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Sequence requirements for the recognition of tyrosine-based endocytic signals by clathrin AP-2 complexes

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We recently determined that fusion proteins containing tyrosine-based endocytic signals bind to the μ2 subunit of AP-2, the complex that drives clathrin coat formation and mediates endocytosis from the plasma membrane. Here we analyze the selectivity of peptide recognition by μ2 and by AP-2 using combinatorial selection methods and surface plasmon resonance. Both μ2 and AP-2 are shown to interact with various sequences of the form tyrosine–polar–polar–hydrophobic (YppØ) found on receptors that follow the clathrin pathway. The optimal sequence for interaction with μ2 and with AP-2 has tyrosine as an anchor and prefers arginine at position Y+2 and leucine at position Y+3. In contrast, no preferred sequence is detected surrounding the YppØ signal, indicating that recognition of the YppØ endocytic signal does not require a prefolded structure. We conclude that sorting into the endocytic pathway is governed by a surprisingly simple interaction between the μ2 chain and a tyrosine-containing tetrapeptide sequence.

Keywords: adaptors/clathrin-associated proteins/coated vesicles/endocytosis/protein sorting

Introduction

Many cell surface membrane proteins bear on their cytoplasmic tails endocytic signals that direct internalization by clathrin-coated pits (reviewed in Trowbridge *et al.*, 1993; Thomas and Roth, 1994). One of these signals, identified by sequence correlation and mutational analyses, is a short stretch of four to six amino acid residues with an essential tyrosine (Y). Mutational analyses of Y-based endocytic motifs have demonstrated that two of the residues in the motif, the anchor Y and a hydrophobic residue at Y+3, are critical for function (Jadot *et al.*, 1992; Trowbridge *et al.*, 1993; Thomas and Roth, 1994). The receptor for the Y motif is presumed to be AP-2, the clathrin-associated protein complex that is located at the plasma membrane (Robinson, 1989). This role for AP-2 was originally suggested by *in vitro* binding studies (Pearse, 1988; Glickman *et al.*, 1989; Sosa *et al.*, 1993; Boll *et al.*, 1995). More recently it was shown that AP-2 associates with various growth factor receptors on cells (Sorkin and Carpenter, 1993; Boll *et al.*, 1995; Gilboa *et al.*, 1995; Nesterov *et al.*, 1995; Sorkin *et al.*, 1995) and that there is a direct interaction between AP-2 and the EGF receptor (Sorkin *et al.*, 1995) dependent on a region in the cytoplasmic domain of the receptor containing the sequence YRAL (Boll *et al.*, 1995; Nesterov *et al.*, 1995; Sorkin *et al.*, 1995).

We have recently shown that the μ2 chain of AP-2 can interact with peptide segments bearing the Y-based signal (Ohno *et al.*, 1995). We have therefore suggested that μ2 is responsible for recognizing the endocytic signal and that this interaction directs receptors into AP-2-containing coated pits. It has not yet been possible, however, to examine whether μ2 is selective only for the known endocytic motif and thus whether it is μ2 that accounts for the selectivity of AP-2–receptor tail interactions. We now show that μ2 can be used directly to select peptide sequences and that the sequences selected by μ2 in two combinatorial methods resemble the observed tetrapeptide consensus sequence for directing endocytic uptake. Furthermore, we find that this short motif is sufficient for binding, since the four residue endocytic signal can be embedded in an essentially random sequence without noticeable loss of affinity. Thus, a short peptide segment binding to μ2 determines the specificity for inclusion of receptors into endocytic clathrin-coated pits.

Results

Selection by free μ2 of the tyrosine-based endocytic signal from an oriented combinatorial peptide library

Previous studies using the yeast two hybrid system demonstrated that interaction of endocytic signals with μ2 is dependent on the presence of a critical Y residue (Ohno *et al.*, 1995). In order to determine the requirements for other residues surrounding the Y, we screened an oriented, Y-fixed combinatorial peptide library containing ~1 000 000 000 different peptides for binding to recombinant μ2. In this library, Y was always present at position eight of the 15 residue peptides and four residues on either side of the Y had degenerate sequence and contained approximately equal amounts of 16 of the possible 20 amino acids. Peptides specifically bound to μ2 were affinity purified using a GST–Δμ2 fusion protein bound to glutathione–agarose beads. Of the eight positions randomized in the library, two showed strong preferences: R at position Y+2 and a large hydrophobic residue, especially L, at Y+3 (Figure 1). Consistent with this pattern were results from an R-fixed library, which yielded

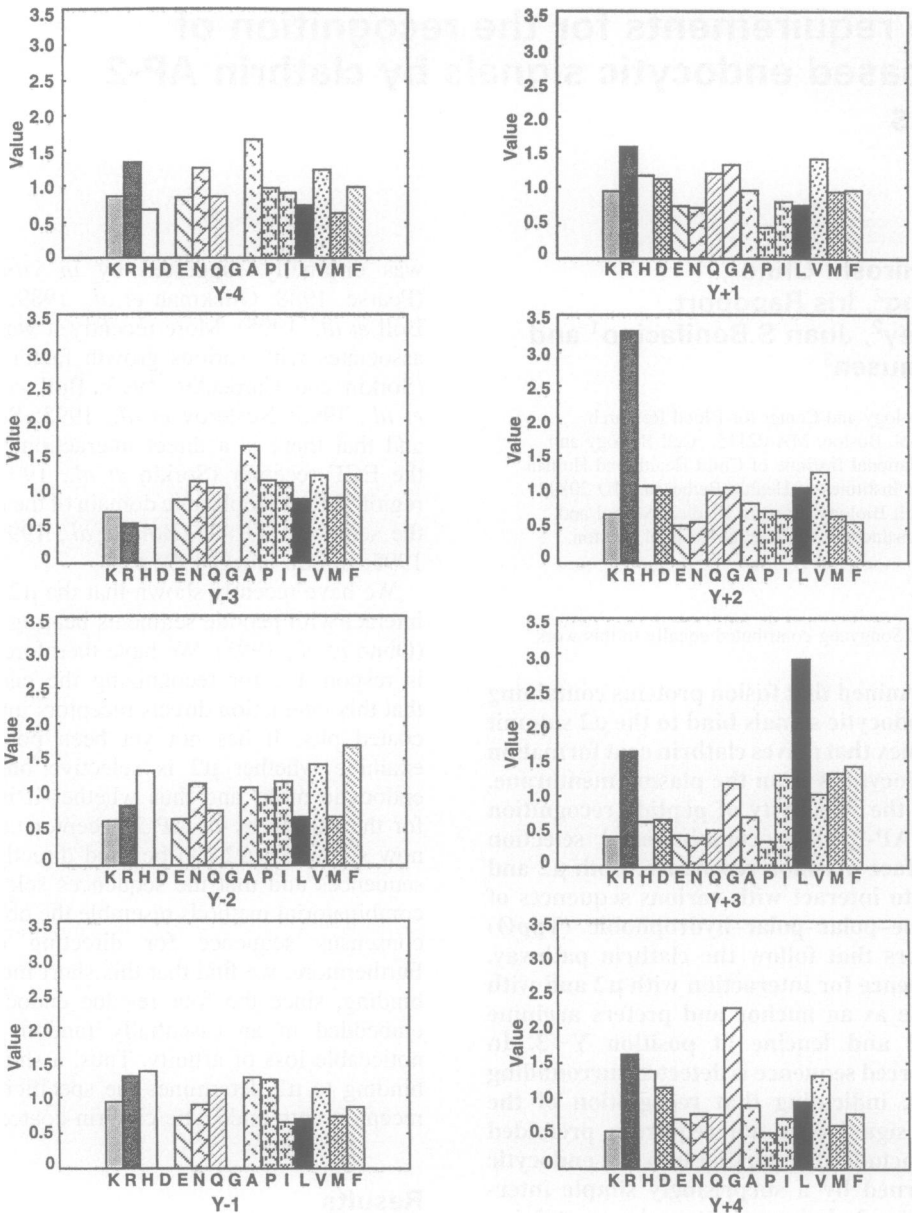

Fig. 1. Selection from a combinatorial library of tyrosine-containing peptides that bind μ2. A solution containing the oriented Y-fixed combinatorial peptide library was passed through a column containing GST–Δμ2 and the retained peptides were subjected to N-terminal microsequence analysis. The sequencing cycles correspond to the four positions before and the four positions after the fixed tyrosine. These positions have a random composition of amino acid residues in the peptide library. Values >1.5 (R at Y+2 and L at Y+3) indicate strong selection. A small but significant selection of M, F and V at Y+3 was also detected. A representative result from three experiments is presented. Amino acid residues are indicated by their single letter abbreviations.

a strong preference for Y at R–2 (data not shown). The Y-fixed library also showed weak but significant bias toward G at Y+4, but the peptides ended almost immediately after this position, so the preference may or may not be significant. It is important to bear in mind that the sequence preferences revealed by this approach are statistical and that they do not show how preferences at one position correlate with residues present at other randomized positions (Songyang *et al.*, 1993), i.e. the selection does not yield sequences from unique peptides, but provides instead the amino acid composition at any given position of the selected peptide mixture.

Selection by free μ2 of the tyrosine-based endocytic signal from a combinatorial yeast two hybrid screen

To determine whether the μ2 chain only binds Y-containing sequences, we used a second combinatorial approach: a yeast two hybrid screen with the truncated Δμ2 chain as 'bait' and a portion of the TGN38 cytoplasmic tail with a randomized sequence of four amino acids instead of the YQRL sorting motif as 'prey'. Nineteen clones were isolated that interacted with μ2 in this assay. We found that most interacting clones have a YppØ sequence at the position of the original Y motif (Figure 2). At the position

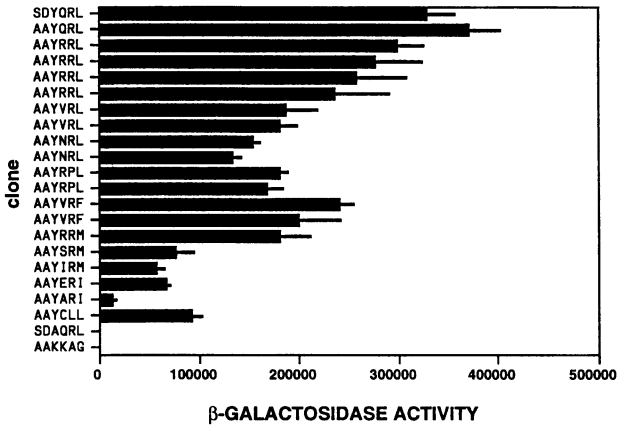

Fig. 2. Selection from a combinatorial DNA library of clones that interact with μ2. A plasmid library encoding a portion of the cytoplasmic tail of TGN38 with the Y motif replaced by a random tetrapeptide sequence was screened for clones interacting with Δμ2 using the yeast two hybrid system. Positive clones were selected by growth on histidine-deficient plates and by β-galactosidase assays (Ohno *et al.*, 1995). The data show the β-galactosidase activity (average ± SD) from various yeast transformants. The positive controls contain 24 residues from the cytoplasmic tail of TGN38 plus SDYQRL or AAYQRL; the negative controls contain 24 residues from the cytoplasmic tail of TGN38 plus SDAQRL or a randomly picked clone with AAKKAG added instead of SDYQRL. In control experiments performed to detect potential sequence bias in the library used as prey, 15 clones were randomly selected and sequenced. All clones had different inserts and all amino acids were represented in the randomized tetrapeptide positions, with the exception of Y, F, N and H (not shown).

corresponding to the anchor Y of the YQRL signal, Y was always selected; the most common residue at the Y+1 and Y+2 positions was R, with the preference being particularly strong at Y+2. Eleven of 19 clones had L at the Y+3 position; the others had F, M or I. Thus, in the context of the TGN38 tail, the canonical Y motif is the only tetrapeptide selected for μ2 chain binding. The concordance of results from the peptide library and the yeast two hybrid approaches is striking, especially since in one case the sequence context is random, whereas in the other it is the TGN38 tail. The relative affinities of interaction between μ2 and the interacting sequences indicated in Figure 2 were estimated by comparing the β-galactosidase activity in the surviving clones with the activity elicited by the TGN38 tail containing YQRL used as a positive control and with the TGN38 tail containing AQRL or KKAG as negative controls. As shown in Figure 2, the interaction is more favorable in the presence of polar residues at positions Y+1 and Y+2. At position Y+3, the preference appears to be L > M, F, I. In a few instances, receptors have Y endocytic signals which do not contain basic residues at Y+1 or Y+2 (see Table I). It is possible that their affinity for AP-2 complexes *in vivo* is relatively low, since the level of β-galactosidase activity was significantly lower in the absence of basic residues at Y+1 or Y+2.

Recognition of the tyrosine signal by AP-2

Do the preferences revealed by these two combinatorial methods reflect affinity differences and does the specificity exhibited by μ2 hold for the complete AP-2 complex? To answer these questions, we used surface plasmon resonance to study the interactions between peptides bearing the Y motif and AP-2 complexes, using synthetic peptides to compete with a GST–TGN38 tail fusion protein for binding to immobilized AP-2. We first established that recognition of the tail of TGN38 by AP-2 is specific by demonstrating that it only recruits GST–TGN38 and not GST alone (Figure 3A). As a further control for the specificity of the assay we compared the ability of immobilized AP-1 and AP-2 to recruit GST–TGN38 and found that AP-1 binds less well to the Y-based endocytic signal (Figure 3B). This result is in agreement with the preferential recognition by AP-2 rather than by AP-1 of the cytoplasmic tail of the EGF receptor, a membrane protein that is also internalized from the plasma membrane by clathrin-coated vesicles (Boll *et al.*, 1995). As shown in Figures 3 and 4A, the 14 residue peptide pTGN38(YQRL), which has the C-terminal sequence of the TGN38 tail, including the YQRL motif, competes with the GST–TGN38 fusion protein for binding to AP-2 with a K_i of ~1 μM. The 11 residue peptide p--YQRL, which contains an arbitrary sequence surrounding the YQRL signal, has a similar K_i value, but the shorter six residue peptide SDYQRL does not compete at all (Figure 4A). These findings suggest that the YQRL sequence determines most or all of the specificity for AP-2, but requires additional surrounding residues, of no particular sequence, for proper presentation. The Y residue is crucial, as indicated by the failure to compete of peptide pTGN38(AQRL) (which contains alanine instead of tyrosine; Figure 4A) or of peptide p–pYQRL (which contains a phosphotyrosine instead of tyrosine; Figure 4D). This observation is consistent with the absence of β-galactosidase activity seen in the negative control of the yeast two hybrid experiment (SDAQRL; Figure 2) and with the decreased rates of endocytosis of various proteins, such as TGN38, furin, lamp-1 and transferrin receptor, that result from replacement of the Y with A (Collawn *et al.*, 1990; McGraw and Maxfield, 1990; Jadot *et al.*, 1992; Bos *et al.*, 1993; Humphrey *et al.*, 1993; Trowbridge *et al.*, 1993; Schafer *et al.*, 1995; Voorhees *et al.*, 1995). We have also changed each of the three residues in the competing peptide independently, using the arbitrary sequence peptide as a backbone (Figure 4B and C). Peptide p--YARL has a somewhat elevated K_i value (~3 μM); peptide p--YQAL has a significantly increased K_i value (~100 μM); peptide p--YARA does not show measurable competition. Changing L to I at Y+3 has only a small effect (K_i ~20 μM), but changing the Y to F increases the K_i value substantially (~70 μM). Thus, the relative importance of residues at various positions in the motif, as measured in this inhibition assay, correlates well with the results of the combinatorial selections and with the effects of similar changes on endocytosis *in vivo* (Jadot *et al.*, 1992; Trowbridge *et al.*, 1993; Thomas and Roth, 1994).

Use of the Y-oriented combinatorial peptide library did not reveal preferences upstream of the Y. We incorporated the sequence NPV N-terminal to the anchor Y (p--NPVYQRL) to create a peptide containing the LDL receptor motif NPVY, which has been postulated to favor recognition by AP-2 through formation of a tight β-turn in the free peptide (Collawn *et al.*, 1990; Bansal and Gierasch, 1991). This modification actually decreases recognition by μ2 (Figure 4D). Moreover, a peptide

Table I. Sequence comparison among the optimal sequences selected by the μ2 chain using the combinatorial peptide and yeast two hybrid methods and the Y motifs recognized by the clathrin endocytic pathway

I	Preferred motif selected by μ2 from the oriented Y-fixed peptide combinatorial library	YXRL(M, F, V)
II	Preferred motif selected by μ2 from the yeast-two hybrid DNA combinatorial library	YXRL(M, F, I)
III	Y motifs from proteins internalized by the clathrin endocytic pathway	
	Proteins containing endocytic signals with basic residues at Y+1 or Y+2	
	TGN38	YQRL
	Transferrin receptor	YTRF
	Cation-independent manose 6-phosphate receptor	YSKV
	Epidermal growth factor receptor	YRAL
	Cation-dependent manose 6-phosphate receptor	YRGV
	Lysosomal acid phosphatase	YRHV
	Simian immunodeficiency virus transmembrane protein	YRPV
	Furin	YKGL
	Hemagglutinin HA+8	YKSF
	Proteins containing endocytic signals with no basic residues at Y+1 or Y+2	
	Fc γ receptor	YETL
	Fc γ receptor	YTGL
	Asialoglycoprotein receptor	YQDL
	Lamp-1	YQTI
	Lamp-2	YEQF
	pIg receptor	YSAF

containing the portion of the LDL receptor with the endocytic motif FDSNPVYQKT (pLDL) does not compete at all with GST–TGN38. Thus, NPXY-containing signals either have very low affinity for μ2 or are recognized by another site.

Discussion

We have used two different combinatorial selection methods to establish the minimal sequence requirements for the recognition of an endocytic motif by recombinant μ2. The results from the two methods are distinctly complementary, since one (oriented combinatorial peptide library) involves use of a selection of synthetic peptides *in vitro* and the other (combinatorial yeast two hybrid) involves the selective interaction of fusion proteins in the nucleus of a cell. Although not all positions were randomized at the same sites in the two methods, where they did overlap (the Y tetrapeptide motif), they gave the same results.

Another important conclusion is that the sequence requirements for interaction of the Y-based signals with AP-2 complex (revealed by surface plasmon resonance) and with recombinant μ2 (revealed by the combinatorial selection methods) are the same. By exploring the consequence of varying the composition of the tetrapeptide motif in the interaction with μ2 and AP-2 it has been possible to analyze the relative contribution of different residues to the contact. These experiments establish that μ2 is selective for the Y motif and refine our earlier work based on an alanine scan across the Y motif of TGN38, where we established that the tyrosine at the anchor position and the residue at Y+3 are the key for effective interaction between the Y-based signal and isolated μ2 (Ohno *et al.*, 1995). Thus, peptides bearing the Y-based endocytic signal interact directly with μ2 and no other subunits of AP-2 are required to establish specificity.

Does the Y motif always serve as an endocytic signal when present in the cytoplasmic domain of a membrane protein? Clearly it can do so only if part of an unfolded or extended segment, since in a folded domain both the Y and the L would probably be buried in the hydrophobic

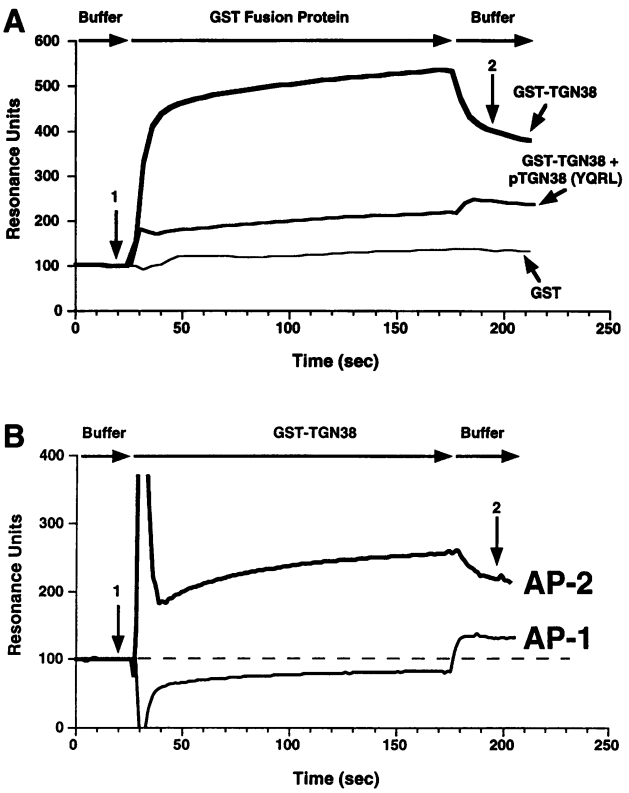

Fig. 3. Interaction between the cytoplasmic tail of TGN38 and purified AP-2 complexes. (**A**) Analysis of the binding of the GST–TGN38 tail to purified AP-2 complexes was done using surface plasmon resonance. The recruitment by similar amounts of AP-2 of GST, GST–TGN38 or GST–TGN38 co-injected with 100 μM competitor peptide pTGN38(YQRL) was recorded as the relative response in resonance units before (arrow 1) and after (arrow 2) injection of the GST fusion proteins. (**B**) Comparison of the recruitment of GST–TGN38 by AP-1 and AP-2 directly immobilized to the surface of the chip.

interior. Indeed, our results show that μ2 recognizes this Y motif in various sequence contexts and in short peptides, demonstrating that the interaction does not need a pre-folded surface. Mutational analysis of the LDL receptor indicates that its endocytic signal spans a segment of

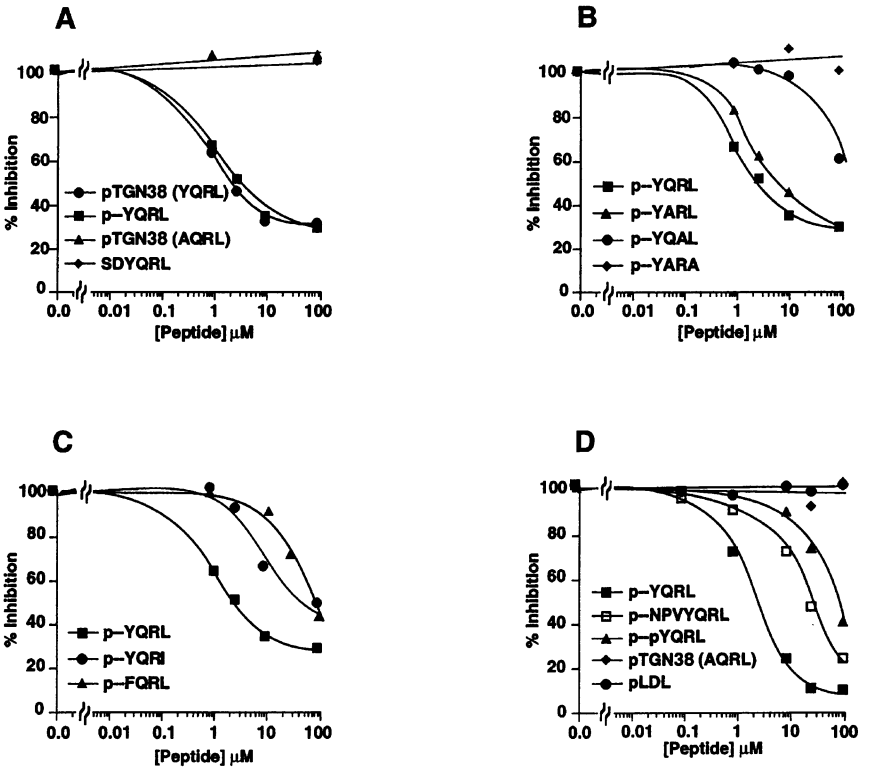

Fig. 4. Competition by synthetic peptides of GST–TGN38 binding to AP-2. For each experimental point, the amount of GST–TGN38 specifically bound to AP-2 in the presence of increasing amounts of competing peptides was determined by surface plasmon resonance and expressed as the difference in resonance units between arrows 1 and 2, as indicated in Figure 3. Each point is expressed as a percentage of the control binding of GST–TGN38 to AP-2 (no competing peptide added). These results are representative of two or more independent experiments performed with different batches of AP-2 and GST–TGN38. (**A**) Competition of the binding by peptides pTGN38(YQRL), p--YQRL, pTGN38(AQRL) and SDYQRL. (**B**) Competition of the binding by peptides p--YQRL, p--YARL, p--YQAL and p--YARA. (**C**) Competition of the binding by peptides p--YQRL, p--YQRI and p--FQRL. (**D**) Competition of the binding by peptides p--YQRL, p--NPVYQRL, p--pYQRL, TGN38(AQRL) and pLDL.

about six amino acids N-terminal to the key Y (Chen *et al.*, 1990). NMR studies have suggested that this motif prefers to adopt a tight turn conformation in solution (Bansal and Gierasch, 1991). The properties of this LDL motif are clearly distinct from the tetrapeptide C-terminal to an anchor Y revealed by the experiments reported here. It is possible that the LDL receptor type signal is recognized by another part of the AP-2 complex or by an intermediary protein that in turn binds AP-2.

Recognition of a phosphotyrosine motif by many SH2 domains also involves interaction of the domain with a four amino acid motif of the type phosphoYXXØ, with few restrictions on the sequence of the surrounding peptide (for an overview see Songyang *et al.*, 1993). High resolution structures of SH2–peptide complexes reveal that the specificity for interaction is provided by contacts between the SH2 domain and side chains in the motif (Eck *et al.*, 1993; Waksman *et al.*, 1992). The good correlation between the results described here and the composition of Y motifs used as endocytic signals (Table I) indicate a similarly direct recognition of the Y motif by AP-2 complexes. The details of the interaction between μ2 and the endocytic signal or between SH2 and its motif are obviously different, because there is no significant sequence identity between μ2 and SH2 domains and, more importantly, because change of phosphoY for Y in the peptide p--pYQRL abolishes its association with AP-2. This lack of binding could be part of a mechanism that ensures proper recognition of the tyrosine motif by AP-2 and at the same time prevents 'false' recognition of SH2 binding sites found in the cytoplasmic tails of many membrane-bound growth factor receptors.

There are homologs of AP-2 subunits (Pevsner *et al.*, 1994; Newman *et al.*, 1995; Stepp *et al.*, 1995) that might function in complexes in other membrane traffic pathways, such as TGN to endosomes, early endosomes to late endosomes or endosomes to basolateral membrane, for which the requirements for recognition have yet to be determined. Likewise, the rules of selection of the AP-1 complex, thought to control part of the traffic out of the TGN, are still unknown. Although isolated μ1 also recognizes Y-based endocytic signals (Ohno *et al.*, 1995), it probably does so with less efficiency than μ2 because AP-1 binds the endocytic signals of TGN38 (this work) and of EGF-R (Boll *et al.*, 1995) with lower affinity than AP-2. Recent comparative studies on the traffic pattern of wild-type and mutant forms of the lysosomal protein lamp-1 indicate that sorting from the TGN (presumably mediated by AP-1 complex) is strongly dependent on the position of its Y motif with respect to the transmembrane part of the protein (Rohrer *et al.*, 1996). Since endocytosis (presumably by AP-2) was not affected in the same mutants, it is possible that different types of APs will have varying requirements for accessibility and presentation of the sorting signals, providing a further element for discrimination. The approaches used here are likely to be useful in identifying the trafficking motifs and the proteins responsible for selectivity in these other pathways.

Materials and methods

Combinatorial peptide library

The design and synthesis of the Y-fixed oriented combinatorial peptide library of sequence MAXXXXYXXXXAKKK, where X indicates any amino acid residue with the exception of S, T, Y, C and W, have been described previously (Songyang *et al.*, 1993, 1994).

Selection of peptides by μ2 from the combinatorial peptide library

A GST–Δμ2 fusion construct expressing the C-terminal portion of human μ2 (residues 122–435) was obtained from Dr J.Setleman (Harvard Medical School). Expression of the GST fusion protein was achieved by induction for 4 h with 100 mM IPTG at room temperature followed by their purification using glutathione–agarose beads (Sigma Chemical Co., St Louis, MO). GST-Δμ2 (~200 μg) was captured on 60 μl glutathione–agarose beads and incubated with 1 mg tyrosine-fixed oriented combinatorial library in 300 μl binding buffer [150 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.5 M LiCl, 1 mg/ml bovine serum albumin (BSA), 0.01% NP40, pH 7.2]. After 1 h at 4°C the beads were washed three times with 1 ml of the same buffer but without LiCl, BSA and NP40. Bound peptides were eluted with 30% acetic acid, lyophilized and sequenced on an amino acid sequencer (Applied Biosystems 477A). G and D were not scored for the Y–4 to Y–1 sequencing cycles due to the appearance of contaminating peaks carried over from the peptide mixture that eluted in the HPLC gradient at the same position as these residues. The intensity of the contamination signals becomes insignificant after four to five sequencing cycles, allowing a reliable scoring of G and D after Y–1. The high values for A at Y–4 and R at Y+3 are probably due to a lag from the previous cycle. To calculate the selectivity, values obtained after each sequencing cycle were divided by the corresponding results obtained using GST alone (Songyang *et al.*, 1993).

Combinatorial DNA library

A DNA fragment encoding the cytoplasmic tail of TGN38 minus the last 10 amino acids and with a *Not*I site at the C-terminus was made by polymerase chain reaction and ligated into the *Eco*RI and *Xho*I sites of the pGBT9 yeast plasmid, resulting in the construct pGBT-TGNΔ-*Not*I. A random 12mer nucleotide library of sequence 5′-ataagaatgcggccgctnn-nnnnnnnnnntgactgcagtc-3′ was annealed via 3′ palindromic sequences, converted to double-stranded DNA by mutually primed synthesis, digested with *Not*I and *Pst*I and ligated into the same sites of pGBT-TGNΔ-*Not*I to create the tetrapeptide library with 3.1×10^6 independent clones. The sequence encoded by the resulting clones was HNKRKII-AFALEGKRSKVTRRPKASAAXXXX, where X indicates any amino acid residue.

Yeast two hybrid selection by μ2 of interacting sequences from the combinatorial DNA library

The yeast strain HF7c containing the plasmid pACTII-3M9, which encodes the GAL4 activation domain–Δμ2 fusion (Ohno *et al.*, 1995), was transformed with the GAL4bd pGBT-TGNΔ-*Not*I tetrapeptide library. Out of 8×10^5 transformants from two independent transformations, 19 library clones were obtained which were positive in two reporter assays, correction of histidine auxotrophy and β-galactosidase activity. The clones tested positive in the presence of pACTII-3M9 but not in the presence of the empty plasmid pACTII.

Cultures of yeast transformants (5–7 colonies/culture) were prepared according to the manufacturer's protocol (Matchmaker Two Hybrid System; Clontech). β-Galactosidase activities were measured by using a luminescence β-galactosidase assay (Clontech) and a luminometer (Monolight 2010; Analytical Luminescence Laboratory). Yeast cells were resuspended at 10 OD_{600}/ml and 10–50 μl were used for measurements done in triplicate and repeated three times. The data represent β-galactosidase activities per 0.1 OD_{600}/ml cells.

Surface plasmon resonance

Surface plasmon resonance experiments were done at 25°C with a BIAcore instrument (Pharmacia, Piscataway, NJ) at a flow rate of 4 μl/min using AP binding buffer I (100 mM NaMES, 150 mM NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3, 0.5 mM DTT, 0.1% Triton X-100, pH 7.0) (Boll *et al.*, 1995) or AP binding buffer II (100 mM NaMES, 150 mM NaCl, 1 mM EDTA, 0.02% NaN_3, 0.5 mM DTT, 0.1% Triton X-100, pH 7.0). AP-2 complexes were obtained from bovine brain coated vesicles (Boll *et al.*, 1995) and for each surface plasmon resonance experiment similar amounts (~2500–3000 resonance units) were captured upon injection of 5–10 μl purified AP-2 (~0.1 mg/ml) on a sensor chip coated on its surface with the monoclonal antibody 9A specific for the hinge region of the β2 chain of AP-2. The antibody was produced in collaboration with Dr K.Clairmont (Bayer Co.). The GST–TGN38 fusion was generated from a polymerase chain reaction product corresponding to the cytoplasmic tail of human TGN38 that was ligated into the *Eco*RI and *Sal*I sites of pGEX- 5X-1 (Pharmacia, Piscataway, NJ). The complete sequence of the tail is HNKRKIIAFALEGKRSKVTRRPKASDYQRL-NLKL. GST or GST–TGN38 (in AP binding buffer) was injected at ~0.02 mg/ml in the presence or absence of competing peptides. At the end of every experiment the sensor chip was subjected to regeneration by flushing with 10 μl 10 mM NaOH, 0.5% SDS at 60 μl/min (Boll *et al.*, 1995). Surface plasmon resonance experiments were also performed with identical results by using sensor chips containing AP-1 or AP-2 directly immobilized (5000–12000 RU) to its surface by chemical cross-linking (Boll *et al.*, 1995). At the end of an experiment bound GST–TGN38 was released from AP-2 by flushing with 20 μl 100 mM Tris, 200 mM NaCl, pH 10.0, at a flow rate of 30 μl/min. To remove aggregates, the samples were spun at 4°C before surface plasmon resonance experiments, for 20 min at 100 000 r.p.m. in a TLA100.4 rotor using a TLX109 centrifuge (Beckman Instruments).

The sequences of the synthetic peptides were as follows: pTGN38-(YQRL), KVTRRPKASD**YQRL**; pTGN38(AQRL), KVTRRPKASD-**AQRL**; p--YQRL, KAFA**YQRL**GAK; p--YARL, KAFA**YARL**GAK; p--YQAL, KAFA**YQAL**GAK; p--YARA, KAFA**YARA**GAK; p--YQRI, KAFA**YQRL**GAK; p--FQRL, KAFA**FQRL**GAK; SDYQRL, SD**YQRL**; p--pYQRL, KAFA**pYQRL**GAK; p--NPVYQRL, KN**PVYQRL**GAK; pLDL, SIN**FDNPVY**QKTTE. The bold amino acids indicate the position of the Y-based endocytic motif as defined by site-directed mutagenesis.

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References

Bansal,A. and Gierasch,L.M. (1991) *Cell*, **67**, 1195–1201.
Boll,W., Gallusser,A. and Kirchhausen,T. (1995) *Curr. Biol.*, **5**, 1168–1178.
Bos,K., Wraight,C. and Stanley,K.K. (1993) *EMBO J.*, **12**, 2219–2228.
Chen,W.J., Goldstein,J.L. and Brown,M.S. (1990) *J. Biol. Chem.*, **265**, 3116–3123.
Collawn,J.F., Stangel,M., Kuhn,L.A., Esekogwu,V., Jing,S., Trowbridge,I.S. and Tainer,J.A. (1990) *Cell*, **63**, 1061–1072.
Eck,M.J., Shoelson,S.E. and Harrison,S.C. (1993) *Nature*, **362**, 87–91.
Gilboa,L., Ben-Levy,R., Yarden,Y. and Henis,Y.I. (1995) *J. Biol. Chem.*, **270**, 7061–7067.
Glickman,J.N. Conibear,E. and Pearse,B.M. (1989) *EMBO J.*, **8**, 1041–1047.
Humphrey,J.S., Peters,P.J., Yuan,L.C. and Bonifacino,J.S. (1993) *J. Cell Biol.*, **120**, 1123–1135.
Jadot,M., Canfield,W.M., Gregory,W. and Kornfeld,S. (1992) *Proc. Natl Acad. Sci. USA*, **267**, 11069–11077.
McGraw,T.E. and Maxfield,F.R. (1990) *Cell Regul.*, **1**, 369–377.
Nesterov,A., Kurten,R.C. and Gill,G.N. (1995) *J. Biol. Chem.*, **270**, 1–8.
Newman,L.S., McKeever,M.O., Okano,H.J. and Darnell,R.B. (1995) *Cell*, **82**, 773–783.
Ohno,H. *et al.* (1995) *Science*, **269**, 1872–1875.
Pearse,B.M. (1988) *EMBO J.*, **7**, 3331–3336.
Pevsner,J., Volknandt,W., Wong,B.R. and Scheller,R.H. (1994) *Gene*, **146**, 279–83.
Robinson,M.S. (1989) *J. Cell Biol.*, **108**, 833–842.
Rohrer,J., Schweizer,A., Russell,D. and Kornfeld,S. (1996) *J. Cell Biol.*, **132**, 565–576.
Schafer,W., Stroh,A., Berghofer,S., Seiler,J., Vey,M., Kruse,M., Kern,H.F., Klenk,H. and Garten,W. (1995) *EMBO J.*, **14**, 2424–2435.
Songyang,Z. *et al.* (1993) *Cell*, **72**, 767–778.
Songyang,Z. *et al.* (1994) *Mol. Cell. Biol.*, **14**, 2777–85.
Sorkin,A. and Carpenter,G. (1993) *Science*, **261**, 612–615.
Sorkin,A., McKinsey,T., Shih,W., Kirchhausen,T. and Carpenter,G. (1995) *J. Biol. Chem.*, **270**, 619–625.

Sosa,M.A., Schmidt,B., von Figura,K. and Hille-Rehfeld,A. (1993) *J. Biol. Chem.*, **268**, 12537–12543.
Stepp,J.D., Pellicena-Palle,A., Hamilton,S., Kirchhausen,T. and Lemmon,S.K. (1995) *Mol. Biol. Cell*, **6**, 41–58.
Thomas,D.C. and Roth,M.G. (1994) *J. Biol. Chem.*, **269**, 15732–15739.
Trowbridge,I.S., Collawn,J.F. and Hopkins,C.R. (1993) *Annu. Rev. Cell Biol.*, **9**, 129–161.
Voorhees,P., Deignan,E., van Donselaar,E., Humphrey,J., Marks,M.S., Peters,P.J. and Bonifacino,J.S. (1995) *EMBO J.*, **14**, 4961–4975.
Waksman,G. *et al.* (1992) *Nature*, **358**, 646–653.

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