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Screening and Identification of The Proteins Interacting With NLS-RARα Protein*

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Abstract Acute promyelocytic leukemia (APL) is characterized by the generation of the prototypic promyelocytic leukemia-retinoic acid receptor alpha (PML-RARα), an oncogenic fusion protein due to chromosomal translocation. In a human myeloid cell line, PML-RARα is cleaved by neutrophil elastase (NE) to produce the mutational PML [nuclear localization signal (NLS) deletion] and RARα (NLS-RARα, containing NLS of PML), both of which may play an important role in APL pathogenesis. The yeast two-hybrid technique was used to screen the intracellular proteins interacting with NLS-RARα, which may be involved in NLS-RARα signaling. The NLS-RARα coding sequence was amplified by polymerase chain reaction method and was cloned into the bait plasmid pGBKT7 vector, which, after the confirmation by sequencing, was transformed into yeast AH109 and the subsequent expression of bait plasmid was proved by Western-blot. The transformed yeast AH109 was mated with yeast YI87 (containing leukemia cDNA library plasmids pACT2) in medium. Diploid yeast was plated on synthetic dropout nutrient medium containing X-α-gal for screening. After being reintroduced into yeast AH109 and sequenced to verify the expression of ORF, eight positive colonies were obtained, among which one containing JTV-1 was cloned. The interaction between NLS-RARα and JTV-1 was further supported by indirect immunofluorescence, GST pull-down and co-immunoprecipitation, respectively. These findings brought some new clues for the further exploration of NLS-RARα signaling to APL.

Key words NLS-RARα protein, leukemia, yeast two-hybrid, protein-protein interaction
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More than 90% patients of acute promyelocytic leukemia (APL) have t(15; 17) translocation to generate the PML-retinoic acid receptor alpha (PML-RARα) fusion protein that leads to the deregulation of wild type PML and RARα function. However, the molecular mechanisms by which PML-RARα predisposes early myeloid cells to eventual leukemic transformation are not yet completely understood. There is strong evidence that Neutrophil elastase (NE) plays an important role in the proceeding of PML-RARα. NE as an early myeloid-specific serine protease is important for the development of APL in mice [1], suggesting that NE expression may therefore help to define the susceptible hematopoietic cell for PML-RARα actions. PML-RARα is cleaved by NE in early myeloid cells dominantly within the carboxyl-terminal domain to produce mutational PML and RARα mutant [2], which suggested that the interaction of PML-RARα with NE may play some roles in the development of APL. However, the functions of the two proteins are not yet fully understood. In this report, we designed a bait plasmid containing the NLS-RARα sequence to screen the proteins interacting with NLS-RARα in leukemia cDNA library by the yeast two-hybrid technique, and the findings is believed to shed some light on the research of the function of NLS-RARα in leukemogenesis.

1 Materials and methods

1.1 Materials

The plasmid pcDNA3.1-PML-RARα was gifted

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by Doctor Andrew A. Lane (Washington University). All of the yeast strains and plasmids for yeast two-hybrid experiments, including Matchmaker GAL4 two-hybrid system 3 were from Clontech Co.. Yeast strain Y187 containing pACT2 with the leukemia cDNA library were generated in our laboratory[3]. All the restriction endonucleases, PCR kit and DNA marker were ordered from TaKaRa Co., tryptone, yeast extractive and glucose were from Oxide Co., yeast transformation kit, and all kinds of selection media were from Clontech Co., both BD monoclonal antibody, Myc monoclonal antibody and HA polyclonal antibody were from Clontech Co., Cy3-Conjugated Goat Anti-Rabbit IgG and FITC-Conjugated Goat Anti-Mouse IgG were from Millipore Co., Lipofetamine™ 2000 was ordered from Invitrogene Co., GST gene fusion system was from Pharmacia Co., chemoluminescence Kit was produced by Viagene Co., the Co-IP reactant was from Clontech Co. and Calbiochem Co..

1.2 Amplification of the NLS-RARα and construction of bait plasmid

Take pcDNA3.1-PML-RARα as DNA template, NLS-RARα was generated by PCR amplification. The sequences of the primers containing the *Eco*R Ⅰ and *Sal* Ⅰ restriction enzyme sites were the following: sense primer, 5′ CAGGAATTCGCTGTGGTACAGT-CAGTG 3′; antisense primer, 5′ AGGGTCGACT-CCATGTGGCGTGGG 3′.

The 100 ng of template was added to a PCR mixture in a final volume of 20 μl containing 4 μl 5× buffer, 2.5 μmol of each of dATP, dCTP, dGTP and dTTP, 10 pmol of each set of the sense / antisense primers, and 0.6U of PrimerSTAR™ HS DNA polymerase. The PCR conditions were: predenature at 98℃ for 5 min, then 98℃ for 15 s，58℃ for 40 s，72℃ for 2 min, altogether for 35 cycles, 72℃ for 10 min. After reclaimed NLS-RARα sequence was released from PCR products by digestion with *Eco*R Ⅰ and *Sal* Ⅰ, and then was cloned into the bait expression vector pGBKT7. The structure of insert was confirmed by restriction enzyme analysis and direct sequencing.

1.3 Transformed the bait plasmid into AH109 and Western blotting analysis

The bait plasmid was transformed into yeast strain AH109 by lithium acetate method, according to Yeast Protocols Handbook (Clontech). Then the yeast AH109 containing pGBKT7-NLS-RARα was cultured on SD/-Trp/X-α-gal, SD/-His/-Trp/X-α-gal, SD/-Ade/-Trp/X-α-gal and SD/-Trp/-Leu to exclude the toxicity and autonomous transcriptional activity. The yeast AH109 containing pGBKT7-53 was used as control. Western blotting was performed to confirm the expression of the fusion protein using BD monoclonal antibody. The yeast AH109 cells transformed with pGBKT7 were used as negative control.

1.4 Yeast two-hybrid screening

The transformed AH109 mated with yeast Y187 containing pACT2 with leukemia cDNA library for 22～24 h. The cells were spread on 50 large (150 mm) plates containing 100 ml of SD/-Trp/-Leu/-His medium. After growing for 5～15 days, the yeast colonies were transferred to the QDO plates containing X-α-gal, to check for expression of the reporter gene (blue colonies) to exclude false positives. The yeast AH109 containing pGBKT7-53 mated with yeast strain Y187 containing pGADT7-T provided a positive control for interacting proteins. The yeast AH109 containing pGBKT7-53 mated with yeast strain Y187 containing PGBKT7-Lam provided a negative control.

1.5 Plasmids isolation from yeast and retransformed

Yeast plasmid was isolated from positive yeast colonies, and sixteen positive plasmids were obtained and transformed into DH5α. Then plasmid which inserted with cDNA library would be obtained from DH5α. In order to exclude false positives, the plasmids of positive colonies and pGBKT7-NLS-RARα were retransformed into yeast strain AH109 and then plated on SD/-Trp/-Leu/-His/-Ade/X-α-gal to test the specificity of interactions.

1.6 Bioinformatic analysis of positive plasmids

The structure of insert was confirmed by direct sequencing. After sequencing of the positive colonies, the sequences were compared with GenBank to analyze the structure and function of the genes.

1.7 Indirect immunofluorescence analysis

The eukaryotic expression vector pCMV-HA-NLS-RARα and pCMV-Myc-JTV1 were constructed. The HEK293 cells used were maintained in Dulbecco′s modified Eagle′s medium containing 10% fetal bovine serum (FBS) in a 37℃ incubator with 5% CO_2 atmosphere. One day before transfection, plated 2×10^5 cells in one well of 24-well format contained coverslip (coating polylysine). The pCMV-HA-NLS-RARα and pCMV-Myc-JTV1 plasmids were cotransfected into HEK293 cells using the Lipofetamine™ 2000 in a 1∶2

ratio. Briefly, cells were fed with 0.2 ml fresh medium without FBS for another 1 h before transfection. The 0.2 ml of transfection mixture (containing 0.5 μg pCMV-HA-NLS-RARα and 0.55 μg pCMV-Myc-JTV1, 2 μl Lipofetamine™ 2000) added into the medium after standing at room temperature for 0.5 h and incubated with the cells at 37℃ for 4.5 h. Then the transfected cells were fed with 1 ml fresh medium containing 5% FBS for 48 h. The transfected cells rinsed thrice with phosphate buffered saline (PBS), fixed in polyoxymethylene for 20 min, washed 3 times with PBS for 5 min each time. Then permeated with 0.5% Triton X-100 at 37℃ for 15 min and incubated with 5% bovine serum albumin for 30 min. Cells were incubated with 2 primary antibodies (HA polyclonal antibody (1∶500) from rabbit and Myc monoclonal antibody from mouse (1∶100)) at 37℃ for 1.5 h, and then washed 7 times with PBS. The PBS with no primary antibody was used as negative control. The cells then incubated with Cy3-conjugated goat anti-rabbit IgG (1∶400) plus FITC-conjugated goat anti-mouse IgG (1∶300) at 37℃ for 1 h, and then washed 7 times with PBS. Cells were incubated with DAPI for 3 min, and then washed 3 times with PBS. Specimens were mounted with 50% glycerine, and observed under laser-scanning confocal microscopy with an excitation wavelength.

1.8 Detecting interaction between NLS-RARα and JTV1 *in vitro* GST pull-down and Western blot

The fusion protein expression plasmids (pCMV-Myc-JTV1) were transfected into HEK293 cells using the Lipofetamine™ 2000 in a 1∶2.5 ratio. After 48 h, collected the cells and the fusion protein was extracted. The pGEX4T-2-NLS-RARα plasmid was transformed into JM109. After induced in 0.5 mmol/L IPTG at 30℃ for 4 h, the bacteria were collected, broken and harvested. After added into the glutathione Sepharose 4B, the harvested bacteria were rocked at room temperature for 2 h. The lysates were harvested and centrifuged at 12 000 *g* at 4℃ for 1 min, decanted the supernatant. The sedimentum was mixed with the cell lysates containing Myc-JTV1 fusion protein at 4℃ for 16～18 h. Next day, the precipitate was harvested by centrifugation and washed five times using PBS. The pellets were run on SDS-PAGE, and then the gel was identified *via* Western blotting. The pGEX4T-2 plasmid was used as negative control.

1.9 Detecting interaction between NLS-RARα and JTV1 *in vivo* (co-immunoprecipitation and Western blot)

The pCMV-HA-NLS-RARα and pCMV-Myc-JTV1 plasmids were cotransfected into HEK293 cells using the Lipofetamine™ 2000 in a 1∶2.5 ratio. 48 h after cotransfected, the transfected cells rinsed thrice with phosphate buffered saline (PBS) in an ice-bath, and then schizolysised with 500 μl of a lysis buffer at ice-bath for 30 min. The cell lysates were harvested, centrifuged at 14 000 *g* at 4℃ for 30 min. The supernatant obtained was mixed with 2 μg anti-HA polyclonal antibody and placed on a shaker for overnight at 4℃. 40 μl mixture of protein G/A agarose beads suspension were added to it, rocked at 4℃ for 8 h. The beads were washed five times with lysis buffer. 20 μl of 2 × sample loading buffer was added to the beads, and the mixture was boiled for 5 min. The extracts were then separated by SDS-PAGE. The proteins were transferred to PVDF membranes which were probed by Western blotting. The pCMV-HA-NLS-RARα/pCMV-Myc-JTV1 was transfected to HEK293 respectively provided a negative control.

2 Results

2.1 Recombinant plasmids

The full length sequence of NLS-RARα was generated by PCR amplification. The NLS-RARα fragment was ligated into pGBKT7 plasmid at the *Eco*R Ⅰ / *Sal* Ⅰ site.

2.2 Expression of the bait protein

Yeast strain AH109 transformed with pGBKT7-NLS-RARα stably expressed the fusion protein with molecular mass of 80 ku (Figure 1).

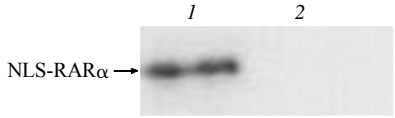

Fig. 1 Western blot analysis of bait protein in yeast
1: pGBKT7-NLS-RARα fusion protein; *2*: Negative control.

2.3 Screening of the leukemia cDNA library

The NLS-RARα protein was used as the bait for screening leukemia cDNA library. Sixteen positive clones grew in the SD/-Trp/-Leu/-His/-Ade/X-α-gal. To confirm the true interaction with NLS-RARα protein in yeast, the sixteen clones were transformed into AH109 and were further tested. Eight positive clones, which grew on SD/-Trp/-Leu/-His/-Ade/X-α-gal media became blue (Figure 2).

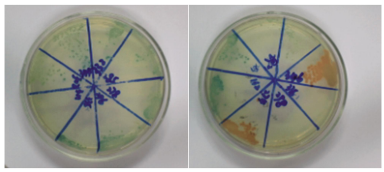

Fig. 2 Positive clones interactive with NLS-RARα protein lining on QDO containing X-α-gal (blue ones)

2.4 Analysis of coding sequence of positive clones

The nucleotide sequences of the eight clones from this cDNA library were sequenced and analyzed, the sequences were compared with those in GenBank. The data is presented in Table 1.

Table l Comparison between positive clones and similar sequences in GenBank

High similarity to known genes	GenBank Number	Homolog	Function
JTV-1	100814397	100%	Probable core protein of the multisynthetase complex that serves as a template for the assembly of the supramolecular structure. Mediates ubiquitination of FUBP1 and its degradation by the proteasome
CKS1B	4502856	99%	Binding to the catalytic subunit of the cyclin dependent kinases and is essential for their biological function
GLUL	74271825	95%	Glutamine synthetase
UBQLN1	44955932	100%	A ubiquitin-like protein
COPS5	38027922	97%	A regulator in multiple signaling pathways
ERH	4758301	95%	May be an important transcription regulator
ISCA1	52426767	97%	Implicated in iron-sulfur cluster biogenesis
AGBL5	118421079	94%	Unknown

2.5 HA-NLS-RARα co-localizes with Myc-JTV1 in HEK293 cells

The eukaryotic expression vector pCMV-HA-NLS-RARα and pCMV-Myc-JTV1 were constructed and cotransfected into HEK293 cells successfully. 48 h after transfected, to know the possible interaction of HA-NLS-RARα protein and Myc-JTV1 protein, their intracellular localization in cells was analyzed using indirect immunofluorescence. As shown in Figure 3, HA-NLS-RARα protein was localized in nucleus (Figure 3a), Myc-JTV1 protein was localized in nucleus majority and in cytoplasm partial (Figure 3b). Figure 3d, shown in yellow color, is implying that there is possible interaction between the two proteins in nucleus.

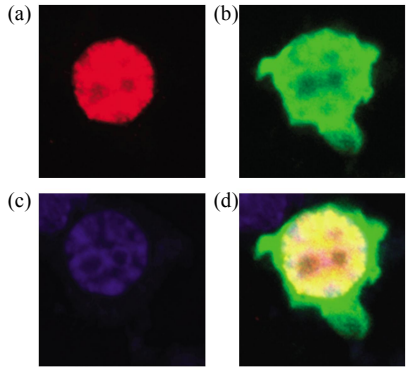

Fig. 3 Indirect immunofluorescence result

(a) Analysis of the localization of HA-NLS-RARα with anti-HA and anti-rabbit IgG antibodies (red). (b) Analysis of the localization of Myc-JTV1 with anti-Myc and anti-mouse IgG antibodies (green). (c) Nucleus stained with DAPI (blue). (d) Merge.

2.6 Interaction between NLS-RARα and JTV1 *in vitro*

After pGEX4T-2-NLS-RARα and pGEX4T-2 were transformed into JM109, GST-NLS-RARα and GST were expressed in the supernatant. After added into the glutathione Sepharose 4B, the sedimentum was mixed with the cell lysates containing Myc-JTV1 fusion protein respectively. The precipitate was harvested by centrifugation and washed five times using PBS. The pellets were identified *via* Western blotting, the result showed the interaction between GST-NLS-RARα and JTV1 *in vitro* (Figure 4).

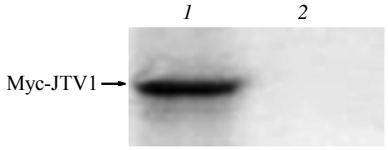

Fig. 4 Western blot analysis for GST pull-down with Myc monoclonal antibody

1: GST-NLS-RARα and JTV1; *2*: GST and JTV1.

2.7 Interaction between NLS-RARα and JTV1 *in vivo*

In order to confirm that these two proteins are interacted with each other *in vivo*, co-immunoprecipitation was carried out. The blot was probed with Myc monoclonal antibody, and be recognized by the secondary antibody, as we expected (Figure 5).

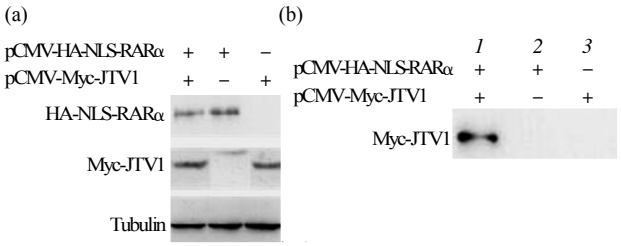

Fig. 5 Western blot analysis for CO-IPs with Myc monoclonal antibody

(a)Western blot analysis for whole-cell lysate. (b)Western blot analysis for CO-IPs with Myc monoclonal antibody, *1*: pCMV-HA-NLS-RARα and pCMV-Myc-JTV1 cotransfection; *2*, *3*: pCMV-HA-NLS-RARα/ pCMV-Myc-JTV1 was transfected respectively.

3 Discussion

The production of the oncogenic fusion protein PML-RARα is a principal cause of APL. In APL, PML-RARα leads to the deregulation of wild type PML and RARα function, thus inducing the differentiation block [4, 5], and epigenetic alterations could actively contribute to the development of APL[6]. PML-RARα may need to be cleaved to become fully oncogenic [1]. Neutrophil elastase (NE) was shown to cleave PML-RARα at several positions, and it was demonstrated that mice deficient for this protein developed APL at a greatly reduced rate when mated with PML-RARα knock-in mice. The cleavage products of NE were mutational PML protein (53 ku) and NLS-RARα protein (69 ku). The interaction of PML-RARα with neutrophil elastase can cause toxic insult to early myeloid cells, or it can confer a proliferative advantage, depending on the dose of PML-RARα that is expressed [2], but their action mechanism is still unknown. The NLS-RARα protein is one of the cleavage products that can be localized within the nucleus *via* the action of its NLS, which suggested different function of NLS-RARα from that of RARα protein in cells. Therefore, the identification of the intracellular proteins directly-interacting with PML-RARα is helpful to elucidate the possible pathway of PML-RARα signaling.

In our study, the bait plasmid pGBKT7-NLS-RARα was transformed into yeast strain AH109, which was subsequently mated with yeast strain Y187 containing leukemia cDNA library, to select the diploid yeast cells. After reintroduced into yeast strain AH109, eight positive clones were obtained and verified. One of such colonies containing JTV1 was further cloned, indirect immunofluorescence result implying that there are possible interaction, and the interaction between JTV1 and NLS-RARα were verified by GST pull-down *in vitro* and co-immunoprecipitation *in vivo* successfully. JTV1 as a DNA-repairing gene encoding p38 protein promotes apoptosis [7]. It is a factor associated with a protein complex consisting of several different aminoacyl-tRNA synthetases, which acts as a scaffold for the assembly and stability of the multi-tRNA synthetase complex [8]. The interaction between NLS-RARα and p38 may influence the structure and the turnover of the multi-tRNA synthetase complex, and thus to affect cell growth and differentiation.

Besides JTV1, other proteins in Table 1 also have important roles. CKS1B (cyclin kinase subunit 1B) gene maps an amplicon at chromosome 1q21 region. Over-expression of CKS1B, mainly due to gene amplification, imparts a poor prognosis in multiple myeloma (MM)[9, 10]. Glutamate-ammonia ligase (GLUL) is expressed throughout the body and plays an important role in controlling body pH and in removing ammonia from the circulation. UBQLN1 (an ubiquitin-like protein) associates with both proteasomes and ubiquitin ligases, and are thought to functionally link the ubiquitination machinery to the proteasome to affect *in vivo* protein degradation [11]. COPS5 is one of the eight subunits of COP9 signalosome, a highly conserved protein complex that functions as an important regulator in multiple signaling pathways [12]. ERH may be an important transcription regulator that also functions in the control of cell-cycle progression [13]. ISCA1 implicated in iron-sulfur cluster biogenesis [14]. How the interactions between NLS-RARα and the above-mentioned interacting proteins affect the development of acute promyelocytic leukemia needs to be further studied.

Currently there are no reports about the interaction between JTV1 and PML-RARα/RARα. Our experimental results can provide some useful information to understand PML-RARα/RARα signaling in APL pathogenesis.

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NLS-RARα蛋白相互作用蛋白的筛选与验证 *

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摘要 急性早幼粒细胞白血病(APL)具有特征性染色体易位，产生的 PML-RARα 融合基因在其发生发展中有重要作用. PML-RARα 融合蛋白在细胞内被中性粒细胞弹性蛋白酶(neutrophil elastase，NE)切割为 PML 突变蛋白(核定位信号 NLS 缺失)和 RARα 突变体 (NLS-RARα，包含有 PML 的核定位信号)，这两段蛋白质在 APL 的发生中可能具有重要作用. 为进一步研究 NLS-RARα 的生物学功能，运用酵母双杂交技术在白血病 cDNA 文库中筛选与其作用的蛋白质. 首先 PCR 技术扩增 NLS-RARα 编码序列，克隆至诱饵载体 pGBKT7，测序鉴定后将其转化酵母 AH109. 免疫印迹检测到诱饵蛋白表达后，将含有诱饵载体的 AH109 与含有白血病 cDNA 文库的酵母 Y187 交配，在含有 X-α-gal 的营养缺陷性培养基上选择和筛选二倍体酵母. 经回转实验和测序分析验证得到 8 个与 NLS-RARα 相互作用的蛋白质. 为进一步验证这些相互作用，克隆其中的 JTV-1 蛋白，利用间接免疫荧光，GST pull-down 和免疫共沉淀技术成功验证了它与 NLS-RARα 的相互作用. 为进一步探讨 APL 的发生机制提供了新的线索.

关键词 NLS-RARα 蛋白，白血病，酵母双杂交，蛋白质相互作用

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