http://www.abbs.info e-mail:abbs@sibs.ac.cn
ISSN 0582-9879 ACTA BIOCHIMICA et BIOPHYSICA SINICA 2003, **35**(1):13-17
CN 31-1300/Q

Expression, Purification and *in vitro* N-myristoylation of Human Src N-terminal Region

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Abstract The DNA fragment encoding N-terminal region of human c-Src was amplified from Caco-2 cell total RNA by RT-PCR and cloned into vector pMFHT to obtain His-tag fusion expression plasmid pMF-SrcHT, which was based on T7 expression system. The fusion protein SrcHT was highly expressed in *E.coli* BL21(DE3) harboring the pMF-SrcHT and purified from bacterial lysate by Ni-IDA affinity chromatography. The assays using [^{3}H]-labeled substrate demonstrate that the purified fusion protein SrcHT can be effectively N-myristoylated by recombinant human myristoyl-CoA: protein N-myristoyltransferase (NMT) *in vitro*. This work is a basis for further biochemical studies and development of new anti-cancer chemotherapeutic drugs based on specific inhibition of N-myristoylation of human Src.

Key words Src; His-tag; fusion expression; N-myristoylation

V-Src, a transforming product of Rous sarcoma virus, is a tyrosin kinase. Its cellular homologue (c-Src) has a widespread cellular distribution. The family of Src-related protein tyrosine kinases includes nine members. There are common multiple regulatory domains for the Src family proteins to respond to a number of receptor-mediated signals with changes of both kinase activity and intracellular localization[1]. It has been shown that the membrane association of Src protein requires addition of myristic acid to the N-terminal glycine via an amide linkage[2].

N-myristoylation is a biochemical modification of proteins in which the myristic acid (C14: 0) is co-translationally linked to NH2-terminal glycine residues of various cellular and viral proteins[3]. The enzyme responsible for transferring myristate onto the N-terminus of the protein substrates is myristoyl-CoA: protein N-myristoyltransferase[4] (NMT, EC 2.3.2.97). A large number of cellular N-myristoylproteins with diverse functions have been identified. With the essential role of N-myristoylproteins in many physiological and pathological events such as signal transduction, carcinogenesis and viral replication and assembly, N-myristoylation of proteins by NMT, therefore, has been recognized as possible chemotherapeutic target for anti-viral, anti-fungal and anti-neoplastic therapy[5].

Replacement of the N-terminal glycine in Src with either alanine or glutamic acid preventing myristoylation and morphological transformation indicates that myristoyl moiety is essential for transforming activity of Src protein kinase[6], which is activated in human colon carcinoma, compared with that in normal colon tissues or cultures of normal colon mucosal cells[7]. Moreover, increased NMT activity has also been observed in rat and human colonic tumors[8]. Accordingly, N-myristoylation of Src has been proposed as a target for developing chemotherapeutic drugs against colon cancer. The N-terminal 16 residues of Src have been synthesized for studying N-myristoylation and its correlated functions[9]. In this paper, His6-tagged N-terminal region of Src was expressed and purified from *E. coli* as a substrate of NMT, which provides a basis for biochemical studies and exploration of new anti-cancer chemotherapeutic drugs based on specific inhibition of N-myristoylation of Src.

1 Materials and Methods

1.1 Materials

1.1.1 *Plasmid and bacteria* Expression plasmid pMFHT[10] containing His-tag coding sequence is constructed in our lab. *E. coli* XL1-Blue and BL21(DE3) are used as hosts for cloning and protein expression.

1.1.2 *Enzymes and reagents* Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Boehringer Mannheim or Gibco BRL Company. Agarose was purchased from Gibco BRL Company. Pseudomonas acyl CoA synthetase, LiCoA, acrylamide, bisacrylamide, IPTG (isopropylthio-β-*D*-galactoside), PMSF (phenylmethylsulfonyl fluoride), imidazole and iminoacetic acid (IDA)-Sepharose 6B were purchased from Sigma Company. [9,10(n)-^{3}H] myristic acid, AmplifyTM and Hyperfilm were obtained from Amersham Company. Trizol reagent was from Gibco BRL Company. M-MLV Reverse Transcriptase was from Promega Company. *Taq* DNA polymerase was from Sino-American Biotechnology Company.

1.2 Methods

1.2.1 *Cell culture* Caco-2 cells are propagated in DMEM (Gibco BRL) containing 20% fetal bovine serum, 100 u/mL penicillin and 100 mg/L streptomycin at 37 ℃ and in 10% CO_2.

1.2.2 *RT-PCR* Primer 1(5'-GGACCATGGGTAGCAACAAG-3') as the forward primer with a *Nco*I site (underlined) and primer 2(5'-AGGGAATTCGCCTGGATGGAGTCG-3') as the reverse primer with an *Eco*RI site (underlined) were designed for amplifying DNA fragment encoding N-terminal region of human Src (147 amino acids), and synthesized in Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences. The total RNA from Caco-2 cells was prepared according to single step acid guanidinium thiocyanate phenol chloroform method (Trizol Regent, Life Technologies, Inc). The first-strand cDNA synthesis was performed by annealing 4 μg of total RNA from Caco-2 cells with 0.5 μg of oligo(dT) (12 – 18 in length) in a total volume of 20 μl and reverse transcribing with 200 u of M-MLV

Reverse Transcriptase at 37 ℃ for 60 min. After this reaction, the DNA fragment encoding N-terminal region of human Src was amplified from 1 μl aliquot of the mixture in a PCR reaction containing 10 mmol/L Tris-HCl(pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L for each dNTP, 0.4 μmol/L of primer 1 (forward primer) and primer 2 (reverse primer) and 1 u of *Taq* polymerase. The reaction mixture was denatured at 94 ℃ for 45 s, annealed at 50 ℃ for 45 s, and polymerized at 72 ℃ for 45 s. Thirty cycles were performed and followed by a 10-min extension at 72 ℃.

1.2.3 *Construction of expression plasmid pMF-SrcHT* The amplified DNA fragments encoding N-terminal region of human Src were inserted into upstream of His-tag sequence on an expression vector of pMFHT under the control of T7 promoter. The expression plasmid pMF-SrcHT was obtained by analysis of restriction digestion and DNA sequencing. All of the DNA manipulation or identification including the digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments and ligation with T4 DNA ligase were performed as described by Sambrook *et al*.[11].

1.2.4 *Expression of the fusion protein SrcHT* *E. coli* BL21(DE3) harboring the fusion expression plasmid pMF-SrcHT was grown to A_{600}= 0.4 – 0.6 in LB (Luria-Bertani medium) containing 100 mg/L ampicillin at 37 ℃, and then induced to produce the fusion SrcHT by adding IPTG to a final concentration of 0.5 mmol/L and the incubation was extended for additional 3 h. The cells were harvested centrifugation for 10 min at 5000 g and samples were analyzed by 15% SDS-PAGE according to the methods of LaemmLi[12].

1.2.5 *Purification of the fusion protein SrcHT* The recombinant SrcHT was purified using one-step Ni-IDA affinity chromatography[13]. Briefly, the induced *E. coli* BL21(DE3) cells harboring the expression plamid pMF-SrcHT were harvested by centrifugation for 10 min at 5000 *g*, resuspended in buffer A (20 mmol/L Tris-HCl pH 7.9, 0.5 mol/L NaCl, 10% glycerol, 1 mmol/L PMSF, 40 mmol/L imidazole) and sonicated on ice for 30 min for 30 times (with 1 min interval per time). The supernatant fraction was obtained from the sonicated bacterial lysate by centrifugation for 30 min at 10 000 *g* and applied to Ni-IDA agarose column at a constant flow-rate of 4 mL per min. Then, the non-specific bound proteins were removed with buffer A (20 mmol/L Tris-HCl pH 7.9, 0.5 mol/L NaCl, 10% glycerol, 1 mmol/L PMSF, 40 mmol/L imidazole) and the recombinant SrcHT was eluted with buffer B (20 mmol/L Tris-HCl pH 7.9, 0.5 mol/L NaCl, 10% glycerol, 1 mmol/L PMSF, 200 mmol/L imidazole). The eluted proteins were identified by 15% SDS-PAGE analysis described as above.

1.2.6 *In vitro N-myristoylation assay* Firstly, [^{3}H]-labeled myristoyl-CoA was synthesized as described by Towler *et al.*[14] and the reaction mixture containing 20 mmol/L Tris-HCl (pH 7.4), 1 mmol/L dithiothreitol, 10 mmol/L MgCl2, 0.1 mmol/L EGTA, 5 mmol/L ATP, 10 mmol/L LiCoA, 1 μmol/L [9,10-^{3}H] myristic acid (5.2 μCi), and 0.3 u/mL pseudomonas acyl CoA synthetase was allowed to incubate at 30℃ for 30 min. Then, the NMT assay was carried out in a final 20 μl volume of NMT buffer containing 30 mmol Tris-HCl (pH 7.4), 0.5 mmol/L EDTA, 0.45 mmol/L 2-mercaptoethanol, 10 μmol/L peptide, 1% Triton X-100, and the purified recombinant human NMT. The NMT reaction was performed by adding 10 μl synthesized [^{3}H]-myristoyl-CoA in each reaction firstly, then with incubation at 30 ℃ and extension for 30 min and was stopped by boiling for 5 min. Finally, the boiled NMT reaction mixture was subjected to 15% SDS-PAGE and the [^{3}H]-myristoyl-peptides were analyzed by autoradiography.

2 Results and Discussion

2.1 Construct for expression of the fusion protein SrcHT

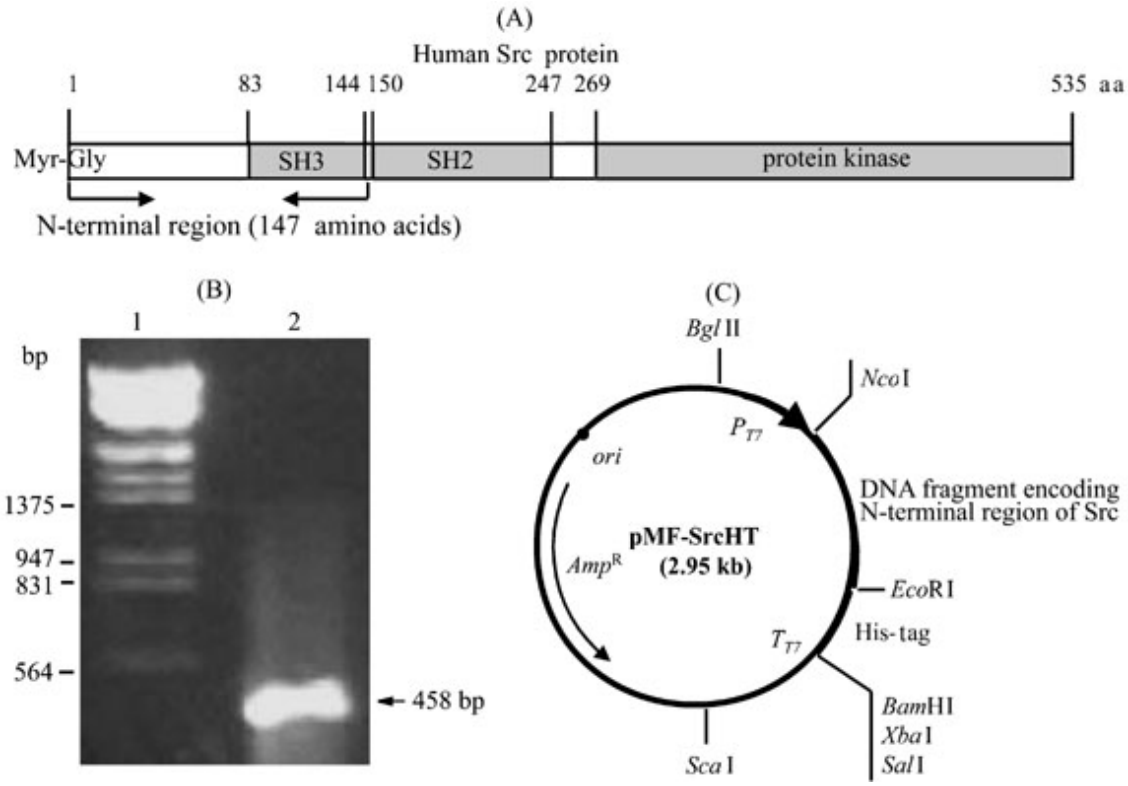

Fig.1 Construction of expression plasmid for the fusion protein SrcHT

(A) Diagram of human Src protein and its N-terminal region (147 amino acids, arrowed oppositely) containing the Gly residue at N-end. (B) Separation of amplified DNA fragment encoding N-terminal region of human Src. DNA fragment encoding N-terminal region of human Src (lane

2) was amplified from Caco-2 cell total RNA by RT-PCR and separated by 1.5% agarose gel electrophoresis with the size control of molecular weight markers (lane 1). (C) Construct for expression of the fusion protein SrcHT. DNA fragment encoding N-terminal region of human Src was inserted into upstream of His-tag sequence in expression vector controlled under T7 promoter and terminator.

N-terminal region (147 amino acids) of human Src containing N-end of Gly residue [Fig.1(A)] was designed to use as substrate of NMT. The relative DNA fragment was amplified from Caco-2 cell total RNA by RT-PCR with a set primers specific for human Src gene (GenBank accession No. AH002989). The 458 bp DNA fragment thus obtained was separated by 1.5% agarose gel electrophoresis [Fig.1(B)]. DNA fragment encoding N-terminal region of human Src was fused to upstream of His-tag sequence on expression vector pMFHT to yield expression plasmid pMF-SrcHT [Fig.1(C)]. The construct is used to express a 182-amino acid fusion protein SrcHT which consists of N-terminal region of human Src (2 – 148 amino acids) and an extension of 34-amino-acid C-terminus (149 – 182) containing His-tag (152 – 157 amino acids) encoded by vector sequence (Fig.2).

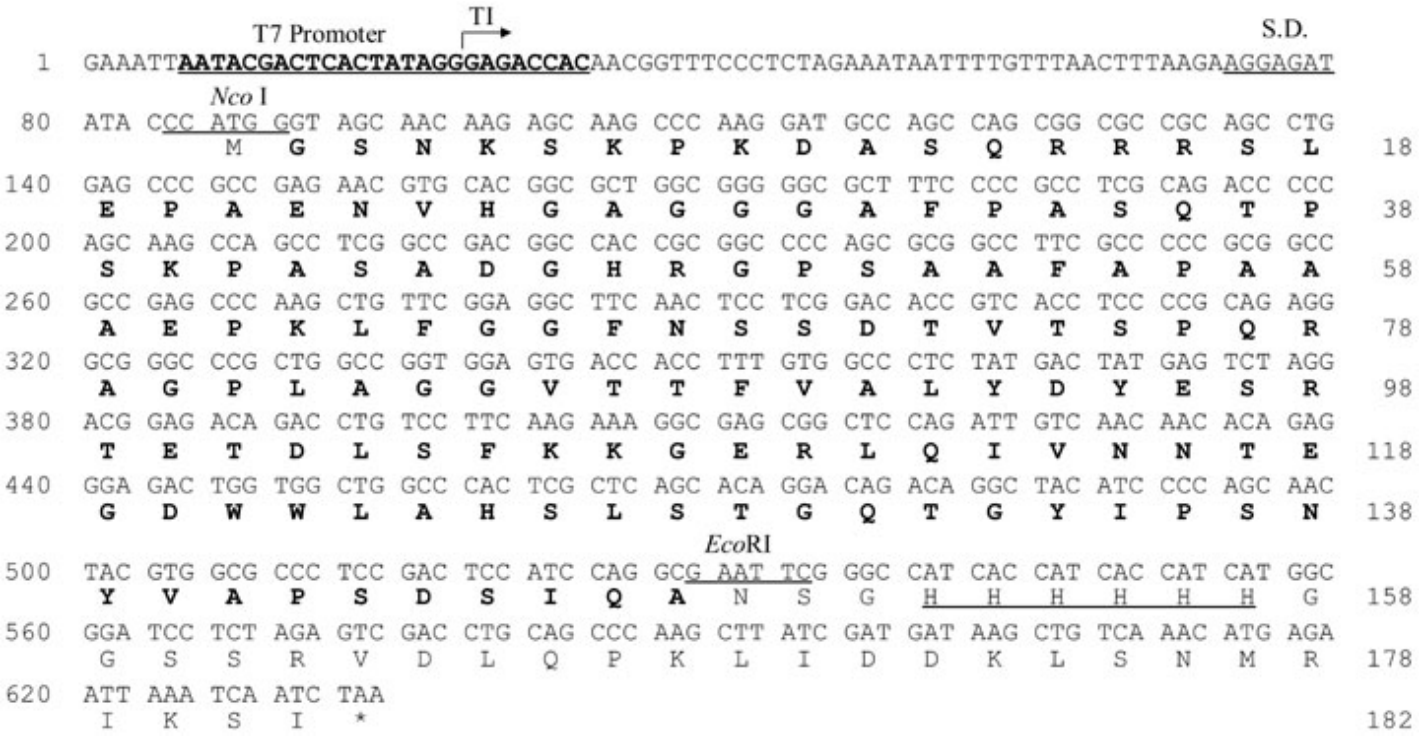

Fig.2 Sequences of the DNA fragment inserted into expression vector and the amino acids of human Src N-terminal region

The DNA fragment encoding human Src N-terminal region (2-148 amino acids) was inserted into the expression vector by *Nco*I and *Eco*RI site (underlined). T7 promoter sequence (bolded and underlined), transcription initiation (TI, arrowed) and Shine Dalgarno sequence (S.D., underlined) are the vector sequences presented at upstream of coding sequence for fusion protein SrcHT. The amino acids of human Src N-terminal region (2 – 148 amino acids) are bolded. The 34-amino-acid (149 – 182) C-terminal extension containing His-tag (152 – 157 amino acids, underlined) is encoded by vector sequence.

2.2 Expression and purification of the fusion protein SrcHT

After the expression plasmid pMF-SrcHT was transformed into *E. coli* BL21(DE3), SrcHT was highly expressed after induced by IPTG (Fig.3, lane 1 and 2). In order to determine whether the fusion protein is soluble or not, the cell pellet was resuspended by sonication on ice with sonication buffer. It is found that SrcHT was mainly in soluble form (Fig.3, lane 3 and 4). The lysate supernatant was applied to Ni-IDA agrose column for affinity chromatography, and SrcHT was purified in one step. Thirty four milligram of the fusion protein SrcHT has been successfully purified from 1 liter of induced bacteria by one-step affinity chromatography. SDS-PAGE results indicate that purity of the purified SrcHT is more than 95% (Fig.3, lane 5). The expressed and purified SrcHT is a good preparation for its N-myristoylation.

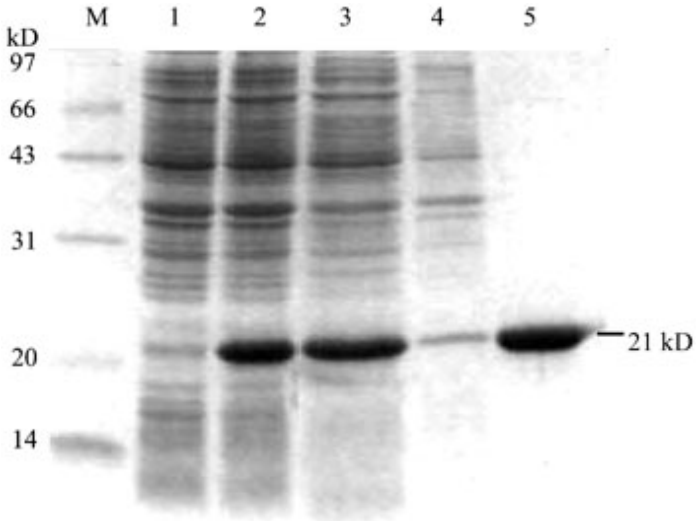

Fig.3 SDS-PAGE analysis of expression and purification of fusion protein SrcHT

The bacterial lysates were prepared from un-induced (lane 1) and induced (lane 2) *E. coli* BL21(DE3) cells harboring pMF-SrcHT by sonication. Then, the supernatant (lane 3) and pellet (lane 4) were obtained from the above induced bacterial lysate by centrifugation. Finally, fusion protein SrcHT (lane 5) is purified from the supernatant by Ni-IDA affinity chromatography. All of the above samples were directly subjected to 15% SDS-PAGE with the size control of molecular weight marker (lane M).

2.3 *In vitro* N-myristoylation of the fusion protein SrcHT

In vitro N-myristoylation assay of the purified SrcHT was performed with α subunit of mouse cAMP-dependent protein kinase (mCα4H)[15] as positive control. The results illustrate that the purified SrcHT [Fig.4(A), lane 2] can be efficiently N-myristoylated by NMT as same as the control of protein substrate mCα4H [Fig.4(A) lane 3], suggesting that they contains the N-end-glycine [Fig.4(B)] which is essential for N-myristoylation of proteins[16] and C-terminal fusion His-tag sequence do not affect its N-myristoylation. Since myristoylation of Src has been proposed as a potential target for developing chemotherapeutic drugs against colon cancer[7,8], the fact that the fusion protein SrcHT has been successfully expressed and purified as an effective substrate of N-mystoyltransferase will provide a good basis for studying the biochemical function and specific inhibitors for N-myristoylation of human Src.

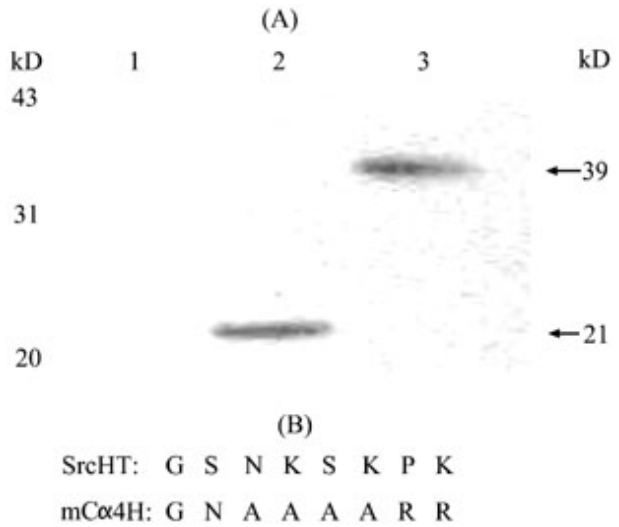

Fig.4 *In vitro* N-myristoylation assay

(A) Autoradiography of [^{3}H]-labeled-myristoyl-peptides. *In vitro* N-myristoylation assay of the purified SrcHT was carried out by using mCα4H as positive control. [^{3}H]-myristoyl peptides were separated by 15% SDS-PAGE and then exposed by autoradiography, which indicated the negative control without NMT substrate (lane 1), expressed SrcHT (lane 2) and positive control with mCα4H (lane 3). (B) N-terminal amino acids of SrcHT and mCα4H as NMT substrates.

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Received: July 22, 2002 Accepted: September 6, 2002
This work was supported by grants from the National Nature Science Foundation of China (No.39770875 and 39425005) and the Foundation of Shanghai Science and Technology Commission (No.97XD14022)
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