

# The Ras Target AF-6 is a Substrate of the Fam Deubiquitinating Enzyme

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**Abstract.** The Ras target AF-6 has been shown to serve as one of the peripheral components of cell–cell adhesions, and is thought to participate in cell–cell adhesion regulation downstream of Ras. We here purified an AF-6-interacting protein with a molecular mass of ~220 kD (p220) to investigate the function of AF-6 at cell–cell adhesions. The peptide sequences of p220 were identical to the amino acid sequences of mouse Fam. Fam is homologous to a deubiquitinating enzyme in *Drosophila*, the product of the *fat facets* gene. Recent genetic analyses indicate that the deubiquitinating activity of the *fat facets* product plays a critical role in controlling the cell fate. We found that Fam accumulated

at the cell–cell contact sites of MDCKII cells, but not at free ends of plasma membranes. Fam was partially colocalized with AF-6 and interacted with AF-6 in vivo and in vitro. We also showed that AF-6 was ubiquitinated in intact cells, and that Fam prevented the ubiquitination of AF-6.

These results indicate that AF-6 forms a complex with and serves as one of the substrates for Fam, and suggest that the degradation of peripheral components of cell–cell adhesions may be regulated by Fam.

**Key words:** AF-6 • Fam • deubiquitinating enzyme • ubiquitination • cell–cell adhesions

**R**AS (Ha-Ras, Ki-Ras, N-Ras) is a signal-transducing guanine nucleotide-binding protein for tyrosine kinase-type receptors such as epidermal growth factor receptors and the Src family (for reviews see Satoh et al., 1992; McCormick, 1994). Ras has GDP-bound inactive and GTP-bound active forms, the latter of which make physical contact with targets (Marshall, 1995a). Intensive investigations revealed that the Raf kinase family, consisting of c-Raf-1 (for reviews see Blenis, 1993; Daum et al., 1994), A-Raf (Vojtek et al., 1993), and B-Raf (Moodie et al., 1994; Jaiswal et al., 1994; Catling et al., 1994; Yamamori et al., 1995), is one of the direct targets for Ras. The activated Raf phosphorylates mitogen-activated protein (MAP)<sup>1</sup> kinase kinase and activates it. Consequently, the activated MAP kinase kinase activates MAP kinase, leading to the expression of certain genes

such as *c-fos* (for reviews see Cano and Mahadevan, 1995; Marshall, 1995b). Several molecules interacting with activated Ras in addition to Raf have been identified in mammals. These include phosphatidylinositol-3-OH kinase (Rodriguez-Viciano et al., 1994), Ral guanine nucleotide dissociation stimulator (Kikuchi et al., 1994; Spaargaren and Bischoff, 1994), and Rin1 (Han and Colicelli, 1995). We previously identified the acute lymphoblastic leukemia-1 (ALL-1) fusion partner from chromosome 6 (AF-6) as a Ras target (Kuriyama et al., 1996). AF-6 was identified as the fusion partner of ALL-1 protein (Prasad et al., 1993). The ALL-1/AF-6 chimeric protein is a critical product of the t(6;11) abnormality associated with some human leukemia. AF-6 has the postsynaptic density protein PSD-95/discs-large tumor suppressor protein Dlg/ZO-1 (PDZ) domain, which is thought to localize AF-6 at the specialized sites of plasma membranes such as cell–cell contact sites.

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1. *Abbreviations used in this paper.* ALL-1, acute lymphoblastic leukemia-1; ALLM, *N*-acetyl-Leu-Leu-methionine; ALLN, *N*-acetyl-Leu-Leu-norleucine; HA-Ub, hemagglutinin-tagged ubiquitin; GST, glu-

tathione-S-transferase; MAP, mitogen-activated protein; MBP, maltose-binding protein; *p*-APMSF, (*p*-amidino-phenyl)methanesulfonyl fluoride; SH3, src homology region 3; Ub, ubiquitin; Ubps, ubiquitin-specific proteases; Ub-PEST, ubiquitin- $\alpha$ NH-MHISPPPEPESEEEEEHYC; ZO-1, zonula occludens-1.

We have recently found that AF-6 accumulates at tight junctions in epithelial cells such as MDCKII cells and at cell–cell adhesions in nonepithelial cells such as Rat1 fibroblasts and PC12 rat pheochromocytoma cells (Yamamoto et al., 1997). AF-6 interacts with ZO-1 in vitro. ZO-1 interacts with the Ras-binding domain of AF-6, and this interaction is inhibited by activated Ras. Overexpression of activated Ras in Rat1 cells results in perturbation of cell–cell contacts, followed by a decrease of the accumulation of AF-6 and ZO-1 at the cell surface (Yamamoto et al., 1997). These observations indicate that AF-6 serves as one of the peripheral components of tight junctions in epithelial cells and of cell–cell adhesions in nonepithelial cells, and that AF-6 may participate in the regulation of cell–cell contacts including tight junctions via direct interaction with ZO-1 downstream of Ras. To understand the function of AF-6 at cell–cell contact sites, we here attempted to identify AF-6–interacting molecules. We purified an AF-6–interacting protein with a molecular mass of ~220 kD, and identified it as the bovine counterpart of mouse Fam protein (Wood et al., 1997).

Fam is homologous to a deubiquitinating enzyme in *Drosophila*, the product of the *fat facets* (*faf*) gene (Fischer-Vize et al., 1992; Huang et al., 1995; Huang and Fischer-Vize, 1996). The *faf* gene is specifically required for normal eye development in *Drosophila*. The ubiquitin-proteasome pathway plays an important role in the complete degradation of abnormal and short-lived regulatory proteins that include transcriptional activators such as c-fos, NFκB-IκB complex, and p53, or growth factor receptors such as platelet-derived growth factor receptor (PDGFR) and fibroblast growth factor 1 receptor (FGFR-1; Hochstrasser, 1995; Hicke, 1997). This pathway requires ATP and a covalent conjugation of the ubiquitin (Ub) molecules. The conjugation involves a series of enzymatic reactions: Ub itself is first activated with ATP by the Ub-activating enzyme (E1), secondly transferred onto a Ub carrier protein (E2), and in a third step transferred onto a Ub ligase (E3). Multiubiquitinated substrates are then recognized by the 26S proteasome and rapidly degraded into short peptides.

A large superfamily of genes encoding deubiquitinating enzymes, ubiquitin-specific proteases (Ubps), was recently identified (Hochstrasser, 1995). The deubiquitinating enzymes can generally be divided into two main types. The first one is thought to remove ubiquitin from Ub-conjugated degradation products produced by the proteasomes, and thereby to accelerate the proteasome-dependent proteolysis. This type is present in all cells, and is thought to have little substrate specificity. The second one is thought to remove Ub from multiubiquitinated substrates, and thereby to prevent their proteasome-dependent proteolysis before they reach the proteasome. This type shows a tissue-specific expression pattern, and is thought to have substrate specificity (Hochstrasser, 1995). Recent genetic analyses indicate that Faf belongs to the second type, and that the deubiquitinating activity of Faf is essential for regulating a cell communication pathway essential for normal eye development (Huang et al., 1995; Huang and Fischer-Vize, 1996). In situ hybridization analyses revealed that mouse Fam transcripts exist in the rapidly expanding cell population of gastrulating and neurulating embryos, and

in postmitotic cells of the central nervous system as well as in the apoptotic regions between digits (Wood et al., 1997), although the role and the physiological substrate of mammalian Fam are unknown.

In this study, we found that Fam accumulated at cell–cell contacts, and that AF-6 interacted with Fam both in vivo and in vitro. We also found that AF-6 was ubiquitinated in intact cells and that Fam prevented the ubiquitination of AF-6.

## Materials and Methods

### Materials and Chemicals

The Fam cDNA clone was generated as described previously (Wood et al., 1997). MDCKII cells, EL cells, and the mouse antibody against ZO-1 were kindly provided by Drs. A. Nagafuchi and S. Tsukita (University of Kyoto, Kyoto, Japan) (Itoh et al., 1991; Nagafuchi et al., 1994). pMT123 (HA-ubiquitin expression plasmid) was kindly provided by Dr. D. Bohmann (European Molecular Biology Laboratory, Heidelberg, Germany) (Treier et al., 1994). Rabbit polyclonal antibodies against AF-6 (914–1129 aa; #3), AF-6 (1130–1612 aa; #4), Fam (1–20 aa; N20), Fam (2441–2554 aa; C114), and Fam (1165–1967 aa; K2) were generated as described previously (Harlow and Lane, 1988). FITC-conjugated anti-rabbit IgG antibody, Texas red-conjugated anti-mouse IgG antibody, and [<sup>35</sup>S]methionine were purchased from Amersham Corp. (Buckinghamshire, United Kingdom). Mouse monoclonal antibody against β-catenin was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antibodies against α-catenin and β-catenin were purchased from Sigma Chemical Co. (St. Louis, MO). Polyvinylidene difluoride membranes (Problott, 0.45-μm pore size) were purchased from PE Applied Biosystems (Foster, CA). *Achromobacter* protease I was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Proteasome inhibitor ALLN (*N*-acetyl-Leu-Leu-norleucinal) and calpain inhibitor II ALLM (*N*-acetyl-Leu-Leu-methional) were purchased from Peptide Institute, Inc. (Osaka, Japan). All materials used in the nucleic acid study were purchased from Takara Shuzo Corp. (Kyoto, Japan). Other materials and chemicals were obtained from commercial sources.

### Plasmid Construction

The *Escherichia coli* expression plasmid pGEX-3X-AF-6 (1130–1612 aa) was constructed by subcloning the PvuII and EcoRI fragments of AF-6 into the SmaI and EcoRI sites of pGEX-3X. The plasmids for in vitro translation of Fam were constructed by amplifying the cDNA fragments encoding Fam (1–669 aa, 670–1213 aa, 1210–2100 aa, and 2097–2554 aa) by PCR from the full-length Fam cDNA in pBluescript SK(–). The fragment of Fam (1–669 aa) was amplified using the sense primer containing a BamHI site (5′-AATTGGATCCATGACAGCCAGACTCGTG-3′) and the antisense primer containing a BamHI site (5′-ATTAGGATCCTCACAGCTGGCCATCCTTCA-3′). The fragment of Fam (670–1213 aa) was amplified using the sense primer containing a BamHI site (5′-AATTGGATCCCTGTGGCTGTGTGCTCCAC-3′) and the antisense primer containing a BamHI site (5′-ATTAGGATCCTCAGCATGCATTCAGATGATGGG-3′). The fragment of Fam (1210–2100 aa) was amplified using the sense primer containing a BamHI site (5′-AATTGGATCCTGCATGCTTAGAAACGTGTC-3′) and the antisense primer containing a BamHI site (5′-ATTAGGATCCTTACTCAGAGAATCGATTGAAAC-3′). The fragment of Fam (2097–2554 aa) was amplified using the sense primer containing a BamHI site (5′-AATTGGATCCCGATTCTCTGAGTACCTTTTG-3′) and the antisense primer containing a BamHI site (5′-ATTAGGATCCTTAATATGTGCATTTCACTGATC-3′). The fragments (1–669 aa, 670–1213 aa, and 2097–2554 aa) were cloned into the BamHI site of pRSET-A, and the fragment (1210–2100 aa) was cloned into the BamHI site of pBluescript SK(–). The *Escherichia coli* expression plasmid pMal-c2-Fam (1476–1918 aa) was constructed by inserting the blunt-ended PstI and BstXI fragment of Fam into pMal-c2. The mammalian expression plasmid pEF-BOS-AF-6-myc was constructed as follows: the cDNA fragments encoding AF-6 were subcloned into pBluescript SK(–) having a sequence encoding a myc epitope tag (MEQKLISEEDL), and AF-6-myc cDNA fragment was inserted into the XbaI site of pEF-BOS. For the preparation of pEF-BOS-HA-Fam-CAT (1210–2410 aa), the frag-

ment (1210–2410 aa) was subcloned into pBluescript SK(-), and the fragment was inserted into the BamHI site of pEF-BOS-HA.

## Cell Culture

MDCKII and Rat1 cells were grown in DME containing 10% calf serum, penicillin, and streptomycin in an air-5% CO<sub>2</sub> atmosphere at constant humidity. EL cells were grown in DME containing 10% FBS and 100 µg/ml of G418 in an air-5% CO<sub>2</sub> atmosphere at constant humidity. COS7 cells were grown in DME containing 10% FBS, penicillin, and streptomycin in an air-5% CO<sub>2</sub> atmosphere at constant humidity.

## Preparation of Bovine Brain Cytosolic Fraction

100 g of bovine brain gray matter was cut into small pieces with scissors and suspended in 300 ml of homogenizing buffer A (25 mM Tris/HCl at pH 7.5, 1 mM EDTA, 1 mM DTT, 10 mM MgCl<sub>2</sub>, 10 µM [*p*-amidino-phenyl]methanesulfonyl fluoride [*p*-APMSF], 1 µg/ml leupeptin, 10% sucrose). The suspension was homogenized with a Potter-Elvehjem Tefron-glass homogenizer and filtered through four layers of gauze. The homogenate was centrifuged at 20,000 *g* for 30 min at 4°C and then at 100,000 *g* for 60 min at 4°C as described (Yamamoto et al., 1995). The supernatant was stored at -80°C as the cytosolic fraction.

## GST-AF-6 (1130–1612 aa) Affinity Column Chromatography

Glutathione-S-transferase (GST)-AF-6 (1130–1612 aa) was expressed in *Escherichia coli* BL21 (DE3) and purified according to the manufacturer's instructions. Glutathione-Sepharose 4B (1 ml) was coated with GST-AF-6 (1130–1612 aa; 30 nmol). The brain cytosolic fraction was then preabsorbed to remove the native GST with glutathione-Sepharose 4B and loaded onto the GST-AF-6 (1130–1612 aa) affinity column. The column was washed with 10 ml of buffer B (20 mM Tris/HCl at pH 7.5, 1 mM EDTA, 1 mM DTT, 5 mM MgCl<sub>2</sub>, 10 µM *p*-APMSF, 1 µg/ml leupeptin) followed by washing with 10 ml of buffer B containing 75 mM NaCl. The protein bound to the affinity column was eluted 10 times with 1 ml of buffer B containing 10 mM reduced glutathione.

## Peptide Sequence Analysis of p220

The 10-mM reduced glutathione eluates from the first to seventh fractions were dialyzed three times against distilled water and concentrated by freeze-drying. The concentrated samples were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Iwamatsu, 1992). The immobilized p220 was reduced and *S*-carboxymethylated, followed by in situ digestion with *Achromobacter* protease I and Asp-N. The digested peptides were fractionated by C18 column chromatography and subjected to amino acid sequencing (Iwamatsu, 1992).

## Hydrolysis Assay of Ub-PEST

For purification of Fam, the brain cytosolic fraction was loaded onto the GST-AF-6 (1130–1612 aa) affinity column. Fam was eluted by adding buffer B containing 200 mM NaCl from the GST-AF-6 affinity column. Ubiquitin- $\alpha$ NH-MHISPPPESEEEEEHYC (Ub-PEST) was radiolabeled with Na<sup>125</sup>I using IODO-BEADS (Markwell, 1982). Fam (1.6 µg) was incubated for various periods at 37°C in a final volume (50 µl) containing 1 µg of <sup>125</sup>I-labeled Ub-PEST, 100 mM Tris/HCl at pH 7.5, 1 mM EDTA, 1 mM DTT, and 5% (vol/vol) glycerol. The reaction was terminated by adding 50 µl of acetone, and the mixture was frozen overnight at -80°C. The pellet fractions were boiled in sample buffer for SDS-PAGE, and were subjected to SDS-PAGE detected by Coomassie Brilliant Blue staining and by autoradiography (Woo et al., 1995).

## Immunofluorescence and Laser Scanning Confocal Microscopy

MDCKII cells plated on 13-mm round glass coverslips were fixed with methanol for 10 min. The fixed cells were incubated for 2 h with mouse monoclonal antibodies against ZO-1 or  $\beta$ -catenin, and were washed three times for 10 min with PBS. The cells were then incubated for 2 h with anti-Fam antibody (K2) and washed three times for 10 min with PBS. The cells were incubated for 1 h with FITC-conjugated anti-rabbit IgG antibody and Texas red-conjugated anti-mouse IgG antibody, and washed three

times for 10 min with PBS. The distributions of Fam, ZO-1, and  $\beta$ -catenin were examined using a laser scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with an argon laser and a helium-neon laser for double fluorescence at 488 and 543 nm (emission filter; BP510–525 and LP590).

## Coimmunoprecipitation Assay

MDCKII cells were grown in 100-mm tissue culture dishes. After being washed with PBS, the cells were lysed with 1 ml of buffer C (50 mM Tris/HCl at pH 8.0, 1 mM EDTA, 75 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.2% Triton X-100, 10 µM *p*-APMSF, 10 µg/ml leupeptin). The lysate was removed from the dishes with a rubber policeman. The lysate was sonicated, incubated in a 1.5-ml tube for 15 min on ice, and then clarified by centrifugation at 12,000 *g* for 30 min at 4°C. The soluble supernatant was incubated with 10 µg of anti-Fam antibody (K2), anti-AF-6 antibody (#3), or preimmunoserum. The immunocomplexes were then precipitated with protein A-Sepharose 4B (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). The immunocomplexes were washed six times with buffer C, eluted by boiling in sample buffer for SDS-PAGE and subjected to immunoblot analysis using anti-Fam antibody (N20) or anti-AF-6 antibody (#4).

## In Vitro Binding Assay (In Vitro-translated Fam)

In vitro translation of pRSET-Fam (1–669 aa), pRSET-Fam (670–1213 aa), pBluescript SK(-)-Fam (1210–2100 aa), and pRSET-Fam (2097–2554 aa) were performed using a TNT T7-coupled reticulocyte lysate system (Promega Corp., Madison, WI) under the conditions described in the manufacturer's instruction manual. Glutathione-Sepharose 4B beads (31 µl) were coated with GST fusion proteins and washed with 310 µl of buffer B. The coated beads were added to 40 µl of the in vitro-translated products labeled with [<sup>35</sup>S]methionine containing 1 mg/ml BSA, and were incubated for 1 h at 4°C with gentle mixing. The beads were washed six times with 102 µl of buffer B, and the bound proteins were coeluted with GST fusion proteins three times by adding 102 µl of buffer B containing 10 mM reduced glutathione. The eluates were subjected to SDS-PAGE and vacuum-dried. The [<sup>35</sup>S]-labeled bands corresponding to in vitro-translated Fam were visualized with an image analyzer (BAS-2000; Fuji Photo Film Co., Tokyo, Japan).

## In Vitro Binding Assay (Maltose-binding Protein [MBP]-Fam)

The deubiquitinating catalytic domain of Fam (1476–1918 aa) was expressed as a MBP fusion protein, and was purified with amylose resin (New England Biolabs, Inc., Beverly, MA). MBP-Fam (1476–1918 aa; 0.05, 0.125, 0.25, 1.25, 2.5, and 7.5 nmol) was mixed with glutathione-Sepharose 4B beads (30 µl) coated with 0.3 nmol of either GST or GST-AF-6 (1130–1612 aa) in 250 µl of buffer B. The beads were washed six times with 100 µl of buffer B, and were then washed three times with 100 µl of buffer B containing 50 mM NaCl. The bound MBP-Fam (1476–1918 aa) was coeluted with GST or GST-AF-6 (1130–1612 aa) by adding 100 µl of buffer B containing 10 mM glutathione three times. Portions (30 µl each) of the three fractions of the glutathione-eluates were subjected to SDS-PAGE, followed by Coomassie Brilliant Blue staining. MBP-Fam (1476–1918 aa) was visualized and estimated with a densitograph (ATTO, Tokyo, Japan).

## Effect of Proteasome Inhibitor on Rat1 and EL Cells

Rat1 and EL cells were seeded in 100-mm tissue culture dishes at a cell density of 1 × 10<sup>6</sup> cells/dish, and were cultured for 24 h. The cells were then treated with 25 µM ALLN or ALLM for 3 h. ALLN and ALLM were dissolved in DMSO to a final concentration of 10 mM. The cells were washed twice with PBS and lysed in 360 µl of buffer D (50 mM Tris/HCl at pH 7.5, 100 mM KCl, 4 mM EGTA, 1 mM NaF, 1 mM sodium vanadate, 0.25% Triton X-100, 10 µM *p*-APMSF, 1 µg/ml leupeptin, 25 µM ALLN, and 300 mM sucrose). The cell lysates of Rat1 and EL cells were subjected to SDS-PAGE and immunoblotted with anti-AF-6 (#4) antibody, polyclonal antibody against  $\beta$ -catenin, or anti- $\alpha$ -catenin antibody.

## Ubiquitination Assay of AF-6 In Vivo

COS7 cells were seeded on 60-mm tissue culture dishes at a cell density of 6 × 10<sup>5</sup> cells/dish, and were cultured for 16 h. COS7 cells were transfected

with pEF-BOS-AF-6-myc (12  $\mu$ g) and pMT123 (HA-ubiquitin; 3  $\mu$ g) by the standard DEAE-dextran method (Lopata et al., 1984). To examine the effect of the proteasome inhibitor ALLN, COS7 cells were grown for 30 h after transfection, and were then treated with 25  $\mu$ M ALLN for 18 h. The cells were washed twice with PBS and lysed in 360  $\mu$ l of buffer D. An immunoprecipitation analysis with anti-AF-6 antibody (#3) was performed as described above. The samples were subjected to SDS-PAGE followed by immunoblot analysis using anti-HA antibody.

### Deubiquitination Assay of the Ubiquitinated AF-6 by Fam

The plasmids pEF-BOS-AF-6-myc (12  $\mu$ g) and pMT123 (3  $\mu$ g) were transfected with or without pEF-BOS-HA-Fam-CAT (5  $\mu$ g) in COS7 cells. Immunoprecipitation and an immunoblot analysis were carried out as described above.

### Other Procedures

SDS-PAGE was performed as described (Laemmli, 1970). Protein concentrations were determined with BSA as the reference protein as described (Bradford, 1976). Immunoblot analyses were carried out as described (Harlow and Lane, 1988).

## Results

### Identification of AF-6 (1130–1612 aa)-interacting Molecules

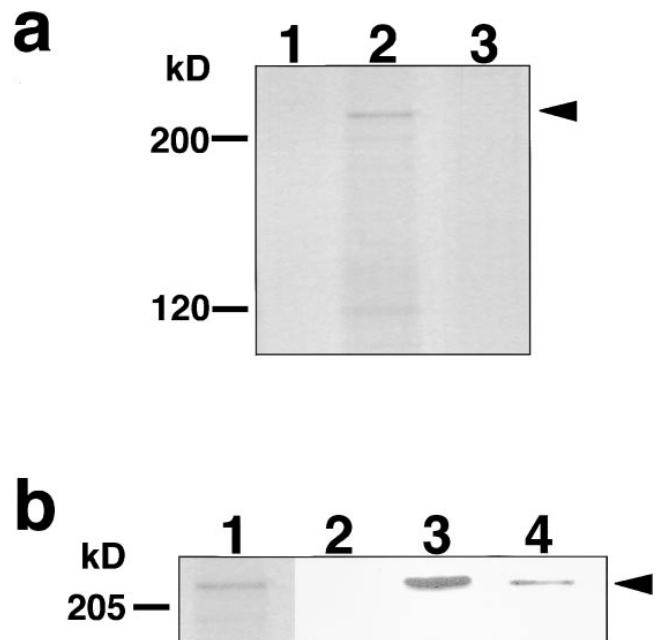
To detect molecules interacting with AF-6 (1130–1612 aa), which includes a proline-rich region, the bovine brain cytosolic fraction was loaded onto affinity columns coated with GST, GST-AF-6 (1130–1612 aa), or GST-CD44. The proteins bound to the affinity columns were coeluted with GST-AF-6 (1130–1612 aa) by adding glutathione. The glutathione-eluted fractions were subjected to SDS-PAGE followed by silver staining. A protein with a molecular mass of  $\sim$ 220 kD (p220) was detected in the glutathione eluate from the GST-AF-6 (1130–1612 aa) affinity column, but not from the GST or GST-CD44 affinity columns (Fig. 1 *a*), indicating that p220 specifically interacts with AF-6 (1130–1612 aa) directly or indirectly. To identify the AF-6 (1130–1612 aa)-interacting molecule, p220 was subjected to amino acid sequencing as described in Materials and Methods. Two peptide sequences derived from p220 were determined. These were LSVPATFMLVSLD and NDYFEF. Both peptide sequences were identical to the deduced amino acid sequence of mouse Fam, which is one of the deubiquitinating enzymes (Wood et al., 1997).

Fam shows homology with Ubps-type deubiquitinating enzyme in *Drosophila*, the product of the *fat facets* (*faf*) gene. Recent genetic analyses indicate that the *faf* gene is required for normal eye development and embryogenesis, suggesting that the deubiquitinating activity of the *faf* product plays a critical role in controlling the cell fate. The amino acid sequence of Fam shows  $\sim$ 50% amino acid identity and 70% similarity with that of Faf (Wood et al., 1997). Fam has the cysteine and histidine domains characteristic of Ubps. Fam shows a high similarity to several Ubps from yeast to mammals in these two regions (Wood et al., 1997). The calculated molecular mass of mouse Fam is 290 kD, but the apparent molecular mass of bovine Fam estimated by SDS-PAGE is  $\sim$ 220 kD. To confirm that p220 is Fam, an immunoblot analysis was performed on p220 from the glutathione-eluted fraction with two anti-Fam antibodies. Anti-Fam antibody (N20) against 20 aa of

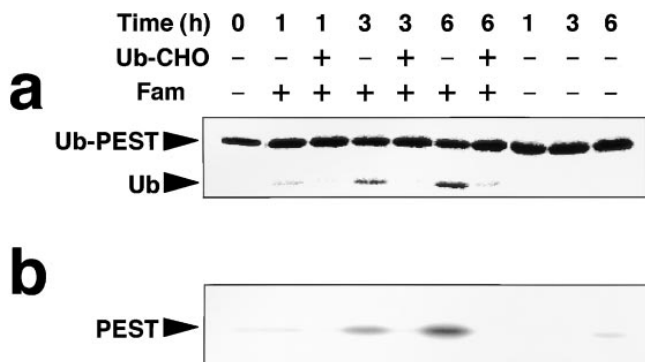
the amino-terminal site and anti-Fam antibody (C114) against 114 aa of the carboxy-terminal site were generated. Since p220 was recognized by both anti-Fam antibodies (N20 and C114) as shown in Fig. 1 *b*, we judged that p220 was the full length of Fam, but not the degradation product of Fam. We therefore concluded that p220 was the bovine counterpart of mouse Fam, and hereafter refer to it as Fam.

### Deubiquitinating Activity Catalyzed by Fam

First we examined whether Fam has deubiquitinating activity. We used the 200-mM NaCl eluate from the GST-AF-6 (1130–1612 aa) affinity column as a source of Fam. Fam was incubated with the  $^{125}$ I-labeled ubiquitin-conjugated PEST sequence (Ub-PEST) for various periods of time. As shown in Fig. 2, Fam could release ubiquitin from Ub-PEST (*a*), and could produce the hydrolyzed  $^{125}$ I-PEST peptides from Ub-PEST (*b*). In the Fam minus control of Fig. 2, the 200-mM NaCl buffer that did not contain any enzyme was used. These results indicate that Fam is able to generate free ubiquitin from Ub-PEST. It is well-known that ubiquitin-aldehyde (Ub-CHO) inhibits Ubps. Release of ubiquitin from Ub-PEST or production of the PEST peptides was almost abolished in the presence of Ub-CHO. The deubiquitinating activity was not detected in the 200-mM NaCl eluate from the GST affinity column



**Figure 1.** Purification of AF-6-interacting protein. (*a*) The bovine brain cytosol was loaded onto glutathione-Sepharose 4B columns coated with GST, GST-AF-6 (1130–1612 aa), or GST-CD44. The bound proteins were coeluted with the respective GST fusion proteins by adding glutathione. Aliquots of the eluates were resolved by SDS-PAGE, followed by silver staining. Lane 1, GST; lane 2, GST-AF-6 (1130–1612 aa); lane 3, GST-CD44. The arrowhead denotes the position of p220. (*b*) Protein p220 was immunoblotted with the anti-Fam antibodies. Lane 1, the position of p220 by silver staining; lane 2, with preimmuneserum; lane 3, with the anti-Fam antibody (N20); lane 4, with the anti-Fam antibody (C114). The arrowhead denotes the position of p220. The results shown are representative of three independent experiments.

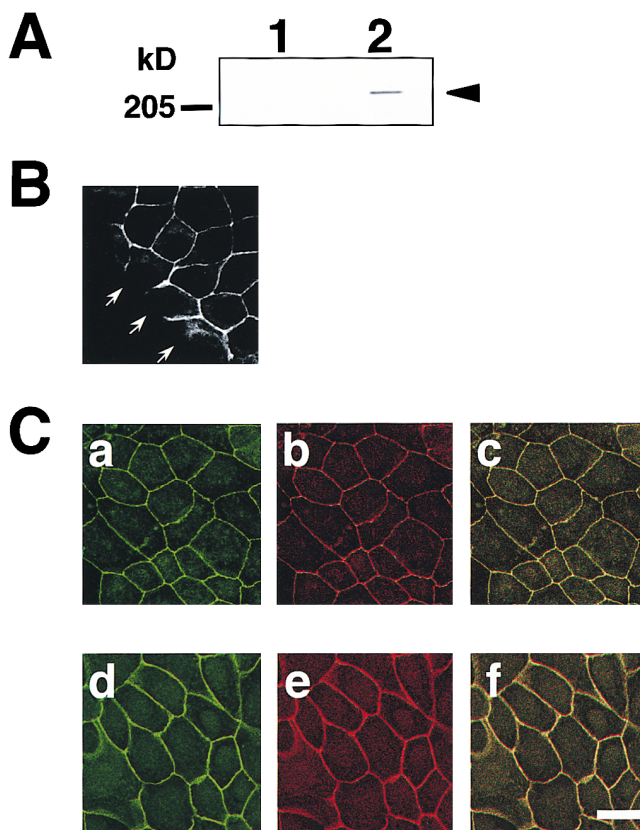


**Figure 2.** Hydrolysis of  $^{125}\text{I}$ -labeled Ub-PEST by Fam. The  $^{125}\text{I}$ -labeled Ub-PEST was incubated alone or with 1.6  $\mu\text{g}$  of Fam purified by GST-AF-6 (1130–1612 aa) affinity column in the absence or presence of ubiquitin-aldehyde (Ub-CHO) for 1, 3, or 6 h at 37°C. After the incubation, the samples were subjected to SDS-PAGE, followed by Coomassie Brilliant Blue staining (a), and by autoradiography (b). The release of ubiquitin from Ub-PEST (a) or the production of the hydrolysis of  $^{125}\text{I}$ -labeled PEST peptides from Ub-PEST (b). The results shown are representative of three independent experiments.

(data not shown). These results demonstrated that Fam has deubiquitinating activity.

#### Distributions of Fam in Confluent MDCKII Cells

To understand the functions of Fam, we examined its intracellular distribution in MDCKII epithelial cells that show characteristics of polarized epithelial cells and form the junctional complex, including the tight junction and adherens junction at cell–cell contact sites (Gonzalez-Mariscal et al., 1985). The immunoblot analysis of cell lysates from MDCKII cells showed that anti-Fam antibody recognized a single band corresponding to a molecular weight of  $\sim 220$  kD (Fig. 3 A) as in the case of bovine brain extract (Fig. 1 b). Antibody preincubation with the recombinant Fam abolished the immunoreactivity (data not shown). The immunoreactivity of Fam was specifically localized at sites where a cell contacted a neighboring cell, and not at free ends of plasma membranes (Fig. 3 B). The cytoplasm exhibited a relatively low level of immunoreactivity. To further examine whether Fam exists in the apical or basal site of the lateral membrane, cellular distribution of Fam was compared with that of ZO-1 and  $\beta$ -catenin (Fig. 3, C, b and e). ZO-1 was concentrated at the apical sections, whereas  $\beta$ -catenin was found at more basal sections as described previously (Nagafuchi and Takeichi, 1989; Itoh et al., 1993). In contrast, Fam immunoreactivity was colocalized at the immunofluorescence microscopic level with ZO-1 at the apical sites, and with  $\beta$ -catenin at the basal sites (Fig. 3, C, c and f). Since we have previously shown that AF-6 interacts with ZO-1 and is colocalized with ZO-1 at cell–cell contact sites including tight junctions (Yamamoto et al., 1997), part of the Fam that is localized at the same sites with ZO-1 may be colocalized with AF-6. We also found that accumulation of Fam is induced by the formation of cell–cell adhesions by using a  $\text{Ca}^{2+}$  switch assay in MDCKII cells (data not shown). These results suggest that Fam is partly colocalized with

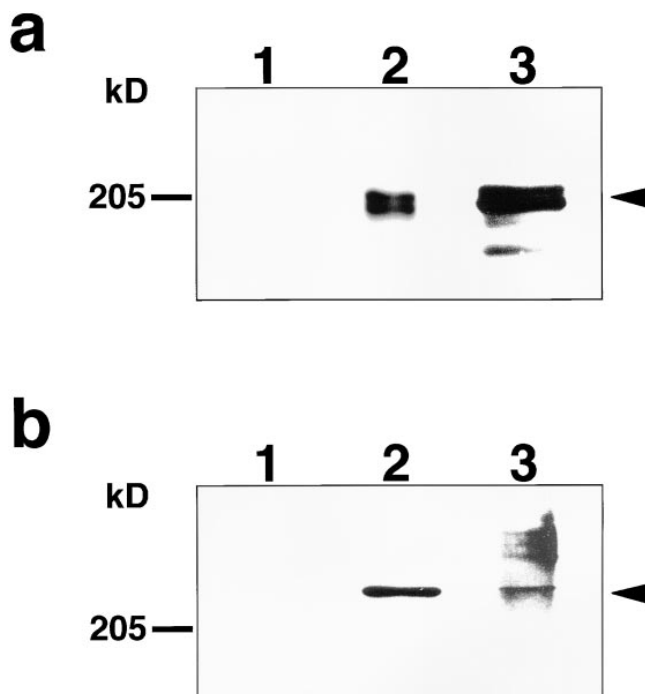


**Figure 3.** Confocal microscope images of confluent MDCKII cells showing the distributions of Fam, ZO-1 and  $\beta$ -catenin. (A) Immunoblot analysis of MDCKII cell lysates with preimmune serum (lane 1) and with anti-Fam antibody (K2; lane 2). The arrowhead denotes the position of Fam. (B) Subconfluent MDCKII cells stained with anti-Fam antibody (K2). Arrows indicate free ends of plasma membrane. (C) Confluent MDCKII cells doubly stained with a rabbit polyclonal antibody against Fam (K2; a and d) and mouse monoclonal antibodies against ZO-1 (b) or  $\beta$ -catenin (e). Fam immunoreactivity is shown in green, and ZO-1 and  $\beta$ -catenin immunoreactivities are shown in red. ZO-1 was concentrated at the apical sections (b), whereas  $\beta$ -catenin was found at more basal sections (e). The yellow area indicates colocalization of Fam and ZO-1 at the apical sites (c), or Fam and  $\beta$ -catenin at the basal sites (f). The results shown are representative of three independent experiments. Bar, 10  $\mu\text{m}$ .

AF-6 at apical sites of the lateral membrane in confluent MDCKII cells.

#### Interaction of Fam and AF-6 In Vivo

Because Fam was partly colocalized with AF-6 at apical sites of the lateral membrane, we examined whether Fam interacts with AF-6 in vivo. When Fam was immunoprecipitated with anti-Fam antibody from confluent MDCKII cells, AF-6 was coimmunoprecipitated with Fam (Fig. 4 a). AF-6 was not coimmunoprecipitated with control preimmuneserum. AF-6 appeared to associate with Fam with a stoichiometry of about one AF-6 per ten Fam under these conditions. Similarly, Fam was also coimmunoprecipitated with AF-6 (Fig. 4 b) when AF-6 was immunoprecipitated with anti-AF-6 antibody from confluent MDCKII cells.



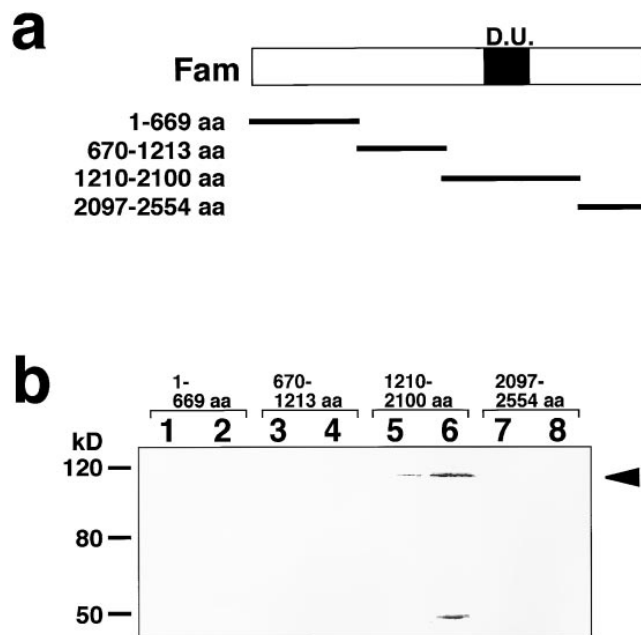
**Figure 4.** Coimmunoprecipitation of Fam with AF-6. MDCKII cells were lysed and solubilized with buffer C (see Materials and Methods). This sample was incubated with preimmuneserum (lane 1), with anti-Fam antibody (K2; lane 2), or with anti-AF-6 antibody (#3; lane 3). The immunocomplexes were then precipitated with protein A-Sepharose 4B, and subjected to immunoblot analysis using anti-AF-6 antibody (#4; *a*) or anti-Fam antibody (N20; *b*). The arrowheads denote the positions of AF-6 (*a*) and Fam (*b*), respectively. The results shown are representative of three independent experiments.

These results indicate that Fam interacts with AF-6 *in vivo*.

### Interaction of Fam and AF-6 *In Vitro*

We examined which domain of Fam interacts with AF-6 using *in vitro*-translated Fam such as Fam (1–669 aa), Fam (670–1213 aa), Fam (1210–2100 aa), and Fam (2097–2554 aa; Fig. 5 *a*). Affinity beads coated with GST or GST-AF-6 (1130–1612 aa) were mixed with the *in vitro*-translated Fam (1–669 aa), Fam (670–1213 aa), Fam (1210–2100 aa), and Fam (2097–2554 aa), and the interacting proteins were then coeluted with GST fusion proteins by adding glutathione. As shown in Fig. 5 *b*, Fam (1210–2100 aa) bound to GST-AF-6 (1130–1612 aa), but Fam (1–669 aa), Fam (670–1213 aa), and Fam (2097–2554 aa) did not. Fam (1210–2100 aa) did not bind to control GST.

To test the specificity of the binding, we carried out a kinetic study of the binding of Fam to AF-6. We first examined whether GST-AF-6 (1130–1612 aa) could bind to MBP-Fam (1476–1918 aa) involving the deubiquitinating catalytic domain. Affinity beads coated with GST or GST-AF-6 (1130–1612 aa) were mixed with MBP-Fam (1476–1918 aa). The bound MBP-Fam (1476–1918 aa) was then coeluted with the GST fusion proteins by adding glutathione, and the eluted MBP-Fam (1476–1918 aa) was detected by Coomassie Brilliant Blue staining. MBP-Fam



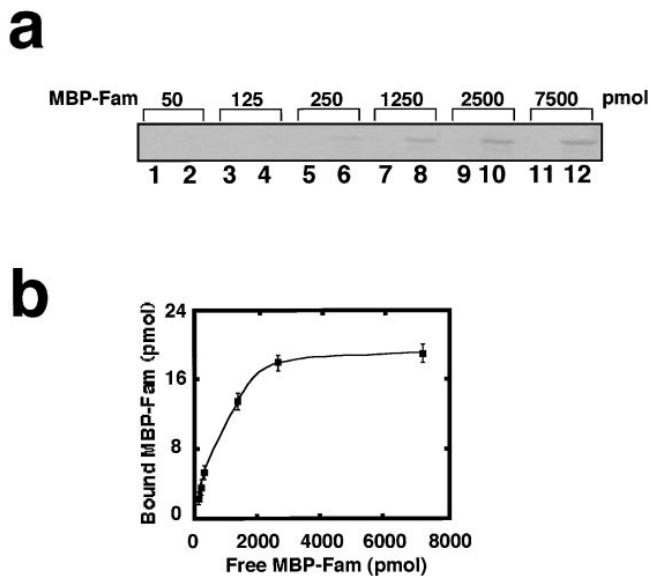
**Figure 5.** Interaction of *in vitro*-translated Fam (1210–2100 aa) with GST-AF-6 (1130–1612 aa). (*a*) Domain diagram of Fam and the recombinant fragments used for the *in vitro* binding assay. D.U., catalytic site of deubiquitinating enzyme including cysteine and histidine domains. Bold lines show the recombinant fragments used for the *in vitro* binding assay. (*b*) *In vitro*-translated Fam (1–669 aa; lanes 1 and 2), Fam (670–1213 aa; lanes 3 and 4), Fam (1210–2100 aa; lanes 5 and 6), and Fam (2097–2554 aa; lanes 7 and 8) were mixed with GST (lanes 1, 3, 5, and 7) or GST-AF-6 (1130–1612 aa; lanes 2, 4, 6, and 8)-coated glutathione-Sepharose 4B beads. The interacting proteins were coeluted with GST fusion proteins by adding glutathione. The eluates were subjected to SDS-PAGE and vacuum-dried. The *in vitro*-translated Fam fragments were visualized with an image analyzer. The arrowhead denotes the position of the *in vitro*-translated Fam (1210–2100 aa). The results shown are representative of three independent experiments.

(1476–1918 aa) was detected in the eluates from the GST-AF-6 (1130–1612 aa) affinity beads, but only slightly in those from the GST affinity beads (data not shown). In addition, as shown in Fig. 6, MBP-Fam (1476–1918 aa) bound to GST-AF-6 (1130–1612 aa) in a dose-dependent manner, and this binding was saturable when the amounts of MBP-Fam (1476–1918 aa) were increased (Fig. 6, *a* and *b*). The apparent  $K_d$  value for binding MBP-Fam (1476–1918 aa) to GST-AF-6 (1130–1612 aa) was estimated to be  $\sim 810$  nM under the conditions used. These results indicate that mainly 1476–1918 aa of Fam is responsible for binding Fam to AF-6.

### Ubiquitination of AF-6 *In Vivo*

As described above, AF-6 interacts with Fam, and the AF-6-interacting domain of Fam involves the deubiquitinating catalytic domain of Fam. This raises the possibility that AF-6 is the substrate of Fam, and that AF-6 is Ub-conjugated and subjected to the ubiquitin-proteasome pathway. First, to determine whether AF-6 is degraded by the proteasome-dependent proteolysis pathway, Rat1 fibroblasts and EL cells were treated with the proteasome inhibitor.

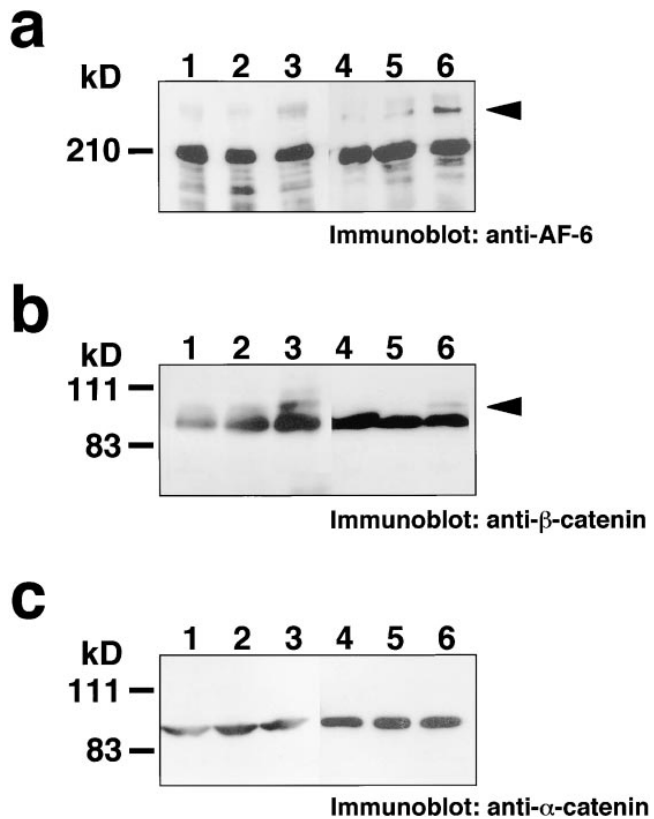




**Figure 6.** Interaction of MBP-Fam (1476–1918 aa) and GST-AF-6 (1130–1612 aa) in vitro. The result of the kinetic study of the binding of MBP-Fam (1476–1918 aa) with GST-AF-6 (1130–1612 aa) are shown. Various amounts of MBP-Fam (1476–1918 aa) were mixed with GST (lanes 1, 3, 5, 7, 9, and 11) or GST-AF-6 (1130–1612 aa) (lanes 2, 4, 6, 8, 10, and 12)-coated glutathione-Sepharose 4B beads. The interacting proteins were coeluted with GST fusion proteins by adding glutathione. (a) The eluted MBP-Fam (1476–1918 aa) was detected by Coomassie Brilliant Blue staining. (b) The bound MBP-Fam (1476–1918 aa) was visualized and estimated with a densitograph. The values shown are means  $\pm$  SEM of triplicate experiments.

EL cells are L cells stably expressing E-cadherin (Nagafuchi et al., 1994). It is well-known that the peptide aldehyde ALLN inhibits the proteasome-dependent proteolysis pathway, leading to an accumulation of proteins that are usually metabolized by the proteasome pathway (Coux et al., 1996). ALLM is the related peptide aldehyde and a calpain inhibitor, but it does not inhibit the proteasome pathway. Recently some groups reported that the turnover of  $\beta$ -catenin is regulated by the ubiquitin–proteasome pathway (Aberle et al., 1997; Orford et al., 1997). They showed that treating certain cell lines with ALLN resulted in accumulation of the higher molecular weight  $\beta$ -catenin. It has been reported that such a modification in  $\alpha$ -catenin was not observed when cells were treated with ALLN. We obtained similar results as shown in Fig. 7, b and c, when Rat1 and EL cells were treated with ALLN. To determine whether AF-6 shows the similar modification, we immunoblotted the cell lysates with anti-AF-6 antibody. The higher molecular weight forms of AF-6 were detected by the treatment with ALLN, but little was detected by the treatment with ALLM under the same conditions (Fig. 7 a). These results suggest that AF-6 is degraded by the proteasome pathway.

Next, to determine whether AF-6 is ubiquitinated, we performed an assay to detect ubiquitinated proteins (Treier et al., 1994; Aberle et al., 1997). AF-6 and hemagglutinin-tagged ubiquitin (HA-Ub) were transiently expressed in COS7 cells. The immunoprecipitates were then collected with anti-AF-6 antibody and subjected to an im-

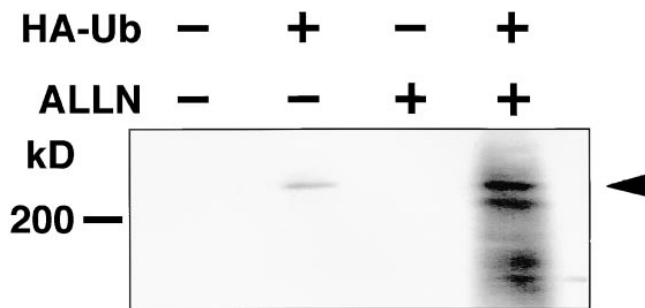


**Figure 7.** Regulation of AF-6 by the ubiquitin–proteasome pathway. Rat1 and EL cells were treated with the vehicle (DMSO; lanes 1 and 4), 25  $\mu$ M ALLM (lanes 2 and 5), or 25  $\mu$ M ALLN (lanes 3 and 6) for 3 h. Cell lysates of Rat1 (lanes 1–3) and EL (lanes 4–6) cells were immunoblotted with anti-AF-6 antibody (#4; a), anti- $\beta$ -catenin antibody (b), and anti- $\alpha$ -catenin antibody (c). The arrowheads denote the position of the ubiquitinated AF-6 (a) and the ubiquitinated  $\beta$ -catenin (b), respectively. The results shown are representative of three independent experiments.

munoblot analysis with anti-HA antibody. As shown in Fig. 8, HA-Ub-conjugated AF-6 was detected in cells expressing HA-Ub, but not in control cells. We examined the effect of ALLN on AF-6 ubiquitination. HA-Ub-conjugated proteins were more strongly detected in the cells treated with ALLN than in the untreated cells (Fig. 8). The band below HA-Ub-conjugated AF-6 was as strong as HA-Ub-conjugated AF-6 in the lane of ALLN plus. The lower band was probably the degradation product of the full-length AF-6, because the lower band as well as the upper band were detected only when the AF-6 cDNA was transfected. It may be noted that the intensity of the lower band was more strongly enhanced by ALLN, though we can not give the precise reasons for this phenomenon. Taken together, these results indicate that AF-6 is ubiquitinated in vivo and suggest that the ubiquitinated AF-6 is degraded by the proteasome pathway.

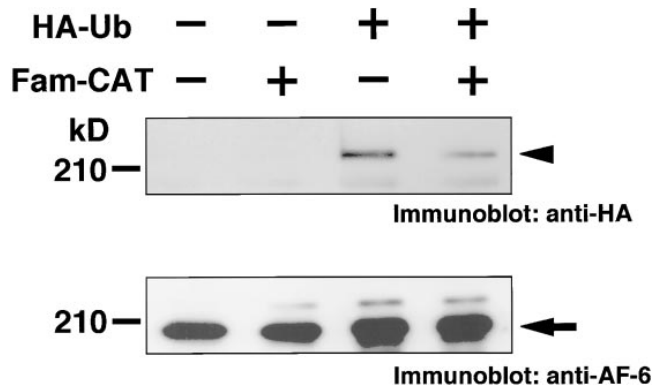
#### Deubiquitination of the Ubiquitinated AF-6 by Fam-CAT

For the determination of whether Fam can release ubiquitin from the ubiquitinated AF-6 in vivo, AF-6 and HA-Ub were expressed with or without Fam-CAT in COS7



**Figure 8.** Ubiquitination of AF-6 in COS7 cells. AF-6 and HA-tagged ubiquitin (*HA-Ub*) were expressed in COS7 cells. COS7 cells were treated with or without 25  $\mu$ M ALLN for 18 h. The immunoprecipitates were then collected with anti-AF-6 antibody (#3) and subjected to an immunoblot analysis with anti-HA antibody. The arrowhead denotes the position of HA-Ub-conjugated AF-6. The results shown are representative of three independent experiments.

cells. We used Fam-CAT, which includes the deubiquitinating catalytic domain of Fam (1210–2410 aa), because we could not detect the expression of full-length Fam, probably due to its low expression level. As described above, AF-6 was immunoprecipitated from the cell lysates with anti-AF-6 antibody, and the immunoprecipitates were subjected to an immunoblot analysis with anti-HA antibody. In the absence of Fam-CAT, HA-Ub-conjugated AF-6 was observed (Fig. 9) as shown in Fig. 8. When AF-6 was coexpressed with Fam-CAT, the amount of HA-Ub-conjugated AF-6 was reduced while the amount of immunoprecipitated AF-6 was not affected (Fig. 9). These results indicate that Fam inhibited the ubiquitination of AF-6, and suggest that AF-6 is one of the substrates of Fam.



**Figure 9.** Deubiquitination of the ubiquitinated AF-6 by Fam-CAT. AF-6 and HA-tagged ubiquitin (*HA-Ub*) were expressed with or without Fam-CAT (the deubiquitinating catalytic domain of Fam) in COS7 cells. The immunoprecipitates were then collected with anti-AF-6 antibody (#3), and subjected to an immunoblot analysis with anti-HA antibody or anti-AF-6 antibody (#4). The arrowhead denotes the position of HA-Ub-conjugated AF-6, and the arrow denotes the position of the immunoprecipitated AF-6. The results shown are representative of three independent experiments.

## Discussion

### Interaction of AF-6 and Fam

We here identified Fam as an AF-6 (1130–1612 aa)-interacting protein. The carboxyl terminal domain of AF-6 (1130–1612 aa) has the proline-rich region. Since it has been reported that the proline-rich region interacts with certain proteins containing the src homology region 3 (SH3) or WW domain (Sudol, 1996), we first thought that AF-6 (1130–1612 aa) may interact with proteins containing the SH3 or WW domain. The present results, however, showed that the AF-6 (1130–1612 aa) containing the proline-rich region interacts with Fam, which has neither an SH3 nor a WW domain, and that the AF-6-interacting domain of Fam involves the cysteine and histidine domains characteristic of Ubps.

As described above, Fam is partly colocalized with AF-6 at cell–cell contact sites, but not at free ends of the plasma membrane. We also found that accumulation of Fam is induced by formation of cell–cell adhesions by using a  $Ca^{2+}$  switch assay in MDCKII cells (data not shown). These observations raised the possibility that AF-6 may function as an anchoring molecule for recruiting Fam to cell–cell contact sites. Fam is, however, widely distributed at other lateral membranes where AF-6 is not localized. In this case, Fam may be recruited to lateral membranes by interacting with other cell–cell adhesion molecules. In this regard, we have recently found that Fam interacts with  $\beta$ -catenin, but not with  $\alpha$ -catenin and the cytoplasmic domain of E-cadherin *in vitro* (unpublished results). Investigations to identify the Fam-interacting molecules are in progress; their findings may clarify the mechanisms by which Fam is recruited to cell–cell contact sites.

### Ubiquitination of AF-6

Since the AF-6-interacting domain of Fam involves the deubiquitinating catalytic domain of Fam, we speculate that AF-6 is the substrate of Fam, and is subjected to the ubiquitin–proteasome pathway. As described above, when Rat1 and EL cells were treated with the proteasome inhibitor ALLN, the higher molecular weight forms of AF-6 were detected. We also performed an assay for detecting the ubiquitinated proteins (Treier et al., 1994; Aberle et al., 1997) and found that AF-6 was ubiquitinated *in vivo*. Many proteins involved in cell cycle control, transcription activation, cell growth, antigen presentation, and so on are known to be regulated by the ubiquitin–proteasome pathway. These proteins include c-fos, NF $\kappa$ B-I $\kappa$ B complex, p53 and growth factor receptors such as PDGFR and FGFR-1, and it has been gradually clarified how these proteins are subjected to the ubiquitin–proteasome pathway (Hochstrasser, 1995; Hicke, 1997). It remains to be clarified how AF-6 is ubiquitinated and subsequently degraded by the proteasome pathway.

### Deubiquitination of AF-6 by Fam

Fam belongs to the deubiquitinating enzyme, and we showed that Fam has deubiquitinating activity *in vitro* (Fig. 2). Indeed, we found that Fam-CAT decreases the amount of HA-Ub-conjugated AF-6 in COS7 cells (Fig.



9), suggesting that Fam can release ubiquitin from AF-6, although we cannot exclude the possibility that Fam simply inhibits the ubiquitination of AF-6. Whether Fam-CAT is expressed or not in Fig. 9, the AF-6 level was similar. We first thought that the AF-6 level might increase when Fam-CAT was expressed. However, HA-Ub-conjugated AF-6 was only ~5% of the total AF-6 under our conditions. Thus, we think that we can not detect a big change of the AF-6 level in this assay.

Since Fam is widely distributed at lateral membranes, it is possible that Fam deubiquitinates components of cell-cell adhesions including  $\beta$ -catenin. We have recently found that Fam interacts with  $\beta$ -catenin in vitro as described above. However, we could not examine whether  $\beta$ -catenin is the substrate of Fam because exogenous  $\beta$ -catenin was hardly ubiquitinated in COS7 cells under our assay conditions. Further studies are necessary to resolve this problem and to clarify the substrate spectrum of the Fam deubiquitinating enzyme.

### Possible Roles of Fam at Cell-Cell Adhesions

Polarized epithelial cells form the junctional complex, including tight junctions and adherens junctions, at cell-cell contact sites (Gonzalez-Mariscal et al., 1985). In epithelial and endothelial cells, ZO-1 is directly associated with occludin, which has four transmembrane domains in its amino-terminal half, in tight junctions (Itoh et al., 1993; Furuse et al., 1993). In nonepithelial cells, ZO-1 is concentrated at adherens junctions, and is associated with a cadherin/catenin complex via a direct interaction with  $\alpha$ -catenin (Itoh et al., 1993; Itoh et al. 1997). Since AF-6 directly interacts with ZO-1 and Fam, we propose that the roles of Fam in epithelial cells and nonepithelial cells are as follows: Fam is probably recruited to tight junctions or adherens junctions via direct interaction with AF-6. When cells move and enter the mitotic phase, cell-cell adhesions appear to be perturbed or dynamically rearranged. These perturbations or rearrangements of cell-cell adhesions may be regulated by the ubiquitin-proteasome pathway. Fam probably maintains the stability of cell-cell adhesions by deubiquitinating the components of cell-cell adhesions. It remains to be determined whether Fam deubiquitinates the components of cell-cell adhesions.

The transformation of epithelial and fibroblastic cells by activated Ras results in the perturbation of cell-cell adhesions. We observed that the formation of the AF-6/ZO-1 complex is specifically inhibited or changed by activated Ras, as described previously (Yamamoto et al., 1997). Since this alteration by activated Ras may prevent recruitment of Fam to the cell-cell adhesions or inhibit the deubiquitinating activity of Fam, Fam may become unable to contribute to the stability of cell-cell adhesions. It remains to be clarified whether the function of Fam is regulated by Ras signaling and is implicated in the Ras-induced transformation.

### The Interaction of AF-6-Fam Pathway with Ras Signaling

AF-6 shows strong sequence homology with *Drosophila* Canoe, and shares a common domain organization with Canoe (Kuriyama et al., 1996). The *Drosophila* compound

eye consists of 800 identical facets that are each made up of eight photoreceptors (R1-R8) and four cone cells. Canoe is implicated in cone cell formation in the developing compound eye in *Drosophila* (Matsuo et al., 1997). The fates of cone cells are thought to be determined by cell-cell contacts. The phenotypic effect of Canoe mutations on the cone cells depends on the state of Ras (Matsuo et al., 1997), and Canoe is shown to interact with the activated Ras, indicating that Canoe serves as a target of Ras, as described for AF-6 (Kuriyama et al., 1996; Matsuo et al., 1997).

Here we showed that AF-6 interacts with Fam in vitro and in vivo. Fam is homologous to a deubiquitinating enzyme in *Drosophila*, the product of the *fat facets* (*faf*) gene (Huang et al., 1995; Wood et al., 1997). *Faf* is essential for regulating of a cell communication pathway in the early stage of eye development. *Faf* regulates the number of photoreceptor cells in each facet of the compound eye (Fischer-Vize et al., 1992; Huang and Fischer-Vize, 1996). When the deubiquitinating activity of *Faf* is abolished by mutagenesis in *Drosophila*, the mutant fly has an abnormal eye morphology, suggesting that the deubiquitinating activity of *Faf* is necessary for the normal eye development (Huang et al., 1995). It was recently reported that Ras1 interacts genetically with *Faf* in *Drosophila* eye development (Li et al., 1997). *Faf* has an additional function in the later stage of eye development involving Ras1. These observations in *Drosophila* raise the possibility that both *Faf* and Canoe function downstream of Ras and that *Faf* interacts genetically with Canoe, although further genetic analyses are required to determine the relation of the Ras1-Canoe and Ras1-*Faf* pathways.

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