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Characterization of SHP-1 protein tyrosine phosphatase transcripts, protein isoforms and phosphatase activity in epithelial cancer cells☆

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ABSTRACT

We identified 7 SHP-1 (*PTPN6*) transcripts using epithelial cancer-derived cell lines. Four were shown to utilize the epithelial promoter 1 to transcribe a full-length, a partial (exon 3) or complete (exons 3 & 4) deletion of the N-SH2 domain, and also a non-coding transcript having a stop codon caused by a frame shift due to intron 2 retention. Three additional transcripts were shown to utilize the hematopoietic promoter 2 to transcribe a full-length, a partial (exon 3) deletion of the N-SH2 domain and a non-coding transcript with intron 2 retention. We show that endogenous proteins corresponding to the open-reading-frame (ORF) transcripts are produced. Using GST-fusion proteins we show that each product of the ORF SHP-1 transcripts has phosphatase activity and isoforms with an N-SH2 deletion have increased phosphatase activity and novel protein–protein interactions. This study is the first to document utilization of promoter 2 by SHP-1 transcripts and a noncoding transcript in human epithelial cells.

1. Introduction

The intricate balance of phosphotyrosine residues on proteins is controlled by the opposing actions of protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP) [1–5]. Perturbation of PTP signaling can lead to abnormal accumulations of phosphorylated proteins which can disrupt normal cell proliferation, differentiation, growth, and adhesion.

SHP-1 (also designated as SHPTP-1, SHP, HCP and PTP1C, PTPN6) is a 68 kDa cytosolic PTP predominantly expressed in hematopoietic and epithelial cells [6–8]. The human SHP-1 gene is located on chromosome 12p13 [9,10] and consists of 17 exons and 16 introns, spanning 17 kb of DNA (6). SHP-1 contains two tandem src-homology 2 (SH2) domains, three potential SH3-binding motifs (PxxP) for binding, a catalytic phosphatase domain and a C-terminal tail with two sites for tyrosine phosphorylation [11–15]. In physiological conditions SHP-1 engages in auto-inhibition due to intramolecular interaction between its N-terminus SH2 (N-SH2) domain and its C-terminal tail [5,16]. N-terminal deletion of SHP-1 can result in excessive phosphatase activity [17], which can disrupt normal signaling pathways and may contribute to tumorigenesis [18].

Two promoters have been reported for SHP-1: promoter 1 (P1) or epithelial promoter (EP) which directs the transcription of exon 1 encoding the amino acid sequence, Methionine–Leucine–Serine–Arginine–Glycine (MLSRG) and promoter 2 (P2) or hematopoietic promoter (HP) which initiates the transcription of exon 2 encoding the amino sequence, Methionine–Valine–Arginine (MVR) at the N-terminus [6]. P1 has been suggested to be predominantly expressed by epithelial cells whereas P2 is active in cells of the hematopoietic lineage [6]. The exclusivity of these promoters, however has recently been questioned [8].

Loss of SHP-1 leads to extensive hematopoietic disruptions in mice including aberrant expansion and tissue accumulation of myeloid/monocytic cells that results in death in motheaten (me/me) mice that do not express SHP-1 at about 2-3 weeks or in motheaten-viable (mev/mev) mice that express aberrant SHP-1, at 9-12 weeks [17–19]. An absence of SHP-1 expression has been observed in the development of several human cancers including B and T cell lymphoma and T-cell chronic lymphocyte leukemia (TCLL) and chronic myelogenous leukemia (CML) [18,20,21]. In addition, growth of CML or malignant hematopoietic cell lines can be suppressed with introduction of wild-type SHP-1 [18,20–25]; these findings implicate SHP-1 as a potential tumor suppressor and as a critical regulator of a broad range of hematopoietic cell functions [20–25]. The role of SHP-1 in epithelial cells, however, and its

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implication in epithelial tumorigenesis still remains unclear. Enhanced expression of SHP-1 has been documented in several epithelial cancers, including prostate [26], breast [27] and ovarian cancers [7,8]. The differences in SHP-1 expression may be an outcome of the cell type versus its role in cancer progression and development [25-27]. However, recent work has shown that SHP-1 is a negative regulator of proliferation in malignant breast adenocarcinoma using its N-terminal domain [28]. In prostate cancer, proper binding between SHP-1 and somatostatin is required to negatively regulate cancer growth [26].

In this study, we examined SHP-1 transcripts in different human colon, breast and other epithelial cancer-derived cells to obtain a better understanding of the possible role of SHP-1 in these cancers. We show for the first time P1 and P2 utilization in human epithelial cells. In addition to the three transcripts previously reported [6]; we identified an additional 4 transcripts, 2 having open reading frames and 2 noncoding transcripts having a frame shift due to retention of an intron, that was previously reported for hematopoietic SHP-1 [18]. This study is also the first to show endogenous protein expression representing the variant SHP-1 mRNA as well as protein binding patterns of variant SHP-1 proteins encoded by alternatively-spliced transcripts. SHP-1 alternative splicing and production of protein isoforms may have functional consequences and subsequent biological impact in human epithelial cells.

2. Results and discussion

2.1. Identification of multiple SHP-1 transcripts in epithelial cancer cells

We have re-examined the SHP-1 transcripts present in epithelial cancer-derived cell lines. Previously, it was thought that the selective expression of SHP-1 is governed by two specific promoter regions: promoter 1 (P1) or epithelial promoter (EP) which is located upstream from exon 1 (exon 1a in ref. [29]) or by promoter 2 (P2) also known as hematopoietic promoter (HP) situated upstream from exon 2 (exon 1b in ref. [29]) (Fig. 1). In contrast to a previous report (Banville et al. [6]) that only promoter 1 is used in epithelial cancer cells, in our study we identified SHP-1 mRNA transcripts containing exon 1 and exon 2, indicating that both promoters are utilized in epithelial cells (Fig. 2A). Furthermore, we have characterized 4 additional, novel SHP-1 transcripts (Figs. 1, 2B and C) which add to the previous studies conducted by Banville et al. [6] who showed 3 SHP-1 transcripts using A431 cancer-derived cells. In our studies, we used multiple cancer-derived cell lines, including from breast and colon cancers, and forward primers specifically designed for P1 or P2 promoter use. Under the control of P1, we identified a full-length SHP-1 transcript and transcripts lacking either exon 3 or both exons 3 and 4, which correspond to those found by Banville et al. [6] (Fig. 2B). In addition to A431, we identified novel SHP-1 transcripts in epithelial cancer cell lines not previously examined, including colon cancer cell line Colo 205, benign breast-derived cell line MCF-10A, and the breast cancer cell lines MCF-7, MB231, and SK-BR-3 (Fig. 2). Our results also indicated that multiple transcripts were differentially expressed, depending on the cancer cell used (Fig. 2B). Under the control of P2, we found SHP-1 transcripts corresponding to wild-type and exon 3 deleted SHP-1 mRNA (Fig. 2). We did not find a transcript lacking both exons 3 and 4 under the control of the P2 promoter. Again, as with P1 promoter, we found differential expression of transcripts under the control of P2 in different cancer-derived cells (Fig. 2). These are the first SHP-1 transcripts and SHP-1 variants identified that specifically use promoter 2 in human epithelial cells. Additionally, we have identified two novel SHP-1 transcripts, one using the epithelial promoter and one using the hematopoietic promoter, that retain intron 2 (Fig. 2; largest bp product; 685 bp using P1 and 710 bp using P2), resulting in a non-coding transcript, similar to those we found earlier in KiT 225 leukemia-derived and the HuT 78 lymphoma-

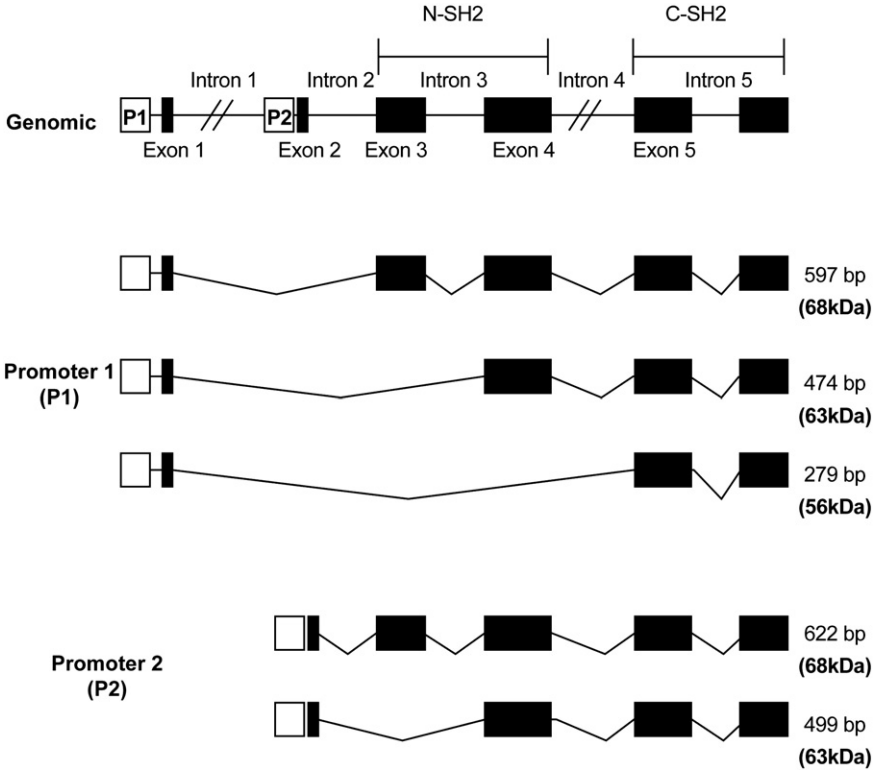

Fig. 1. Schematic diagram of N-terminal region of genomic SHP-1. Shows the positions of introns and exons 1 thru 6 and representations of the multiple SHP-1 mRNA transcripts detected by RT-PCR in various human epithelial cancer cell lines. The sizes of the corresponding PCR products in base pairs (bp) are indicated. These transcripts have open reading frames and translate a full-length 68 kDa protein and truncated proteins of 63 kDa and 56 kDa, with partial and complete deletion of the SHP-1 N-SH2 domain, respectively.

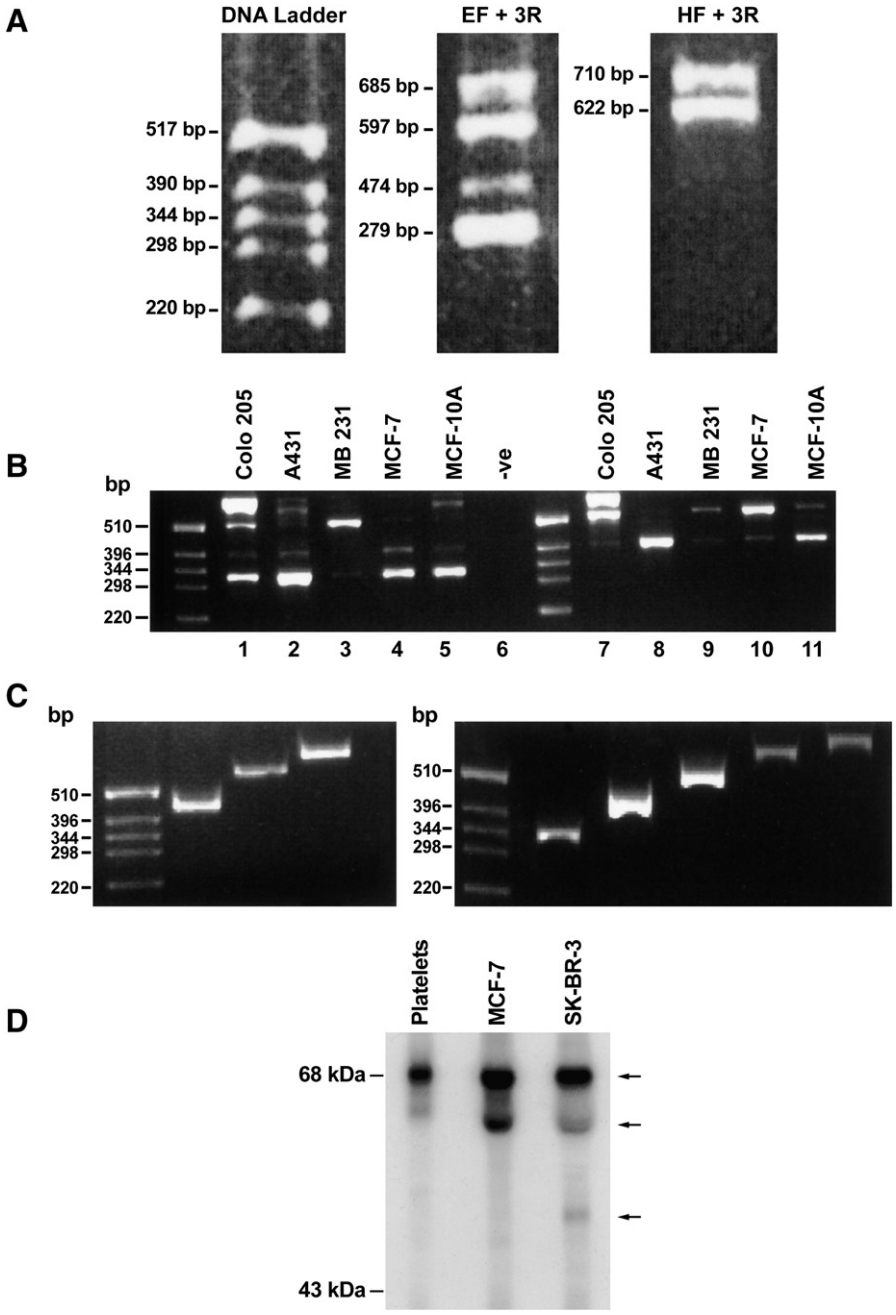

Fig. 2. SHP-1 mRNA from various epithelial cancer cells. Using RT-PCR, products are shown for: (A) SHP-1 transcripts from SK-BR-3 human breast cancer cells, using forward primers specific for either the epithelial promoter 1 (EF) or the hematopoietic promoter 2 (HF) and the common reverse primer (3R). (B) SHP-1 transcripts from various human epithelial cancer cell lines using epithelial promoter (lanes 1–5) or hematopoietic promoter (lanes 7–11). Negative control is water (lane 6). (C) Representative of PCR products from transcripts generated from hematopoietic promoter (left) or from epithelial promoter (right), that were cloned into pGEM-T Easy vectors, isolated and sequenced. (D) SHP-1 proteins were immunoprecipitated from lysates made from platelets (control) or MCF-7 and SK-BR-3 cancer cell lines (10^8 cells). Immune complexes were run on 10% SDS-PAGE under non-reducing conditions. Arrows indicate proteins of 68 kDa, 63 kDa and 56 kDa, relative to molecular weight standards.

derived cell lines [17]. Our findings provide evidence for the usage of both promoters 1 and 2, and intron 2 retention (highlighting the similarity between SHP-1 mRNA expression in human epithelial and hematopoietic cells). Furthermore, our finding of an epithelial SHP-1 transcript lacking exons 3 and 4 support results reported by Martin et al. where an analogous SHP-1 transcript using promoter 1 was identified [17,23].

Although we confirm the original work by Banville et al. [6], we have discovered additional and novel transcripts not previously reported. As we did not confirm the presence of polyA in our transcripts, it is possible that the novel transcripts are artifacts due to the use of transformed cells. However, we identified these transcripts in a number of different cancer cell lines, used promoter-specific primers, cloned and sequenced a number of the fragments and found consistency in the results from cell line to cell line making it unlikely that these were artifacts.

2.2. Endogenous proteins corresponding to SHP-1 mRNA found in cancer cell lines

We next examined whether endogenous proteins are produced from the three SHP-1 transcripts which have open reading frames (ORFs). Although Banville et al. [6] stated without showing any results, that variant proteins could not be identified, they did not provide a

detailed method or results with controls. They apparently used only Western blot of cell lysate and did not use immunoprecipitation of SHP-1 followed by Western blotting as was done in our study. In addition, these authors state that they used a polyclonal anti-SHP-1 in their protein studies but did not indicate where this antibody was from or any details of this polyclonal anti-SHP-1. For both immunoprecipitation and Western immunoblotting, we used a specific monoclonal anti-SHP-1 directed to the C-terminal portion of the molecule allowing for detection of SHP-1 proteins truncated in their N-terminal region.

Using our approach, we were able to identify endogenously produced proteins of 68 kDa using MCF-7 and SK-BR-3 breast cancer-derived cell lines but also proteins of 63 kDa (MCF-7 and SK-BR-3) and 56 kDa (SK-BR-3) that correspond to the predicted molecular size of the SHP-1 transcript lacking exon 3 of the N-SH2 domain and the truncated SHP-1 transcript lacking both exons 3 and 4 (Figs. 1 and 2D). The use of non-reducing conditions allowed the clear identification of the 63 kDa and 56 kDa proteins without interference from the heavy chain of the immunoprecipitating antibody (Fig. 2D). Although SDS-PAGE and Western immunoblotting is a standard method to document particular proteins using reducing conditions, under non-reducing conditions as was used in our experiments, sequencing would have further helped to prove the protein specificity; however, this was not done.

2.3. Phosphatase activity of SHP-1 isoforms

We then investigated the SHP-1 activity of the three different protein isoforms. Wild-type (wt), exon 3-deleted, exons 3- and 4-deleted, and phosphatase dead (C453S) SHP-1 constructs were cloned into pGEX-4T-2 glutathione S-transferase (GST) expression vectors and the resulting GST-fusion proteins purified. Using these fusion proteins to evaluate the phosphatase activity of our SHP-1 constructs indicated no significant differences (data not shown). This was perplexing as we anticipated that the construct lacking exons 3 and 4 would have increased phosphatase activity due to a loss of autoinhibition of the enzymatic activity and previous publications that supported this [23,29–35]. Indeed, murine SHP-1 proteins lacking the N-SH2 domain have demonstrated a 5-fold increase in phosphatase activity when compared to wild-type SHP-1 [17,23]. These anomalies led us to believe that the low level of exon 3-deleted GST fusion protein and steric interference by the GST may preclude accurate testing of the activity of the SHP-1 isoforms. We therefore assessed the phosphatase activity of SHP-1 constructs using thrombin-cleaved-GST SHP-1 proteins.

Thrombin-cleaved-GST SHP-1 proteins were immunoprecipitated using monoclonal anti-SHP-1 antibodies. We aimed at achieving an equal protein distribution to ensure that concentrations were not a factor when assessing differences in phosphatase activity. In this experiment, we were not able to obtain enough exon 3-deleted protein to accurately measure phosphatase activity compared to wild-type SHP-1. However, we were able to obtain adequate protein for wild-type and exons 3- and 4-deleted SHP-1 and observed a 3-fold increase in phosphatase activity of SHP-1 protein lacking exons 3 and 4 when compared to wild-type SHP-1 protein (Fig. 3, these results were statistically significant ($p < 0.05$)). Thus, as in murine SHP-1 [17,23], human SHP-1 when lacking the N-SH2 domain has greatly increased phosphatase activity.

2.4. Phosphatase activity of SHP-1 variants expressed in HEK 293 cells

To further examine the phosphatase activity of SHP-1 variants under more physiologic conditions, we used transfection of each SHP-1 cDNA having an Xpress tag into HEK 293 cells and then anti-Xpress immunoprecipitation of the expressed proteins. The transfectants were divided equally for subsequent protein expression analysis and phosphatase activity assessment. Western immunoblotting using monoclonal anti-SHP-1 allowed the detection of the SHP-1 proteins corresponding to the sizes predicted by the multiple mRNA transcripts (full-length wild-type & C453S: 68 kDa; exon 3 deletion: 63 kDa; exons 3 and 4 deletion: 56 kDa) (Fig. 4A). SHP-1 protein constructs were demonstrating differential expression levels when transfected into HEK 293 cells. We therefore used a densitometry analysis to empirically ensure equal distribution of cells for transfection (Fig. 4A). We consistently observed reduced expression of the exon 3-deleted SHP-1 protein construct in HEK 293 transfects (Figs. 4A and B). However, we were able to obtain enough protein expression of exon 3-deleted SHP-1 to demonstrate that it has phosphatase activity similar to wild-type SHP-1 (Fig. 4C). Our results, as in Fig. 3B, again showed that the phosphatase activity of exons 3- and 4-deleted SHP-1 protein was approximately 4-fold higher when compared to wild-type SHP-1 proteins (Figs. 4A and B). These results were statistically significant ($p < 0.05$). Taken together, results from Figs. 3 and 4 confirm the results of Martin et al., who found that murine SHP-1 lacking the N-SH2 domain has a 5-fold higher specific phosphatase activity than wild-type when transfected in COS-1 cells [17,23].

In addition to truncation of the N-SH2 domain, it is possible that posttranslational modifications may have influenced the function/activity of these proteins. Although both tyrosine and serine phosphorylation of SHP-1 have been proposed to affect its activity and function [36–38], this work was done in hematopoietic cells and these phosphorylation events appear to occur only in the C-terminal region of SHP-1; thus, may not play a role in our studies, although we did not investigate phosphorylation.

2.5. Differential protein binding activity of SHP-1 variants

We next examined the protein binding partners for the different SHP-1 proteins. Protein–protein interactions are responsible for regulating various signaling pathways that drive normal cellular functions. Improper interactions may disrupt signaling pathways and promote neoplastic transformations. Our SHP-1 variant protein constructs exhibited structural alteration (in particular the partial or complete absence of the N-terminal SH2 binding domain) which may lead to novel and uncharacterized protein–protein interactions. We measured protein–protein interactions of wild-type, exon 3-deleted, exons 3- and 4-deleted and C453S SHP-1 constructs in KiT 225 cells (lacking endogenous SHP-1 (18)) using ^{35}S-biosynthetic labelling and a GST-fusion protein pull-down assay. Using Western blotting (Fig. 5A) to ensure that the same amount of each GST-fusion protein was used in each pull-down, we incubated this amount with lysate from the biosynthetically labeled KiT 225 cells to compare ^{35}S-proteins pulled down by each GST-fusion construct.

When compared to wild-type SHP-1, exon 3-deleted SHP-1 demonstrated several additional protein interactions that were absent in wtSHP-1 (Fig. 5). We observed a prominent ~90 kDa band as well as a large amount of protein at ~30 kDa that exclusively interacted with exon 3-deleted SHP-1. It is possible that these additional proteins are ones involved in causing the unstable nature in the expression of this protein in vitro; perhaps involved in increasing degradation and/or ubiquitination. Exon 3- and 4-deleted SHP-1 proteins exhibited significantly reduced interactions, consistent with the role of the entire N-SH2 domain as being responsible for the interaction of SHP-1 with phosphotyrosine-containing proteins [30]. However, a weak protein of ~38 kDa was observed with exon 3- and 4-deleted SHP-1. This protein is likely the adaptor protein CrkL which we have previously shown binds to SHP-1 via its SH3 domains to PxxP motifs present within the C-SH2 and PTP domains of SHP-1 [14]. As expected, dominant negative C453S SHP-1 exhibited a comparable array of protein partners as with wild-type SHP-1 (Fig. 5B) as this construct has been shown to act as a substrate trap due to its lack of phosphatase activity.

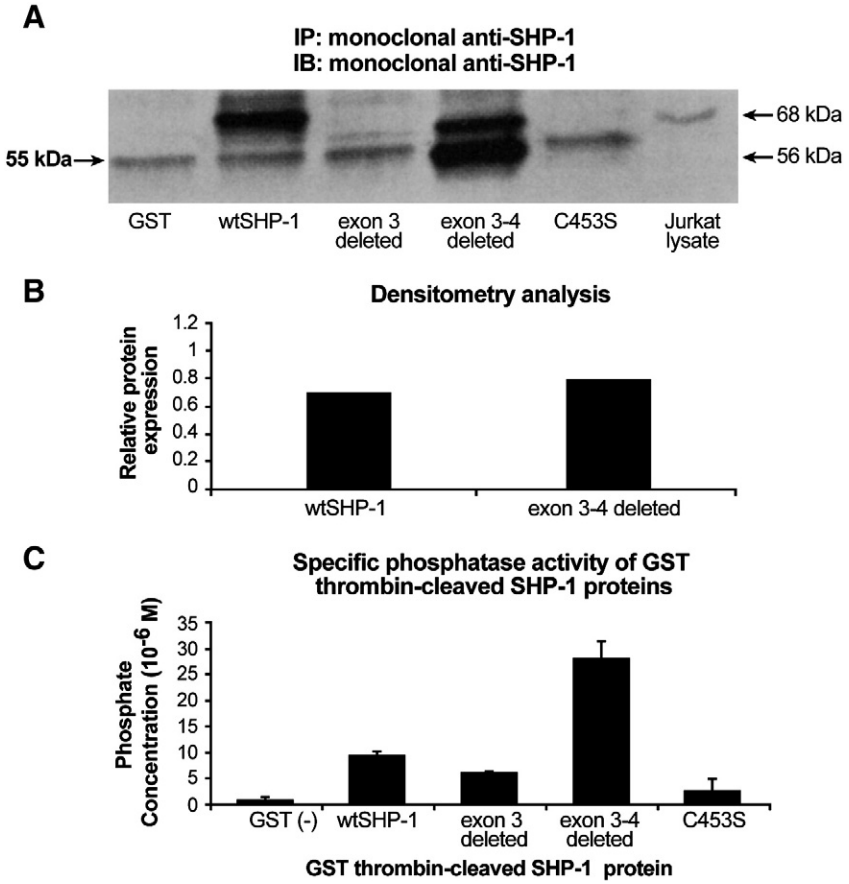

Fig. 3. Phosphatase activity of variant SHP-1 proteins. (A) Western blot of thrombin-cleaved-GST SHP-1 proteins. Thrombin-cleaved SHP-1 was immunoprecipitated using monoclonal anti-SHP-1 specific for the C-terminus and Western blotted under non-reducing conditions. (B) Densitometry analysis of the variant SHP-1 proteins as well as the C453S phosphatase dead control is shown as well as (C) phosphatase activity using a malachite green assay.

3. Conclusions

We have identified multiple, novel SHP-1 transcripts in epithelial cancer cells. The transcripts we have identified use either promoter 1 or promoter 2 to generate full-length wild-type SHP-1 or truncated transcripts lacking either exon 3 or both exons 3 and 4. In addition we have shown that a noncoding transcript is generated by either promoter where intron 2 is retained resulting in a frame shift and stop codon, as we previously have shown for SHP-1 transcripts in hematopoietic cells [18]. This is the first evidence that SHP-1 is generated by both promoters in epithelial cells. We also have provided data that proteins corresponding to each of the open-reading frame transcripts are produced in epithelial cancer cell lines. Furthermore, we show that these truncated proteins have differential phosphatase activity and have different protein binding partners.

Previously, only promoter 1 was shown to be active in production of SHP-1 transcripts of full-length or lacking exon 3 or exons 3 and 4, and SHP-1 proteins were only found for the full-length transcript [6]. Our work indicates that both promoters are active in epithelial cells and proteins of 68 kDa, 63 kDa and 56 kDa, corresponding to each of the open reading frame transcripts, can be detected in epithelial cancer cells. As truncated SHP-1 proteins were identified in both MCF-7 and SK-BR-3 breast cancer cell lines, these proteins may have a role in tumorigenesis, as previously suggested for truncated SHP-1 proteins that have been identified in hematopoietic cells [18,39,40]. Recently, it has been shown that SHP-1 expression is related to the proliferation of breast cancer cells [28]. Thus, the role of truncated SHP-1 in epithelial cancers requires further study to determine if these proteins have different functional outcomes in certain cancers. This is an important question as we demonstrated a differential expression of the truncated proteins in colon, breast and other cancer cells (Fig. 2D). Also, these proteins bind different substrates and have differential phosphatase activity; thus, may play a role in carcinogenesis.

This study is the first to show promoter 2 usage in epithelial cells. Hyper-methylation and transcriptional inactivation of promoter 2 have been observed in several epithelial cells [40]. However, in diseases such as psoriasis and various skin lesions such as squamous cell carcinoma, promoter 2 is hypo-methylated and elevated SHP-1 expression has been observed, suggesting that promoter 1 and 2 utilization is common in epithelial cells [41]. Hypo-methylation of the SHP-1 promoter 2 region has also been observed with the onset of several hematopoietic tumors such as CML and several leukemias, indicating that promoter 2 may be easily susceptible to modifications, in particular hypo-methylation. The elevation or reduction of SHP-1 expression may be a result of a cell specific characteristic.

Banville et al., [6] suggested that significant structural alterations may lead to functional changes of SHP-1 proteins. Specifically, abnormal phosphatase activity and uncharacteristic protein–protein interactions have been hypothesized as a consequence of SHP-1 structural alterations [6,39]. In fact, murine SHP-1 proteins lacking the N-SH2 domain have demonstrated a 5-fold increase in phosphatase activity when compared to wild-type SHP-1 [27,30]. As with murine SHP-1, in our studies, SHP-1 variants lacking exons 3 and 4 (the N-SH2 domain) demonstrated elevated phosphatase activity in vitro. In addition, SHP-1 variants lacking exon 3 also exhibited novel protein–protein interactions. These results suggest that structural alterations of SHP-1 may lead to loss of regulatory function of normal signaling pathways, and this could lead to a pathologic condition such as tumorigenesis [40].

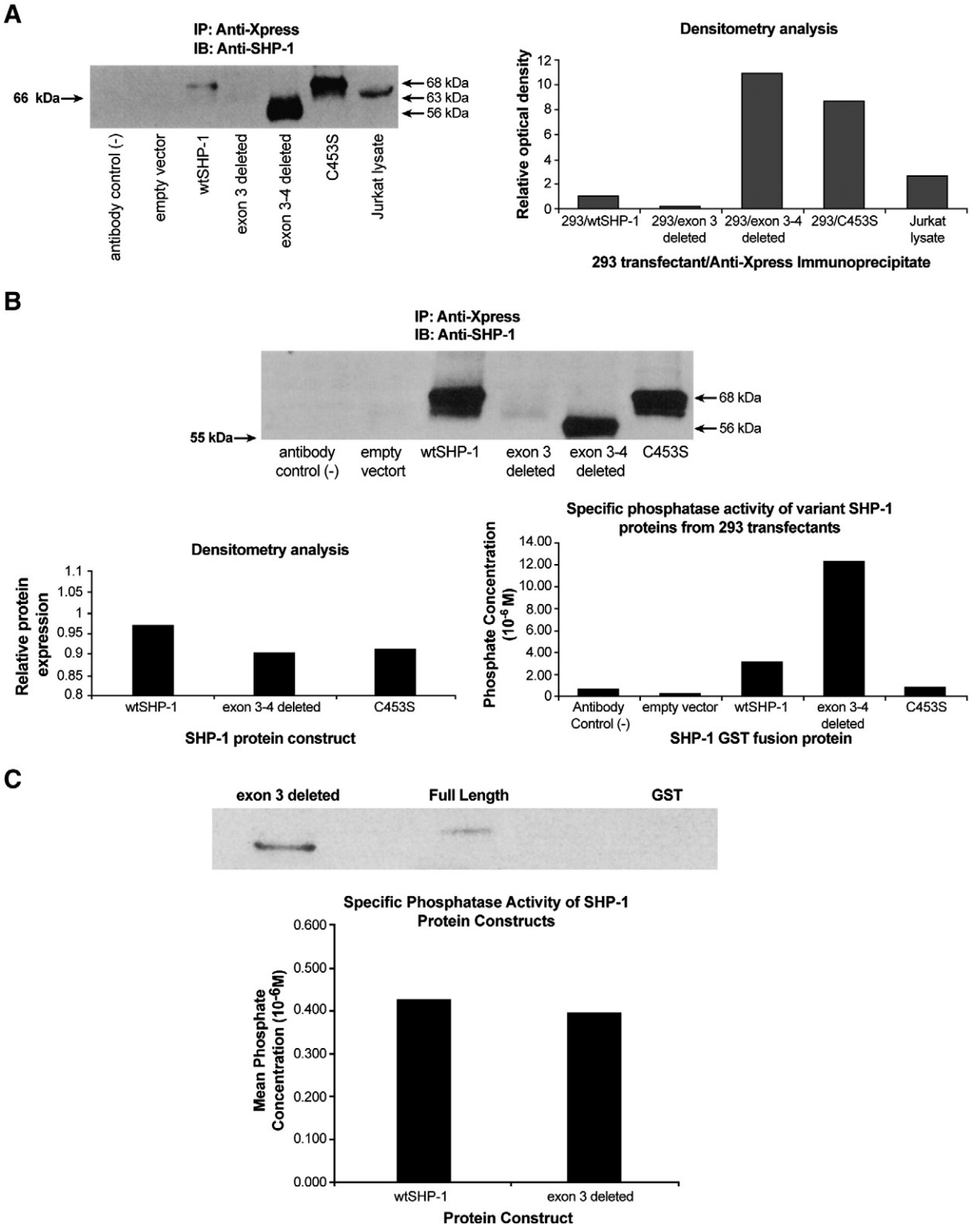

Fig. 4. Transfection/expression of SHP-1 variant cDNAs. (A) Transfectants expressing Xpress were immunoprecipitated using anti-Xpress and Western immunoblotted using anti-SHP-1 under non-reducing conditions. Jurkat cells served as a positive control and anti-Xpress alone bound to Protein G-Sepharose and empty vector served as negative controls. Densitometry analysis is also shown. (B) Repeat of (A) but also showing phosphatase activity of isolated proteins. (C) Isolation and characterization of the exon 3-deleted protein showing Western immunoblot results and phosphatase activity.

4. Materials and methods

4.1. Cells

The Jurkat human T cell leukemia cell line was obtained from American Type Culture Collection (E6-1, ATCC #TIB-152), KiT 225 human acute T-cell leukemia-derived cells, lacking SHP-1 protein [18], were a gift from Dr. G. B. Mills (MD Anderson Cancer Center, Houston, TX, USA), SK-BR-3 mammary gland epithelial adenocarcinoma cells were obtained from ATCC (ATCC#: HTB-30), human embryonic kidney (HEK) 293 epithelial cells were obtained from the NIH AIDS Research and Reference Reagent Program (#18-036).

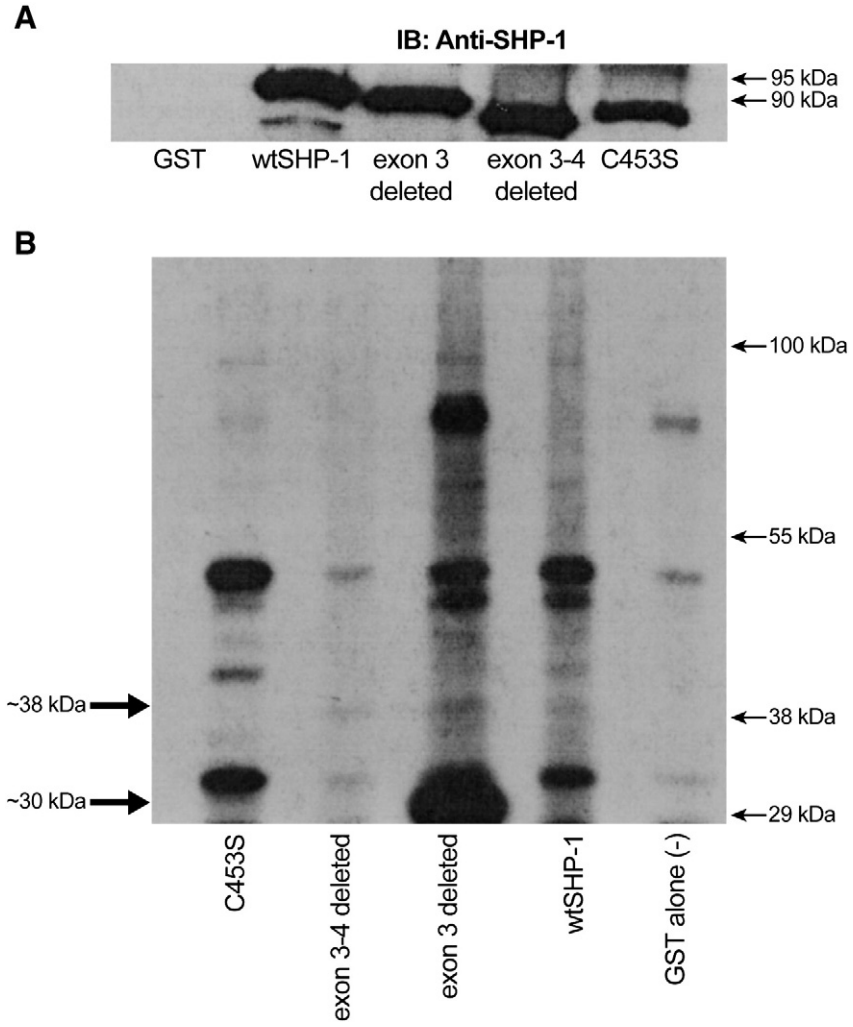

Fig. 5. Protein binding partners for variant SHP-1 proteins. (A) GST fusion proteins used in pull-down on Western immunoblot showing expected sizes and equal levels of GST-fusion proteins. (B) KiT 225 cells were incubated with ^{35}S-methionine and ^{35}S-cysteine, lysed and the biosynthetically radiolabeled proteins pulled down using the GST-fusion proteins. GST alone was a negative control. Molecular size standards are indicated on the right while arrows on the left indicate the 38 kDa protein (likely CrkL) that is bound by the exon 3-4 deleted protein lacking the N-SH2 domain and the 30 kDa protein that is a major protein bound to the exon 3-deleted variant SHP-1.

Additional human epithelial cell lines used in these studies include were from ATCC: Colo 205 (#CCL-222), MCF-7 (#HTB-22), A431 (#CRL-1555), MD-MB-231 (MB 231, #HTB-26) and MCF-10A (#CRL-10317).

4.2. Antibodies, Western immunoblots, immunoprecipitation and membrane stripping

Mouse monoclonal anti-SHP-1 antibody recognizing the C-terminus (BD Biosciences, Mississauga, Ontario, cat # 611334) or a rabbit polyclonal anti-SHP-1 #170 (a generous gift from Dr. K. Siminovitch) were used at 1:1000 dilution. Cells were lysed using a radioimmune precipitation assay (RIPA) buffer, resolved by sodium dodecyl sulfate-acrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech Inc.)[18,42]. Western blotting was performed as previously [14,18] using primary monoclonal anti-SHP-1 followed by goat-anti-mouse horseradish peroxidase (HRP) used as a secondary antibody. Protein bands were visualized using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia Biotech Inc.). Immunoprecipitation was done as previously described [14]. Antibody complexes were first prepared by initial binding to Protein G PLUS-Agarose beads (Santa Cruz Biotechnology, Inc.) Prepared antibody complexes were added to cell lysates or GST thrombin-cleaved SHP-1 fusion proteins to mediate immunoprecipitation. Immune complexes were used in Western blotting and/or subjected to phosphatase assays.

For immunoprecipitation of endogenous SHP-1 proteins, we used a slightly different method that we have described previously [42]. Fifty million cells were lysed with RIPA and the lysate mixed with Protein A-Sepharose beads (GE Healthcare Life Sciences Inc., Baie D'Urfe, Quebec) which had been pre-bound, first with rabbit-anti-mouse Ig (RAM; Western Blotting Enterprises, Oakville, ON) followed by binding to RAM with monoclonal anti-SHP-1 (or monoclonal anti-Xpress (Invitrogen™, Carlsbad, CA)). Non-reducing conditions were used for SDS-PAGE when investigating SHP-1 proteins to eliminate any interference from the heavy chains of the immunoprecipitating antibody allowing for clear identification of SHP-1 proteins of 63 kDa and 56 kDa.

4.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing

mRNA from various cell lines was isolated using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech Inc.) and cDNA was synthesized using First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech Inc.) Human SHP-1 cDNAs were amplified using

the following primers: hematopoietic forward (HF) primer (5′-ctctcc ggaagcccc cag gat g-3′); epithelial forward (EF) primer (5′-gag gagg aagtggct gat tac-3′); reverse primer 3R specific to exon 5 (5′-cacat caaggtcatgtgc gag-3′). The forward and reverse primer pairs used were specific for the amplification of the N-terminus SH2 domain region spanning exon 1 or exon 2 to exon 5 (Fig. 1).

5. SHP-1 cDNA construct synthesis and mutagenesis

PCR products of multiple SHP-1 transcripts were cloned into pGEM-T Easy vectors. Full-length human SHP-1 was cloned using PCR (wtSHP-1 forward primer: 5′-GATCAGGAATTCCCAGGATGGTGAGGT-3′) based on previously published sequence from normal primary peripheral blood mononuclear cells [18]. Forward primers were designed that would anneal at the 5′ EcoRI site within the pGEM-T Easy vector covering the N-terminal sequence (first exon) of the full length SHP-1 sequence. The promoter 2/exon 3 deletion forward primer sequence (5′-GATCAGGAATT CCCAGGATGGTGAGGGTGGGGGATCAG-3′) was designed by incorporating the 5′ flanking EcoRI site, exon 2 (MVR) and 5′ sequences of exon 4. The promoter 1/exon 3 and 4 deletion forward primer sequence (5′-CAGGAATTCCCTACAGAGAGATGCTGTCCCGTGGGTGGTACCATGGCCACAT GTCT-3′) incorporates the 5′ flanking EcoRI site, exon 1 (MLSRG) and 5′ sequences of exon 5. A reverse primer anneals at the SHP-1 C-terminal sequences (5′-CCACCTGAGGACAGCACCGCT-3′). PCR products were cloned into the pGEX-4T-2 glutathione S-transferase (GST) expression vector (Amersham Pharmacia Biotech Inc.) and pcDNA3.1/HisA mammalian expression vector (Invitrogen) at their EcoRI sites. Full-length SHP-1/C453S mutant was generated by oligonucleotide-directed mutagenesis in the SHP-1 phosphatase recognition site for ATP; Cys/Ser (Cys → Ser). Specifically, SHP-1/C453S was made using a forward primer: 5′-CATCGTGCAC **TCC**AGCGCCGGCATCGGCCGCACAGGC-3′. The PCR products were cloned into the pGEX-4T-2 GST expression vector at the corresponding restriction sites located downstream from GST. The SHP-1/C453S cDNA insert was subcloned into the EcoRI and XhoI sites of pcDNA3.1/HisA mammalian expression vector for subsequent transfection into cells.

5.1. Expression, purification and thrombin cleavage of GST fusion proteins

Wild-type, exon 3 deletion, exons 3 and 4 deletion and C453S SHP-1 cDNA constructs were cloned into pGEX-4T-2. GST-SHP-1 fusion proteins were amplified in *Escherichia coli* strain BL21 (DE3; Stratagene, La Jolla, CA) and induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). GST fusion proteins were purified using 50% glutathione sepharose 4B beads. A thrombin cleavage site (Leu–Val–Pro–Arg–Gly–Ser) was present in all pGEX-4T-2 GST expression vectors. GST fusion protein–glutathione sepharose 4B beads complexes were treated with thrombin protease (Amersham Pharmacia Biotech Inc.). Reaction buffers were collected for subsequent immunoprecipitation assay.

5.2. Malachite green phosphatase assay

Phosphatase assays were performed on thrombin-cleaved GST-fusion SHP-1 proteins (wild-type and variants), TDL monoclonal anti-SHP-1 immunoprecipitates of GST thrombin-cleaved SHP-1 proteins (wild-type and variants) and HEK 293 transfectants bound on Protein G-Agarose beads using a Malachite green phosphatase assay kit (Upstate Cell Signaling Solutions Catalog #17-125). Proteins bound on glutathione or protein G-agarose beads were washed with Malachite green assay buffer. Phosphopeptides (RRLIEDAEpYAARG) were added and incubated with Malachite green detection reagent (99% Malachite Green (Solution A) Upstate Cell Signaling Solutions Catalog #20-105; 1% Malachite Green Additive (Solution B) Upstate Cell Signaling Solutions Catalog #20-103). Phosphatase activities were measured by SpectraMax Plus^{384} and SOFTmax® PRO reader software. Sample absorbance readings were converted to phosphate concentration based on the Beer's Law.

5.3. Transfection

SHP-1 cDNA constructs cloned into pcDNA3.1/HisA mammalian expression vectors and were transfected into HEK 293 epithelial cells using the Effectene Transfection Kit (*QIAGEN*). Transfected cells were cultured in complete medium at 37 °C under 5% CO_2 for 48 h before harvesting.

5.4. Metabolic ^{35}S-Labelling/GST pull-down assay

KiT 225 cells were labeled with 2 mCi of ^{35}S-Cys and ^{35}S-Met and then lysed with RIPA buffer. To perform the GST pull-down assay, pre-cleared lysates were added to glutathione sepharose 4B beads bound GST alone (control) and GST fusion proteins. GST pulled-down proteins were separated by SDS-PAGE on a polyacrylamide gel. The gels were fixed with 7% acetic acid and soaked in Amplify NAMP 100 (Amersham Pharmacia Biotech Inc.) fluorographic enhancer solution. Gels were dried using a BioRad Model 583 gel dryer at 80 °C and radiofluorescence was captured on Kodak BioMax MR film.

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Appendix A. Supplementary data

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