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Interactome analysis reveals molecular mechanisms underlying the association between selenium binding protein 1 expression and the malignant features of tumor cells

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SUMMARY

The potential biological and clinical significance of selenium binding protein 1 (SBP1) has been suggested in various types of cancer. To evaluate the role of SBP1 and reveal the molecular basis for its function, we examined the SBP1 protein complex. A gene transfection assay revealed that overexpression of SBP1 promoted proliferation and migration of A549 lung adenocarcinoma cells. Halo-tag-based affinity purification coupled with liquid chromatography-tandem mass spectrometry identified 23 components of the SBP1 protein complex. The functional classification of these 23 proteins suggests that the SBP1 complex participates in critical biological events including cell structure, protein translation, stress response, chaperone, and apoptosis. Moreover, the SBP1 complex includes several proteins that are aberrantly expressed in cancers. These findings indicate that SBP1 may function coordinately with these multiple proteins to facilitate cancer progression. A comprehensive study of the multiple proteins associated with SBP1 together with an examination of individual proteins will be required to elucidate the roles of aberrant SBP1 regulation in cancer progression.

Key words: SBP1, mass spectrometry, proteome, interactome

INTRODUCTION

Cancer is a clinically diverse disease. Analysis of molecular background data as well as clinical and pathological observations will aid in the development of innovative methods for improved clinical outcomes. Proteomic approaches have resulted in the identification of proteins underlying cancer diversity, leading to the development of molecular biomarkers that support clinical and pathological observations^{1,2}. Proteins often function in a coordinate manner and these associations can be revealed by studying protein complexes. However, protein-protein interactions cannot be predicted by studying DNA sequences and mRNA expression; direct observations of the proteome are required to determine the status of protein complexes. These protein interaction studies constitute a unique proteomics approach. Recent advances in mass spectrometry have facilitated increased sensitivity in the identification of protein complex components,

thus revealing novel functional properties of target proteins under different physical and pathological conditions.

Selenium binding protein 1 (SBP1) is a member of the selenium-binding protein family that mediates intracellular transport of selenium and intra-Golgi proteins^{3,4}. Previous studies have indicated that the expression of SBP1 is associated with the prognosis of patients with lung, colorectal, stomach, breast, and liver cancer⁵⁻¹⁰. Molecular data regarding the function of SBP1 in cancer are still limited; therefore, further investigations exploring the significance of aberrant SBP1 expression in cancer are of great interest¹¹.

In the present study, we examined the SBP1 protein complex in lung adenocarcinoma cells to elucidate its molecular properties.

MATERIALS AND METHODS

Cell culturing

A549 lung adenocarcinoma cells (American Type Culture

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Collection, CCL-185), were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum. The cells were maintained in an incubator at 37°C in an atmosphere of 5% CO₂.

Plasmid construction

SBP1 (GenBank accession no. DQ890731) cloned cDNA (ORS08411) was obtained from Kazusa DNA Research Institute (Chiba, Japan). The N-terminal Halo-His6-tagged SBP1 and non-tagged SBP1 inserts were generated by PCR, sequenced, and inserted into the pNL1.1[Nluc] vector (Promega, Madison, WI) and the pCMV-Tag2B vector (Agilent Technologies, Santa Clara, CA), respectively.

SBP1 transfection

The Halo-His6-tagged SBP1, non-tagged SBP1, and non-insert expression vectors were transfected into A549 cells using the Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA). Cultured cells were collected 24 h post transfection.

Western blotting

The expression levels of SBP1 were measured using western blotting. Briefly, protein samples were separated using SDS-PAGE, transferred to PVDF membranes, then incubated with an anti-SBP1 antibody (1:500 dilution; ARP48234 P050, Santa Cruz, CA). Next, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000 dilution; anti-rabbit IgG, NA9340V, Healthcare, Uppsala, Sweden). SBP1 was detected using an enhanced chemiluminescence system (ECL Prime, GE Healthcare) and LAS-3000 image analyzer (FUJIFILM, Tokyo, Japan).

Proliferation assay

Transfected A549 cells were seeded into 96-well cell culture plates at a density of 1500 cells/well. The effect of SBP1 overexpression on cell proliferation was monitored using the Cell Counting Kit-8 (Dojindo Technologies, Kumamoto, Japan) at 24 and 72 h post transfection.

Wound-healing assay

Transfected A549 cells were grown in 6-well cell culture plates. Monolayers were wounded with a pipette tip. The wounds were imaged immediately following scratching and 48 h later (at the same location) using a BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan). Wound dimensions were measured at the time of scratching and 48 h later. Cell motility was defined as the fold-change in wound dimensions between the two time points.

Pull-down assay

Transfected A549 cells (10-cm dish, 80% confluency) were mixed with 500 µl of lysis buffer (50 mM HEPES-KOH [pH 8.0], 300 mM NaCl, 0.005% NP-40, 1 mM 2-

mercaptoethanol, and Protease Inhibitor Cocktail [Promega Corp, Madison, WI]) and sonicated. Subsequent to centrifugation, the lysates were mixed with 10 µl of HaloLink Resin (Promega) and rotated for 4 h at 4°C. The lysates were then centrifuged, the supernatant was discarded, and the beads were washed five times with lysis buffer. The pellet was then mixed with 500 µl of lysis buffer containing 15 units of Halo TEV Protease (Promega) and shaken overnight at 4°C. Subsequent to centrifugation, the supernatant was mixed with 10 µl of Ni-NTA Agarose (Qiagen) and 5 µl of 1 M imidazole (pH 8.0) and rotated for 2 h at 4°C. The pellet were washed five times with lysis buffer containing 10 mM imidazole (pH 8.0) and boiled with 20 µl of 1 × SDS sample buffer for 5 min at 95°C.

Identification of proteins

Pull-down purified proteins were separated by SDS-PAGE using a gradient gel (Supersep TM Ace 191-15031, Wako Pure Chemical Industries, Ltd). To visualize the total purified proteins, the gels were stained with Silver Stain KANTO III (Kanto Regents, Tokyo, Japan). The separated proteins were extracted as peptides from the gels by in-gel digestion. Briefly, the gels were cut into 48 sections using a grid cutter (Gel Company, Tubingen, Germany). The gels were extensively washed with a solution containing acetonitrile and ammonium bicarbonate and treated with trypsin (Promega) at 37°C overnight. The tryptic digests were recovered from the gel sections, concentrated under vacuum, and resolubilized in 0.1% trifluoroacetic acid. The final trypsin digests were subjected to LC-MS/MS (LTQ OrbitrapXL hybrid ion trap mass spectrometer, Thermo Fisher Scientific Inc., Waltham, MA). A Mascot search (version 2.3.2; Matrix Science, London, UK) of the SWISS PROT database (Homo sapiens, 471 472 sequences in the Sprot_57.5 FASTA file) was conducted to identify the proteins based on peptide ion peak mass. Proteins with Mascot scores >34 were considered as a positive identification. The search parameters were as follows: trypsin digestion allowing up to three missed tryptic cleavages; fixed modifications with carbamidomethyl; variable modifications of oxidation, acetylation and phosphorylation; 1+, 2+, and 3+ peptide charge; peptide mass tolerance of 2.0 Da; and MS/MS tolerance of 1.0 Da for all tryptic-mass searches.

RESULTS

The functional significance of SBP1 expression was examined in A549 lung adenocarcinoma cells. The functional significance of SBP1 expression was examined by assessing cell proliferation and mobility in A549 lung adenocarcinoma cells. Transient transfection with SBP1 increased the expression level of SBP1 in A549 cells, compared with control cells (Fig. 1A). Moreover, overexpression of SBP1 significantly promoted cell proliferation (Fig. 1B) as well as cell mobility in A549 cells (Fig. 1C).

Fig. 1. Over-expression of SBP1 promoted proliferation of lung adenocarcinoma cells.

A. Western blotting analysis shows the SBP1 expression (lane 2) in A549 after transfection. Lane 1 is empty vector. B. Cell proliferation was measured after transfection. C. For the wound healing assay, wound dimensions were measured and cell motility was calculated as the fold-change in dimensions between the initial wound and the wound at 48 h. Our findings indicate that cell mobility was significantly promoted by the overexpression of SBP1 in A549 cells. The examination was repeated three times, and mean and standard deviation (error bars) were calculated. (*: Student *t* test between transfected SBP1 vs. empty vector, $p < 0.05$).

To elucidate the molecular functions of SBP1 we examined the SBP1 protein complex. The process of Halo-tag-based protein complex purification is illustrated in Fig. 2A. A549 cells were transiently transfected with the Halo-His6-tagged SBP1 expression vector or the empty vector. To identify the large number of isolated proteins^{13–18}, the proteins were initially separated according to their molecular weight by SDS-PAGE in order to reduce protein sample complexity. Next, the gel was cut into 48 sections and the peptides were recovered subsequent to in-gel trypsin digestion. The peptides in each two adjacent gel sections were combined and 24 peptide samples were subjected to mass spectrometric protein identification. Fig. 2B shows the proteins separated on SDS-PAGE gels including separated protein complexes; non-specific proteins were observed in both the original A549 cells and cells transfected with the control vector. These non-specific proteins were subtracted from those

Fig. 2. Identification of SBP1-binding proteins.

A. Overview of the Pull-down assay of SBP1-binding proteins. Two-step purification of SBP1 complex proteins using HaloLink resin and Ni-NTA agarose was performed on lysates obtained from transiently transfected A549 cells. B. Experimental workflow for the identification of the proteins in the SBP1 protein complex.

identified in Halo-tagged SBP1-transfected cells and thus excluded from further analysis. Consequently, 23 proteins were identified as SBP1 binding proteins by mass spectrometry (Table 1). The proteins identified with high scores include members of the tubulin family, elongation factors, and heat shock proteins. The high identification scores may reflect the high abundance of these proteins.

DISCUSSION

The study of protein complexes exhibiting aberrantly regulated expression in cancer may reveal new insights into tumor cell biology. Moreover, functional studies of these proteins are crucial for clinical application. Although the correlation between specific proteins and disease phenotypes are evaluated statistically in patients, the number of cases available for research is often limited and additional samples are not always obtainable for validation studies. Molecular background data regarding aberrant protein regulation may support their clinical application and facilitate the inclusion of additional cases in validation studies. Furthermore, novel biomarker candidates and therapeutic targets could be identified by investigating factors that structurally and functionally interact with proteins known to be significantly associated with clinically detected targets. Since proteins may function coordinately, the identification of protein complex components is the first step towards elucidating cancer

Table 1. Identified proteins and supporting peptide data

Gene symbol	protein description	protein score	protein matches significant	peptide score	peptide sequence	Functional properties
HORN	Hornerin	40	1	44.21	GPYSGSGHSSGLGHR	Cell envelop organization
PDS5B	Sister chromatid cohesion protein PDS5 homolog B	35	1	35.13	QIAWIK	DNA replication
NR6A1	Nuclear receptor subfamily 6 group A member 1	35	1	35.13	QIAWIK	Orphan nuclear receptor
HS90A	Heat shock protein HSP 90-alpha	38	1	38.05	ELISNSSDALDK	Chaperone
HS902	Putative heat shock protein HSP 90-alpha A2	38	1	38.05	ELISNSSDALDK	Chaperone
HS71L	Heat shock 70 kDa protein 1L	86	2	35.5	VEIANDQGNR	Stress response
HSP72	Heat shock-related 70 kDa protein 2	86	2	50.61	IINEPTAAAIAYGLDK	Stress response
ELMO1	Engulfment and cell motility protein 1	36	1	35.5	VEIANDQGNR	Stress response
TCPH	T-complex protein 1 subunit eta	44	1	35.83	GALKQNK	Cell migration
TCPE	T-complex protein 1 subunit epsilon	35	1	43.77	LPIGDVATQVFADR	Chaperone
TBA1B	Tubulin alpha-1B chain	247	5	34.51	IADGYEQAAR	Chaperone
				57.88	DVNAAIATIK	Cell structure
				34.7	LISQVSSITASLR	
				58.82	AVFVDLEPTVIDEVR	
				36.29	VGINYQPTTVVPGDLAK	
				59.09	TIGGGDDSFNTFFSETGAGK	
TBB2A	Tubulin beta-2A chain	233	4	36.99	LAVNMVPFPR	Cell structure
				46.39	EVDEQMLNVQNK	
				94.88	GHYTEGAELDSVLDVVR	
				54.89	SGPFGQIFRPDVFVFGQSGAGNNWAK	
TBB2B	Tubulin beta-2B chain	233	4	36.99	LAVNMVPFPR	Cell structure
				46.39	EVDEQMLNVQNK	
				94.88	GHYTEGAELDSVLDVVR	
				54.89	SGPFGQIFRPDVFVFGQSGAGNNWAK	
TBB2C	Tubulin beta-2C chain	233	4	36.99	LAVNMVPFPR	Cell structure
				46.39	EVDEQMLNVQNK	
				94.88	GHYTEGAELDSVLDVVR	
				54.89	SGPFGQIFRPDVFVFGQSGAGNNWAK	
TBB5	Tubulin beta chain	233	4	36.99	LAVNMVPFPR	Cell structure
				46.39	EVDEQMLNVQNK	
				94.88	GHYTEGAELDSVLDVVR	
				54.89	SGPFGQIFRPDVFVFGQSGAGNNWAK	
NLRC4	NLR family CARD domain-containing protein 4	37	1	36.71	LTDTEIR	Apoptosis
K0774	Uncharacterized protein KIAA0774	36	1	35.75	LSSTIQR	Protein translation
EF1A1	Elongation factor 1-alpha 1	131	3	36.97	STTTGHLIYK	Protein translation
				57.45	EHALLAYTLGVK	
				36.69	YYVTIIDAPGHR	
EF1A3	Putative elongation factor 1-alpha-like 3	131	3	36.97	STTTGHLIYK	Protein translation
				57.45	EHALLAYTLGVK	
				36.69	YYVTIIDAPGHR	
FILA2	Filaggrin-2	36	1	35.6	SVVTVIDVFYK	Epidermal barrier

biology. Previously, we investigated the proteins interacting with nucleophosmin, a prognostic biomarker for Ewing's sarcoma discovered by proteomics¹⁹, and identified novel prognostic proteins²⁰, suggesting the utility of interactome approach to biomarker discovery. Although a previous study demonstrated the interaction of SBP1 with glutathione peroxidase 1 selenoprotein in colorectal cancer cells¹², comprehensive studies of the SBP1 protein complex in cancer have yet to be conducted. Based on these findings, we conducted an interactome study for SBP1.

Several SBP1 complex proteins are associated with malignant features of cancer cells (Table 1). HSP72 is expressed in primary and metastatic breast cancer tissue²¹ and overexpression of HSP72 is correlated with poor prognosis in esophageal squamous cell carcinoma²². Engulfment and cell motility protein 1 (ELMO1) is aberrantly regulated in breast cancer chemotaxis and invasion²³ and highly expressed in rhabdomyosarcoma²⁴ and gliomas²⁵. Overexpression of T-complex protein 1 (TCP) has been reported in colorectal cancer²⁶, hepatocellular carcinoma²⁷, and various types of malignancies²⁸. NLR family CARD domain-containing protein 4 (NLRC4) is associated with a variety of diseases including cancer²⁹. Increased expression of eEF1A is significantly correlated with poor prognosis of prostate cancer³⁰ and eEF1A may contribute to the mechanisms underlying anti-cancer drug resistance³¹.

A second group of identified proteins included factors involved in the pathogenesis of diseases other than cancers. Hornerin (HORN) and filaggrin-2 (FLA2) are members of the S100 fused-type protein (SFTP) family involved in barrier formation and skin function³². Hornerin is expressed in psoriatic skin³³ and aberrant regulation of FLA2 has been reported in atopic dermatitis³⁴. HORN expression is uniquely decreased in invasive ductal carcinomas in breast cancer³⁵.

The results of this study show that overexpression of SBP1 promoted cell proliferation and migration (Fig. 1). These observations are inconsistent with previous clinical observations. Chen *et al.* reported that reduced expression of SBP1 was associated with poor outcome in lung adenocarcinoma⁵, suggesting a tumor suppressive function for SBP1. Previously, we reported that inhibition of SBP1 by siRNA increased cell proliferation and motility by inhibiting apoptosis in liver cancer cells¹⁰. Moreover, Pohl *et al.* reported that overexpression of SBP1 inhibited cell proliferation and migration by inducing apoptosis in colorectal cancer cells³⁶. They also demonstrated the suppressive effects of SBP1 on cancer cell growth in nude mice³⁶. Although the association of SBP1 with favorable prognosis was observed in various cancers, the actual effects of SBP1 on the cells were examined in only a limited number of studies. Therefore future studies should examine whether the apparent inconsistencies in the results are tissue type dependent and also identify the molecular mechanisms regulating SBP1 function in the cells. Our study clearly indicates the utility of inter-

actome approach to the detailed molecular mechanisms underlying the association of proteins with the malignant features of tumor cells, and worth considering the approach to elaborate the biological significances of biomarker candidates in cancer.

CONCLUSIONS

The SBP1 complex proteins may function coordinately thus contributing to the phenotypes of lung adenocarcinoma. Although ectopic expression of SBP1 promoted malignant features of lung adenocarcinoma in our study, SBP1 expression has often been correlated with favorable prognosis in various cancers including lung adenocarcinoma. These apparent discrepancies require further investigation. The newly identified proteins could be the focus of future studies determining their relevance as cancer biomarkers. The interactome approach should be considered in cancer biomarker study.

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