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The μ2 Subunit of the Clathrin Adaptor AP-2 Binds to FDNPVY and YppØ Sorting Signals at Distinct Sites

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The endocytic sorting signal on the low-density lipoprotein receptor for clathrin-mediated internalization is the sequence FDNPVY in the receptor's cytosolic tail. We have used a combination of surface plasmon resonance and crosslinking with a photoactivated peptide probe to demonstrate the interaction between FDNPVY-containing peptides and the μ2 chain of purified AP-2 clathrin adaptors (the complexes responsible for plasma membrane sorting). We show that recognition of the FDNPVY signal is mediated by a binding site in the μ2-subunit that is distinct from the site for the more general YppØ sorting signal, another tyrosine-based sequence also recognized by μ2-adaptin. These results suggest the possibility that low-density lipoprotein receptor uptake may be modulated specifically and independently of other proteins in the clathrin pathway.

Key words: Alzheimer's, AP-2 adaptor, clathrin, endocytosis, membrane traffic

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Low-density lipoproteins (LDL) are a major carrier of cholesterol in mammals. Accumulation of LDLs in blood vessels causes the formation of atherosclerotic plaques, which can lead to heart failure or to stroke. LDL levels are regulated, in part, by receptor-mediated endocytosis; LDL particles are bound by the LDL receptor, actively internalized into cells, and transported to the lysosome for destruction (1). Since the pioneering work of Brown and Goldstein, it has been known that the LDL receptor enters by the clathrin-dependent pathway (2). This form of receptor-mediated endocytosis requires a short peptide sequence in the cytosolic tail of the receptor. It was originally proposed that this endocytic motif had the general form NPXY (N denotes asparagine, P is proline, X refers to any amino acid, and Y is tyrosine) because other proteins, known to be internalized through the clathrin pathway, including the Alzheimer's β-amyloid precursor protein (β-APP), also have elements of this motif in their cytosolic tails (3). Mutagenesis studies with the LDL receptor have subsequently shown, however, that the hexapeptide, FDNPVY, is the sequence element recognized for its efficient endocytosis (4). Further studies have also demonstrated the existence of a more widely used tyrosine-based sorting motif, of the form YppØ (Y denotes tyrosine, p is a polar residue, and Ø refers to amino acids with a large hydrophobic side chain) [for recent review see (5)].

A large number of membrane proteins that traffic through the clathrin pathway are sorted into coated pits by clathrin adaptors, a group of proteins that recognize signals in the cytosolic portion of membrane proteins and also interact with clathrin [for recent reviews, see (5–7)]. The endocytic adaptor AP-2 is a heterotetrameric complex composed of α, β2, μ2 and σ2 subunits. The μ2-adaptin recognizes tyrosine-based sorting signals of the form YppØ (8–11); the β2-adaptin contains binding sites for a second sorting signal, the dileucine-based motif, and also for clathrin (4,12–19). It was initially proposed that AP-2 would recognize the FDNPVY sequence through the same interaction used for the canonical YppØ motif (20). Unrecognized at the time, however, this group used a recombinant tail that included a dileucine motif to show association with AP-2. Subsequent biochemical studies showed that peptides bearing the FDNPVY motif of the LDL receptor do not compete with YppØ-containing peptides for binding to μ2 (9). Indeed, in cells overexpressing the transferrin receptor, which carries a canonical YppØ endocytic signal (YTRF), there is a decrease in the rate of internalization of other YppØ-containing proteins, but no inhibition of LDL receptor internalization (21). Thus, the endocytic sorting motif found in the cytosolic tail of the LDL receptor, FDNPVY, is distinct from the more common YppØ motif and is unique to members of the LDL receptor family (4,18,19). More recently, from results obtained using nuclear magnetic resonance, it was proposed that sorting of the LDL receptor into coated pits does not require association with clathrin adaptors but instead results from a direct contact of the hexapeptide FDNPVY on the LDL receptor with the N-terminal domain of the clathrin heavy chain (22). Since clathrin is common to the endocytic and exocytic pathways, however, this suggestion would not explain the fact that the FDNPVY motif in the LDL receptor is not used for sorting by clathrin-

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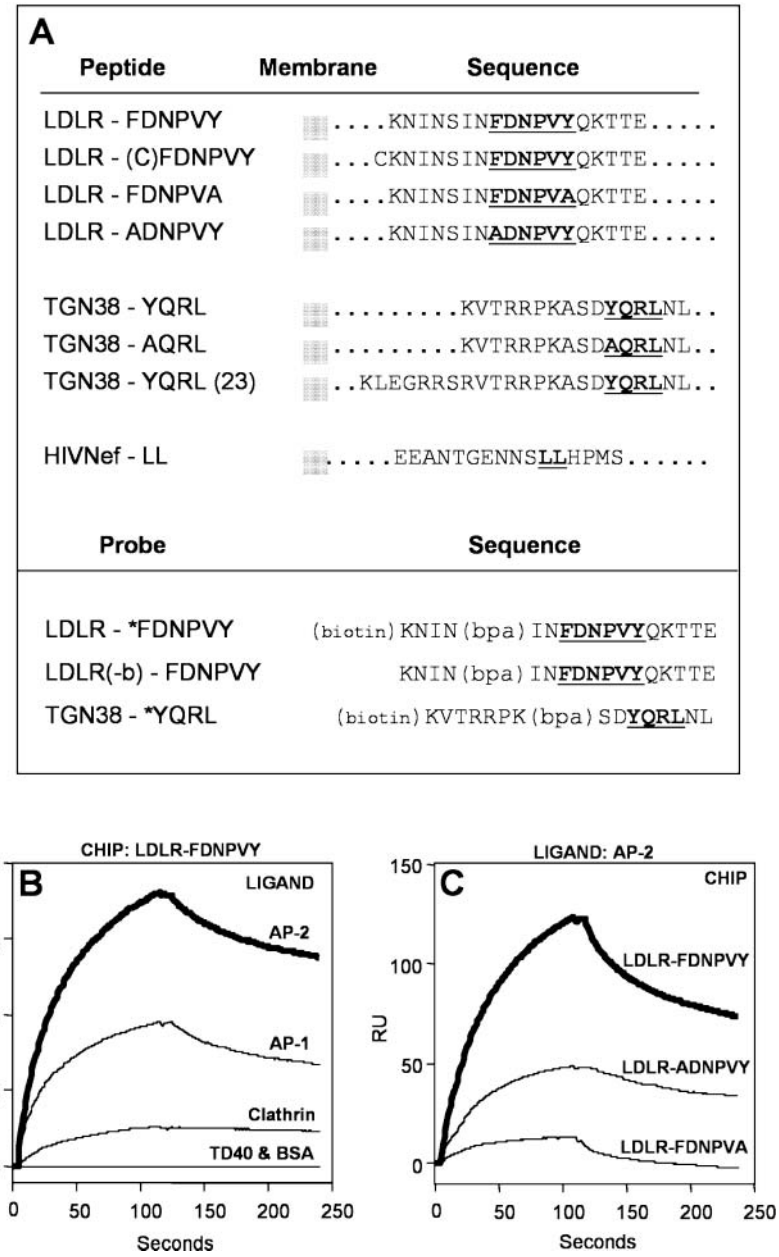


Figure 1: Interaction between AP-2 adaptors and the internalization motif FDNPVY of the LDL receptor. (A) Schematic representation of the synthetic peptides used for SPR and UV-crosslinking experiments containing FDNPVY, YppØ and LL sorting signals used in the study. The indicated motifs were defined in previous studies by analyzing the effect of point mutations on the traffic of the LDL receptor (LDLR) (5,20) and TGN38 (37,38) transmembrane proteins, and on Nef (45,47), a myristoylated protein encoded by the HIV-1 retrovirus. The sequences represent portions of the corresponding cytoplasmic portions of these proteins that surround these motifs. The solubility of the LDL-*FDNPVY peptide increased considerably by inclusion of benzoylphenylalanine (bpa) at serine 799, upstream of the FDNPVY motif, and allowed its use as a competitor for specificity determination in the crosslinking experiments. TGN38-YQRL (23) contains arginine instead of lysine residues at positions +4 and +8, thus minimizing chemical crosslinking of internal portions of the peptide with the chip. (B) Real-time surface plasmon resonance (SPR) analysis of the binding of clathrin coat components to a sensor chip containing immobilized peptidesbearing the FDNPVY endocytic motif of the LDL receptor (LDLR-FDNPVY). Samples containing 200 nM AP-1, AP-2, clathrin, or a recombinant fragment corresponding to the N-terminal domain of clathrin (TD40) were tested for their ability to bind to the surface of the chip. The chip contained 500 resonance units (RU) of the immobilized 18-mer LDLR-FDNPVY. The protein samples were injected at 20 μl/min for 120 s followed by a 120-s washout period; in order to compare the tracings from proteins of different sizes to each other, they were normalized to the molecular weight of AP-2. (C) Effect of varying the sequence of the LDL receptor endocytic motif on its ability to bind AP-2 adaptors. 200 nM AP-2 (the ligand) were injected at 20 μl/min for 120 s and used to evaluate its binding capacity to different chips containing the peptides LDLR-FDNPVY (490 RU immobilized), LDLR-ADNPVY (560 RU immobilized), or LDLR-FDNPVA (470 RU immobilized).

based traffic at the trans-Golgi network (in which sorting of proteins containing certain YppØ and dileucine-signals is mediated by the exocytic clathrin adaptor AP-1). Moreover, the FDNPVY motif in all members of the LDL receptor family is situated close to the membrane, usually 12 amino acids from the transmembrane domain (4). A 12-residue segment could span no more that 30–40 Å, even if completely extended, while the N-terminal domain of clathrin within the coat lies out of reach, about 100 Å from the membrane (23,24). Thus, the geometry of the proposed interaction seems unlikely.

We therefore decided to reinvestigate whether a peptide corresponding to part of the cytosolic tail of the LDL receptor and containing the FDNPVY motif could interact with either AP-2 or clathrin. We used two independent methods: one based on surface plasmon resonance (SPR) spectroscopy (8,9,25–29) and the other on photoactivated crosslinking techniques (9,15,30) that have been used successfully to detect relatively weak associations between sorting signals and adaptors. In particular, SPR is suitable for detecting short-lived interactions that are hard to see with more conventional 'pull-down' or chromatographic methods. We demonstrate here that AP-2 interacts through the μ2 subunit with peptides, derived from the cytosolic tail of the LDL receptor, that contain the FDNPVY endocytic sorting signal. This interaction involves a site in μ2-adaptin distinct from the one that recognizes the more common YppØ tyrosine-based sorting signal.

Results

The clathrin adaptor AP-2 interacts with the FDNPVY sorting signal

We immobilized on a SPR chip an 18-residue peptide (LDLR-FDNPVY) containing the FDNPVY endocytic motif of the LDL receptor, and asked whether we could detect an interaction with isolated protein components from coated vesicles. The sequence of the LDLR-FDNPVY peptide (Figure 1A) corresponds to residues 6–23 of the LDL receptor cytoplasmic tail, that includes the sequence required to support endocytosis of the LDL receptor (4). The SPR tracings in Figure 1(B) indicate that AP-2 binds specifically to the LDLR-FDNPVY chip, whereas equal amounts of AP-1 bind substantially less. In contrast, the LDLR-FDNPVY peptide barely binds clathrin purified from coated vesicles, and it does not bind the clathrin N-terminal domain [TD40, a fragment known to be correctly folded in a β-propeller conformation (17,31)] or bovine serum albumin (used as a negative control), even at concentrations of up to 5 μM (not shown). To examine the specificity of the interaction between the LDL receptor peptide and AP-2, we used two modified LDL receptor peptides in which single amino acids in the FDNPVY motif were replaced by alanine (Figure 1A). It is known that LDL receptor mutants containing FDNPVA or ADNPVY, instead of FDNPVY, have lower rates of receptor-mediated endocytosis and significantly impaired lysosomal degradation of the internalized LDL particles (9% or 37% of the control value, respectively) (4). Using chips with the altered sequences also caused a significant reduction in AP-2 binding (Figure 1C). We therefore believe we can rule out the proposed role of clathrin N-terminal domain as a significant element in the endocytic machinery responsible for LDL receptor sorting by recognition of the FDNPVY motif (22). Attempts to immobilize peptides with the sequence FDNAVY or FDAPVY corresponding to other LDL mutants also impaired in endocytosis (4) were not successful because of their tendency to aggregate.

The binding site for the FDNPVY sorting signal is located in the AP-2 core

The binding sites in clathrin adaptors that recognize the YppØ or the dileucine sorting signals are located within the 'AP-core' (8,15), the part of the complex that remains after enzymatic proteolysis consisting of the N-terminal fragments of α and β2 and the intact μ2 and σ2 subunits (32,33). To test whether the AP-2 core is also involved in the recognition of the FDNPVY motif, we subjected clathrin adaptors to limited trypsin digestion (Figure 2A) and asked whether AP-cores can still recognize the LDL receptor motif. As shown by the SPR tracing in Figure 2(B), AP-2 cores bind to the LDLR-FDNPVY chip, to about the same extent as intact AP-2s (compare the amplitude in the SPR tracings in Figure 1B,C with Figure 2B). As expected, the association of AP-2 cores to the LDLR-FDNPVA or LDLR-ADNPVY chips was significantly reduced (Figure 2B), confirming the specificity of the interaction.

The μ2 subunit of AP-2 interacts with the FDNPVY sorting signal

Because μ2-adaptin binds directly to peptides bearing YppØ sorting signals, and because both the YppØ and the FDNPVY signals are tyrosine based, we asked whether the free μ2 subunit of AP-2 could also interact with the immobilized LDLR-FDNPVY peptide. Two constructs of μ2 were used for SPR experiments, His-Δμ2 (hexa histidine-tag fused to residues 122–435 of rat brain μ2 (34)) and His-ΔΔμ2 (hexa histidine-tag fused to residues 158–435). His-Δμ2 and His-ΔΔμ2 contain the binding site for YppØ (8), fold correctly and have atomic structures that are essentially identical as determined by X-ray crystallography (11,35).

Both constructs, His-Δμ2 and His-ΔΔμ2, bind to TGN38-YQRL, a 16-residue peptide corresponding to the well-studied endocytic motif at the C-terminus of the trans-Golgi TGN38 protein (36,37), as expected from published data (8,9,11,38) and from data shown here (Figure 3A). Likewise, each one of these fragments bound equally well to the LDLR-FDNPVY chip, although with a lower binding capacity than the TGN38-YQRL chip (Figure 3B). The calculated association constant (k_a) for the interaction of His-Δμ2 with the LDLR-FDNPVY peptide was 7×10^5 ($M^{-1} \times s^{-1}$) and the dissociation constant (kDa) was 1.2×10^{-2} (s^{-1}). Likewise, for AP-2 the association and dissociation constants were

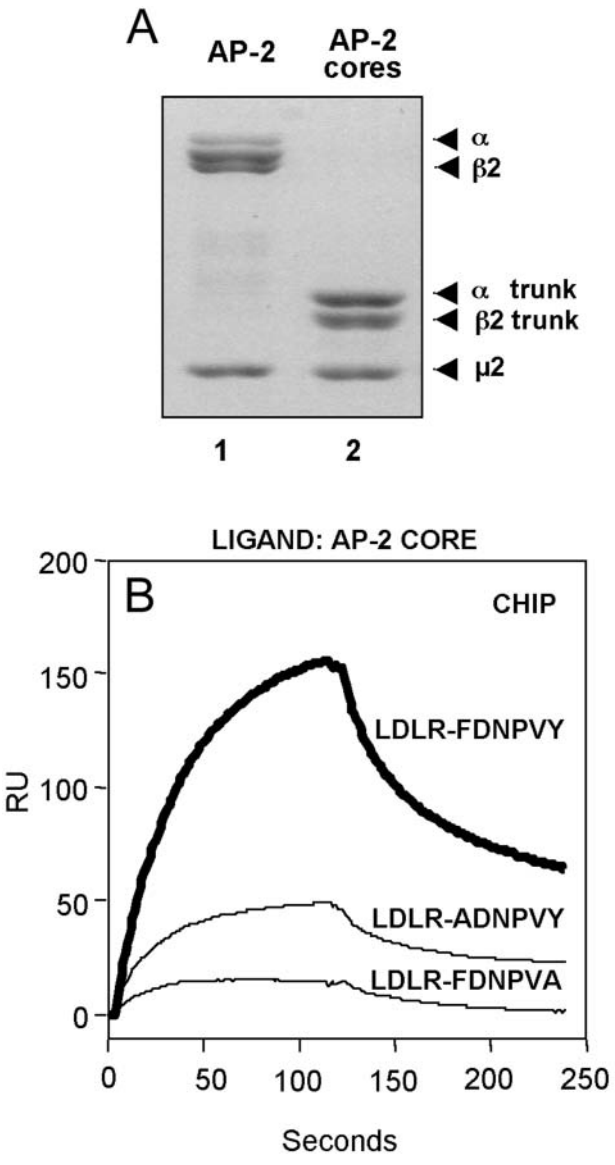


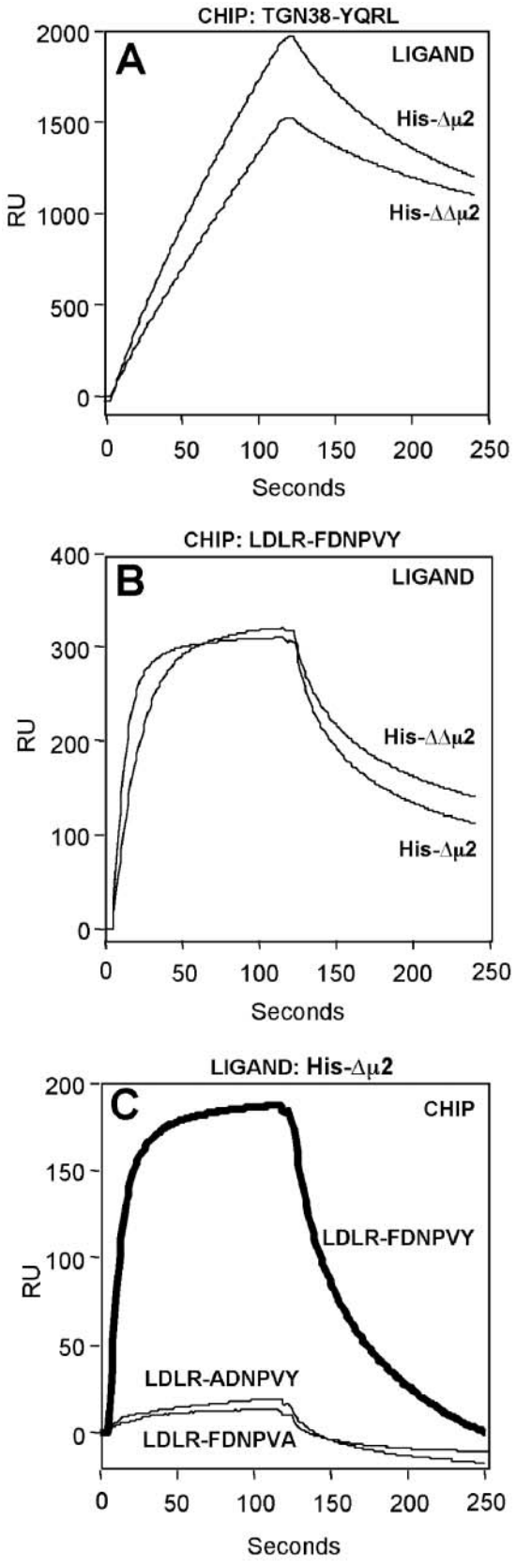
Figure 2: AP-2 cores bind to the LDLR-FDNPVY peptide. (A) Generation of AP-2 cores. The protein bands of AP-2 before (lane 1) or after (lane 2) limited digestion with trypsin were visualized by 10% SDS-PAGE fractionation and Coomassie Blue staining. The bands for intact α, β1 and μ2-adaptins in AP-2 and the remaining N-terminal (trunk) domains of α and β together with the μ2 contained within the AP-2 core are shown. (B) Binding of AP-2 cores to the LDLR-FDNPVY peptide is specific. The SPR tracings correspond to the recruitment of 200 nM AP-2 cores to three different chips containing either wild-type peptide LDLR-FDNPVY or mutant peptides LDLR-ADNPVY and LDLR-FDNPVA.

7.4×10^3 ($M^{-1} \times s^{-1}$) and 2×10^{-3} (s^{-1}). Due to the poor solubility of the LDL-based peptides, we could not reach sufficient concentrations to include them as competitors during the dissociation phase, and thus prevent rebinding of the released proteins back to the chip. Therefore, these values should only be considered as approximate, since the measured dissociation constants are likely to be significantly lower than the real values. His-ΔΔμ2 also binds to the LDL-FDNPVY chip, suggesting that the μ2 binding site for the LDLR-FDNPVY peptide lies in the 158–435 fragment and not in the segment between residues 122 and 157 that is disordered in the X-ray crystal structure of His-Δμ2 and AP-2 cores (11,39). Further confirmation that μ2 is the subunit of AP-2 responsible for selective recognition of the FDNPVY motif is presented in Figure 3(C). It shows that, like intact or cleaved AP-2, the His-Δμ2 construct binds less efficiently to chips with the LDLR-ADNPVY and LDLR-FDNPVA peptides.

As an alternative to the SPR experiments, we used an independent approach involving peptides bearing a photo-activatable crosslinker to verify that the μ2 subunit interacts with the LDL-based peptide in a specific way. This method was successfully used before to investigate the regulation involved in the recognition of YppØ sorting signals by μ2 in clathrin adaptors and to establish that their β subunits recognize dileucine-based sorting signals (15,30). The results presented in Figure 4(A) (lanes 1–3) are completely consistent with the SPR data. His-Δμ2 crosslinked with the LDL-*FDNPVY photo-reactive peptide (lane 1), and formation of the crosslinked species was prevented by the nonbiotinylated form of the LDL-probe (lane 2). As a control for specificity, we used excess amounts of the TGN38-YQRL peptide bearing the canonical YppØ motif and found that it does not reduce formation of the crosslinked product with LDL-*FDNPVY (lane 3); the same concentration of TGN38-YQRL, however, completely blocked the generation of crosslinked species of His-Δμ2 and the photo-reactive peptide TGN38-*YQRL (lane 7). Use of AP-2 with LDL-*FDNPVY led to the generation of a major crosslinked species involving μ2, together with a weaker labeling of α and β-adaptin, whose staining intensity varied between experiments.

The binding sites in μ2 for FDNPVY and YppØ are not the same

One indication that μ2 has two distinct binding sites, one for the FDNPVY motif and the other for the YppØ motif, comes from the observation that soluble TGN38-YQRL peptide is effective in blocking the recruitment of His-Δμ2 to the TGN38-YQRL chip (Figure 4B), whereas it has no effect on the recruitment of His-Δμ2 to the LDL-FDNPVY chip (Figure 4C). A second line of evidence comes from using a mutated form of μ2 that cannot interact with peptides bearing YppØ motifs (11,40). Peptides bearing the endocytic motifs YQRL (from TGN38) or YRAL (from the epidermal growth factor receptor) bind to μ2 in a site with contributions from residues along the β-1 and β-16 strands in subdomain A of μ2 (11). A key residue in μ2 required to support this interaction is W421, which forms hydrophobic contacts with the arginine at Y + 2 of the YppØ motif, leading to the preferential association with μ2. Indeed, cells expressing μ2 in which W421 has been changed to alanine assemble AP-2 adaptors carrying the mutated μ2 subunit but are deficient in receptor-mediated endocytosis of transferrin (40). Therefore, we constructed the mu-



tated His-Δμ2 (W421A) and asked whether this protein would be hindered in its capacity to bind not only to the TGN38-YQRL peptide but also to the LDL-FDNPVY peptide. As expected, His-Δμ2 (W421A) did not bind to the TGN38-YQRL chip (Figure 4B). We found, however, that His-Δμ2 (W421A) still bound to the LDL-FDNPVY chip, to a similar extent as His-Δμ2 (Figure 4C).

These results were confirmed in two ways with the crosslinking approach (Figure 4A). Using a concentration of TGN38-YQRL peptide that blocks the crosslinking reaction with TGN-*YQRL (Figure 4A, lane 7), we were not able to prevent the crosslinking reaction between LDL-*FDNPVY and His-Δμ2 (compare lanes 3 and 1), suggesting that the interaction occurs at different sites in μ2. In the second approach, we used His-Δμ2 (W241A) and found that, as expected, it totally failed to react with the TGN38-*YQRL photoactive peptide (lane 8); in contrast, His-Δμ2 (W241A) interacted perfectly well with the LDL-*FDNPVY photo-active peptide (lane 4). Taking the results from the SPR and crosslinking experiments together, we conclude that μ2 contains distinct binding sites for the FDNPVY and YppØ motifs.

The interaction of the FDNPVY motif with AP-2 is enhanced by the presence of soluble peptides containing the YQRL motif

Recently it was found that a 14-mer TGN38-based peptide containing its YQRL motif elicits an enhancement of the interaction between AP-2 and synaptotagmin, a transmembrane protein found in synaptic vesicles, thereby facilitating its incorporation into coated vesicles (41). It is possible that interaction of peptides bearing YppØ motifs with the μ2 subunit in AP-2 has a general 'activation' effect on the adaptor to facilitate cargo loading during the formation of coated pits (41). There is no significant sequence similarity between the cytosolic portions of the LDL receptor and synaptotagmin, and it is therefore unlikely that both proteins contact the same binding site in AP-2. Although the TGN38-YQRL peptide does not inhibit the binding of AP-2 to the LDLR-FDNPVY chip, to our surprise, addition of TGN38-YQRL peptide actually stimulated recruitment of AP-2 to the LDLR-FDNPVY

Figure 3: The μ2-adaptin recognizes the FDNPVY internalization motif of the LDL receptor. (A) The SPR tracings show the recruitment of 200 nM of the recombinant forms of μ2 His-Δμ2 or His-ΔΔμ2 to a chip containing 600 RUs of immobilized TGN38-YQRL peptide. (B) His-Δμ2 and His-ΔΔμ2 bind equally well to the peptide containing the FDNPVY internalization motif. The SPR tracings illustrate the relative association of 200 nM His-Δμ2 or His-ΔΔμ2 to a chip containing 500 RU of immobilized LDLR-FDNPVY. (C) Binding of His-Δμ2 to the LDLR-FDNPVY peptide is specific. The SPR tracings illustrate the recruitment of 200 nM His-Δμ2 to a chip containing wild-type peptide LDLR-FDNPVY and the minimal association to two different chips containing the mutant peptides LDLR-ADNPVY or LDLR-FDNPVA.

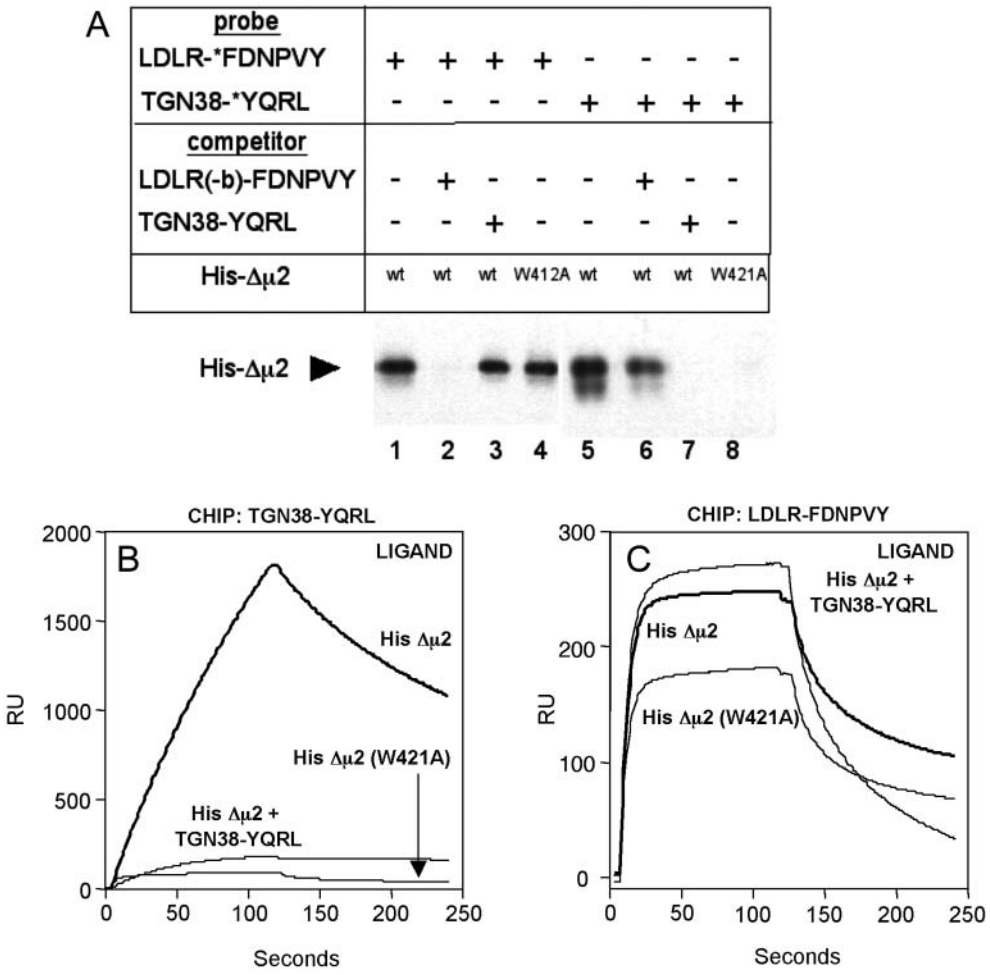


Figure 4: Distinct sites in μ2-adaptin recognize the FDNPVY internalization motif or the canonical YppØ endocytic signal. (A) His-Δμ2 has two different binding sites, one for FDNPVY and the other for YQRL sorting signals. The blot shows the appearance of crosslinked products between His-Δμ2 (lanes 1–3, 5–7) or His-Δμ2 (W421A) (lanes 4, 8) and the photo-reactive peptide probes LDL-*FDNPVY (lanes 1–4) and TGN38-*YQRL (lanes 5–8). (B) Interaction of His-Δμ2 with TGN38-YQRL is specific and depends on the specific contact between the YQRL sequence and its binding site in μ2. Two SPR tracings correspond to the recruitment of His-Δμ2 to immobilized TGN38-YQRL peptide carried in the absence (thick tracing) or presence (thin tracing) of 200 μM TGN38-YQRL peptide (added as a competitor). The third SPR tracing (thin) shows that the mutated μ2-adaptin His-Δμ2 (W421A) does not bind to the chip with TGN38-YQRL because it is impaired in its recognition of YppØ endocytic motifs. (C) Association of His-Δμ2 to the LDLR-FDNPVY peptide does not require the YppØ binding site in μ2. The SPR tracings illustrate that His-Δμ2 and His-Δμ2 (W421A) bind similarly to the LDLR-FDNPVY chip. The presence of 200 mM TGN-YQRL peptide does not influence binding of His-Δμ2 to the LDL-FDNPVY chip.

chip (Figure 5A). As expected, the association of AP-2 with the chip containing the YQRL motif was inhibited (Figure 5B). There was no effect of TGN38-YQRL on the association of clathrin or its N-terminal domain with the LDLR-FDNPVY chip (not shown).

The stimulatory effect requires the presence of an active YQRL motif. This was verified by using two peptides known not to bind to μ2: the TGN38-AQRL peptide, in which the Y of the YQRL motif is replaced with alanine (9,42,43), and a peptide derived from the disordered loop at the C-terminus of HIV-1 Nef, which lacks the YppØ motif but includes a di-leucine sorting signal recognized by β-adaptin (44–46). Neither of these peptides induced the enhancement of AP-2 binding seen in the presence of TGN38-YQRL (Figure 5A). Like intact AP-2, the core showed an increase of LDLR-FDNPVY binding in the presence of TGN38-YQRL peptides and no stimulation by the peptides known not to bind AP-2 (not shown). Soluble TGN38-YQRL did not, however, enhance binding of His-Δμ2 to LDLR-FDNPVY (Figure 4C).

This stimulatory effect might involve a substantial conformational change in the adaptor induced by the presence of the TGN38-YQRL peptide (41). Indeed, in the presence of

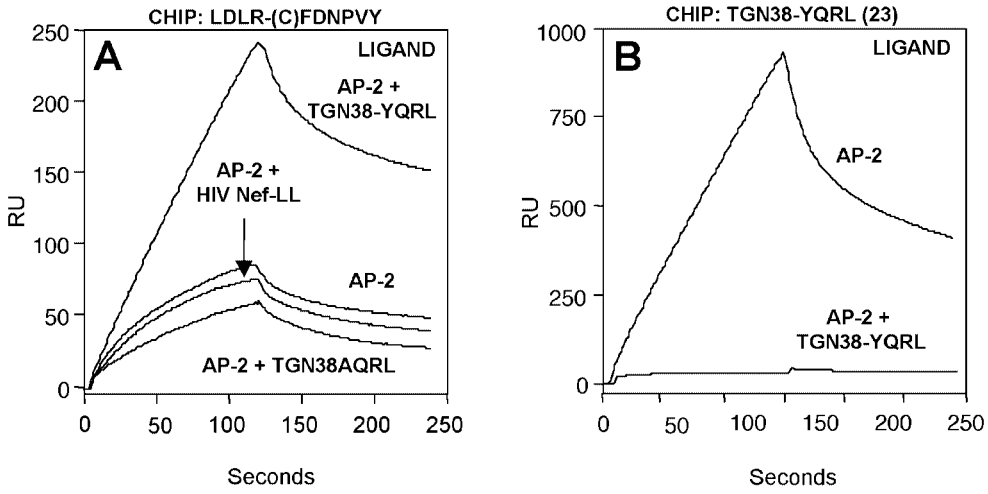


Figure 5: Peptide bearing the YQRL endocytic signal stimulates the interaction between AP-2 adaptors or AP-2 cores and the LDLR-FDNPVY chip. (A) Stimulatory effect of TGN38-YQRL peptide on the recruitment of AP-2 to a chip containing the FDNPVY motif. LDLR-(C)FDNPVY peptides were immobilized to the chip by a thiol-coupling reaction. The tracings reflect the relative association of 0.2 μM AP-2 to the chip in the presence of 200 μM TGN38-YQRL, TGN38-AQRL or HIV Nef-LL peptides. (B) TGN38-YQRL peptide does not stimulate the recruitment of AP-2 to a chip containing the YQRL motif. The SPR tracings show the binding of AP-2 to a chip containing immobilized TGN38-YQRL (23) peptide carried out in the absence or presence of 200 μM of free TGN38-YQRL. This experiment was performed with the slightly longer TGN38-YQRL (23) peptide immobilized to the chip, instead of TGN38-YQRL, because it improved binding of AP-2 to the chip. Similar binding results were obtained with a chip with immobilized TGN38-YQRL (not shown).

the TGN38-YQRL peptide, AP-2 becomes less susceptible to enzymatic proteolysis. This is illustrated by the SDS-PAGE data in Figure 6, which show a significant decrease in the extent of tryptic cleavage at the hinges separating the N-terminal trunk and the C-terminal ear domains of not only the α-adaptin, as was shown earlier (41), but also at the equivalent position in β-adaptin. This effect requires the interaction of μ2, since TGN38-AQRL or HIV Nef-LL (data not shown) did not change the access of the hinges to trypsin (Figure 6). It is known that the isolated 158–435 segment of μ2 does not appear to undergo a major conformational change upon binding of YppØ-containing peptides as demonstrated by the same crystal structure in the presence or absence of bound peptides bearing the YQRL or YRAL endocytic sequences (11,35). Thus, the stimulation seen here for the association of AP-2 with the LDL-peptide, or of its association with synaptotagmin as reported previously (41), might involve a conformational change in the adaptor triggered by the association of the YQRL-peptide with μ2.

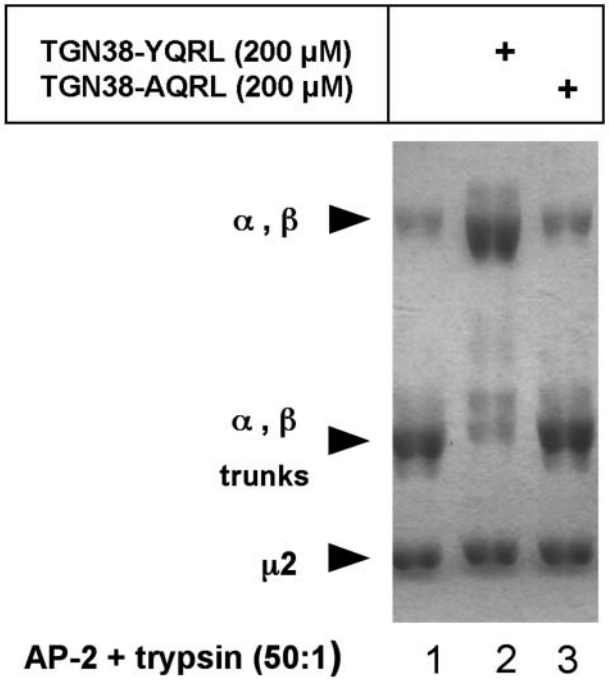


Figure 6: TGN38-YQRL peptide inhibits the proteolysis of AP-2. Twenty micrograms (at 1 mg/ml) AP-2 was incubated with 0.5 μg (at 10 μg/ml) trypsin for 30 min at room temperature without peptide (lane 1), with 0.2 mM TGN38-YQRL (lane 2) or TGN38-AQRL (lane 3). Proteins were resolved by 12% SDS-PAGE and bands detected by Coomassie Blue staining.

Discussion

Our data show that the AP-2 adaptor and, more specifically, its μ2 subunit interact with a peptide bearing the FDNPVY endocytic signal found in the cytosolic tail of the LDL receptor. Moreover, we document here that μ2 contains two distinct binding sites: one for the canonical YppØ endocytic signal and the other for the LDL receptor tyrosine-based motif.

Beyond the original observation by Pearse that a very small fraction of iodinated AP-2 is retained in an affinity support containing an immobilized portion of the LDL receptor cytosolic tail (20), it has been hard to detect interaction using methods such as coimmunoprecipation from cells, pull-downs with recombinant proteins or yeast-two-hybrid approaches. Here we detect and study the interaction between the LDL receptor internalization motif and μ2 of AP-2 with two independent methods.

Since the binding site on μ2 for FDNPVY is distinct from that for YppØ [this work and also (9)], it is possible to imagine independent regulation in the internalization of the LDL-receptor class of molecules from the much more common class of proteins that use YppØ for sorting. Recruitment and assembly of the coat components at the plasma membrane is a dynamic process that undoubtedly involves a large number of regulatory steps (5–7). The interaction of AP-2 with clathrin or with phosphoinositides, especially those phosphorylated in the inositol ring at the D-3 position, also leads to a considerable increase in the affinity of interaction between AP-2 and YppØ endocytic motifs (30). AP-2 recruitment to plasma membranes of neuronal and non-neuronal membranes is also enhanced by peptides bearing the YppØ motif (41). This recruitment is probably mediated by synaptotagmin, an AP-2 binding protein proposed to function as a docking site for AP-2 in synaptic membranes, which binds better to AP-2 in the presence of peptides with the YppØ-tyrosine-based motif. Thus, the binding of YppØ endocytic motifs to the μ2 chain of AP-2 appears to increase AP-2 affinity for at least two types of molecules: synaptotagmin and, as reported now here, to a peptide corresponding to part of cytosolic tail of the LDL receptor. Therefore, there is significant cross-talk among a number of interacting molecules, orchestrated by the adaptor complexes.

These observations are consistent with the general model proposed by us and by others linking cargo loading with clathrin coat formation (30,41). These interactions might help to cluster the cargo, or might help to ensure that a vesicle does not form unless it can capture a sufficient number of cargo molecules. Indeed, other observations suggest that coat formation is linked to cargo capture: electron microscopy of cells in tissue culture shows that about 60–70% of their clathrin-coated profiles are labeled with markers for the LDL and transferrin and epidermal growth factor receptors (47–49).

The intracellular traffic of the LDL receptor is far from simple, and is clearly under the control of a number of steps through the endocytic as well as the secretory pathway. The stimulatory results documented here on the association of the LDL peptide containing the FDPNPY sequence and AP-2 are consistent with the possibility that the uptake of the LDL receptor can be subjected to specific regulation within the clathrin pathway. This regulatory step could complement the well-established modulation of the number of LDL receptors available for endocytosis that is exerted by transcriptionally based long-term control in response to available cellular cholesterol (18,50).

During biosynthesis, LDL receptors that transit the TGN are not sorted by the ubiquitously expressed AP-1A adaptors (51). Our *in vitro* data are consistent with these observations in that the ubiquitous AP-1A adaptor (equivalent to the neuronal AP-1 used here) binds with less affinity than AP-2 to the LDL receptor internalization motif. In fact, the polarized secretory traffic of the receptor to the basolateral plasma membrane involves a second sorting signal located elsewhere in the cytosolic tail of the receptor, which differs from the FDNPVY internalization motif (52,53). Basolateral localization is mediated by a specialized, less abundant μ1b species found in AP-1B, an adaptor characteristic of polarized epithelial cells, through a mechanism of interaction that still needs to be resolved (51).

Another way to control the traffic of the LDL receptor might involve recognition of its FDNPVY motif by proteins containing phosphotyrosine binding (PTB)-domains, since these domains can recognize an NPXY motif whose tyrosine is phosphorylated. Since mutations in a PTB-domain containing protein lead to autosomal recessive hypercholesterolemia (ARH) protein, it was suggested that the ARH protein interacts with the LDL receptor through the NPVY motif, thereby regulating the intracellular traffic of the receptor (54).

In conclusion, we demonstrate that μ2 contains two distinct binding sites: one for peptides containing the FDNPVY internalization motif of the LDL receptor, and a second one that recognizes the more common YppØ tyrosine-based sorting signal. Moreover, the interaction of AP-2 with peptides bearing the FDNPVY motif seems to be modulated by a conformational change in AP-2, involving its association with YQRL-containing peptides.

Materials and Methods

Proteins

Clathrin, AP-1, and AP-2 were purified from bovine-brain-coated vesicles as described (15,55). Clathrin was kept at 4°C in 500 mM TRIS, 1 mM EDTA, 0.02% NaN_3. APs were kept at 4°C in 100 mM MES pH 7.0, 150 mM NaCl, 1 mM EDTA, 0.02% NaN_3, 0.5 mM DTT (AP buffer). Limited tryptic proteolysis of AP-2 results in the release of the carboxyl-terminal domains (the ear and hinge) of the large β1/β2 and α subunits of AP-2 from the AP-core (containing the N-terminal domains of α and β-adaptins, together with μ2 and σ2). AP-2 cores were generated upon incubation of AP-2 (0.4 mg/ml) with 5 μg/ml (final concentration) of trypsin at 25°C for 30 min (12,32,33). Proteolysis was ended by addition of 75 μl benzamidine-agarose beads (Pharmacia) and end-over-end rotation for 10 min at 4°C. The beads were separated from the protein solution using a spin column (Bio-Spin; Bio-Rad, Hercules, CA, USA). The extent of proteolysis was verified by 10% SDS-PAGE and staining with Coomassie Blue. The AP-2 cores were stored in AP buffer at 4°C in the presence of 2 mM (final concentration) of benzamidine.

Recombinant TD40 corresponds to the N-terminal domain of rat brain clathrin (residues 1–393) (17), and contains six histidines added to the N-terminus. TD40 was expressed in *Escherichia coli and* purified by Ni-NTA chromatography and gel filtration (Superdex 75) in 20 mM MES, pH 8.0, 150 mM NaCl, 6 mM $MgCl_2$, and 0.5 mM DTT. His-Δμ2 and His-ΔΔμ2 were generated by polymerase chain reaction from a plasmid template containing the complete rat brain μ2 open reading frame (34). His-Δμ2 and His-ΔΔμ2 span residues 122–435 and 158–435, respectively, and were inserted between the NcoI and EcoRI sites of the pProEX^{TM}HTA expression vector (Life Technologies, Grand Island, NY, USA). The proteins were expressed in *E. coli and* purified by Ni-NTA chromatography. The recombinant proteins were stored at −80 °C in 50 mM TRIS-HCl pH 8.0, 500 mM LiCl, 3 mM β-mercaptoethanol, 200 mM imidazole and 20% glycerol (His-Δμ2 or His-ΔΔμ2). Prior to SPR experiments, the glycerol in the buffer of the protein solutions was removed by gel filtration (Sephadex G-25, NAP5 columns, Pharmacia, Piscataway, NJ, USA), followed by 50-fold dilution of the protein sample in AP buffer. Aggregates were eliminated by high-speed centrifugation at 100 000 r.p.m. for 20 min and 4 °C (TLA-100.4 rotor, Optima TLX centrifuge, Beckman, Fullerton, CA, USA).

Surface plasmon resonance

Most of the results shown were obtained at 25 °C with a BIAcore 3000 instrument (Pharmacia Biosensor), although a BIAcore 2000 was used with similar results to follow the interaction of the sample proteins with sensor chips containing immobilized peptides. The SPR experiments were carried in AP buffer supplemented with 1 mg/ml carboxymethyl-dextran and with 0.02% Triton X-100. The peptides were synthesized by standard M-MOC procedures, dissolved in water and kept at −80 °C. The peptides were immobilized to a final amount of 400–600 resonance units (RUs) onto the surface of CM5 sensor chips (research grade; Pharmacia Biosensor) using EDC/NHS. In some cases we immobilized the LDLR-FDNPVY peptide with a cysteine at its N-terminal end (LDLR-(C)FDNPVY) by coupling it to a PDEA-derivatized chip. Use of relatively low amounts of immobilized peptides (400–600 RUs), in conjunction with a flow rate of 20 μl/ml and a relatively low concentration (200 nM) of soluble protein (injected as 40-μl samples), consistent with a reasonable and reproducible binding signal, allowed us to minimize possible kinetic artifacts due to steric hindrance, crowding and aggregation. Similar conditions have been used to study the interaction between APs and peptides containing sorting signals (26,28,29). Similarly to their results, we observed that a fraction of APs bound irreversibly to the chip, perhaps reflecting a small portion of APs that forms dimers or higher aggregates. All experiments were carried out in the presence of 1 mg/ml carboxy-methylated dextran. No differences in the binding profiles were detected in the absence of carboxy-methylated dextran. None of the proteins bound to any significant level to chips derivatized in the absence of peptide, ruling out the possibility that the observed associations were nonspecific and charge dependent. Association and dissociation rate constants were calculated using BIAevaluation 3.1 software from injections into flow cells immobilized with 150–200 RU of peptide. The sensorgrams represent the binding of ligand after subtraction of changes in refractive index caused by bulk flow of protein and/or peptides through the flow cells.

When the protein samples were co-injected with soluble peptide (dissolved in AP buffer), they were preincubated for 15 min at room temperature before injection and were present during the dissociation phase. After each injection, the surfaces of the chips were regenerated by injection of 8–10-s pulses (80 μl/min) of 10 mM NaOH and 0.25% SDS until the pre-injection baseline was restored. The results presented in the figures are representative of at least three independent experiments performed using different sensor chips and different protein preparations. The stimulation effect of TGN38-YQRL on the binding of AP-2 to the FDNPVY motif denoted in Figure 5 represents the results of 20 different experiments.

UV-induced crosslinking reaction and detection of crosslinked products

The UV-induced crosslinking reaction and detection of products was carried out as previously described (15,30). Sixteen microliters of a solution containing HisΔμ2 or HisΔμ2 (W421A) (2 μM final concentration) were mixed with 2 μl of the photoreactive peptides (1 μM LDLR-*FDNPVY or 0.2 μM TGN38-*YQRL final concentrations). Competition experiments were done by including 2 μl of the nonbiotinylated peptides LDL(−b)-FDNPVY (60 μM final concentration) or TGN38-YQRL (200 μM final concentration). Crosslinking was triggered by exposure to UV-illumination for 5 min in wet ice. The UV illumination was also carried out with samples frozen in dry ice, with identical results (not shown).

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