J. Biochem. **143,** 731–745 (2008)
doi:10.1093/jb/mvn030

Human Calpain 7/PalBH Associates with a Subset of ESCRT-III-related Proteins in its N-terminal Region and Partly Localizes to Endocytic Membrane Compartments

Chiharu Yorikawa[1], Emi Takaya[1], Yohei Osako[1], Ryohei Tanaka[1], Yoshinori Terasawa[1], Takao Hamakubo[2], Yasuhiro Mochizuki[2], Hiroko Iwanari[2], Tatsuhiko Kodama[2], Tatsuya Maeda[3], Kiyotaka Hitomi[1], Hideki Shibata[1] and Masatoshi Maki[1,*]

[1]Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601; [2]Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro, Tokyo 153-8904; and [3]Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

Received December 20, 2007; accepted February 13, 2008; published online March 3, 2008

Calpain 7 (also known as PalBH) is a mammalian homologue of the *Aspergillus*, atypical calpain PalB. Knowledge of the biochemical properties of calpain 7 is limited and its function is not yet known. In this study, we investigated the interactions of calpain 7 with all 11 ESCRT-III-related proteins, named charged multivesicular body proteins (CHMPs), and the subcellular localization of calpain 7. Pulldown assays using stable HEK293T transfectants of Strep-tagged calpain 7 revealed interactions of calpain 7 with a subset of FLAG-tagged CHMPs, among which CHMP1B was selected for further analyses. The N-terminal region containing a tandem repeat of MIT domains of calpain 7 was found to be necessary and sufficient for interaction with CHMP1B. Direct interaction was confirmed by a pulldown assay using recombinant proteins. Fluorescence microscopic analysis using HeLa cells revealed that overexpression of GFP-fused CHMPs or a dominant-negative construct of SKD1/Vps4B caused accumulation of epitope-tagged calpain 7 in a punctate pattern in the perinuclear area. Subcellular fractionation revealed that the most of endogenous calpain 7 is present in the cytosol but a small portion is present in particulate fractions. Punctate fluorescence signals of monomeric GFP-fused calpain 7 partly merged with those of endocytosed tetramethylrhodamine-labelled EGF. These results suggest that calpain 7 plays roles in the endosomal pathway by interacting with a subset of ESCRT-III-related proteins.

Key words: calpain, CHMP, endosome, ESCRT, MIT domain.

Abbreviations: CBB, Coomassie Brilliant Blue R-250; CHMP, charged multivesicular body protein; ESCRT, endosomal sorting complex required for transport; FBS, fetal bovine serum; GST, glutathione-*S*-transferase; MIT, microtubule interacting and trafficking; mAb, monoclonal antibody; mGFP, monomeric green fluorescent protein; mRFP, monomeric red fluorescent protein; MVB, multivesicular body; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PEF, penta-EF-hand; Rh-EGF, tetramethylrhodamine-labelled epidermal growth factor; shRNA, short hairpin RNA; Trx, thioredoxin; Vps, vacuolar protein sorting; WB, western blotting.

Typical calpains, represented by m- and μ-calpains, are calcium-dependent cysteine proteases that are found in animal cells. Their structure–function relationships and activation mechanisms have been extensively studied (*1–3*). The m- and μ-calpains are heterodimeric enzymes with specific catalytic subunits (~80 kDa) and a common regulatory subunit (~30 kDa). Both large and small subunits have five EF-hands (penta-EF-hand domain, PEF) at their C-termini and belong to the PEF Ca^{2+}-binding protein family, including ALG-2 (*4*, *5*). In addition to or in place of typical calpains, eukaryotic cells from lower to higher organisms harbour atypical calpains that lack PEF domains but possess unique domains, comprising the calpain superfamily (*2*, *3*). One such atypical calpain is PalB, which was first identified in the filamentous fungus *Aspergillus nidulans* (*6*). PalB is one of six gene products (PalA, PalB, PalC, PalF, PalH and PalI) that are required for the alkaline adaptation pathway, and PalB is essential for activation of a transcription factor, PacC, by limited proteolysis (*7*, *8*).

PalA associates with PacC by recognizing a motif designated YPXL/I (*9*). Possessing a Bro1 domain, PalA interacts also with Snf7 (also named Vps32, vacuolar protein sorting 32), a component of ESCRT-III (endosomal sorting complex required for transport-III) (*8*). Yeast genetics have revealed that multi-protein complexes of ESCRT-I, -II and -III are essential for formation of MVB (multivesicular body) luminal vesicles and

*To whom correspondence should be addressed. Tel: +81-52-789-4088, Fax: +81-52-789-5542, E-mail: mmaki@agr.nagoya-u.ac.jp

sorting of ubiquitinated cargo proteins into these vesicles (*10*). Thus, it is thought that PalA recruits PacC to endosomal membranes where pH sensor proteins (PalH and PalI) endocytosed from plasma membranes are transported. Recently, PalC was also found to possess a Bro1 domain and to directly associate with Snf7 (*11*). A similar pH signalling pathway is found in the budding yeast *Saccharomyces cerevisiae*, and orthologues of the *Aspergillus* alkaline adaptation factors, including Cpl1 (also named Rim13)/PalB, Rim20/PalA, YGR122w/PalC and Rim101/PacC (*7*, *8*, *12* and references therein), have been identified. ESCRTs have been shown to play critical roles in yeast for Rim101 processing (*13–15*). Results of genome-wide interaction analyses by yeast two-hybrid screening suggested that Cpl1 is an interacting partner of Snf7 (*16*). However, information on the biochemical properties of PalB/Cpl1 is limited and recruitment of the proteases to endosomes has not been clearly demonstrated. Humans have a total of eleven ESCRT-III and related proteins called charged multivesicular body proteins (CHMPs) (charged MVB proteins), all of which possess Snf7 domains and are classified into seven subfamilies (CHMP1-7) (*17*, *18*). CHMP1 and CHMP2 have two isoforms (CHMP1A, CHMP1B, CHMP2A and CHMP2B) and CHMP4 has three isoforms (CHMP4a, CHMP4b and CHMP4c).

Various human tissues express the mRNA of a PalB homologue named PalBH, designated calpain 7 in this article (*19*). Exogenously overexpressed epitope-tagged calpain 7 exhibited nuclear localization in COS7 cells and did not show any proteolytic activities against known calpain substrates (*19*). The function of calpain 7 in mammalian cells remains unkown. A bioinformatic database search has revealed that calpain 7 possesses a tandem repeat of MIT (microtubule interacting and trafficking) domains (*20*). Some CHMP proteins have been shown to associate with MIT domains of two different AAA-type ATPases (Spastin and Vps4) (*21*, *22*) and with those of two distinct classes of deubiquitinating enzymes, AMSH and UBPY (*23*, *24*). In this report, we present for the first time evidence that the N-terminal region containing two MIT domains of calpain 7 interacts with a subset of CHMPs, including CHMP1A, CHMP1B and CHMP4b. Monomeric GFP (mGFP)-fused calpain 7 was recruited to aberrant endosomes that were formed by overexpression of an ATPase-defective dominant negative mutant of SKD1/Vps4B. Moreover, mGFP-calpain7 partly localized with endocytosed EGF at membrane compartments. Although substrates of calpain 7 could not be identified, these findings suggest that calpain 7 functions in the endosomal pathway.

MATERIALS AND METHODS

Antibodies and Reagents—The following mouse monoclonal antibodies (mAbs) were used: anti-Strep-tag II mAb (IBA GmbH), anti-FLAG mAb (clone M2, Sigma, St Louis, MO, USA), anti-GFP (green fluorescent protein) mAb (clone B-2, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-α-tubulin mAb (clone DM 1A, Sigma), anti-calnexin mAb (clone 37, BD Biosciences, San Jose, CA, USA), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mAb (Chemicon, Billerica, MA, USA), anti-CD107a/Lamp-1 mAb (Pharmingen, Palo Alto, CA, USA) and anti-calnexin mAb (clone 37, BD Biosciences). Rabbit anti-GFP antiserum (A6455) and anti-m-calpain (ab93165) polyclonal antibody (pAb) were obtained from Invitrogen/Molecular Probes, Carlsbad, CA, USA and Abcam, Cambridge, UK, respectively. Antiserum against recombinant human calpain 7 was raised in rabbits using a GST-fused N-terminal fragment containing MIT domains (GST-calpain7MIT) as an antigen, and pAb was affinity purified. Mouse mAb against human calpain 7 was raised by immunizing mice with recombinant baculovirus particles displaying an N-terminal region (78–157 a.a.) of calpain 7 as fusion protein to the viral surface glycoprotein gp64 as previously described (*25*, *26*). Positive hybridoma cells were screened by ELISA and western blotting (WB) using recombinant viral proteins, by WB using purified recombinant GST-calpain7MIT and lysates of HEK293 cells that expressed mGFP-calpain7 and then by immunostaining of HeLa cells that expressed mGFP-calpain7. One of the obtained clones, designated clone Y0717, was selected for the present study. Peroxidase-conjugated goat anti-mouse and anti-rabbit IgG antibodies were obtained from Jackson Immunoresearch Laboratories, West Groove, PA, USA. Cy3-labelled and Alexa 488-labelled goat anti-mouse IgGs used for indirect immunofluorescence analyses were obtained from GE Healthcare, Uppsala, Sweden and Molecular Probes, respectively. Blasticidine S hydrochloride and G418 were obtained from Funakoshi, Tokyo, Japan and Nacalai Tesque, Kyoto, Japan, respectively.

Plasmid Constructions—Cloning of human calpain 7/PalBH cDNA was described previously (*19*). For exogenous expression of Strep-calpain7, mGFP-calpain7, FLAG-calpain7 and untagged calpain 7, DNA fragments corresponding to calpain 7 were amplified by PCR and inserted into mammalian expression vectors of pEXPR-IBA105-A (*17*), pmGFP-C1 (monomeric EGFP, modified according to Ref. *27*), and pCMV3xFLAG-A (*28*) and pIRES1neo (Clontech, Palo Alto, CA, USA), respectively. To construct pcalpain7-mGFP, a calpain 7 cDNA fragment mutated at the stop codon was amplified by PCR using a pair of primers (forward, AATGAGCTCTGCGCCA TGGACGCCACAGCACTGG; reverse, CCGGTACCTGAA GTTGTGTGATCTTGATG, restriction sites underlined). The *Sac*I/*Kpn*I fragment was inserted into the *Sac*I/*Kpn*I site of pmGFP-N-SGG (*17*). For bacterial expression of GST-calpain7MIT, a cDNA fragment encoding 1–165 a.a. was amplified by PCR using a pair of primers (forward, ACGAATTCATGGACGCCACAGCACTGG; reverse, CCG GATCCTAACTTGTTGAACTGATTTTGC, restriction sites underlined). The *Eco*RI/*Bam*HI fragment was first inserted into the *Eco*RI/*Bam*HI site of pGBKT7 to construct a yeast two-hybrid screening vector, and then the *Eco*RI/*Sal*I fragment of pGBKT7-calpain7MIT was inserted to the *Eco*RI/*Sal*I site of pGEX4T-1. Construction of the expression vectors for FLAG-CHMP4a, -CHMP4b, -CHMP4c, -CHMP6, -CHMP7, CHMP4b-GFP and CHMP6-GFP was described previously (*17*, *28–30*).

The mammalian expression plasmids for FLAG-CHMP1A, -CHMP1B, -CHMP2A, -CHMP2B, -CHMP3

and -CHMP5 were constructed by conventional methods using restriction enzyme digestion, isolation of fragments, and ligation into a 3xFLAG-fused vector, pCMV3xFLAG-B. A fragment of the full-length CHMP1B cDNA was inserted into a pEXPR-IBA105-A vector and pEGFP-N1 vector (Clontech), and the resultant plasmids were designated pStrep-CHMP1B and pCHMP1B-GFP, respectively. The pCHMP1B-GFP was further converted to pCHMP1B-mGFP by using a PCR-based site-directed mutagenesis kit, QuikChange, from Stratagene, Cedar Creek, TX, USA. A CHMP1A cDNA fragment was inserted into a pmGFP-N1 vector to generate pmGFP-CHMP1A. An N-terminal deletion mutant of CHMP1B (CHMP1BΔNT) encoding 68–199 a.a. was constructed by PCR using pcDNA3/FLAG-CHMP1B as a template and a pair of primers that were designed on the basis of the registered sequence of CHMP1B cDNA (NCBI accession No. NM_020412) for the forward primer (TTGAATTCATGAGTGCGCGAGTCGATGC, containing an additional *Eco*RI site, underlined) and the vector sequence for the reverse primer (TAGA AGGCACAGTCGAGG). To express an N-terminal deletion mutant of CHMP1B, CHMP1BΔNT, in *Escherichia coli* as a thioredoxin (Trx)-fused protein, the *Eco*RI/*Xho*I fragment of CHMP1BΔNT cDNA was inserted into the *Eco*RI/*Sal*I site of a 6xHis-containing Trx-fusion vector, pET32-a (Novagen). Venus cDNA was amplified by PCR using pCS2-Venus (*31*) (a kind gift from Dr Miyawaki, RIKEN at Saitama, Japan) as a template and the following pair of primers containing *Sac*II recognition sites: forward, ATCCCGCGGTGGTGAGCAAGGGCGAGGAGCT; reverse, TCGCCGCGGACTTGTACAGCTCGTCCATGCCG. The PCR product was digested and then ligated with *Sac*II-digested pEXPR-IBA105B to construct pStrep-Venus. Construction of pmRFP-SKD1^{E235Q} was described previously (*17*).

Cell Culture and Selection of Stable Transfectants—HEK293, HEK293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under humidified air containing 5% CO_2. To obtain stable transfectants that constitutively overexpress Strep-tagged calpain 7 (Strep-calpain7OE/HEK293T), HEK293T cells were co-transfected with pStrep-calpain7 and pUCSV-BSD (Funakoshi) and selected by culturing in the presence of blasticidine (20 μg/ml) for 2 weeks. To obtain HEK293 stable transfectants that constitutively overexpress untagged calpain 7 (calpain7OE/HEK293), HEK293 cells transfected with the expression vector (pIRES1neo-calpain7) were selected by culturing in the presence of G418 (1.5 mg/ml) for 2 weeks. To generate HEK293 cells with reduced expression of calpain 7 by the RNA interference method, HEK293 cells were transfected with a short hairpin RNA (shRNA) expression vector (pSM2c-calpain7 shRNA, V2HS_254358, Open Biosystems) and calpain 7-knocked-down stable cell lines (calpain7KD/HEK293) were selected and maintained in the presence of puromycin (2 μg/ml). All stable transfectants described earlier were cloned by selecting antibiotic-resistant colonies using cloning rings.

Strep-pulldown Assay—At 24 h after transfection with expression vectors by the conventional calcium phosphate precipitation method, HEK293, HEK293T or Strep-calpain7OE/HEK293T cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.3), and harvested cells were lysed in buffer A (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2% Triton X-100) supplemented with protease inhibitors [0.2 mM phenylmethylsulfonylfluoride (PMSF), 0.1 mM pefabloc, 25 μg/ml leupeptin, 1 μM E-64, and 1 μM pepstatin]. Supernatants (cleared lysates) obtained by centrifugation at 10,000*g* were incubated with Strep-Tactin beads (IBA GmbH) for 2 h at 4°C with gentle mixing. After the beads had been recovered by a low-speed centrifugation (600*g*) for 1 min and washed three times with buffer A containing 0.1% sodium deoxycholate, the bead-bound proteins (pulldown products) were subjected to SDS–PAGE followed by WB analyses. Proteins transferred to polyvinylidene difluoride (PVDF) membranes (Immunobilon-P, Millipore, Bedford, MA, USA) were probed with appropriate antibodies. Immunoreactive bands were visualized by the chemiluminescence method using Super Signal West Pico Chemiluminescent Substrate (Pierce).

Co-immunoprecipitation Assay—One day after HEK293 cells (2×10^6) had been seeded, cells were transfected with 5 μg of expression plasmid DNA. After 24 h, cells were harvested with PBS and lysed in buffer A containing protease inhibitors described earlier. Cleared lysates were incubated with anti-calpain 7 antiserum at 4°C for 2 h and then further incubated for 2 h after addition of Protein G-Sepharose 4 Fast Flow (Amersham Biosciences, Buckinghamshire, UK). The Sepharose beads were then washed three times with buffer A containing 0.1% sodium deoxycholate, and bound proteins were subjected to WB analysis using appropriate antibodies as described earlier.

Expression and Purification of Recombinant Proteins and GST-pulldown Assay—Escherichia coli BL21 cells were transformed with GST-fusion protein expression plasmids (pGEX4T-1, pGST-calpain7MIT). Expression of GST fusion proteins was induced with 0.1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) for 3 h at 30°C, and the proteins were purified by binding to glutathione-Sepharose 4B (GE Healthcare) according to the manufacturer's instructions. For expression of Trx-fusion proteins, *E. coli* BL21(DE3)pLysS cells were transformed with either pET-32a or pET32-CHMP1BΔNT, and induction of expression was performed by adding IPTG (final 0.25 mM) and incubation for 3 h at 30°C. Proteins were purified by binding to TALON Metal Affinity Resins (Clontech) according to the manufacturer's instructions. Trx-CHMP1BΔNT was eluted from columns with 50 mM Tris–HCl, pH 8.0, 300 mM NaCl and 300 mM imidazole. A fixed amount (10 μg) of GST or GST-calpain7MIT was immobilized on glutathione-Sepharose beads and mixed gently with 10 μg of Trx-fusion proteins in 90 μl of binding buffer B (10 mM HEPES–NaOH, pH 8.0, 142.5 mM KCl, 5 mM 2-mercaptoethanol, 0.02% NP-40 and 5% glycerol) containing either 2 mM $MgCl_2$ and 2 mM $CaCl_2$ or 1 mM EDTA at 37°C for 1 h. After Sepharose beads had been pelleted by brief

centrifugation and washed three times with each binding buffer, bound protein complexes were separated on a 10% gel by SDS–PAGE. Protein bands were detected by Coomassie Brilliant Blue R-250 (CBB).

Immunofluorescence Microscopic Analysis—One day after HeLa cells (1×10^4) had been seeded onto $18 \times 18\,mm^2$ coverslips in 35 mm dishes, the cells were transfected with 0.5 μg of respective expression plasmid DNA using FuGENE 6 (Roche Applied Science, Basel, Switzerland). After 24 h, cells were fixed in 4% paraformaldehyde/PBS at room temperature for 15 min, washed with PBS and then permeabilized in 0.1% Triton X-100/PBS at room temperature for 5 min followed by washing with PBS. After blocking with 0.1% gelatin/PBS at 37°C for 30 min, the cells were processed for immunocytochemistry as described previously (*32*). The fluorescence signals were analysed with a confocal laser-scanning microscope, LSM5 PASCAL (Zeiss, Göttingen, Germany).

Subcellular Fractionation—HEK293 cells were harvested, suspended in a hypotonic lysis buffer (10 mM HEPES–KOH, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM pefabloc, 25 μg/ml leupeptin, 1 μM E-64, 1 μM pepstatin, 0.1 mM PMSF and 5 mM 2-mercaptoethanol), and homogenized by passing 20 times through a 26-gauge needle as described previously (*33*). After homogenization, a solution of NaCl was added to the homogenate to a final concentration of 0.15 M and either $CaCl_2$ or EGTA was added (final concentrations: 1 mM and 2 mM, respectively), and then subcellular fractions were obtained by differential centrifugation at 4°C.

Epidermal Growth Factor (EGF) Uptake Assay—HeLa cells that had been seeded on coverslips were either transfected with pmGFP-calpain7 or pmGFP-calpain7^{C290S} or not trasfected with DNA. After 24 h, the cells were incubated with DMEM without fetal bovine serum (FBS) for 1 h at 37°C and then with 0.5 μg/ml tetramethylrhodamine (Rh)-EGF (Molecular Probes, Inc., Eugene, OR, USA) in 0.5 mg/ml of bovine serum albumin/DMEM for 1 h at 4°C. After the cells had been washed and incubated in DMEM containing 5% FBS for 10 min or 120 min at 37°C, they were washed with PBS, fixed in 4% (w/v) paraformaldehyde/PBS, washed with PBS and then observed under a confocal laser-scanning microscope for transfected cells or after immunostaining with anti-calpain 7 mAb for untransfected cells.

RESULTS

Screening of Calpain 7-interacting CHMP Family Members—To determine whether calpain 7 interacts with CHMPs, we performed Strep-pulldown assays by first establishing an HEK293T cell line that stably expresses Strep-calpain7 (Strep-calpain7OE/HEK293T) and then transfecting the cells with each expression plasmid of eleven different FLAG-tagged CHMPs. See Fig. 1 for schematic representations of major protein expression constructs used in this study. Parental HEK293T cells were used as a negative control. The cleared cell lysates were incubated with Strep-Tactin beads, and the proteins bound to the beads (pulldown products) were analysed by WB using anti-FLAG mAb. As shown in Fig. 2, immunoreactive bands for FLAG-CHMP1A, -CHMP1B and -CHMP4b were clearly detected in the pulldown products of Strep-calpain7, whereas those signals for FLAG-CHMP2A, -CHMP4c and -CHMP7 were faint. Bands for FLAG-CHMP2B, -CHMP3, -CHMP4a, -CHMP5 and -CHMP6 were not detected under the conditions used.

Analyses of Interactions Between Calpain 7 and CHMP1B—Selecting CHMP1B for further interaction analyses, we performed a co-immunoprecipitation assay. Cleared lysates of HEK293 cells that had been transfected with the expression plasmid of either FLAG-CHMP1B or FLAG-CHMP6 (used as a negative control) were incubated with anti-calpain 7 antiserum and the immunocomplexes were subjected to WB with anti-FLAG mAb. As shown in Fig. 3A, a clear band was detected for FLAG-CHMP1B but not for FLAG-CHMP6 above the band of immunoglobulin light chain (IgG-L), suggesting a specific interaction between calpain 7 and CHMP1B in HEK293 cells. In order to further confirm specific binding of calpain 7 to CHMP1B, we performed a reciprocal Strep-pulldown assay by transiently expressing Strep-CHMP1B and analysing the pulldown products by WB with anti-calpain 7 antiserum. As shown in Fig. 3B, endogenous calpain 7 was pulled down with Strep-CHMP1B but not with Strep-Venus used as a negative control. Faster migrating bands indicated by open arrow heads (Fig. 3A and B) were frequently observed and they may represent proteolysed products of calpain 7 because these bands were more abundantly detected in the calpain 7-overexpressed cells but not detected in the calpain 7-knocked down cells (Supplementary Fig. S1). Since a mutant of Strep-calpain7^{C290S} (a mutant with amino acid substitution of the putative catalytic cysteine residue, Cys290, to Ser) also showed a similarly faster migrating band by WB with anti-calpain 7 pAb (data not shown), calpain 7 may be proteolysed by proteases other than calpain 7 itself.

Interaction of Calpain 7 MIT Domains with CHMP1B—To determine whether MIT domains in calpain 7 interact with CHMP1B, we prepared various deletion mutants of calpain 7 that were fused with monomeric green fluorescent protein (mGFP) and performed co-immunoprecipitation analyses using anti-GFP antiserum for immunoprecipitation (Fig. 4A). Immunoreactive bands of FLAG-CHMP1B by WB were detected for mGFP-calpain7 wild-type (WT) and mGFP-calpain7MIT but not for mGFP-calpain7ΔMIT. Signals decreased to the background level in the case of single MIT domain constructs (mGFP-calpain7MITa and mGFP-calpain7MITb). Next, we examined whether CHMP1B directly interacts with calpain7 MIT domains by a GST-pulldown assay using purified recombinant proteins *in vitro* (Fig. 4B). We used an N-terminal deletion mutant of thioredoxin-fused CHMP1B (Trx-CHMP1BΔNT) because recombinant full-length CHMP1B protein was insoluble in *E. coli* but became soluble by removing a part of the basic α-helical region as reported previously (*34*). Trx or Trx-CHMP1BΔNT was mixed with glutathione-Sepharose beads that carried either GST (a negative control) or GST-calpain7MIT and incubated in the presence of either divalent metal ions (2 mM $MgCl_2$ and 2 mM $CaCl_2$) or 1 mM EDTA.

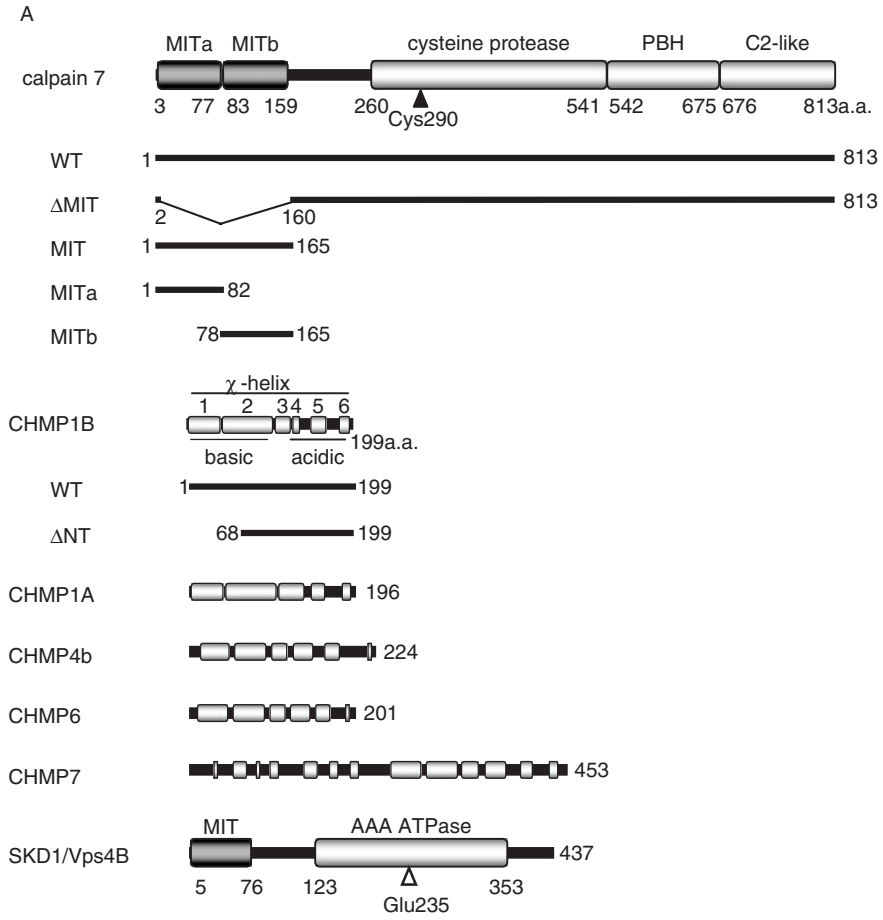

Fig. 1. **Schematic representations of calpain 7, CHMPs and SKD1/Vps4B.** Calpain 7 possesses two MIT domains (MITa, MITb) at its N-terminus and a calpain-like cysteine protease domain (Cys290, a putative catalytic cysteine residue), a PBH domain and a C2-like domain at its C-terminus. CHMP1B, predicted to have six α-helices (indicated by cylinders), has a bias in the distribution of charged residues: rich in basic and acidic residues in its N-terminal half (6–97 a.a.) and C-terminal half (123–187 a.a.), respectively. Predicted α-helices in CHMP1A, CHMP4b, ChMP6 and CHMP7 are also presented. AAA-type ATPase SKD1/Vps4B has an MIT domain and an ATPase domain. Substitution of Glu235 to Gln (E235Q) in the Walker B magnesium-binding motif inhibits ATP hydrolysis and exhibits dominant-negative effects (*49*). Truncated mutants of calpain 7 and CHMP1B were also used for interaction analyses. The numbers below the bars indicate positions in amino acid residues.

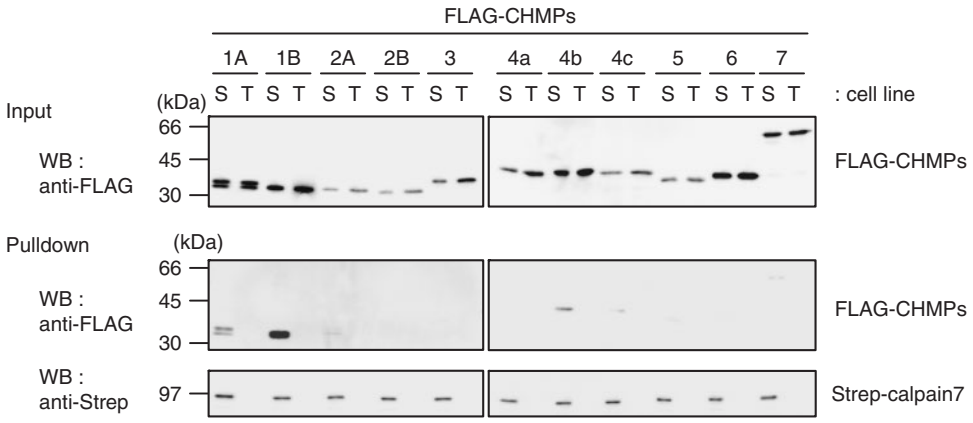

Fig. 2. **Screening of calpain 7-interacting CHMPs by Strep-pulldown assays.** A cell line of HEK293T cells that constitutively express Strep-calpain7 was established (Strep-calpain7OE/HEK293T) and used for a Strep-pulldown assay. At 24 h after cells had been transfected with each of the expression vectors for eleven FLAG-CHMP family members, transfectants were lysed and the cleared lysates were incubated with Strep-Tactin Sepharose beads. The cleared lysates (*Input*) and the bead-bound fractions (pulldown products, *Pulldown*) were analysed by WB with anti-Strep-tag II mAb and anti-FLAG mAb. *S*, Strep-calpain7OE/HEK293T; *T*, parental HEK293T.

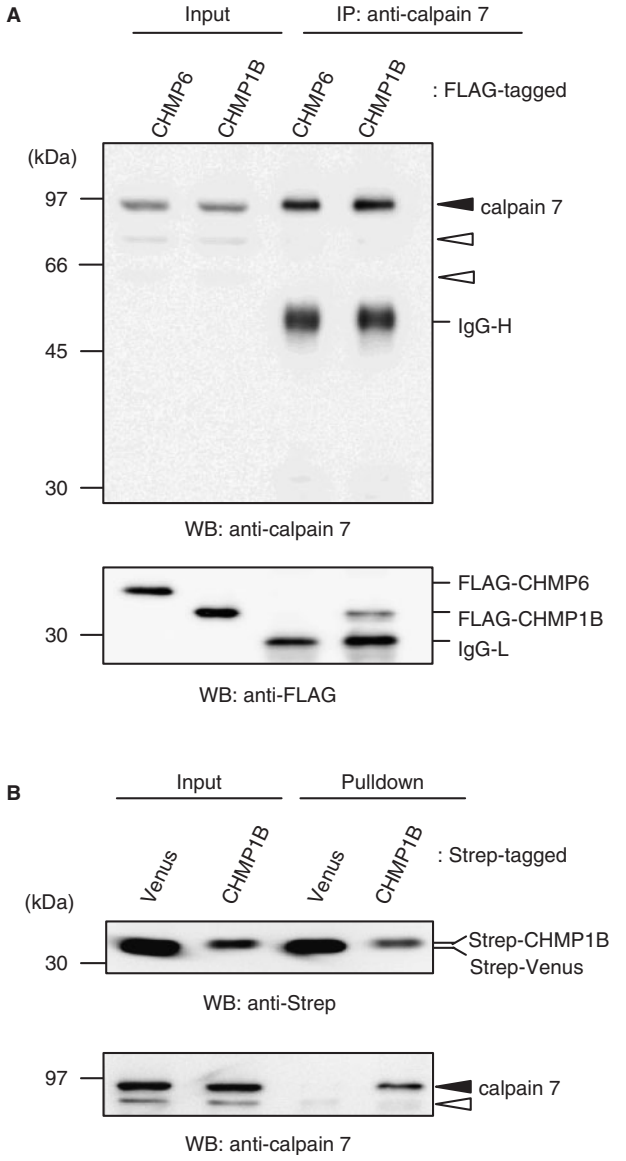

Fig. 3. **Analyses of interactions between calpain 7 and CHMP1B.** (A) Co-immunoprecipitation of FLAG-CHMP1B with calpain 7. HEK293 cells were transfected with either pFLAG-CHMP1B or pFLAG-CHMP6. At 24 h after transfection, the cells were lysed and the cleared lysates (*input*) were subjected to immunoprecipitation (IP) with anti-calpain 7 antiserum followed by 10% gel SDS–PAGE and WB analysis using anti-calpain 7 antiserum (*upper panel*) and anti-FLAG mAb (*lower panel*). Input, 0.2%; IgG-H and IgG-L, immunoglobulin heavy chain and light chain, respectively. (B) Strep-pulldown assay. At 24 h after HEK293 cells had been transfected with either pStrep-Venus as a negative control or pStrep-CHMP1B, the cells were lysed and the cleared lysates were subjected to a Strep-pulldown assay. The cleared lysate (*Input*) and pulldown products (*Pulldown*) were resolved on a 10% gel by SDS–PAGE followed by WB with anti-Strep-tag II mAb (*upper panel*) and with anti-calpain 7 antiserum (*lower panel*).

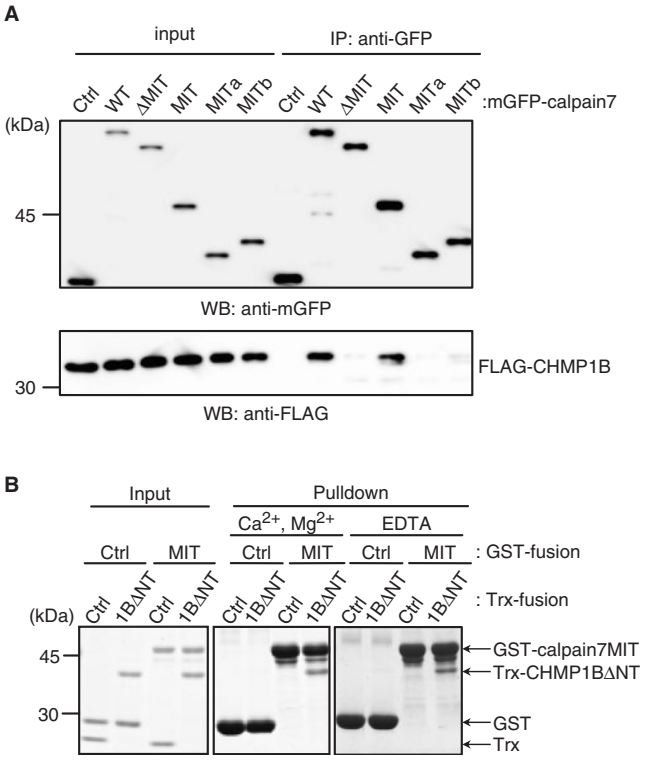

Fig. 4. **Binding of CHMP1B to calpain 7 MIT domains.** (A) Co-immunoprecipitation assay. HEK293T cells were transfected with an expression plasmid for each of the various mGFP-fused calpain 7 constructs, and the cleared cell lysates were incubated with anti-GFP antiserum and Protein G Sepharose beads. After washing, beads were incubated with the cleared lysate of HEK293T cells expressing FLAG-CHMP1B, pelleted, washed and subjected to WB with anti-GFP Ab (*upper panel*) and anti-FLAG mAb (*lower panel*). Input, 0.1%. (B) *In vitro* binding assay using recombinant proteins. GST (negative control) or GST-calpain7MIT that had been immobilized on glutathione-Sepharose beads was incubated with Trx or Trx-CHMP1BΔNT at 37°C for 1 h in the presence of either divalent metal ions (2 mM $MgCl_2$ and 2 mM $CaCl_2$) or 1 mM EDTA. Then the beads were pelleted by low-speed centrifugation and washed with the respective binding buffer. The initial protein mixtures (*input*) and proteins in the pellets (*pulldown*) were resolved on a 10% gel by SDS–PAGE and subjected to CBB staining.

The proteins bound to the beads were separated by SDS–PAGE and visualized by CBB staining. As shown in Fig. 4B, Trx-CHMP1BΔNT was pulled down with GST-calpain7MIT but not with GST. Trx was not pulled down in either case. The presence of divalent metal ions did not affect the binding.

Co-localization of Epitope-tagged Calpain 7 with ESCRT-III-accumulating Compartments—To investigate the subcellular localization of calpain 7 and CHMPs, we co-expressed FLAG-calpain7 with each of several CHMPs that were fused with GFP or mGFP in HeLa cells and immunostained the cells with anti-FLAG mAb and Cy3-labelled anti-mouse IgG antibody. FLAG-calpain7 co-expressed with mGFP (used as a control) showed diffuse cytoplasmic staining (Fig. 5A, FLAG-calpain7; 5B, mGFP; 5C, merged image). Independent overexpressions of mGFP-CHMP1A, CHMP1B-mGFP, CHMP4b-GFP and CHMP6-GFP in HeLa cells showed punctate patterns and they partly co-localized with endosomal maker proteins such as EEA1 and Lamp-1 (*28*, *30*; data not shown). Co-expression with mGFP-CHMP1A,

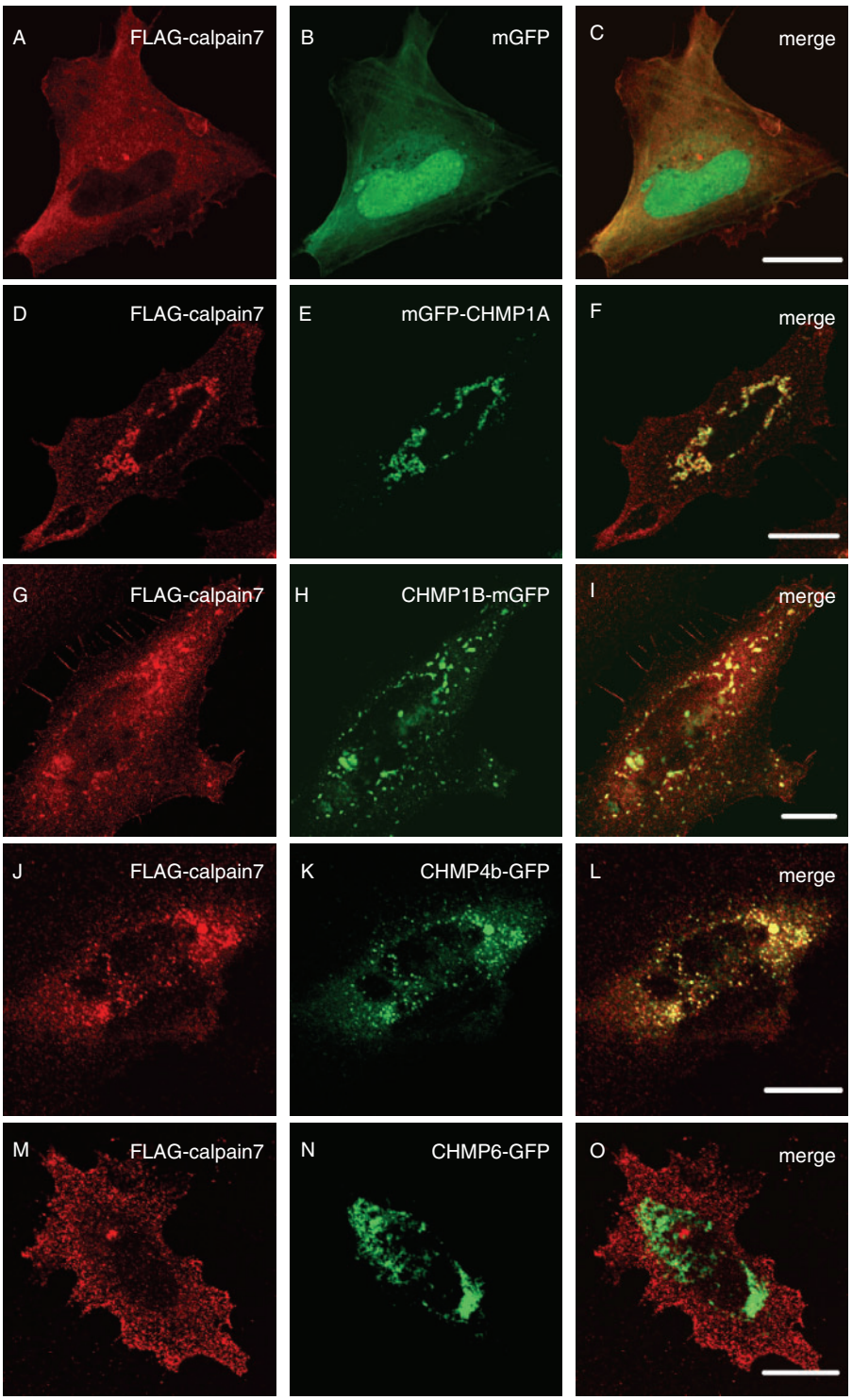

Fig. 5. **Fluorescence microscopic analyses of subcellular distributions of FLAG-calpain7 and GFP-fused CHMPs in HeLa cells.** HeLa cells were co-transfected with pFLAG-calpain7 and an expression plasmid for either mGFP (A–C) or each of the GFP-fused CHMP family members: mGFP-CHMP1A (D–F), CHMP1B-mGFP (G–I), CHMP4b-GFP (J–L) and CHMP6-GFP (M–O). One day after transfection, the cells were processed for immunocytochemistry using anti-FLAG mAb and Cy3-conjugated goat anti-mouse IgG pAb. The fluorescence signals of Cy3 (red: *A*, *D*, *G*, *J* and *M*) and GFP (green: *B*, *E*, *H*, *K* and *N*) were analysed with a confocal laser scanning microscope, LSM5 PASCAL (Zeiss). Merged images are shown in the right column panels. The scale bars represent 20 μm.

CHMP1B-mGFP or CHMP4b-GFP caused accumulation of FLAG-calpain7 in a punctate pattern in the perinuclear area, and both overexpressed proteins showed significant overlapping in the distribution (Fig. 5D–L). On the other hand, the punctate pattern of CHMP6-GFP localization did not match with that of FLAG-calpain7 (Fig. 5M–O).

Although the cytoplasmic distribution patterns of mGFP-calpain7 and mGFP-calpain7^{C290S} in HeLa cells were similar to that of FLAG-calpain7, they showed a slightly greater tendency for punctate distribution in the perinuclear area than did FLAG-calpain7 (Supplementary Fig. S2). However, neither mGFP-calpain7 nor mGFP-calpain7^{C290S} showed co-localization with endosomal marker proteins tested in the present study (data not shown). Interestingly, while mGFP-calpain7 was excluded from nucleus, mGFP-calpain7MIT showed predominant nuclear localization and mGFP-calpain7ΔMIT exhibited a diffuse pattern throughout the cell including the nucleus (Supplementary Fig. S2). Protein molecules involved in endosomal sorting, including ESCRT-III proteins and their interacting proteins, are known to accumulate on endosomal membranes in cells expressing an ATPase-defective dominant-negative mutant of SKD1/Vps4B (SKD1^{E235Q}), and aberrant endosomes (called E235Q compartments) are formed (*29*, *35–37*). Fluorescence signals of overexpressed mRFP-fused dominant-negative SKD1 (mRFP-SKD1^{E235Q}) exhibited a punctate pattern in the perinuclear area and merged with those of mGFP-calpain7 (Fig. 6D–F) but not with mGFP (Fig. 6A–C). While mGFP-calpain7MIT partly localized to the E235Q compartments (Fig. 6G–I), the localization of an MIT-deletion mutant of mGFP-fused calpain 7 (mGFP-calpain7ΔMIT) to the E235Q compartments was not clear (Fig. 6J–L).

Subcellular Distribution of Endogenous Calpain 7—To further investigate the distribution of calpain 7 within cells, we performed subcellular fractionation of HEK293 cells by differential centrifugation and detected endogenous calpain 7 by WB analysis using an anti-calpain 7 pAb. A representative result of three experiments is shown in Fig. 7. Most of calpain 7 was detected in the cytosolic fraction (S), but a small portion was also detected in each pellet of 1,000*g* (P1), 10,000*g* (P2) and 100,000*g* (P3). Marker proteins of late endosome/lysosome (Lamp-1), calnexin (endoplasmic reticulum) and cytosol (GAPDH) showed the expected distribution patterns except for detection of small amounts of Lamp-1 and GAPDH in the P1 fraction, suggesting presence of unbroken cells. Calcium ions, which were added to a final concentration of 1 mM after homogenization, did not influence the distribution of calpain 7. In contrast, a dramatic change was observed in the distribution of ALG-2, which is known to associate with Alix and TSG101 and Ca^{2+}-dependently co-localize to aberrant endosomes with overexpressed SKD1^{E235Q} (*5*, *32*, *35*). The amounts of m-calpain in the P2 and P3 fractions increased slightly in the presence of Ca^{2+}. Inclusion of Ca^{2+} into the lysis buffer before homogenization did not significantly affect the distribution of calpain 7, but more ALG-2 was recovered in the P1 fraction as previously reported (data not shown; *33*).

Subcellular distribution of endogenous calpain 7 in HeLa cells was also analysed by immunofluorescence microscopy using an anti-calpain 7 mAb (clone Y0717) that was raised in this study. The observed immunofluorescence signals showed a diffuse cytoplasmic distribution with a fine punctate pattern in the perinuclear area (Fig. 8). Specificity of the antibody was evaluated by the RNA interference method. Transfection of a shRNA expressing vector for calpain 7 suppressed fluorescence signals in HeLa cells as shown in the two representative microscopic fields (Fig. 8A–C and D–F, cells marked with asterisks). Such reduction in fluorescence signals was not observed in the surrounding cells that were presumed to be not transfected (Fig. 8A–F, cells not marked with asterisks) or in the cells that were processed for transfection with non-sensing shRNA (data not shown). Cytoplasmic staining of endogenous calpain 7 was also observed for HEK293 cells but not for cells of calpain 7 knocked-down (calpain7KD) HEK293 cell line that were pre-mixed and co-cultured with parental HEK293 cells in the ratio of 1:5 or 5:1 (Fig. 8G–L). Next, we examined whether distribution of endogenous calpain 7 is also influenced by overexpression of mGFP-CHMP1B and mRFP-SKD1^{E235Q} in HeLa cells. Overexpression of mGFP-CHMP1B caused accumulation of endogenous calpain 7 and these two proteins co-localized in the perinuclear area (Fig. 9A–C). On the other hand, overexpressed mRFP-SKD1^{E235Q} did not apparently co-localize with endogenous calpain 7 (Fig. 9D–F).

Partial Co-localization of Calpain 7 with Endocytosed EGF—After EGF binds to its receptor (EGFR) on the cell surface, it is internalized into endosomes and finally delivered into lysosomes to be degraded. To obtain evidence supporting the hypothesis that calpain 7 plays roles in the endosomal pathway, we traced endocytosed tetramethylrhodamine-labelled EGF (Rh-EGF). HeLa cells that had been transfected with either pmGFP-calpain7 or pmGFP-calpain7^{C290S} were incubated with Rh-EGF at 4°C for 1 h, washed, and then allowed to uptake Rh-EGF at 37°C for either 10 or 120 min. At 10 min, endocytosed Rh-EGF showed a punctate pattern, and fluorescence signals of mGFP-calpain7 (Fig. 10A–C) and mGFP-calpain7^{C290S} (Fig. 10D–F) partly merged with those of Rh-EGF. The mGFP-calpain7^{C290S} mutant appears to localize with Rh-EGF-containing compartments more efficiently than does mGFP-calpain7. Co-localization between endogenous calpain 7 and Rh-EGF was obscure, but some puncate fluorescence signals partly merged, suggesting localization of calpain 7 in the vicinity of endocytosed Rh-EGF (Fig. 10G–I and higher magnification, J–L). At 120 min, most of the fluorescence signals of endocytosed Rh-EGF were no longer detectable both in the untransfected cells and in the cells that expressed mGFP-calpain7 or mGFP-calpain7^{C290S} (data not shown), suggesting no dominant-negative effects by the overexpressed calpain 7 constructs.

DISCUSSION

An MIT domain of AAA-type ATPase Vps4 is a small protein module (~80 a.a.) of a three-helix bundle (*22*, *38*),

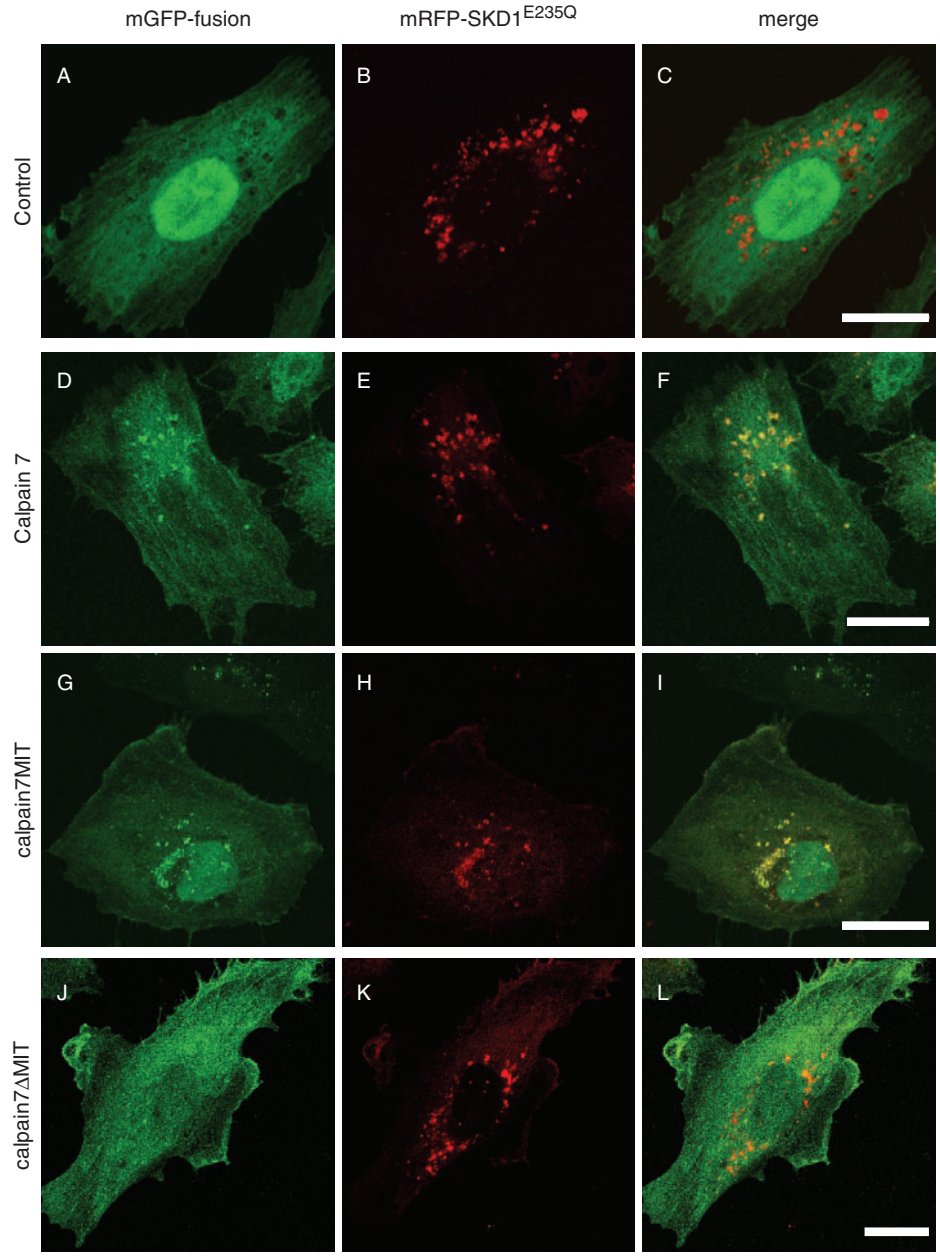

Fig. 6. **Co-localization of mGFP-calpain7 with mRFP-SKD1^{E235Q}.** HeLa cells co-transfected with the expression vectors of mRFP-SKD1^{E235Q} and mGFP (A–C), mGFP-calpain7 (D–F), mGFP-calpain7MIT (G–I) or mGFP-calpain7ΔMIT (J–L) were subjected to confocal fluorescence microscopic analysis. Fluorescence signals of green and red and their merged images are shown in the left, middle and right column panels, respectively. The scale bars represent 20 μm.

and a similar sequence is found in several proteins whose functions have been shown or predicted to be involved in endosomal trafficking or microtubule interaction (*20–24*). In the present study, we demonstrated for the first time that human calpain 7/PalBH interacts with a subset of ESCRT-III (endosomal sorting complex required for transport-III) components and related proteins named CHMPs through a tandem repeat of MIT domains that are present in its N-terminal region. The results of Strep-pulldown assays suggest that calpain 7 binds CHMP1A, -1B, -2A, -4b, -4c and -7, among which it binds CHMP1B most efficiently (Fig. 2). Moreover, the results of an *in vitro* pulldown assay using recombinant proteins of GST-calpain7MIT and Trx-CHMP1BΔNT indicate that these proteins physically associate directly (Fig. 4B). In similar GST-pulldown assays, Trx-CHMP4cΔNT was not detected in the pulldown products by CBB staining but was detected by WB using anti-Trx antibody (data not shown). In the yeast two-hybrid assay, positive interactions between calpain 7MIT and individual ESCRT-related proteins were observed only with CHMP1B and CHMP4c with apparently equal strengths of interactions (data not shown). The differences in the results of positive interactions between the mammalian

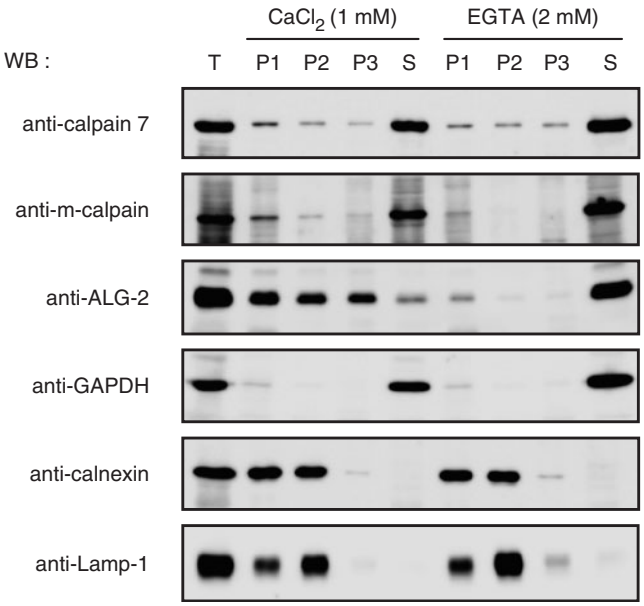

Fig. 7. **Subcellular fractionation of endogenous calpain 7 in HEK293 cells.** Subcellular fractionation of HEK293 cells was performed as described in MATERIALS AND METHODS section. After homogenization, a solution of NaCl was added to the homogenate to a final concentration of 150 mM and either $CaCl_2$ or EGTA was added (final concentrations: 1 mM and 2 mM, respectively), and then subcellular fractions were obtained by differential centrifugation at 4°C: a nuclear and cell debris pellet (P1), 1,000*g* for 5 min; a crude mitochondrial and organelle-enriched pellet (P2), 10,000*g* for 15 min; a microsomal pellet (P3), 100,000*g* for 60 min; cytosolic fraction, final supernatant (S). Sample volumes for SDS–PAGE were adjusted to compare the relative amounts of the proteins in the subcellular fractions. WB analysis was performed with anti-calpain 7 pAb, anti-m-calpain pAb, anti-ALG-2 pAb, anti-GAPDH mAb, anti-calnexin mAb and anti-Lamp-1 mAb.

system and yeast system may be due to the difference in the region of calpain 7 used in these assays (full-length versus MIT).

Efficient binding of CHMP1B to MIT domains has also been reported for spastin (*21*), Vps4 (*22*), AMSH (*23*) and UBPY (*24*). Calpain 7 has a tandem repeat of MIT domains (MITa and MITb) (Fig. 1). Deletion of either MITa or MITb caused an apparent loss of binding to CHMP1B (Fig. 4A) or significant retardation in the growth rate on a selection agar plate in the yeast two-hybrid assay (data not shown), suggesting requirement of both domains for efficient binding. Recent structural studies have revealed that C-terminal α-helical regions of a subset of CHMPs (CHMP1A, -1B and -2B) are essential and sufficient for binding to Vps4 MIT domains (*39*, *40*). Since other CHMPs also associate with Vps4, the possibility of binding to other regions has not been excluded (*39*, *40*). It remains to be clarified in future studies how the two MIT domains of calpain 7 cooperate for binding to CHMP1B and other CHMPs and whether they interact with the C-terminal region of CHMP1B as in the case of Vps4 MIT.

Futai *et al*. (*19*) previously reported that overexpressed C-terminally myc-tagged calpain 7/PalBH localized in the nucleus of transfected COS7 cells. In the present study, however, fluorescence microscopic analyses of both transiently expressed N-terminally tagged FLAG- and mGFP-calpain7 in transfected HeLa cells (Figs 5, 6 and 10 and Supplementary Fig. S2) and endogenous calpain 7 in untrasfected HeLa cells (Figs 8 and 9) revealed that calpain 7 localized mostly in the cytoplasm. Since the epitope for anti-calpain 7 mAb is located within MITb, N-terminally cleaved calpain 7 may not have been detected either by WB (Supplementary Fig. S1) or immunostaining (Figs 8–10). We constructed C-terminally mGFP-fused calpain7 (calpain7-mGFP) and examined its subcellular localization. As shown in panel C of Supplementary Fig. S2, a cytoplasmic distribution was observed for calpain7-mGFP as in the case for mGFP-calpain7, indicating that the major localization area of calpain 7 is the cytoplasm, not the nucleus. Moreover, biochemical subcellular fractionation analysis of endogenous calpain 7 using anti-calpain 7 antibody revealed that most of calpain 7 is recovered in the cytosolic (S) fraction and that the amount of calpain 7 present in the P1 fraction (cell debris and crude nuclear fraction) is very small (Fig. 7). However, we did observe fluorescence signals of mGFP-calpain7 in the nucleus as well as in the cytoplasm in COS7 cells (Supplementary Fig. S3B and C). The cytoplasmic mGFP-calpain7 partly co-localized with Golgi marker proteins, GS15 and GM130, in COS7 cells (Supplementary Fig. S3D–I). Thus, the subcellular localization of calpain 7 may depend on expression levels, cell types and phases of cell cycles.

Upon Ca^{2+} stimulation, conventional calpains are known to translocate to membranes and become activated (*1*, *3*, *41*). A C2-like domain named domain III has been suggested to be one of elements for this translocation process, and domains III of m-, μ- and *Drosophila*-calpains have been demonstrated to bind Ca^{2+} and phospholipids (*42*, *43*). While clapain 7 lacks a C-terminal PEF domain, it retains domain III and possesses a unique domain named PBH (*2*, *3*). We investigated whether distribution of calpain 7 is influenced by the presence of Ca^{2+} during biochemical subcellular fractionation. The amounts of calpain 7 recovered in the particulate fractions were not affected by the presence of Ca^{2+} (Fig. 7). This may be explained by the fact that domain III of calpain 7 lacks a cluster of acidic residues found in the conventional calpains, suggesting no Ca^{2+}-binding capacity of this atypical calpain. Since the presence of Ca^{2+} caused only a slight increase in the amount of m-calpain in the particulate fractions, we cannot exclude the possibility that the lysis procedure used in the present study might have released loosely bound calpains from membranes. Our preliminary experiments suggest that calpain 7 associates weakly with a PEF protein, ALG-2, by three different assays: yeast two-hybrid, GST-ALG-2 pulldown and overlay using biotin-labeled ALG-2 (data not shown). While ALG-2 was recovered in the cytosolic fraction in the presence of EGTA and in the particulate fractions in the presence of Ca^{2+}, the distribution of calpain 7 was not significantly influenced by the presence of Ca^{2+} (Fig. 7). Thus, binding of ALG-2 to calpain 7 seems unphysiological, but we cannot exclude the possibility that weakly associating ALG-2 regulates a catalytic activity of calpain 7.

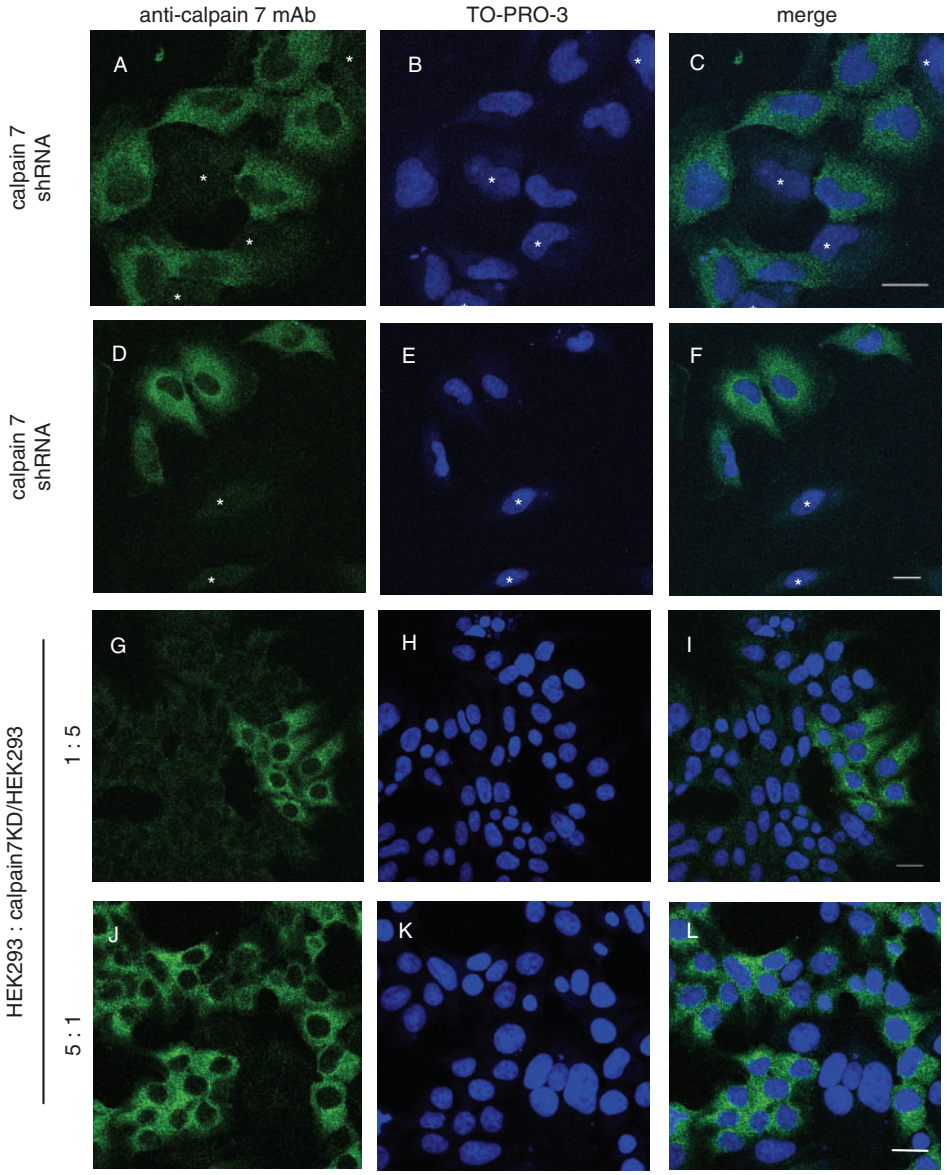

Fig. 8. **Fluorescence microscopic analyses of endogenous calpain 7 in HeLa cells.** Specificity of anti-calpain 7 mAb (clone Y0717) for immunostaining was evaluated by the RNA interference method. (A–F) After HeLa cells were transfected with shRNA-expressing plasmid for depletion of calpain 7, cells were stained with anti-calpain 7 mAb (A and D) and TO-PRO-3 for DNA (B and E). Merged images are shown in C and F. Knocked-down cells are indicated with asterisks. (G–L) parental HEK293 cells and cells of a calpain 7-knocked-down cell line (calpain7KD/HEK293) were pre-mixed at the ratio of 1:5 (G–I) or 5:1 (J–L), co-cultured and stained with anti-calpain 7 mAb (G and J) and TO-PRO-3 for DNA (H and K). Merged images are shown in *I* and *L*. Scale bars, 20 μm

Although most of calpain 7 was found to be present in the cytosolic fraction, a small amount of calpain 7 was consistently found in the particulate membrane fractions both in the presence and absence of Ca^{2+} (Fig. 7). Since the control cytosolic protein (GAPDH) was essentially negligible in these fractions, some calpain 7 proteins appear to associate with organelle membranes through interactions with a few isoforms of CHMPs and unknown proteins. Immunostaining of endogenous calpain 7 in HeLa cells revealed a diffuse cytoplasmic distribution with a fine punctate pattern (Fig. 10). The puncta of mGFP-calpain7, mGFP-calpain7^{C290S} or endogenous calpain 7 did not co-localize with markers of endosomes (EEA1, Lamp-1, clathrin, sorting nexin 2 and Rab5) or Golgi (GM130 and GS15) in HeLa cells (data not shown). Some punctate signals of mGFP-calpain7 and GFP-calpain7^{C290S} merged with endocytosed fluorescence signals of Rh-EGF (Fig. 10C and F), suggesting transient association of calpain 7 with endocytic membrane compartments that were not characterized in this study. Fluorescence signals corresponding to endogenous calpain 7 barely merged with Rh-EGF but were observed in its vicinity (Fig. 10L). Since the fluorescence signals corresponding to endogenous calpain 7 were weak, improvement of fixation and staining are necessary for more detailed analyses. Interestingly,

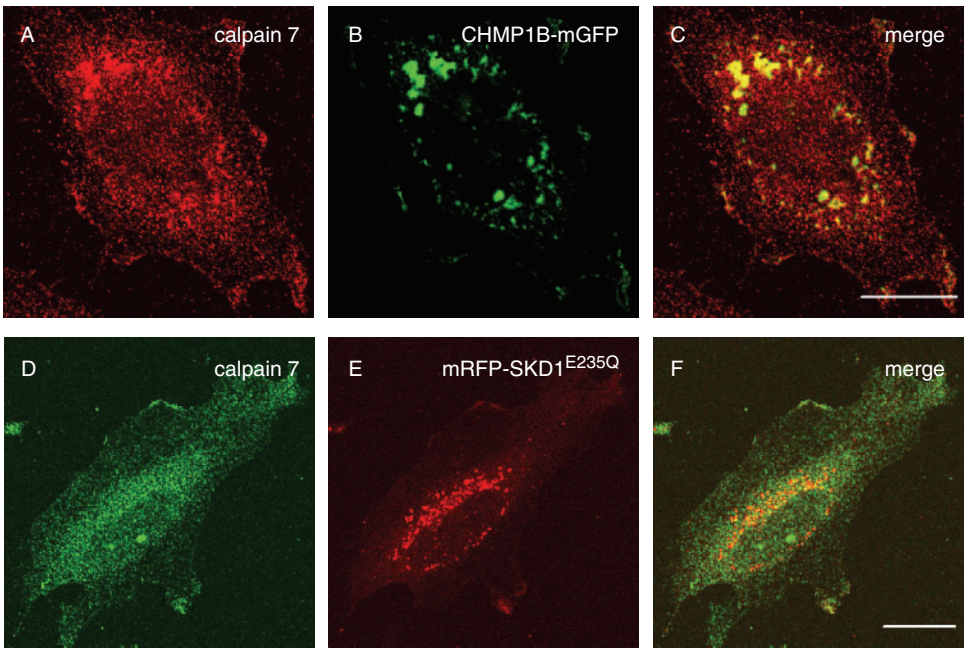

Fig. 9. **Effects of overexpressions of mRFP-SKD1E^{235Q} and CHMP1B-mGFP on endogenous calpain 7.** HeLa cells were transfected with expression vectors for either CHMP1B-mGFP (A–C) or mRFP-SKD1E^{235Q} (D–F) and subjected to immunostaining with anti-calpain 7 mAb. Cy3-conjugated goat anti-mouse IgG pAb (A) or Alexa Fluor 488-conjugated goat anti-mouse IgG pAb (D) was used as a secondary antibody. Fluorescence signals of Cy3 (A) mGFP (B) Alexa Fluor-488 (D) and mRFP (E) are visualized. (C) Merged image of (A) and (B); (F) merged image of (D) and (E). Scale bars, 20 μm.

mGFP-calpain7^{C290S} showed reproducibly better merging with Rh-EGF than did mGFP-calpain7. It is possible that potential proteolytic cleavage of putative substrates on the membranes of endocytic compartments causes an immediate release of untagged endogenous calpain 7 and a slower release of mGFP-calpain7 from the membranes. On the other hand, endocytosed transferrin did not show co-localization with mGFP-calpain7 (data not shown). While the endocytosed transferrin receptor is recycled to the plasma membrane via recycling endosomes, the endocytosed EGF receptor is ubiquitinated and delivered to lysosomes through MVB, where ESCRTs play roles in sorting of ubiquitinated cargoes into intraluminal vesicles of MVB (*10*). Thus, partial co-localization of mGFP-calpain7 and mGFP-calpain7^{C290S} with Rh-EGF suggests association of calpain 7 with endocytic membrane compartments that are situated on the way to lysosomes. Time-lapse tracing of mGFP-calpain7 or mGFP-calpain7^{C290S} and Rh-EGF in future studies will clarify merging of the two fluorescence signals.

Overexpression of GFP-fused CHMP1A, CHMP1B, CHMP4b, but not CHMP6, and the ATPase-defective dominant-negative mutant of SKD1/Vps4B (mRFP-SKD1^{E235Q}) resulted in punctate distributions of epitope-tagged calpain 7 in the cytoplasm by co-localization with these proteins (Figs 5 and 6). While endogenous calpain 7 also co-localized with CHMP1B-mGFP, it did not apparently co-localize with mRFP-SKD1^{E235Q} (Fig. 9). Since calpain 7 binds CHMP1B directly through its MIT domains, efficiency of co-localization may not be significantly influenced by the concentration of calpain 7 itself as long as CHMP1B is overexpressed in the cell. On the other hand, co-localization of calpain 7 to aberrant endosomes with mRFP-SKD1^{E235Q} may greatly depend on the ratios of calpain 7, CHMPs and SKD1E^{235Q}, because both calpain 7 and SKD1E^{235Q} possess MIT domains and compete for binding to CHMPs. Thus, overexpression of calpain 7 protein may be required to co-localize with SKD1E^{235Q} to the aberrant endosomes.

For MVB sorting of ubiquitinated cargoes, four yeast homologues of CHMPs (Vps2/CHMP2, Vps24/CHMP3, Snf7/CHMP4 and Vps20/CHMP6) have been shown to be recruited from the cytosol to endosomes and form ESCRT-III, which is disassembled by the ATPase function of Vps4 (*10*, *18*, *44*). Yeast calpain-like protein, Cpl1 (also named Rim13, orthologue of calpain 7), requires both Snf7/CHMP4 and its myristoylated interacting partner, Vps20/CHMP6 (*10*, *30*) as well as ESCRT-I and –II proteins for processing of a transcription factor in response to pH environments (*7*, *8*, *12*), but other CHMP orthologues are dispensable (*13*, *14*). An ESCRT-III subcomplex (Snf7-Vps20) formed on the endosomal membrane is thought to serve as a scaffold for Cpl1 function by recruiting the substrate via indirectly interacting with an adaptor protein Rim20 (*13–15*). Regardless of a positive interaction by a high-throughput yeast two-hybrid assay (*16*), there are no biochemical data showing that Cpl1 associates directly with Snf7, an interacting protein of Bro1 domain-containing protein Rim20. Moreover, a database search using a simple modular architecture research tool (SMART, http://smart.embl-heidelberg.de/) revealed absence of a conspicuous MIT domain in its N-terminal region in spite of the presence of a weak similarity with that of calpain 7. It remains to be established whether interaction of calpain 7 with CHMP1B, an ESCRT-III-related protein,

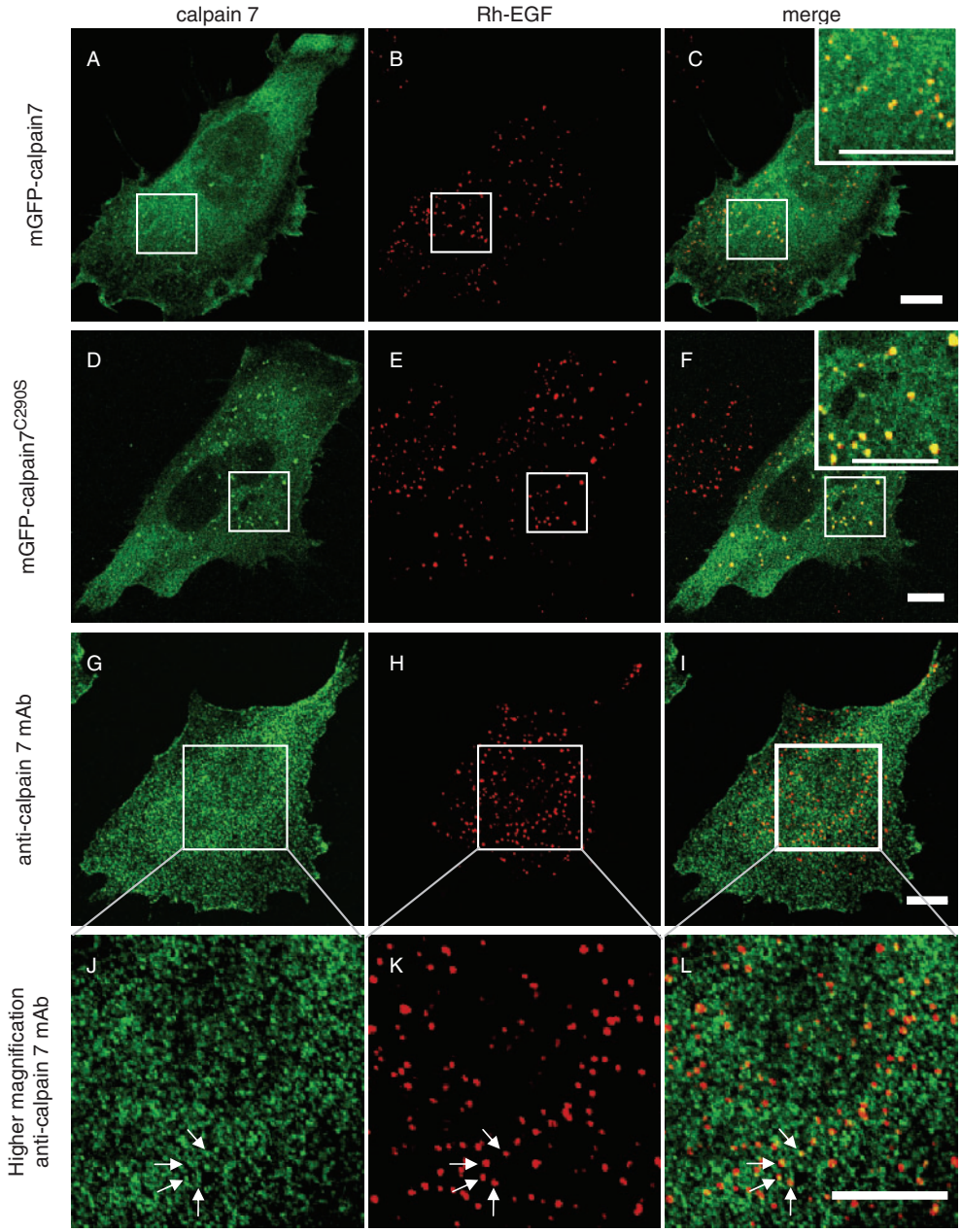

Fig. 10. **Partial co-localization of mGFP-calpain7 with endocytosed EGF.** HeLa cells that had been transfected with an expression vector for either mGFP-calpain7 (A–C) or mGFP-calpain7^{C290S} (D–F) or not transfected with DNA (G–I) were incubated at 4°C for 1 h in the presence of tetramethylrhodamine (Rh)-EGF, washed, and then incubated at 37°C for 10 min. Cells were then washed with PBS and fixed in 4% (w/v) paraformaldehyde/PBS, washed with PBS, and then observed under a confocal laser-scanning microscope for transfected cells (A–F) or after immunostaining with anti-calpain 7 mAb for untransfected cells (G–I). (A) mGFP-calpain7; (B) Rh-EGF; (C) merged image of *A* and *B*; (D) mGFP-calpain7^{C290S}; (E) Rh-EGF; (F) merged image of *D* and *E*; (G) anti-calpain 7 mAb; (H) Rh-EGF; I, merged image of G and H. (J–L) higher magnification of images shown in *G–I*. The scale bars represent 20 μm.

is an analogy to Cpl1-Snf7 connection for proteolytic processing of as-yet-unknown substrates on the membranes of endocytic compartments.

Human CHMP1A (previously named CHMP1, chromatin modifying protein 1) was first identified as a conserved partner of the PcG protein Polycomblike (Pcl) (*45*). Doublet bands were detected for CHMP1A by WB and that the two CHMP1A forms showed distinct distributions (35 kDa protein in the nuclear matrix and 32 kDa protein in the cytoplasm). Thus, the generation of two forms of CHMP1A, most probably by post-translational modification, was suggested to regulate dual functions: vesicular trafficking in the cytoplasm (*46*) and chromatin modification in the nucleus (*45*). As shown in Fig. 2, we also observed doublet bands of ~35 kDa for FLAG-CHMP1A. Since calpain 7 was found to interact with CHMP1A, we expected CHMP1A to be a specific substrate for calpain 7. Contrary to our

expectation, however, the expression levels of calpain 7 did not affect the ratio of the intensities of the doublet bands of CHMP1A (Supplementary Fig. S4), suggesting that CHMP1A is processed by other enzymes. Studies are in progress to determine whether calpain 7 cleaves novel MIT-interacting proteins that have been screened by the yeast two-hybrid system (Takaya, Osako, Shibata and Maki, unpublished data) or proteins that interact with three mammalian Bro1 domain-containing proteins (Alix, HD-PTP and Brox) (*19*, *25*, *47*, *48*). Calpain 7-knocked-down and calpain 7-overexpressing cell lines would be useful to search for calpain 7 substrates by comparing proteins in the two cell lines using a proteomics approach.

Supplementary data are available at *JB* online.

We thank members of the laboratory of Molecular and Cellular Regulation for valuable suggestions. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (M.M.) and a Grant-in-Aid for JSPS Fellows (to C.Y.). This study was supported in part by NFAT project of New Energy and Industrial Technology Development Organization (NEDO) (T.H, Y.M., H.I., and T.K.).

REFERENCES

1. Goll, D.E., Thompson, V.F., Li, H., Wei, W., and Cong, J. (2003) The calpain system. *Physiol. Rev.* **83**, 731–801
2. Sorimachi, H. and Suzuki, K. (2001) The structure of calpain. *J. Biochem.* **129**, 653–664
3. Suzuki, K., Hata, S., Kawabata, Y., and Sorimachi, H. (2004) Structure, activation, and biology of calpain. *Diabetes* **53** (Suppl 1), S12–S18
4. Maki, M., Kitaura, Y., Satoh, H., Ohkouchi, S., and Shibata, H. (2002) Structures, functions and molecular evolution of the penta-EF-hand Ca^{2+}-binding proteins. *Biochim. Biophys. Acta* **1600**, 51–60
5. Maki, M. and Shibata, H. (2007) The penta-EF-hand protein ALG-2 and its interacting ptoteins. *Calc. Binding Proteins* **2**, 4–10
6. Denison, S.H., Orejas, M., and Arst, H.N. Jr. (1995) Signaling of ambient pH in *Aspergillus* involves a cysteine protease. *J. Biol. Chem.* **270**, 28519–28522
7. Peñalva, M.A. and Arst, H.N. Jr. (2002) Regulation of gene expression by ambient pH in filamentous fungi and yeasts. *Microbiol. Mol. Biol. Rev.* **66**, 426–446
8. Peñalva, M.A. and Arst, H.N. Jr. (2004) Recent advances in the characterization of ambient pH regulation of gene expression in filamentous fungi and yeasts. *Annu. Rev. Microbiol.* **58**, 425–451
9. Vincent, O., Rainbow, L., Tilburn, J., Arst, H.N. Jr., and Peñalva, M.A. (2003) YPXL/I is a protein interaction motif recognized by *Aspergillus* PalA and its human homologue, AIP1/Alix. *Mol. Cell. Biol.* **23**, 1647–1655
10. Katzmann, D.J., Odorizzi, G., and Emr, S.D. (2002) Receptor downregulation and multivesicular-body sorting. *Nat. Rev. Mol. Cell Biol.* **3**, 893–905
11. Galindo, A., Hervas-Aguilar, A., Rodriguez-Galan, O., Vincent, O., Arst, H.N. Jr., Tilburn, J., and Peñalva, M.A. (2007) PalC, one of two Bro1 domain proteins in the fungal pH signalling pathway, localizes to cortical structures and binds Vps32. *Traffic* **8**, 1346–1364
12. Futai, E., Maeda, T., Sorimachi, H., Kitamoto, K., Ishiura, S., and Suzuki, K. (1999) The protease activity of a calpain-like cysteine protease in Saccharomyces cerevisiae is required for alkaline adaptation and sporulation. *Mol. Gen. Genet.* **260**, 559–568
13. Xu, W., Smith, F.J. Jr., Subaran, R., and Mitchell, A.P. (2004) Multivesicular body-ESCRT components function in pH response regulation in Saccharomyces cerevisiae and Candida albicans. *Mol. Biol. Cell* **15**, 5528–5537
14. Hayashi, M., Fukuzawa, T., Sorimachi, H., and Maeda, T. (2005) Constitutive activation of the pH-responsive Rim101 pathway in yeast mutants defective in late steps of the MVB/ESCRT pathway. *Mol. Cell Biol.* **25**, 9478–9490
15. Boysen, J.H. and Mitchell, A.P. (2006) Control of Bro1-domain protein Rim20 localization by external pH, ESCRT machinery, and the Saccharomyces cerevisiae Rim101 pathway. *Mol. Biol. Cell* **17**, 1344–1353
16. Ito, T., Tashiro, K., Muta, S., Ozawa, R., Chiba, T., Nishizawa, M., Yamamoto, K., Kuhara, S., and Sakaki, Y. (2000) Toward a protein-protein interaction map of the budding yeast: a comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. *Proc. Natl Acad. Sci. USA* **97**, 1143–1147
17. Horii, M., Shibata, H., Kobayashi, R., Katoh, K., Yorikawa, C., Yasuda, J., and Maki, M. (2006) CHMP7, a novel ESCRT-III-related protein, associates with CHMP4b and functions in the endosomal sorting pathway. *Biochem. J.* **400**, 23–32
18. Williams, R.L. and Urbe, S. (2007) The emerging shape of the ESCRT machinery. *Nat. Rev. Mol. Cell Biol.* **8**, 355–368
19. Futai, E., Kubo, T., Sorimachi, H., Suzuki, K., and Maeda, T. (2001) Molecular cloning of PalBH, a mammalian homologue of the *Aspergillus* atypical calpain PalB. *Biochim. Biophys. Acta.* **1517**, 316–319
20. Ciccarelli, F.D., Proukakis, C., Patel, H., Cross, H., Azam, S., Patton, M.A., Bork, P., and Crosby, A.H. (2003) The identification of a conserved domain in both spartin and spastin, mutated in hereditary spastic paraplegia. *Genomics* **81**, 437–441
21. Reid, E., Connell, J., Edwards, T.L., Duley, S., Brown, S.E., and Sanderson, C.M. (2005) The hereditary spastic paraplegia protein spastin interacts with the ESCRT-III complex-associated endosomal protein CHMP1B. *Hum. Mol. Genet.* **14**, 19–38
22. Scott, A., Gaspar, J., Stuchell-Brereton, M.D., Alam, S.L., Skalicky, J.J., and Sundquist, W.I. (2005) Structure and ESCRT-III protein interactions of the MIT domain of human VPS4A. *Proc. Natl Acad. Sci. USA* **102**, 13813–13818
23. Tsang, H.T., Connell, J.W., Brown, S.E., Thompson, A., Reid, E., and Sanderson, C.M. (2006) A systematic analysis of human CHMP protein interactions: additional MIT domain-containing proteins bind to multiple components of the human ESCRT III complex. *Genomics* **88**, 333–346
24. Row, P.E., Liu, H., Hayes, S., Welchman, R., Charalabous, P., Hofmann, K., Clague, M.J., Sanderson, C.M., and Urbé, S. (2007) The MIT domain of UBPY constitutes a CHMP binding and endosomal localisation signal required for efficient EGF receptor degradation. *J. Biol. Chem.* **282**, 30929–30937
25. Lindley, K.M., Su, J.L., Hodges, P.K., Wisely, G.B., Bledsoe, R.K., Condreay, J.P., Winegar, D.A., Hutchins, J.T., and Kost, T.A. (2000) Production of monoclonal antibodies using recombinant baculovirus displaying gp64-fusion proteins. *J. Immunol. Methods* **234**, 123–135
26. Tanaka, T., Takeno, T., Watanabe, Y., Uchiyama, Y., Murakami, T., Yamashita, H., Suzuki, A., Aoi, R., Iwanari, H., Jiang, S.Y., Naito, M., Tachibana, K., Doi, T., Shulman, A.I., Mangelsdorf, D.J., Reiter, R., Auwerx, J., Hamakubo, T., and Kodama, T. (2002) The generation of monoclonal antibodies against human peroxisome proliferator-activated receptors (PPARs). *J. Atheroscler. Thromb.* **9**, 233–242

27. Zacharias, D.A, Violin, J.D., Newton, A.C., and Tsien, R.Y. (2002) Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* **296**, 913–916
28. Katoh, K., Shibata, H., Suzuki, H., Nara, A., Ishidoh, K., Kominami, E., Yoshimori, T., and Maki, M. (2003) The ALG-2-interacting protein Alix associates with CHMP4b, a human homologue of yeast Snf7 that is involved in multivesicular body sorting. *J. Biol. Chem.* **278**, 39104–39113
29. Katoh, K., Shibata, H., Hatta, K., and Maki, M. (2004) CHMP4b is a major binding partner of the ALG-2-interacting protein Alix among the three CHMP4 isoforms. *Arch. Biochem. Biophys.* **421**, 159–165
30. Yorikawa, C., Shibata, H., Waguri, S., Hatta, K., Horii, M., Katoh, K., Kobayashi, T., Uchiyama, Y., and Maki, M. (2005) Human CHMP6, a myristoylated ESCRT-III protein, interacts directly with an ESCRT-II component EAP20 and regulates endosomal cargo sorting. *Biochem J.* **387**, 17–26
31. Nagai, T., Ibata, K., Park, E. S., Kubota, M., Mikoshiba, K., and Miyawaki, A. (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol.* **20**, 87–90
32. Shibata, H., Yamada, K., Mizuno, T., Yorikawa, C., Takahashi, H., Satoh, H., Kitaura, Y., and Maki, M. (2004) The penta-EF-hand protein ALG-2 interacts with a region containing PxY repeats in Alix/AIP1, which is required for the subcellular punctate distribution of the amino-terminal truncation form of Alix/AIP1. *J. Biochem.* **135**, 117–128
33. Shibata, H., Suzuki, H., Yoshida, H., and Maki, M. (2007) ALG-2 directly binds Sec31A and localizes at endoplasmic reticulum exit sites in a Ca^{2+}-dependent manner. *Biochem. Biophys. Res. Commun.* **353**, 756–763
34. Shim, S., Kimpler, L.A., and Hanson, P.I. (2007) Structure/function analysis of four core ESCRT-III proteins reveals common regulatory role for extreme C-terminal domain. *Traffic* **8**, 1068–1079
35. Katoh, K., Suzuki, H., Terasawa, Y., Mizuno, T., Yasuda, J., Shibata, H., and Maki, M. (2005) The penta-EF-hand protein ALG-2 interacts directly with the ESCRT-I component TSG101, and Ca^{2+}-dependently co-localizes to aberrant endosomes with dominant-negative AAA ATPase SKD1/Vps4B. *Biochem. J.* **391**, 677–685
36. Yoshimori, T., Yamagata, F., Yamamoto, A., Mizushima, N., Kabeya, Y., Nara, A., Ishido, M., Ohashi, M., Ohsumi, M., and Ohsumi, Y. (2000) The mouse SKD1, a homologue of yeast Vps4p, is required for normal endosomal trafficking and morphology in mammalian cells. *Mol. Biol. Cell* **11**, 747–763
37. Fujita, H., Yamanaka, M., Imamura, K., Tanaka, Y., Nara, A., Yoshimori, T., Yokota, S., and Himeno, M. (2003) A dominant negative form of the AAA ATPase SKD1/VPS4 impairs membrane trafficking out of endosomal/lysosomal compartments: class E vps phenotype in mammalian cells. *J. Cell Sci.* **116**, 401–414
38. Takasu, H., Jee, J.G., Ohno, A., Goda, N., Fujiwara, K., Tochio, H., Shirakawa, M., and Hiroaki, H. (2005) Structural characterization of the MIT domain from human Vps4b. *Biochem. Biophys. Res. Commun.* **334**, 460–465
39. Obita, T., Saksena, S., Ghazi-Tabatabai, S., Gill, D.J., Perisic, O., Emr, S.D., and Williams, R.L. (2007) Structural basis for selective recognition of ESCRT-III by the AAA ATPase Vps4. *Nature* **449**, 735–739
40. Stuchell-Brereton, M.D., Skalicky, J.J., Kieffer, C., Karren, M.A., Ghaffarian, S., and Sundquist, W.I. (2007) ESCRT-III recognition by VPS4 ATPases. *Nature* **449**, 740–744
41. Suzuki, K. and Sorimachi, H. (1998) A novel aspect of calpain activation. *FEBS Lett.* **433**, 1–4
42. Tompa, P., Emori, Y., Sorimachi, H., Suzuki, K., and Friedrich, P. (2001) Domain III of calpain is a Ca^{2+}-regulated phospholipid-binding domain. *Biochem. Biophys. Res. Commun.* **280**, 1333–1339
43. Shao, H., Chou, J., Baty, C.J., Burke, N.A., Watkins, S.C., Stolz, D.B., and Wells, A. (2006) Spatial localization of m-calpain to the plasma membrane by phosphoinositide biphosphate binding during epidermal growth factor receptor-mediated activation. *Mol. Cell Biol.* **26**, 5481–5496
44. Babst, M., Sato, T.K., Banta, L.M., and Emer, S.D. (1997) Endosomal transport function in yeast requires a novel AAA-type ATPase, Vps4p. *EMBO J.* **16**, 1820–1831
45. Stauffer, D.R., Howard, T.L., Nyun, T., and Hollenberg, S.M. (2001) CHMP1 is a novel nuclear matrix protein affecting chromatin structure and cell-cycle progression. *J. Cell Sci.* **114**, 2383–2393
46. Howard, T.L., Stauffer, D.R., Degnin, C.R., and Hollenberg, S.M. (2001) CHMP1 functions as a member of a newly defined family of vesicle trafficking proteins. *J. Cell Sci.* **114**, 2395–2404
47. Ichioka, F., Takaya, E., Suzuki, H., Kajigaya, S., Buchman, V.L., Shibata, H., and Maki, M. (2007) HD-PTP and Alix share some membrane-traffic related proteins that interact with their Bro1 domains or proline-rich regions. *Arch. Biochem. Biophys.* **457**, 142–149
48. Ichioka, I., Kobayashi, R., Katoh, K., Shibata, H., and Maki, M. (2008) Brox, a novel farnesylated Bro1 domain-containing protein that associates with charged multivesicular body protein 4 (CHMP4). *FEBS J.* **275**, 682–692
49. Scott, A., Chung, H.Y., Gonciarz-Swiatek, M., Hill, G.C., Whitby, F.G., Gaspar, J., Holton, J.M., Viswanathan, R., Ghaffarian, S., and Hill, C.P. (2005) Sundquist WI. Structural and mechanistic studies of VPS4 proteins. *EMBO J.* **24**, 3658–3669