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Expression, purification and biochemical characterization of the N-terminal regions of human TIG3 and HRASLS3 proteins

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ABSTRACT

Tarzarotene-induced gene 3 (TIG3) and HRAS-like suppressor (HRASLS3) are members of the HREV107 family of class II tumor suppressors, which are down-regulated in various cancer cells. TIG3 and HRASLS3 also exhibit phospholipase activities. Both proteins share a common domain architecture with hydrophilic N-terminal and hydrophobic C-terminal regions. The hydrophobic C-terminal region is important for tumor suppression. However, the function of the hydrophilic N-terminal region remains elusive. To facilitate biochemical characterizations of TIG3 and HRASLS3, we expressed and purified the N-terminal regions of TIG3 and HRASLS3, designated TIG3 (1–134) and HRASLS3 (1–133), in a bacterial system. We found that the N-terminal regions of TIG3 and HRASLS3 have calcium-independent phospholipase A_2 activities. Limited proteolysis revealed that TIG3 (1–132) is a structural domain in the N-terminal region of TIG3. Our data suggest that the hydrophobic C-terminal regions might be crucial for cellular localization, while the hydrophilic N-terminal regions are sufficient for the enzymatic activity of both TIG3 and HRASLS3.

Introduction

Tarzarotene-induced gene 3 (TIG3)[1], also known as retinoid-inducible gene 1 (RIG1) and retinoic-acid receptor responder 3 (RARRES3), is a member of HREV107 family proteins, class II tumor suppressors, in human. TIG3 induces cellular apoptosis, differentiation and growth suppression [1–6]. Low-level or decreased expression of TIG3 has been noted in multiple carcinomas and psoriasis [7–10]. Examples of carcinomas associated with the low-level expression of TIG3 include ovarian carcinoma, hepatocellular carcinoma, cholanggiocarcinoma, skin cancer, and poorly differentiated colorectal adenocarcinoma. TIG3 inhibits the Ras signaling pathway by suppressing Ras activation in HtTA cervical cancer cells [2]. Interferon-γ-induced TIG3 down-regulates HER2 by repression of promoter activity, resulting in down-regulation of the PI3K/Akt/mTOR/VEGF pathway in ovarian cancer cells [11].

Human HRAS-like suppressor 3 (HRASLS3), another HREV107 family member, also suppresses growth in HRAS-transformed cells and exhibits reduced expression levels in various tumor cells such as ovarian carcinoma, testicular germ cell tumors and nasopharyngeal carcinoma [12–15]. HRASLS3 binds to the regulatory subunit A of protein phosphatase 2A (PR65α) and sequesters the catalytic subunit (PR36) from it, inhibiting its catalytic activity [16].

TIG3 and HRASLS3 share a common architecture featuring four domains: proline-rich motif, H-box, NC domain in the N-terminal region (1–133) and membrane-anchoring domain at the C-terminal region [17–19]. The exact function of the N-terminal region remains unclear while the C-terminal region is reported to be essential to localization at the Golgi, regulating the tumor suppression functioning of the HREV107 family proteins [2,6].

Recent studies have suggested that TIG3 and HRASLS3 manifest phospholipase A (PLA) activity, implicating that the phospholipid metabolism might be closely related to their tumor suppressor function [20–22]. TIG3 and HRASLS3 show a relatively high (~51%) sequence identity, which reflects the structural and func-

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[1] *Abbreviations used:* TIG3, tarzarotene-induced gene 3; HRASLS3, HRAS-like suppressor; RIG1, retinoid-inducible gene 1; RARRES3, retinoic-acid receptor responder 3; IPTG , isopropyl-D-thiogalactopyranoside.

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tional similarity between them. Despite of their functional significance in carcinogenesis, no detailed three-dimensional structures for the HREV107 family proteins have been elucidated yet. To facilitate the biochemical characterizations of both TIG3 and HRASLS3, we expressed several constructs of human TIG3 and HRASLS3 in *E. coli* and purified the N-terminal regions of the TIG3 and HRASLS3. Limited proteolysis revealed a structural domain in the N-terminal region of both TI3 and HRASLS3. We were able to determine that the PLA activity resides in the N-terminal regions of both proteins.

Materials and methods

Cloning

Constructs covering the N-terminal regions of TIG3 and HRASLS3 were prepared by subcloning the corresponding inserts into pET28b(+) (Novagen) at the NdeI/XhoI restriction enzyme sites. The resulting constructs have an extra, 21-residue (MGSSHHHHHH SSGLVPRGSH) protein sequence including a six-residue poly histidine tag on the amino-terminus of the recombinant proteins. Other constructs of HRASLS3 were prepared using the pHis vector (modified from Novagen's pET32a) with NdeI/XhoI restriction enzyme sites. These constructs contain a 10-residue poly histidine tag (LEHHHHHHHH) at the carboxyl-terminus of the recombinant proteins. The constructs were summarized in Table 1.

Expression and purification

The recombinant proteins were overexpressed in *E. coli* strain Rosetta 2 (DE3) (Novagen). Cells were grown in Terrific Broth (Bio Basic) to an OD600 of 0.7 at 37 °C and protein expression was induced by 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) at 37 °C. Cell growth continued at 37 °C for 5 h after the protein induction, and the cells were harvested by centrifugation. Cell pellets of TIG3 (1–134) were resuspended in ice-cold lysis buffer (25 mM Tris–HCl pH 7.4, 138 mM NaCl, 2 mM KCl, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride and 0.8 μM lysozyme) and kept at −80 °C. All of the protein purification steps were performed under the ice-cold condition. The frozen cells were rapidly thawed and then homogenized by sonication. The crude lysate was centrifuged at 36,000*g* for 1 h at 4 °C. The supernatant was applied to an Ni–NTA column (Qiagen) and washed with wash buffer (20 mM Tris–HCl pH 7.9, 60 mM imidazole, 500 mM NaCl and 10% (v/v) glycerol), after which the protein was eluted with elution buffer (20 mM Tris–HCl pH 7.9, 500 mM imidazole, 500 mM NaCl and 10% (v/v) glycerol). The fractions containing TIG3 were pooled, dialyzed against buffer A (20 mM Hepes pH 6.8, 40 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol) and applied to an SP-Sepharose FF column. The protein was eluted with a linear gradient of 40–1000 mM NaCl (0–100% buffer B, 20 mM Hepes pH 6.8, 1000 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol) over 10 column volumes. The eluted proteins were concentrated and applied to a Superdex 75 prep-grade column (GE Healthcare) with a buffer of 20 mM Tris–HCl pH 7.5, 100 mM NaCl and 1 mM dithiothreitol. In the HRASLS3 (1–133) purification, the Ni–NTA and Superdex 75 prep-grade columns were employed under the same buffer condition. The homogeneity of the purified proteins was analyzed by SDS–PAGE.

Table 1
Summary of constructs.

Genes	Vectors	Fusion tags	Constructs (amino acids)	Expression	Solubility
TIG3	pET28b	6 × His (N-term.)	1–164 (full-length)	+	–
			1–134	+++	+++
			1–132	+++	+++
			1–128	+++	+++
			1–126	+++	+++
			1–121	+++	–
			1–110	+++	–
			11–110	++	–
			11–134	++	–
HRASLS3	pET28b	6 × His (N-term.)	1–132	+++	+++
	pHis	8 × His (C-term.)	1–133	+++	+++
			11–133	+++	+
			11–110	+++	–
			1–110	+++	+

–, Insoluble; +, low; ++, medium; +++, high level expression.

Limited proteolysis

To determine the structural domain boundary, the purified TIG3 was incubated with porcine trypsin (Promega) for 2 h at 37 °C. The trypsin-digested TIG3 was analyzed by N-terminal amino acid sequencing and mass analysis using the Procise491 protein sequencing system (Applied Biosystems) and an Auto Flex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany), respectively.

Phospholipase A_2 activity assay

To assess the catalytic activity of the purified recombinant proteins, the PLA_2 activity was tested using an EnzChek® Phospholipase A_2 Assay Kit (Invitrogen). PLA_2 assays were performed according to the manufacturer's experimental protocol, except that PLA_2 stock was dissolved with a buffer of 20 mM Tris–HCl pH 7.5, 100 mM NaCl and 1 mM dithiothreitol. The assay was performed using a total volume of 100 μl per well. Recombinant TIG3 (1–134) and HRASLS3 (1–133) were mixed with the substrate-liposome mix at a ratio of 1:1. PLA_2 from honeybee venom was used as the positive control. The assay was performed at 28 °C, and the fluorescence was measured using a microplate reader (Spectra Max Gemini EM, Molecular Devices) for excitation at 480 nm and emission at 515 nm, after 30 min incubation.

Results

Expression and purification of recombinant human TIG3 and HRASLS3

We tested many TIG3 and HRASLS3 constructs (Table 1, Fig. 1A) for soluble expression in the bacterial system and chose the largest soluble construct for purification. Full-length TIG3 was expressed as insoluble, which might be due to the presence of the hydrophobic membrane-anchoring domain. Constructs lacking the N-terminal 10 residues (11–110, 11–134 for TIG3 and 11–133 for HRASLS3) were also insoluble, suggesting that the N-terminal 10 residues in both TIG3 and HRASLS3 might be important for protein folding. A construct devoid of a few residues at the end of the N-terminal region, TIG3 (1–121), was insoluble. By contrast, constructs extending beyond residue 121 – TIG3 (1–126, 1–128, 1–132, 1–134) and HRASLS3 (1–132, 1–133) – were expressed in the soluble form (Fig. 1A). These results indicate that residues 1–126 in both TIG3 and HRASLS3 are required for protein folding. We chose the longest soluble constructs, TIG3 (1–134) and HRASLS3 (1–133), for purification.

Purification of the N-terminal regions of TIG3 and HRASLS3 was performed in multiple chromatographic steps. Initially, the proteins were purified by nickel affinity chromatography. The purified protein showed an approximately 80% purity (Fig. 1B). The second

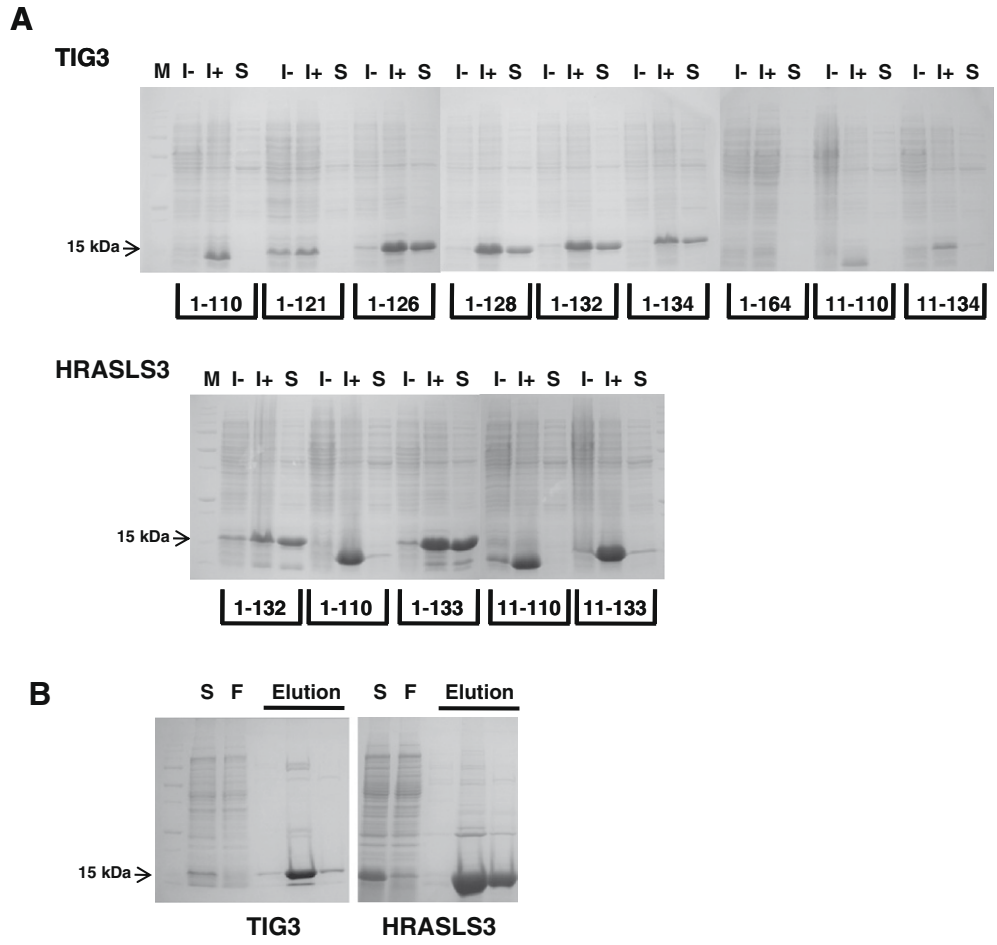

Fig. 1. Overexpression of recombinant TIG3 and HRASLS3. (A) SDS–PAGE analysis of recombinant TIG3 and HRASLS3. M, protein standard marker; I-, control of induction; I+, IPTG induction; S, supernatant fraction after cell lysis. (B) SDS–PAGE analysis of eluted TIG3 and HRASLS3 purified by Ni–NTA affinity column. S, supernatant fraction after cell lysis; F, flow-through during affinity column; Elution; elution fractions of Ni–NTA column.

purification step for TIG3 (1–134) was cation exchange chromatography on a SP-Sepharose FF column, which significantly enhanced the purity of TIG3. The final step was done using size exclusion chromatography on a Superdex 75 16/60 prep-grade column equilibrated with a buffer of 20 mM Tris–HCl pH 7.5, 100 mM NaCl and 1 mM dithiothreitol. SDS–PAGE analysis of the purified proteins confirmed a high purity. HRASLS3 (1–133) was purified by two-step procedure using nickel affinity chromatography followed by size exclusion chromatography on a Superdex 75 16/60 prep-grade column. SDS–PAGE analysis indicated that the purified HRASLS3 indeed was highly pure (Fig. 2A). The final yield of the purified proteins was about 4–5 mg per 1 L TB medium culture and these were sufficient for structural biology application.

Identification of a structural domain in the N-terminal region of TIG3

To identify the structural domain boundary of TIG3 (1–134), the purified TIG3 (1–134) was treated with porcine trypsin and analyzed by SDS–PAGE (Fig. 2A). One thick protein band of 10–15 kDa was observed and further analyzed by N-terminal sequencing and MALDI-TOF mass spectrometry. The N-terminal amino acid sequence of the trypsin-treated fragment of TIG3 (1–134) turned out to be Gly-Ser-His-Met-Ala, and its molecular weight was 15,206 (±100) Da. These results indicate that TIG3 (1–134) was cleaved by trypsin at the thrombin cleavage site of the construct and Lys-132, respectively. We conclude that the structural domain of TIG3 (1–134) corresponds to the residue 1–132 (Fig. 2B).

Phopholipase A_2 activity of TIG3 and HRASLS3

The PLA_2 activity of HRASLS3 has already been reported [20]. According to a more recent report, TIG3, HRASLS3, and HRASLS2 also show $PLA_{1/2}$ activity [21,22]. In the present study, we expressed and purified the N-terminal regions of human TIG3 and HRASLS3 to high purities in the bacterial expression system. We then tested whether these recombinant proteins manifest enzymatic activity. Using a fluorescence-based assay system, we observed that TIG3 (1–134) and HRASLS3 (1–132) do indeed show PLA_2 activity (Fig. 3). TIG3 (1–134) demonstrated a higher PLA_2 activity than did HRASLS3 (1–132), and the activities of both recombinant proteins were calcium-independent (Fig. 3). Our results demonstrate that the N-terminal region is sufficient to exhibit the PLA_2 activity in both TIG3 and HRASLS3. Previous studies have suggested that C-terminal-truncated TIG3 and HRASLS3 lack the tumor suppression activity [2,3,12]. We propose that the main function of the hydrophobic C-terminal regions in both TIG3 and HRASLS3 may be localizing these proteins to the Golgi.

Discussion

TIG3 and HRASLS3 of the HREV107 family are down-regulated in various cancer cells and their cellular functions may be important for cancer development. Recently, it has been reported that TIG3 and HRASLS3 possess PLA activity [20–22], raising the possi-

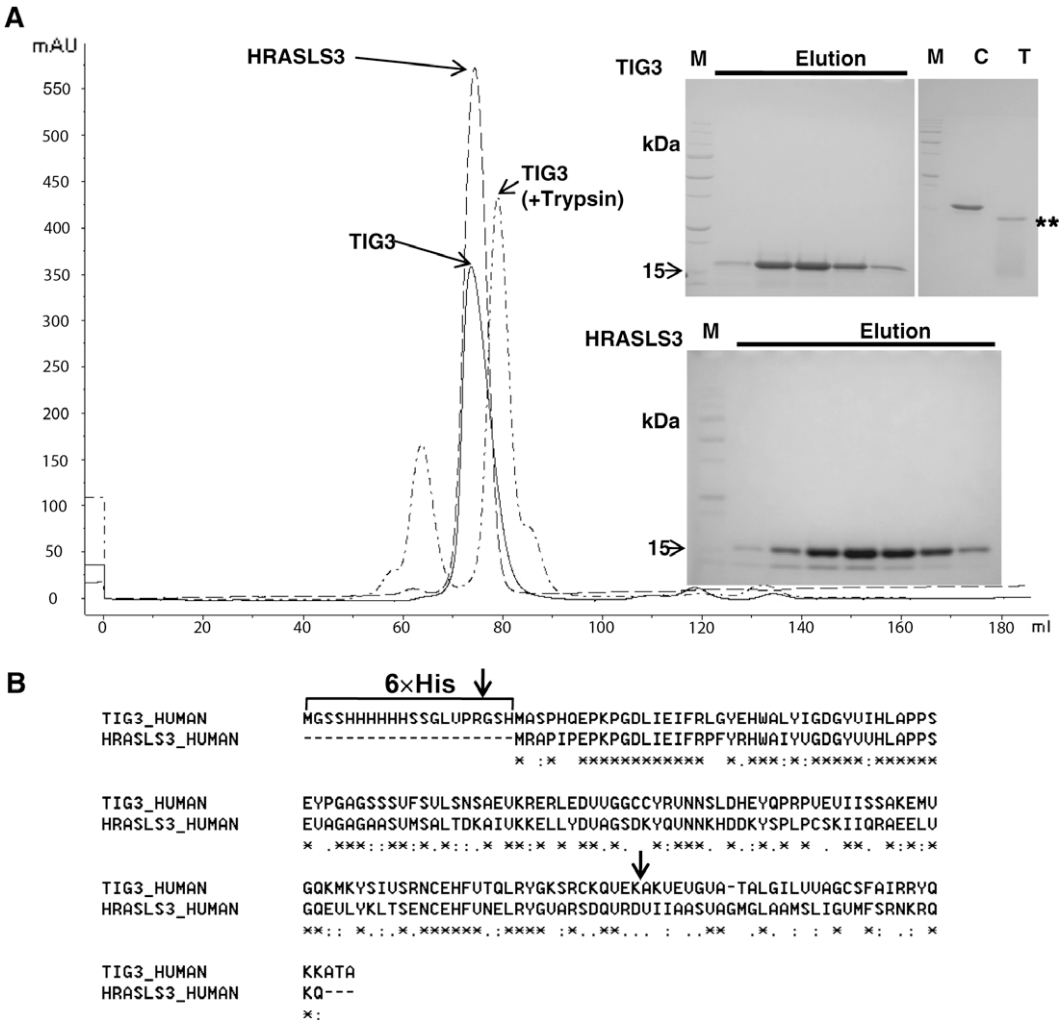

Fig. 2. Purification of the N-terminal regions of TIG3 and HRASLS3. (A) Chromatogram of Superdex 75 16/60 pg column and SDS–PAGE analysis of purified recombinant TIG3, trypsin-treated TIG3 (marked by $**$) and HRASLS3. M, protein standard marker; Elution; elution fractions of Superdex 75 prep-grade column; C, purified TIG3; T, trypsin-digested TIG3. (B) Sequence alignment of human TIG3 and HRASLS3. The 6 × His-containing fusion tag in TIG3 is included for alignment purposes. The structural domain boundaries determined by trypsin digestion are marked by arrows.

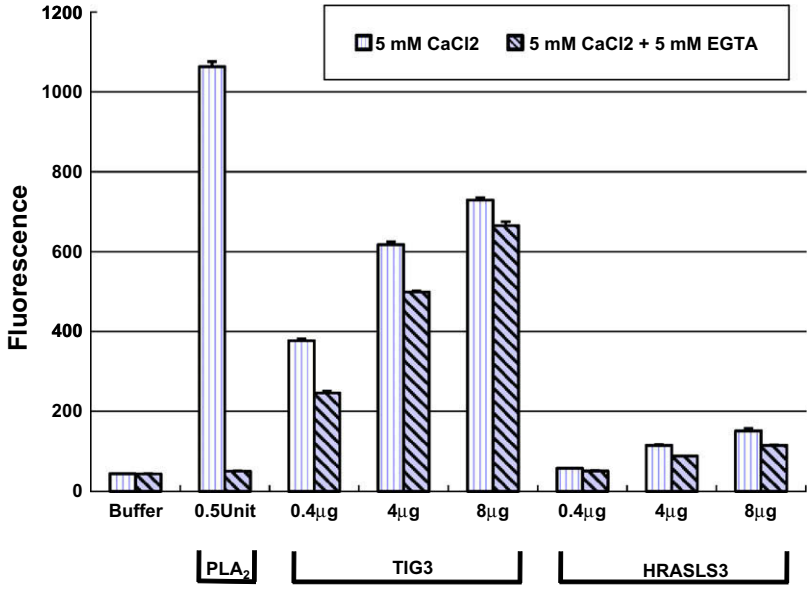

Fig. 3. Phospholipase A_2 (PLA_2) activity of recombinant TIG3 and HRASLS3. The PLA_2 activity assays of TIG3 and HRASLS3 were performed in the presence or absence of EGTA and with different concentration of proteins. Reaction buffer and PLA_2 from honeybee venom were used for the negative and positive controls, respectively. The indicated amounts of purified recombinant TIG3 (1–134) and HRASLS3 (1–133) were added to the reaction buffer containing 5 mM $CaCl_2$ or 5 mM $CaCl_2$ with 5 mM EGTA.

bility that the lipid metabolism by these proteins may be related to their tumor suppression function. Additional support for the possible link between the lipid metabolism and the tumor suppression comes from the finding that the full-length TIG3 could suppress Ras activation at the Golgi, whereas the C-terminal-truncated TIG3 (TIG3ΔC) could not [2,23]. Although the TIG3ΔC, corresponding to TIG3 (1–136), lacks the suppressing function for Ras activation and is distributed homogenously within the cytoplasm, the purified N-terminal region of TIG3 and HRASLS3 possesses the PLA activity. This indicates that the hydrophobic C-terminal region may determine the cellular localization of both proteins rather than harbor the enzymatic activity. The plasma membrane localization by re/depalmitoylation of Ras is important for Ras signaling [24–27]. Therefore, we envisage that the PLA activities of TIG3 and HRASLS3 proteins might modulate the lipid modification of Ras. Taken together, our results suggest that TIG3 and HRASLS3 comprise two functional regions: the N-terminal regions contain the enzymatic activity and the C-terminal regions determine the cellular localization.

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