEX6p-1-NHR3 was transformed into *E. coli* DH5α. Conditions for expression were as follows. Bacteria were grown in LB medium containing 100 μg/ml Amp to A_{600}=0.6, then induced with 1 mM IPTG. After treatment at 16 °C overnight, the cells were collected by centrifugation at 5000 rpm for 10 min and re-suspended in 1×PBS containing 10 mM β-mercaptoethanol, 1 mM PMSF, then sonicated in an ice bath. The lysate was centrifuged at 15,000 rpm for 30 min. The supernatant was separated by SDS-PAGE and stained with Coomassie Brilliant Blue to determine the expression of GST-NHR3. Cleavage of this fusion protein with PreScission protease on column was conducted according to reference [16]. All samples were boiled at 99 °C for 5 min before loading onto SDS-PAGE gel for observation. In order to avoid possible interference of some strong negative charged molecules, after GST affinity chromatography purification, Mono Q anion exchange chromatography was conducted according to the predicted pI 5.66 of NHR3 using ExPASy primary structure analysis tools (available at, http://www.expasy.org). The sample eluted from GST affinity chromatography column was centrifuged at 15,000 rpm for 30 min and diluted to the salt concentration of approximate 50 mM using 20 mM Tris-HCl, pH 8.0, and applied to a Mono Q HR 5/5 column previously equilibrated with the same buffer by AKTA Explorer (Pharmacia Biotech). The column was washed with 10 ml equilibrated buffer and eluted by 30 ml gradient NaCl from 0 to 1.0 M.

Crystallization

The Screen I and II kits (Hampton Research Corp.) were used for crystallization conditions screening by the hanging drop vapor diffusion method with the protein concentration of 20 mg/ml and the buffer was changed to highly purified deioned water. The mixed drop including 1 μl protein solution and 1 μl reservoir solution grew in an airtight system containing 0.5 ml reservoir solution at 18 °C.

Mass spectrum analysis

Sample preparation: NHR3 polypeptide purified by Mono Q affinity chromatography was diluted sufficiently and concentrated against highly purified deioned water using 5 Ku Millipore tube at 2000 *g* by ultra filter centrifugation, and then analyzed with mass spectrometer by Instrumental Analysis Center of Academy of Military Medical Sciences.

CD spectrum analysis

Sample preparation was the same as in mass spectrum analysis and the protein concentration was 0.2 mg/ml. Then far-UV CD spectrum (190–250 nm) was recorded using a Jasco model 715 spectropolarimeter at room temperature.

Results

NHR3 cloning

At the beginning we cloned the opening reading frame of NHR3 and NHR4 into pQE30 vector to express these two domains of AML1-ETO in *E. coli* M15 (prep4), but

found protein degradation during purification. Considering that protein degradation might occur at the linker region between the two domains, we used GOV4 software to deduct a secondary structure of full-length AML1-ETO. Results showed that amino acid 573–657 might be a region mainly consisting of α-helix (data available at http://cn.expasy.org/tools/#secondary). Then we cloned the gene fragment encoding amino acid 573–657 of AML1-ETO into pGEX-6p-1, which covers the region coding amino acid 591–640 reported as NHR3 functional domain [17]. Sequencing was completed by Shanghai Bioasia Inc..

Expression and purification

DNA sequencing verified the correct insertion of coding fragment of NHR3. The resulting recombinant plasmid pGEX-6p-1-NHR3 was transformed into *E. coli* DH5α to express the domain of the interest. The fusion protein GST-NHR3 was determined by SDS-PAGE with a molecular weight of 36 kD (Fig. 1) and found to be identical as expected previously. The SDS-PAGE results also indicated that the expression products were mainly in the supernatant. After PreScission protease cleavage on GST affinity chromatography column, protein with high purity was prepared, increasing the chances for crystallization (Fig. 2). Here Tris-tricine buffer system [18] was used to improve resolution in SDS-PAGE for the reason that the MW of NHR3 was only 10 kD. Anion exchange chromatography was applied after affinity chromatography to avoid the interference of some strong
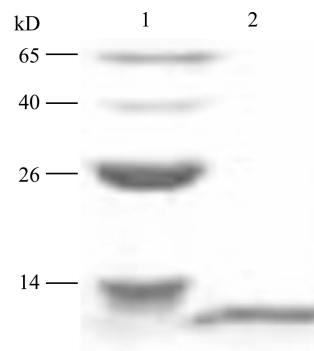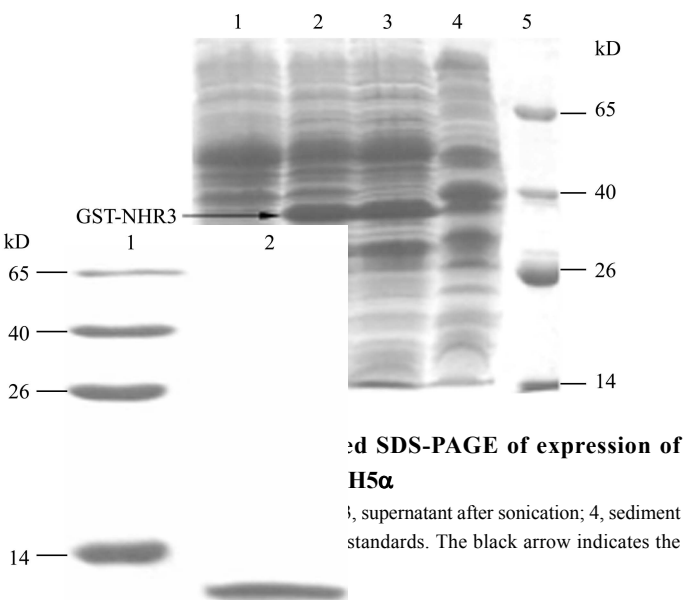

Fig. 2   **SDS-PAGE of purified NHR3 after PreScission protease cleavage on GST affinity chromatography**
1, molecular weight standards; 2, NHR3. Here Tris-tricine buffer system was used.

negative charged molecules (chromatography profile data not shown).

Crystallization

The hanging drop vapor diffusion method was applied for crystallization. Four weeks later, the crystals mainly in rhombus or flat-length stick shape appeared in the drop containing 0.2 M Tris-sodium citrate dihydrate, 0.1 M sodium cacodylate, pH 6.5, 30% iso-propanol (*V/V*) (Fig. 3). In order to differentiate protein crystals from salt crystals, IZIT, a crystal indicator from Hampton Research, was added to the drop. In addition, the crystals were pooled, washed in the well solution at least three times, and applied to SDS-PAGE. Both the IZIT staining and SDS-PAGE results verified they were protein crystals (Fig. 4, 5).

Fig. 3   **Photograph of NHR3 crystal**
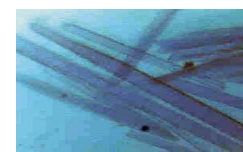The longest dimension is approximately 0.5 mm

Fig. 4   **Result of treatment with crystal indicator IZIT**
The crystals in darker blue than solvent indicate that they are protein crystals.

GST-NHR3

...ed SDS-PAGE of expression of
...H5α

...3, supernatant after sonication; 4, sediment
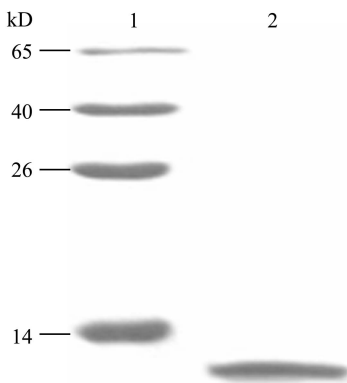...standards. The black arrow indicates the

Fig. 5   Dissolved crystal

Protein was visualized by Coomassie-blue staining. 1, molecular weight standards; 2, dissolved NHR3 crystal. Here Tris-tricine buffer system was used.

Fig. 7   Mass spectrometer of NHR3

CD spectrum

The far-UV CD spectrum shown in Fig. 6 displayed negative bands at 208 and 222 nm. It was a typical α-helix structure and consistent with software prediction. The calculation result showed that the percentage of α-helix was 25.9%, random coil 23.2%, and turn 50.9%.

Mass spectrum

10097 u was the predictive molecular weight of NHR3 domain with three additional residues derived from pGEX-6p-1 vector as the result of PreScission protease cleavage. Mass spectrometric analysis determined the sample molecular weight as 10096.75 u, in consistent with the expected mass 10097 u, within the range of systematic error for ESI mass spectrum. Fig. 7 also indicated that besides the largest peak of 10096.75 u, there also appeared three comparative smaller peaks of 10363, 10629, 10895 u and each was 266 u larger than the previous one. This might be due to NHR3's association with other small molecules.
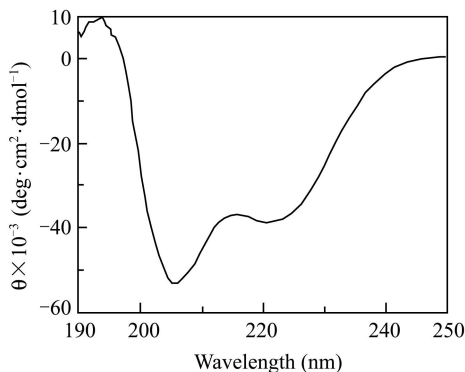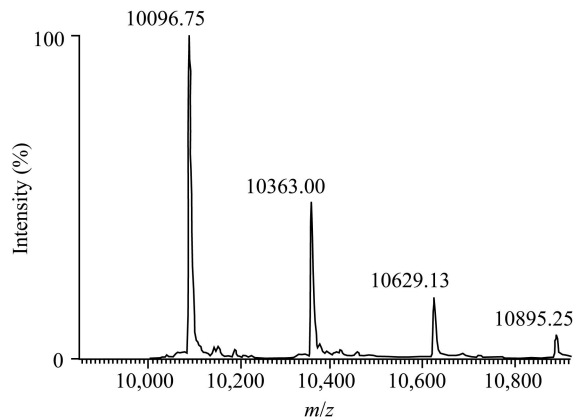
Discussion

To our knowledge, NHR3 was taken for an unknown domain, but recent studies found that only fusion protein GST•NHR3+NHR4 could interact with N-CoR whereas GST•NHR4 could not [15]. This might shed lights on the possible interaction between NHR3 and N-CoR. We expected to investigate the role of NHR3 via solving its structure since there has been no report on structure information of NHR1 to NHR4 yet.

We ever expressed NHR3+NHR4 domains with pQE30 vector in *E. coli* M15(prep4), but found the degradation of target protein. To avoid possible degradation by proteases existent in bacteria, we constructed pGEX-6p-1-NHR3 expression vector via secondary structure prediction and dissected functional domains reported, and successfully express, purify and crystallize this domain. This strategy, however, could be applicable to expression, purification and crystallization of other proteins with similar behavior.

Fig. 6   Circular dichroism spectrum of NHR3

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