BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **230,** 81–84 (1997)
ARTICLE NO. RC965887

# Expression of Src-like Adapter Protein mRNA Is Induced by All-trans Retinoic Acid

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Received November 26, 1996

**By using a differential display method, specific bands were selected from ladder PCR products derived from ATRA-dependent differentiated U937 cells, in comparison with those of untreated U937. By screening the cDNA library of ATRA-dependent differentiated U937 cells with one of the PCR products, we cloned the src-like adapter protein (SLAP). Northern blot analysis of U937 cells with or without ATRA treatment indicated that the SLAP mRNA was clearly induced by ATRA. The induction was inhibited by the addition of cycloheximide, indicating that ATRA acted indirectly through synthesis of other proteins. The SLAP mRNA was induced in HL60 and NB-4 but not in K562 or THP-1. Interestingly, these cells in which SLAP mRNA was induced by ATRA all showed ATRA-dependent cell differentiation. The relationship between SLAP and cell differentiation is unclear, but SLAP may transduce a signal for cell differentiation.**


The differential display technique developed by Liang P. and Pardee A.B. is a powerful tool for identifying and cloning differentially expressed genes (1). This method involves the reverse transcription of the mRNAs with oligo-dT primers anchored to the beginning of the poly (A) tail, followed by the polymerase chain reaction (PCR) in the presence of a second 10-mer arbitrary primer. By changing primer combinations, many individual mRNAs were visualized as different-sized PCR products on the gel. Differentially expressed genes were easily identified by analysis of the mRNA samples side by side and recovered to clone their cDNAs from a cDNA library.

A monoblast-like human histiocytic lymphoma cell line, U937, can be induced to differentiate into monocyte-like cells by incubation with all-trans retinoic acid (ATRA) (2). Since we were interested in the genes associated with cell differentiation, we performed the differential display method using mRNA of ATRA-dependent differentiated U937 for cloning differentially expressed genes compared with untreated U937.

We found that expression of src-like adapter protein (SLAP) mRNA was induced in the ATRA-dependent differentiated state of U937 cells.

## MATERIALS AND METHODS

*Cell lines.* U937 (histiocytic lymphoma, human), HL60 (acute myelocytic leukemia, human), K562 (chronic myelocytic leukemia, human), and THP-1 (acute monocytic leukemia, human) were purchased from American Type Culture Collection. NB-4 is a cell line derived from acute promyelocytic leukemia patient with t(15; 17) (3), and was a kind gift from Dr. Michel Lanotte. These cell lines were all cultured in RPMI1640 containing 10 % fetal calf serum.

*Differential display.* RNAmap kit (GenHunter Corporation, Brookline, MA) was used. Briefly, total RNA pretreated with DNase I (MessageClean kit, GenHunter Corporation) was reverse-transcribed with oligo-dT primer, followed by the PCR with the same oligo-dT primer and the second 10-bp arbitrary primer. PCR was performed in the presence of $^{35}$S-dATP under the following condition: 94°C, 30 sec; 40°C, 2 min, and 72°C, 30 sec for 40 cycles. The PCR products were electrophoresed on 6 % urea-containing polyacrylamide gel. The gel was dried and exposed to X-ray film. The PCR band of interset was cut out from the gel, reamplified with the same primer set, cloned into pCR-TRAP vector (GenHunter Corporation), and used as a probe for screening the cDNA library.

*Screening and sequencing of cDNA library.* The lambda phage cDNA library was constructed from the mRNA of ATRA-dependent differentiated U937 by using Superscript lambda system (Gibco BRL) and Gigapack II packaging kit (Stratagene) according to the manufacturer's instruction. The cDNA library was screened using the PCR product labelled with horseradish peroxidase using an enhanced chemiluminescence (ECL) kit (Amersham) as described previously (4). Inserted DNA in the phage was subcloned in pZL1 plasmid (Gibco BRL), and sequenced by an automatic sequencer (Applied Biosystems).

*Northern blot analysis.* Northern blotting was performed as described elsewhere (4). Briefly, total RNA was isolated from cell lines

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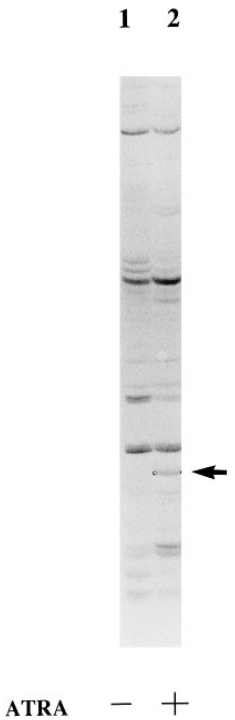


**FIG. 1.** Differential display of U937 cells cultured with or without ATRA. After U937 cells were cultured with or without 1 mM ATRA for 3 days, total RNA was isolated and subjected to the differential display method as described in Materials and Methods. Arrow indicates a differentially expressed PCR band.

with RNAzol B (Cinna/Biotecx Laboratories, USA), fractionated by electrophoresis on 1 % formaldehyde/agarose gel, and blotted onto an Immobilon-N membrane (Millipore, USA). The membrane was hybridized with a probe derived from SLAP cDNA and b-actin cDNA, radiolabelled by using a Multiprime DNA labelling kit (Amersham). Hybridization was performed at 6 × SSC, 68°C for 16 hr, followed by washing at 2 × SSC, 25°C for 30 min.

## RESULTS

*Molecular cloning from the cDNA library of ATRA-dependent differentiated U937 cells.* The ATRA-dependent differentiation of U937 cells was certified by the morphology, nitroblue tetrazolium (NBT) reduction assay, and fluorescence-activated cell sorter (FACS) analysis of the surface markers (CD 11a and CD11c) (data not shown). Using this differentiation model, we selected by the differential display method some PCR bands which were specific in the ATRA-dependent differentiated U937 cells (one of them is shown in Figure 1). By screening of ATRA-stimulated U937 cDNA library with the PCR band (as a probe), we obtained the plasmid (named 4-4) containing an approximately 3-kb DNA insert. Sequencing of the DNA insert of plasmid 4-4 revealed that the sequence of this cDNA shows 100 % homology to that of src-like adapter protein (SLAP) reported recently by Angrist M. et al. (5). The sequence

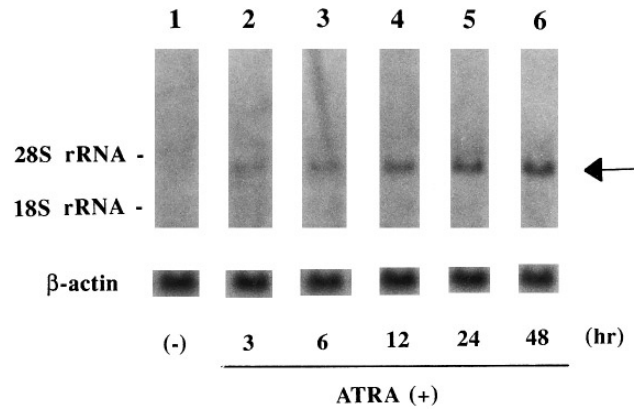


**FIG. 2.** Northern blot analysis of U937 cells cultured with or without ATRA. U937 cells were cultured with 1 mM ATRA for indicated hours. Ten micrograms of total RNA was applied on each lane. Arrow indicates mRNA of SLAP.

reported by them contained 1,074 bp of 5′-side of SLAP cDNA containing entire open reading frame (ORF). On the other hand, the plasmid 4-4 had the entire sequence from 5′-non-coding region and ORF to poly-A tail of SLAP cDNA and the sequence has been deposited in DNA database (Accession No. D89077).

*ATRA-dependent induction of SLAP mRNA.* Northern blot analysis of U937 cells with or without ATRA treatment revealed that the expression of the SLAP mRNA was clearly induced by ATRA in a time-dependent manner (Figure 2). This induction was inhibited by the addition of cycloheximide, indicating that the induction was indirect and through synthesis of other proteins (Figure 3). Of the cell lines HL60, NB-4, K562, and THP-1 examined, the expression of SLAP mRNA was clearly induced in the former two cell lines (Figure 4). Interestingly, HL60 and NB-4 are also differentiated by ATRA (2,3) but differentiation of K562 and THP-1 are not induced by ATRA. U937 cells are also

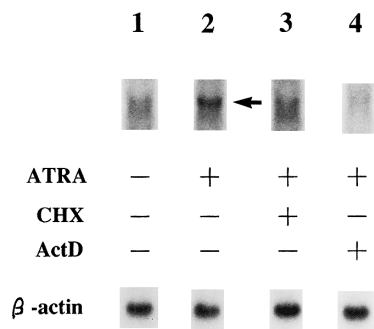


**FIG. 3.** Northern blot analysis of U937 cells cultured with ATRA and cycloheximide. U937 cells were cultured in the following conditions for 12 hours: lane 1, ATRA (−); lane 2, ATRA 1 mM; lane 3, ATRA 1 mM plus cycloheximide (CHX) 2 mg/ml; and lane 4, ATRA 1 mM plus actinomycin D (ActD) 0.08 mg/ml. Ten micrograms of total RNA was applied on each lane. Arrow indicates mRNA of SLAP.

differentiated toward monocytic cells by TPA. However, TPA did not induce the expression of SLAP mRNA (Figure 5). These findings indicated that the expression of SLAP mRNA was not related to monocytic differentiation but to ATRA-stimulation. This was in agreement with the findings in HL60 cells that the expression of SLAP mRNA was found in ATRA-dependent granulocytic differentiation, but not in TPA- or DMSO-dependent monocytic differentiation (Figure 5).

## DISCUSSION

The entire sequence of SLAP cDNA was cloned by comparison of the ATRA-dependent differentiated U937 cells with untreated U937 cells by a differential display method. The mouse 5′-side sequence of SLAP cDNA has been recently cloned as a molecule that interacts with the cytoplasmic domain of the Eck receptor tyrosine kinase by using a yeast two-hybrid system by Pandey A. et al. (6), and the 5′-side sequence of human homologue of SLAP has already reported by Angrist M. et al. (5). SLAP is classified as an adapter protein like Grb2, Crk, and Nck in that it possesses SH2 and SH3 domains but no catalytic tyrosine kinase domain (7). The SH2 domains of the molecules bind to phosphotyrosyl peptide motifs and mediate the assembly of signaling complexes (8,9), and the SH3 domain binds to small proline-rich peptide motifs to mediate protein-protein interactions (10,11), thus transducing signals in the cell. The SH3 domain in Grb-2 protein has been reported to link receptor tyrosine kinase to Sos protein (activator of Ras protein) (12). The characteristic features of SLAP strikingly resemble those of the corresponding SH2 and SH3 domains in the Src family. The SH2 domains of Grb2, Crk, and Nck adapter proteins share a 27,25, and 27 % homology to that of the Src family whereas SLAP is 51 % identical in the region. Similarly, the SH3 domains of the other adapter proteins share an approximately 33 % homology compared with a 50 % homology in SLAP. This suggests that SLAP may play an important role as an adapter protein in the signal transduction of Src family.

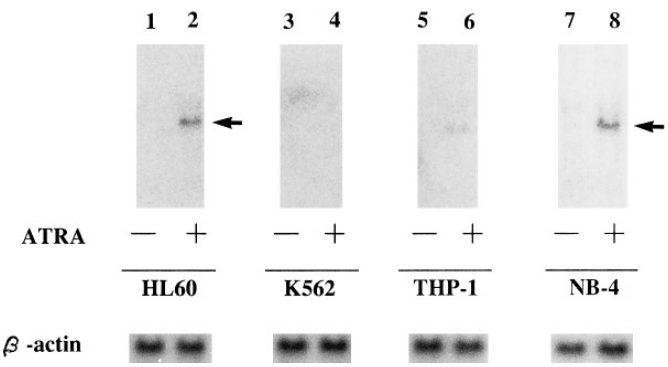


**FIG. 4.** Northern blot analysis of SLAP in cell lines. After HL60, K562, THP-1, and NB-4 cells were cultured with or without 1 mM ATRA for 3 days, total RNA was isolated. Ten micrograms of total RNA was applied on each lane. Arrow indicates mRNA of SLAP.

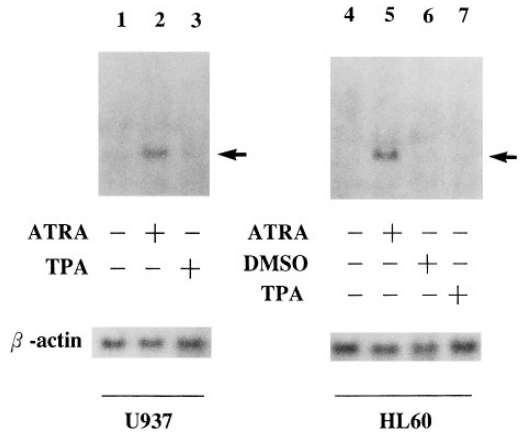


**FIG. 5.** Northern blot of SLAP in U937 and HL60 cells under various conditions. U937 and HL60 cells were cultured for 3 days under the following conditions: untreated (lane 1, 4), 1 mM ATRA (lane 2, 5), 1.3% (v/v) dimethyl sulfoxide (DMSO) (lane 6), and 0.161 mM 12-O-tetradecanoylphorbol-13-acetate (TPA) (lane 3, 7) Ten micrograms of total RNA was applied on each lane. Arrow indicates mRNA of SLAP.

ATRA activates the expression of many genes (13,14), acts as an inducer of differentiation for many cell lines (15), and has recently been evaluated effective for the differentiation-inducing treatment of acute promyelocytic leukemia (APL) (16). Interestingly, in the ATRA-sensitive APL cell line, NB-4, the expression SLAP mRNA was induced by ATRA stimulation (Figure 4). Besides NB-4, in U937 and HL60 (which are also known to be ATRA-sensitive on the differentiation), SLAP mRNA expression was also upregulated, although in ATRA-dependent-differentiation-insensitive K562 and THP-1, SLAP mRNA was not clearly induced (Figure 4). Altough K562 and THP-1 are not differentiated by ATRA, ATRA is reported to have active effects on these cell lines (14,17). These findings suggests that SLAP may transduce a signal for cell differentiation. Although the function of SLAP still remains to be clarified, our findings help to elucidate the differentiation-inducing function of ATRA.

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