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The Connecdenn Family, Rab35 Guanine Nucleotide Exchange Factors Interfacing with the Clathrin Machinery*[S]

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Rabs constitute the largest family of monomeric GTPases, yet for the majority of Rabs relatively little is known about their activation and recruitment to vesicle-trafficking pathways. We recently identified connecdenn (*DENND1A*), which contains an N-terminal DENN (differentially expressed in neoplastic *versus* normal cells) domain, a common and evolutionarily ancient protein module. Through its DENN domain, connecdenn functions enzymatically as a guanine-nucleotide exchange factor (GEF) for Rab35. Here we identify two additional connecdenn family members and demonstrate that all connecdenns function as Rab35 GEFs, albeit with different levels of activity. The DENN domain of connecdenn 1 and 2 binds Rab35, whereas connecdenn 3 does not, indicating that Rab35 binding and activation are separable functions. Through their highly divergent C termini, each of the connecdenns binds to clathrin and to the clathrin adaptor AP-2. Interestingly, all three connecdenns use different mechanisms to bind AP-2. Characterization of connecdenn 2 reveals binding to the β2-ear of AP-2 on a site that overlaps with that used by the autosomal recessive hypercholesterolemia protein and βarrestin, although the sequence used by connecdenn 2 is unique. Loss of connecdenn 2 function through small interference RNA knockdown results in an enlargement of early endosomes, similar to what is observed upon loss of Rab35 activity. Our studies reveal connecdenn DENN domains as generalized GEFs for Rab35 and identify a new AP-2-binding motif, demonstrating a complex link between the clathrin machinery and Rab35 activation.

Clathrin-mediated endocytosis (CME)[3] is a major mechanism for internalization of proteins and lipids. Clathrin-coated vesicles (CCVs) form at the plasma membrane and deliver their cargo to early endosomes from were it either recycles or is targeted for degradation. Central to the formation of CCVs at the plasma membrane is the clathrin adaptor protein 2 (AP-2). AP-2 is a heterotetramer consisting of two large subunits, α and β2, a medium sized μ2 subunit, and a small σ2 subunit. The α and β2 subunits each contain an N-terminal trunk domain, which along with μ2 and σ2 form the core of AP-2. In addition, α and β2 contain C-terminal, ~30-kDa ear (or appendage) domains, separated from the trunk by flexible linkers. The core of AP-2 targets it to the plasma membrane, whereas the β2-ear and linker bind and promote the polymerization of clathrin. The α- and β2-ear domains serve as recruitment hubs, binding accessory proteins and bringing them to sites of CCV formation (1).

Despite low sequence homology, the α- and β2-ears have a similar bi-lobed structure, consisting of N-terminal sandwich and C-terminal platform subdomains (2–4). Each of the four subdomains binds to specific peptide motifs, allowing the ears to recruit a wide variety of accessory proteins. The presence of four distinct binding sites, each of which has a different binding affinity, is likely important for the correct temporal ordering during the recruitment of endocytic accessory proteins. Additionally, proteins will often contain more than one binding motif conferring higher affinity binding thorough avidity effects (5, 7, 12).

Endocytic accessory proteins that bind the ears have multiple roles in CCV formation. For example, a subset of these proteins, known as clathrin-associated sorting proteins, targets cargo to clathrin-coated pits. One such protein, ARH, binds to clathrin and the β2-ear and mediates the CME of the low density lipoprotein receptor (9, 14, 15). Clinically, mutations in ARH result in inherited hypercholesterolemia (16, 17). Likewise, G-protein-coupled receptors (GPCRs) rely on the clathrin-associated sorting protein βarrestin for CME. This ensures that only activated GPCRs are endocytosed as activation-induced phosphorylation of the GPCR causes a conformational change in βarrestin, allowing βarrestin access to its β2-ear binding site (18–20). Despite this complexity, our knowledge of the endocytic adaptor proteins involved in CME and cargo recruitment is likely far from complete.

Once a CCV is uncoated, it is transported to its target organelle where it undergoes fusion. The Rab family of monomeric GTPases, along with their effectors control many aspects of organelle function, including transport, tethering, and fusion (21, 22). Rabs oscillate between active GTP- and inactive GDP-bound states. Guanine nucleotide exchange factors (GEFs)

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Experimental Procedures, Table 1, and Figs. 1–4.

The nucleotide sequences reported are available in the Third Party Annotation section of the DDBJ/EMBL/GenBank™ Databases under the accession numbers TBA:BK006958–BK006960.

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[3] The abbreviations used are: CME, clathrin-mediated endocytosis; CCV, clathrin-coated vesicle; AP-2, adaptor protein-2; GPCR, G-protein-coupled receptor; GEF, guanine nucleotide exchange factor; siRNA, small interference RNA; MBP, maltose-binding protein; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; CHC, clathrin-heavy chain; TD, terminal domain; WT, wild type; CD, connecdenn.

mediate the exchange of GDP to GTP, allowing Rabs to bind effectors. Although Rabs constitute the largest group of monomeric GTPases, relatively few Rabs have assigned GEFs, preventing a better understanding of their complex roles in membrane trafficking (21, 23). One Rab that had been relatively undefined functionally is Rab35. However, a recent study localized Rab35 to the plasma membrane and endocytic compartments where it was implicated in a fast recycling pathway (24). Additionally, Rab35 regulates a recycling pathway in T cells involved in the formation of the immunological synapse, and it has been linked to the fast recycling of major histocompatibility complex class II-peptide complexes (25, 26). In PC12 cells, Rab35 is linked to the actin cytoskeleton in neurite outgrowth, whereas in *Drosophila melanogaster* Rab35 controls actin bundling during bristle formation (11, 27).

We previously identified connecdenn (encoded by the gene *DENND1A*) as a component of the machinery for CME (28). Connecdenn utilizes multiple peptide motifs in its C-terminal region to engage the α-ear of AP-2, whereas the N-terminal region contains a differentially expressed in neoplastic *versus* normal cells (DENN) domain. DENN domains are found in a wide variety of proteins of seemingly unrelated functions, including myotubularin-related 5 and 13, DENN/MADD/Rab3GEP, Rab6 interacting protein 1, and suppressor of tumorigenicity 5, many of which have been related to human diseases (29–32). The DENN domain invariably consists of three modules, an upstream (uDENN), DENN and downstream (dDENN) module, separated by linkers of varying lengths, however the structure and function of this domain is poorly characterized (33). Interestingly, a link between connecdenn and Rab35 came with the observation that in *Caenorhabditis elegans*, the connecdenn homologue RME-4 binds to Rab35 and recruits it to CCVs for subsequent transport to endosomes where it controls yolk receptor recycling (34). Moreover, we have recently determined that the DENN domain of connecdenn (herein referred to as connecdenn 1) functions as a GEF for Rab35, allowing for the transport of active Rab35 to endosomes where it controls the recycling of specific endosomal cargo (35). Here we identify two additional DENN domain proteins, and, although they have limited homology with connecdenn 1 outside of the DENN domain, we demonstrate that they form a conserved family. Like connecdenn 1 (35), connecdenn 2 and 3 also function as Rab35 GEFs, but the three DENN domains display distinctly different levels of activity and ability to bind Rab35. Although the C termini of the connecdenns are highly divergent, they have each evolved a unique method to bind AP-2 ears, highlighting the importance of coupling Rab35 activation and recruitment to the clathrin machinery. Interestingly, connecdenn 2 uses a novel β2-ear-binding motif that is predictive of the AP-2 binding capacity of other proteins. Loss of connecdenn 2 results in an enlargement of early endosomes similar to what we recently observed following knockdown of either connecdenn 1 or Rab35 (35), demonstrating that both connecdenn 1 and 2 have important roles in regulating the activation of Rab35 and its subsequent function at early endosomes.

EXPERIMENTAL PROCEDURES

Antibodies, DNA Constructs, and siRNA—Information regarding antibodies and expression constructs can be found in the supplemental information. siRNA targeting human connecdenn 2 was purchased from Sigma (sequence #1 starts at nucleotide 645, sequence #2 at nucleotide 698). The non-targeting sequence corresponds to the siRNA Universal Negative Control #1 from Sigma.

Knockdown, Immunofluorescence, and Transferrin Uptake—Detailed information regarding the use of siRNA for protein knockdown as well as on immunofluorescence and transferrin uptake can be found in the supplemental information.

Tissue Extraction—Adult rat tissue was homogenized in buffer 1 (20 mM HEPES, pH 7.4, supplemented with protease inhibitors: 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin) and centrifuged at 800 × *g* for 10 min. Equal protein aliquots of the post-nuclear supernatants were analyzed by SDS-PAGE and Western blot. CCVs were purified from rat brain and stripped in 0.5 M Tris as previously described (36).

Pulldown Assays—For pulldown assays from tissue extracts, frozen adult rat brain was homogenized in buffer 1 and centrifuged at 800 × *g* for 10 min, the supernatant was collected, and Triton X-100 was added to a 1% final concentration. The samples were incubated for 15 min at 4 °C, then centrifuged at 205,000 × *g* for 30 min. The supernatant was adjusted to a final concentration of 2 mg/ml in buffer 1 with 100 mM NaCl and 1% Triton X-100. For recombinant proteins, FLAG- and green fluorescent protein-tagged fusion proteins were expressed in HEK-293T cells. At 48 h post transfection, cells were washed with phosphate-buffered saline, scraped into buffer 1 with 0 or 100 mM NaCl, sonicated, and Triton X-100 was added to 1% final concentration. After 15-min incubation at 4 °C, the lysates were centrifuged at 20,000 × *g* for 15 min, and protein expression levels in the supernatant were determined by Western blot. For purified protein, connecdenn 2 tagged with maltose-binding protein (MBP) was expressed in *Escherichia coli* BL21. Bacterial lysates were incubated with amylose resin, and, after washing, the beads were eluted with buffer 1 containing 10 mM D-maltose. The eluate was centrifuged at 205,000 × *g* for 30 min, and the supernatant was adjusted to a final concentration of 0.1 μg/ml in buffer 1 and brought to 1% Triton X-100 and 100 mM NaCl. For competition assays with purified MBP-ARH, protein was expressed and purified as above, then concentrated to a final concentration of 2 μg/μl, and added to the pulldown assays at the molar ratios indicated in the figure. Aliquots of 1 ml of the Triton-soluble brain extract, transfected cell lysates, or purified MBP fusion protein were incubated with GST fusion proteins pre-coupled to glutathione-Sepharose beads. Samples were incubated for ~3 h at 4 °C, washed three times with ice-cold buffer 1 containing 1% Triton X-100 and 0 or 100 mM NaCl, and samples were eluted in SDS-PAGE sample buffer, resolved by SDS-PAGE, and processed for Western blotting. For details on nucleotide state-dependent pulldown assays, see the supplemental information.

Immunoprecipitation Assays—Triton-solubilized rat brain homogenate was prepared as for pulldown experiments in

buffer 1 with a final concentration of 30 mM NaCl, and immunoprecipitation was performed as previously described (6).

In Vitro GDP/GTP Exchange Assays—GST-tagged Rab35 GTPase and connecdenn 1, 2, and 3 DENN domains were expressed in HEK-293T cells. At 48 h post transfection, cells were collected in phosphate-buffered saline with protease inhibitors, sonicated, and Triton X-100 was added to 1% final concentration. The lysates were incubated for 15 min at 4 °C and spun at 205,000 × *g* for 30 min. The supernatant was incubated with glutathione-Sepharose beads for 1 h at 4 °C, washed three times in thrombin cleavage buffer (50 mM Tris, pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM dithiothreitol), and the purified fusion proteins were then cleaved from the GST tag by overnight incubation with 3 units of thrombin at 4 °C. The thrombin was cleared with benzamidine-Sepharose. Cleaved GTPases were then exchanged into GEF loading buffer (20 mM Tris, pH 7.5, 100 mM NaCl), and cleaved DENN domains were exchanged into GEF incubation buffer (20 mM Tris, pH 7.5, 100 mM NaCl, and 5 mM $MgCl_2$). For time curve assays, 15 μM of purified GTPase was loaded with 40 μM GDP by incubation for 10 min at 30 °C in GEF loading buffer with 5 mM EDTA, and loaded GDP was then stabilized by the addition of 10 mM $MgCl_2$. Exchange reactions were carried out at room temperature in 130 μl of total volume containing 1.25 μM loaded GTPase, 150 nM DENN domain, 0.5 mg/ml bovine serum albumin, 5 μM GTPγS, 0.2 mCi/mmol [^{35}S]GTPγS, 0.5 mM dithiothreitol in GEF incubation buffer. At the indicated times, 15 μl of the reaction was removed, added to 1 ml of ice-cold wash buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$), and passed through nitrocellulose filters. The filters were rapidly washed with 5 ml of wash buffer and counted using a liquid scintillation counter (Beckmann Coulter LS6500 Scintillator). For substrate concentration assays, 32 μM of purified GTPase was loaded with 80 μM GDP as above and then diluted to the indicated substrate concentration. Exchange reactions were carried out in 15 μl of total volume containing the indicated concentration of loaded GTPase, 150 nM DENN domain, 0.5 mg/ml bovine serum albumin, 5 μM GTPγS, 0.2 mCi/mmol [^{35}S]GTPγS, 0.5 mM dithiothreitol in GEF incubation buffer. After 1 min, 1 ml of ice-cold wash buffer was added to stop the reaction, and samples were counted as above. Data were plotted in GraphPad Prism 4, curve fit by a nonlinear regression one-phase association. For time-course experiments, $n = 2$, mean ± S.E. For substrate concentration experiments, $n = 3$, mean ± S.E.

RESULTS

The Connecdenn Family—We originally identified connecdenn 1 as a DENN domain-bearing protein enriched on CCVs (28). Connecdenn 1 is encoded by the gene DENN/MADD domain containing 1A (*DENND1A*). Two additional genes, annotated *DENND1B* and *DENND1C*, encode proteins with 38 and 32% overall identity, respectively, with connecdenn 1 (Fig. 1*A* and supplemental Fig. 1*A*). *DENND1A* and *DENND1B* are found on chromosomal regions 9q33.2 and 1q31.3, respectively that have a shared microsynteny with Crumbs homologue 2 and Crumbs homologue 1 (37). Our examination of the chromosomal region 19p13.3 revealed that *DENND1C* is adjacent to Crumbs homologue 3. Thus, *DENND1B* and *DENND1C* appear to be paralogues of connecdenn 1/*DENND1A*. We have therefore named these proteins "connecdenn 1–3."

Connecdenn 1 is expressed predominantly in brain and testis, although it can be detected in multiple additional tissues and cell lines (Fig. 1*B*) (28, 35). At least two splice variants are predicted from databases (supplemental Fig. 1*B*) with our antibody directed to a sequence specific to the longer form. Connecdenn 2 is ubiquitously distributed and runs as a prominent doublet (Fig. 1*B*). At least four splice variants are predicted with alternative C-terminal regions, with or without a 20-residue region in the DENN domain (supplemental Fig. 1*B*). The protein doublet detected on Western blot may represent the long form with and without the DENN domain insert. Connecdenn 3 is expressed in every tissue tested with prominent expression in brain and weak expression in skeletal muscle (Fig. 1*B*).

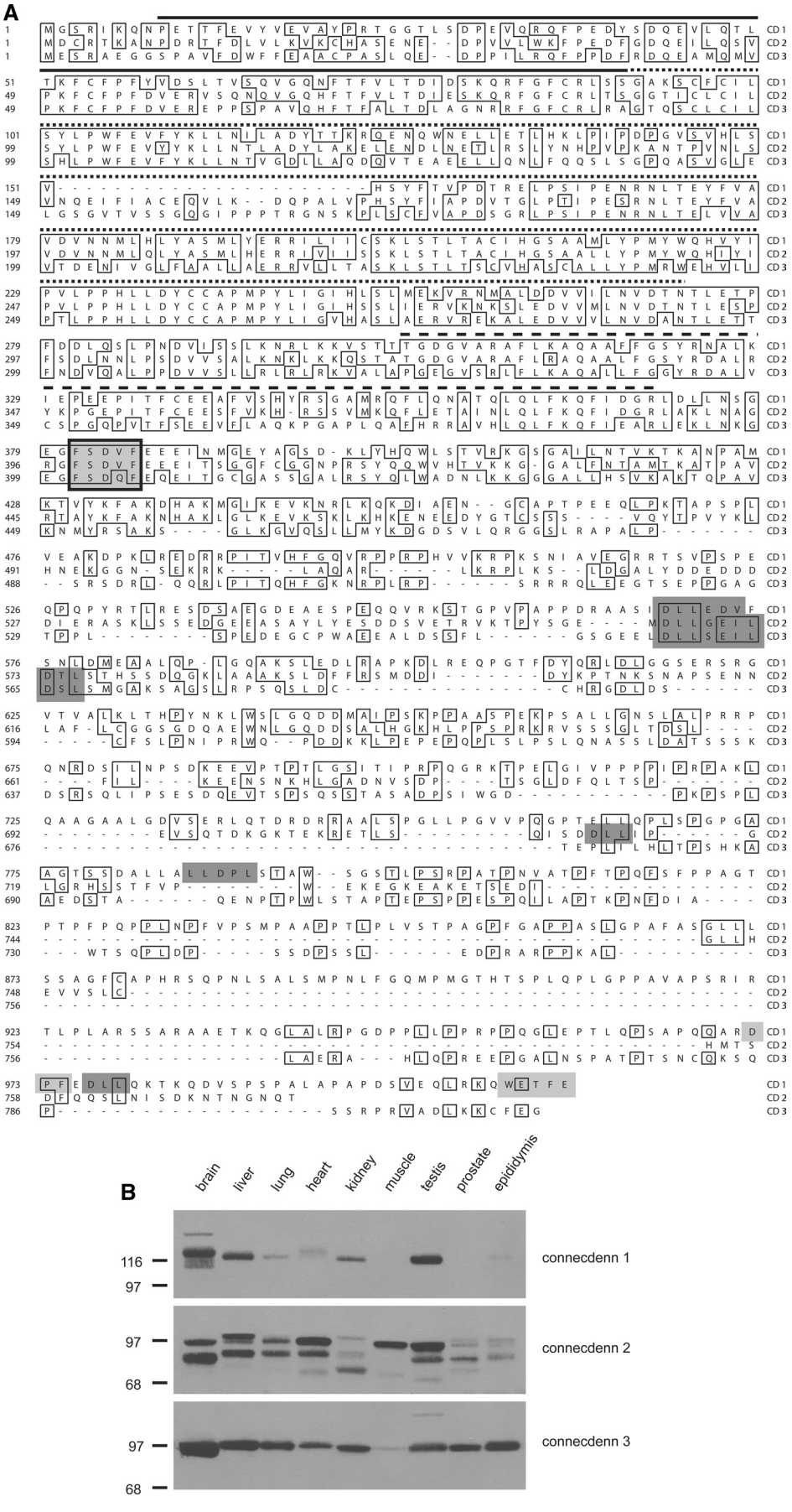
Connecdenn Family DENN Domains Function as GEFs for Rab35—The DENN domain of connecdenn 1 binds to Rab35 in the nucleotide-free form and activates it by functioning as a GEF (35). Whether or not DENN domains can be generalized as GEFs is unknown. Generally, nucleotide-free binding to their substrate GTPase is a hallmark feature of GEFs. We thus tested the other connecdenns for Rab35 binding and found that, like connecdenn 1, the DENN domain of connecdenn 2 binds stably to Rab35 in the nucleotide-free but not GTPγS-loaded state (Fig. 2*A*). No binding to Rab3 or Rab6 was seen for either connecdenn under either condition (Fig. 2*A*). For connecdenn 3, there is only a small degree of Rab35 binding, and it is nucleotide-independent. Interestingly, however, all three DENN domains demonstrate robust GEF activity toward Rab35 (Fig. 2*B*). Connecdenn 1 is faster, converting almost all GDP-loaded Rab35 to the GTP form within 1 min (Fig. 2*B*). Under identical conditions, the connecdenn 2 DENN domain requires ~6 min, whereas connecdenn 3 had not quite reached maximal exchange after 10 min (Fig. 2*B*). To better compare the activities we measured the reaction rate per minute of each DENN domain against increasing concentrations of Rab35 substrate. This allowed us to fully appreciate that the DENN domain of connecdenn 1 has more robust GEF activity than that of connecdenn 2 and 3 (Fig. 2*C*). The DH-PH module of intersectin 1-long, a GEF for Cdc42 (38), had no activity against Rab35 (Fig. 2*C*). The efficient nature of the DENN domains as GEFs toward Rab35 is in contrast with the Rab5 GEF Rabex-5 (35). Rabex-5 has relatively weak activity on Rab5 until in a complex with the effector Rabaptin-5 (39, 40). These data clearly indicate that all three connecdenns function as GEFs for Rab35, with different levels of exchange activities. Moreover, they suggest that Rab35 binding and GEF activity are distinct properties of the DENN domain.

The Connecdenns Are CCV-associated Proteins—Connecdenn 1 is enriched on CCVs (28) (Fig. 3*A*). Because, like connecdenn 1, connecdenn 2 and 3 also contain clathrin-heavy chain (CHC)-binding motifs and an AP-2-binding F*X*D*X*F motif (Fig. 1*A*), we predicted that all connecdenns would be present on CCVs. Indeed connecdenn 2 and 3 are detected on CCVs (Fig. 3*A*), although unlike connecdenn 1, they also display a substantial cytosolic (S2) pool. Whereas connecdenn 1 is recruited to CCVs via C-terminal interac-

tions' with the clathrin machinery, its DENN domain mediates a stable membrane interaction with the vesicle once the clathrin and AP-2 coat has been removed (35). Interestingly, stable association with uncoated vesicles is a property unique to connecdenn 1 (Fig. 3*B*).

Scanning the C-terminal regions of connecdenn 1–3 revealed that each protein contains multiple potential motifs for binding to the terminal domain of the CHC (CHC-TD) (Fig. 1*A* and supplemental Fig. 2*A*). Each protein contains a single type II motif (42) and a recently described type III motif (43). In addition, connecdenn 1 and 2 contain two DLL motifs, whereas connecdenn 3 contains a single DLL (supplemental Fig. 2*A*; see Ref. 44). We thus performed pulldown studies using the CHC-TD with tagged forms of full-length connecdenn 1–3 and found all three connecdenns bind CHC-TD (supplemental Fig. 2*A*). Thus, all connecdenns are clathrin-binding proteins that are associated with CCVs.

Connecdenn Family Proteins Bind to AP-2—Connecdenn 1 uses a strong W*XX*F-acidic motif and a weaker F*XD*X*F* motif to bind the AP-2 α-ear sandwich and platform domains, respectively (see Fig. 7) (28). Connecdenn 2 and 3 conserve the F*XD*X*F* motif (Fig. 1*A*), we thus tested for their potential binding to the α-ear. As previously described (28), connecdenn 1 has robust binding to wild-type (WT) α-ear (Fig. 3*C*). Connecdenn 2 also binds the α-ear with no binding to the platform mutant R916A (Fig. 3*C*), which is known to disrupt F*XD*X*F* interactions (3, 5). Intriguingly, connecdenn 3 also binds the α-ear but, like connecdenn 1, the R916A mutation has little effect while the Q782A mutation in the sandwich sub-domain, which disrupts W*XX*F-acidic motif interactions (8), dramatically reduces binding. Deletion analysis revealed that connecdenn 3 uses a site between amino acids 650 and 675 to bind the α-ear sandwich (see Fig. 7 and supplemental Fig. 2*B*). Interestingly, the



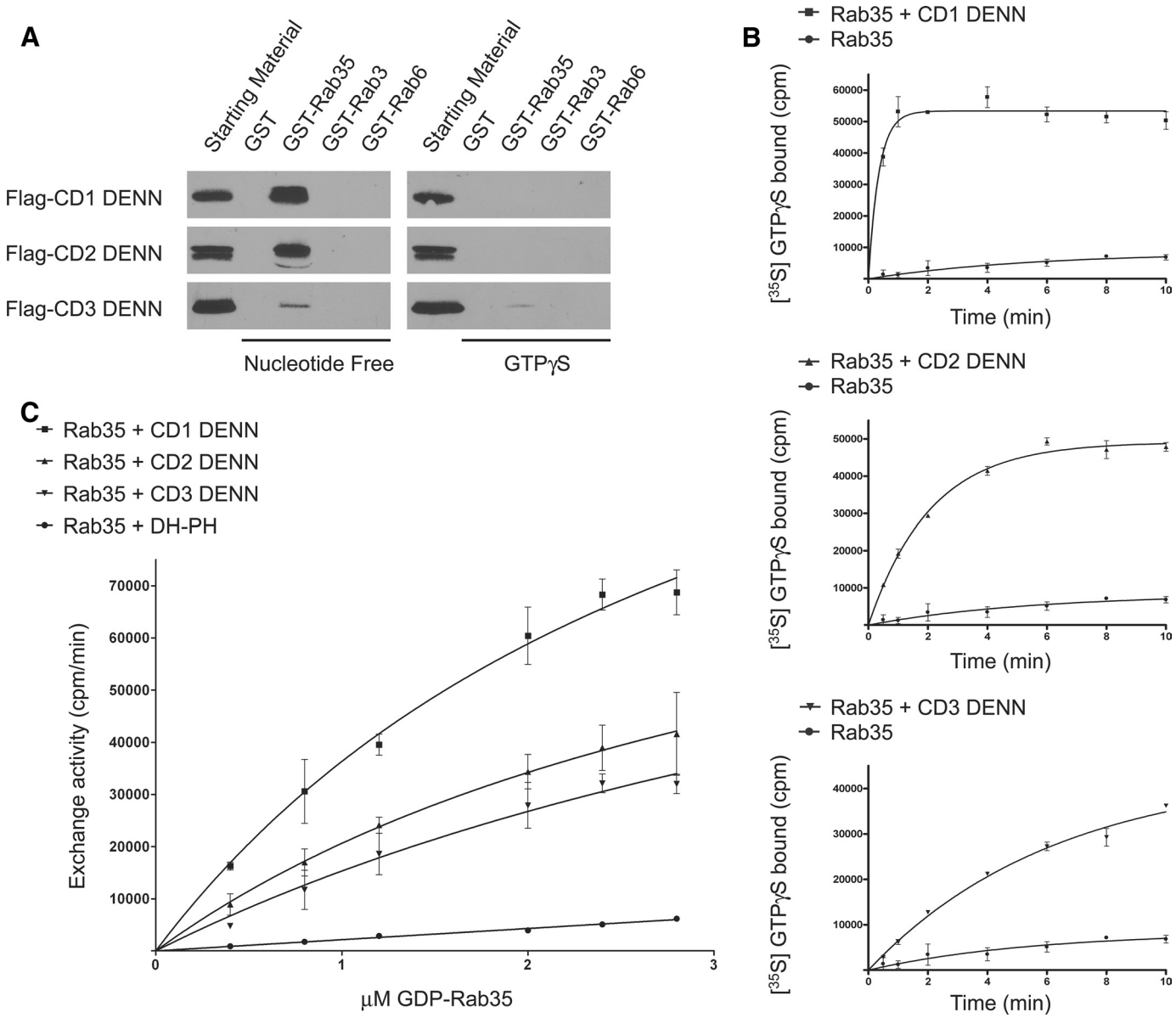


FIGURE 2. **Generalized GEF activity of connecdenn DENN domains.** *A*, GST, GST-Rab35, GST-Rab3, and GST-Rab6, maintained in the nucleotide-free state with 5 mM EDTA or pre-loaded with GTPγS, were used to affinity-purify FLAG-tagged DENN domain of connecdenn (*CD*) 1–3 from HEK-293T cell lysates. Specifically bound proteins were detected by anti-FLAG antibody. Cell lysate (starting material) equivalent to 1/10th that added to beads was analyzed in parallel. *B*, GEF activity of 150 nM purified DENN domains of CD 1–3 or basal exchange activity of Rab35 alone measured as the relative incorporation of GTP-[γ-^{35}S] onto 1.25 μM GDP-loaded Rab35 as a function of time. *C*, rate of GDP to GTP exchange activity per minute of 150 nM purified DENN domains of CD1 (*square*), CD2 (*triangle*), and CD3 (*inverted triangle*), or DH-PH domains of intersectin long (*circle*) as a function of concentration of GDP-loaded Rab35.

650–675 region contains no sequences resembling a W*XX*F-acidic motif, the only known motif for the α-ear sandwich site, suggesting that connecdenn 3 contains a new sandwich-binding motif. It is unlikely that binding is indirect given that the HEK-293T cell lysates used have extremely high levels of tagged connecdenn 3 relative to endogenous protein. The exact nature of the binding site is currently unknown.

Connecdenn 2 Defines a New β2-Ear-binding Motif—We used the β2-ear as a control in our binding studies, and unexpectedly, connecdenn 2 bound this domain (Fig. 3*C*). However, none of the known β2-ear-binding motifs (10, 12, 13, 45) are present in connecdenn 2. To identify the region of connecdenn 2 involved in binding we used a series of C-terminal deletion constructs and narrowed the binding site to a region between amino acids 467 and 550 (supplemental Fig. 3*A*). We then generated a series of 20-amino acid overlapping fragments spanning this region. The construct 497–516 bound FLAG-β2-ear, but not FLAG-α-ear, whereas no other con-

FIGURE 1. **The connecdenn (CD) family.** *A*, alignment of human connecdenn 1/*DENND1A* (gi55749779), connecdenn 2/*DENND1B* (gi218563726), and connecdenn 3/*DENND1C* (gi74750652). Identical amino acids are *boxed*. The uDENN, DENN, and dDENN modules of the DENN domain are overscored with *solid*, *dotted*, and *dashed lines*, respectively. The *outlined gray box* indicates conserved AP-2 α-ear platform binding F*X*D*X*F motifs. *Dark gray shading* indicates clathrin-binding boxes and DLL motifs, and *light gray shading* indicates AP-2 α-ear-binding DPF and W*XX*F-acidic motifs. *B*, equal protein extracts (200 μg) of rat tissues blotted with antibodies against connecdenn 1, 2, and 3. Molecular masses are indicated in kilodaltons.

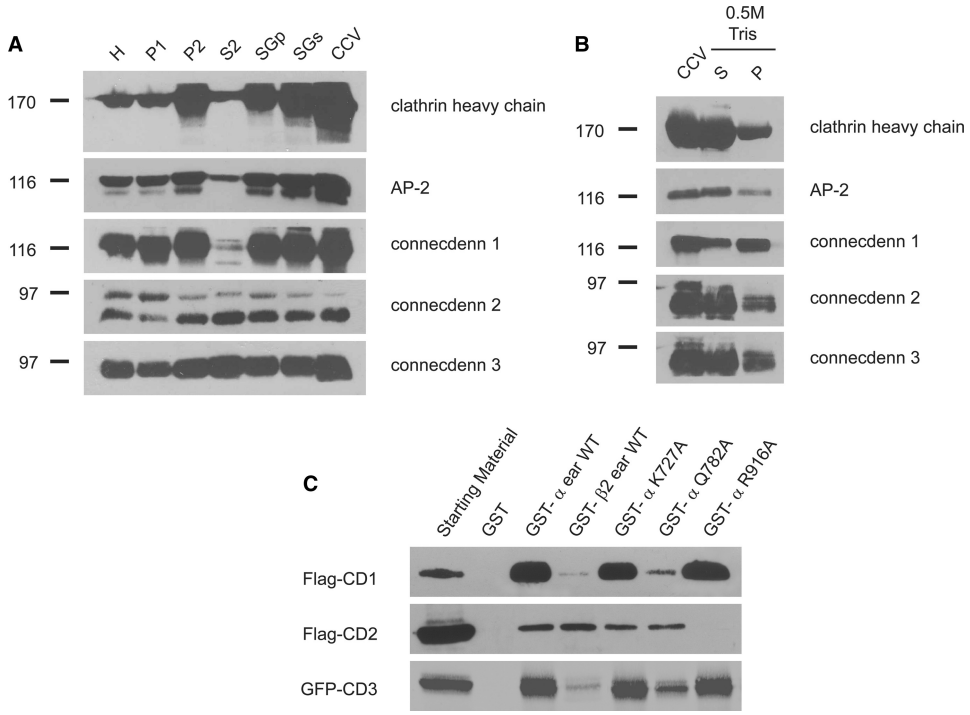


FIGURE 3. **Connecdenn 1, 2, and 3 are CCV-associated proteins with unique AP-2-binding properties.** *A*, equal protein aliquots of rat brain subcellular fractionations leading to highly enriched CCVs blotted with the indicated antibodies (homogenate (*H*), pellet (*P*), supernatant (*S*), and sucrose gradient (*SG*)). *B*, CCVs were stripped of their coats by two successive rounds of incubation with 0.5 M Tris, stripped CCVs were centrifuged, and the supernatant (*S*) and pellet (*P*) fractions were analyzed. *C*, soluble lysates of FLAG-CD1, FLAG-CD2, and green fluorescent protein (GFP)-CD3 from transfected HEK-293T cells were incubated with equimolar amounts of GST or GST fused to wild-type α- and β2-ear and the indicated α-ear point mutants, precoupled to glutathione-Sepharose beads. Western blotting against the appropriate tag revealed specifically bound proteins. Aliquots of cell lysate (starting material) equivalent to 1/10th that added to the beads were analyzed in parallel.

struct exhibited any interaction (Fig. 4*A* and data not shown). Comparing the 497–516 sequence with known β2-ear-binding motifs revealed some limited similarities with the binding motif found in ARH, although out of the essential residues (D/E)$_{n}X_{1-2}$F*XX*(F/L)*XXX*R (9, 10, 12), only the leucine and arginine are conserved (Fig. 4*B*). To analyze whether the β2-ear-binding motif in connecdenn 2 is a variant of that in ARH, we generated point mutants comparable to the three critical residues in ARH that abolish binding to AP-2 (9) (Fig. 4*B*, *asterisks*). Interestingly, only the mutation K500A, which aligns with the critical ARH residue Phe-259 (Fig. 4*B*) reduced binding to the β2-ear (Fig. 4*C*). Thus, connecdenn 2 uses a unique, uncharacterized motif to bind the β2-ear of AP-2.

Connecdenn 2 Binds Directly to the β2-Ear Platform on a Site Overlapping with That for ARH—A purified MBP-tagged fusion protein encoding the region of connecdenn 2 containing the β2-ear-binding site binds the purified β2-ear but not the α-ear or the CHC-TD, confirming that binding is direct (supplemental Fig. 3*B*). We thus sought to determine the binding site on the β2-ear. ARH binds to a deep hydrophobic groove on the β2-ear platform (10). Critical β2-ear residues involved in binding include Tyr-888, Trp-841, Glu-848, and Glu-902 (9, 10, 14). We thus tested if any of these residues are involved in connecdenn 2 binding. Similar to ARH (9, 10), Tyr-888 is essential while W841A greatly reduces but does not eliminate connecdenn 2 binding (Fig. 4*D*). However, unlike ARH, where mutations in Glu-848 and Glu-902 reduce binding, we saw no reduction in the binding of connecdenn 2 to these mutants (Fig. 4*D*). As these glutamic acids form one of three binding pockets, with Tyr-888 and Trp-841 critical for the other two pockets, it appears that connecdenn 2 uses two of the three pockets used by ARH. This situation is similar to the α-ear where DP(F/W) motifs bind to a groove in the platform, whereas F*XDXF* motifs utilize an extended version of the platform groove (5). To confirm that connecdenn 2 binding utilizes part of the same surface of the β2-ear platform as ARH, we performed competition experiments using MBP-tagged ARH 248–270. Indeed, ARH was able to effectively block binding of connecdenn 2 to the β2-ear (Fig. 4*E*). Together, these data demonstrate that the new motif in connecdenn 2 binds directly to the β2-ear platform using an overlapping but non-identical site to that used by ARH.

Identification of Key residues in the Connecdenn 2-Binding Motif—An alanine scan of connecdenn 2 residues 487–511 revealed that the majority of residues have no impact, whereas a series of lysines are important for β2-ear binding (Fig. 4*F* and data not shown). Mutation of Lys-502 has the most deleterious effect, almost completely abolishing the interaction. Although the most proximal upstream and downstream lysines, Lys-500 and Lys-509 greatly reduce binding, mutation of the more N-terminal Lys-489 causes only a slight reduction in binding. Mutation of either Arg-501 or Arg-507 has no effect, supporting that binding is unlikely due to a more general charge effect. This appears to be the first example of an AP-2 ear-binding motif relying on a series of lysines, a "K-motif," and is quite different from canonical binding motifs, which typically rely on hydrophobic phenylalanine or tryptophan residues.

We next sought to identify whether the K-motif is predictive of AP-2 binding. Rap1-GTP-interacting adaptor molecule, also known as APBB1IP or PREL1, contains several proline-rich motifs, a Ras-association like domain, and a pleckstrin homology domain (46, 47). Rap1-GTP-interacting adaptor molecule also contains a sequence similar to the AP-2 binding K-motif (supplemental Fig. 3*C*) and two clathrin binding DLL motifs and a type III clathrin box (LLGEM). We therefore performed a pulldown assay using the K-motif sequence in Rap1-GTP-interacting adaptor molecule, and indeed it binds FLAG-β2-ear (supplemental Fig. 3*D*). Thus, the K-motif represents a second mechanism by which proteins engage the β2-ear platform.

Localization of Connecdenn 2—To confirm a physiological interaction between connecdenn 2 and AP-2, we performed native co-immunoprecipitation analyses from brain using an

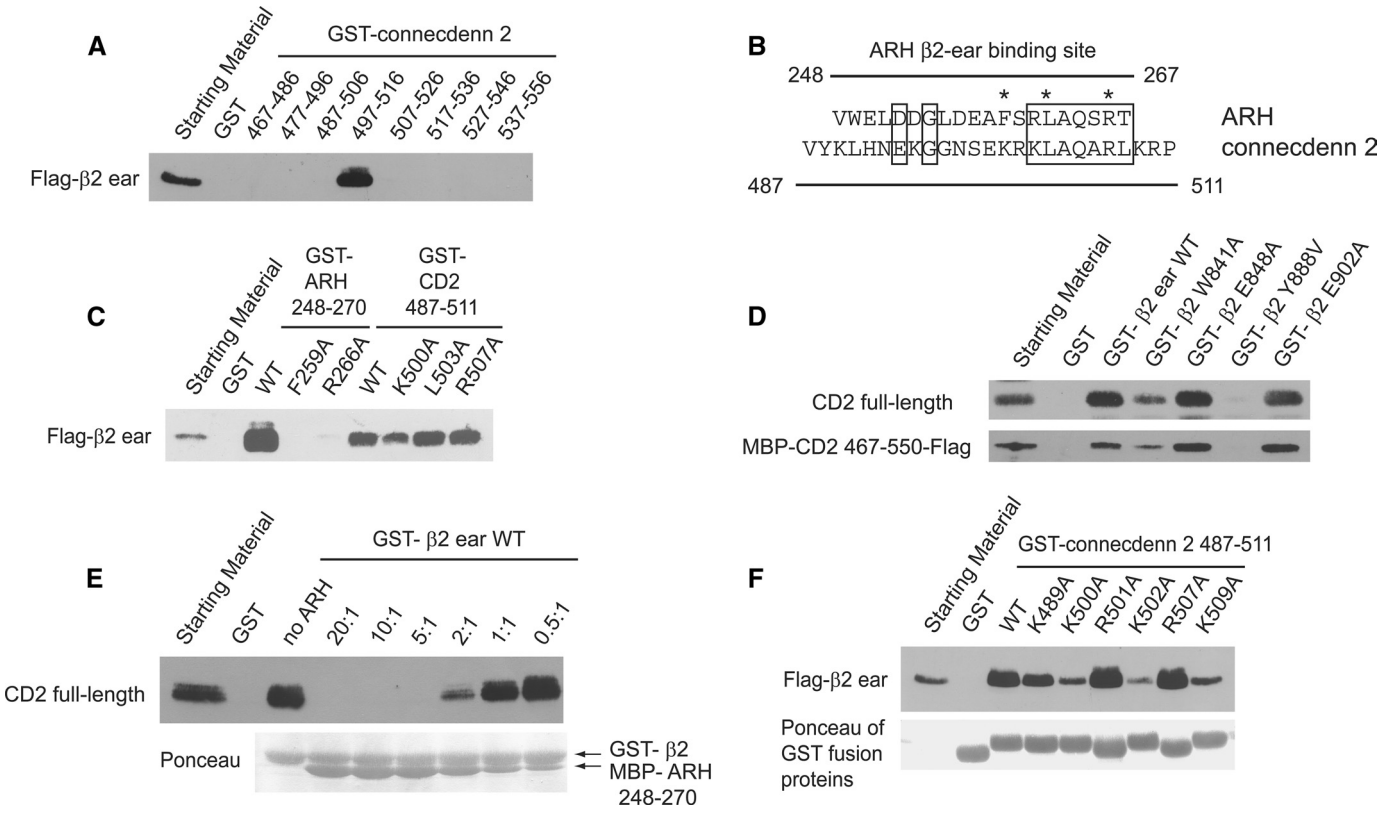


FIGURE 4. **Connecdenn 2 binds directly to the β2-ear platform in a manner distinct from ARH.** *A*, soluble lysates of HEK-293T cells expressing FLAG-β2-ear were incubated with equimolar amounts of GST or the indicated regions of connecdenn 2 fused to GST, precoupled to glutathione-Sepharose beads. *B*, alignment of the regions of ARH and connecdenn 2 that bind the β2-ear ear. Areas of similarity are *boxed*, critical residues in ARH required for β2 binding are indicated by *asterisks*. *C*, soluble lysates of HEK-293T cells expressing FLAG-β2-ear were incubated with equimolar amounts of GST or GST fusion proteins encoding the indicated regions of ARH and connecdenn (*CD*) 2, either in the wild-type (*WT*) form or containing the indicated point mutations, precoupled to glutathione-Sepharose beads. *D*, purified MBP-CD2 467–550-FLAG or soluble rat brain extract were incubated with GST or equimolar amounts of GST fused to the β2-ear WT and β2-ear with the indicated platform point mutations, precoupled to glutathione-Sepharose beads. *E*, soluble rat brain extract was incubated with GST or GST fused to the β2-ear WT, precoupled to glutathione-Sepharose beads in the presence of indicated molar ratios of purified MPB-ARH 248–270. The Ponceau-stained transfer reveals binding of MBP-ARH to GST-β2-ear WT. *F*, soluble lysates of HEK-293T cells expressing FLAG-β2-ear were incubated with GST or equimolar amounts of GST fusion proteins encoding either WT connecdenn 2 487–511 or the same construct containing the indicated point mutations, precoupled to glutathione-Sepharose. The Ponceau-stained transfer of the GST fusion proteins reveals equal loading. For *A*, *C*, *D*, and *F*, an aliquot of the lysate (starting material) equivalent to 1/10th that added to the beads was analyzed in parallel. Western blotting against the FLAG epitope or endogenous CD2 revealed specifically bound protein.

anti-α-adaptin antibody that disrupts interactions with α-ear-platform-binding proteins (6). Connecdenn 2 co-immunoprecipitated with AP-2 (Fig. 5*A*), demonstrating that connecdenn 2 interacts physiologically with AP-2 outside of its α-ear-platform binding F*X*D*X*F motif. While the degree of co-immunoprecipitation is small, it is similar to that for Eps15, another protein that binds the β2-ear (6). In COS-7 cells, FLAG-tagged connecdenn 2 shows an overall punctate distribution, with a portion of the punctae co-localizing with AP-2 (Fig. 5*B*). However, while connecdenn 2 enriches on CCVs, there is also a substantial cytosolic (S2) pool (Fig. 3*A*). This suggests that the interaction between connecdenn 2 and AP-2 may be transient in nature, such as is the case for Eps15 (10), or alternatively that its recruitment onto CCVs may be subject to regulation, as is the case for other β2-ear-binding proteins, such as βarrestins and PIPKγ661, which in their inactive state are found in a cytoplasmic pool, only associating with AP-2 upon activation (13, 20, 41, 45, 59).

Connecdenn 2 Knockdown Leads to an Enlargement of Early Endosomes—Rab35 is transported on CCVs to early endosomes where it functions in receptor recycling (34, 35). A portion of the FLAG-tagged connecdenn 2 punctae co-localize with Myc-tagged Rab35 S22N, a point mutant analogous to Ras S17N that greatly reduces nucleotide affinity, consistent with the biochemical interaction between the connecdenn 2 DENN domain and nucleotide free Rab35 (supplemental Fig. 4 and Fig. 2*A*). Loss of either connecdenn 1 or Rab35 leads to disruption of the recycling of specific cargo from early endosomes with an enlargement of the early endosome compartment (35). We therefore examined the effect of connecdenn 2 knockdown on early endosome morphology. Two siRNA sequences targeting connecdenn 2 gave an efficient knockdown, while levels of CHC and EEA1 remained unchanged (Fig. 6*A*). Interestingly, knockdown of connecdenn 2 resulted in the same perinuclear clustering and enlargement of early endosomes as seen with knockdown of either connecdenn 1 or Rab35 (Fig. 6*B*) (35). To verify that cargo were still being transported to these enlarged endosomes, we performed a transferrin labeling experiment at 16 °C, which allows transferrin to undergo CME and be transported to early endosomes, but prevents recycling thereby trapping the transferrin in early endosomes. This demonstrates that

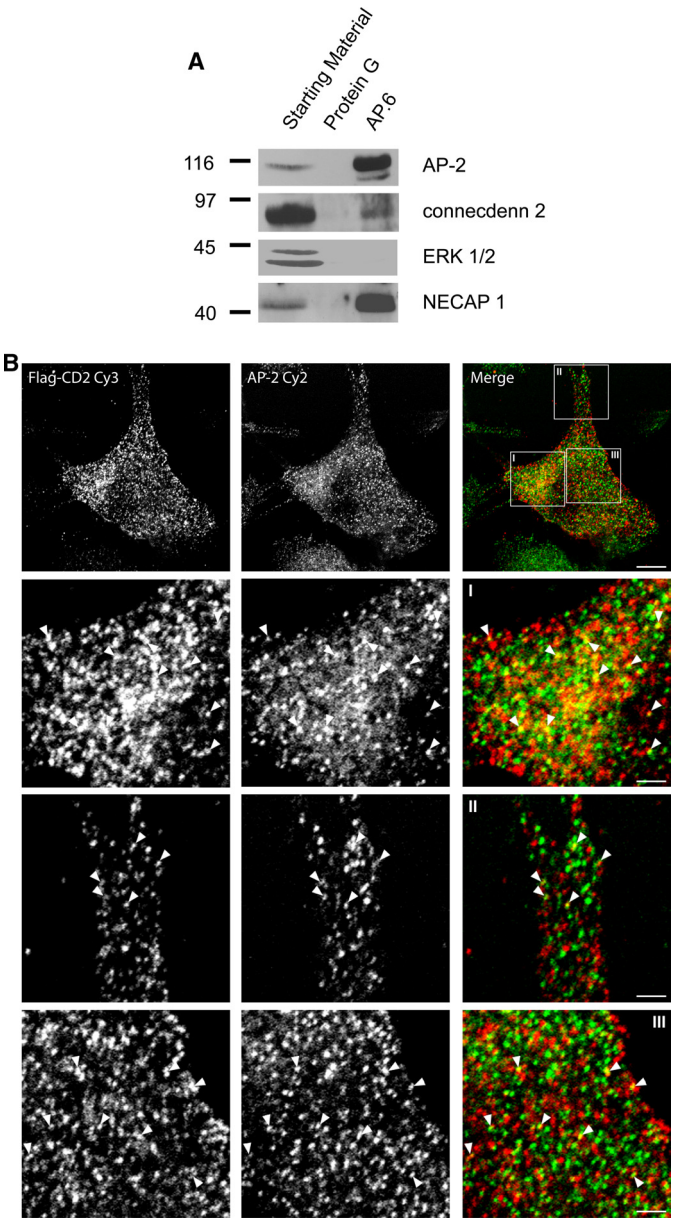


FIGURE 5. **Connecdenn 2 co-immunoprecipitation and co-localization with AP-2.** *A*, soluble rat brain extract was incubated with protein G-Sepharose alone or protein G-Sepharose precoupled to a monoclonal antibody to α-adaptin (*AP.6*). Immunoprecipitated proteins were processed for Western blot with the indicated antibodies. Aliquots of soluble brain extract (starting material) equivalent to 1/10th that added to the immunoprecipitation were analyzed in parallel. *B*, COS-7 cells were transfected at low levels with FLAG-tagged full-length connecdenn 2 and processed for immunofluorescence with a polyclonal antibody against the FLAG-tag (*red*) and monoclonal antibody against AP-2 (*AP.6*, *green*). *Arrowheads* on higher magnification panels indicate examples of co-localizing punctae. *Scale bars*: lower magnification, 10 μm; higher magnification, 2.5 μm.

CME is not perturbed and allowed us to further visualize the enlargement and redistribution of early endosomes upon connecdenn 2 knockdown (Fig. 6*B*). No changes were seen in AP-2 staining or in the morphology of the *trans*-Golgi network. Because knockdown of either connecdenn 1 or 2 results in the same endosome enlargement phenotype, this suggests that they do not serve a redundant role in Rab35 activation, nor did we see a decrease in connecdenn 1 expression upon connecdenn 2 knockdown, confirming that the connecdenn 2 phenotype is not an indirect result of loss of connecdenn 1 (data not shown). Taken together, these results demonstrate that connecdenn 2 is a CCV-associated protein that, through its new β2-ear-binding motif, can be found in a complex with AP-2 and that its DENN domain is a GEF for Rab35 providing a mechanism to couple Rab35 activation to the clathrin machinery. This suggests that connecdenn 2 functions as a clathrin-associated sorting protein for Rab35, whereby during CME connecdenn 2 binds AP-2 and inactive Rab35, then activates Rab35 for its subsequent function at endosomes. Loss of connecdenn 2 results in an enlargement and perinuclear cluster of early endosomes, as does loss of Rab35 activity, indicating that connecdenn 2 has an important role in Rab35 activation and endosomal trafficking.

DISCUSSION

The DENN domain is an evolutionarily ancient protein module found in all eukaryotes (48) with multiple proteins encoded by at least 16 genes in humans. The importance of the module is highlighted by its involvement in human disease. For example, Charcot-Marie-Tooth 4B2 neuropathy is caused by a deletion in the dDENN module of myotubularin-related 13, a pseudo-phosphatase member of the myotubularin family of lipid phosphatases (31, 49). DENN domains have crucial roles in plants, as a serine to phenylalanine mutation in the DENN domain of the protein SCD1 in *Arabidopsis thaliana* impairs secretory vesicle trafficking resulting in impaired cytokinesis and polarized cell expansion (50). Only a fraction of the predicted DENN domain proteins have been characterized in any detail. However, for those that have been studied, there is some evidence linking their function to various aspects of the function of Rab GTPases. For example, Rab6-interacting protein 1 binds to Rab6 and Rab11A (51). However, a region outside the DENN domain mediates binding (52). DENN/MADD/Rab3GEP acts as a Rab3 GEF, although enzymatic activity was only observable with full-length protein, and it is suggested that the GEF activity is outside the DENN domain (53, 54). Interestingly, the uDENN module of DENN/MADD/Rab3GEP binds Rab3 in a GTP form, suggesting the DENN domain mediates an effector interaction and not a GEF function (55). Thus, the function of the DENN domain has remained obscure.

We recently ascribed the first enzymatic activity to a DENN domain, demonstrating that the purified DENN domain of connecdenn 1 is a GEF for Rab35 (35). We have now expanded the repertoire of Rab35 GEFs to include the DENN domains of connecdenn 2 and 3. Interestingly, GEF activity and ability to bind Rab35 in the nucleotide-free state are separable features of the DENN domain, because connecdenn 3 shows no nucleotide-dependent binding yet displays GEF activity. Generally, nucleotide-free binding is a hallmark of GEFs, however there are other previously reported exceptions (56–58). As the DENN domain consists of three separate modules it is possible that each module mediates a separate function. This could confer important functional differences to the connecdenns. Connecdenn 1 appears to recruit Rab35 to CCVs for subsequent targeting of the

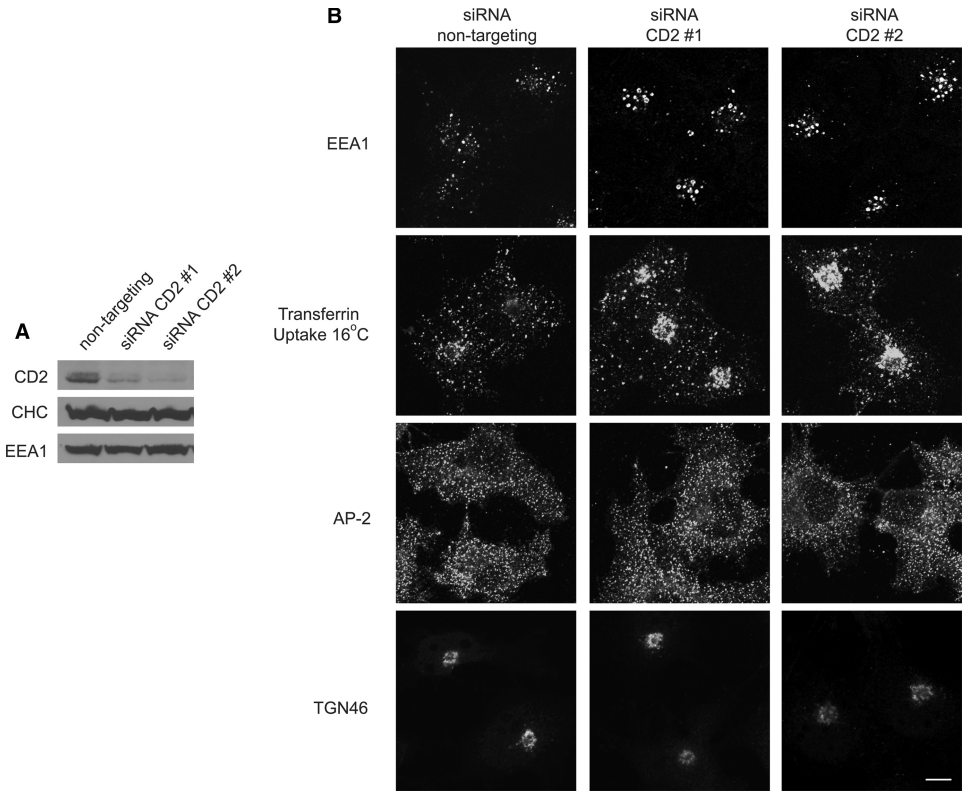


FIGURE 6. **Knockdown of connecdenn 2 causes an enlargement and redistribution of early endosomes.** *A*, equal lysates from COS-7 cells transfected with a non-targeting siRNA or siRNA targeting connecdenn 2 (*siRNA CD2 #1* and *#2*) were analyzed by Western blot for expression levels of connecdenn 2 (*CD2*), clathrin heavy chain (*CHC*), and early endosomal antigen 1 (*EEA1*). *B*, COS-7 control and knockdown cells were processed for immunofluorescence to examine the morphology of early endosomes using the marker EEA1, and transferrin uptake at 16 °C, which causes a block in the endocytosis of transferrin at the early endosomes. Staining for AP-2 and the *trans*-Golgi marker TGN46 reveals no changes to those compartments. *Scale bar*, 10 μm.

GTPase to endosomes (34, 35). Connecdenn 2 is also able to bind inactive Rab35 and engage the clathrin machinery, yet does so in a manner completely different from connecdenn 1. The early endosomal phenotype seen upon connecdenn 2 knockdown is similar to that seen following connecdenn 1 and Rab35 knockdown, therefore connecdenn 2 likely represents a second distinctive mechanism to recruit Rab35 to CCVs for the targeting, activation, and function of Rab35 at early endosomes. Given the lack of binding between connecdenn 3 and Rab35, it seems unlikely that connecdenn 3 would serve a similar function. Although GTPase binding and GEF activity may be entirely separable features, we cannot rule out that binding is related to GEF activity. Notably, connecdenn 3 has the least robust GEF activity; therefore, it is possible that the ability to bind Rab35 keeps the GTPase and GEF in proximity for the exchange reaction to occur. Our demonstration of the generality of GEF activity in different DENN domains raises the possibility that other DENN domains could serve as GEFs toward other Rabs or other small GTPases, suggesting a conserved enzymatic activity for these domains. It is also possible that connecdenns have GEF activity toward other Rabs not yet tested. Of interest, the *C. elegans* connecdenn homologue, RME-4, shows endocytic defects in coelomocytes, whereas Rab35 mutants do not, indicating that RME-4 might regulate another Rab GTPase required in coelomocytes (34). Given the differing tissue distributions of connecdenn 1–3, the different isoforms could have tissue-specific functions, either in controlling Rab35-mediated recycling, or recycling through another Rab, as likely is the case in *C. elegans*.

Intriguingly, although the family of connecdenns has conserved the N-terminal DENN domain enzymatic activity, they have all evolved highly divergent C termini in terms of amino acid sequence. Yet the C-terminal regions have a conserved function in terms of binding the clathrin machinery, albeit through unrelated mechanisms (Fig. 7). Like connecdenn 1, connecdenn 3 interacts with the α-ear sandwich, although not through a canonical W*XX*F-acidic motif. The motif used by connecdenn 2 represents a distinctive method to engage an AP-2 ear. Although it uses a conserved site on the β2 platform, it does so in a unique way, using a series of lysines and not the typical nonpolar phenylalanine or tryptophan. Both ARH and βarrestins also utilize a critical basic residue, arginine, in addition to critical nonpolar residues (9, 18). Although connecdenn 2 does not rely on any critical nonpolar residues, lysine does have amphipathic properties due to the long hydrophobic carbon chain and its positively charged amino group that forms hydrogen bonds, accordingly lysine is often involved in protein binding.

The ability to engage different sites on the AP-2 α- and β2-ears through different motifs is likely of important functional relevance. Indeed, although all three isoforms of connecdenn enrich on CCVs, connecdenn 2 is present in a large soluble pool, whereas the majority of connecdenn 1 enriches on membrane fractions. Connecdenn 2 also exhibits a much smaller degree of co-localization and co-immunoprecipitation with AP-2 than connecdenn 1 (28), suggesting that the interaction of connecdenn 2 with CCVs is transitory and subject to regulation, a likely explanation for why it has evolved a unique β2-ear platform binding motif. Such regulation has been shown previously for the β2-ear platform, whereby GPCR activation and phosphorylation are required for soluble βarrestin to translocate from a cytoplasmic pool to the cell surface and engage the β2-ear platform for the subsequent CME of the activated GPCRs. This regulation is mediated through an isoleucine-valine-phenylalanine motif found within the β2-ear binding motif of βarrestins (20). Conversely, dissociation of the βarrestin/β2-ear complex is regulated by Src-dependent phosphorylation of the β2-ear (59). It will be intriguing to see whether connecdenn 2

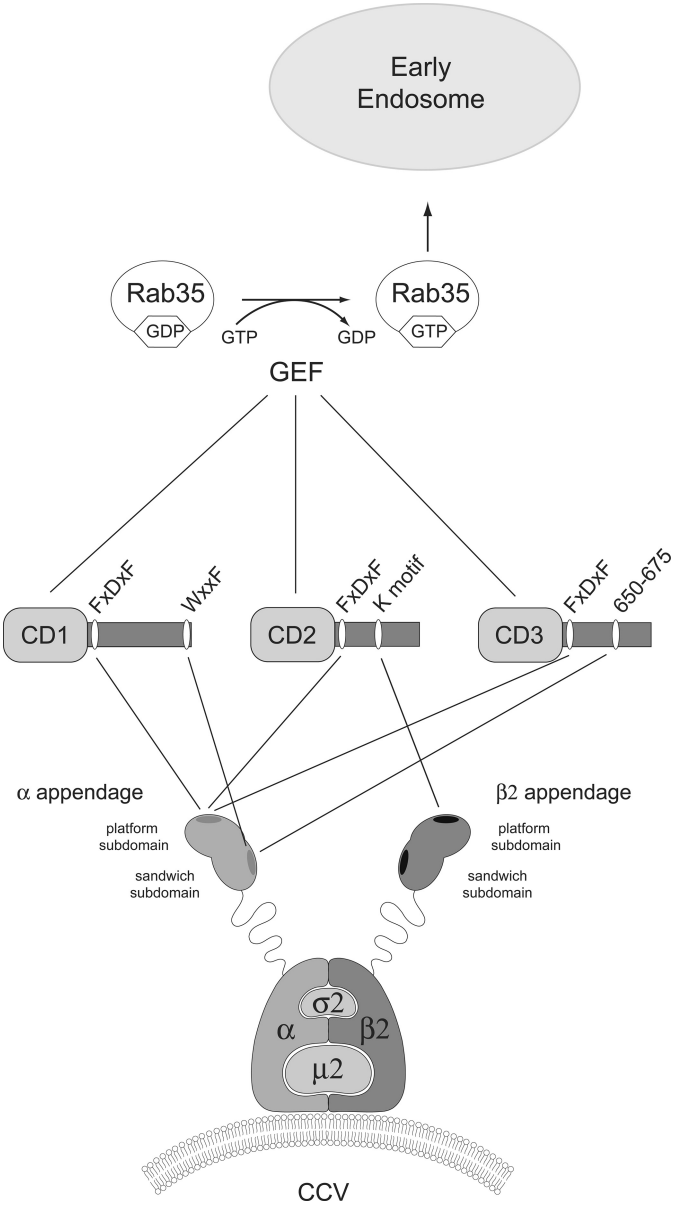


FIGURE 7. **Interaction model of the distinct mechanisms by which the connecdenn family couple Rab35 activation and binding to AP-2.**

engagement of the β2-ear platform is also subject to regulation, thereby creating a regulated mechanism by which to recruit and activate Rab35.

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