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Identification of a family of endocytic proteins that define a new α-adaptin ear-binding motif

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Endocytosis by clathrin-coated vesicles (CCVs) is an important mechanism mediating protein internalization. Here, we show that two proteins identified through a proteomics analysis of CCVs are new components of the endocytic machinery. The proteins, named NECAP (adaptin-ear-binding coat-associated protein) 1 and 2, are paralogues that display no sequence similarity or common domains with any known protein. Both are enriched in CCV coats, and further analysis of the brain-enriched isoform, NECAP 1, shows its partial localization to clathrin-coated pits and direct binding to the globular ear domain of the α-adaptin subunit (α-ear) of the adaptor protein 2 (AP-2) complex. Intriguingly, this interaction is mediated by a new motif, WVQF, that uses a distinct α-ear interface relative to known α-ear-binding partners. Disruption of this interaction blocks clathrin-mediated endocytosis. Together, our studies identify a new family of endocytic proteins that define a unique AP-2-binding motif.

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INTRODUCTION

The internalization of nutrients, signalling receptors and other cell-surface regulatory proteins via clathrin-mediated endocytosis is essential for normal cellular function (Conner & Schmid, 2003). Central to this process is the adaptor protein 2 (AP-2), a heterotetramer composed of two large subunits, α and β2; a medium-sized μ2 subunit and a small σ2 subunit (Robinson & Bonifacino, 2001). The β2 subunit binds to clathrin through a short peptide motif, whereas cargo proteins interact with the μ2 and β2 subunits through specific endocytic targeting sequences (Robinson & Bonifacino, 2001). AP-2 so functions to concentrate endocytic cargo into clathrin-coated pits (CCPs). AP-2 also serves as a nexus for the recruitment of a surprisingly diverse array of endocytic accessory proteins including amphiphysin I, epsin 1 and 2, Eps15, disabled-2, auxilin and the huntingtin-interacting protein (HIP) 1. These proteins bind directly to the globular ear domain at the carboxy terminus of the AP-2 α-subunit (α-ear; Owen *et al*., 1999; Rosenthal *et al*., 1999; Traub *et al*., 1999; Metzler *et al*., 2001; Mishra *et al*., 2002), and they have multiple functions including catalysing clathrin assembly, linking endocytic vesicles to the actin cytoskeleton and recruiting regulatory endocytic enzymes such as synaptojanin, dynamin and intersectin-l (Slepnev & De Camilli, 2000; McPherson, 2002). Interestingly, each of these proteins contains one or more copies of two distinct consensus α-ear-binding motifs, DPF/W and FXDXF (Owen *et al*., 1999; Traub *et al*., 1999; Brett *et al*., 2002).

The identification of consensus motifs or protein modules for protein–protein interactions often provides important clues regarding the specific cellular process to which a new protein can be linked. However, cDNA and genomic databases contain a large number of sequences that encode hypothetical proteins without recognizable domains or motifs. In the absence of such information, the association of a protein with a specific subcellular compartment can provide a framework on which to infer the function of the protein. An important technique in this regard is subcellular proteomics, which combines subcellular fractionation with mass spectrometry (MS) to identify proteins in isolated organelles. We have previously used subcellular proteomics to identify novel proteins of rat brain CCVs (Wasiak *et al*., 2002). Here, we characterize two potential open-reading frames from this screen, NECAP (adaptin-ear-binding coat-associated protein) 1 and 2. Our results show that the NECAPs are new components of the endocytic machinery and use a unique mode of AP-2 interaction.

RESULTS AND DISCUSSION

NECAP 1 and 2 are paralogues with a *Drosophila* orthologue of unknown function (Fig. 1A). They contain no modular domains and show no sequence similarity to any known protein. Polyclonal antibodies against recombinant proteins reveal that NECAP 1, which migrates as a doublet at 40 kDa, is expressed primarily in brain (Fig. 1B). The doublet may result from post-translational modification as ectopically expressed protein shows an identical band pattern (data not shown). NECAP 2, which is detected as a prominent band at 29 kDa and a weaker band at 32 kDa, has a broader tissue distribution that is variable between the two bands (Fig. 1C). It is unknown whether the detected variants are a result of post-translational modification or

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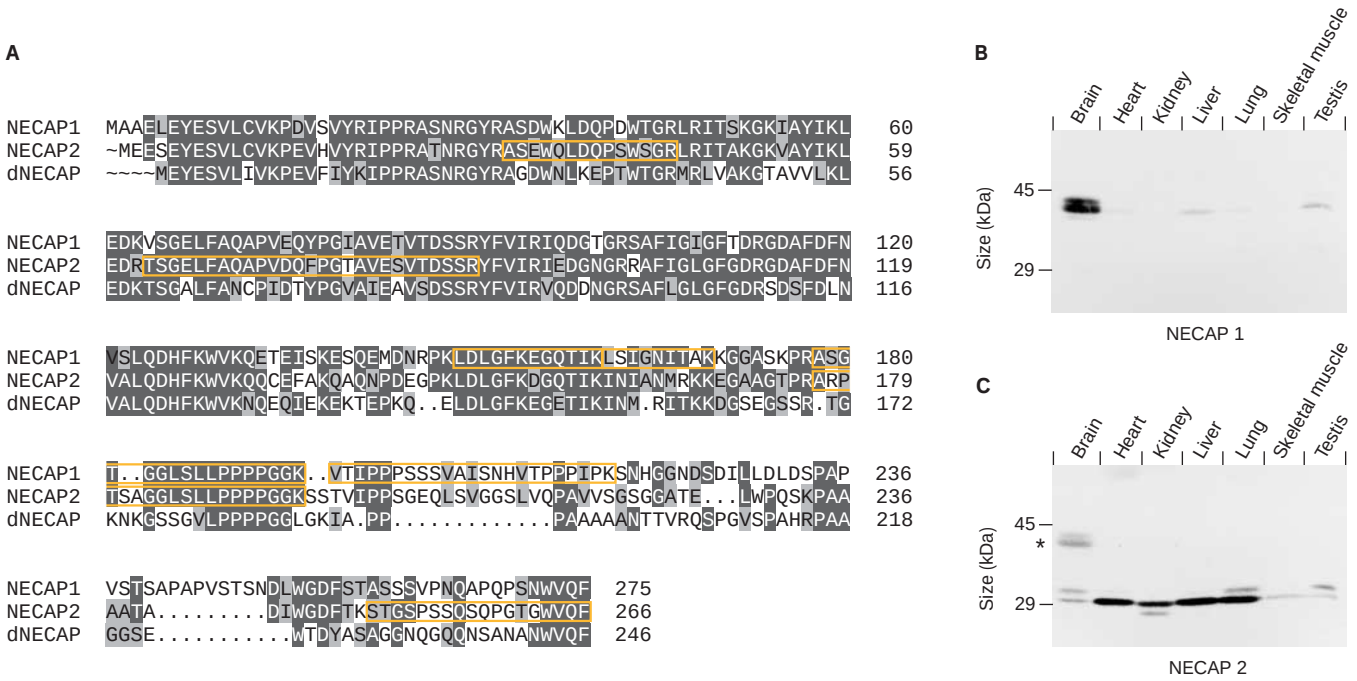


Fig. 1 | Identification and tissue distribution of NECAPs. (**A**) Alignment of murine NECAP 1 and 2 with the *Drosophila* NECAP orthologue (dNECAP, gi 7293286). Identical and conserved amino acids are shaded black and grey, respectively. Yellow boxes denote peptides identified by tandem mass spectrometry. The sequence data are available from GenBank/EMBL/DDBJ under accession numbers BK000656 and BK000657. The level of (**B**) NECAP 1 and (**C**) NECAP 2 in the indicated tissues was determined by western blot. The asterisk indicates NECAP 1 expression in brain, which is detected weakly by the NECAP 2 antibody. NECAP, adaptin-ear-binding coat-associated protein.

tissue-specific splicing. Although the predicted molecular weights for NECAP 1 and 2 are 29.6 kDa and 28.6 kDa, respectively, Flag-tagged full-length proteins migrate on SDS–polyacrylamide gel electrophoresis (PAGE) at approximately 40 kDa and 30 kDa, similar to the endogenous proteins (data not shown). The aberrant migration of NECAP 1 may be due to its low isoelectric point of 5.97 relative to the isoelectric point of 7.72 for NECAP 2.

Subcellular fractionation shows that both NECAP isoforms are enriched on CCVs from rat brain and partition with clathrin into coat fractions stripped from CCVs (Fig. 2A). To address potential mechanisms targeting the NECAPs to CCVs, we performed affinity-purification experiments from brain extracts using a glutathione *S*-transferase (GST)–NECAP 1 fusion protein. Tandem MS of specifically bound protein bands detected by Coomassie blue staining (data not shown) revealed the presence of the AP-2 subunits α-, β2- and σ2-adaptin, and the β1- and σ1-adaptin subunits of the AP-1 complex, which functions in clathrin-mediated budding at the *trans*-Golgi network (TGN). These interactions were confirmed by western blot with antibodies against the γ- and α-adaptin subunits of AP-1 and AP-2, respectively (data not shown). AP-2 and, to a lesser extent, AP-1 were co-immunoprecipitated with NECAP 1 from cytosolic and solubilized membrane fractions from brain, further verifying their interaction (Fig. 2B). Interestingly, NECAP 1 immunoprecipitates contained additional AP-2-interacting proteins including clathrin, Eps15, epsin 1, and amphiphysin I and II. These proteins are probably indirectly associated with NECAP 1 through AP-2 and, unlike AP-2, they are not enriched in the immunoprecipitates. So, AP-2 can interact simultaneously with both NECAP 1 and other endocytic accessory proteins. The accessory protein endophilin A1, which does not interact directly with NECAP 1 or AP-2, is not present within the complex (Fig. 2B). A significant portion of the total brain pool of AP-2 is constitutively bound to NECAP 1 as immunodepletion of NECAP 1 led to a 19.6 ± 6.5% (mean ± s.e.m., $n = 4$) decrease in AP-2 levels in brain extracts (see, for example, Fig. 2C).

The interaction of NECAP 1 with AP-2 and its enrichment on CCVs suggest that the protein could also be a component of CCPs. Flag-tagged NECAP 1 shows an overall punctate distribution with some additional staining at the cell periphery when expressed in COS-7 cells (Fig. 2D). A fraction of the NECAP 1 punctate colocalized with clathrin and AP-2 at the plasma membrane, although a significant pool of the protein is not localized to CCPs (Fig. 2D).

To identify the region of NECAP 1 that mediates AP-2 interactions, we generated NECAP 1 deletion constructs lacking the last one-third (AB fragment) or the first one-third (BC fragment) of the protein (Fig. 3A). Affinity-selection experiments from brain extracts showed AP-2 binding selectively to the BC fragment (Fig. 3B), suggesting that the major AP-2-binding site(s) is located in the C region. When expressed in COS-7 cells, the BC fragment but not the AB fragment significantly blocked transferrin uptake (Fig. 3C,D). The inhibitory effect of the BC fragment on endocytosis is unlikely to be caused by overexpression of the AP-2-binding site, given that full-length NECAP 1 did not affect transferrin uptake, even when expressed at high levels (Fig. 3C,D). We hypothesize that the BC fragment has a dominant-negative effect on endocytosis by preventing endogenous NECAPs—which are

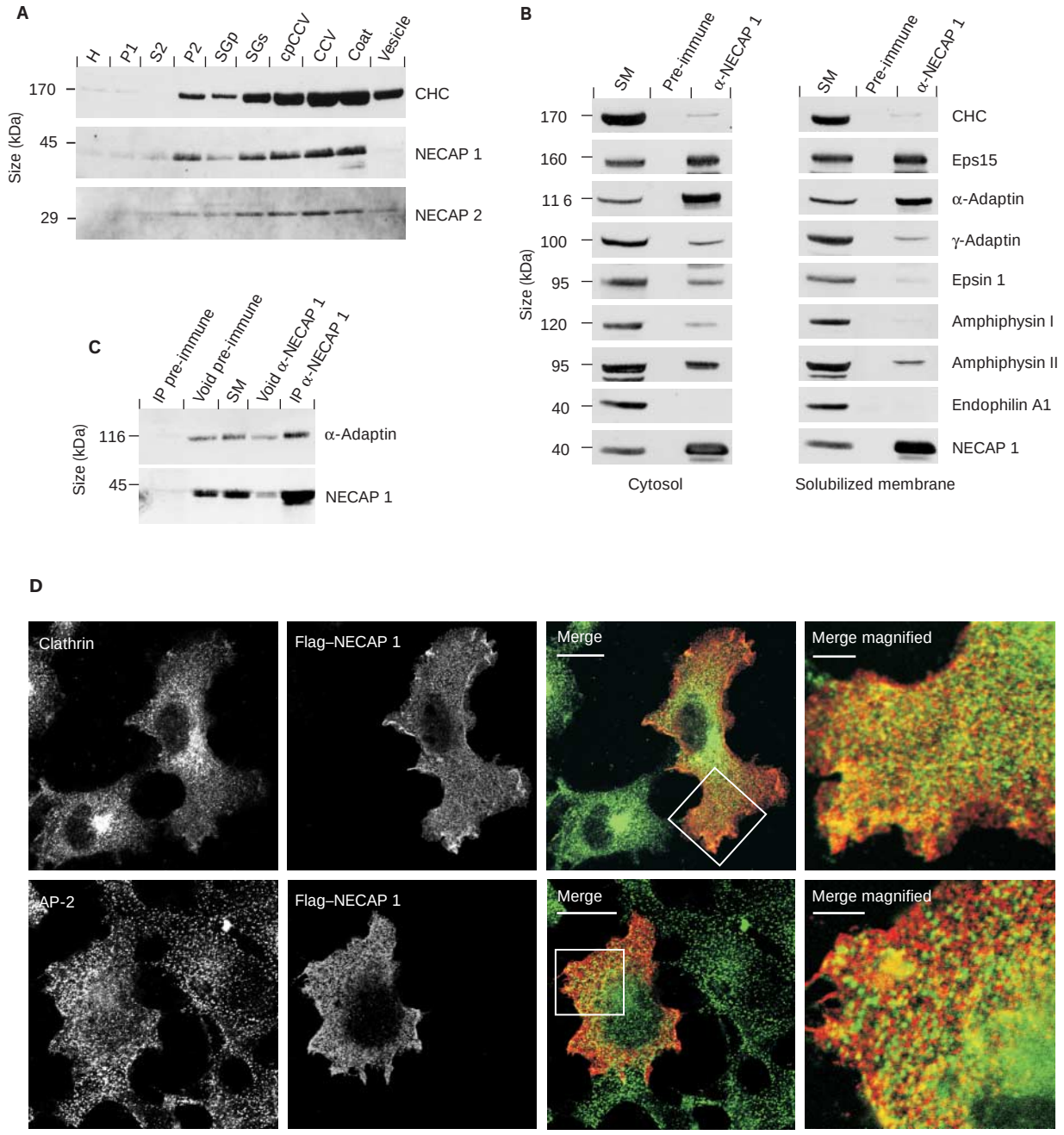
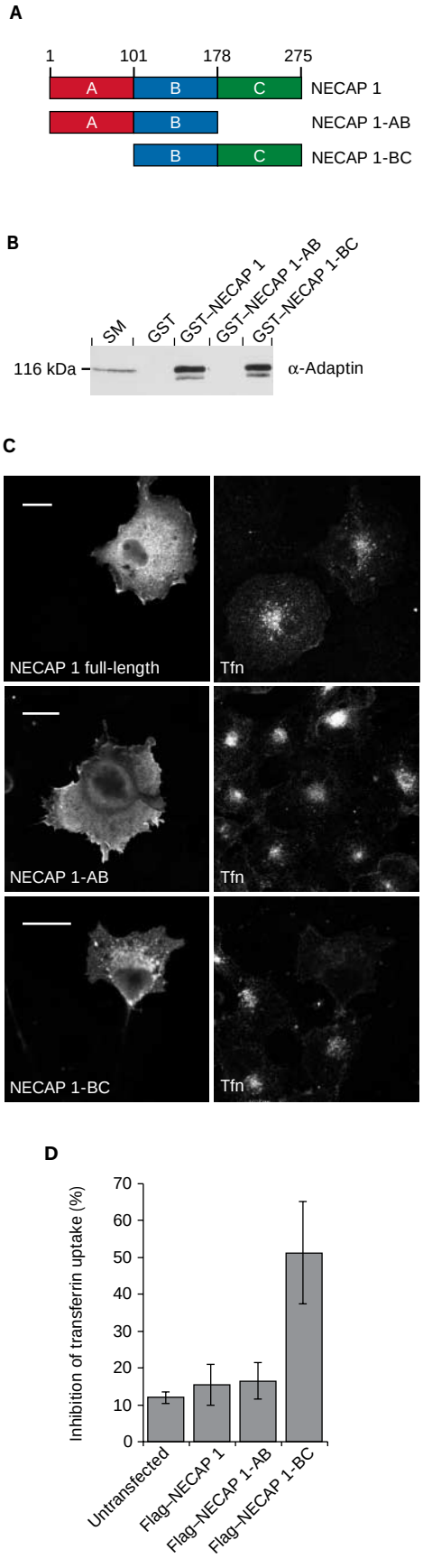


Fig. 2 | NECAP 1 in endocytic protein complexes. (**A**) Subcellular fractions from a preparation leading to highly enriched CCVs were western blotted with NECAP 1 and 2 and clathrin heavy-chain (CHC) antibodies. CCVs were stripped to obtain coats and vesicles. (**B**) NECAP 1 polyclonal antibody or pre-immune NECAP 1 sera were incubated with cytosolic or solubilized membrane fractions prepared from rat brain. Antibody was precipitated by the addition of protein-A–sepharose beads, and immunoprecipitated proteins were processed for western blotting with the antibodies indicated. Starting material (SM) is an aliquot of extract equal to one-tenth of that used for immunoprecipitation. (**C**) NECAP 1 immunoprecipitations were performed from Triton X-100-solubilized rat brain extract as in (**B**), with the addition of a protein aliquot of the unbound material (void) equal to that of the SM analysed in parallel. (**D**) COS-7 cells were transfected with Flag-tagged NECAP 1 and processed by indirect immunofluorescence with Flag-epitope polyclonal antibody (red) and monoclonal antibodies against clathrin (X22) and AP-2 (α-adaptin, AP.6) (green). Moderately transfected cells were analysed. Colocalization (yellow) is visualized through superimposition (merge). Higher-magnification images (merge magnified) are from the boxed areas in the merge. Scale bars, 20 μm (merge) and 5 μm (merge magnified). CCV, clathrin-coated vesicles; cpCCV, cushion pellet clathrin-coated vesicles; H, homogenate; NECAP, adaptin-ear-binding coat-associated protein; P, pellet; S, supernatant; SGp, sucrose gradient pellet; SGs, sucrose gradient supernatant.

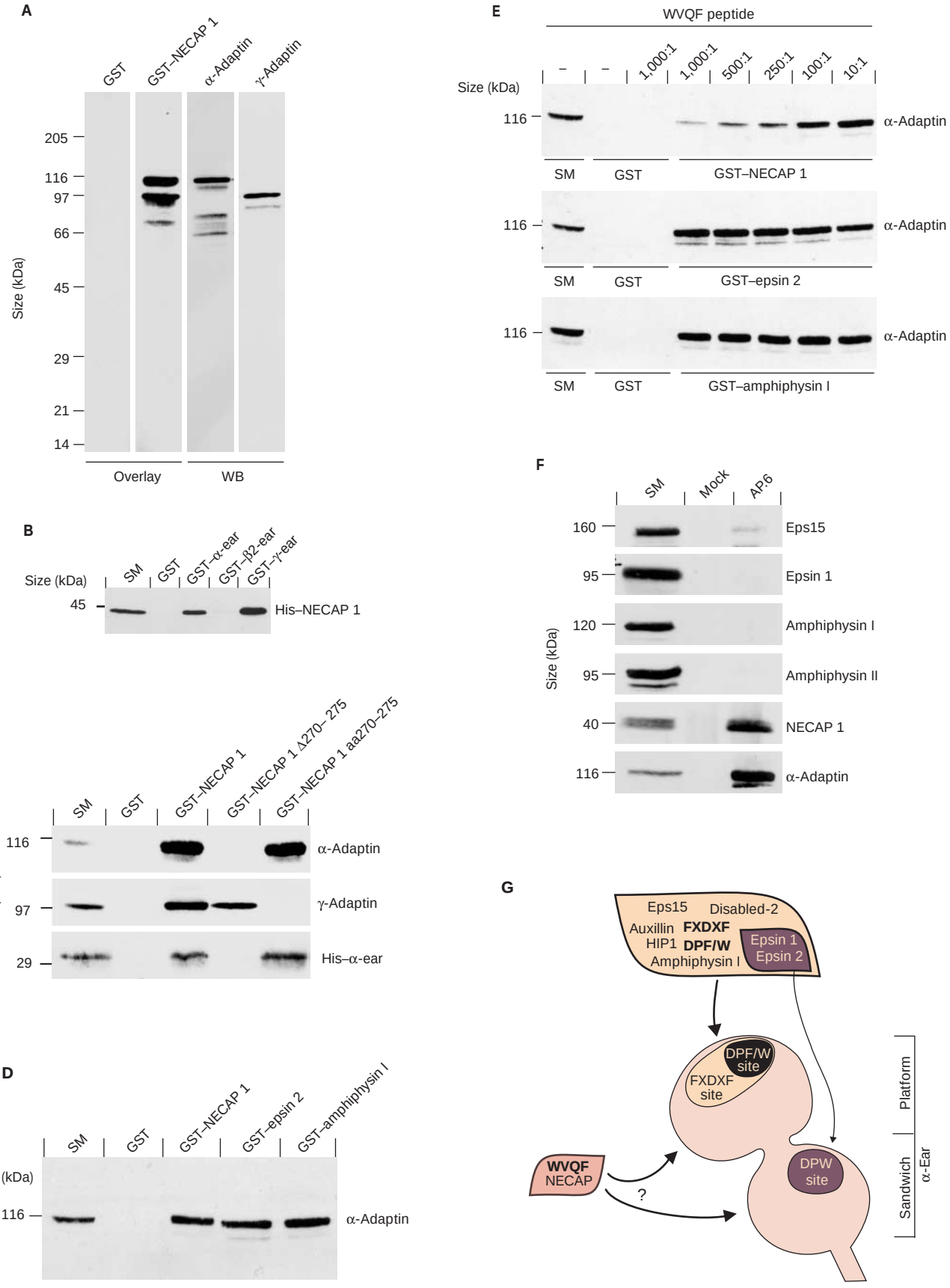


both detected in COS-7 and other cultured cells (data not shown)—from binding to AP-2. However, the exact mechanism of this dominant-negative effect remains uncertain.

To explore the nature of the NECAP 1–AP-2 interaction, we first used GST–NECAP 1 in overlay assays on coat fractions stripped from CCVs. GST–NECAP 1 bound to proteins of approximately 116 kDa and 97 kDa that co-migrate with α- and γ-adaptin, respectively (Fig. 4A). Affinity-selection experiments revealed binding of purified, recombinant His-tagged NECAP 1 to the α- and γ-adaptin ears with no binding to the β2-ear (Fig. 4B).

◀ **Fig. 3** | NECAP 1 functions in endocytosis. (**A**) Domain models of NECAP 1 deletion constructs. Amino acid numbers are indicated on top. (**B**) Glutathione *S*-transferase (GST), GST–NECAP 1 and GST–NECAP 1 deletion constructs were pre-coupled to glutathione–sepharose and incubated with soluble rat brain extracts. Affinity-selected α-adaptin was detected by western blot. An aliquot of brain homogenate (starting material, SM) equal to one-tenth of that added to the fusion proteins was analysed in parallel. (**C**) COS-7 cells were transfected with Flag-tagged constructs encoding NECAP 1 and NECAP 1 deletion constructs as indicated. Endocytosis was assayed by monitoring the uptake of Cy3-labelled transferrin (Tfn). Scale bar, 20 μm. (**D**) Quantification of the percentage of cells that failed to take up transferrin (mean ± s.e.m., $n = 4$). NECAP 1, adaptin-ear-binding coat-associated protein 1.

Fig. 4 | Identification of a new AP-2-binding motif in NECAP 1. (**A**) Coat proteins stripped from clathrin-coated vesicles were separated on SDS–PAGE and transferred to nitrocellulose. Nitrocellulose strips were overlaid with glutathione *S*-transferase (GST) or GST–NECAP 1, or western blotted with α-adaptin and γ-adaptin antibodies. (**B**) GST fusion proteins encoding the ear domains of α-, β2- and γ-adaptin and GST alone were pre-coupled to glutathione–sepharose and incubated with purified His6-tagged NECAP 1. Affinity-selected His–NECAP 1 was detected with the His6 epitope tag antibody. The starting material (SM) is an aliquot of His–NECAP 1 equal to one-tenth of that added to the fusion proteins. (**C**) GST fusion proteins encoding full-length NECAP 1, NECAP 1 lacking the last six amino acids (Δ270–275), or the last six amino acids of NECAP 1 alone (aa 270–275), as well as GST, were pre-coupled to glutathione–sepharose and incubated with soluble rat brain extracts or purified His–α-ear. Affinity-selected proteins, along with an aliquot of the SM equal to one-tenth of that added to the fusion proteins, were western blotted with antibodies against α- and γ-adaptin or the His6 epitope tag. (**D**) Equimolar amounts of the indicated GST fusion proteins were pre-coupled to glutathione–sepharose and incubated with soluble rat brain extracts. Affinity-selected proteins were processed as in (**C**). (**E**) Equimolar amounts of the indicated GST fusion proteins were pre-coupled to glutathione–sepharose and incubated with 0.5 mg of soluble rat brain extract without (–) or in the presence of increasing concentrations of a peptide encoding the last 11 amino acids of NECAP 1. The molar ratio of the peptide to fusion protein is indicated. Affinity-selected proteins were western blotted as described in (**C**). (**F**) Monoclonal antibody to α-adaptin (AP.6) was incubated with a Triton X-100-solubilized rat brain extract. Antibody was precipitated by the addition of protein-G–agarose beads and immunoprecipitated proteins were processed for western blotting with the antibodies indicated. The SM is an aliquot of extract equal to one-tenth of that used for immunoprecipitation. Mock samples were treated identically, except that AP.6 antibody was excluded. (**G**) Model of distinct modes of interaction with α-ear. NECAP 1, adaptin-ear-binding coat-associated protein 1. ▶



Together, these data show that NECAP 1 binds directly to AP-1 and AP-2 through the ear domains of their γ- and α-adaptin subunits, respectively.

To identify the AP-2-binding motif in NECAP, we examined the alignment of the NECAPs and their *Drosophila* orthologue in the C region between amino acids 178 and 275 of NECAP 1 and noticed a conserved six-amino-acid stretch at the extreme C terminus (Fig. 1A). Remarkably, NECAP 1 constructs deleted for these six amino acids showed no binding to AP-2 from brain extracts, whereas interactions with AP-1 were not affected (Fig. 4C). Moreover, when coupled to GST, the six-amino-acid stretch showed comparable AP-2 binding to full-length protein but did not interact with AP-1 (Fig. 4C). Purified His–α-ear showed parallel binding to native AP-2 (Fig. 4C). So, the last six amino acids of NECAP 1 are fully responsible for AP-2 binding mediated through the α-ear. NECAP 2 shows similar AP-2-binding properties as NECAP 1 (data not shown). The tetrapeptide WVQF at the extreme C terminus is conserved between NECAP 1 and 2 and their *Drosophila* orthologue (Fig. 1A). So, the WVQF sequence appears to define a minimal consensus AP-2-binding motif.

The two established α-ear-binding motifs DPF/W and FXDXF share a characteristic core of an acidic residue and a bulky hydrophobic residue separated by one amino acid (Owen *et al*., 1999; Traub *et al*., 1999; Brett *et al*., 2002). Because the WVQF motif does not share this biochemical property, we sought to compare the AP-2 binding of NECAP 1 to that of proteins using these other motifs. We have previously generated GST fusion proteins encoding the most C-terminal 511 amino acids of mouse epsin 2 (Hussain *et al*., 1999), which contains multiple copies of the DPW tripeptide, and the insert domain of amphiphysin I (Ramjaun *et al*., 1997), which contains an FXDXF and DPF motif. These fusion proteins were used in affinity-selection assays with equimolar amounts of GST–NECAP 1. All proteins bound equally well to AP-2 (Fig. 4D), suggesting that the ability of NECAP 1 to interact with AP-2 through its WVQF motif is similar to that of previously described α-ear-binding proteins.

We next generated a peptide (CQAPQPSN**WVQF**) corresponding to the last 11 amino acids of NECAP 1 with an amino-terminal cysteine. AP-2 binding to GST–NECAP 1 was slightly reduced when the peptide was introduced into the binding assays at a 100-fold molar excess to the GST fusion protein and was strongly reduced at 250- to 1,000-fold excess (Fig. 4E). By contrast, the peptide did not affect the binding of AP-2 to the epsin 2 and amphiphysin I fusion proteins, even at a 1,000-fold molar excess (Fig. 4E). The lack of competition of amphiphysin I and epsin 2 with the WVQF peptide suggests that the unique AP-2-binding motif identified in NECAP 1 uses a site on the α-ear that is distinct from the known sites for α-ear-binding partners. This idea was further validated through immunoprecipitation analyses using an anti-α-adaptin antibody (AP.6) that disrupts interactions of AP-2 with known α-ear-binding proteins. Immunoprecipitation of AP-2 with AP.6 failed to co-immunoprecipitate epsin 1, as well as amphiphysin I and II, and Eps15 was only weakly detected (Fig. 4F), possibly because of binding to β2-adaptin (Owen *et al*., 2000). By contrast, the AP.6 antibody led to a strong co-immunoprecipitation of NECAP 1 with AP-2 (Fig. 4F).

The α-ear is composed of a proximal sandwich domain and a distal platform domain (Fig. 4G; Owen *et al*., 1999; Traub *et al*., 1999). The binding site on the α-ear for the FXDXF motif partially overlaps with the site for the DPF/W motif located in the platform domain, and the sandwich domain contains a site that specifically uses the DPW motif (Brett *et al*., 2002). A large number of endocytic accessory proteins are recruited to CCPs and CCVs through interactions with the α-ear, and all of these binding partners use DPF/W or FXDXF motifs (Fig. 4G). These accessory proteins function at distinct steps in the formation of CCVs, and their recruitment must therefore be coordinated spatially and temporally (Brodin *et al*., 2000). The sequential recruitment and transient association of many of these proteins with CCVs can be explained by their competition for common sites on the α-ear (Fig. 4G).

The NECAPs may therefore have a privileged α-ear interaction, suggesting a more stable association with AP-2 (Fig. 4G). This is consistent with the enrichment of NECAP 1 and 2 on CCVs and its stable association with AP-2 as judged by co-immunoprecipitation analysis. So, the dominant-negative effect of NECAP 1 on transferrin receptor endocytosis is probably not a result of competition with known AP-2-binding partners. The unique nature of the AP-2–NECAP interaction may reflect a role for the NECAPs in direct regulation of AP-2 function. Alternatively, NECAP function may be needed at multiple steps of endocytosis and the proteins therefore require a more stable association with clathrin coats mediated through interactions with AP-2. Interestingly, the WVQF motif can also be found within the endocytic protein stonin 2. Detailed characterization of the new motif could lead to a new class of accessory proteins that exert unexpected functions in endocytosis.

METHODS

Antibodies and cDNA constructs. cDNA clones for NECAP 1 (gi27229051) and NECAP 2 (gi13384758) were used as PCR templates to amplify DNA encoding full-length proteins. N-terminal GST, His6 and Flag epitope tags were added by cloning the PCR products into pGEX-4T1 (Pharmacia), pFO1 (derived from pET15b, generous gift of Mirek Cygler) and pFLAG–CMV-2 (Sigma), respectively. Rabbits were immunized with GST–NECAP fusion proteins using standard protocols. All other antibodies and constructs are described in supplementary information online.

Binding assays. Triton X-100-solubilized rat brain extracts were prepared in buffer A (10 mM HEPES, pH 7.4, protease inhibitors) containing 150 mM NaCl as previously described (Wasiak *et al*., 2002). Aliquots (2 mg) were incubated for 1 h at 4 °C with GST fusion proteins pre-coupled to glutathione–sepharose. In other cases, pre-coupled GST fusion proteins were incubated for 2 h at 4 °C with 1-μg aliquots of purified His–NECAP 1 or His–α-ear in buffer A containing 1% Triton X-100 and 500 mM or 150 mM NaCl, respectively. Overlays were performed as described previously (McPherson *et al*., 1994).

Immunoprecipitation assays. For immunodepletion experiments, rat brain was homogenized in buffer A containing 1% Triton X-100 and centrifuged at 205,000*g*. Aliquots of the supernatant (0.5 mg) were processed for immunoprecipitation. For AP.6 immunoprecipitations, brain homogenates were prepared as above in buffer A with 1% Triton X-100 and 33 mM NaCl. For other immunoprecipitations, rat brains were homogenized in buffer A and centrifuged at 800*g* for 5 min; the supernatant was then centrifuged at 205,000*g* for 30 min. The supernatant (cytosol) was adjusted to 1% Triton X-100 and 33 mM NaCl. The pellet was resuspended in buffer A with 1% Triton X-100 and then recen-

trifuged at 205,000*g*. The supernatant from this step (solubilized membrane) was adjusted to 33 mM NaCl. The various extracts were incubated for 1 h at 4 °C with 20 μl protein-A–sepharose (NECAP 1 immunoprecipitation) or protein-G–agarose beads (AP.6 immunoprecipitation) and the beads were then removed by centrifugation. The supernatants were incubated for 3 h at 4 °C with 20 μl of pre-immune serum or 20 μl of NECAP 1 serum and protein-A–sepharose beads, or with 5 μg AP.6 antibody and protein-G–agarose or protein-G–agarose alone (mock). In all cases, the beads were subsequently washed in buffer A containing 1% Triton X-100 and the appropriate concentration of NaCl and specifically bound proteins were processed for western blotting.

Subcellular fractionation. CCVs were isolated from rat brain (Takei *et al*., 1995; Wasiak *et al*., 2002) and their coats were stripped as described previously (Legendre-Guillemin *et al*., 2002). Adult rat tissues were homogenized in buffer A and centrifuged at 800*g* for 5 min. The protein content of the post-nuclear supernatants was determined and equal protein aliquots were analysed by SDS–PAGE and western blot.

Endocytosis assays. COS-7 cells were plated on poly-L-lysine coated coverslips and transfected with Flag-tagged NECAP 1 expression constructs using FuGENE 6 (Roche). Forty-eight hours after transfection, cells were serum-starved for 30 min, incubated with Cy3-labelled transferrin at 25 μg ml^{-1} for 30 min at 37 °C and then processed for immunofluorescence with anti-Flag antibody. Flag-positive cells were selected independently of signal intensity and without us knowing the status of the transferrin signal. Transfected cells were assessed for endocytosis by examining the accumulation of Cy3-transferrin in the recycling endosome. Cells with no transferrin detectable in the perinuclear region of the recycling endosome were considered blocked. Cells that had transferrin levels comparable to those of non-transfected cells, as well as cells with reduced perinuclear transferrin staining, were counted as active for endocytosis. The numbers given in Fig. 3D represent the percentage of cells blocked for uptake within the total pool of transfected cells for each construct.

Competition assays. Triton X-100-solubilized rat brain extracts were prepared in buffer A with 300 mM NaCl as described above. Aliquots of the extract (0.5 mg) were incubated for 2 h or overnight at 4 °C with 100 pmol each of GST fusion proteins of NECAP 1, amphiphysin I and epsin 2 coupled to glutathione–sepharose. In other experiments, a NECAP 1-specific peptide was added to the binding assays at a range of molar ratios relative to the fusion proteins as indicated on the figure. For all experiments, the beads were subsequently washed in buffer A containing 1% Triton X-100 and 300 mM NaCl, and specifically bound proteins were processed for SDS-PAGE and western blotting.

Immunofluorescence. COS-7 cells were plated on poly-L-lysine-coated coverslips, transfected with Flag-tagged NECAP 1 expression constructs using FuGENE 6 (Roche) and subsequently processed for immunofluorescence. Cells with moderate levels of Flag-NECAP 1 expression were analysed by confocal microscopy.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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