Production of BBF2H7-derived small peptide fragments via endoplasmic reticulum stress-dependent regulated intramembrane proteolysis

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Abstract
Intramembrane cleavage of transmembrane proteins is a fundamental cellular process to produce important signals that elicit biological responses. These proteolytic events are known as regulated intramembrane proteolysis (RIP). ATF6 and BBF2H7 are transmembrane basic leucine zipper transcription factors and are subjected to RIP by site-1 protease (S1P) and site-2 protease (S2P) sequentially in response to endoplasmic reticulum (ER) stress. However, the detailed mechanisms responsible for RIP of the transcription factors, including the precise cutting sites, are still unknown. In this study, we demonstrated that S1P cleaves BBF2H7 just before the RXXL S1P recognition motif. Conversely, S2P cut at least three different sites in the membrane (next to Leu380, Met381, and Leu385), indicating that S2P cleaves the substrates at variable sites or via a multistep process. Interestingly, we found BBF2H7-derived small peptide (BSP) fragments located between the S1P and S2P cleavage sites in cells exposed to ER stress. Major type of BSP fragments was composed of 45 amino acid including partial transmembrane and luminal regions and easily aggregates like amyloid β (Aβ) protein. These results advance the understanding of poorly characterized ER stress-dependent RIP. Furthermore, the aggregable peptides produced by ER stress could link to the pathophysiology of neurodegenerative disorders.

Abbreviations: RIP, regulated intramembrane proteolysis; S1P, site-1 protease; S2P, site-2 protease; ER, endoplasmic reticulum; BSP, BBF2H7-derived small peptide; iCLiP, intramembrane-cleaving protease; SREBP, steroid regulatory element-binding proteins; bHLH-ZIP, basic-helix-loop-helix-leucine zipper; SCAP, SREBP cleavage-activating protein; INSIG, insulin-induced gene; COPII, coat protein II; APP, amyloid precursor protein; Aβ, amyloid β; bZIP, basic leucine zipper; Hh, hedgehog; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; α-MEM, α-modified Eagle's medium; BCA, bicinchoninic acid; KLH, keyhole limpet hemocyanin; Tg, thapsigargin; BBF2H7-N, BBF2H7 N-terminal fragments; BBF2H7-C, BBF2H7 C-terminal fragments; CHX, cycloheximide; LC-MS/MS, liquid chromatography-tandem mass spectrometry; GST, glutathione S-transferase; RseP, regulator of σE protease; SPPL2b, signal peptide peptidase-like 2b; Tm, tunicamycin; DTT, di-thiothreitol; 2-ME, 2-mercaptoethanol; TEM, transmission electron microscopy.

Koji Matsuhisa and Atsushi Saito are contributed equally in this study.

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INTRODUCTION

Certain types of transmembrane protein can be cleaved within the transmembrane region to disengage from the membrane. The relieved fragments function as important signal molecules that elicit biological responses. This mechanism is called regulated intramembrane proteolysis (RIP).\(^1\) Extensive studies have shown that proteolytic cleavage by RIP proceeds in two steps.\(^1\) A first protease cleaves at the juxtamembrane sequence, subsequently the cleavage by a second protease within the transmembrane region to liberate the fragment from the membrane.\(^3\) Four distinct and evolutionarily conserved intramembrane-cleaving protease (iCLiP) families have been identified; the zinc metalloprotease site-2 protease (S2P),\(^2\) the aspartyl protease-like (including γ-secretase),\(^3\) the serine protease family of rhomboids,\(^4\) and the glutamyl protease Ras and a-factor converting enzyme 1.\(^5\) These proteases are all polytopic integral membrane proteins that harbor their predicted catalytic residues within the hydrophobic environment of the membrane.\(^6,7\) Accordingly, catalysis of the peptide bond of a substrate protein by an iCLiP is understood to occur within the plane of the membrane.\(^6,7\)

The RIP of sterol regulatory element-binding proteins (SREBPs) has well-analyzed its characteristics. SREBPs are membrane-bound transcription factors responsible for feedback regulation of gene expressions required for fatty acid and cholesterol synthesis and uptake.\(^8,9\) The N-terminal segment is a transcription factor of the basic-helix-loop-helix-leucine zipper (bHLH-ZIP) family and projects into the cytosol. It is anchored to membranes by two membrane-spanning segments separated by a short hydrophilic loop that projects into the endoplasmic reticulum (ER) lumen. The C-terminal domain protrudes into the cytosol and interacts with the escort factor SREBP cleavage-activating protein (SCAP).\(^10\) Insulin-induced gene (INSIG) proteins bind to cholesterol-loaded SCAP, and retain the SCAP/SREBP complex in the ER.\(^11,12\) When cells are acutely deprived of sterols, SCAP/SREBP dissociates from INSIG. The liberated SCAP/SREBP complex binds to coat protein II (COP II) and moves to the Golgi apparatus. In the Golgi apparatus, site-1 protease (S1P) cleaves SREBPs within the short luminal loop between the first and second membrane-spanning helices.\(^13\) The resultant N-terminal fragments are the substrates for S2P, which cleaves within the membrane-spanning helix.\(^13\) Precise S2P cleavage site was determined by the analysis of mutant SREBP2 proteins.\(^14\) However, it has not been investigated where the native substrates are cleaved by S2P.

Amyloid precursor protein (APP) is also one of the substrates for RIP.\(^15\) APP is a type I oriented membrane protein with its N-terminus within the lumen/extracellular space and its C-terminus within the cytosol. The N-terminal large part of its ectodomain is shed by β-secretase. This cleavage is followed by γ-secretase cleavage within its transmembrane domain, leading to the liberation of amyloid β (Aβ) peptides.\(^15\) Aβ peptide is a hydrophobic protein located between the β- and γ-secretase cleavage sites of APP. The cleavage of γ-secretase is heterogeneous and gives rise to Aβ species of 37-43 amino acids in length. Aβ easily forms a cross-β structure, leading to aggregation. It is well known that aggregated Aβ in the brain is critically involved in the pathogenesis of Alzheimer’s disease.\(^16\)

ATF6 and OASIS family members are transmembrane basic leucine zipper (bZIP) transcription factors and are known to be activated by RIP in response to ER stress. These molecules are type II transmembrane proteins which target their N-terminus and C-terminus to the cytosol and ER lumen, respectively.\(^17,19\) A S1P consensus cleavage site is contained in their ER luminal domain.\(^17,19\) When cells are exposed to ER stress, these proteins are translocated from the ER to the Golgi apparatus and are then cleaved within their luminal and transmembrane regions by S1P and S2P, respectively.\(^17,19\) The cleaved N-terminal fragments containing the bZIP domain
are translocated to the nucleus to bind to ER stress-response element or cyclic AMP-response element sites.20,21

BBF2H7, one of the OASIS family members, is highly expressed in neurons, chondrocytes, and certain tumor tissues.19,22-24 BBF2H7 is activated via RIP by S1P and S2P like other OASIS family members.19 The N-terminal fragments that are cleaved in response to ER stress act as the transcription factor and promote expression of the COP I components.19,22 The pathway plays crucial roles in chondrogenesis by activation of extracellular matrix protein secretion. The cleaved C-terminus is secreted from the cells into the extracellular space, where it acts as a signaling molecule to promote cell proliferation of neighboring cells via the activation of hedgehog (Hh) signaling.23 Hh signaling modulates diverse events including cell proliferation.25 The secreted C-terminus traps Hh ligands, and then the C-terminus-Hh ligand complex efficiently binds to Hh receptor Ptc1 at the cell surface.23 The pathway mediated by the C-terminal fragment plays a role in the proliferation of chondrocytes in developing cartilage.23 On the other hand, the secreted C-terminus also promotes cell proliferation in certain types of cancer cells, such as glioblastoma, invasive ductal breast carcinoma, cervical squamous carcinoma, and prostate adenocarcinoma.24 Recent studies showed that the uncontrolled activation of Hh signaling leads to survival and proliferation of cancer cells and tumor formation.26,27 Therefore, Hh signaling is one of the most attractive targets for the development of anticancer drugs.28,29 Actually, various components of the Hh pathway are the focus of attention as therapeutic targets. Nevertheless, multiple genetic and molecular mechanisms resulting in resistance to existing Hh pathway-targeting drugs pose major limitations to anti-Hh therapies.30 Since the functions of BBF2H7 are controlled via RIP by S1P and S2P, modulating RIP of BBF2H7 may be a possible treatment for Hh signaling-dependent cancers. However, so far, the precise cleavage mechanisms of BBF2H7 have not been determined.

Although the cutting enzyme is different from the RIP of APP, ER stress transducers, including BBF2H7, also receive two-step cleavage in their luminal and transmembrane domains like APP. In addition, predicted lengths of the amino acid sequence between the transmembrane domain and the luminal cleavage site of the ER stress transducers are similar to those of APP. Therefore, it is possible that approximately 40-50 amino acid small peptides are also produced in RIP of ER stress transducers by S1P and S2P. The hydrophobic property of the small peptides containing the partial transmembrane region may be involved in the formation of the aggregates and may injure cells. Thus, it is significant to investigate the production of the small peptides derived from ER stress transducers.

In this study, we identified the RIP cleavage sites of BBF2H7. Our investigation revealed that BBF2H7 is cleaved by S2P in at least three sites in its transmembrane domain. Furthermore, we found that the small peptide fragments located between the S1P- and S2P cleavage sites of BBF2H7, named BBF2H7-derived small peptide (BSP) fragments, were produced in an ER stress-dependent manner. The peptide fragments had an aggregative property, and formed fibrils like Aβ peptide.

2 MATERIALS AND METHODS

2.1 Cell culture, reagents, plasmids, and siRNA

HEK293T and Hela cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) at 37°C in a 5% CO₂, 95% humidified air atmosphere. SK-N-SH cells were maintained in α-modified Eagle's medium (α-MEM; Gibco) supplemented with 10% FBS. M19 cells, which were kindly provided by Dr. Joseph L. Goldstein,2 were maintained in DMEM/F12 (a mixture of DMEM and Ham's F-12 medium; Gibco) supplemented with 10% FBS. For cell treatments, 1 μM thapsigargin (Wako, Osaka, Japan), 0.5 μM A23187 (Wako), 3 μg/mL tunicamycin (Wako), 1 mM dithiothreitol (Wako), 10 μM MG132 (Wako), and 100 μg/mL cycloheximide (Wako) were used. Major species of BSP fragment (CFAVAFGSFFQGYGPYPSATKMALPSQHPLEPYTASVVRSNLL) was synthesized by Peptide Institute (Osaka, Japan). Aβ1-42 peptide was purchased from Peptide Institute. Apelin-36 was purchased from Cayman chemical Company (Ann Arbor, MI, USA).

The pcDNA3.1(+) vectors expressing BBF2H7, BBF2H7-S1Pmut. (R427A/L430V), and FLAG-BBF2H7 were previously constructed.19 pCMV vectors expressing Myc-S1P and HSV-S2P were purchased from the American Type Culture Collection (ATCC). pcDNA3.1(+) vector expressing HA-S2P was generated by PCR using the following primer sets: 5′-GCGGCCGCCACCATGAGGACCTACCGGTTCGCCTCGTAAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGAGGAACTGA...
2.2 | Protein preparation and western blotting

Proteins were extracted from the cells in a cell lysis buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and Protease inhibitor cocktail set V (Wako) at 4°C. The lysates were incubated on ice for 15 minutes. After centrifugation at 15000 g for 15 minutes, the protein concentrations of the supernatants were determined using a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific). Equal amounts of proteins (10 μg) were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For immunoblotting, the following antibodies were used: anti-HA (1:1000; Cell Signaling Technology, 2367), anti-FLAG M2 (1:2000, Sigma, F3165), anti-V5 (1:1000, Cell Signaling Technology, 13202), anti-GST (1:1000, ROCKLAND, 600-102-200), anti-β-actin (1:10000, Sigma, A5441), and anti-ubiquitin (1:1000, Cell Signaling Technology, 3936S). Rabbit polyclonal anti-BBF2H7-N antibody (1:2000) was generated as described previously. Rabbit polyclonal antibody recognizing BSP fragments (1:1000) was raised against synthesized peptides corresponding to 398-426 of BBF2H7 fused to Keyhole limpet hemocyanin (KLH) at the N-terminus, respectively, and affinity-purified using a HiTrap NHS-activated HP column (GE healthcare). The samples were subjected to SDS-PAGE followed by western blotting analysis.

2.3 | Immunofluorescence staining

Hela and M19 cells were grown on coverslips and fixed in 4% paraformaldehyde for 30 minutes. After the fixation, cells were permeabilized in 0.1% Triton-X 100 for 5 minutes, followed by treatment with 10% goat serum for 60 minutes. These procedures were performed at room temperature (25°C). The following antibodies were used: anti-FLAG M2, anti-Myc (1:200; MBL, M192-3), anti-HA (1:200; Cell Signaling Technology, 2367), anti-Calnexin (1:200, Enzo Life Sciences, ADI-SPA-860), anti-GM130 (1:200, Cell Signaling Technology, 12480), and anti-Golgin97 (1:200, Cell Signaling Technology, 13192). Cells were visualized under a FV1000D confocal microscope (Olympus, Tokyo, Japan).

2.4 | Immunoprecipitation

Cells were lysed in a cell lysis buffer [10 mM Tris-HCl pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and Protease inhibitor cocktail set V (Wako)] for 15 minutes. Cell lysates were incubated with anti-BBF2H7-C (1 μg/1 × 10^6 cells, kindly provided by Mitsubishi Tanabe Pharma Corporation) or anti-BSP fragment (10 μg/1 × 10^6 cells, generated in this study) antibodies and Protein G Agarose Beads (Millipore) at 4°C overnight. The beads were rinsed three times with a wash buffer (10 mM Tris-HCl pH7.4, 5 mM EDTA, 150 mM NaCl, 0.1% Triton X-100). Immunoprecipitates were boiled with Laemmli SDS-PAGE sample buffer, followed by performing western blotting using the following antibodies: anti-BBF2H7-C (1:1000) and anti-BSP fragment (1:1000) antibodies.

2.5 | Amino acid sequencing by LC-MS/MS

For analysis of cleavage sites of BBF2H7, BBF2H7 N- or C-terminal fragments were collected from GST-BBF2H7- or BBF2H7-GST-transfected HEK293T or M19 cells treated with 1 μM Tg and 10 μM MG132 for 12 hours by Glutathione sepharose beads (GE healthcare). Collected proteins were electrophoresed by SDS-PAGE, followed by silver staining using Silver Stain MS kit (Wako). Then, target bands were digested with trypsin and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

2.6 | Amino acid sequencing by Edman degradation analysis

For analysis of N-terminal sequence of BSP fragments, the peptides were collected by immunoprecipitation using rabbit anti-BSP fragment antibody from cell lysates of BBF2H7-transfected HEK293T cells treated with 1 μM Tg and 10 μM MG132 for 12 hours. Collected BBF2H7-derived peptides were separated by electrophoresis and recollected by electrophoresis. Recollected peptides were concentrated by Speedvac, followed by performing electrophoresis by SDS-PAGE and being transferred to PVDF membrane. Transferred sample was cut out from the membrane, and sequenced by Edman degradation (Procise491cLC, ABI).

2.7 | Transmission electron microscopy (TEM) for observation of fibril formation of peptides

Fibril formations of Aβ1-42 and BSP fragments were analyzed using a modification of previously published protocols. Briefly, the synthetic BSP fragments were dissolved in dimethyl formamide to a concentration of 10 mM. The peptides were diluted to a final concentration of 100 μM in 50 mM potassium phosphate buffer (pH 7.4). Diluted peptides were then incubated at 37°C for 0, 12, 24, 48, and 72 hours. After incubation, peptides were centrifuged for 10 minutes at 15000 rpm, and then precipitated fibrils were analyzed by a TEM.
Precipitated fibrils were suspended in 10 μl of distilled water. Samples (3 μL) were applied to carbon-coated Slidefilm SLC-C15 (STEM) and incubated for 3 minutes at room temperature. Excess samples were absorbed with filter paper, after which an equal volume of uranyl acetate solution was added. After incubation for 2 minutes at room temperature, the solution was removed and the grid was air-dried. Samples were examined using a JEOL JEM-1400 transmission electron microscope.

3 | RESULTS

3.1 | Intramembrane proteolysis of BBF2H7

BBF2H7 has the RNLL sequence overlapping with potential recognition sites for S1P (RXXL) in its luminal domain, and it has been demonstrated that BBF2H7 is cleaved by S1P. However, the S2P cleavage site of BBF2H7 has not been identified (Figure 1A). To confirm cleavage of BBF2H7 in an ER stress-dependent manner, we carried out western blotting using anti-BBF2H7 N- and C-terminus antibodies. The 85 and 80 kDa full-length BBF2H7 [BBF2H7(F)] [glycosylated (higher) and unglycosylated forms (lower)] were gradually increased in HEK293T cells transfected with an expression vector for BBF2H7 by treatment with thapsigargin (Tg, which is an ER stressor) (Figure 1B). The amounts of 60 and 55 kDa BBF2H7 N-terminal fragments (BBF2H7-N) were also increased, synchronized with those of full-length BBF2H7. BBF2H7 is a notably unstable protein that is easily degraded via the ubiquitin-proteasome pathway under normal conditions. ER stress conditions enhance its stability because of avoidance from the degradation system. The stabilized BBF2H7 is translocated to the Golgi apparatus, followed by cleavage by S1P and S2P. The double bands of the N-termini were located to the Golgi apparatus, followed by cleavage by the degradation system. The stabilized BBF2H7 is translocated to the Golgi apparatus, followed by cleavage by S1P and S2P.

3.2 | BBF2H7 is cleaved at cis-Golgi apparatus by S1P and S2P

Although S1P and S2P are known to localize in the Golgi apparatus, the sub-Golgi localization of these proteases and detailed segments subjected to RIP are still unexplored. We analyzed the subcellular localization of S1P and S2P by performing immunofluorescence staining using Hela cells transfected with S1P tagged with myc (myc-S1P) or S2P tagged with HA (HA-S2P) (Figure 1F). Immunoreactivities of myc-S1P and HA-S2P were mostly overlapped with those of GM130 (a cis-Golgi apparatus marker). In addition, the signals of myc-S1P were partially detected in calnexin-positive ER. Immunoreactivities of both myc-S1P and HA-S2P were never detected in Golgin-97-positive trans-Golgi apparatus (Figure S1A). These data indicate that S1P and S2P are mainly localized in cis-Golgi apparatus, but S1P is partially localized in the ER. It is possible that small amounts of BBF2H7 are cleaved by S1P in the ER, followed by cleavage by S2P in the cis-Golgi apparatus. To examine this possibility, we chased the subcellular localization of BBF2H7 using M19 cells expressing BBF2H7 tagged with FLAG at the N-terminus with cycloheximide (CHX). In those cells, FLAG-BBF2H7 is cleaved by S1P at its luminal domain, but is never cut by S2P at its transmembrane region because of S2P deficiency. Eventually, FLAG-tagged N-terminal fragments should be retained at the cellular segment where BBF2H7 receives proteolytic cleavage by S1P. Western blotting showed that almost all of the full-length FLAG-BBF2H7 disappeared and small amounts of the FLAG-N-terminal fragments cleaved by S1P were detected at 24 hours after treatment with CHX (Figure 1G). Immunoreactivities of FLAG-BBF2H7 were observed in calnexin-positive ER before the treatment with CHX (Figure 1H). FLAG-N-terminal fragments strictly accumulated in the GM130-positive cis-Golgi apparatus after treatment with CHX for 24 hours, but not in the ER. Furthermore, these signals did not overlap with those of Golgin-97 (Figure S1B). The results suggest that BBF2H7 is cleaved by S1P and S2P in the cis-Golgi apparatus. Small levels of S1P signals in the ER could derive from the immature S1P forms which do not have proteolytic activities and are not translocated to the Golgi apparatus.
3.3 Determination of intramembrane cleavage sites of BBF2H7

Intramembrane cleavage sites of the bZIP transmembrane transcription factors, including BBF2H7 and ATF6, have not been investigated. We tried to sequence the cleaved ends of BBF2H7 N- and C-termini with LC-MS/MS analysis. Constructions of BBF2H7 for the sequencing and the purification procedures are depicted in Figure 2A. HEK293T cells were transfected with BBF2H7 tagged with glutathione

(A) ... (B) ... (C) ... (D) ... (E) ... (F) ... (G) ... (H) ...
BBF2H7 is cleaved by S1P and S2P in the cis-Golgi under ER stress conditions. A, Schematic structure of BBF2H7 and the fragments produced by ER stress. S1P: site-1 protease, S2P: site-2 protease. B, Western blot analysis of N-terminal fragments of BBF2H7 in HEK293T cells exogenously expressing BBF2H7. Cells were treated with 1 µM thapsigargin (Tg) for indicated times, and lysates were subjected to western blot analysis with an anti-BBF2H7 N-terminus antibody. Mock indicates empty vector. BBF2H7(F); full length of BBF2H7, BBF2H7(S1P), and BBF2H7(S2P); N-terminal fragments of BBF2H7 cleaved by only S1P and received cleavage by S2P, respectively. C, Western blot analysis of C-terminal fragments in HEK293T cells exogenously expressing BBF2H7. Cells were treated with Tg described as (B), and lysates were immunoprecipitated by anti-BBBF2H7 C-terminus antibody, followed by western blot analysis using the same antibody. BBF2H7(C): C-terminal fragments of BBF2H7. Asterisk indicates nonspecific bands. D, Western blot analysis of BBF2H7 N-terminal fragments in HEK293T cells expressing BBF2H7 wild-type or mutated in the S1P recognition site (BBF2H7_S1Pmut.). Cells were exposed to 1 µM Tg for indicated times. E, Western blot analysis of C-terminal fragments in HEK293T cells exogenously expressing BBF2H7. Cells were treated with Tg described as (B), and lysates were subjected to western blot analysis with an anti-BBBF2H7 N-terminus antibody. Mock indicates empty vector. BBF2H7(N): N-terminal fragments of BBF2H7. F, Immunofluorescence staining analysis using M19 cells expressing FLAG-tagged BBF2H7 (FLAG-BBF2H7). Cells were treated with 100 µg/mL cycloheximide (CHX) for indicated times. BBF2H7(N): N-terminal fragments of BBF2H7. H, Immunofluorescence staining analysis using M19 cells expressing FLA-GST-BBF2H7. Cells were treated with 100 µg/mL CHX for 24 hours. Bars: 10 µm.

S-transferase (GST) at its N- or C-termini (GST-BBF2H7 or BBF2H7-GST, respectively), followed by treatment with Tg for 12 hours. The N-terminal fragments of GST-BBF2H7 (GST-BBF2H7-N) and the C-terminal fragments of BBF2H7-GST (BBF2H7-C-GST) were purified using glutathione beads. 100 kDa full-length GST-BBF2H7 [GST-BBF2H7(F)] and 75 kDa N-terminal fragments [cleaved by S2P; GST-BBBF2H7-N(S2P)] were detected from purified fraction of cell lysates of HEK293T cells expressing GST-BBF2H7 (Figure 2B). 100 kDa full-length BBF2H7-GST [BBF2H7-GST(F)] and 40 kDa C-terminal fragments [cleaved by S1P; BBF2H7-C-GST(S1P)] were also purified from HEK293T cells expressing BBF2H7-GST (Figure 2C). The purified BBF2H7-C-GST(S1P) was digested with trypsin and subjected to LC-MS/MS analysis to determine the amino acid sequence. Only one potential peptide cleaved by S1P was detected by tryptic digestion of BBF2H7-C-GST(S1P). The N-terminal end of the peptide was Ile431, which is not a trypsin digestion site (Figure 2D). Ile431 is next to the C-terminal residue of the S1P recognition site (RNLL) in BBF2H7.

LC-MS/MS analysis following the trypsin digestion of purified GST-BBF2H7-N(S2P) showed two peptide fragments that were presumably cleaved by S2P. The C-terminal end of one peptide was Leu380 (Figure 2E). Another peptide contained Met381 at the C-terminal end. The C-terminal ends of both peptides were not tryptic digestion sites. In addition, they were not detected by the trypsin digestion of GST-BBF2H7(F) (data not shown), indicating that the C-terminal end of these peptides was cleaved by proteases other than trypsin. Akiyama et al. reported that RIP by a regulator of E protease (RseP), an Escherichia coli ortholog of mammalian S2P, is not inhibited by substitution of amino acid residues in and around the cleavage site of the substrates. They proposed that RseP prefers a partially destabilized helical conformation of a transmembrane sequence for proteolytic action. Cleavage sites of substrates for mammalian S2P are predicted to be decided in a similar manner. Site-directed mutations of potential cleavage sites within BBF2H7 may not inhibit the cleavage of this protein by S2P. Hence, we used S2P-deficient (M19) cells to confirm that the two cleavage sites detected by MS (Figure 2E) are ascribed to S2P. These peptides cleaved at the Leu380-Met381 peptide bond and Met381-Val382 peptide bond were not detected in the analysis of GST-BBF2H7-N purified from M19 cells expressing GST-BBF2H7 (Figure S2), indicating that the C-terminal ends of the two peptides were cleaved by S2P. From the LC-MS/MS analysis, we concluded that BBF2H7 is cleaved at two different sites in the transmembrane region: One between Leu380 and Met381, and another between Met381 and Val382. These expected multiple sites cleaved by S2P implicate that S2P cleaves BBF2H7 at variable cutting sites or through multistep cleavage in the transmembrane region. The peptide sequences detected by the LC-MS/MS analysis are illustrated in Figure 2F.

3.4 Production of the intermediate small peptide from BBF2H7

Theoretically, the cleavage of BBF2H7 by S1P and S2P produces three types of fragments: N-terminal and C-terminal fragments, and 40-50 amino acid BSP fragments located at the luminal domain, and the partial transmembrane region between S1P and S2P cleavage sites (Figure 3A). To examine whether BSP fragments are produced in response to ER stress, we raised antibodies against the putative luminal domain of BSP fragments in rabbits. We carried out immunoprecipitation followed by western blotting using the antibody (Figure 3B). As expected, we detected BSP fragments as approximately 6 kDa double bands. These fragments were detected at 12 hours and gradually increased in response to
FIGURE 2  Analysis of the cleavage sites of BBF2H7 by liquid chromatography-tandem mass spectrometry (LC-MS/MS). A, Schema of the LC-MS/MS analysis for the determination of the cleavage sites of BBF2H7 using glutathione S-transferase (GST)-tagged constructs. B, C, Western blot analysis for the purified BBF2H7 tagged with GST in its N-terminus (GST-BBF2H7-N) (B) and in its C-terminus (BBF2H7-C-GST) (C). GST-BBF2H7(F): full length of GST-BBF2H7, GST-BBF2H7-N(S2P): N-terminal fragment of GST-BBF2H7 cleaved by S2P, BBF2H7-GST(F): full length of BBF2H7-GST, BBF2H7-C-GST(S1P): C-terminal fragment of BBF2H7-GST cleaved by S1P, asterisk: C-terminal fragment of BBF2H7-GST cleaved by S2P. D, E, Identified sequences for the BBF2H7-C-GST (D) and the GST-BBF2H7-N (E) by LC-MS/MS analysis. Det. Mass: determined molecular mass, Calc. Mass: calculated molecular mass, Mox: oxidized methionine. F, Location of detected sequences from GST-tagged BBF2H7 by LC-MS/MS analysis. Arrow heads indicate tryptic digestion sites. Red and blue lines and green line indicate peptide sequences detected by the LC-MS/MS analysis of GST-BBF2H7-N and BBF2H7-C-GST, respectively. Transmembrane region of BBF2H7 is highlighted in gray.
the treatment with Tg. The double bands implicate that variable lengths of BSP fragments may be produced by ER stress. Furthermore, the amounts of full-length BBF2H7 and three types of BBF2H7-derived fragments (N- and C-terminal and BSP fragments) are simultaneously increased in an ER stress-dependent manner (Figures 1B,C and 3B). The temporal consistency of the ER stress-induced increase of these fragments suggests RIP-mediated production of these BBF2H7-derived fragments in response to ER stress.

Next, we sequenced major species of BSP fragments with Edman degradation analysis (Figure 3C). HEK293T cells transfected with BBF2H7 were treated with Tg for 12 hours. BSP fragments were collected from the cells by immunoprecipitation with an anti-BSP fragment antibody.
The collected peptides were purified by a two-step procedure using SDS-PAGE and electroelution. The purified peptides were electrophoresed by SDS-PAGE, then transferred to a PVDF membrane, and subjected to Edman degradation analysis. Consequently, we determined the N-terminal sequence of BSP fragments as XFAVA (X is an unknown residue in this experiment) (Figure 3D). We could not detect the amino acid at the N-terminal end of the BSP fragments. It is well known that cysteine residues are difficult to detect in Edman degradation analysis because of the instability of the released cysteine.35 Accordingly, an unidentified residue generally represents unmodified cysteine. The N-terminal sequence of the BSP fragments corresponds to the transmembrane region of BBF2H7 (from Cys386 to Ala390) if X is postulated as cysteine (Figure 3E). The identified N-terminus was different from the intramembrane cleavage sites detected in Figure 2. This inconsistency suggests that BBF2H7 may be cleaved by S2P through at least three cutting sites detected by the LC-MS/MS analysis and Edman degradation analysis (Figure 3E). Collectively, we concluded that BBF2H7 is cleaved by S1P at the luminal region and by S2P at variable cutting sites (at least three positions) at the transmembrane region. The variable cleavage in the transmembrane region is also observed in RIP by other iCLiPs such as γ-secretase and signal peptide peptidase-like 2b (SPL2b).31,36,37 Our result suggests that iCLiPs may not cleave specific peptide bonds. Moreover, we found that BSP fragments are produced by ER stress-dependent RIP of BBF2H7. Major species of BSP fragments consists of 45 amino acid residues containing the partial transmembrane region (from Cys386 to Leu430).

3.5 Production of BSP fragments via cleavage of BBF2H7 by S1P and S2P

Next, we investigated the biochemical characteristics of BSP fragments which are produced by the cleavage of BBF2H7. Approximately, 6 kDa BSP fragments were increased in HEK293T cells transfected with BBF2H7 by treatment with ER stressors (Tg or A23187) for 12 hours, corresponding to the increase in cleaved N-termini (Figure 4A). In contrast, BSP fragments were not observed from BBF2H7S1Pmut. transfected cells even though the treatment of cells with Tg. We could not detect BSP fragments in S2P-deficient M19 cells transfected with BBF2H7 (Figure 4B). Production of the BSP fragments was recovered by transfection with HA-S2P into M19 cells. These results revealed that BSP fragments are produced in response to ER stress through cleavage by S1P and S2P, and the cleavage site between Leu385 and Cys386 detected by Edman sequencing is cleaved by S2P.

Interestingly, an approximately 12 kDa band, in addition to the 6 kDa BSP fragment monomers, was detected in HEK293T cells transfected with BBF2H7 by treatment with some ER stressors [Tg, A23187, tunicamycin (Tm), and dithiothreitol (DTT)] (Figure 4C). The molecular weight of the band was double that of the monomeric BSP fragments. The 12 kDa band was gradually increased by all of the ER stressors that we examined. However, we could not detect any BSP fragments in the cells transfected with BBF2H7S1Pmut. These findings indicate that BSP fragments produced by RIP in response to ER stress form SDS-resistant dimers.

Endogenous BBF2H7 is degraded by the ubiquitin-proteasome system under normal conditions32; therefore, it is difficult to detect BSP fragments derived from endogenous BBF2H7. We tried to detect endogenous BSP fragments by treating the cells with a proteasome inhibitor, MG132. The amounts of ubiquitinated proteins detected as smeared bands following Tg treatment did not change when compared with those of nontreated cells (Figure 4D). In contrast, the amount of ubiquitinated proteins increased significantly following treatment with MG132. These data indicate that MG132 definitely blocks the proteolytic activities of the proteasome. Treatment with Tg promoted the cleavage of full-length BBF2H7 and the production of the N-termini. The amounts of full-length BBF2H7 and N-termini increased more when treated with both Tg and MG132 as compared with those cells treated with only Tg, which is consistent with previous research.32 The endogenous monomeric BSP fragments were detected in cells treated with Tg and MG132 but not in the cells treated with Tg alone. The knockdown of BBF2H7 canceled the production of endogenous BSP fragments. Collectively, we conclude that BSP fragments are produced endogenously when proteasomal degradation is inhibited.

3.6 Aggregative property and fibril formation of BSP fragments

Kyte-Doolittle analysis showed the highly hydrophobic characteristics of BSP fragments (45 amino acid major species identified in Figure 3E) in the N-terminal region located in the partial transmembrane domain of BBF2H7 (Figure 5A). As shown in Figure 4C, BSP fragments exhibited homodimerization, suggesting the potential aggregative property. Thus, we assessed the aggregative property of BSP fragments in detail. The 10 pmol synthetic peptide, based on the identified 45 amino acid sequence shown in Figure 3C, was detected as approximately 6 kDa monomeric fragments by western blotting under reduced conditions [using 2-mercaptoethanol (2-ME)] (Figure 5B). The molecular weight was equal to those of monomeric BSP fragments extracted from HEK293T cells expressing BBF2H7. The synthetic BSP fragments did not form the oligomer in this dose, although BSP fragments extracted from the cells exhibited oligomerization. Under nonreduced conditions (denatured without 2-ME at 37°C), 10 pmol synthetic BSP fragments formed 12
kDa homodimers as well as the 6 kDa monomer (Figure 5C). 16 kDa trimeric and 22 kDa tetrameric forms were observed by the high amount of nonreduced synthetic BSP fragments (100 pmol). The monomeric forms of 10 pmol nonreduced BSP fragments were decreased, and trimeric forms appeared in the incubation at 37°C for 24 hours. The 100 pmol non-reduced BSP fragments were detected as highly aggregated smeared bands of high molecular size after incubation. These data indicate that synthetic BSP fragments readily aggregate at 37°C. The aggregation propensity is induced in a dose-dependent manner. The oligomers were decreased, but partially remained under reduced condition (denatured with 2-ME at 37°C), suggesting that these oligomers may be partially formed through the intermolecular disulfide bonds mediated by the N-terminal cysteine (Cys386). The oligomerization patterns of reduced and boiled BSP fragments (denatured with 2-ME at 100°C) were similar to those of the reduced BSP fragments. These results indicate that the oligomerization of BSP fragments is resistant to heat denaturation. Additionally, we evaluated the aggregation of synthetic BSP fragments by blue native-PAGE (Figure 5D). The synthetic BSP fragments were detected as aggregated smeared bands of high molecular size, although Δmembrane fragments that are lacking the N-terminal transmembrane region of BSP fragments (from Pro400 to Leu430) exhibited no smeared bands. These findings indicate that BSP fragments have an aggregative property and easily oligomerize via the highly hydrophobic N-terminal transmembrane region. Furthermore, we conducted morphological analysis of synthetic BSP fragments using transmission electron microscopy (TEM) (Figure 5E). The 10 μM synthetic BSP fragments were incubated in PBS at 37°C for 12, 24, 48, and 72 hours. The fragments were not aggregative before the incubation. The fibril-like structures gradually increased as time proceeded. In contrast, the hydrophilic synthetic peptide, Apelin-36 (negative control) (Figure S3), never formed the fibril-like structures. The BSP fibrils elongated until 72 hours incubation (Figure 5E and F). The elongated fibrils intertwined, resulting in the formation of large bundle structures (see higher magnification of Figure 5E). The width of
FIGURE 5  Small peptide fragments derived from BBF2H7 have highly aggregative properties, and form amyloid-like fibrils. A, Kyte-Doolittle hydrophobicity plot for BSP fragments. Amino acid position is presented on the X-axis. Kyte-Doolittle hydrophobicity scores (window size n = 9) for individual amino acids are on the Y-axis. B, Western blot analysis of the intracellularly produced BSP fragments. The BSP fragments were collected from BBF2H7-transfected HEK293T cells treated with 1 µM Tg and 10 µM MG132 for 12 hours by immunoprecipitation using an anti-BSP fragment antibody. C, Dimer, trimer, and tetramer formation of BSP fragments. Synthesized BSP fragments with or without incubation (37°C, 24 hours) were subjected to western blotting analysis with the anti-BSP fragment antibody under nonreduced, reduced, or reduced-and-boiled conditions. D, Blue native-PAGE for the synthetic BSP fragments and control peptides lacking the transmembrane region (Δmembrane), followed by western blot analysis with the anti-BSP fragment antibody. E, Negative-stain TEM micrographs of BSP fragments and control peptides. Scale bar: 200 nm. F, Length of the fibrils of BSP fragments incubated at 37°C for the indicated times (means ± SD, n = 5). G, Length, width, and periodicity of the fibrils of amyloid β (Aβ) and BSP fragments incubated at 37°C for 72 hours (means ± SD, n = 5). H, Highly magnified negative-stain TEM micrographs of fibrils formed by BSP fragments and Aβ1-42. Scale bar: 50 nm
the fibrils was approximately 10 nm, and the length was approximately 600 nm (Figure 5G). These features are a little larger than those of amyloid fibrils formed by the Aβ peptide. In addition to the difference in size, the BSP fibrils did not show the periodical turns observed in Aβ fibrils (Figure 5G and H). Collectively, BSP fragments have a unique potential to form fibril structures, but the characteristics of these fibrils are different from those of amyloid fibrils formed by the Aβ peptide.

4 | DISCUSSION

We have successfully determined the intramembrane cleavage sites of BBF2H7. Interestingly, BBF2H7 is cleaved in at least three sites in its transmembrane domain by S2P (Figure 3E). RseP also showed multiple cleavages.34 RseP cleaves the first transmembrane domain of lactose permease LacY between Phe15 and Phe16, Phe16 and Phe17, and Phe17 and Phe18.34 These positions are in the central region of its transmembrane domain.34 BBF2H7 is also cleaved at its medial region of the transmembrane domain (next to Leu385, Figure 3E). Thus, the proteolytic characteristics are conserved in terms of the multiple cleavage and the cleaving positions in the membrane between S2P and RseP.

Previously, S2P cleavage site was determined only in SREBP2 (at Leu484-Cys485 bond).14 In this study, we identified three intramembranous cleavage sites of BBF2H7 (between Leu380 and Met381, Met381 and Val382, and Leu385 and Cys386) (Figure 3E). However, ATF6 and three OASIS family members (Luman, AlbZIP, and CREBH) do not contain Leu-Met, Met-Val, or Leu-Cys sequences.17,38-40 Therefore, S2P may determine the cleavage position without depending on the amino acid sequence. Gamma-secretase and SPPL2b also cleave variable intramembranous region of their substrates.31,36,37 For example, γ-secretase cleaves APP between Val and Ile, Ile and Ala, Ala and Thr, Leu and Val, Ile and Val, and Thr and Leu, while Notch-1 is cleaved by γ-secretase between Ala and Ala, Ala and Phe, Phe and Val, and Gly and Val.41,42 Different cutting sites for each substrate suggest that RIP by iCLiPs may cleave various peptide bonds. Akiyama et al. have reported that replacement of helix-destabilizing residues in the substrate with helix-promoting residues interfered with its cleavage by RseP.34,43 These reports imply that BBF2H7 may be cleaved at the position where the helix structure is loosened. Structural analysis of BBF2H7 is needed to unravel how S2P decides the cleavage positions.

The cleaved C-terminal end of BBF2H7 N-terminal fragments did not correspond to the N-terminal end of BSP fragments, indicating that the intramembrane region of BBF2H7 is cleaved by S2P in a multistep manner. APP is known to be cleaved by γ-secretase in a similar multistep manner. The ε-cleavage occurs at the C-terminal region of the intramembrane domain. The central region of the transmembrane domain subsequently receives γ-cleavage. It is known that both γ- and ε-cleavages occur at variable sites (at least two patterns: next to Val40 and Ala42, and next to Thr48 and Leu49, respectively).41 The initial ε-cleavage sites determine the subsequent γ-cleavage sites, and that provides various types of Aβ species.41 Major species of BSP fragments are produced via cleavage in the medial transmembrane region of BBF2H7 by S2P (Figure 3E). To refer to cleavage of APP, the primary cleavage sites near the membrane-cyttoplasm boundary of BBF2H7 may decide the subsequent cleavage sites in the middle of the transmembrane domain, resulting in the generation of variable BSP fragments. Successive intramembrane proteolysis by S2P will be supported by the detection of short peptides corresponding to amino acid sequences from Val382 to Leu385 (VVVL), or Met381 to Leu385 (MVVVL). However, we failed to detect intermediates containing these sequences in LC-MS/MS and Edman degradation analyses. These results may suggest the instability of the intermediates because of immediate multistep cleavage by S2P. Another possibility is that the intermediates might not be produced in RIP by S2P because multiple cleavages occur simultaneously. We are generating antibodies that recognize VVVL and MVVVL sequences to detect the intermediates produced in RIP of BBF2H7. Further analyses using the generated antibodies will reveal the precise processes of intramembrane cleavage by S2P.

We found that small peptide fragments are produced from bZIP transmembrane transcription factors via cleavage by S1P and S2P. High aggregation propensity of BSP fragments is supported by the following results: 1) BSP fragments showed high hydrophobicity by Kyte-Doolittle analysis, 2) synthetic BSP fragments exhibited SDS-resistant oligomerization, and 3) synthetic BSP fragments facilitated the formation of fibrils during incubation at 37°C. Neurodegenerative disorders are known to be triggered by accumulation of aggregated proteins. One of those are Aβs. Amyloid fibrils are formed through the interaction between β-sheet structures of the Aβ.44 However, the predicted structure of BBF2H7 does not contain β-sheet structures.19 α-synuclein, which contributes to Parkinson's disease through the aggregation and formation of fibrils, forms random-coil- or β-sheet structures.45 The β-sheet form of α-synuclein shows higher aggregation propensity than those of random-coil form.45 Various buffer conditions, including high ionic strength and the presence of Cu2+ ions, cause conformational changes between the random-coil- or β-sheet structures.45 It is possible that the structure of BSP fragments may also change to β-sheet structures in response to various cellular conditions and form fibrils involved in the interaction of each β-sheet structure. Numerous studies have indicated that ER stress is involved in the onset of neurodegenerative disorders.46-48 Excessive and prolonged ER stress may trigger abnormal production and accumulation
of BSP fragments. Amyloid fibrils, which are found in patients with Alzheimer’s disease, that accumulate inside or outside of cells cause cytotoxicity and are associated with the pathology. In this study, we found that BSP fragments also exhibit high aggregation propensity and form fibrils. Hyper and prolonged production of BSP fragments by ER stress may cause neurotoxicity, leading to modification of the pathogenesis of neurodegenerative disorders such as Alzheimer’s disease. To answer these hypotheses, it is essential to define the intra- and extracellular localization and dynamics of BSP fragments. Anti-BSP fragment antibodies that specifically recognize the BSP fragments and not full-length BBF2H7 are necessary for elucidating the precise localization and dynamics in brains. However, the anti-BSP fragment antibodies used in this study also recognize full-length BBF2H7. We are currently raising antibodies that can specifically recognize BSP fragments. Future studies will be required to analyze the detailed characteristics including intra- and extracellular accumulation, cytotoxicity, the degradation pathway in brains, and links to neurodegenerative disorders using these newly generated antibodies that only recognize the BSP fragments.

It is known that secreted C-terminal fragment of BBF2H7 contributes proliferation of cancer cells. RIP is an essential process to produce the BBF2H7-derived fragments. Therefore, inhibition of the RIP could be a potential approach for treatment of cancers. However, S1P- or S2P-specific inhibitors have not been developed. Several peptidomimetic γ-secretase inhibitors were developed by modifying γ-secretase cleavage site of APP. These compounds act as transition-state analogs, and effectively block the production of Aβ. Although both serine protease S1P and metalloprotease S2P are different types of protease from γ-secretase, inhibitors against these types of protease were also developed by modifying peptides corresponding to cutting sites of each substrates. Peptidomimetics containing the cleavage sites of BBF2H7 identified in this study may inhibit S1P or S2P in the same manner and be useful for the treatment of tumors overexpressing BBF2H7. However, S1P and S2P are also involved in the activation of SREBPs, ATF6, and other OASIS family members. To avoid severe side effects, such as dysregulations of lipid metabolism and ER stress response, further analysis for RIP of BBF2H7 is needed to design a highly specific inhibitor.

In conclusion, we have demonstrated that bZIP transmembrane transcription factors receive multistep variable cutting. The small peptide fragments produced by the variable cleavage in response to ER stress demonstrated high aggregation propensity. Our present study suggests a novel molecule linking ER stress with various ER stress-related diseases, including neurodegenerative diseases.

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AUTHOR CONTRIBUTIONS
K. Matsuhisa, A. Saito, L. Cai, and F. Sakaue performed experiments. A. Saito and K. Imaizumi supervised the project. K. Matsuhisa, A. Saito, and K. Imaizumi designed the experiments, analyzed the data, and wrote the manuscript. L. Cai, M. Kaneko, T. Okamoto, and R. Asada helped to construct expression vectors. M. Matsumoto carried out LC-MS/MS analysis. F. Urano, K. Yanagida, M. Okochi, Y. Kudo, and K. Nakayama provided substantial input into the writing of the manuscript.

CONFLICT OF INTEREST
All authors declare no competing financial interests within this article.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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