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Identification of Annexin A1 as a Novel Substrate for E6AP-Mediated Ubiquitylation

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ABSTRACT

E6-associated protein (E6AP) is a cellular ubiquitin protein ligase that mediates ubiquitylation and degradation of p53 in conjunction with the high-risk human papillomavirus E6 proteins. However, the physiological functions of E6AP are poorly understood. To identify a novel biological function of E6AP, we screened for binding partners of E6AP using GST pull-down and mass spectrometry. Here we identified annexin A1, a member of the annexin superfamily, as an E6AP-binding protein. Ectopic expression of E6AP enhanced the degradation of annexin A1 in vivo. RNAi-mediated downregulation of endogenous E6AP increased the levels of endogenous annexin A1 protein. E6AP interacted with annexin A1 and induced its ubiquitylation in a Ca^{2+}-dependent manner. GST pull-down assay revealed that the annexin repeat domain III of annexin A1 is important for the E6AP binding. Taken together, our data suggest that annexin A1 is a novel substrate for E6AP-mediated ubiquitylation. Our findings raise the possibility that E6AP may play a role in controlling the diverse functions of annexin A1 through the ubiquitin-proteasome pathway. J. Cell. Biochem. 106: 1123–1135, 2009.

KEY WORDS: E6AP; ANNEXIN A1; UBIQUITIN; DEGRADATION

The ubiquitin/26S proteasome pathway plays important roles in the control of many basic cellular processes, such as cell cycle progression, signal transduction, transcriptional regulation, DNA repair, and the regulation of inflammation responses [Hershko and Ciechanover, 1998]. Ubiquitin is a 76-aa polypeptide that is highly conserved among eukaryotic organisms. The ubiquitin-proteasome pathway consists of an enzymatic cascade that ubiquitylates proteins, thereby targeting them for proteasomal degradation. The E1 ubiquitin-activating enzyme binds ubiquitin through a thioester linkage in an ATP-dependent manner [Ciechanover et al., 1981; Haas and Rose, 1982]. The activated ubiquitin is then transferred to the E2 ubiquitin-conjugating enzyme. E2 works in conjunction with the E3 ubiquitin-protein ligase, which is responsible for conferring substrate specificity [Hershko et al., 1986]. E3 mediates the transfer of ubiquitin to the target protein. The polyubiquitylated substrates are rapidly recognized and degraded by the 26S proteasome [Ciechanover, 1998; Ciechanover et al., 2000].

E6-associated protein (E6AP) was initially identified as the cellular factor that stimulates ubiquitin-dependent degradation of the tumor suppressor p53 in conjunction with the E6 protein of cervical cancer-associated human papillomavirus (HPV) types 16 and 18 [Huibregtse et al., 1993a; Scheffner et al., 1994]. The E6–E6AP complex functions as an E3 ubiquitin ligase in the ubiquitylation of p53 [Scheffner et al., 1993]. E6AP is the prototype of a family of ubiquitin ligases called HECT domain ubiquitin ligases, all of which contain a domain *h*omologous to the

Abbreviations used: E6AP, E6-associated protein; HPV, human papillomavirus; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; MS, mass spectrometry; HCV, hepatitis C virus; MAb, monoclonal antibody; PAb, polyclonal antibody; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; CHX, cycloheximide.

T. Shimoji and K. Murakami contributed equally to this work.

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*E*6AP carboxyl *t*erminus [Huibregtse et al., 1995]. Known substrates of the E6–E6AP complex include the tumor suppressor p53 [Scheffner et al., 1993], the PDZ domain-containing protein scribble [Nakagawa and Huibregtse, 2000] and NFX1-91, a transcriptional repressor of the gene encoding hTERT [Gewin et al., 2004]. Interestingly, E6AP is not involved in the ubiquitylation of p53 in the absence of E6 [Talis et al., 1998]. Several potential E6-independent substrates for E6AP have been identified, such as HHR23A and HHR23B (the human orthologs of *Saccharomyces cerevisae* Rad23) [Kumar et al., 1999], Blk (a member of the Src family kinases) [Oda et al., 1999], Mcm7 (which is involved in DNA replication) [Kuhne and Banks, 1998], trihydrophobin 1 [Yang et al., 2007], and AIB1 (a steroid receptor coactivator) [Mani et al., 2006].

Some patients with Angelman syndrome, a severe neurological disorder linked to E6AP, have mutations within the catalytic cleft that have been shown to reduce E6AP ubiquitin ligase activity [Kishino et al., 1997; Cooper et al., 2004]. Despite the significant progress in the study of Angelman syndrome-associated E6AP mutations, none of the identified E6AP substrates have been directly linked to the disorder. The physiological functions of E6AP are poorly understood at present.

In an attempt to identify novel substrates of E6AP, we identified annexin A1 (formerly known as lipocortin 1) as an E6AP-binding protein. Annexin A1 is a 37-kDa member of the annexin superfamily of Ca^{2+} and phospholipid-binding proteins [Lim and Pervaiz, 2007]. Annexin A1 is involved in the inhibition of cell proliferation, anti-inflammatory effects, and the regulation of cell differentiation. In addition, annexin A1 is involved in the regulation of cell death signaling, phagocytosis of apoptosis, and the process of carcinogenesis [Buckingham et al., 2006; Lim and Pervaiz, 2007]. Annexin A1 is phosphorylated by various kinases such as tyrosine kinase, pp60c-src [Varticovski et al., 1988], protein kinase C [Oudinet et al., 1993], epidermal growth factor receptor protein kinase [Haigler et al., 1987], and hepatocyte growth factor receptor kinase [Skouteris and Schroder, 1996].

In this study, we have examined the possibility that the stability of annexin A1 is regulated through E6AP-dependent ubiquitylation. Our study revealed that E6AP mediates ubiquitin-dependent degradation of annexin A1 in a Ca^{2+}-dependent manner. Our results raise the possibility that E6AP may have a role in controlling the diverse functions of annexin A1.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

Human embryonic kidney (HEK) 293T cells, and human cervical carcinoma C33-A cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA), and 10% (v/v) fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS) at 37°C in a 5% CO_2 incubator. HEK 293T cells and C33-A cells were transfected with plasmid DNA using FuGene 6 transfection reagents (Roche, Mannheim, Germany). The *Spodoptera frugiperda* (Sf) 9 cells were cultured in TC100 (JRH Biosciences) supplemented with 10% (v/v) FBS and 100 μg/ml kanamycin at 26°C in an incubator. The *Trichoplusia ni* (Tn) 5 cells were cultured in Ex-Cell 405 (JRH Biosciences) at 26°C in an incubator.

PLASMIDS AND RECOMBINANT BACULOVIRUSES

To express annexin A1 as a FLAG-tagged fusion protein in mammalian cells, annexin A1 fragment was amplified from pKK-trc-lipo-155 (a kind gift from Dr. Browning, Biogen) by polymerase chain reaction (PCR) using two oligonucleotides, 5′-TATCCCGG-GAACCACCATGGCAATGGTATCAGAATTCC-3′ and 5′-TATGCGG-CCGCTTACTTATCGTCGTCATCCTTGTAATCGTTTCCTCCACAAAG-AGCC-3′. The FLAG-tag sequence was fused to the C-terminus of the annexin A1 gene in frame. The amplified PCR fragment was digested with *Sma*I and *Not*I, purified, and subcloned into pCAGGS [Niwa et al., 1991], resulting in pCAG-annexin A1-FLAG. To express E6AP and the active-site cysteine-to-alanine mutant of E6AP in mammalian cells, pCAG-HA-E6AP isoform II and pCAG-HA-E6AP C-A were used [Shirakura et al., 2007]. The C-A mutation was introduced at the site of E6AP C843 [Kao et al., 2000]. To express Nedd4, pCAG-HA-Nedd4 was constructed. To make a fusion protein consisting of glutathione S-transferase (GST) fused to the N-terminus of E6AP in *Escherichia coli* (*E. coli*), pGEX-E6AP was used [Shirakura et al., 2007]. Recombinant baculoviruses expressing GST-E6AP were described previously [Shirakura et al., 2007]. To express hexahistidine (His)-tagged annexin A1 in *E. coli*, annexin A1 fragment was amplified from pKK-trc-lipo-155 by PCR using two oligonucleotides, 5′-TATCCCGGGAACCACCATGGCAATGG-TATCAGAATTCC-3′ and 5′-ATAGCGGCCGCGTTTCCTCCACAAA-GAGCC-3′. The PCR fragment was purified and digested with *Sma*I and *Not*I. pET21b was digested with *Nde*I, blunt ended with a DNA blunting kit (Takara, Japan), and digested with *Not*I. Then, the PCR fragment of annexin A1 was ligated into the pET21b fragment, resulting in pET21b-annexin A1. To map the E6AP-binding site on annexin A1 protein, a series of expression plasmids for GST-annexin A1 fusion proteins were constructed by amplifying annexin A1 gene fragments with PCR using sense primers containing *Sma*I site and antisense primers containing a *Not*I site. The amplified PCR fragments were subcloned into pGEM T-Easy (Promega, Madison, WI) and verified by sequencing. Then, the annexin A1 gene fragments were digested with *Sma*I and *Not*I and ligated into the *Sma*I–*Not*I site of pGEX 4T-1 (GE Healthcare, Uppsala, Sweden). The annexin A1 (1-41) gene fragment was amplified from pET21b-annexin A1 by PCR using two oligonucleotides, 5′-TATCCCGG-GAACCACCATGGCAATGGTATCAGAATTCC-3′ and 5′-ATATAGC-GGCCGCTTAGGTAGGATAGGGGCTCACCGCT-3′. The PCR primers used to amplify the annexin A1 fragments were as follows:

Annexin A1 (42–346): 5′-TATCCCGGGAACCACCATGTTCAAT-CCATCCTCGGATGTCG-3′ and 5′-ATATAGCGGCCGCTTAGTTT-CCTCCACAAAGAGCC-3′.
Annexin A1 (42–113): 5′-AAACCCGGGTATGTTCAATCCATCCT-CGGATGTCG-3′ and 5′-TTTGCGGCCGCTTATTTTAGCAGAGC-TAAAACAAC-3′.
Annexin A1 (114–195): 5′-AAACCCGGGTATGACTCCAGCG-CAATTTGATGC-3′ and 5′-TTTGCGGCCGCTTAATTCACACCAA-AGTCCTCAG-3′.

Annexin A1 (196–274): 5′-AAACCCGGGTATGGAAGACTTGG-CTGATTCAG-3′ and 5′-TTTGCGGCCGCTTAGCTTGTGGCGCAC-TTCACG-3′.
Annexin A1 (275–346): 5′-AAACCCGGGTATGAAACCAGCTTT-CTTTGCAGAG-3′ and 5′-ATATAGCGGCCGCTTAGTTTCCTCCA-CAAAGAGCC-3′.
Annexin A1 (42–195): 5′-AAACCCGGGTATGTTCAATCCATCC-TCGGATGTCG-3′ and 5′-TTTGCGGCCGCTTAATTCACACCAAA-GTCCTCAG-3′.
Annexin A1 (114–274): 5′-AAACCCGGGTATGACTCCAGCGCA-ATTTGATGC-3′ and 5′-TTTGCGGCCGCTTAGCTTGTGGCGCAC-TTCACG-3′.
Annexin A1 (196–346): 5′-AAACCCGGGTATGGAAGACTTGG-CTGATTCAG-3′ and 5′-ATATAGCGGCCGCTTAGTTTCCTCCAC-AAAGAGCC-3′.

The sequences of the inserts were extensively verified using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). To express GST, GST-E6AP, and MEF-E6AP in the baculovirus expression system, recombinant baculoviruses were recovered using a BaculoGold transfection kit (Pharmingen, San Diego, CA) as described previously [Shirakura et al., 2007].

ANTIBODIES

The mouse monoclonal antibodies (MAbs) used in this study were anti-HA MAb (12CA5) (Roche), anti-HA 16B12 MAb (HA.11; BabCO), anti-Annexin I MAb (BD Biosciences, San Jose, CA), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) MAb (Chemicon, Temecula, CA), anti-GST MAb (Santa Cruz Biotechnology, Santa Cruz, CA), anti-ubiquitin MAb (Chemicon), anti-E6AP MAb (E6AP-330) (Sigma), and anti-β-actin MAb (Ab-1) (Calbiochem, San Diego, CA). The polyclonal antibodies (PAbs) used in this study were anti-HA rabbit PAb (Y-11; Santa Cruz Biotechnology), anti-FLAG rabbit PAb (F7425; Sigma), anti-E6AP rabbit PAb (H-182; Santa Cruz Biotechnology), and anti-GST goat PAb (Amersham Bioscience, Buckinghamshire, UK).

IDENTIFICATION OF E6AP-BINDING PROTEINS WITH MALDI-TOF MASS SPECTROMETRY

To screen for potential E6AP-binding proteins, GST pull-down assays were performed using GST-E6AP and ten 225 cm^2-flasks (Corning, New York, NY) of confluent C-33A cells as the source of protein. The cells were lysed in 15 ml of the cell lysis buffer (100 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.5% Triton X-100 [ICE Biomedicals, Aurora, OH], Complete protease inhibitor cocktail [Roche]). The samples were incubated at 4°C for 1 h, and centrifuged at 13,000*g* for 30 min. The supernatants were collected and pre-cleared with 250 μl of 50% slurry glutathione-Sepharose 4B beads (Amersham Bioscience) to remove proteins that can nonspecifically bind to glutathione-Sepharose 4B beads. The supernatants were then pre-cleared with 250 μg of GST immobilized on glutathione-Sepharose 4B beads to remove proteins which can bind to GST. Then, the supernatant was collected, mixed with 250 μg of GST-E6AP or GST immobilized on glutathione-Sepharose 4B beads, and incubated for 1 h at 4°C. The beads were collected and washed with the cell lysis buffer three times. To remove the bound proteins from GST-E6AP, the bound proteins were released with the releasing buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100) five times. The released proteins were mixed with 20% (w/v) trichloroacetic acid (TCA) and incubated at 4°C for 30 min. After centrifugation, the TCA-precipitated samples were washed with ice-cold acetone four times, dried, and lysed in SDS-polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer. The samples were separated by 7.5% SDS–PAGE and stained with Coomassie brilliant blue (CBB). The specific protein bands were excised from the gel and subjected to in-gel trypsin digestion. The tryptic peptide mixtures were analyzed by MALDI-TOF/MS analysis [Kaji et al., 2000]. Prior to MALDI-TOF/MS analysis, the peptide mixtures were desalted using C18 Zip Tips (Millipore, Bedford, MA) according to the manufacturer's instructions. The peptide data were collected in the reflection mode and with positive polarity, using a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile (PE Biosystems, Foster City, CA) and 0.1% trifluoroacetic acid as the matrix. Spectra were obtained using a Voyger DE-STR MALDI-TOF mass spectrometer (PE Biosystems). The database-fitting program MS-Fit at the website (http://jpsl.ludwig.edu.au/ucsfhtml3.4/msfit.htm) of the University of California, San Francisco was used to interpret the MS spectra of the protein digests.

EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

E. coli BL21 (DE3) cells were transformed with plasmids expressing GST fusion protein or His-tagged protein and grown at 37°C. Expression of the fusion protein was induced by 1 mM isopropyl-β-D-thiogalactopyranoside at 25°C for 4 h. Bacteria were harvested, suspended in lysis buffer (phosphate-buffered saline [PBS] containing 1% Triton X-100, Complete protease inhibitor cocktail, EDTA free [Roche]), and sonicated on ice.

Hi5 cells were infected with the recombinant baculoviruses to produce GST-E6AP or GST. GST-E6AP and GST-fusion proteins were purified on glutathione-Sepharose beads (Amersham Bioscience) according to the manufacturer's protocols. His-tagged proteins were purified on Ni-NTA beads (Qiagen, Hilden, Germany) according to the manufacturer's protocols. MEF-E6AP and MEF-E6AP C-A [Shirakura et al., 2007] were purified on anti-FLAG M2 agarose beads (Sigma) according to the manufacturer's protocols.

IMMUNOPRECIPITATION AND IMMUNOBLOT ANALYSIS

Cells were lysed in IP buffer (100 mM Tris–HCl, 100 mM NaCl, pH 7.4, 0.5% Triton X-100, 0.5 mM $CaCl_2$, plus Complete protease inhibitor cocktail, EDTA free) at 4°C for 15 min. Extracts were clarified by centrifugation at 13,000*g* for 20 min, and soluble lysates were pre-cleared with protein G Sepharose (GE Healthcare). The samples were incubated with anti-FLAG M2 agarose (Sigma) and rotated at 4°C for 5 h. The beads were washed five times with IP buffer, and bound proteins were eluted with Laemmli sample buffer. Samples were separated by 10% SDS–PAGE. Immunoblot analysis was performed essentially as described previously [Harris et al., 1999]. The membrane was visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

IN VIVO UBIQUITYLATION ASSAY

In vivo ubiquitylation assays were performed essentially as described previously [Shirakura et al., 2007]. Where indicated, cells were treated with 25 μM MG132 (Calbiochem) or with dimethylsulfoxide (DMSO; control) for 30 min prior to collection. FLAG-annexin A1 was immunoprecipitated with anti-FLAG MAb. Immunoprecipitates were analyzed by immunoblotting, using either anti-HA PAb or anti-annexin A1 MAb to detect ubiquitylated annexin A1.

IN VITRO UBIQUITYLATION ASSAY

In vitro ubiquitylation assays were performed essentially as described previously [Shirakura et al., 2007]. For in vitro ubiquitylation of annexin A1, purified GST-annexin A1 was used as a substrate. Purified GST was used as a negative control. Assays were done in 40-μl volumes containing 20 mM Tris–HCl, pH 7.6, 50 mM NaCl, 5 mM ATP, 8 μg of bovine ubiquitin (Sigma), 0.1 mM DTT, 200 ng of mouse E1, 200 ng of E2 (UbcH7), and 0.5 μg of MEF-E6AP, in the presence or absence of $CaCl_2$ as indicated. The reaction mixtures were incubated at 37°C for 120 min followed by immunoblotting.

SIRNA TRANSFECTION

HEK 293T cells (3×10^5 cells in a 6-well plate) were transfected with 40 pmol of either E6AP-specific small interfering RNA (siRNA; Sigma), or scramble negative-control siRNA duplexes (Sigma) using HiPerFect transfection reagent (Qiagen) following the manufacturer's instructions. The E6AP-siRNA target sequences were as follows:

siE6AP-1 (sense) 5′-GGGUCUACACCAGAUUGCUTT-3′; scramble negative control (siCont-1) (sense) 5′-UUGCGGGUCUAAUCACCGATT-3′ [Shirakura et al., 2007]; E6AP-2 (sense), 5′-CAACUCCUGCUCUGAGAUATT-3′; and scramble negative control (siCont-2), 5′-AGACCUACCCGAUUACUGUTT-3′ [Kelley et al., 2005].

ANNEXIN A1 PROTEIN AND E6AP-BINDING ASSAYS

To map the E6AP binding site on annexin A1 protein, GST pull-down assays were performed. A series of recombinant GST-annexin A1 proteins were expressed in *E. coli* and purified using glutathione-Sepharose 4B beads. Equivalent amounts of purified proteins, as estimated by CBB staining, were used for the binding assays. For pull-down assays, purified MEF-E6AP was incubated with GST-annexin A1 proteins immobilized on glutathione-Sepharose 4B beads in 1 ml of the binding buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, and 5 mM $CaCl_2$) at 4°C for 4 h. The beads were washed four times with binding buffer, and the pull-down complexes were separated by SDS–PAGE on 10% polyacrylamide gels and analyzed by immunoblotting with anti-FLAG MAb.

CYCLOHEXIMIDE (CHX) HALF-LIFE EXPERIMENTS

To examine the half-life of annexin A1 protein, transfected HEK 293T cells were treated with 50 μg/ml CHX at 44 h post-transfection. The cells at time-point zero were harvested immediately after treatment with CHX. Subsequent time points were incubated in medium containing CHX at 37°C for 3, 6, and 9 h as indicated.

CONFOCAL IMMUNOFLUORESCENCE MICROSCOPY

Cells were transfected with pCAG-HA-E6AP C-A and pCAG annexin A1-FLAG using TransIT-LT1 (Takara) according to the manufacturer's instructions. Transfected cells grown on collagen-coated coverslips were washed with PBS, fixed with 4% paraformaldehyde for 30 min at 4°C, and permeabilized with PBS containing 2% FCS and 0.3% Triton X-100. Cells were incubated with anti-HA mouse MAb and anti-FLAG rabbit PAb as primary antibodies, washed, and incubated with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR) and Alexa 555 Fluor goat anti-rabbit IgG (Molecular Probes) as secondary antibodies. Then the cells were washed with PBS, mounted on glass slides, and examined with an LSM510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

RESULTS

IDENTIFICATION OF ANNEXIN A1 AS A BINDING PARTNER FOR E6AP

To identify novel substrates for E6AP, we screened for E6AP-binding proteins using pull-down experiments with GST-E6AP. Whole cell lysates from C33-A cells were prepared as described above and incubated with immobilized GST-E6AP or GST alone. After the separation of bound proteins by SDS–PAGE, CBB staining of the gels revealed at least 15 specific bands precipitating with the GST-E6AP. The protein bands were excised from the gel and subjected to in-gel trypsin digestion. The tryptic peptide mixtures were analyzed by MALDI-TOF/MS as described above. Masses obtained using MALDI-TOF were analyzed using the MS-Fit program. This procedure identified seven individual proteins (Fig. 1A,a–g), such as a heat shock protein and a translation elongation factor. One of these bands, migrating at 37 kDa (Fig. 1A,e), was identified as annexin A1 based on six independent MS spectra (Fig. 1B). To verify the interaction of annexin A1 with E6AP, we repeated the pull-down experiments by incubating immobilized GST-E6AP with lysate from C-33A cells. Immunoblot analysis confirmed the proteomic identification of annexin A1 (Fig. 1C).

IN VIVO INTERACTION BETWEEN ANNEXIN A1 AND E6AP

To determine whether the interaction between annexin A1 and E6AP could take place in vivo, annexin A1-FLAG expression plasmid was introduced into HEK 293T cells together with either HA-E6AP expression plasmid or HA-Nedd4 (another HECT domain ubiquitin ligase) [Staub et al., 1996] expression plasmid. A catalytically inactive form of E6AP in which the active site cysteine residue has been substituted with alanine (C843A) was used to avoid potential degradation of interacting proteins. Cells were lysed and annexin A1-FLAG was immunoprecipitated with FLAG-beads. As shown in Figure 2A, HA-E6AP but not HA-Nedd4 was co-immunoprecipitated with annexin A1-FLAG, indicating that E6AP actually interacts with annexin A1 in the cells. We confirmed that the active form of HA-E6AP was also coimmunoprecipitated with annexin A1-FLAG (data not shown).

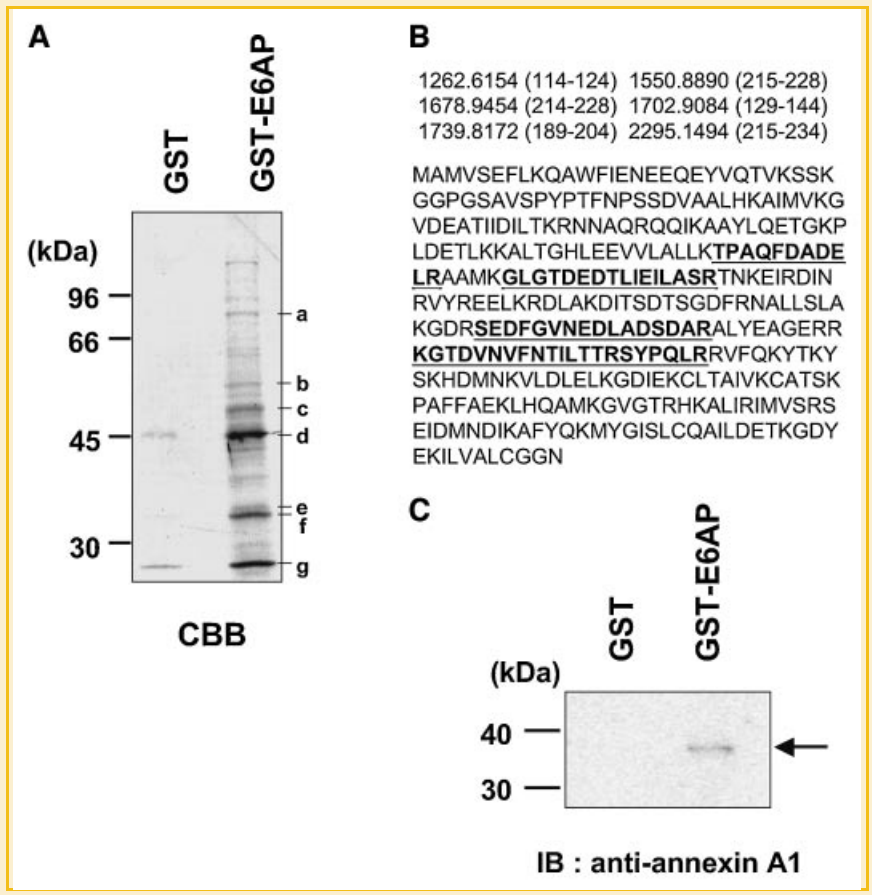

Fig. 1. Identification of annexin A1 as a binding partner for the E6AP. A: GST-E6AP on glutathione-Sepharose beads was incubated with whole-cell extract from C-33A cells. Bound proteins were detected by SDS–PAGE and CBB staining. Molecular weight markers are indicated, as well as the position of p37 (e), which likely corresponds to annexin A1. B: Peptide masses were identified by MALDI-TOF/MS and corresponding amino acids of annexin A1 (trypsin cleavage). Annexin A1 (accession no. 12654863) was identified through MALDI-TOF/MS as a candidate protein interacting with GST-E6AP. The database-fitting program MS-Fit was used to interpret the MS spectra of the protein digests. Six out of 22 masses obtained through the MALDI-TOF analysis corresponded to the theoretical values for annexin A1 cleavage (upper panel, amino acids corresponding to tryptic fragments in brackets) and represented 18% of the proteins' fragments (lower panel, peptides in bold print). The molecular weight search score, MOWSE, was 3.94E + 03. C: The identity of the band shown in panel A as annexin A1 was confirmed by Western blotting with anti-annexin A1 mouse MAb.

To determine whether annexin A1 and E6AP co-localize in the cells, immunofluorescence microscopy analysis was performed in two different cell lines, HEK 293T cells and C-33A cells. The immunofluorescence study showed that E6AP partially co-localized with annexin A1 in the cytoplasm of both types of cells (Fig. 2B).

To determine whether endogenous E6AP interacts with endogenous annexin A1 in vivo, C-33A cells were lysed and subjected to immunoprecipitation with anti-annexin A1 antibody or anti-E6AP antibody. Endogenous E6AP was co-immunoprecipitated with anti-annexin A1 antibody, but not with control antibody (Fig. 2C, left panel, upper lane). Moreover, endogenous annexin A1 was co-immunoprecipitated with anti-E6AP antibody, but not with control antibody (Fig. 2C, right panel, lower lane). These results suggest that endogenous E6AP can interact with endogenous annexin A1 in C-33A cells.

E6AP DECREASES STEADY-STATE LEVELS OF ANNEXIN A1 PROTEIN

To determine whether E6AP functions as an E3 ubiquitin ligase for the ubiquitylation of annexin A1, we assessed the effects of E6AP on annexin A1 protein in HEK 293T cells. The annexin A1-FLAG expression plasmid together with the plasmid for HA-tagged wild-type E6AP, catalytically inactive mutant E6AP, E6AP C-A, or Nedd4, was introduced into HEK 293T cells, and the levels of annexin A1 proteins were examined by immunoblotting. The steady-state levels of annexin A1 protein decreased with an increase of the E6AP plasmids (Fig. 3A,B). However, neither E6AP C-A nor Nedd4 decreased the steady-state levels of the annexin A1 protein, suggesting that E6AP enhances the degradation of annexin A1 protein.

E6AP ENHANCES THE DEGRADATION OF ANNEXIN A1 PROTEIN

To determine whether the E6AP-induced reduction of the annexin A1 protein is due to an increase in the rate of degradation of annexin A1 protein, we examined the degradation of annexin A1 using the protein synthesis inhibitor CHX. Annexin A1 together with wild-type E6AP or inactive mutant E6AP C-A was expressed in HEK 293T cells. At 44 h after transfection, the cells were treated with either 50 μg/ml CHX alone or 50 μg/ml CHX plus 25μM MG132 to inhibit

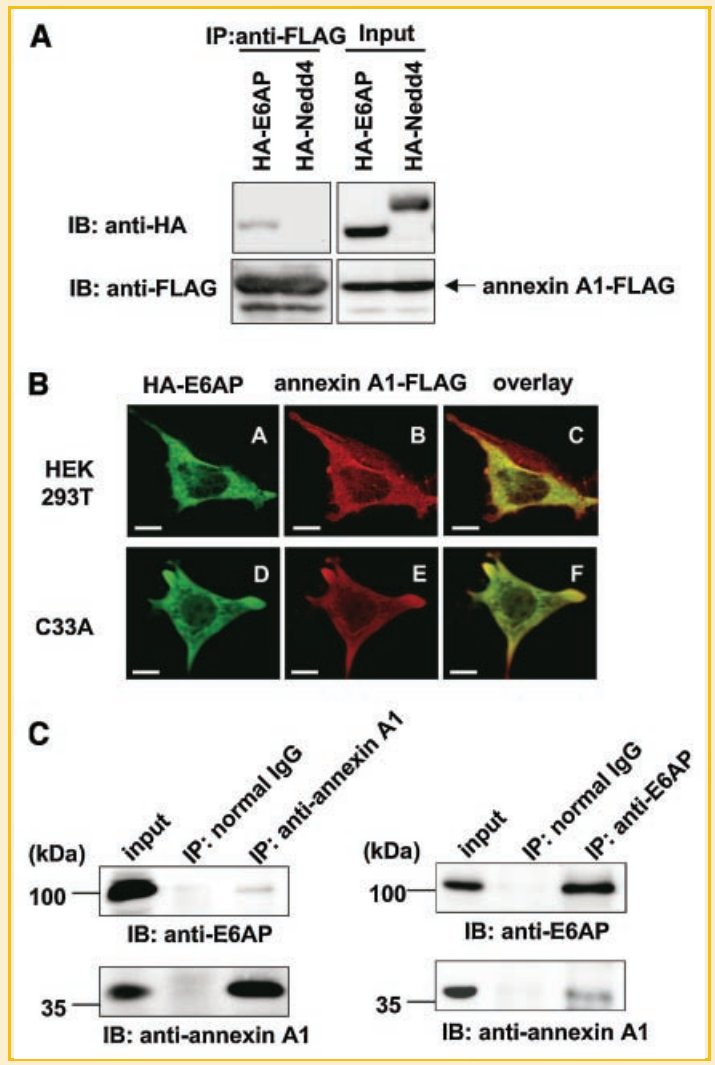

Fig. 2. In vivo interaction between annexin A1 and E6AP. A: HEK 293T cells were co-transfected with pCAG-annexin A1-FLAG together with pCAG-HA-E6AP C-A or pCAG-HA-Nedd4. The cell lysates were immunoprecipitated with FLAG beads and analyzed by immunoblotting with anti-HA PAb or anti-FLAG PAb. B: HEK 293T cells and C-33A cells were transfected with either HA-E6AP plasmid or annexin A1-FLAG plasmid, grown on coverslips, fixed, and processed for double-label immunofluorescence for HA-E6AP or annexin A1-FLAG. All the samples were examined with an LSM510 laser scanning confocal microscope (bar, 10 μm). C: C33A cells were lysed in the cell lysis buffer. The cell lysates were immunoprecipitated with anti-annexin A1 mouse MAb or control normal mouse IgG and analyzed with anti-E6AP mouse mAb or anti-annexin A1 mouse MAb as indicated (left panel). The cell lysates were immunoprecipitated with anti-E6AP mouse mAb or control normal mouse IgG and analyzed with anti-E6AP mouse mAb or anti-annexin A1 mouse mAb as indicated (right panel).

proteasome function. Cells were collected at 0, 3, 6, and 9 h following the treatment and analyzed by immunoblotting (Fig. 4A). Overexpression of E6AP resulted in rapid degradation of the annexin A1 protein, whereas the annexin A1 protein was stable in the cells transfected with inactive mutant E6AP C-A. Treatment of the cells with MG132 inhibited the degradation of annexin A1 (Fig. 4A). These results suggest that E6AP enhances proteasomal degradation of annexin A1.

KNOCKDOWN OF ENDOGENOUS E6AP BY SIRNA RESULTS IN ACCUMULATION OF ENDOGENOUS ANNEXIN A1 PROTEIN

To determine whether or not E6AP is critical for the degradation of endogenous annexin A1 protein, the expression of E6AP was knocked down by siRNA and the expression of annexin A1 and E6AP was analyzed by immunoblotting. We used two different siE6AP duplexes, siE6AP-1 and siE6AP-2, to knockdown the endogenous E6AP. Transfection of either siE6AP-1 or siE6AP-2 into HEK 293T cells resulted in a decrease in E6AP levels by 70–95% (Fig. 4B, the first panel), indicating that both siRNAs against E6AP resulted in a remarkable decrease in the protein level of E6AP. Knockdown of endogenous E6AP resulted in an accumulation of the endogenous annexin A1 protein, but no accumulation of the endogenous annexin A2 protein (Fig. 4B, the second and third panels), suggesting that the ubiquitylation and degradation of endogenous annexin A1 is specifically inhibited by knockdown of endogenous E6AP in vivo. These results suggest that endogenous E6AP plays a role in the proteolysis of endogenous annexin A1.

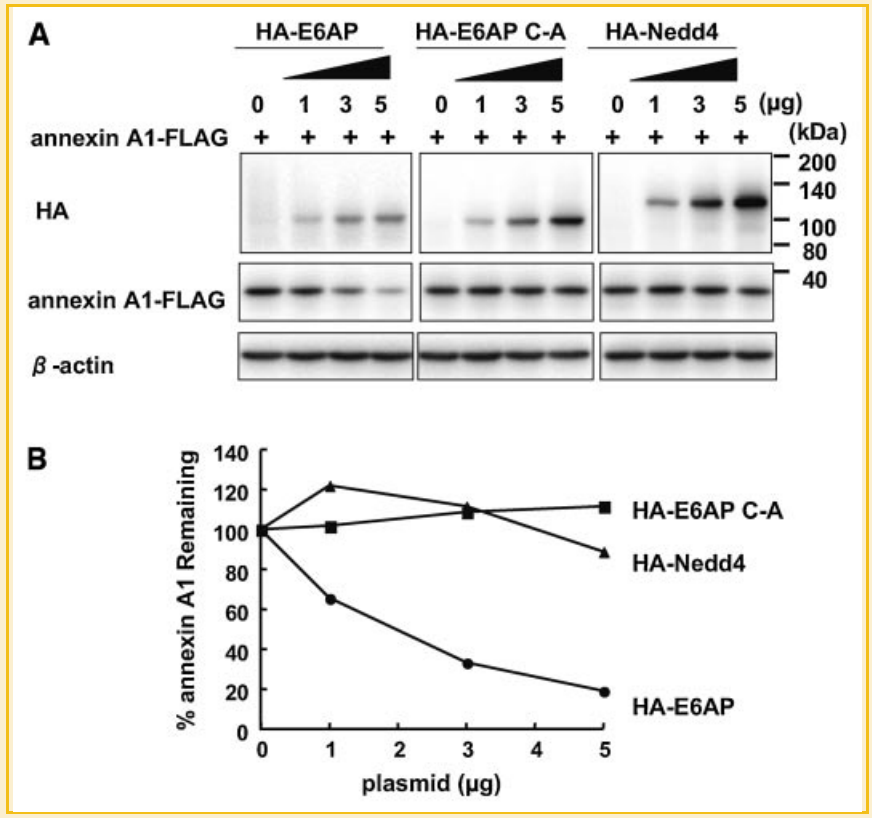

Fig. 3. E6AP decreases steady-state levels of annexin A1 protein in HEK 293T cells. HEK 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG annexin A1-FLAG along with either pCAG-HA-E6AP, pCAG-HA-E6AP C-A, or pCAG-HA-Nedd4 as indicated. At 48 h post-transfection, protein extracts were separated by SDS-PAGE and analyzed by immunoblotting with anti-HA PAb (top panel), anti-FLAG MAb (middle panel), and anti-β-actin MAb (bottom panel). B: Quantitation of data shown in panel A. Intensities of the gel bands were quantitated using the NIH Image 1.62 program. The level of β-actin served as a loading control. Circles, E6AP; squares, E6AP C-A; triangles, Nedd4.

E6AP MEDIATES UBIQUITYLATION OF ANNEXIN A1 IN VIVO

To determine whether E6AP can induce ubiquitylation of annexin A1 in cells, we performed in vivo ubiquitylation assays. HEK 293T cells were transfected with annexin A1-FLAG plasmid and either E6AP or Nedd4 plasmid, together with a plasmid encoding HA-tagged ubiquitin to facilitate the detection of ubiquitylated annexin A1 protein. Cell lysates were immunoprecipitated with anti-FLAG MAb and immunoblotted with anti-HA PAb to detect ubiquitylated annexin A1 protein. Only a faint ubiquitin signal was detected in the cells co-transfected with empty plasmid or Nedd4 plasmid (Fig. 5A, lanes 4 and 6). In contrast, co-expression of E6AP led to readily detectable ubiquitylated forms of the annexin A1 as a smear of higher-molecular-weight bands (Fig. 5A, lane 5). Immunoblot analysis with anti-FLAG PAb confirmed that annexin A1-FLAG proteins were immunoprecipitated and that higher-molecular-weight bands conjugated with HA-ubiquitin were indeed ubiquitylated forms of the annexin A1 proteins (Fig. 5B, lane 5). These results suggest that E6AP enhances ubiquitylation of annexin A1 in the cells.

E6AP MEDIATES UBIQUITYLATION OF ANNEXIN A1 IN VITRO

To reconstitute the E6AP-mediated ubiquitylation of annexin A1 in vitro, we performed an in vitro ubiquitylation assay of the annexin A1 using purified MEF-E6AP and GST-annexin A1 as described above. When the in vitro ubiquitylation reaction was carried out either in the absence of MEF-E6AP or in the presence of MEF-E6AP C-A, no ubiquitylation signal was detected (Fig. 5C, lanes 4 and 5). However, inclusion of purified MEF-E6AP in the reaction mixture resulted in ubiquitylation of GST-annexin A1 (Fig. 5C, lane 6), while no ubiquitylation was observed in the absence of ATP (Fig. 5C, lane 7). No signal was detected when GST was used as a substrate (data not shown). These results indicate that E6AP directly mediates ubiquitylation of annexin A1 protein in an ATP-dependent manner.

Ca^{2+}-DEPENDENT INTERACTION BETWEEN ANNEXIN A1 AND E6AP

We next assessed the effects of Ca^{2+} on the interaction between annexin A1 and E6AP. We performed the pull-down experiments by incubating immobilized GST-E6AP or GST alone with purified His-tagged annexin A1 in the presence or absence of 1 mM $CaCl_2$. After precipitation and SDS–PAGE, the bound annexin A1 was detected by immunoblotting with anti-annexin A1 antibody. GST-E6AP, but not GST, was able to precipitate annexin A1 only in the presence of Ca^{2+} (Fig. 6A, lane 4). These interactions were dependent on the concentration of Ca^{2+}, as increasing concentrations of Ca^{2+} resulted in an increase of binding of annexin A1 to E6AP (Fig. 6B). These

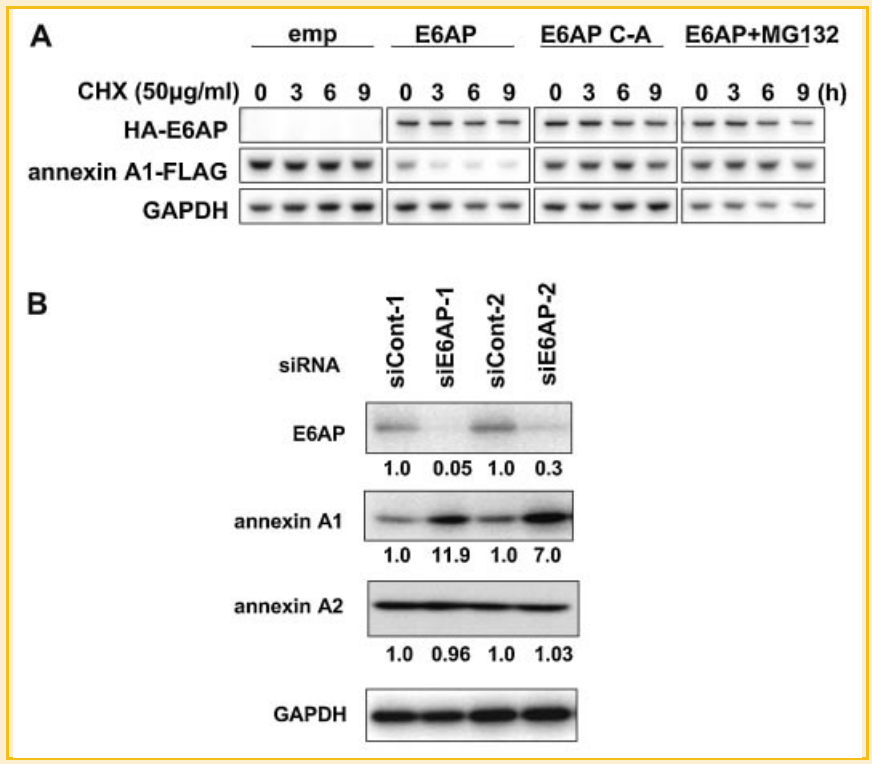

Fig. 4. E6AP-dependent degradation of annexin A1 protein. A: HEK 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG-annexin A1-FLAG plus 4 μg of empty vector, pCAG-HA-E6AP, or pCAG-HA-E6AP C-A. The cells were treated with 50 μg/ml CHX at 44 h after transfection. Cell extracts were collected at 0, 3, 6, and 9 h after treatment with CHX, followed by immunoblotting. Data are representative of three independent experimental determinations. B: Knockdown of endogenous E6AP by siRNA resulted in the accumulation of endogenous annexin A1 in HEK 293T cells. HEK 293T cells (3×10^5 cells/6-well plate) were transfected with 40 pmol of E6AP-specific duplex siRNA (or scramble negative control). Two sets of siRNAs (siE6AP-1 and siCont-1, siE6AP-2 and siCont-2) were used as described in Materials and Methods Section. The cells were harvested at 120 h after siRNA transfection. The relative levels of protein expression were quantitated using the NIH Image 1.62 program and are indicated below in the respective lanes. Data are representative of three independent experimental determinations.

results indicate that E6AP binds annexin A1 in a Ca^{2+}-dependent manner.

UBIQUITYLATION OF ANNEXIN A1 BY E6AP IS CA^{2+}-DEPENDENT

The HECT-type ubiquitin ligases transfer ubiquitin molecules to the substrates through direct interaction. Therefore, the E6AP-annexin A1 interaction is considered to be necessary for E6AP-mediated annexin A1 ubiquitylation. To determine if the molecular interaction is required for E6AP-mediated annexin A1 ubiquitylation, we performed in vitro ubiquitylation assay in the presence or absence of 1 mM $CaCl_2$. When in vitro ubiquitylation reaction was carried out in the presence of Ca^{2+}, the higher-molecular-weight species of GST-annexin A1 were detected with anti-GST PAb (Fig. 6C, lane 2), indicating that annexin A1 is polyubiquitylated by E6AP in vitro. However, no ubiquitylation signal was detected when the ubiquitylation reaction was carried out in the absence of Ca^{2+} (Fig. 6C, lane 1), indicating that the E6AP-annexin A1 interaction is required for E6AP-mediated annexin A1 ubiquitylation.

To further investigate whether the ubiquitylation of annexin A1 is dependent on the presence of Ca^{2+}, we examined the effects of EGTA on the E6AP-mediated ubiquitylation of annexin A1. Polyubiquitin chains were synthesized even in the presence of a high concentration of EGTA (Fig. 6D), indicating that E6AP was active even in the presence of EGTA. However, increasing amounts of EGTA resulted in decreases in the ubiquitylation of annexin A1 (Fig. 6E), suggesting that chelating the Ca^{2+} in the reaction mixture with EGTA inhibits the ubiquitylation of annexin A1. These findings suggest that the ubiquitylation of annexin A1 by E6AP is dependent on the presence of Ca^{2+}.

E6AP-BINDING DOMAIN FOR ANNEXIN A1 PROTEIN

To map the E6AP-binding domain on annexin A1 protein, GST pull-down assays were performed using a panel of annexin A1 deletion mutants expressed as GST-fusion proteins. Figure 7A shows a schematic representation of annexin A1 and known motifs in annexin A1. A series of deletion mutants of annexin A1 as GST fusion proteins (Fig. 7A) were expressed in *E. coli*. Purified MEF-E6AP was used to determine E6AP-binding domain. GST pull-down assays revealed that the core domain of annexin A1 (42-346), but not the N-terminal tail of annexin A1 (1–41), bound to E6AP (Fig. 7B, lanes 4 and 3). GST pull-down assays also showed that annexin A1 (114–274) and annexin A1 (196–346), but not annexin A1 (42–195), were able to bind to E6AP (Fig. 7B, lanes 5–7). As shown in Fig. 7C, GST-annexin A1 (196–274) bound to E6AP. These findings suggest that annexin repeat domain III is important for E6AP binding.

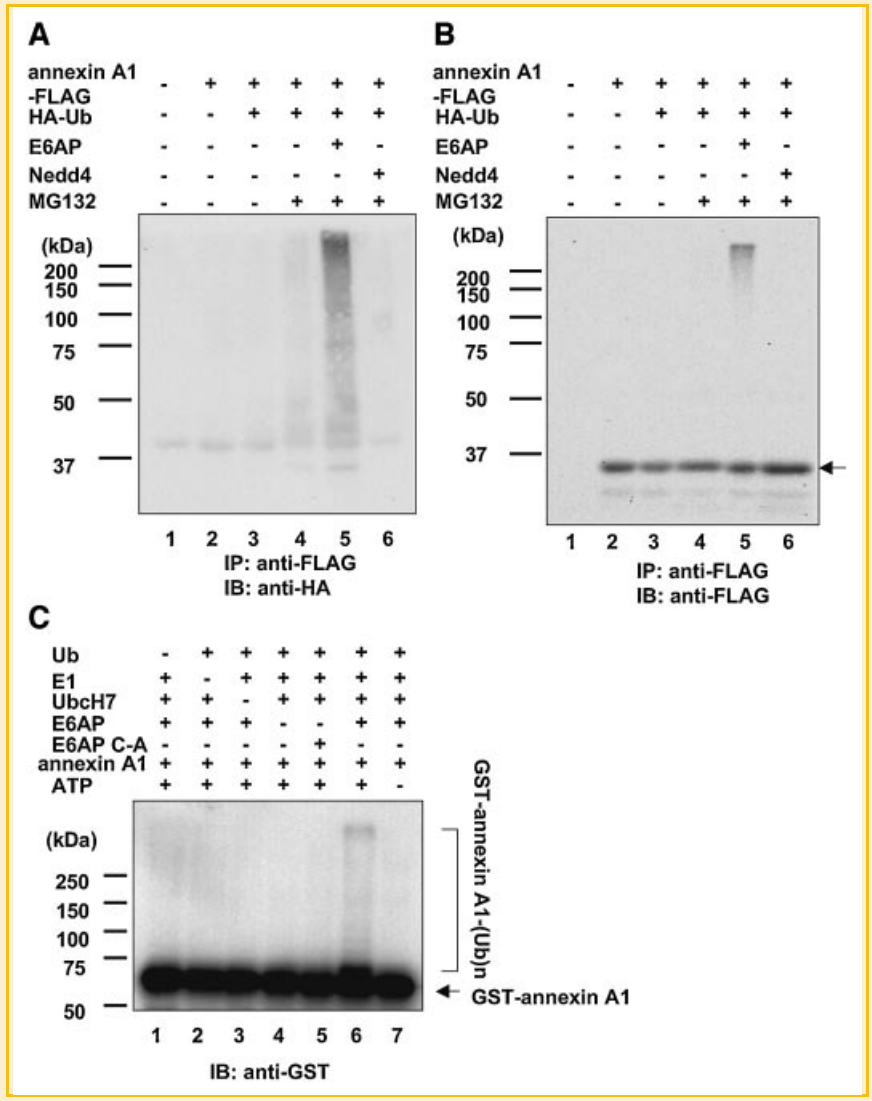

Fig. 5. E6AP-dependent ubiquitylation of annexin A1 protein in vivo and in vitro. HEK 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG annexin-FLAG together with 2 μg of plasmid encoding E6AP as indicated. Each transfection also included 2 μg of plasmid encoding HA-ubiquitin. The cell lysates were immunoprecipitated with FLAG beads and analyzed by immunoblotting with anti-HA PAb (A) or anti-FLAG PAb (B). The Western blot shows the presence of a ubiquitin smear. The arrow indicates annexin-FLAG. IB, immunoblot; IP, immunoprecipitation. C: In vitro ubiquitylation of annexin A1 by E6AP. For in vitro ubiquitylation of annexin A1 protein, purified GST-annexin A1 was used as a substrate. Assays were done in 40-μl volumes containing each component as indicated. The reaction mixture is described in the Experimental Procedures. The reaction mixture contained 1 mM $CaCl_2$. The reaction was terminated by addition of SDS–PAGE loading buffer and followed by immunoblotting with anti-GST PAb. The arrow indicates GST-annexin A1. Ubiquitylated species of GST-annexin A1 proteins are marked by brackets.

DISCUSSION

In the present study, we have identified annexin A1 as a novel substrate for E6AP using four lines of evidence: (1) E6AP bound to annexin A1 in vivo and in vitro; (2) overexpression of E6AP enhanced proteasomal degradation of annexin A1 in vivo; (3) knockdown of endogenous E6AP by siRNA resulted in the accumulation of endogenous annexin A1 in vivo; and (4) E6AP enhanced the polyubiquitylation of annexin A1 in vivo and in vitro. These results provide evidence that E6AP mediates the ubiquitylation and proteasomal degradation of annexin A1. We have shown that E6AP bound to annexin A1 only in the presence of Ca^{2+} and that these interactions were enhanced by increasing concentrations of Ca^{2+}. Annexin A1 was polyubiquitylated by E6AP only in the presence of Ca^{2+}. Chelating Ca^{2+} with EGTA inhibited E6AP-mediated polyubiquitylation of annexin A1. The E6AP-binding domain on annexin A1 was mapped to the core domain, especially the annexin repeat domain III. These results suggest that the conformational change of annexin A1 induced by Ca^{2+} binding allows E6AP to bind to annexin repeat domain III of annexin A1 and to mediate its ubiquitylation and degradation.

Post-translational modifications, such as Ca^{2+} binding, phosphorylation, and lipidation, have roles in the regulation of annexin A1. Solito et al. [2006] showed that the translocation of annexin A1 from the cytoplasm to the cell surface is regulated by phosphorylation and lipidation. Annexin A1 is phosphorylated by several

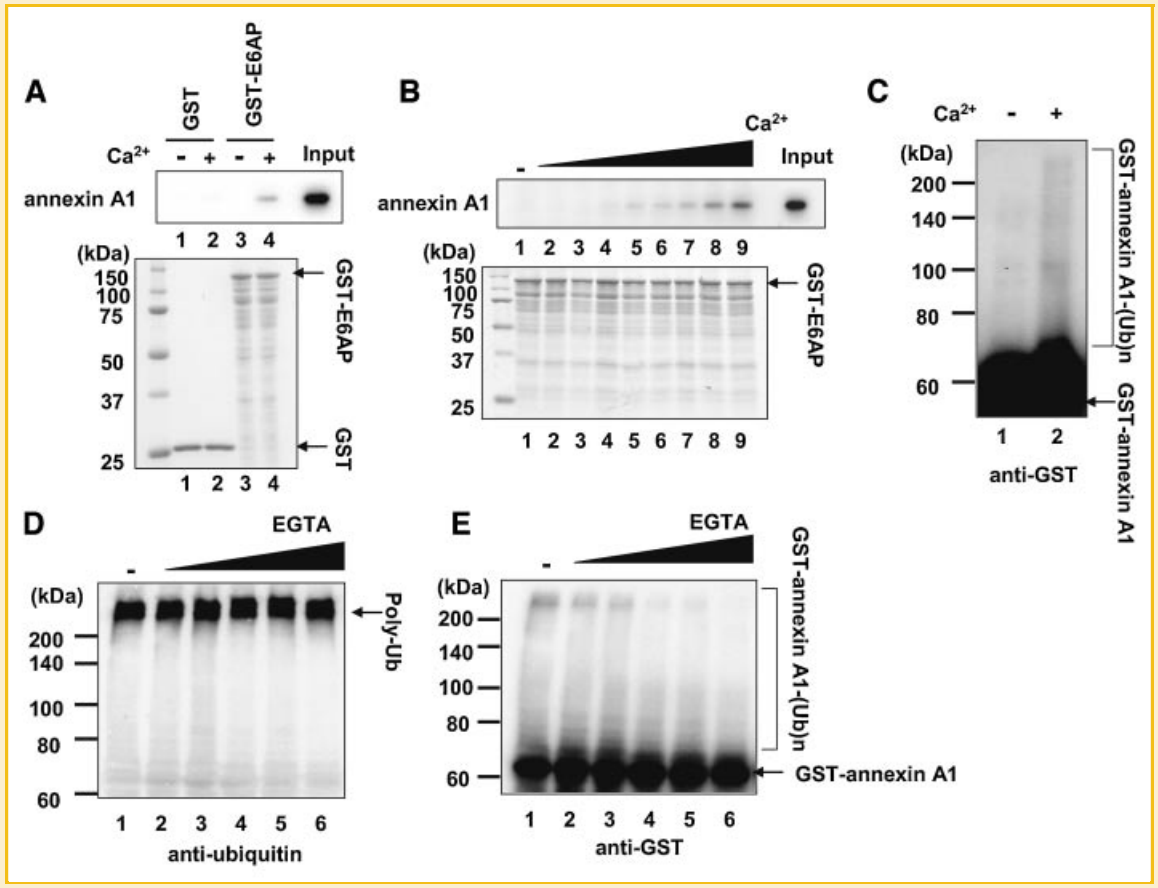

Fig. 6. E6AP mediates ubiquitylation of annexin A1 in a Ca^{2+}-dependent manner. A: In vitro binding of annexin A1 and E6AP. Immobilized GST-E6AP or GST alone was incubated with purified His-annexin A1 in the presence or absence of 1 mM $CaCl_2$ in the binding solution. Immunoblotting to detect bound annexin A1 was performed using anti-annexin A1 antibody. B: Ca^{2+}-dependent interaction between annexin A1 and E6AP. The GST pull-down assays described in (A) were repeated in the presence of increasing concentrations of $CaCl_2$ in the binding solution as follows: lane 1 (0 μM), 2 (10 μM), 3 (100 μM), 4 (250 μM), 5 (500 μM), 6 (750 μM), 7 (1 mM), 8 (2.5 mM), and 9 (5 mM). C: For in vitro ubiquitylation of annexin A1 protein, purified GST-annexin A1 was used as a substrate. Assays were done in 40-μl volumes in the presence or absence of 1 mM $CaCl_2$. The reaction mixture is described in Materials and Methods Section. The reaction was terminated by addition of SDS–PAGE loading buffer and followed by immunoblotting with anti-GST PAb. Arrow indicates GST-annexin A1. Ubiquitylated species of GST-annexin A1 proteins are marked by brackets. D,E: The in vitro ubiquitylation assays were performed in the presence of various concentrations of EGTA in the reaction mixture containing 1 mM $CaCl_2$. The concentrations of EGTA were as follows: lane 1 (0 mM), 2 (0.1 mM), 3 (0.5 mM), 4 (1 mM), 5 (5 mM), and 6 (10 mM). D: Immunoblotting to detect whole polyubiquitylated proteins with anti-ubiquitin MAb. E: Immunoblotting to detect polyubiquitylated GST-annexin A1 with anti-GST PAb.

protein kinases, such as epidermal growth factor receptor protein kinase, protein kinase C, and hepatocyte growth factor receptor kinase to mediate proliferation [Lim and Pervaiz, 2007], suggesting that phosphorylation plays some roles in the regulation of annexin A1 function. The findings presented in this study suggest that the ubiquitin-proteasome pathway plays a role in the regulation of annexin A1 function. Our data also suggest that E6AP preferentially recognizes the Ca^{2+}-binding form of annexin A1 and targets it for proteasomal degradation. The main biological property of annexin A1 is the binding to the phospholipid membrane in a Ca^{2+}-dependent manner [Lim and Pervaiz, 2007]. X-ray crystallography studies of annexin A1 have suggested that a calcium-driven conformational switch of the N-terminal and core domains of annexin A1 involves the membrane aggregation properties of annexin A1 [Rosengarth et al., 2001; Rosengarth and Luecke, 2003]. It will be intriguing to examine the role of E6AP in membrane aggregation. Further investigations will be required to elucidate the role of E6AP in the regulation of annexin A1 functions.

Targeting of a substrate via the ubiquitin system involves specific binding of the protein to the appropriate E3 ubiquitin ligase. There are several modes for specific substrate recognition, such as (1) NH2-terminal residue (N-end rule pathway), (2) allosteric activation, (3) recognition of phosphorylated substrate, (4) phosphorylation of E3, (5) phosphorylation of both the ligase and its substrate, (6) recognition in trans via an ancillary protein, (7) abnormal/mutated/misfolded proteins, and (8) recognition via hydroxylated proline [Glickman and Ciechanover, 2002]. E6AP specifically recognizes active forms of Blk, indicating that tyrosine phosphorylation of the regulatory tyrosine of Blk plays a role in specific substrate recognition [Oda et al., 1999]. Here we propose a novel mechanism of specific substrate recognition in the ubiquitin system, in which E6AP recognizes annexin A1 via a Ca^{2+}-induced conformational change. E6AP plays a direct catalytic role in the

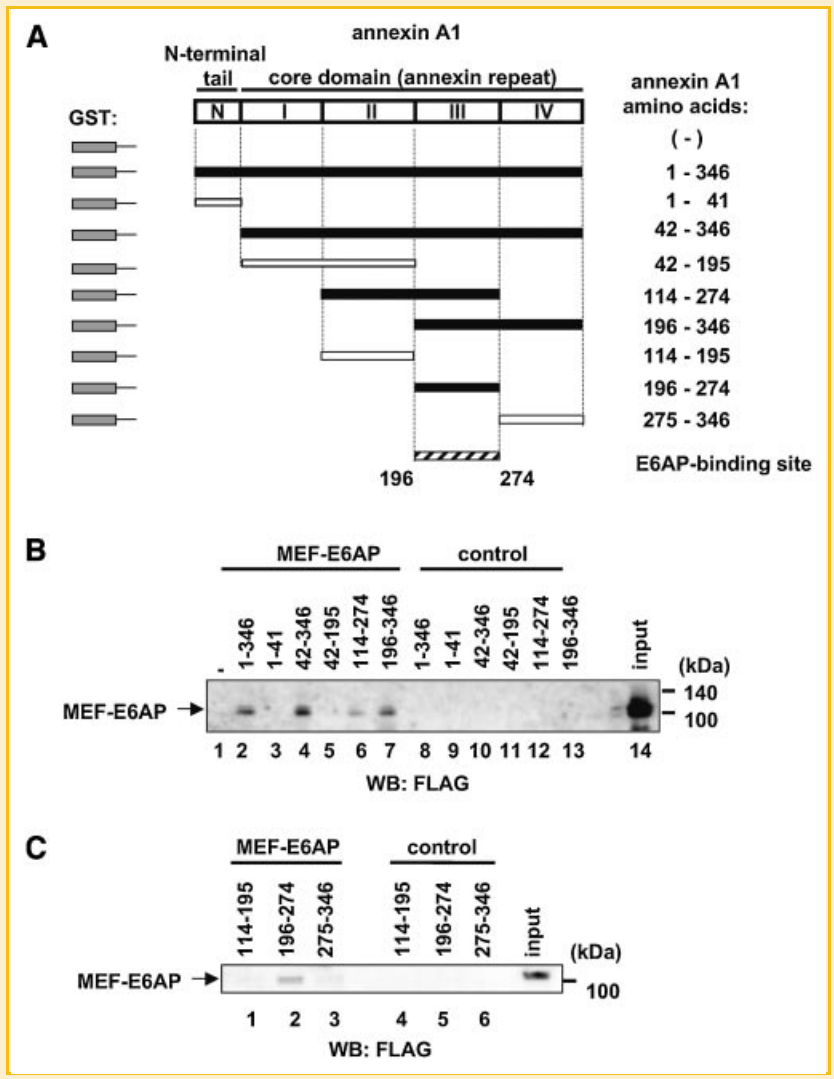

Fig. 7. Mapping of the E6AP-binding domain for annexin A1 protein. A: Structure of annexin A1. Shown is a schematic representation of the region of annexin A1 protein. N-terminal tail (aa 1–41), core domain (aa 42–346), and annexin repeat domains I (aa 42–113), II (aa 114–195), III (aa 196–274), and IV (aa 275–346) are shown. Schematic representation of GST-annexin A1 proteins. GST proteins contain the annexin A1 amino acids indicated to the right. The shaded box in each represents the GST sequence. Closed boxes represent proteins that are bound specifically to MEF-E6AP, and open boxes represent those that are not bound. B,C: In vitro binding of MEF-E6AP to GST-annexin A1 proteins. Purified recombinant MEF-E6AP was assayed for association with GST (–) or the GST-annexin A1 proteins using the binding buffer with 1 mM $CaCl_2$. GST pull-down was performed to assay for the association of E6AP with annexin A1. Control experiments were performed without MEF-E6AP. The association of MEF-E6AP was detected by immunoblotting with anti-FLAG MAb.

final attachment of ubiquitin to substrate proteins. Our findings suggest that Ca^{2+}-induced conformational change of annexin A1 may function as a degradation signal for annexin A1.

Ubiquitylated annexin A2 is enriched in the cytoskeleton fraction of mouse Krebs II cells [Lauvrak et al., 2005]. It remains unclear whether the ubiquitylated annexin A2 is degraded by proteasome. The apical membrane localization of Nedd4, a member of HECT-type ubiquitin ligases, is mediated by an association of its C2 domain with the apically targeted annexin XIIIb [Plant et al., 2000]. However, it is unknown whether annexin XIIIb is a substrate of Nedd4. To our knowledge, this is the first study to identify a specific E3 ubiquitin ligase for the ubiquitylation of an annexin family protein. All annexins share a core domain composed of four similar repeats, each approximately 70 amino acids long. Each repeat is composed of five α helices and usually contains a characteristic type-2 motif for binding calcium ions with the sequence GxGT-[38 residues]-D/E [Moss and Morgan, 2004]. The core domains of most vertebrate annexins reveal conservation of their secondary and tertiary structures despite the presence of only 45–55% amino-acid identity among individual annexins [Moss and Morgan, 2004]. It will be required to investigate whether other annexins are regulated by E6AP or other E3 ubiquitin ligases.

E6AP is hijacked by the HPV16E6 to target the tumor suppressor p53 in cervical cancer. Moreover, E6AP is mutated in Angelman syndrome and mediates ubiquitin-dependent degradation of HCV core protein, suggesting that E6AP plays important roles in sporadic and hereditary human diseases including cancer, neurological disorders, and infectious diseases [Kishino et al., 1997; Scheffner

and Staub, 2007; Shirakura et al., 2007]. Physical and functional association of E6AP with viral proteins, such as HPV16E6 [Huibregtse et al., 1993b] and HCV core protein [Shirakura et al., 2007], have been demonstrated. It is possible that the viral proteins redirect E6AP away from annexin A1, increasing the stability of annexin A1, and thereby contributing to viral pathogenesis. It would be interesting to investigate whether these viral proteins affect E6AP-dependent degradation of annexin A1. The association of E6AP with the viral protein (HPV16E6 or HCV core protein) could provide a feasible target for molecular approaches in the treatment of cervical cancer or HCV-related diseases.

In conclusion, we have demonstrated that E6AP interacts with annexin A1 protein and mediates its ubiquitin-dependent degradation. We propose that E6AP may play a role in regulating the diverse functions of annexin A1 protein. Identification of the specific E3 ubiquitin ligase may provide a link between the annexin family proteins and the ubiquitin-proteasome pathway. Elucidating the regulation of annexin A1 may provide a novel clue in the treatment of the E6AP-related diseases.

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