Heliyon 6 (2020) e05804

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Identification of novel substrates of a disintegrin and metalloprotease 17 by specific labeling of surface proteins

Kazuki Omoteyama [a,*], Toshiyuki Sato [a], Masaaki Sato [a], Atsuhiro Tsutiya [a], Mitsumi Arito [a], Naoya Suematsu [a], Manae S. Kurokawa [b], Tomohiro Kato [a]

[a] Clinical Proteomics and Molecular Medicine, St. Marianna University Graduate School of Medicine
[b] Disease Biomarker Analysis and Molecular Regulation, St. Marianna University Graduate School of Medicine

ARTICLE INFO

Keywords:
ADAM17
Ectodomain shedding
Cell surface protein-specific labeling
Proteomics

ABSTRACT

A disintegrin and metalloprotease 17 (ADAM17) catalyzes the cleavage and release of the ectodomains of its substrates at the cell surface in a process termed ectodomain shedding. However, not all ADAM17 substrates have been identified. Here, we used cell surface protein-specific labeling and proteomic approaches to detect and identify ADAM17 substrates. HeLa cell surface proteins were labeled with a fluorescent dye and cultured with or without TAPI-2, an ADAM17 inhibitor. Labeled proteins released into the culture medium were detected by 2-dimensional gel electrophoresis (2DE). Protein spots showing decreased intensity in response to TAPI-2 were selected as substrates of ADAM17 or their binding proteins, and identified by mass spectrometry. *ADAM17* knockdown was preformed to examine the behavior of identified proteins. Of 347 proteins detected by 2DE, 49 showed lower intensity in TAPI-2 (+) than in TAPI-2 (-) samples ($p < 0.05$), and were considered as candidate substrates of ADAM17. Mass spectrometric analysis of 14 protein spots showing >50% decreased intensity identified clusterin as a novel ADAM17 substrate, in addition to known substrates such as desmoglein-2. Western blot analysis showed that *ADAM17* knockdown decreased the levels of clusterin fragments cleaved and released from the cell surface. The results identified clusterin as a novel ADAM17 substrate. The method used to identify clusterin could be used to identify the substrates of other sheddases involved in ectodomain shedding.

1. Introduction

The ectodomains of certain membrane proteins are released by membrane-type metalloproteinase-mediated proteolysis in a process termed "ectodomain shedding" [1]. Among these proteases, a disintegrin and metalloprotease 17 (ADAM17) catalyze the proteolytic cleavage and release of the ectodomain of membrane-bound tumor necrosis factor-alpha (TNF-α) as a soluble cytokine [2]. Various substrates of ADAM17, such as desmoglein-2 and L1 cell adhesion molecule (L1CAM), have been identified to date [3, 4]. Because the released ectodomains of the substrates of ADAM17 can act as ligands to activate cells, abnormal expression of ADAM17 may be involved in the pathophysiology of some diseases. For example, TNF-α promotes articular inflammation in patients with rheumatoid arthritis (RA) [5]. ADAM17 expression in synovial tissue is significantly higher in patients with RA than in those with osteoarthritis [6], suggesting that it contributes to the overproduction of soluble TNF-α in the synovial tissues of patients with RA.

Therefore, discovering novel substrates for ADAM17 would improve our understanding of the pathophysiology of some diseases. However, identifying novel substrates for ADAM17 is technically challenging. Because known substrates of ADAM17 do not have common amino acid sequences [7], it is difficult to predict ADAM17 substrates based on their amino acid sequences. Furthermore, recombinant ADAM17 fails to cleave several full-length substrates in *in vitro* assays [8]. One explanation for this is that a specific structural modification of the cell surface is required for digestion of certain substrates of ADAM17 [9]. This structural change may determine substrate specificity, suggesting that soluble ADAM17 and cell surface ADAM17 have different substrates.

To shed light on this issue, we selected one of the most well-studied sheddases, ADAM17, and one of the most commonly established cell lines, HeLa. ADAM17 is expressed in HeLa cells and its level is increased in certain cancers, including cervical cancer [10]. However, not all of the roles of ADAM17 in cancer have been identified [10]. We performed a comprehensive analysis to identify substrates of ADAM17 using cell surface protein-specific labeling, an ADAM17 inhibitor, and a proteomic

* Corresponding author.
E-mail address: omoteyama@marianna-u.ac.jp (K. Omoteyama).

https://doi.org/10.1016/j.heliyon.2020.e05804
Received 21 August 2020; Received in revised form 5 November 2020; Accepted 18 December 2020

approach. The cell surface proteins of living cells were labeled with a membrane-impermeable fluorescent dye and cultured in the presence or absence of the ADAM17 inhibitor, TAPI-2. Candidate ADAM17 substrates were selected by comparing the expression of labeled proteins using two-dimensional gel electrophoresis (2DE) and were identified by mass spectrometry. We identified clusterin as a novel candidate substrate for ADAM17 and confirmed its identity by western blotting. The results suggest that this method is effective for identifying substrates for various proteases that mediate ectodomain shedding in addition to ADAM17.

2. Materials and methods

2.1. Cell culture and cell surface protein-specific labeling

The human cervical carcinoma cell line HeLa was maintained in Dulbecco's modified Eagle's medium (GIBCO, CA, USA) supplemented with 10% fetal bovine serum (FBS; Wako Pure Chemical Industries, Japan), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich, MO, USA) in a humidified atmosphere with 5% CO_2 at 37 °C. The medium was replaced twice a week. Cell surface protein-specific labeling was performed using sulfo-cyanine3-NHS ester (sulfo-Cy3-NHS; ab146458, Abcam, UK), sulfo-cyanine5-NHS ester (sulfo-Cy5-NHS; ab146459, Abcam), Cy3-NHS ester (Cy3-NHS; IC3-OSu, Dojindo, Japan), and sulfo-NHS-LC-LC-biotin (21338; Thermo Fisher Scientific, MA, USA). Briefly, living cells were incubated with 50 μM sulfo-Cy3-NHS, sulfo-Cy5-NHS, Cy3-NHS, or sulfo-NHS-LC-LC-biotin at 4 °C for 30 min, washed with PBS, and then incubated in FBS-free medium supplemented with 15 μM $ZnCl_2$. To inhibit ADAM17 activity, TAPI-2 (Cayman Chemical, MI, USA) was added to the medium at a concentration of 100 μM.

2.2. Confocal laser-scanning microscopy

Living cells were plated in an 8 chamber slide (AGC techno glass, Japan) and incubated with or without sulfo-Cy3-NHS and Cy3-NHS at 4 °C. After washing, the cells were fixed with 4% paraformaldehyde and mounted with SlowFade Gold Antifade Mountant with DAPI® (Thermo Fisher Scientific). Cells were visualized by confocal laser-scanning microscopy (LSM 510 META; Zeiss, Germany).

2.3. Time-course of the detection of released cell surface proteins

Internal control samples were prepared by incubating confluent sulfo-Cy3-NHS-labeled cells from a 150 mm dish in 15 mL culture medium (CM) for 24 h and transferring the medium to a conical tube. Cells and debris were removed by centrifugation, and the proteins in the CM were concentrated by membrane ultrafiltration (Vivaspin 10 kDa; GE Healthcare, IL, USA). The volume was reduced from 15 mL to 250 μL (internal control). For the preparation of analysis samples, confluent sulfo-Cy5-NHS-labeled cells in a 60 mm dish were incubated in 1 mL CM for 0.5–24h and then the medium was transferred to a sample tube. After adding 10 μL of the internal control, the medium was concentrated by membrane ultrafiltration, resulting in a reduction in volume from 1 mL to 20 μL (analysis sample). Equal amounts of the analysis samples were separated by SDS-PAGE, and fluorescence signals were detected by image scanning. The Cy5-fluorescent intensity was normalized using Cy3-fluorescent intensity. The Cy5 intensity was calculated from three independent experiments and the average intensity of the samples after 0.5 h incubation was defined as 1.0.

2.4. Two-dimensional gel electrophoresis (2DE)

The preparation of internal control samples and analysis samples was performed as described under 2.3. Briefly, confluent sulfo-Cy5-NHS-labeled cells in a 60 mm dish were incubated in 1 mL CM for 1 h. Proteins were precipitated from the culture media using trichloroacetic acid (TCA; final concentration, 10%). Then, the proteins were washed with ethanol and dissolved in 100 μL urea lysis buffer (8 M Urea, 4% CHAPS). All proteins collected from one dish were considered as one sample for analysis. Internal control samples were prepared by incubating confluent sulfo-Cy3-NHS-labeled cells from a 100 mm dish in 10 mL CM for 24 h. Proteins in the medium were concentrated by membrane ultrafiltration, transferred to a microtube, and precipitated from the CM of sulfo-Cy3-NHS-labeled cells using TCA. The proteins were washed with ethanol and dissolved in 100 μL urea lysis buffer. For 2DE analysis, equal amounts of the sulfo-Cy3-NHS-labeled sample, as an internal control, and the sulfo-Cy5-NHS-labeled samples were mixed and subjected to isoelectric focusing electrophoresis (IEF) in 13 cm-long, pH 3–11 non-liner gradient gels (GE Healthcare). The IEF-separated proteins were further separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were detected using an image analyzer (Typhoon 9400 Imager, GE Healthcare). The intensity of the detected protein spots was quantified using the Progenesis Same Spots® software (Nonlinear Dynamics, UK). Briefly, the Cy5-fluorescent intensity of protein spots was normalized to the Cy3-fluorescent intensity of the identical spots. Then, the normalized Cy5-intensity was used to compare the TAPI-2 (+) condition with the TAPI-2 (-) condition.

2.5. Protein identification

Protein samples (1 mg) labeled with sulfo-Cy3-NHS were subjected to IEF and 10% SDS-PAGE as described above. Gel fragments corresponding to protein spots of interest were subjected to in-gel digestion with trypsin. The generated peptides were extracted from the gel fragments into a solution containing 5% TFA and 50% acetonitrile, desalted using a Zip Tip C18 (Millipore Corporation, MA, USA), and analyzed by matrix-assisted laser desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS; Ultraflex, Bruker Daltonics, Germany). Peptide masses identified by MS and MS/MS analyses were compiled to search the Swiss-Prot database using the Mascot software program (Matrix Science, UK).

2.6. Protein sample preparation from ADAM17 knock down cells

To knock down *ADAM17*, cells were transfected with small interfering RNA (siRNA) against *ADAM17* and control siRNA (sc-36604, sc-37007; Santa Cruz Biotechnology, CA, USA). Briefly, 20 pmol siRNA were mixed with 6.6 μL of Lipofectamine RNAiMAX (13778; Thermo Fisher Scientific) in 0.6 mL of DMEM. After diluting the mixture to a volume of 3 ml with antibiotic-free culture medium, the mixture was added to 50 % confluent cells in a 60 mm dish. Cell surface proteins were labeled with sulfo-NHS-LC-LC-biotin at 48 h after siRNA transfection, and cells were cultured for 24 h. Biotinylated proteins released into the CM were isolated using CaptAvidin beads (C21386; Thermo Fisher Scientific).

2.7. Western blot analysis

Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes. The membranes were incubated in a solution containing mouse anti-ADAM17 antibody (sc-390859, Santa Cruz Biotechnology), mouse anti-L1CAM antibody (sc-53386, Santa Cruz Biotechnology), mouse anti-desmoglein-2 antibody (sc-80663, Santa Cruz Biotechnology), rabbit anti-galectin-3 antibody (ab76245, Abcam), rabbit anti-galectin-3 binding protein (G3BP) antibody (ab217572, Abcam), rabbit anti-clusterin antibody (42143S, Cell Signaling Technology, MA, USA), and mouse anti-beta-actin (ACTB) antibody (sc-47778, Santa Cruz Biotechnology). After washing, bound antibodies were reacted with Alexa Fluor 647-conjugated goat anti-rabbit IgG H&L (ab150079, Abcam) or Alexa Fluor 647-conjugated goat anti-mouse IgG H&L (ab150115, Abcam). The fluorescent signals were detected using an image analyzer (Typhoon 9400 Imager).

2.8. Statistical analysis

Results are presented as the mean ± standard deviation of triplicate samples, and differences were assessed by Student's t-test. P-values <0.05 were considered statistically significant.

3. Results

3.1. Sulfo-cy3-NHS specifically labels cell surface proteins

We tested whether sulfo-Cy3-NHS, a membrane-impermeable dye, could specifically label cell surface proteins on living cells. Living HeLa cells adhered to a chamber slide were treated with sulfo-Cy3-NHS and the Cy3-NHS of a membrane-permeable dye as a negative control. Labeled proteins were visualized by confocal laser-scanning microscopy. Sulfo-Cy3-NHS-labeled proteins were detected only on the cell surface, whereas Cy3-NHS-labeled proteins were mainly detected in the cytoplasm (Figure 1A). These data indicate that sulfo-Cy3-NHS specifically labels cell surface proteins on living cells.

3.2. Time-course measurement of ectodomain shedding

To detect the ectodomains of cell surface proteins released to the CM, the amounts of labeled proteins in the CM were measured at different time points. HeLa cells treated with sulfo-Cy5-NHS were cultured for up to 24 h. Labeled proteins released to the CM were visualized by SDS-PAGE followed by image scanning. Labeled proteins were detected as early as 30 min after incubation, and the amount of released labeled proteins increased in a time-dependent manner for up to 24 h (Figure 1B).

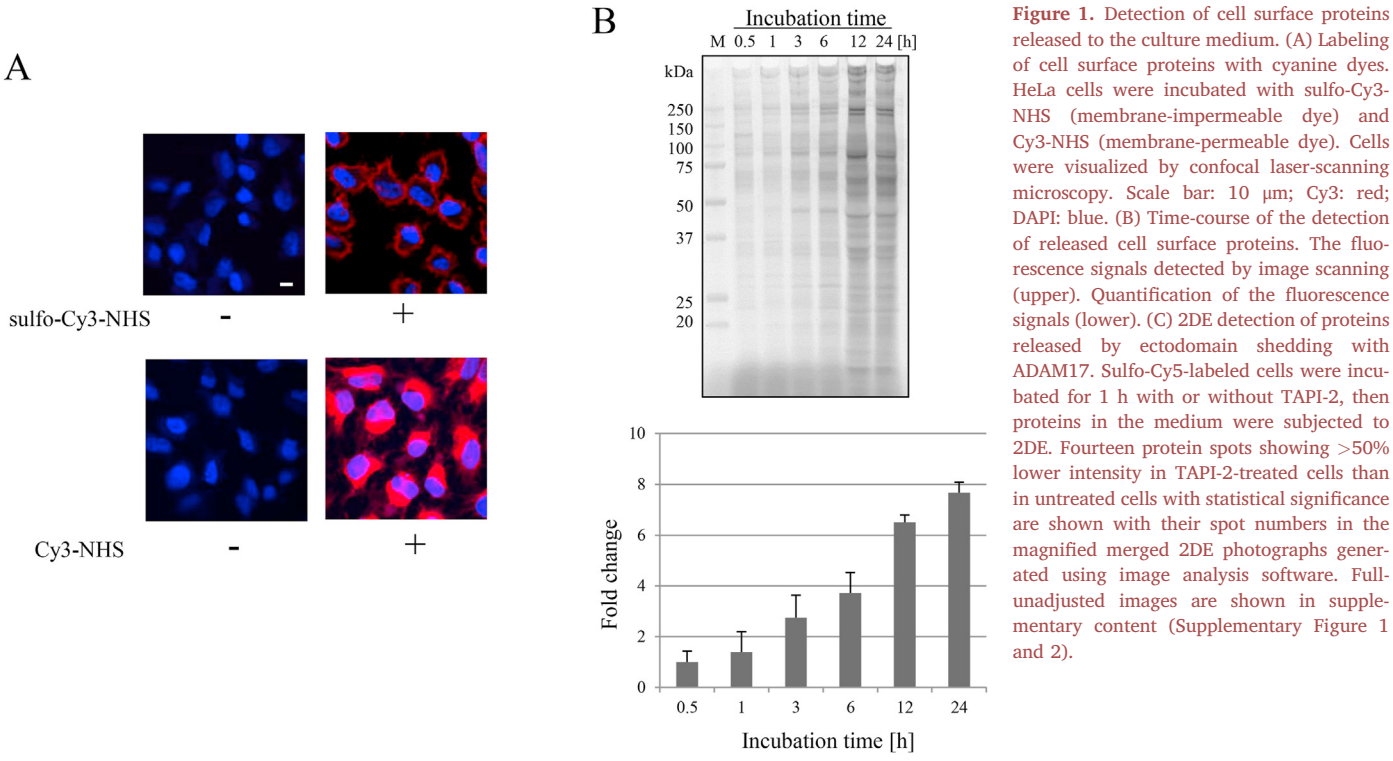

Figure 1. Detection of cell surface proteins released to the culture medium. (A) Labeling of cell surface proteins with cyanine dyes. HeLa cells were incubated with sulfo-Cy3-NHS (membrane-impermeable dye) and Cy3-NHS (membrane-permeable dye). Cells were visualized by confocal laser-scanning microscopy. Scale bar: 10 μm; Cy3: red; DAPI: blue. (B) Time-course of the detection of released cell surface proteins. The fluorescence signals detected by image scanning (upper). Quantification of the fluorescence signals (lower). (C) 2DE detection of proteins released by ectodomain shedding with ADAM17. Sulfo-Cy5-labeled cells were incubated for 1 h with or without TAPI-2, then proteins in the medium were subjected to 2DE. Fourteen protein spots showing >50% lower intensity in TAPI-2-treated cells than in untreated cells with statistical significance are shown with their spot numbers in the magnified merged 2DE photographs generated using image analysis software. Full-unadjusted images are shown in supplementary content (Supplementary Figure 1 and 2).

3.3. Comprehensive detection of proteins released by ectodomain shedding

Comprehensive detection of surface proteins released by ectodomain shedding mediated by ADAM17 was achieved by culturing sulfo-Cy5-labeled cells in the presence and absence of the ADAM17 inhibitor TAPI-2, and by comparing the profiles of released labeled proteins by 2DE (Figure 1C). The most effective culture time for comparing the TAPI-2 (+) and TAPI-2 (-) conditions was 1 h. As shown in Table 1, of 347 protein spots detected, 49 showed decreased intensity in response to TAPI-2 treatment ($p < 0.05$). The fold-change was calculated as the ratio of the staining intensity in the TAPI-2 (+) condition to that in the TAPI-2 (-) condition [i.e., TAPI-2 (+)/TAPI-2 (-)]. Proteins corresponding to the 49 spots were considered as candidate substrates of ADAM17 or substrate-binding proteins.

3.4. Identification of candidate ADAM17 substrates by mass spectrometry

Of the 49 spots showing decreased intensity, we focused on 14 proteins that showed a >50% decrease in intensity in response to TAPI-2 treatment (Figure 1C). As shown in Table 2, we identified the proteins of 7 of the 14 spots. Spots 159, 877, and 1082 were identified as desmoglein-2, L1 cell adhesion molecule (L1CAM), and galectin-3 binding protein (G3BP), respectively. Spots 429, 432, 433, and 1033 were identified as clusterin. Desmoglein-2 and L1CAM were previously reported to be ADAM17 substrates [3, 4], whereas clusterin and G3BP are not known ADAM17 substrates. The present data thus identified clusterin and G3BP as novel candidate substrates of ADAM17.

3.5. Confirmation studies of desmoglein-2 and L1CAM

To further examine the identity of the selected protein spots as ADAM17 substrates or their binding proteins, we first confirmed that desmoglein-2 and L1CAM are ADAM17 substrates [3, 4] by western blotting. A band of approximately 150 kDa corresponding to the theoretical molecular mass of full-length desmoglein-2 was detected in the whole cell (WC) lysate, and a band of approximately 90 kDa, which is the molecular mass of cleaved desmoglein-2 [3], was detected in the CM (Figure 2A). Similarly, a band of approximately 220 kDa corresponding to the theoretical molecular mass of full-length L1CAM was detected in the WC, and a band of approximately 210 kDa, which is the molecular mass of cleaved L1CAM [4], was detected in the CM (Figure 2B). These results suggest that full-length desmoglein-2 and L1CAM were cleaved by shedding, and that the ectodomain was released into the CM. Next, we tested the effect of siRNA-mediated knockdown of *ADAM17* on desmoglein-2 and L1CAM ectodomain shedding. As shown in Figure 2C, ADAM17 expression was suppressed at 24 h after siRNA transfection, and this effect was maintained for 96 h after transfection. Cell surface proteins were labeled with sulfo-NHS-LC-LC-biotin at 48 h after siRNA transfection, and cells were cultured for 24 h. Biotinylated proteins released into the CM were isolated using CaptAvidin beads. The results showed that siRNA-mediated knockdown of *ADAM17* decreased the levels of the desmoglein-2 and L1CAM ectodomains in the CM (Fig. 2D and E).

3.6. Detailed analysis of clusterin and G3BP

The potential role of clusterin as an ADAM17 substrate was investigated next. Western blot analysis detected a band of approximately 65 kDa, which corresponded to the theoretical molecular mass of full-length clusterin, in the WC lysate, and a lower molecular mass band of approximately 37 kDa in the CM (Figure 3A). These results indicate that clusterin was cleaved by shedding, and a fragment was released to the CM. In addition to spots 429, 432, 433, and 1033, which were identified as clusterin by mass spectrometry, 2D-western blot analysis of the CM identified spots 983, 1039, and 1053 as clusterin (Figure 3B). These seven clusterin spots were scattered between p*I* 4.8 and 5.2, and molecular masses of 35–41 kDa, suggesting different post-translational modifications and/or various digestion events. To determine whether the release of clusterin from the cell surface to the medium was mediated by ADAM17, the expression of clusterin in the CM of *ADAM17*-knockdown cells was analyzed by western blotting. Biotin-labeled proteins released from the cell surface were detected using the avidin/biotin method, and clusterin was identified by western blotting. As shown in Figure 3C, siRNA-mediated knockdown of *ADAM17* decreased the levels of clusterin in the CM. Taken together, these data indicate that clusterin may be a novel substrate of ADAM17.

Next, we investigated whether G3BP is an ADAM17 substrate or a binding protein. A band of approximately 110 kDa, which corresponds to the theoretical molecular mass of full-length G3BP, was detected in the WC lysate and in the CM by western blotting (Figure 3D). These results indicate that G3BP was not cleaved by ADAM17. Because G3BP binds to galectin-3 [11], we investigated whether galectin-3 is a substrate of ADAM17. A band of approximately 28 kDa, the molecular mass of full-length galectin-3, was detected by western blotting in both the WC and CM fractions (Figure 3E), indicating that galectin-3 is not a substrate of ADAM17. Furthermore, siRNA-mediated knockdown of *ADAM17* decreased the amount of G3BP released from the cell surface (Figure 3F). Taken together, these data indicate that G3BP is not a substrate of ADAM17, whereas it binds to substrates of ADAM17.

4. Discussion

In this study, a combination of cell surface protein-specific labeling, 2DE analysis of the effects of ADAM17 inhibition, and mass spectrometric analysis were used to analyze and identify substrates of ADAM17. Since highly specific inhibitors of ADAM17 have not yet been established, we used TAPI-2, which has relatively high specificity for ADAM17 [12]. By combining use of TAPA-2 and use of siRNA for ADAM17, it was possible to eliminate both the ADAM17-non-specific effects of TAPI-2 and the off-target effects of siRNA. The results can be summarized as follows: (1) the 2DE analysis of cell surface proteins in the CM showed that inhibition of ADAM17 decreased the expression of 49 of the 347 detected protein spots. These 49 proteins were considered as candidate substrates of ADAM17 or their binding proteins. (2) Analysis of 10 spots identified four proteins, desmoglein-2, L1CAM, clusterin, and G3BP, of which desmoglein-2 and L1CAM were previously reported as ADAM17 substrates. (3) The cleavage and release of clusterin from the cell surface was

Table 1. The intensities of protein spots that were significantly altered by TAPI-2 treatment.

The total number of detected protein spots	The number of protein spots with different intensities ($p < 0.05$)	Fold differences	Numbers of the protein spots
347	62	≥2.0	1
		≥1.5	5
		>1.0	13
		<1.0	49
		≤1/1.5	45
		≤1/2.0	14

Table 2. Identification of protein spots by MS.

	Spot	Difference	Observed		Theoretical		Protein	Accession No.	NPM	Mascot score	Coverage (%)	Sequence confirmed
		TAPI-2	Mass [kDa]	p*I*	Mass [kDa]	p*I*						(Mascot ion score)
1	432	-3.91	37	5.1	52.5 (65^{glyco})	5.9	clusterin	P10909	9	140	17	^{326}ELDESLQVAER^{336} (31)
												^{409}LFDSDPITVTVPVEVSR^{425} (45)
2	1082	-3.81	110	4.3	65 (110^{glyco})	5.1	G3BP	Q08380	12	149	22	^{138}ELSEALGQIFDSQR^{151} (42)
												^{377}TLQALEFHTVPFQLLAR^{393} (60)
3	433	-3.36	36	5.2	52.5 (65^{glyco})	5.9	clusterin	P10909	9	212	16	^{326}ELDESLQVAER^{336} (81)
												^{409}LFDSDPITVTVPVEVSR^{425} (62)
												^{438}ALQEYR^{443} (19)
4	159	-3.21	95	4.8	122 (150^{glyco})	5.1	desmoglein-2	Q14126	11	136	11	^{407}GQIIGNFQAFDEDTGLPAHAR^{427} (77)
5	783	-3.15	200	5.1			N.D.					
6	1053	-3.07	35	5.2	52.5 (65^{glyco})	5.9	clusterin	P10909				
7	877	-2.88	210	5.0	140 (220^{glyco})	5.8	L1CAM	P32004	17	153	12	^{248}LQLNYLGNYIPR^{259} (22)
												^{500}HTLGHLLSLDSYR^{512} (11)
												^{635}AQFDLAFVVR^{644} (6)
8	983	-2.55	39	5.0	52.5 (65^{glyco})	5.9	clusterin	P10909				
9	1039	-2.51	41	4.8	52.5 (65^{glyco})	5.9	clusterin	P10909				
10	429	-2.46	38	5.1	52.5 (65^{glyco})	5.9	clusterin	P10909	9	68	12	^{409}LFDSDPITVTVPVEVSR^{425} (46)
11	1033	-2.42	40	4.9	52.5 (65^{glyco})	5.9	clusterin	P10909	9	86	14	^{168}QQTHMLDVMQDHFSR^{182} (16)
												^{183}ASSIIDELFQDR^{194} (3)
												^{215}RPHFFFPK^{222} (25)
12	109	-2.19	190	5.2			N.D.					
13	810	-2.08	205	5.0			N.D.					
14	1109	-2.06	200	5.3			N.D.					

p*I*: isoelectric point, NPM: number of peptides matched, M: oxidated methionine, glyco: glycosylation, N.D.: not detected.

mediated by ADAM17, whereas G3BP was not identified as a substrate of ADAM17, but rather as a protein bound to a substrate of ADAM17.

Biotin is a widely used reagent for labeling cell surface proteins; however, biotin labeling is not suitable for the quantitative comparison of multiple samples by 2DE. We therefore used membrane-impermeable dyes such as sulfo-Cy3-NHS and sulfo-Cy5-NHS to label surface proteins in living cells. This strategy enabled the quantitative comparison of released proteins between cells with active and inactive ADAM17 by 2DE. Although the amount of labeled proteins released to the CM increased in a time-dependent manner up to 24 h (Figure 1B), the most effective incubation period for comparing protein profiles between the TAPI-2 (+) and TAPI-2 (-) conditions by 2DE in our preliminary experiments was 1 h (data not shown). Long-term culture may mask the effects of the slowdown of the reaction rate by competitive inhibition when the amount of substrate is limited. Even in the presence of sufficient TAPI-2, labeled substrates on the cell surface are digested by ADAM17 when the reaction time is extended. For these reasons, experiments were performed using cells cultured for 1 h. Of the 347 protein spots detected by 2DE, 49 (14.1%) showed decreased staining intensity in response to TAPI-2 treatment ($p < 0.05$). The proteins contained in these 49 spots were considered candidate substrates of ADAM17 or their binding proteins. More than 80 molecules, including membrane-tethered cytokines, growth factors, and cell surface receptors, have been reported as ADAM17 substrates [13]. ADAM17 is an important enzyme regulating ectodomain shedding in HeLa cells. In this study, TAPI-2 increased the staining intensity of 13 protein spots ($p < 0.05$) (Table 1). One possible explanation for this result is that the membrane-bound sheddases responsible for digesting the 13 cell surface proteins are also substrates of ADAM17. In this case, an ADAM17 inhibitor would upregulate the sheddases on the cell surface, resulting in an increased release of the ectodomains of their substrates. This point should be investigated in the future.

We analyzed 14 of the 49 protein spots showing a marked decrease in staining intensity in the presence of TAPI-2. The proteins of 10 of the 14 spots were identified as desmoglein-2, L1CAM, clusterin, and G3BP.

Desmoglein-2 and L1CAM were previously reported as substrates of ADAM17 [3-4]. In addition to the proteomic identification, the ectodomains of desmoglein-2 and L1CAM released into the CM were detected by western blotting (Figure 2), and the results showed that their levels were decreased by siRNA-mediated knockdown of *ADAM17*. The detection of known ADAM17 substrates indicates that our method is reliable for the identification of substrates of ADAM17, and could be valuable for investigating other sheddases and shedding mechanisms.

Clusterin is detected on the surface of some types of cells [14, 15]. Here, we detected cleaved clusterin in the CM, and showed that its expression was decreased by siRNA-mediated knockdown of *ADAM17* (Figure 3). To the best of our knowledge, this is the first study demonstrating that the cleavage and release of clusterin are mediated by ADAM17. Full-length clusterin contains 449 amino acids, and is cleaved between Arg227 and Ser228 in the Golgi apparatus [16]. The resulting two clusterin fragments form a heterodimer, which is incorporated into high-density lipoprotein cholesterol [17]. However, clusterin can evade the secretion pathway under certain stress conditions [18]. The fragment ions detected in our MS/MS analysis were scattered in the area from Gln168 to Arg443. This suggests that clusterin is cleaved by ADAM17 between amino acids 1–167 instead of being cleaved in the Golgi apparatus. To clarify this point, we attempted to demonstrate the digestion of clusterin by ADAM17, and to identify the cleavage site using recombinant clusterin and ADAM17. However, the recombinant full-length clusterin was not cleaved by ADAM17 (data not shown). This phenomenon may be a specific feature of ADAM17, as supported by a previous study showing that physiological substrates of ADAM17, including pro-TGF-α, L-selectin, p55 TNFR, and p75 TNFR, are not cleaved by recombinant ADAM17 *in vitro* [8]. Therefore, it is difficult to demonstrate conclusively that clusterin is a substrate of ADAM17.

G3BP is a secreted glycoprotein that binds to galectin-3 at the cell surface [10, 19]. In this study, we showed that full-length G3BP was released to the CM by ADAM17; however, G3BP and galectin-3 were not cleaved by ADAM17 (Figure 3). Galectin-3 binds to the galactose residues of some substrates of ADAM17. This point needs further investigation.

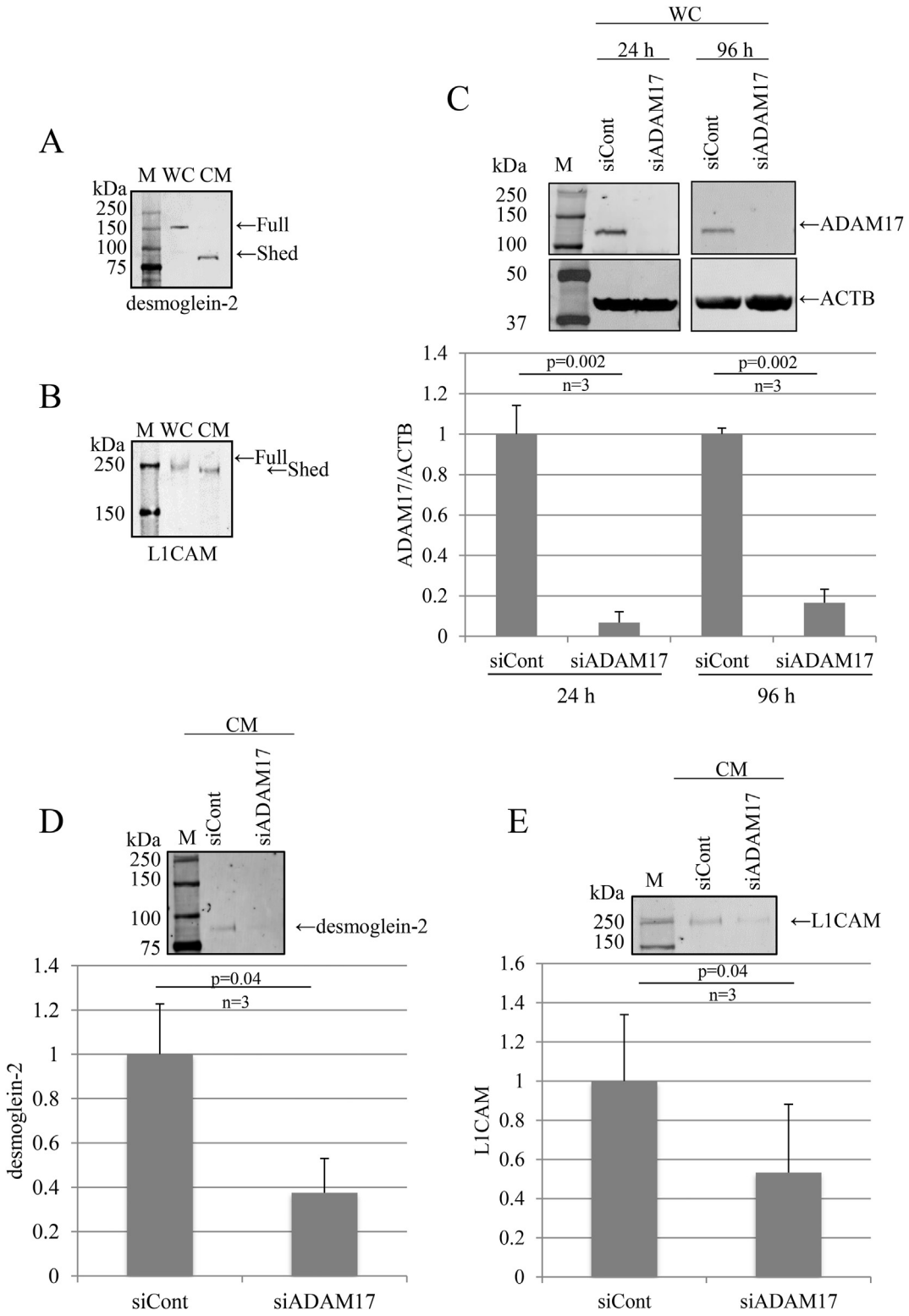

Figure 2. Effects of *ADAM17* knockdown on the shedding of desmoglein-2 and L1CAM. Detection of desmoglein-2 (A) and L1CAM (B) by western blotting. M: molecular weight markers. WC: whole cell lysate. CM: culture medium. (C) siRNA-mediated knockdown of *ADAM17*. At 24 and 96 h after siRNA transfection, ADAM17 and ACTB were detected by western blotting. ADAM17 levels were normalized to the level of ACTB. Detection of desmoglein-2 (D) and L1CAM (E) in the culture medium. Protein samples (10 μg) were loaded into each well. Full-unadjusted images are shown in supplementary content (Supplementary Figure 3).

The combination of the two techniques, cell surface protein labeling and 2DE, successfully identified clusterin as a substrate of ADAM17, in addition to detecting several known substrates of ADAM17. By employing other sheddase inhibitors and/or gene knockdowns, it should be possible to use this methodology to identify the substrates of other sheddases.

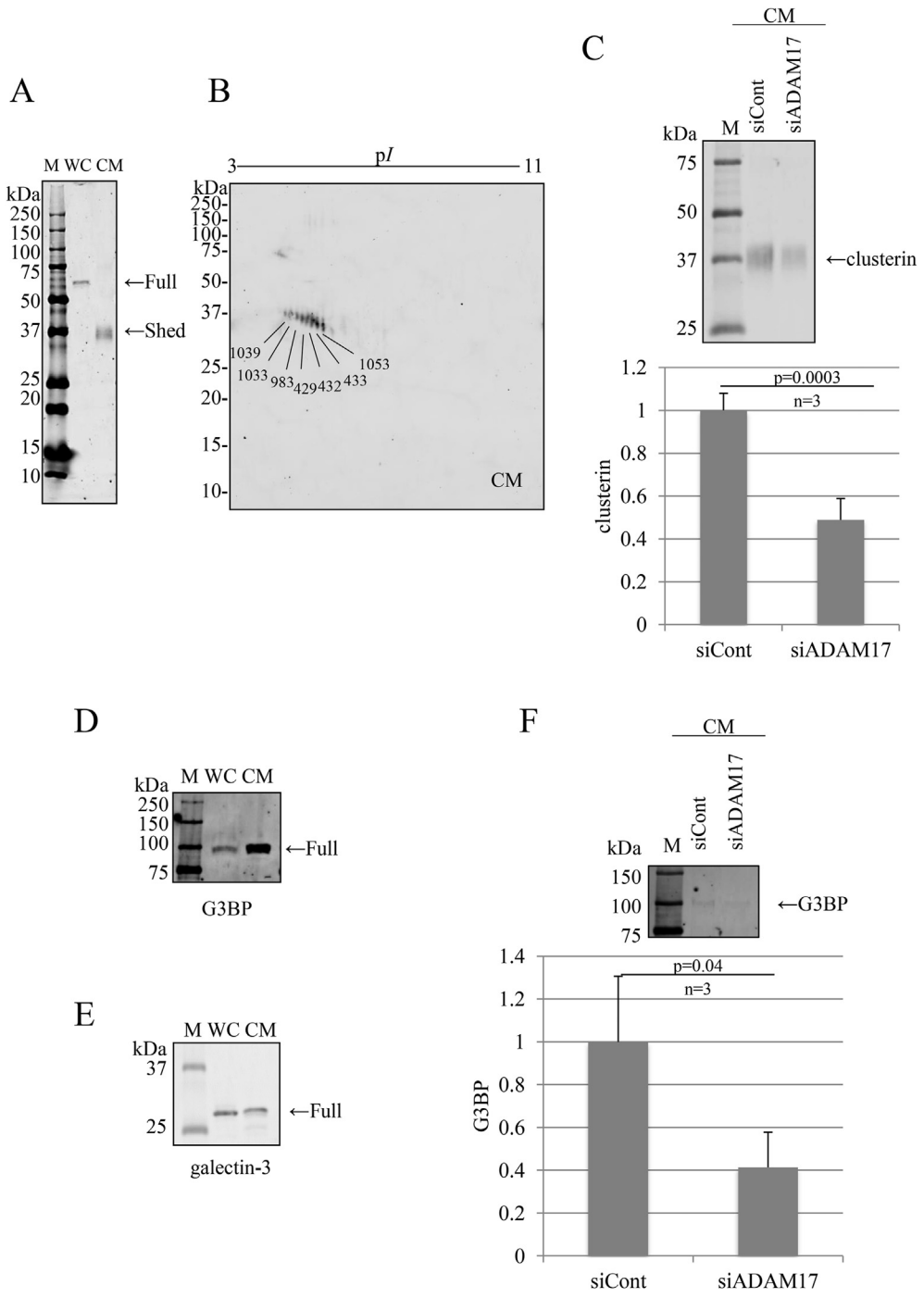

Figure 3. Effects of *ADAM17* knockdown on the shedding of clusterin and G3BP. Detection of clusterin by 1D- (A) and 2D-western blotting (B). (C) Detection of clusterin in the culture medium. Protein samples (10 μg) were loaded into each well. Western blot (upper). Quantification of the bands (lower). Detection of G3BP (D) and galectin-3 (E) by western blotting. (F) Detection of G3BP in the culture medium. Eluted protein samples (10 μg) were loaded into each well. Western blot (upper). Quantification of the bands (lower). Full-unadjusted images are shown in supplementary content (Supplementary Figure 3).

Declarations

Author contribution statement

Kazuki Omoteyama: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Toshiyuki Sato, Masaaki Sato, Atsuhiro Tsutiya, Mitsumi Arito: Contributed reagents, materials, analysis tools or data.

Naoya Suematsu, Manae S. Kurokawa: Analyzed and interpreted the data.

Tomohiro Kato: Analyzed and interpreted the data; Wrote the paper.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2020.e05804.

References

[1] J.J. Peschon, J.L. Slack, P. Reddy, K.L. Stocking, S.W. Sunnarborg, D.C. Lee, W.E. Russell, B.J. Castner, R.S. Johnson, J.N. Fitzner, R.W. Boyce, N. Nelson, C.J. Kozlosky, M.F. Wolfson, C.T. Rauch, D.P. Cerretti, R.J. Paxton, C.J. March, R.A. Black, An essential role for ectodomain shedding in mammalian development, Science 282 (1998) 1281–1284.

[2] R.A. Black, C.T. Rauch, C.J. Kozlosky, J.J. Peschon, J.L. Slack, M.F. Wolfson, B.J. Castner, K.L. Stocking, P. Reddy, S. Srinivasan, N. Nelson, N. Boiani, K.A. Schooley, M. Gerhart, R. Davis, J.N. Fitzner, R.S. Johnson, R.J. Paxton, C.J. March, D.P. Cerretti, A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells, Nature 385 (1997) 729–733.

[3] J.J. Bech-Serra, B. Santiago-Josefat, C. Esselens, P. Saftig, J. Baselga, J. Arribas, F. Canals, Proteomic identification of desmoglein 2 and activated leukocyte cell adhesion molecule as substrates of ADAM17 and ADAM10 by difference gel electrophoresis, Mol. Cell Biol. 26 (2006) 5086–5095.

[4] S. Beer, M. Oleszewski, P. Gutwein, C. Geiger, P. Altevogt, Metalloproteinase-mediated release of the ectodomain of L1 adhesion molecule, J. Cell Sci. 112 (1999) 2667–2675.

[5] Y. Nozaki, Y. Nagare, C. Ashida, D. Tomita, A. Okada, A. Inoue, K. Kinoshita, M. Funauchi, I. Matsumura, Infliximab dose adjustment can improve the clinical and radiographic outcomes of rheumatoid arthritis patients: REVIVE study results, Biologics 12 (2018) 171–182.

[6] S. Ohta, M. Harigai, M. Tanaka, Y. Kawaguchi, T. Sugiura, K. Takagi, C. Fukasawa, M. Hara, N. Kamatani, Tumor necrosis factor-alpha (TNF-alpha) converting enzyme contributes to production of TNF-alpha in synovial tissues from patients with rheumatoid arthritis, J. Rheumatol. 28 (2001) 1756–1763.

[7] J. Tucher, D. Linke, T. Koudelka, L. Cassidy, C. Tredup, R. Wichert, C. Pietrzik, C. Becker-Pauly, A. Tholey, LC-MS based cleavage site profiling of the proteases ADAM10 and ADAM17 using proteome-derived peptide libraries, J. Proteome Res. 13 (2014) 2205–2214.

[8] R. Stawikowska, M. Cudic, M. Giulianotti, R.A. Houghten, G.B. Fields, D. Minond, Activity of ADAM17 (a disintegrin and metalloprotease 17) is regulated by its noncatalytic domains and secondary structure of its substrates, J. Biol. Chem. 288 (2013) 22871–22879.

[9] A. Sommer, F. Kordowski, J. Büch, T. Maretzky, A. Evers, J. Andrä, S. Düsterhöft, M. Michalek, I. Lorenzen, P. Somasundaram, A. Tholey, F.D. Sönnichsen, K. Kunzelmann, L. Heinbockel, C. Nehls, T. Gutsmann, J. Grötzinge, S. Bhakdi, K. Reiss, Phosphatidylserine exposure is required for ADAM17 sheddase function, Nat. Commun. 7 (2016) 11523.

[10] Q. Xu, M. Ying, G. Chen, A. Lin, Y. Xie, N. Ohara, D. Zhou, ADAM17 is associated with EMMPRIN and predicts poor prognosis in patients with uterine cervical carcinoma, Tumour Biol. 35 (2014) 7575–7586.

[11] H. Inohara, A. Raz, Identification of human melanoma cellular and secreted ligands for galectin-3, Biochem. Biophys. Res. Commun. 201 (1994) 1366–1375.

[12] J. Arribas, L. Coodly, P. Vollmer, T.K. Kishimoto, S. Rose-John, J. Massagué, Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors, J. Biol. Chem. 271 (1996) 11376–11382.

[13] F. Zunke, S. Rose-John, The shedding protease ADAM17: physiology and pathophysiology, Biochim. Biophys. Acta Mol. Cell Res. 1864 (2017) 2059–2070.

[14] T.T. Hochgrebe, D. Humphreys, M.R. Wilson, S.B. Easterbrook-Smith, A reexamination of the role of clusterin as a complement regulator, Exp. Cell Res. 249 (1999) 13–21.

[15] M.K. O'Bryan, B.F. Murphy, D.Y. Liu, G.N. Clarke, H.W. Baker, The use of anticlusterin monoclonal antibodies for the combined assessment of human sperm morphology and acrosome integrity, Hum. Reprod. 9 (1994) 1490–1496.

[16] N.H. Choi-Miura, Y. Takahashi, Y. Nakano, T. Tobe, M. Tomita, Identification of the disulfide bonds in human plasma protein SP-40,40 (apolipoprotein-J), J. Biochem. 112 (1992) 557–561.

[17] H.V. de Silva, W.D. Stuart, C.R. Duvic, J.R. Wetterau, M.J. Ray, D.G. Ferguson, H.W. Albers, W.R. Smith, J.A. Harmony, A 70-kDa apolipoprotein designated ApoJ is a marker for subclasses of human plasma high density lipoproteins, J. Biol. Chem. 265 (1990) 13240–13247.

[18] P. Nizard, S. Tetley, Y. Le Dréan, T. Watrin, P. Le Goff, M.R. Wilson, D. Michel, Stress-induced retrotranslocation of clusterin/ApoJ into the cytosol, Traffic 8 (2007) 554–565.

[19] S. Dong, R.C. Hughes, Macrophage surface glycoproteins binding to galectin-3 (Mac-2-antigen), Glycoconj. J. 14 (1997) 267–274.