Regulation of Amino Acid Transporter ATA2 by Ubiquitin Ligase Nedd4-2*

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Takahiro Hatanaka1, Yasue Hatanaka1,5, and Mitsutoshi Setou19

From the 1Mitsubishi Kagaku Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194-8511, Japan, 5PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan, and 6National Institute of Physiological Sciences, 5-1 Higashiyama, Myodaiji-cho, Okazaki, Aichi 444-8787, Japan

We report here that ubiquitin ligase Nedd4-2 regulates amino acid transporter ATA2 activity on the cell surface. We first found that a proteasome inhibitor MG132 increased the uptake of α-(methylamino)isobutyric acid, a model substrate for amino acid transport system A, in 3T3-L1 adipocytes as well as the preadipocytes. Transient expression of Nedd4-2 in Xenopus oocytes and Chinese hamster ovary cells down-regulated the ATA2 transport activity induced by injected cRNA and transfected cDNA, respectively. Neither the Nedd4-2 mutant with defective catalytic domain nor c-Cbl affected the ATA2 activity significantly. RNA-mediated interference of Nedd4-2 increased the ATA2 activity in the cells, and this was associated with decreased polyubiquitination of ATA2 on the cell surface membrane. Immunofluorescent analysis of Nedd4-2 in the adipoocytes stably transfected with the enhanced green fluorescent protein (EGFP)-tagged ATA2 showed the co-localization of Nedd4-2 and EGFP-ATA2 in the plasma membrane but not in the perinuclear ATA2 storage site, supporting the idea that the primary site for the ubiquitination of ATA2 is the plasma membrane. These data suggest that ATA2 on the plasma membrane is subject to polyubiquitination by Nedd4-2 with consequent endocytic sequestration and proteasomal degradation and that this process is an important determinant of the density of ATA2 functioning on the cell surface.

Amino acid transport system A is a Na+-dependent active transport system for neutral amino acids expressed in most tissues (1). A unique characteristic of this system is its ability to recognize N-alkylated amino acids as substrates (2). α-(Methylamino)isobutyric acid (MeAIB) is commonly used as a model substrate for system A. Among the various amino acid transport systems known to function in mammalian cells, system A is best known for its regulation (3–5). Recently, several groups including us have established the molecular identity of the amino acid transport system A (6–13). These studies have identified three distinct transporter proteins that are responsible for system A transport activity in mammalian cells, and all three transporters are capable of mediating the Na+-coupled uptake of the system A model substrate MeAIB. The three transporters are known as amino acid transporter A (ATA)1 (also known as SNAT1), ATA2 (SNAT2), and ATA3 (SNAT4). These transporters belong to the solute-linked carrier family SLC38 (14). ATA1 and ATA2 possess similar functional characteristics but show distinct tissue expression pattern. ATA1 is expressed primarily in the placenta and brain, whereas ATA2 is expressed ubiquitously in mammalian tissues. ATA3 is functionally distinguishable from ATA1 and ATA2, and its expression is restricted to the liver. It is generally believed that ATA2 represents system A, which is known for its regulatory features. There is also evidence to indicate that ATA2 corresponds to system A activity in adipocytes (15, 16). Recently, we reported the regulation of ATA2 in adipocytes by insulin and in diabetes. In detail, we showed that insulin accelerated the translocation of ATA2 from the trans-Golgi network storage site to the plasma membrane and that the steady-state levels of ATA2 mRNA decreased in diabetes. The insulin-modulated translocation does not occur via a common endosomal pathway that is available for other plasma membrane proteins but via a pathway that is specific for ATA2. It is the balance between insertion into and sequestration from the plasma membrane that determines the density of the transporter on the cell surface that is responsible for measurable transport function. Very little is known at this time on the molecular events involved in the degradation of ATA2 subsequent to sequestration from the plasma membrane.

Membrane proteins are frequently degraded in lysosomes, but there are some examples of transporter proteins being degraded by the ubiquitin-proteasome system (18–20). The ubiquitin-proteasome system is responsible for the disposal of many short-lived proteins in eukaryotic cells, and the process is initiated by covalent tagging of the target protein with a polyubiquitin chain on the lysine residue (21).

Here, we investigated the internalization and degradation pathway of ATA2 in 3T3-L1 adipocytes as well as preadipo-

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1 To whom correspondence should be addressed: Mitsubishi Kagaku Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194-8511, Japan. Tel.: 81-42-724-6259; Fax: 81-42-724-6316; E-mail: setou@nips.ac.jp.

2 The abbreviations used are: MeAIB, α-(methylamino)isobutyric acid; ATA, amino acid transporter A; 2DG, 2-deoxy-D-glucose; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; RNAi, RNA-mediated interference; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; CHO, Chinese hamster ovary; siRNA, small interfering RNA; GLUT, glucose transporter.

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cytes. These studies show that an E3 ubiquitin ligase Nedd4-2 ligates ATA2 with the ubiquitin chain as the sorting signal for endocytosis, and then the ubiquitin-conjugated ATA2 is degraded by proteasomes. This is rather a specific internalization and degradation pathway for this membrane transporter than a general bulk pathway such as the lysosomal degradation.

EXPERIMENTAL PROCEDURES

Materials—Mouse ATA2 (mATA2) cDNA was cloned from mouse kidney cDNA library as described previously. 3 3T3-L1 murine fibroblasts were from Health Science Research Resources Bank (Osaka, Japan). CHO-K1 cells were provided from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Mouse Nedd4-2 cDNA, cell culture media, Lipofectamine 2000, and Alexa568-conjugated secondary antibodies were purchased from Invitrogen. [14C]MeAIB was from American Radiolabeled Chemicals (St. Louis, MO). 2-Deoxy-D-[14C]glucose ([14C]-labeled 2DG) was purchased from Moravek Biochemicals (Brea, CA). MG132 was purchased from the Peptide Institute Inc. (Osaka, Japan). The anti-ubiquitin monoclonal antibody (1B3) was purchased from Medical Biological Laboratories (Nagoya, Japan). The horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibody was from Jackson ImmunoResearch (West Grove, PA). Mouse Nedd4 cDNA was purchased from DNAform (Ibaragi, Japan).

Cell Culture—3T3-L1 fibroblasts (preadipocytes) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% calf serum, and differentiation was induced according to established protocols as described previously (22, 23). Briefly, cells were allowed to reach confluence at least 2 days before the induction of differentiation. Differentiation was induced (on day 0) with high glucose DMEM, 10% fetal bovine serum containing 0.25 μM dexamethasone, 5 μg/ml insulin, and 500 μM methylisobutylxanthine. After 48 h (day 2), the cells were fed with high glucose DMEM, 10% fetal bovine serum containing 5 μg/ml insulin. After an additional 48 h (day 4), the cells were re-fed every 2 days with high glucose DMEM, 10% fetal bovine serum. All media were supplemented with 2 mM glutamine, 50 units of penicillin/ml, and 50 μg of streptomycin/ml. Differentiation was monitored by noting the accumulation of lipid droplets, which typically began by day 4 of differentiation. Cells were considered fully differentiated between days 8 and 12. CHO-K1 cells were cultured according to the protocol of the provider.

Uptake Experiments in 3T3-L1 Cells—Before the uptake experiments, 3T3-L1 cells were fed with serum-free DMEM with or without MG132 (10 μM) for 4 h and then incubated with or without insulin (1 μM) in uptake buffer, pH 7.4, for 30 min. Uptake experiments were carried out following the previously described protocol. 3 The uptake buffer was 25 mM Tris/HEPES, pH 8.0, for MeAIB (7, 9) or HEPES/Tris, pH 7.4, for 2DG, and the buffer contained 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, and 0.8 mM MgSO4. The uptake experiment was performed in 3T3-L1 cells at 37 °C for 30 min with [14C]MeAIB or [14C]-labeled 2DG as the substrate. The concentration of the radiola-
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Previously. Uptake experiments were performed by following the protocol in 3T3-L1 cells as described above using the 100 μM MeAIB and 20 min of incubation.

Cell Lysis and Immunoprecipitation—Cells were lysed with Triton X-100 lysis buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, protease inhibitor mixture (Complete, Roche Applied Science). An agarose-conjugated anti-GFP rat monoclonal antibody (Medical Biological Laboratories) was added to lysates and incubated at 4 °C for 1.5 h. The agarose-conjugated antibody was washed thoroughly (four times) with Triton X-100 lysis buffer and subjected to the Western blot analysis using the anti-ubiquitin or anti-GFP antibody. Biotinylation of cell surface proteins was performed by the method described by Rotmann et al. (30) for the study of the internalization of the cationic amino acid transporter with slight modifications. Briefly, the preadipocytes stably expressing EGFP-ATA2 protein were grown to confluence and differentiated into adipocytes in 10-cm dishes. After the experimental treatment, the cells were rinsed with ice-cold phosphate-buffered saline (PBS) containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS+) and incubated in the same solution supplemented with 0.5 mg/ml sulfo succinimidobiotin (EZ-Link sulfo-NHS-SS-Biotin: Pierce) for 30 min at 4 °C. The cells were then rinsed with the quenching solution in the cell surface protein biotinylation and purification kit (Pierce) once and Tris-buffered saline twice to quench any unbound biotin. The cells were then lysed by the addition of 1 ml of radioimmunoprecipitation assay buffer (100 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Complete EDTA-free; Roche Diagnostics) for 30 min at 4 °C. After removal of the cellular debris, protein concentrations of the lysates were determined using the Bradford method. 1 mg of the lysate proteins were batch-extracted overnight at 4 °C using avidin-coated agarose beads (immobilized NeutrAvidin, Pierce) and then released from the beads by incubation in the SDS-PAGE sample buffer (50 mM Tris/HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, 10% glycerol, 0.001% bromphenol blue, 5 min at 95 °C).

Western Blot Analysis—Cell lysates, immunoprecipitated proteins, or cell surface proteins were separated in 6–10% SDS-PAGE and then blotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were probed with the appropriate primary antibodies. The bound primary antibodies were detected with the corresponding horseradish peroxidase-conjugated secondary antibodies. Signal was visualized using ECL kit (Amersham Biosciences).

Stealth siRNA Treatment—We obtained two Stealth siRNA for mouse Nedd4-2 from Invitrogen. Each siRNA is a 25-bp duplex oligoribonucleotide with a sense strand corresponding to nucleotides 1755–1779 or 2336–2360 of the reported mouse Nedd4-2 coding sequence (GenBank™ accession number BC039746). The sense sequences of Nedd4-2 siRNA 1755 and 2336 are 5’-AAC-UCUCUGGAGUACCGAAGCGGC-3’ and 5’-UUCAGAUCC-ACUUGUAUGUGCC-3’, respectively. Nedd4-2-siRNA control siRNA 1755 and 2336 with sense strands 5’-AAACAUCUCCGGUUGAGCAAAGGCC-3’ and 5’-UUCUGACUACA- CGUUAUGUGGCGG-3’ are the control stealth siRNAs for Nedd4-2 siRNA 1755 and 2336, respectively. 3T3-L1 preadipocytes were transfected by 20 nM of the Nedd4-2 siRNA or the control siRNA in the antibiotic-free growth medium using Lipofectamine 2000 per the instructions of the manufacturer. After siRNA treatment (24 h), the medium containing the Nedd4-2 siRNA or the control siRNA was changed to fresh medium. 24 h after the medium change (i.e. 48 h after the initiation of RNAi treatment), the cells were used for MeAIB uptake experiments or immunoprecipitation as described above and in the figure legends.

RESULTS

Proteasome Inhibition Increased ATA2 Transport Activity by Increasing the Amount of ATA2 Protein in 3T3-L1 Adipocytes and Preadipocytes—To determine the effect of the proteasome inhibition on ATA2 transport activity, we treated 3T3-L1 adipocytes or preadipocytes with a proteasome inhibitor MG132 and then measured the uptake of MeAIB, a specific substrate for ATA2 into the cells (Fig. 1A). In adipocytes as well as preadipocytes, the treatment with MG132 for 4 h accelerated MeAIB uptake significantly. The effects of insulin and MG132 were additive. The uptake of 2DG, a specific substrate for the members of the facilitated glucose transporter family (GLUTs) did not show any change after MG132 treatment either with or without insulin (data not shown). We also used the 3T3-L1 cell line stably transfected with the EGFP-tagged ATA2 (EGFP-ATA2 3T3-L1 cells) to observe the changes of intracellular localization of ATA2. The establishment of this cell line has been described previously. In adipocytes as well as preadipocytes, the fluorescent signals associated with EGFP-ATA2 were markedly increased after treatment with MG132 (Fig. 1B). We compared the fluorescence signals under identical conditions in cells treated with or without MG132. We first optimized the experimental conditions in MG132-treated cells to avoid the saturation of signals and then applied identical conditions to nontreated cells. Strong signals were detected at the perinuclear site in MG132-treated cells. Under these conditions, the signals from nontreated cells were barely detectable. We also analyzed the steady-state levels of the ATA2 fusion protein by Western blot using anti-GFP antibody with whole cell lysates from EGFP-ATA2 3T3-L1 adipocytes and preadipocytes treated with or without MG132 (Fig. 1C). The band corresponding to 74 kDa (indicated with the arrow in Fig. 1C) was the intact EGFP-ATA2 protein as described previously, and the signal intensity of this band was increased by MG132 treatment in adipocytes as well as in preadipocytes. In addition to the band corresponding to the size of the intact, unmodified EGFP-ATA2, there were other protein bands with a range of higher molecular sizes whose intensities were also increased markedly in MG132-treated cells.
ATA2 Protein as a Target for the Ubiquitin-Proteasome Pathway in 3T3-L1 Cells—To determine whether EGFP-ATA2 fusion protein is subject to ubiquitination, we treated the 3T3-L1 adipocytes with or without MG132 and then immunoprecipitated the proteins in the cell lysate with anti-GFP antibody. The proteins in the immunoprecipitate were then separated by SDS-PAGE and then immunoblotted with either anti-ubiquitin antibody or anti-GFP antibody. The presence of the ladder positive to the anti-ubiquitin antibody was detected by Western blot with the immunoprecipitate from MG132-treated cells but not from control cells (Fig. 2). The bands detected with the anti-ubiquitin antibody were of the size ranging from 90 to 250 kDa, suggesting varying degrees of ubiquitination of the fusion protein. In contrast, the proteins detected obviously by the anti-GFP antibody were smaller than 100 kDa including the intact EGFP-ATA2. After the long exposure time for ECL detection, the signals of the proteins with larger molecular sizes were faintly detectable in the blot of the immunoprecipitate from the MG132-treated cells (data not shown). This difference of detectability with the anti-ubiquitin or anti-GFP antibody was supposed to be due to the stoichiometry for polyubiquitination of a target protein EGFP-ATA2 and ubiquitins.

Influence of Ubiquitin Ligase on ATA2 Transport Function upon Co-expression in Xenopus Oocytes—To determine the effect of ubiquitination on ATA2, we co-expressed ATA2 with...
either Nedd4-2 (a HECT domain E3) or c-Cbl (a RING domain E3) in *Xenopus* oocytes and then monitored the transport function of ATA2 by measuring the Na\(^+\)-dependent uptake of MeAIB (Fig. 3). This co-expression system has been used by several investigators to determine the molecular identity of the ubiquitin ligase that interacts with any given target protein (31–33). Co-expression of Nedd4-2 with ATA2 down-regulated ATA2-mediated MeAIB uptake, whereas c-Cbl did not affect MeAIB uptake significantly.

**Influence of Ubiquitin Ligase on ATA2 Transport Function upon Co-expression in CHO Cells**—To investigate the interaction between ATA2 and an E3 ubiquitin ligase in further detail, we co-transfected the cDNAs of ATA2 and Nedd4, Nedd4-2, or c-Cbl in CHO cells. ATA2-specific transport activity was measured by MeAIB uptake (Fig. 4). Ligase-defective cysteine-to-serine mutants in the HECT domain (CS mutants) of Nedd4 and Nedd4-2 were used to determine the involvement of the catalytic activity of ligase in observed changes in ATA2 transport function. MeAIB uptake induced by ATA2 cDNA was found to be decreased markedly by co-transfection with Nedd4-2 in CHO cells, an effect not seen with Nedd4-2 CS mutant. The effect of Nedd4 on ATA2 transport function was minimal compared with that of Nedd4-2. We confirmed by Western blot using appropriate antibodies that Nedd, Nedd4-2, their corresponding CS mutants, and c-Cbl were expressed at comparable levels in these cells (data not shown), showing that the observed differences in the transport function of ATA2 were not due to differences in the expression levels of these ligases.

**Effect of Knockdown of Nedd4-2 on the Activity and Ubiquitination of ATA2 in 3T3-L1 Cells**—To evaluate the physiological relevance of endogenous Nedd4-2 in ubiquitination/internalization/degradation of ATA2, we knocked down the endogenous Nedd4-2 in 3T3-L1 preadipocytes by RNAi and then monitored the transport function of ATA2 by uptake measurements and the extent of ubiquitination of the EGFP-ATA2 fusion protein by Western blot (Figs. 5 and 6). We first confirmed the RNAi-induced down-regulation of Nedd4-2 protein by Western blot using cell lysates prepared from cells treated with two independent Nedd4-2-specific siRNAs or with the corresponding nonspecific scrambled siRNAs (Fig. 5A). Knockdown effect for Nedd4-2 siRNA 1755 or 2336 normalized with \(\alpha\)-tubulin was 86 and 88%. The down-regulation of Nedd4-2 by two independent siRNAs resulted in the significant increase in the transport function of ATA2 as evident from the increase in MeAIB uptake (Fig. 5B). Insulin treatment enhanced the extent of increase of MeAIB uptake induced by both Nedd4-2 siRNAs. With the proteins immunoprecipitated by the anti-GFP antibody from the lysates of cells treated with Nedd4-2 siRNA 1755 or its scrambled control siRNA, we performed the Western blot analysis using the anti-ubiquitin antibody and observed the differences of the band pattern (Fig. 6A). Three bands with approximate sizes of 190, 215, and 240 kDa that were detectable with the anti-ubiquitin antibody in lysates from cells treated with nonspecific siRNA were decreased most in lysates from cells treated with Nedd4-2-specific siRNA (Fig. 6A, left, indicated with the arrowheads). Similar results were obtained when the blots were probed with anti-GFP antibody, although the signals were so weak that the long exposure time for ECL detection was needed (Fig. 6A, right). This difference of detectability with the anti-ubiquitin or anti-GFP antibody is due to the stoichiometry of EGFP-ATA2 and ubiquitin for polyubiquitination. We analyzed the ubiquitination of EGFP-ATA2 on the surface membrane in the cells treated with Nedd4-2 siRNA or its scrambled control after MG132 treatment (Fig. 6B). The Western blots of the cell surface membrane proteins with anti-GFP antibody showed that the intensities from protein bands with a range of higher molecular sizes were decreased by RNAi of Nedd4-2. The bands with the size of 190–250 and 155–170 kDa were decreased most.
Co-localization of ATA2 and Nedd4-2 on the Plasma Membrane in 3T3-L1 Adipocytes—To determine the cellular site for the interaction of ATA2 and Nedd4-2 as a pre-requisite for ATA2 ubiquitination, we compared the localization of EGFP-ATA2 and Nedd4-2 when co-expressed transiently in 3T3-L1 cells (Fig. 7). EGFP-ATA2 was detected mainly at the perinuclear site and on the plasma membranes, as described previously.3 Nedd4-2 was present ubiquitously in whole cytoplasm and to a lesser extent on or near the plasma membrane. A merged image shows the proximity of the existence of both EGFP-ATA2 and Nedd4-2 at the plasma membrane.

DISCUSSION

Two groups including us found that insulin stimulates amino acid uptake acutely without relying on de novo synthesis of the transporter protein in adipocytes and muscle cells and that this involves the specific translocation of amino acid transporter
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ATA2 from an intracellular pool to the plasma membrane (43). The reverse pathway, namely the retrieval of the transporter from the plasma membrane and its subsequent degradation, have not been investigated in detail. Recent studies have demonstrated that the ubiquitin-proteasome pathway is involved in the degradation of plasma membrane transporters (18–20). In the present study we tested the hypothesis that the amino acid transporter ATA2 is internalized and degraded by the ubiquitin-proteasome pathway. This is an internalization and degradation pathway specific for ATA2. This pathway is not involved in the degradation of facilitated glucose transporters. ATA2 is an important transporter in the biology of adipocytes; therefore, the identification of a specific intracellular degradation pathway as a determinant of the plasma membrane density of this transporter has important implications in terms of adipocyte function.

We first showed that proteasome inhibition by MG132 causes an increase of carrier-mediated uptake of MeAIB, a model substrate for amino acid transport system A both in intact, nontransfected preadipocytes and adipocytes (Fig. 1A). There is evidence to indicate that ATA2 corresponds to system A activity in 3T3-L1 cells (15). Using EGFP-ATA2 3T3-L1 preadipocytes and adipocytes, which stably express the EGFP-ATA2 fusion protein, we confirmed these findings (Fig. 1B). These data indicate that the inhibition of proteasome even for 4 h is sufficient to have a marked effect on ATA2 degradation. The data from native adipocytes and EGFP-ATA2 3T3-L1 cells are similar, pointing to the comparable nature of the degradation kinetics of the native ATA2 protein and the EGFP-ATA2 fusion protein. In our previous studies using the EGFP-ATA2 3T3-L1 preadipocytes and adipocytes, we have already shown the similarity of localization and insulin-stimulated translocation between the endogenous ATA2 and EGFP-tagged ATA2.3 We also showed by the Western blot analysis the increase in intact EGFP-ATA2 by proteasome inhibition (indicated with the arrow in Fig. 1C). Moreover, treatment of the cells with proteasome inhibitor MG132 led to the detection of the fusion protein with higher molecular sizes, indicating the ubiquitination of ATA2 to a variable extent. We confirmed these data with anti-GFP antibody immunoprecipitates, thus analyzing specifically the ubiquitination of only those proteins which contained GFP (Fig. 2). With the anti-ubiquitin antibody, we were able to detect proteins of larger size than the intact EGFP-ATA2 very clearly in cells treated with MG132, suggesting the existence of ubiquitinated EGFP-ATA2 and the participation of the ubiquitin-proteasome pathway in ATA2 degradation.

There are several steps in the ubiquitin-proteasome protein degradation pathway, and the conjugation of ubiquitin to a target protein is the initiation in the pathway. Three types of enzymatic activities are necessary to catalyze the conjugation of ubiquitin to a target protein; they are a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3) (21, 35). There is only one isoform of E1 in eukaryotic cells but many different E2s and E3s work together, influencing the turnover rate and specificity of the degradation process. E3 binds to E2 as well as the target protein; thus, E3 plays an important role in the determination of substrate specificity in the ubiquitin-proteasome pathway. Two primary classes of E3s have been described. One is distinguished by a HECT domain (e.g. Nedd4 and Nedd4-2) and the other by a RING domain (e.g. c-Cbl) (21, 35, 36). To establish the molecular identity of the E3 that is specific for ATA2, we tested the interaction of three different E3s: Nedd4, Nedd4-2, and c-Cbl. These three E3s are thought to be involved in the endocytosis of membrane proteins via ubiquitination (35, 36). Recently, Boe-heimer et al. (37) reported that co-expression of Nedd4-2 with amino acid transporter SN1 (SLC38A3) down-regulated SN1-mediated amino acid transport in Xenopus oocytes, although details of the mechanism were not examined. Here we show that the co-expression of Nedd4-2, not c-Cbl, with ATA2 decreased the ATA2 activity in the oocytes significantly (Fig. 3). Moreover, to confirm that Nedd4-2 is indeed the E3 specific for ATA2, we performed the co-expression of ATA2 and Nedd4/ Nedd4-2, their mutants in the HECT domain, or c-Cbl in CHO cells (Fig. 4). The HECT E3s participate in the catalytic reaction by forming a thioester bond with ubiquitin via conserved cysteine residue within the HECT domain during the transfer of ubiquitin from E2 to the target protein (21). The catalytically active wild type Nedd4-2 decreased ATA2 activity in EGFP-ATA2-expressing CHO cells, but the catalytically inactive mutant did not. Nedd4 showed only a minimal effect on ATA2 activity even though the protein was expressed at comparable levels as was Nedd4-2. Therefore, we conclude that Nedd4-2 is the principal E3 involved in degradation of ATA2. We confirmed the role of endogenous Nedd4-2 in the degradation of ATA2 via the ubiquitin-proteasome pathway using the RNAi technique (Figs. 5 and 6). RNAi of Nedd4-2 obviously increased ATA2 activity in 3T3-L1 cells. Interestingly, insulin treatment enhanced the extent of increase of MeAIB uptake induced by both Nedd4-2 siRNAs. Insulin stimulates the translocation of ATA2 to the plasma membrane (43); therefore, this result indicates the Nedd4-2 is involved in the sequestration of ATA2 from the membrane to the intracellular degradation site. We also show for the first time that Nedd4-2 localizes to a significant extent on the plasma membrane in 3T3-L1 adipocytes (Fig. 7). Nedd4-2 was not found to be co-localized with ATA2 at the perinuclear site, the intracellular ATA2 storage site. Instead, the co-localization was evident at the plasma membrane, suggesting that the principal site of ATA2 ubiquitination is on or near the plasma membrane. This is also the first evidence show-
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ing the co-localization of ATA2 and Nedd4-2 on the plasma membrane in the cells. Malik et al. (20) reported that Nedd4-2 is partially bound to the plasma membrane in A6 X. laevis distal nephron cell lines. Palmda et al. (38) also showed that Nedd4-2 is localized on or near the apical membrane as well as in the cytoplasm in human intestinal epithelial cells. Recently, Dunn et al. (39) reported that Rsp5, the yeast ortholog of Nedd4/Nedd4-2, binds membrane phosphoinositides and directs ubiquitination.

Ubiquitin coupling can be two different types; monoubiquitination and polyubiquitination. Monoubiquitination occurs when individual ubiquitin molecules are coupled to lysine residues on a target protein so that the final stoichiometry is one ubiquitin per lysine. In contrast, polyubiquitination involves the coupling of a chain of four or more ubiquitin molecules to one lysine residue on a target protein so that the final stoichiometry is four or more ubiquitin molecules per lysine. Monoubiquitinated proteins are degraded in lysosomes, whereas polyubiquitinated proteins are recognized and degraded by the 26 S proteasome (35, 36). Conjugation of an ubiquitin molecule to target proteins generally leads to a distinct SDS-PAGE mobility shift of relative molecular size with 7–8 kDa. The molecular size of EGFP-ATA2 in SDS-PAGE is 74 kDa as described above. Because we can predict the numbers of lysine in the intracellular domain of ATA2 as 12–15, EGFP-ATA2 proteins with the sizes of more than 180–195 kDa should be polyubiquitinated forms, excluding the possibility of monomoubiquitination. EGFP-ATA2 proteins supposed to be ubiquitinated were detected both in the blot of the proteins immunoprecipitated from total lysates and the cell surface membrane proteins treated with Nedd4-2 siRNA or its scrambled control after MG132 treatment (Fig. 6). RNAi of Nedd4-2 decreased the 190-, 215-, and 240-kDa bands most in the proteins immunoprecipitated from the total lysates and 190–250- and 155–170-kDa bands in the cell surface membrane proteins. The 190–240-kDa bands were common as decreased bands by Nedd4-2 siRNA in both the cell surface membrane proteins and the proteins immunoprecipitated from the total lysates. From the molecular size, the 190–250-kDa bands most likely to be polyubiquitinated EGFP-ATA2, as described above. Interestingly, the 90-kDa band was observed both in the proteins immunoprecipitated from total lysates and the cell surface membrane proteins. This band is likely to be EGFP-ATA2 conjugated with two ubiquitin molecules, implying multimonoubiquitinated EGFP-ATA2. This 90-kDa protein was neither so decreased by Nedd4-2 RNAi in the proteins immunoprecipitated from total lysates nor the cell surface proteins. In addition to the endocytic pathway from the plasma membrane, ubiquitination of transmembrane proteins serves as a sorting signal of the proteins to direct their movement between different cellular compartments, for example, trans-Golgi network or endosomes (40). The ubiquitin ligase other than Nedd4-2 may be involved in the multimonoubiquitination of ATA2. The 190–250-, 155–170-, and 90-kDa proteins and the smearing band pattern were obvious only after MG132 treatment in the cell surface membrane proteins. This indicates that multimonoubiquitination of EGFP-ATA2 is not dominant in the cell surface membrane under a general condition and that it was increased by secondary effect after inhibition of proteasomal degradation of EGFP-ATA2 by MG132 caused the change of a dynamic equilibrium between intact EGFP-ATA2 and polyubiquitinated EGFP-ATA2.

Recently, Haynes et al. (41) reported that Rsp5, a yeast ortholog of Nedd4 and Nedd4-2, is involved in endoplasmic reticulum-associated degradation of a mutant form of the yeast vacuolar carboxypeptidase Y. As described above, insulin treatment enhanced the extent of increase of MeAIB uptake induced by Nedd4-2 RNAi. Insulin stimulates the translocation of ATA2 to the plasma membrane (43); therefore, this result indicates that the increased MeAIB uptake by Nedd4-2 RNAi is mainly attributed to the abolishment of polyubiquitination of ATA2 on the cell surface membrane, not to the inhibition of endoplasmic reticulum-associated degradation for ATA2. It is well established that growth hormone receptor is polyubiquitinated by an ubiquitin ligase (probably SOCS2) and internalized from the plasma membrane and degraded by the proteasome (42) in a similar way to ATA2.

The turnover rate of ATA2 appears to be high in 3T3-L1 adipocytes and preadipocytes because treatment of these cells with MG132 even for only 4 h resulted in more than a 2-fold increase of ATA2 activity in the cell surface. In contrast, the turnover rate of GLUT4, another insulin-responsive and important transporter in adipocytes, is comparatively slow (17) even though the insulin-mediated translocation of GLUT4 from intracellular sites to the plasma membrane is much faster than that of ATA2. GLUT4 trafficking is mainly regulated by insulin (34), whereas ATA2 trafficking as well as its expression is regulated by many factors including insulin and diabetes (3–5). The fast turnover of ATA2 suggested in the present study may reflect the ability of ATA2 expression and trafficking to respond differentially to a diversity of the regulatory factors.

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