γ2-Adaptin, a Ubiquitin-interacting Adaptor, Is a Substrate to Coupled Ubiquitination by the Ubiquitin Ligase Nedd4 and Functions in the Endosomal Pathway*

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γ2-Adaptin is a putative member of the clathrin adaptor protein family with unknown physiological function. We previously reported that γ2-adaptin acts as a ubiquitin receptor by virtue of its ubiquitin-interacting motif. Here we demonstrate that this motif mediates a specific physical interaction with the ubiquitin ligase Nedd4 and promotes ubiquitination of γ2-adaptin. By mapping regions of Nedd4 involved in binding to γ2-adaptin, we identified its C2 domain to be essential, whereas the WW and HECT domains are dispensable. Consistent with this, we uncovered that the C2 domain of Nedd4 is ubiquitinated itself and as such is recruited by the ubiquitin-interacting motif of γ2-adaptin for subsequent ubiquitin conjugation. Unlike known coupled ubiquitination reactions, this novel type of interaction leads to mono- and multi/polyubiquitinated γ2-adaptin. In addition, we show that γ2-adaptin functions in the endosomal/multivesicular body (MVB) pathway. Depletion of γ2-adaptin impairs the degradation of internalized epidermal growth factor and results in defective MVB morphology characterized by significantly enlarged vesicles. These defects cannot be rescued by γ1-adaptin, a closely related homolog of γ2-adaptin, which is unable to bind ubiquitin. Together, these results indicate that γ2-adaptin may operate within the MVB sorting system in a manner different from that of classic adaptins.

The covalent attachment of ubiquitin marks proteins for various cellular fates and functions, including proteasomal degradation, endocytosis, endosomal sorting, DNA repair, and virus budding. One way cells interpret and transmit the information conferred by ubiquitin is through proteins that bind ubiquitin noncovalently. These ubiquitin receptors contain one or more ubiquitin binding domains (UBD),2 of which at least sixteen have been identified to date (1, 2). The first discovered UBD was the ubiquitin-interacting motif (UIM) that is found in several ubiquitin receptors controlling endocytic membrane traffic (3). This class of proteins, including eps15, epsin, Hrs, and Stam, regulates the internalization of plasma membrane proteins into the endocytic pathway as well as the sorting of proteins into the multivesicular body (MVB) in a ubiquitin-dependent manner (4–8). A recent addition to this group of UIM-containing receptors is γ2-adaptin, which we originally identified as a hepatitis B virus (HBV) interacting protein in a yeast two-hybrid screen (9, 10).

γ2-Adaptin is classified as a member of the heterotetrameric clathrin adaptor complex (AP) family (11). APs mediate the sorting of protein cargo and their incorporation into clathrin-coated transport vesicles. Four AP complexes have been identified designated AP-1 through AP-4, and they all exhibit a similar organization consisting of two large subunits, a medium-sized subunit, and a small subunit (11, 12). γ2-Adaptin is highly similar to γ1-adaptin (one large subunit of the trans-Golgi network/early endosome adapter AP-1) in both primary sequence and modular domain architecture (13, 14). Despite their relatedness, γ2-adaptin appears to serve a separate yet unknown function distinct from that of γ1-adaptin. This is evidenced by embryonic lethality in mice upon disruption of the γ1-adaptin gene (15) and by the failure of γ1-adaptin to functionally substitute for the essential role of γ2-adaptin played during HBV egress from human liver cells (10). The unique function of γ2-adaptin may rely on its ubiquitin-interacting ability (10), a feature that has not been described so far for other members of the classic adaptor protein complex family.

First evidence for a ubiquitin-dependent action of γ2-adaptin in the endosomal pathway was provided by studies investigating the host cell requirements of HBV assembly. Budding of this enveloped virus depends on the UIM domain of γ2-adaptin which couples the viral structural components at compartments positive for the late endosomal/MVB marker CD63 (10). Interestingly, during HBV egress γ2-adaptin acts in concert with the cellular ubiquitin ligase Nedd4. However, a direct linkage between ubiquitin recognition by γ2-adaptin and ubiquitin conjugation by Nedd4 has not yet been established.

For some UIM-containing proteins, like eps15, epsin, and Hrs, such a link has recently been discovered by the demonstration that these proteins are themselves ubiquitinated. This modification strictly depends on the presence of their UIMs and is, therefore, referred to as coupled ubiquitination (7, 8, 16). In this process, UIM-directed ubiquitin binding appears to...
recruit ubiquitin ligases to promote the ubiquitination of the receptors themselves. Among the two major classes of ubiquitin ligases, the homologous to the E6-AP carboxy terminus (HECT) domain E3 ligases have preferentially been implicated in coupled ubiquitination (7, 17). Nedd4 (neuronal precursor cell expressed developmentally down-regulated), a prototype member of this family, contains a catalytic HECT domain with an active site cysteine that forms a thioester bond with ubiquitin, a Ca^{2+}-dependent phospholipid binding C2 domain, and two to four WW domains interacting with proline-rich sequences, like the canonical PPXY motif (18–20). In the case of Hrs, Nedd4 and the Nedd4-like ligase AIP4 mediate its ubiquitination (7, 21), whereas eps15 has been shown to recruit monoubiquitinated Nedd4 and the RING-type ubiquitin ligase Parkin for its subsequent modification (17, 22). The functional significance of this UIM-regulated ubiquitin network remains to be resolved, but it has been implicated to control the activity of the endocytic ubiquitin receptors and, hence, of the endocytic machinery (7, 16, 23).

Nedd4 family members also ubiquitinate cellular cargos to target them for endocytosis and subsequent incorporation into intraluminal vesicles of the MVBs for either degradation, lysosomal functions, or exosomal release (18, 19). The sorting of ubiquitinated cargo into this class of vesicles requires the coordinated action of at least three hetero-oligomeric complexes, referred to as ESCRT (endosomal sorting complex required for transport) complexes I, II, and III, and associated proteins. Individual subunits of ESCRT-I and ESCRT-II have different UBDs and appear to function as receptors for ubiquitinated cargo and/or ubiquitinated trans-acting components (24–26). This sorting machinery is also exploited by many enveloped viruses, including HBV, for use in viral budding (27–31). Accordingly, the functional inhibition of individual subunits of the ESCRT machinery results not only in malformed, dysfunctional MVB but also in a loss of function in viral budding (24, 25, 27, 32, 33). A similar phenotype in terms of perturbation of endosomal morphology and viral budding has been reported to occur upon overproduction of γ2-adaptin in mammalian cells (29), suggesting a potential role for this UIM-bearing receptor in the endocytic MVB pathway.

To explore the functional role of γ2-adaptin, we here characterized biological features of this protein with focusing on its ubiquitin binding ability. We found that this property allows a specific and productive interaction with the ubiquitin ligase Nedd4. Unlike well established Nedd4/substrate interactions involving the WW domains of the ligase, we identified a novel type of interaction depending on the UIM of γ2-adaptin and on the ubiquitinated C2 domain of Nedd4. We also show that γ2-adaptin functions in the endosomal/MVB system in a manner different from that of γ1-adaptin.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—The expression vectors for human γ1-adaptin and γ2-adaptin containing N-terminal hemagglutinin (HA) tags (13) were kindly provided by K. Nakayama (Kyoto University). Construction of the γ2ΔUIM mutant devoid of the UIM and the γ2Δ528–785 mutant lacking the hinge and ear domains has been described (10). The γ2Δ193–785 mutant encodes the first 192 amino acids of γ2-adaptin. The human Nedd4.1 gene cloned in plasmid pGADN0T (34) was a gift from F. Bouamr (Howard Hughes Medical Institute, New York). For ectopic expression in the N-terminal FLAG-tagged form, the Nedd4.1 gene was subcloned into the expression vector p3xFLAG (Sigma-Aldrich). The Nedd4.C894S mutant carries a cysteine-to-serine substitution in the HECT domain, whereas the Nedd4ΔHECT mutant contains a deletion of this domain (amino acids 534–900). Nedd4.1-specific domain constructs encoding the N-terminal C2 domain (Nedd4.C2; amino acids 1–161) or the central WW domain (Nedd4.WW; amino acids 210–544) in FLAG-tagged versions were kindly provided by E. Gottwein (University of Heidelberg). For mutation of ubiquitin acceptor sites, the nine lysine residues present in the C2-encoding region were replaced by arginines (Nedd4.C2ΔK). Primer sequences and mutagenesis details will be available on request. The vector for HA-tagged ubiquitin (35) was a gift from M. Treier (EMBL, Heidelberg, Germany) provided by D. Sitterlin (University of Versailles).

**Antibodies**—Commercially available antibodies were as follows: mouse antibodies against the HA epitope tag (Berkeley Antibody Co., Inc.), mouse anti-FLAG antibodies (Sigma-Aldrich), rabbit antibodies against human ubiquitin (Dako), rabbit antibodies against human Nedd4.1 (Santa Cruz Biotechnology), mouse antibodies against human heat shock protein Hsc70 (StressGen Biotechnologies), and mouse antibodies against human γ1-adaptin (Sigma-Aldrich). For detection of untagged γ2-adaptin, a rabbit antiserum generated against its C-terminal 19 amino acids was used (9). The mouse antibody against lysobisphosphatidic acid (LBPA) (36) was kindly provided by J. Gruenberg (University of Geneva, Switzerland). Peroxidase-labeled, secondary antibodies were obtained from Dianova, and fluorophor-labeled antibodies were purchased from Molecular Probes.

**Cells and Transfections**—The human hepatocellular carcinoma HuH-7 and the human cervical cancer HeLa cell lines were used. Transient transfections with plasmid DNAs were performed with Lipofectamine™ Plus (Invitrogen) according to the manufacturer’s instructions. Unless otherwise indicated, plasmid DNAs were used at a 1:1 ratio in cotransfection experiments and adjusted to induce moderate levels of protein expression. For transfection of cells with small interfering RNA (siRNA) plus plasmid DNA, the Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) was used. Briefly, 5 × 10^5 cells per well of a 6-well plate were transfected with 40 pmol of siRNA according to the protocol of the supplier. After 24 h cells were retransfected with 1 μg of RNase-free plasmid DNA using Lipofectamine™ Plus, and cells were harvested after an additional 48 h. The γ2-adaptin-specific siRNA was obtained from Qiagen and targets the nucleotide positions 2065–2085 (AAACCCTGCTTTGCTGTTAAT). The Nedd4.1-specific siRNA (Sigma-Aldrich) corresponds to nucleotide positions 2206 to 2224 (CACATCAACATGAGCTGA). As a control, a nonsense siRNA with no known homology to mammalian genes (Sigma-Aldrich) was used.

**Coimmunoprecipitation Analysis**—To probe for γ2-adaptin/Nedd4 complex formation, cells were cotransfected with HA-tagged γ2-adaptin and FLAG-tagged Nedd4 constructs. After transient transfection, cells were washed with Tris-buffered...
saline (TBS, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) supplemented with 10 mM N-ethylmaleimide (NEM) and lysed with TBS containing 10 mM NEM, 0.5% Triton X-100, 0.01% sodium deoxycholate, and 1× protease inhibitor mixture (Sigma-Aldrich) for 20 min on ice. Lysates were centrifuged for 5 min at 15,000 × g and 4 °C and subjected to immunoprecipitation using superparamagnetic polystyrene beads (Dynabeads M-280 Sheep anti-mouse IgG; DYNAL) that had been pre-coated with anti-FLAG or anti-HA antibodies in phosphate-buffered saline (PBS), 0.2% bovine serum albumin overnight at 4 °C with agitation. For cross-linking the bound antibodies, the beads were washed with 0.2 M triethanolamine, pH 8.2, and incubated with diethyl malelinate dihydrochloride (Sigma-Aldrich) in 0.2 M triethanolamine, pH 8.2, for 30 min at 20 °C. After washing in 50 mM Tris-HCl, pH 7.5, the beads were suspended with PBS, 0.1% bovine serum albumin and reacted with the lysates for 3 h at 4 °C. The immune complexes were washed 3 times with PBS, 0.2% Triton X-100 and once with PBS before SDS-PAGE and Western blotting analyses.

Ubiquitin Pulldown and Ubiquitination Assays—The ubiquitin pulldown assay was done as described (10). Briefly, transfected cells were lysed in assay buffer (25 mM Hepes-KCl, pH 7.4, 75 mM NaCl, 0.25% Triton X-100, 2.5 mM magnesium acetate supplemented with protease inhibitor), and cleared lysates were incubated with ubiquitin-agarose (Sigma-Aldrich) for 4 h at 4 °C with rocking. Precipitates were then washed 3 times with 25 mM Hepes-KCl, pH 7.4, 75 mM NaCl, and 0.25% Triton X-100 and once with 125 mM Tris-HCl, pH 6.8.

To probe for ubiquitination, cells were lysed in PBS containing 1% SDS, 10 mM N-ethylmaleimide, and protease inhibitor. After boiling for 10 min at 95 °C, the lysates were adjusted to 1× radioimmune precipitation assay buffer (by 10-fold dilution with PBS containing 1.1% Nonidet P-40 and 0.55% sodium deoxycholate) and cleared by centrifugation at 15,000 × g for 10 min at 4 °C. Samples were subjected to immunoprecipitation using a 10% protein A-Sepharose slurry or tosy1-activated, superparamagnetic polystyrene beads (DYNAL) that had been pre-coated with specific antibodies as outlined above. After immune complex formation for 4 h at 4 °C with agitation, the slurry/beads were washed twice with PBS, 0.1% Nonidet P-40 and once with 125 mM Tris-HCl, pH 6.8, and prepared for SDS-PAGE.

Preparation of Membrane and Cytosolic Fractions—Cells were incubated with 0.1× TBS for 10 min on ice, and swollen cells were disrupted by Dounce homogenization (20 strokes) and adjusted to 1× TBS. After centrifugation for 20 min at 2500 × g and 4 °C, the postnuclear supernatant was layered on 250 mM sucrose, TBS and ultracentrifuged for 45 min at 160,000 × g and 4 °C in a SW60 rotor (Beckman) to separate membrane and cytosolic fractions. Proteins in the cytosolic fraction were precipitated with 10% trichloroacetic acid. The precipitates and pelleted membrane fraction were adjusted to the same volume sample buffer and analyzed by SDS-PAGE.

Immunofluorescence Microscopy—For protein immunostaining, cells grown on coverslips were fixed and permeabilized with ice-cold methanol containing 2 mM EGTA for 15 min at −20 °C. After washing and blocking for 30 min in PBS containing 2% animal serum, cells were incubated with the indicated primary antibodies for 1 h at 37 °C, rinsed with PBS, and then incubated with AlexaFluor-tagged secondary antibodies for 1 h at 37 °C. DNA was stained with Hoechst 33342 (Sigma-Aldrich). Coverslips were washed with PBS and mounted onto slides using Fluoprep mounting medium (Biomereux). For LPBA staining, cells were fixed with 3% paraformaldehyde, PBS for 20 min at room temperature, washed with PBS, and then treated with 50 mM ammonium chloride for 10 min. After blocking with PBS, 0.1% bovine serum albumin, cells were reacted with the anti-LPBA antibody in the presence of 0.05% saponin for 30 min at room temperature. After washing with PBS, cells were incubated with the Alexa-conjugated specific secondary antibody for 30 min and further processed as above. Z-stack images were acquired separately for each channel using a Zeiss Axiovert 200M microscope equipped with a Plan-Apochromat 100× (1.4 NA) and a Zeiss AxioCam digital camera. Axiosvision software 4.6 was used for merging pictures. Z-stack images were optically deconvoluted using the software supplied by Zeiss. Tiffs were assembled into figures using Photoshop CS2 (Adobe).

RESULTS

γ2-Adaptin, but Not γ1-Adaptin, Binds Ubiquitin via Its UIM—The ubiquitously expressed human γ2-adaptin is closely related to γ1-adaptin and shares the domain structure, consisting of an N-terminal head and a C-terminal ear domain that are connected by a hinge region (11, 13, 14) (Fig. 1A). Previously, we identified a UIM motif in the head domain of γ2-adaptin (LSLAVLNSSNV) that resembles the conserved UIM core sequence ΦXXXΑΑΑΑΑΑΑΑ (Φ denotes a large hydrophobic residue, X is any amino acid, and e denotes an acidic residue) (3). Mutational analyses revealed that the substitution of critical residues of the UIM diminished the ubiquitin binding ability of γ2-adaptin, whereas a precise deletion of the UIM completely blocked ubiquitin binding (10). Because the head domains of γ2- and γ1-adaptin show 69% amino acid identity (13), we inspected the sequence of γ1-adaptin and found a similar sequence pattern (LSFALVNGNNI). This prompted us to analyze whether γ1-adaptin could also bind ubiquitin. HuH-7 cells were transfected with a HA-tagged version of γ1-adaptin, and lysates were incubated with ubiquitin-agarose. As controls, HA-tagged wild-type (wt) γ2-adaptin and the UIM-deleted γ2UIM mutant were included in the ubiquitin pulldown assay. Although all three constructs were expressed at the same levels, γ1-adaptin did not interact with ubiquitin (Fig. 1B). As the ubiquitin binding ability is not conserved in γ1-adaptin, this feature may contribute to distinct functions of the two γ-adaptins.
**Coupled Ubiquitination of β2-Adaptin by Nedd4**

**A**

![Diagram](image)

**B**

![Image](image)

**C**

![Image](image)

**D**

![Image](image)

**FIGURE 1.** Ubiquitin binding and ubiquitination properties of γ-adaptins. **A**, a schematic representation of wt γ2-adaptin and its mutants used in this study. The domain architecture of γ2-adaptin with the head, hinge, and ear domains is depicted. **B**, γ2-adaptin, but not γ1-adaptin, binds ubiquitin via the UIM. HA-tagged versions of γ1-adaptin, γ2-adaptin, and the UIM-deficient γ2ΔUIM mutant were expressed in HuH-7 cells, and protein expression levels (Input) were analyzed by HA-specific Western blotting. Lysates were incubated with ubiquitin-agarose, and bound proteins were separated by SDS-PAGE before HA-specific immunoblotting (Pulldown). The amount of γ2-adaptin bound to ubiquitin-agarose corresponds to 16% of the input amount. **C**, γ2-adaptin is ubiquitinated in an UIM-dependent manner. Cells expressing wt γ2-adaptin or γ2ΔUIM were lysed by boiling in SDS. Extracts were analyzed by anti-HA immunoblotting or were subjected to HA-specific immunoprecipitation (IP) before Western blotting (WB) with an anti-ubiquitin antiserum. D, γ2-adaptin is ubiquitinated within its N-terminal head domain. Cells expressing the HA-tagged γ2Δ528-785 or γ2Δ193-785 deletion mutants were lysed under denaturing conditions, and extracts were probed by HA-specific immunoblotting. The star to the left of panels D and C denotes ubiquitinated γ2-adaptin molecules.

**UIM-directed Ubiquitination of γ2-Adaptin**—Recent works have demonstrated that many ubiquitin receptors, especially those that contain UIM motifs, are frequently ubiquitinated themselves (7, 8, 16). To probe whether γ2-adaptin shares this property, cells were transfected with HA-tagged γ2-adaptin and lysed under denaturing conditions to prevent a possible post-lysis removal of ubiquitin. When extracts were analyzed by HA-specific immunoblotting, γ2-adaptin appeared in its expected position of 90 kDa. Importantly, a slower migrating form of γ2-adaptin with an apparent molecular mass of ~96 kDa could be reproducibly detected under these assay conditions (Fig. 1C). To test whether this 96-kDa species might represent ubiquitinated γ2-adaptin, extracts were subjected to HA-specific immunoprecipitation, and the immune complexes were examined by Western blotting using anti-ubiquitin antibodies. These antibodies clearly recognized the 96 kDa form, thus demonstrating that a fraction of γ2-adaptin is modified by monoubiquitination (Fig. 1C). An overexposure of the blot detected not only the 96-kDa form but also slower-migrating bands with a ladder-like appearance (data not shown), suggesting that γ2-adaptin is modified by a mixture of mono-, multi-, and polyubiquitinated moieties. Prompted by this finding, we next analyzed the UIM-deficient γ2ΔUIM mutant in the same experimental setting. Thereby, no evidence for a ubiquitin modification of this mutant was obtained (Fig. 1C), indicating that ubiquitination of γ2-adaptin requires the presence of its UIM.

Previous analyses of UIM-bearing proteins have shown that their ubiquitination often occurs at sites that are N-terminal to the UIMs but not in a UIM itself (1, 16). γ2-Adaptin contains a high number of target lysine residues that hamper precise mapping of its ubiquitination site(s). For an initial mapping, we took use of a deletion mutant (γ2Δ528–785) that lacks the C-terminal hinge and ear domain. This mutant was predominantly synthesized in its calculated mass of about 58 kDa and, importantly, appeared in a second form with a molecular weight enlarged by about 6 kDa, likely resembling ubiquitinated γ2Δ528–785 (Fig. 1D). Hence, γ2-adaptin undergoes ubiquitination in its N-terminal head domain. A larger deletion encompassing not only the hinge/ear regions but also parts of the head domain including the UIM (γ2Δ193–785) consistently gave no signs of ubiquitinated species (Fig. 1D). This adds further proof that ubiquitination of γ2-adaptin is coupled to its ubiquitin interacting capacity.

**UIM-dependent Interaction of γ2-Adaptin with Nedd4.1**—For some ubiquitin receptors, their UIM-directed ubiquitination has been implicated to involve E3 ubiquitin ligases of the Nedd4-like family (7, 17). This observation together with our recent demonstration that γ2-adaptin cooperates with the Nedd4.1 ligase to promote virus egress in HBV-replicating liver cells (10) prompted us to probe for a possible link between these two proteins. To test for an in vivo interaction between γ2-adaptin and Nedd4.1 (termed “Nedd4”), coimmunoprecipitation experiments were performed. HuH-7 cells were cotransfected with HA-tagged γ2-adaptin and FLAG-tagged Nedd4, and cell extracts were probed with epitope-specific antibodies to show efficient synthesis of γ2-adaptin and Nedd4. When immune complexes were isolated with antibodies against the HA-tagged γ2-adaptin and examined by FLAG-specific Western blotting, we could easily identify coprecipitated Nedd4 (Fig. 2). This binding was specific, as only a faint background band was visible in samples prepared from cells transfected with Nedd4 alone. To examine whether the UIM of γ2-adaptin had
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FIGURE 2. γ2-Adaptin interacts with Nedd4 in a UIM-dependent manner. HA-tagged versions of wt or mutant γ2-adaptin were cotransfected with FLAG-tagged Nedd4 in HuH-7 cells. Each cotransfection was done at a 1:1 DNA ratio, and empty plasmid was used as a negative control (Control). Stable synthesis (Input) of the γ2-adaptin constructs and Nedd4 is shown by immunoblotting of lysates with anti-HA (top) and anti-FLAG (middle) antibodies, respectively. The input amounts correspond to 12% of the samples used for immune capture. For coimmunoprecipitation, lysates depicted in lanes 1–4 were reacted with anti-HA antibodies before Western blotting (WB) with the FLAG-specific antibody (bottom). Extracts of cells expressing γ2Δ528–785 or γ2Δ193–785 plus Nedd4 (lanes 5 and 6) were immunoprecipitated (IP) with anti-FLAG antibodies followed by HA-specific Western blotting. IgLC denotes unspecifically stained immunoglobulin G light chains.

an impact on the association with Nedd4, lysates of γ2ΔUIM/Nedd4 cotransfected cells were assayed. Thereby, we observed that the γ2ΔUIM mutant was totally blocked in Nedd4 binding. Consistent with this was the behavior of the two γ2Δ528–785 and γ2Δ193–786 deletion mutants. Although the UIM-positive γ2Δ528–785 construct associated with Nedd4, the UIM-negative γ2Δ193–785 mutant did not (Fig. 2). Of note, to avoid comigration of the γ2-adaptin mutants with precipitating antibody chains, the coimmunoprecipitation assay was reversed in that Nedd4 was captured by antibodies and coprecipitated γ2-adaptin constructs were analyzed by immunoblotting. Together, these data show a stable, UIM-directed interaction between γ2-adaptin and Nedd4.

The C2 Domain of Nedd4, but Not the WW and HECT Domains, Mediates Interaction with γ2-Adaptin—Because the association of γ2-adaptin with Nedd4 involves its UIM and, presumably, binding of the UIM to ubiquitin, we reasoned that the UIM might bind to the ubiquitin moiety linked through a thiolester to the catalytic cysteine residue of Nedd4. A schematic representation of the domain structure of Nedd4 including the location of the active site cysteine within the HECT domain is shown in Fig. 3A (18–20). To test this hypothesis, we constructed two catalytically inactive Nedd4 mutants either by substituting the active cysteine to serine (Nedd4.C894S) or by deleting the HECT domain (Nedd4ΔHECT). The mutants were efficiently synthesized in transfected HuH-7 cells, and surprisingly, they both retained the capacity to interact with γ2-adaptin (Fig. 3B). The removal of the HECT domain even resulted in a stronger γ2-adaptin binding as compared with the active site Nedd4 mutant. These results led us to conclude that the UIM-dependent binding of γ2-adaptin to Nedd4 does not require (i) the ubiquitin attached to the catalytic site of the ligase, (ii) the ligandin HECT structural domain, and (iii) its enzymatic activity. To understand how Nedd4 forms a stable complex with γ2-adaptin, we made use of two other mutants carrying only its N-terminal C2 (Nedd4.C2) or central WW domains (Nedd4.WW) and tested them for an association with γ2-adaptin. The Nedd4.C2 construct still interacted with γ2-adaptin, whereas Nedd4.WW did not (Fig. 3B), suggesting that the C2 domain of Nedd4 plays a key role in binding γ2-adaptin. This finding may also account for the higher affinity of Nedd4ΔHECT to γ2-adaptin as compared with Nedd4.C894S, as the C2 domain has been shown to form an intramolecular interaction between the HECT domain in a subset of HECT-type ubiquitin ligases (37). Preventing such an interaction by deleting HECT of Nedd4 may render its C2 domain more accessible to make contacts with γ2-adaptin.

Nedd4 Is Ubiquitinated within Its C2 Domain—The finding that the γ2-adaptin-Nedd4 complex formation involves ubiquitin and the ligase C2 domain was unexpected and implied that Nedd4 per se and its C2 domain in particular may undergo ubiquitination. To detect such a modification, FLAG-tagged full-length Nedd4 and Nedd4.C2 were individually cotransfected with a plasmid encoding HA-tagged ubiquitin. The immunoprecipitation of lysates prepared in boiling SDS with anti-FLAG antibodies followed by immunoblotting with anti-HA antibodies demonstrated specific ubiquitinated Nedd4 forms that were absent in control-transfected cell lysates (Fig. 4A). However, because of the simultaneous analysis of Nedd4.C2 on the same gel, the resolution of these forms is imperfect. Importantly, an even more pronounced ladder of ubiquitinated forms could be detected for the Nedd4.C2 construct (Fig. 4A), implicating that this domain is a target for ubiquitin modification. To corroborate this finding, a Nedd4.C2 mutant was constructed in which all lysine residues,
the known ubiquitin attachment sites, were substituted by arginines (Nedd4.C2ΔK). The wt and mutant C2 domains were cotransfected with HA-tagged ubiquitin, and lysates were prepared under denaturing conditions. The comparative analysis of the pattern of bands showed that at least three of the slower migrating species of Nedd4.C2 represented ubiquitinated forms, as they were absent in the Nedd4.C2ΔK mutant (Fig. 4B). Based on this finding, we next analyzed whether preventing ubiquitination of Nedd4.C2 might interfere with binding of γ2-adaptin. HA-tagged γ2-adaptin was cotransfected with FLAG-tagged Nedd4.C2 or Nedd4.C2ΔK, and cellular extracts were immunoprecipitated with anti-FLAG antibodies followed by HA-specific immunoblotting. As shown in Fig. 4C, the ubiquitination-defective Nedd4.C2ΔK mutant was almost completely blocked in γ2-adaptin binding activity. Collectively, these data suggest a novel type of interaction that depends on the UIM of γ2-adaptin and the ubiquitinated C2 domain of Nedd4.

FIGURE 3. The C2 domain of Nedd4 binds to γ2-adaptin. A, schematic structure of wt and mutant Nedd4. The C2, WW, and HECT domains with the active site cysteine residue are indicated. Below the Nedd4.C894S, Nedd4ΔHect, Nedd4.C2, and Nedd4.WW mutants are illustrated. B, Nedd4 interacts with γ2-adaptin via C2 but not via WW and HECT. HuH-7 cells were transfected with HA-tagged γ2-adaptin plus the indicated FLAG-tagged Nedd4 mutants. Cell extracts were tested for the expression of the constructs by specific Western blotting (Input), and the migration of the proteins is indicated on the left of the panels. The input amounts correspond to 12% of the samples used for immune capture. For the extracts shown in lanes 1 and 2, the subsequent coimmunoprecipitation assay was done with anti-HA antibodies followed by FLAG-specific Western blotting (WB), whereas extracts depicted in lanes 3 and 4 were subjected to FLAG-specific immunoprecipitation (IP) followed by HA-specific WB.

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γ2-Adaptin Colocalizes with Nedd4 and Recruits Its C2 Domain—For corroboration of these results, we performed deconvolution immunofluorescence microscopy to visualize the distribution of γ2-adaptin and Nedd4 in cotransfected HuH-7 cells. γ2-Adaptin yielded a cytoplasmic vesicular staining with some enrichment in punctuate structures (Fig. 5). Nedd4 was found mostly throughout the cytoplasm if expressed alone, and this distribution was not grossly altered upon coexpression with γ2-adaptin. In line with our coprecipitation analyses, an overlay of the fluorescence patterns revealed an extensive degree of colocalization of Nedd4 and γ2-adaptin. Colocalization was also found for Nedd4.C2, but differently. This protein was largely redistributed and recruited by coexpressed γ2-adaptin and accumulated in punctuate structures (Fig. 5). Concomitantly, the distribution of γ2-adaptin also changed, as it became substantially enriched in these structures. One likely interpretation of this finding might be that the complex formed between γ2-adaptin and Nedd4.C2 is unable to dissociate due to the missing WW and HECT domains of the ligase. In the case of Nedd4.WW that cannot bind γ2-adaptin, we could neither detect a significant colocalization nor a γ2-adaptin-induced sequestration (Fig. 5). We conclude from these data that γ2-adaptin binds and recruits Nedd4 via the C2 domain.
**Coupled Ubiquitination of γ2-Adaptin by Nedd4**

**γ2-Adaptin Is Ubiquitinated by Nedd4**—To examine whether Nedd4 binding leads to ubiquitination of γ2-adaptin, we used siRNA to reduce endogenous levels of Nedd4. HuH-7 cells were cotransfected with untagged γ2-adaptin plus HAtagged ubiquitin and either a control siRNA or a Nedd4-specific siRNA, and cells were lysed under denaturing conditions. As shown in Fig. 6A, the Nedd4 siRNA effectively reduced the amount of ubiquitinated forms of γ2-adaptin compared with control siRNA-treated cells (Fig. 6A). Besides Nedd4.1, mammalian cells express the closely related Nedd4.2 isofrom (19). Because this isofrom should not be targeted by our silencing approach, these data suggest that γ2-adaptin is specifically ubiquitinated by Nedd4.1. To substantiate this finding, we took use of the catalytically inactive, dominant-negative Nedd4.C894S mutant. Upon overexpression of this mutant in cells synthesizing untagged γ2-adaptin and HA-tagged ubiquitin, the degree of γ2-adaptin ubiquitination was dramatically reduced (Fig. 6B).

**Membrane Association of γ2-Adaptin Is Different from That of γ1-Adaptin and Does Not Involve Nedd4 Function**—Classic adaptor proteins, like γ1-adaptin, with functions in intracellular vesicle trafficking are known to transiently interact with the cytosolic side of their donor and acceptor organelle membranes but otherwise are predominantly cytosolic (11, 38). To determine whether or not γ2-adaptin exhibits this characteristic, we compared the subcellular fractionation behavior of endogenous γ1- and γ2-adaptin. HuH-7 homogenates were separated into soluble and membrane-associated fractions and probed by specific immunoblotting. The sufficient separation of the fractions was confirmed by assessing the distribution of the cytosolic heat shock protein Hsc70 that was exclusively found in the cytosolic pool (Fig. 7A). γ1-Adaptin was mainly present in the soluble fraction with minor quantities in the membrane fraction, as expected (38). In contrast, γ2-adaptin was equally distributed in both fractions, indicating that its membrane association is substantially stronger than that of γ1-adaptin (Fig. 7A). To our surprise, its ubiquitinated forms were completely partitioned to the membrane fraction. Similar results were obtained upon ectopic expression of γ2-adaptin (Fig. 7B, lanes 1 and 2). The UIM-deficient γ2ΔUIM mutant yielded almost the same distribution profile as the wt protein, implicating that the UIM-directed ubiquitination of γ2-adaptin is not responsible for its membrane localization (Fig. 7B, lanes 3 and 4). To further define elements important for recruiting...
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| Individual Expression | Coexpression |
|-----------------------|--------------|
| A Nedd4               | D merge      |
| E Nedd4.C2            | H merge      |
| I Nedd4.WW            | L merge      |

**FIGURE 5. γ2-Adaptin colocalizes with Nedd4.** HuH-7 cells were transfected with wt or mutant FLAG-tagged Nedd4 constructs either individually (A, E, and I) or in combination with HA-tagged γ2-adaptin as indicated (B–D, Nedd4 + γ2-adaptin; F–H, Nedd4.C2 + γ2-adaptin; J–L, Nedd4.WW + γ2-adaptin). Cells were fixed and immunostained with mouse anti-FLAG and rat anti-HA antibodies. After staining with AlexaFluor 546-conjugated anti-rat and AlexaFluor 488-conjugated anti-mouse antibodies, cells were analyzed by deconvolution fluorescence microscopy. The staining pattern of the Nedd4 constructs is shown in green, and the fluorescent signal of γ2-adaptin is in red. The fluorescence overlays are shown in the merged images with yellow indicating colocalization. Insets in these images display enlarged sections that are shown in the right column. Bar, 10 μm.

**FIGURE 6. γ2-Adaptin is ubiquitinated by Nedd4.** A, depletion of Nedd4 inhibits γ2-adaptin ubiquitination. HuH-7 cells were transfected with siNedd4 or control siRNA duplexes (siControl) or with siControl and coexpressed with wt γ2-adaptin antibodies followed by HA-specific Western blot. The communoprecipitation was done with anti-γ2-adaptin antibodies followed by HA-specific Western blot.

A second transfection with untagged γ2-adaptin and HA-tagged ubiquitin DNA was performed 1 day later, and cellular lysates were harvested by boiling in SDS after an additional 2 days. Lysates were subjected to Nedd4-ubiquitination. Untagged γ2-adaptin plus HA-tagged ubiquitin were coexpressed with wt Nedd4 (Nedd4.wt) or the dominant-negative Nedd4.C894S mutant (Nedd4.dn). Lysates were examined by specific immunoblotting to probe for efficient protein expression (Input). The commounprecipitation was done with anti-γ2-adaptin antibodies followed by HA-specific Western blot.

### γ2-adaptin to membranes, we analyzed its two C-terminal-truncated γ2A528–785 and γ2A193–785 mutants and found that both mutants displayed the wt-like distribution pattern (Fig. 7B, lanes 5–8). Collectively, these results point to another distinctive feature between γ2-adaptin and γ1-adaptin, as they differ considerably in their partition to membrane fractions. Furthermore, to our surprise the first 192 amino acids of γ2-adaptin are sufficient to mediate its peripheral membrane association.

Although these data pointed toward a dispensability of the UIM for γ2-adaptin membrane association, we nonetheless tested whether Nedd4 had an impact on this property. We, therefore, investigated the distribution profile of γ2-adaptin in Nedd4-depleted cells. Consistent with the results shown above, the knockdown of Nedd4 substantially inhibited γ2-adaptin ubiquitination (Fig. 7C). The distribution of γ2-adaptin to soluble and membrane-associated fractions, however, was virtually unaffected irrespective of whether Nedd4 was depleted or not. Thus, binding of γ2-adaptin to Nedd4 is not necessary for its membrane association.

**γ2-Adaptin Is Required for Normal Endosomal Morphology**—Our previous works have implicated that γ2-adaptin may function in the endosomal/MVB system, as it partially colocalizes with CD63-positive compartments in HBV-replicating liver cells and as it is able to perturb the endosomal morphology if heavily overexpressed (10, 29). To further assess the physiological role of γ2-adaptin, we reduced its endogenous expression in HuH-7 cells using siRNA (Fig. 8A) and examined the distribution of LBPA, a phospholipid enriched in MVBs/late endosomal membranes (36). Depletion of γ2-adaptin altered the morphology of MVBs marked by LBPA in that they were significantly enlarged and clustered (Fig. 8A). A similar endosomal enlargement phenotype was evident in γ2-adaptin-depleted HeLa cells (data not shown), implying that it is not merely restricted to liver cells.

**γ2-Adaptin Is Required for Efficient Degradation of EGF**—The altered morphology of MVBs/late endosomes suggests that depletion of γ2-adaptin may affect some steps of the endosomal membrane traffic. To provide functional data supporting this, we monitored endocytosis of EGF that is bound to its receptor at the cell surface then internalized and delivered via the MVB pathway to the lysosomal lumen for degradation (39). Control- and γ2-adaptin-depleted cells were incubated with Alexa-conjugated EGF for 30 min and
the ubiquitination of γ2-adaptin is specifically mediated by Nedd4.1, as evidenced by the lack of modification in Nedd4.1-depleted cells. Thus, neither the Nedd4.2 isoform nor other HECT-type ubiquitin ligases can functionally substitute for Nedd4.1 in this process. In support for the specific involvement of Nedd4.1, we identified a physical interaction between this ligase and γ2-adaptin in life cells. This interaction involves the UIM of γ2-adaptin pointing toward a dual function of this motif in ubiquitin binding and promoting ubiquitination. In this regard, γ2-adaptin represents a new example of a subset of ubiquitin receptors, including epsin15, epsin, and Hrs, which undergo UIM-directed self-ubiquitination (7, 8, 16). However, unlike these receptors, γ2-adaptin becomes not only monoubiquitinated but also multi/polyubiquitinated. One possible explanation for the apparently distinct outcomes of coupled ubiquitination is that the nature of interactions between the ubiquitin receptors and their ligases engaged may be different. In the case of epsin15 that is exclusively monoubiquitinated by Nedd4, no stable interaction could be detected between the substrate and its ligase (17). This has been interpreted in such that, after catalysis of monoubiquitination, the contact between enzyme and substrate is rapidly lost, thereby restricting further modification events. In contrast, γ2-adaptin tightly associates with Nedd4. Because Nedd4 has been shown to be capable in catalyzing both monoubiquitination and polyubiquitination (40), its high affinity to γ2-adaptin may allow its catalytic activity to direct the addition of mono- plus multi/polyubiquitin chains.

By mapping regions of Nedd4 involved in binding to γ2-adaptin, we found that its catalytic HECT domain in general and its active site cysteine in particular are dispensable. This indicates that the UIM of γ2-adaptin does not recognize the thiolester-conjugated ubiquitin of Nedd4 but, rather, ubiquitin moieties attached to lysine residue(s) of the ligase. In support of this view, we identified the C2 domain of Nedd4 as the critical determinant of complex formation with γ2-adaptin and uncovered its modification with ubiquitin. It, therefore, appears that the UIM of γ2-adaptin targets ubiquitin C2 of Nedd4 for subsequent ubiquitination of the adaptor. These findings confirm and extend recent observations drawn from an in vitro study in which the mechanistic basis for the coupled monoubiquitination of eps15 has been investigated (17). Similar to γ2-adaptin, eps15 can be ubiquitinated by Nedd4 in a process that requires the ubiquitination of the ligase. If ubiquitin was
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A

B

FIGURE 8. Depletion of γ2-adaptin affects the morphology of endosomal compartments and inhibits EGF degradation. A, for analysis of MVB morphology, the distribution pattern of LBPA was monitored in control-treated HuH-7 cells (siControl) and in cells treated with specific siRNA against γ2-adaptin (siγ2) by staining with anti-LBPA antibodies (bottom). The knockdown efficiency of endogenous γ2-adaptin was quantitated at protein level by anti-γ2-adaptin Western blotting (top). The experiment was repeated three times, and representative images are shown. B, for analysis of EGF uptake and degradation, the fate of internalized Alexa 488-EGF was investigated in control-treated HuH-7 cells (siControl) and in cells treated with specific siRNA against γ2-adaptin (siγ2). Cells were stimulated with labeled EGF for 30 min and examined for Alexa 488-EGF and DNA staining either directly (top) or after a chase for 3 h (bottom). Bars, 10 μm.

covalently attached to Nedd4 in vitro, the ability of the ligase to execute monoubiquitination of eps15 was profoundly enhanced. As similar mechanisms apparently apply for the coupled ubiquitination of eps15 and γ2-adaptin, it is tempting to speculate that this may be a general strategy employed by other UIM/UBD-containing proteins. In this respect it will be interesting to decipher the nature of the ubiquitin ligase responsible for ubiquitination of Nedd4. Moreover, our discovery that the ubiquitinated C2 domain of Nedd4 is essential for the recognition and, hence, ubiquitination of γ2-adaptin raises an important question of whether this domain is relevant for other productive Nedd4/substrate interactions. In addition, it will be of interest to determine whether C2 domains of other HECT family members are modified with ubiquitin to regulate substrate selection. One result arguing in favor is provided by the ubiquitin ligase Rsp5, the sole member of the Nedd4 family in Saccharomyces cerevisiae, whose C2 domain has been shown to be required for ubiquitination of endosomal cargo (41). Interestingly, two lysine-rich clusters of the Rsp5 C2 domain critically contribute to cargo recognition and ubiquitination, likely by mediating precise localization of the ligase to endosomal membranes for subsequent function. However, whether this lysine-rich patch is functionally modified with ubiquitin, as it is the case for the Nedd4 C2 domain, has not been experimentally addressed in that study.

The Nedd4 C2 domains are Ca2+/lipid binding modules and are thought to function as membrane recruitment domains involved in protein localization and trafficking (18–20). At first glance it, therefore, appears possible that Nedd4 contributes to the recruitment of γ2-adaptin to membranes. However, our results argue against this possibility because (i) a γ2-adaptin mutant defective in Nedd4 binding still cofractionates with membranes, (ii) the membrane localization of γ2-adaptin is not distorted in Nedd4-depleted cells, and (iii) the Nedd4 C2 domain is redistributed by γ2-adaptin rather than vice versa. Accordingly, one possible function of γ2-adaptin may be to act as an adaptor for Nedd4, recruiting it to membrane compartments for subsequent ubiquitination. In support, we observed that ubiquitinated γ2-adaptin chains are exclusively present in membrane fractions. Such a role of γ2-adaptin to localize or enrich Nedd4 in a specific cellular environment would be consistent with functions of other proteins interacting with Nedd4 family members. For example, annexin XIIIb physically interacts with the C2 domain of Nedd4, thereby recruiting the ligase to the apical plasma membrane of polarized epithelial cells (42). In the case of the lysosomal-associated multispansing membrane protein 5 (LAPTMs5), Nedd4 is recruited to assist in LAPTM5 trafficking from the Golgi to the lysosome (43). Note-worthy, however, neither annexin XIIIb nor LAPTM5 become ubiquitinated by the recruited ligase which is in contrast to γ2-adaptin. It, therefore, remains to be solved whether γ2-adaptin binding to Nedd4, its ubiquitination by Nedd4, or both features are critical for its function.

At present, we do not know the precise function of γ2-adaptin, but the results of this and previous works provide compelling evidence that this protein acts in the endosomal/MVB sorting and trafficking system. We show that the knockdown of γ2-adaptin leads to a prominent decrease in the degradation of endocytosed EGF concomitant with its accumulation in enlarged vesicles. Furthermore, the loss of γ2-adaptin results in defective MVB morphology characterized by considerably enlarged vesicle structures that stained positive for LBPA, a highly specific marker for MVBs (36). These results coincide with our previous studies demonstrating that heavily overexpression of γ2-adaptin perturbed the normal endosomal morphology and induced aberrant, dysfunctional MVBs (29). To explain the perturbing effect of both deficit and excess γ2-adaptin on MVB structure and function, it seems conceivable that an imbalance in the concentration of the adaptor might interfere with productive interactions required for proper MVB maintenance. Intriguingly, the phenotypes induced by up- and down-
regulation of γ2-adaptin are reminiscent to those evoked by functional loss of subunits of the MVB sorting machinery, in particular of ESCRT-III and Vps4 (24–26, 32, 33). Hence, γ2-adaptin may act within the ESCRT machinery by virtue of its ubiquitination and/or ubiquitin- and Nedd4 binding abilities. Several known subunits of this machinery contain ubiquitin recognition modules that are required to sort ubiquitinated cargo into the forming MVB vesicles (24–26) and that may bind ubiquitinated trans-acting components, such as γ2-adaptin, to coordinate cargo transport and vesicle formation. Within this network, the UIM of γ2-adaptin may serve as a docking site for Nedd4, thereby bringing the ligase to endosomal compartments. Nedd4 family members have been implicated to function in endocytic trafficking and both viral and vesicle budding into the MVBs (27), but so far a direct association between the ligases and the ESCRT core machinery, including ESCRT-I, -II, -III, and Vps4, has not been detected (44). This has led to the suggestion that the ubiquitin ligases may interact with the ESCRT cascade via an as yet unidentified bridging factor that might be regulated by ubiquitination (28, 44). Based on the results presented here, it is tempting to speculate that one such factor may be γ2-adaptin.

Although the native function of γ2-adaptin remains to be established, our results add new evidence for γ2-adaptin being a unique entity distinct from γ1-adaptin. Our silencing experiments indicate that the loss of γ2-adaptin cannot be rescued by γ1-adaptin with regard to both the endosomal enlargement phenotype and the delay in EGF degradation. Because γ1-adaptin does not share the ubiquitin binding ability of γ2-adaptin, this character likely prevents its access to the ubiquitin network and presumably to the endosomal/MVB pathway. Unlike γ1-adaptin, γ2-adaptin in turn may not operate as a typical vesicle-forming adaptor but, rather, as a monomeric ubiquitin receptor. In favor of this view, a γ2-adaptin-containing AP complex has not been described, and a yeast two-hybrid screen using γ2-adaptin as bait did not render interactions with other adaptin subunits.3 In this respect, γ2-adaptin would resemble the monomeric GGAs (Golgi-localizing, γ-adaptin ear domain homology, ADP-ribosylation factor-binding proteins) that sort cargo without being major constituents of the transport vesicle (45). GGAs also possess ubiquitin-interacting activity (46, 47), and GGA3 has been shown to become monoubiquitinated by the ring-type ubiquitin ligase hVPS18 in a UBD-dependent manner (48, 49).

In summary, we have unraveled some mechanistic insights of ubiquitin receptor ubiquitination. Whether similar mechanisms apply for coupled ubiquitination of candidate proteins other than γ2-adaptin remains to be determined. The finding that γ2-adaptin appears to act in the MVB sorting pathway is another aspect that deserves further study.

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3 M. Rost, T. Döring, and R. Prange, unpublished data.

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