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Receptors compete for adaptors found in plasma membrane coated pits

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An affinity matrix of LDL receptor cytoplasmic tails binds the HA-II 100/50/16 kd complexes found in plasma membrane coated pits. Other receptors (or their cytoplasmic domains), which are localized in coated pits during endocytosis, inhibit this binding. This includes an 8 residue peptide containing tyrosine, corresponding to the cytoplasmic portion of a mutant influenza haemagglutinin. In contrast, the equivalent peptide lacking tyrosine (like the tail of the native haemagglutinin, a protein excluded from coated pits) does not compete. These results imply that the HA-II complex has a recognition site for a common signal, probably involving a tyrosine residue, carried by the LDL receptor and competing receptors also found in plasma membrane coated pits. The HA-II complex therefore fulfils the role of an 'adaptor', the name proposed for the structural units which mediate the binding of clathrin to receptors in coated vesicles. Another related complex, the HA-I adaptor, which is restricted to Golgi coated pits, probably does not recognize the 'tyrosine signal' on the LDL receptor tail. The HA-I adaptor is likely to contain a recognition site for a different signal carried by receptors, e.g. the mannose-6-phosphate receptor, which are found in Golgi coated pits.

Key words: endocytosis/LDL receptor/mannose-6-phosphate receptor/poly Ig receptor/sorting

Introduction

Coated pits on particular membranes in the cell distinguish between those proteins that are to be transported to another compartment via coated vesicles and those that are to remain behind (Pearse and Bretscher, 1981). On the endocytic cycle at the plasma membrane, the LDL receptor, transferrin receptor and others assemble into coated pits (Anderson *et al.*, 1977; Bleil and Bretscher, 1982) while resident proteins such as Thy1-antigen and influenza haemagglutinin (HA) (Bretscher *et al.*, 1980; Roth *et al.*, 1986) are excluded. In the Golgi region, the mannose-6-phosphate receptor is thought to be a major receptor in the coated pits, binding lysosomal enzymes for delivery to lysosomes (Farquhar, 1985). However the LDL receptor and other membrane proteins destined for the plasma membrane are presumed to be excluded from those coated pits in the Golgi region and to travel constitutively to the plasma membrane (Griffiths *et al.*, 1988). How is this 'sorting' achieved at a molecular level?

It is simplest to assume that it is the receptors themselves which bear the information which determines their route in the cell. For example, the tyrosine residue (at position 807) in the cytoplasmic tail of the LDL receptor plays a crucial role in the localization of that receptor in coated pits and its subsequent endocytosis (Davis *et al.*, 1987). In addition, the replacement of a particular cysteine for a tyrosine residue in the short influenza HA cytoplasmic tail allows this protein to assemble into coated pits and undergo endocytosis (Lazarovits and Roth, 1988).

What role in sorting do the coat proteins of the coated vesicle play? The coat structure is composed of two distinct structural units, the clathrin triskelion and a complex containing 100 and 50 kd polypeptides. Clathrin forms the outer polyhedral cage, making flexible contacts, via its terminal domains, with the inner layer of 100 and 50 kd proteins (Vigers *et al.*, 1986a,b). While the assembly of the whole structure provides the mechanical framework to bud a vesicle, the inner 100/50 kd complex is the obvious candidate for binding to selected receptors. A direct interaction was previously demonstrated between the soluble mannose-6-phosphate receptor and 100/50 kd complexes (Pearse, 1985). Other receptors in the absence of lipid or excess detergent tend to aggregate by themselves and so this approach is not possible for many receptors.

To circumvent the problem of dealing with precipitating receptors, I have expressed the cytoplasmic region of the LDL receptor as a fusion protein in *Escherichia coli* and coupled it to AffiGel 10 to provide an affinity matrix of LDL receptor cytoplasmic tails. Coat 100/50 kd complexes bind to these LDL receptor tails, and I find that other receptors or their tails compete for binding with the LDL receptor tail. This suggests that these molecules are recognized by a common binding domain on the 100/50 kd complexes.

As it is now clear that these 100/50 kd coat complexes mediate the interaction of clathrin cages with many different receptors, I propose to call them 'adaptors'. Previously they have been termed 'assembly polypeptides', or 'coat associated proteins' deriving from earlier work on their effect on clathrin assembly (Zaremba and Keen, 1983; Pearse and Robinson, 1984). The term 'adaptor' was proposed in 1981 (Pearse and Bretscher) for a family of molecular units which would sort out those molecules that are to travel in clathrin cages from those that are to remain behind. These 100/50 kd coat complexes essentially fulfil that predicted role. Coated vesicles thus contain clathrin, adaptors, receptors and ligands.

The first set of adaptors to be purified and characterized were those of the HA-II type (Pearse and Robinson, 1984) which are restricted to plasma membrane coated pits (Robinson and Pearse, 1986; Robinson, 1987). These complexes are dimers containing one copy of an α class 100 kd protein (A or C) and one copy of a β class (B) 100 kd polypeptide (Ahle *et al.*, 1988). As the 100 kd proteins form the bulk of the structural adaptor unit, I propose to call them adaptins. The adaptor unit also comprises at least one copy

of a 50 kd polypeptide and one copy of a light chain of 16 kd (Keen, 1987; Virshup and Bennett, 1988; Ahle *et al.*, 1988).

Recently, a second HA-I-type adaptor unit has been identified which is restricted to coated pits in the Golgi region of the cell (Ahle *et al.*, 1988). This adaptor contains four quite distinct molecular species related to the four polypeptides of the HA-II-type plasma membrane adaptor, namely: one copy of a β class (β') 100 kd polypeptide, one copy of a new γ class 100 kd polypeptide (as defined by a monoclonal antibody), at least one copy of a 47 kd polypeptide and one copy of a light chain of 19 kd. The HA-I and HA-II type adaptors separate on hydroxylapatite chromatography, hence the terminology. As these adaptors have a non-overlapping distribution in the cell it is of interest to determine their binding properties with respect to the LDL receptor, which enters coated pits on the plasma membrane but presumably not those in the Golgi region.

Results

Expression of the LDL receptor cytoplasmic portion in E.coli

The expression vector pLcII(nic$^-$) (Nagai and Thøgersen, 1987) was used in *E.coli* to produce a fusion protein containing the cytoplasmic portion of the LDL receptor. The original objective was to make a hybrid protein containing the N-terminal 31 amino acid residues of λcII protein and 50 residues of the human LDL receptor cytoplasmic tail, with the tetrapeptide Ile-Glu-Gly-Arg in between (to allow subsequent proteolytic cleavage by blood coagulation factor λ_a). In practice, however, this product did not accumulate in *E.coli*. A corresponding fusion protein containing myosin light chain (MLC), whose synthesis is directed by the vector pLcIIFXMLC (Reinach *et al.*, 1986) is produced in quantity. To facilitate expression of the LDL receptor tail, the DNA fragment coding for 112 residues of chicken MLC was inserted into the vector, giving rise to the construct called pLcIIBP2 (Figure 1). pLcIIBP2 directs the expression of the fusion protein BP2 in good yield. Thus the protein BP2 consists of the N-terminal 31 amino acid residues of λcII protein, 112 residues of chicken MLC and 50 residues of the human LDL receptor cytoplasmic tail, interspersed with blood coagulation factor X_a recognition sites. BP2 is easily purified from the inclusion bodies of *E.coli* and is soluble in the buffers required in the binding experiments. Therefore the complete fusion protein was used to generate an affinity column rather than the LDL receptor tail alone, which was difficult to obtain in good yield after activated factor X_a cleavage.

Properties of the LDL receptor tail column

An affinity matrix of LDL receptor cytoplasmic tails was made by coupling the BP2 fusion protein to AffiGel 10. Hopefully many of the crosslinks made to the resin are via the λcII and MLC domains leaving a significant proportion of receptor tails available in a suitable orientation for recognition by coated vesicle proteins. However, these domains may also contribute in some way to the binding properties of the column. To provide a control column, a similar resin was prepared with the pLcIIFXMLC fusion protein which contains the rest of the chicken MLC polypeptide instead of the LDL receptor tail (Reinach *et al.*, 1986).

Adaptors (HA-II type), labelled with ^{125}I Bolton and Hunter reagent (Bolton and Hunter, 1973), bind specifically to the BP2 column (in buffer A pH 7.0 or 50 mM Tris−acetate, pH 7.5, 0.1 M NaCl and 5 mM $MgCl_2$) and are eluted with a solution containing 1 M Tris−HCl, pH 7.0. No such binding is observed under the same conditions using the MLC resin lacking the LDL receptor cytoplasmic portion (Figure 2). Excess unlabelled adaptors compete with the labelled material for binding to the BP2 column (Figure 2). Thus it is reasonable to assume that the BP2 resin is behaving as an affinity matrix of LDL receptor cytoplasmic tails which bind adaptors.

Figure 3 shows samples from typical adaptor binding experiments analysed by SDS−PAGE. The HA-II type adaptors bind specifically to the LDL receptor tail column and appear in the eluate, whereas bovine serum albumin (BSA) and other contaminants occurring in less pure adaptor samples are washed through the column as filtrate. The total capacity of the affinity matrix is modest: of the order of

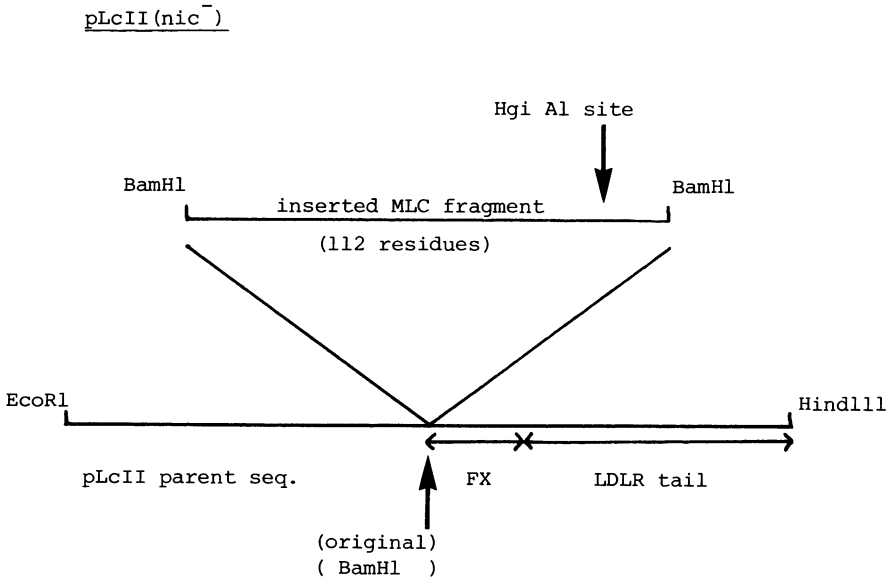

Fig. 1. Construction of the expression plasmid pLcIIBP2.

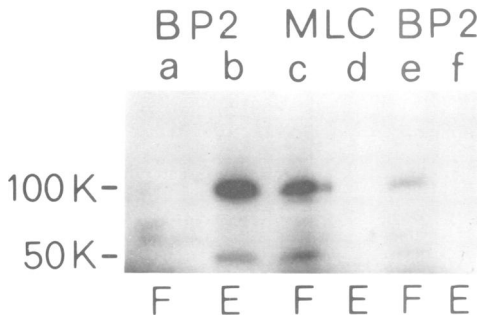

Fig. 2. Specific binding of ^{125}I-labelled HA-II adaptors to LDL receptor cytoplasmic tails, analysed by SDS–polyacrylamide mini gel electrophoresis, followed by autoradiography. (**a**) The filtrate (F) and (**b**) the eluate (E) from a binding experiment to a BP2 column compared to (**c**) the filtrate and (**d**) the eluate from a similar MLC (expressed from pLcIIFXMLC) column. The adaptors bind to the LDL receptor tails on the BP2 column and are released by elution with buffer B. In contrast no binding is observed on the MLC column and the adaptors flow through in the filtrate. In a separate experiment (**e**) is filtrate and (**f**) eluate from a smaller BP2 column when a competing 5-fold excess of cold HA-II adaptors was added in the sample applied to the column. The major portion of the labelled adaptors appears in the filtrate.

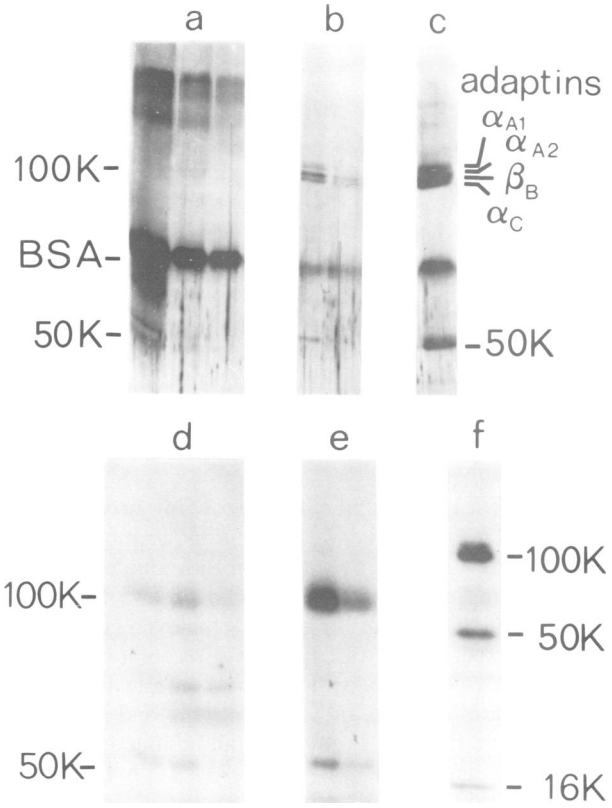

Fig. 3. Affinity binding of ^{125}I-labelled HA-II adaptors to LDL receptor cytoplasmic tails analysed by SDS–PAGE followed by autoradiography. (**a**) Three sequential samples of filtrate from the BP2 column with cold carrier BSA. (**b**) Two sequential samples of eluate from the same experiment. (**c**) Sample of similar eluate after concentration showing the different 100 kd adaptin in bands (α_{A1}, α_{A2}, β_B and α_C) of the HA-II adaptors. (a), (b) and (c) show the silver stained gel. (**d**) and (**e**) show the autoradiograph of the lanes shown in (a) and (b). (**f**) The autoradiograph of the eluate from a similar experiment analysed on a 7–20% gradient polyacrylamide gel to show all the components of the adaptor: the 100 kd adaptins (which form an $\alpha\beta$ heterodimer), the 50 kd protein and the 16 kd light chain.

100 μg of adaptors bind per 1 ml of resin (containing about 800 μg coupled fusion protein). All the 100 kd adaptin polypeptide species of the HA-II type adaptors resolved so far (i.e. bands α_{A1}, α_{A2}, β_B and α_C) appear to bind to the column as shown on the 7.5% polyacrylamide separation. The analysis on gradient gels shows the 50 and 16 kd components of these adaptors.

In the cell, HA-II adaptors occur in plasma membrane coated pits (Robinson, 1987) but not in Golgi associated coated pits. In contrast, HA-I type complexes appear to occur in Golgi associated coated pits but not in plasma membrane coated pits (Ahle *et al.*, 1988). These HA-I adaptor complexes are separated from the HA-II type by hydroxylapatite chromatography (Figure 4). They contain β- and γ- 100 kd adaptin polypeptides (Ahle *et al.*, 1988), a 47 kd species instead of a 50 kd species and a 19 kd light chain instead of a 16 kd light chain. The HA-I adaptors (in comparison to HA-II adaptors in 50 mM Tris–acetate, pH 7.5, 0.1 M NaCl, 5 mM $MgCl_2$) do not appear to bind to the LDL receptor tails and when mixed in about equal proportions with ^{125}I-labelled HA-II type adaptors, do not inhibit the binding of the HA-II complexes to the LDL receptor tails (data not shown).

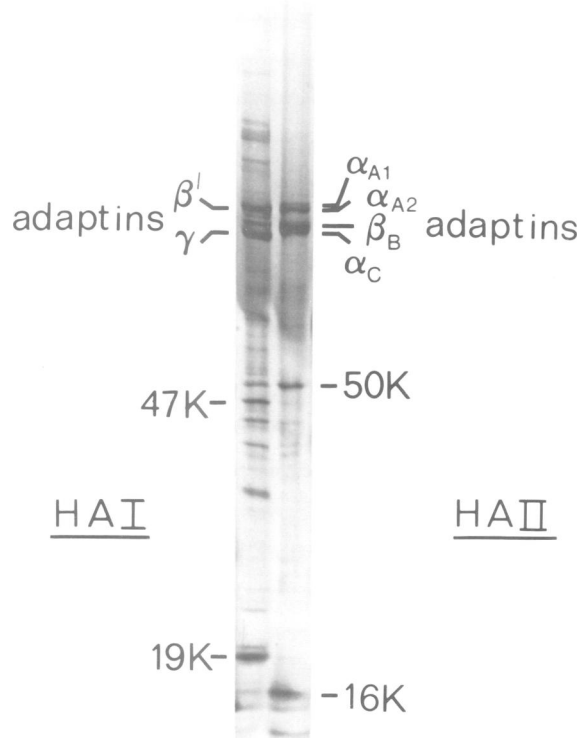

Fig. 4. HA-I adaptors (characteristic of the Golgi region coated pits) and HA-II adaptors (restricted to plasma membrane coated pits) analysed by SDS gradient (7–20%) polyacrylamide gel electrophoresis. The two adaptor fractions were separated by chromatography on hydroxylapatite according to Manfredi and Bazari (1987). The HA-I adaptor complex consists of a heterodimer of a β'-adaptin and a γ-adaptin in combination with the 47 kd protein and a 19 kd light chain (Ahle *et al.*, 1988) while the HA-II adaptor consists of a β_B-adaptin and an α-adaptin (either A or C) in combination with the 50 kd protein and a 16 kd light chain. Whereas the HA-II adaptors bind to the LDL receptor tail as shown in Figure 3, the HA-I adaptors, in the same conditions, show no appreciable binding and pass through the BP2 column in the filtrate.

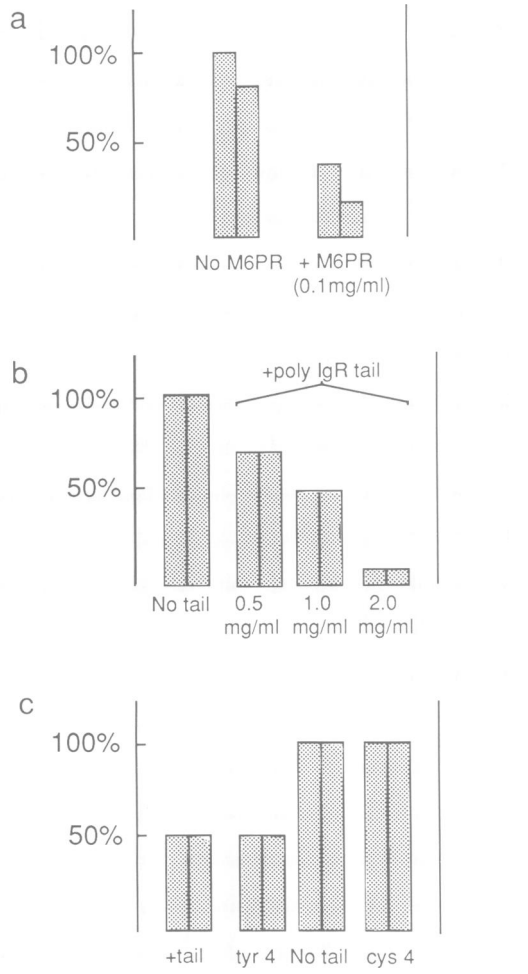

Fig. 5. Competition with other receptor tails. (**a**) The mannose-6-phosphate receptor (pre-incubated with the HA-II adaptors). Duplicate experiments on two separate, approximately equivalent BP2 columns, where 100% binding represents the maximum amount of bound ^{125}I-labelled adaptors observed. (**b**) The poly IgR tail. (**c**) The poly IgR tail (at 1.0 mg/ml) compared to the native influenza HA tail (at 2.5 mg/ml) and the mutant influenza HA tail (at 2.5 mg/ml) where the cysteine residue at position 4 is changed to a tyrosine residue. In (b) and (c) 100% binding represents the amount of ^{125}I-labelled adaptors (in the absence of competitors) bound and eluted from the same BP2 column used throughout a series of competition experiments.

Competition with other receptor tails

The mannose-6-phosphate receptor binds to HA-II type adaptors (Pearse, 1985). This receptor is found in the same plasma membrane coated pits as the LDL receptor. Does this receptor inhibit the binding of the HA-II adaptors to the LDL receptor tails? The results of a competition experiment are shown in Figure 5(a). Preincubation of the mannose-6-phosphate receptor at 0.1 mg/ml with the affinity purified ^{125}I-labelled adaptors before the binding experiment reduced the level of bound adaptors compared to duplicate experiments lacking receptor. At this concentration of receptor (5×10^{-7} M) the binding of adaptors to the LDL receptor appears to be reduced by ~50% (Figure 5a) suggesting that by associating with the adaptors the mannose-6-phosphate receptor blocks binding to the LDL receptor.

The poly Ig receptor is also found in plasma membrane

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                    membrane                          → COOH

Native HA:          NGSLQCRIC(I)
   HA-Tyr:          NGSLQYRIC(I)

LDL receptor:       KNWRLKNINSINFDNPVYQKTTED→

M6PR:               KKERREMVMSRLTNCCRRSANVSYKY→

VSVG:               RVGIHLCIKLKHTKKRQIYTDIEMNRLGK

Circulating
membrane IgG γ2a:   KVKWIFSSVVELKQTISPDYRNMIGQGA
             γ2b:   KVKWIFSSVVELKQKISPDYRNMIGQGA
             γ1 :   KVKWIFSSVVELKQTLVPEYKNMIGQAP
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Fig. 6. Comparison of the cytoplasmic tails of several transmembrane proteins thought to enter coated pits. The HA tails (native and mutant) are written with the C-terminal isoleucine in brackets as this was omitted in the synthesis of the peptides used in the competition experiments. The native influenza HA is excluded from plasma membrane coated pits (Roth *et al.*, 1986) in contrast to the other molecules listed. Membrane IgG is endocytosed in B cells (Watts and Davidson, 1988) and is included in the list as the different classes all exhibit a tyrosine in their cytoplasmic rails. Sequences from: native HA (Gething *et al.*, 1980); LDL receptor (Yamamoto *et al.*, 1984); 215 kd mannose-6-phosphate receptor (Lobel *et al.*, 1987); vesicular stomatitis virus G protein (Rose and Gallione, 1981) and circulating membrane IgG (Yamawaki-Kataoka *et al.*, 1982). The poly Ig receptor has a 100 residue cytoplasmic tail containing two tyrosines (Mostov *et al.*, 1984).

coated pits (Limet *et al.*, 1985) like the LDL receptor. As the pure, soluble poly Ig receptor cytoplasmic portion was kindly made available by Keith Mostov, it was possible to perform a set of competition experiments, tail versus tail. The results are shown in Figure 5(b). Depending on its concentration, the competing poly Ig receptor tail inhibits the binding of the ^{125}I-labelled adaptors to the LDL receptor tail. At 2 mg/ml poly Ig receptor tail ($\sim 10^{-4}$ M), the competition for binding is almost complete.

Competition with the cytoplasmic portion of influenza haemagglutinin

The influenza HA is a membrane spanning protein which is excluded from coated pits on the plasma membrane (Roth *et al.*, 1986). Thus, as a control, an 8 residue polypeptide corresponding to the native HA tail (with the N-terminal residue omitted, Figure 6) was employed in parallel inhibition experiments with the poly Ig receptor tail (Figure 5c). The HA polypeptide should not compete for binding of the adaptors with the LDL receptor tail—and it does not. At 1.0 mg/ml the poly Ig receptor tail inhibited binding to 50% of that in its absence, whereas at 2.5 mg/ml of the native HA polypeptide, no inhibition was observed.

In contrast, a mutant form of the HA which has a tyrosine substituted for a cysteine at position 6 from the membrane in the cytoplasmic tail, does enter coated pits and is endocytosed (Lazarovits and Roth, 1988). Thus the 8 residue cytoplasmic polypeptide, with the tyrosine for cysteine substitution (Figure 6), would be expected to compete with the LDL receptor tail for binding of the HA-II adaptors—and it does appear to do so. At 2.5 mg/ml the tyrosine containing polypeptide inhibited the binding of adaptors to the LDL receptor tail by ~50%, comparable to the inhibition observed with the poly Ig receptor tail at 1.0 mg/ml.

Discussion

The availability of an affinity matrix of LDL receptor tails allows a study of the binding of coat proteins to this receptor *in vitro*. Other receptor tails can be immobilized in a similar fashion for comparison of their adaptor binding properties. This new approach avoids the problem associated with aggregation of purified receptors in binding studies performed in aqueous solution (Pearse, 1985).

The BP2 affinity matrix of LDL receptor cytoplasmic tails binds, specifically, the HA-II adaptors of plasma membrane clathrin coated pits. BSA and contaminants of partially purified adaptors do not bind to the BP2 column. The equivalent resin coupled to a similar fusion protein, lacking the LDL receptor tail, but containing instead the rest of the MLC does not bind adaptors under the same conditions.

Whole receptors, or tails of receptors, known to assemble into plasma membrane coated pits inhibit the binding of these HA-II adaptors to the receptor tail column. A control is provided by the influenza HA, a protein which is excluded from coated pits (Roth *et al.*, 1986). As would be expected, the peptide corresponding to the cytoplasmic portion of HA does not compete for binding. In contrast, the identical peptide containing a tyrosine, substituted for a cysteine at position 4, does compete. This competition is expected, as in the cell, it has been shown that HA carrying this substitution enters coated pits and is endocytosed (Lazarovits and Roth, 1988).

These competition experiments suggest that there is a common tyrosine recognition site on the HA-II adaptor which directs a number of receptors carrying a 'tyrosine signal' into coated pits. In fact several receptors, listed in Figure 6, which enter plasma membrane coated pits do exhibit a tyrosine in their cytoplasmic portions, probably including membrane IgG, shown to endocytose in B cells (Watts and Davidson, 1988).

The abilities of the various competing polypeptides to inhibit the binding of the HA-II adaptors to the LDL receptor tail vary widely. The poly Ig receptor tail competes effectively for adaptor binding at a much lower molar concentration ($\sim 10^{-4}$ M) than the Ha-Tyr polypeptide ($\sim 2.5 \times 10^{-3}$ M). The mannose-6-phosphate receptor was the most potent inhibitor of binding assayed. It competed effectively in the region of 10^{-7} M receptor polypeptide. Previously the soluble receptor has been shown to interact with the HA-II adaptors with a K_d of $\sim 2 \times 10^{-7}$ M (Pearse, 1987). Probably the mannose-6-phosphate receptor binds more effectively as it is a polymeric protein in the conditions of the experiment, although other features of the receptor may modulate the binding.

The observation that HA-I adaptors—those from Golgi coated pits—do not associate with the LDL receptor tail as readily as the HA-II adaptors—those from the plasma membrane—is important for the 'sorting' of receptors to their destinations in the cell. Further evidence that the HA-I adaptor does not recognize the 'tyrosine signal' but some other label comes from a companion study of HA-I and HA-II adaptor binding to the mannose-6-phosphate receptor cytoplasmic tail (J.Glickman and B.M.F.Pearse, in preparation). The puzzle has been how are particular receptors segregated into coated pits on one membrane but supposedly excluded from coated pits on another compartment. The solution appears to be that there are specialized adaptors—restricted by some means to a particular membrane—which recognize a common signal on receptors which enter coated pits on that membrane. Thus the LDL receptor, having a tyrosine signal, binds to HA-II adaptors in forming coated pits on the plasma membrane, but lacking some other label, fails to bind to HA-I adaptors in Golgi associated coated pits. Presumably the mannose-6-phosphate receptor contains such a second site in its cytoplasmic portion which is recognized by these HA-I adaptors. Probably there are other, as yet unidentified, adaptors.

A dilemma remains—why, in the cell, do adaptors not rebind to receptors on uncoated vesicles but return to bind only to those on the parent membrane?

Materials and methods

Construction of the expression plasmid

The clone pLDLR3 (ATCC no. 57004/57005) containing complementary DNA coding for the complete human LDL receptor was obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. A 1077 bp restriction fragment containing the coding sequence for the cytoplasmic region was cut out using *Sty*I and *Nae*I. It was treated with the Klenow fragment of DNA polymerase in the presence of dNTP to obtain flush ends and cloned into the *Sma*I site of M13mp8. A clone containing the insert with the 3′ end of the coding sequence facing the universal priming site was selected. A *Sma*I site was engineered by oligonucleotide site directed mutagenesis (Carter *et al.*, 1984) to replace the sequence AACTGG coding for Asn–Trp at the beginning of the LDL receptor cytoplasmic region. From this construct the 200 bp *Sma*I–*Hin*dIII fragment was excised and recloned into M13mp11FX (Nagai and Thøgersen, 1987) cut with *Hin*dIII and *Stu*I. After checking for the presence of the correct sequence alignments, the *Bam*HI–*Hin*dIII fragment containing the FX sequence and the LDL receptor tail sequence was cloned into pLcII (Nagai and Thøgersen, 1987) cut with *Bam*HI and *Hin*dIII. Finally, the coding sequence, cut with *Bam*HI, for 112 residues of the chicken MLC polypeptide was inserted at the phosphatased *Bam*HI site in between the λcII coding region and the FX and LDL receptor tail coding region. The orientation of the MLC coding sequence in the constructs was determined by restriction digestion with *Bam*HI, *Hin*dIII and *Hgi*AI. A QY13 clone was selected, after transformation, in which the plasmid construct directs the synthesis of a fusion protein containing only one copy of MLC (judged by size) in the correct orientation. This construct is termed pLcIIBP2 and thus directs the temperature sensitive synthesis in *E.coli* of a fusion protein consisting of the N-terminal 31 amino acid residues of λcII protein, 112 residues of chicken MLC polypeptide and 50 residues of the human LDL receptor cytoplasmic tail, interspersed with blood coagulation factor X_a recognition sites (Figure 1).

Generation of the affinity matrix

The expressed fusion protein BP2 accumulates in the inclusion bodies of QY13 *E.coli* cells, which are easily purified (Nagai and Thøgersen, 1987). Whereas much of the material in the inclusion body dissolves in 50 mM Tris–Hcl, pH 7.5, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT) containing 6 M urea and may be discarded, the BP2 protein remains insoluble until 8 M urea is included in the extraction buffer. The 8 M urea extract is dialysed against the same buffer containing 8 M urea for 3 h, and then applied to a small DEAE–cellulose column (Whatman DE-53; ~20 ml resin for a 9 l bacterial preparation). The BP2 protein passes straight through leaving most of the contaminating material stuck to the column. Further purification is achieved by dialysing the BP2 filtrate against 20 mM MES, pH 6.0, containing 0.1% β-mercaptoethanol and 8 M urea and ion exchange chromatography on 10 ml CM-52 cellulose equilibrated in the same buffer. The BP2 protein is eluted in an NaCl gradient from 0 – 0.1 M NaCl in the same buffer. The purified BP2 protein at an OD of 0.7 at 280 nm is then dialysed at 4°C against 50 mM Tris–HCl, pH 8.0, containing 100 mM NaCl to remove the urea. To generate a matrix coated with LDL receptor cytoplasmic tails, the soluble protein BP2 (in 50 mM triethanolamine–HCl, pH 8.0, containing 100 mM NaCl) is coupled to AffiGel 10 at 1 ml of protein solution (at about 1 mg/ml) to 1 ml of swelled resin (coupling efficiency ~80%). A similar resin was prepared by coupling the pLcII FXMLC fusion protein which contains the complete chicken MLC polypeptide but no LDL receptor tail (Reinach *et al.*, 1986).

Adaptor purification

HA-II type adaptors were purified as described previously (Pearse and Robinson, 1984). This preparation was used in most of the experiments

described here. After the identification of the HA-I type of adaptor complex and its localization to the coated pits of the Golgi region (Ahle *et al.*, 1988), the preparation procedure was modified in the following ways. First, the Triton X-100 extraction of the coated vesicles was omitted. Instead a centrifugation step in 12.5% sucrose and 12.5% Ficoll was substituted to separate a crude coated vesicle fraction as described by Campbell *et al.* (1984). Second, after the gel filtration step in 1 M Tris–HCl buffer, pH 7.0, hydroxylapatite chromatography was performed, as described by Manfredi and Bazari (1987), to separate the HA-I adaptors from the HA-II adaptors. The α and β adaptins were identified by immunoblotting with the AC1-M11 (α) and the B1-M6 monoclonal antibodies respectively (Robinson, 1987) and the γ adaptin was identified with mAb 100/3 monoclonal antibody, the kind gift of Ernst Ungewickell (Ahle *et al.*, 1988). When required, the HA-II adaptors were labelled with ^{125}I-labelled Bolton and Hunter reagent (Bolton and Hunter, 1973).

Binding studies

Initially, adaptors were dialysed against reconstitution buffer A (pH 7.0) for binding to the receptor tails [Pearse, 1985: 0.1 M Mes–NaOH (pH 7.0), 0.2 mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3]. The standard elution buffer, buffer B, contained 1 M Tris–HCl, pH 7.0, 1 mM EDTA, 0.1% 2-mercaptoethanol, 0.02% NaN_3. The BP2 affinity column was washed with several volumes of both buffer A and buffer B before final equilibration in buffer A, pH 7.0, containing 0.1 mg/ml BSA. Including 0.5 mg/ml BSA in the buffer instead of 0.1 mg/ml made no difference in the observed binding of adaptors. In some experiments the equilibration buffer contained 50 mM Tris–acetate, pH 7.5, 0.1 M NaCl and 5 mM $MgCl_2$, and the adaptor buffer was adjusted to match. Binding of HA-II adaptors to the column was slightly reduced in these conditions compared to identical runs in buffer A, pH 7.0. Experiments involving HA-I adaptors were performed in the Tris–acetate buffer.

Typically, adaptors at 0.04 mg/ml in equilibration buffer containing 0.1 mg/ml BSA were applied to the BP2 resin (300 λ adaptors to 500 λ resin). The column was washed with 5 volumes of the equilibration buffer lacking BSA and then eluted in 5 volumes of elution buffer B. Columns were further washed in buffer B, re-equilibrated in buffer A and used several times for binding experiments without diminution of binding capacity.

For competition experiments with other receptor tails, the ^{125}I-labelled adaptors were first affinity purified on the BP2 matrix. They were then dialysed against buffer A, pH 7.0, and the competing polypeptides dissolved in that solution. In the case of the mannose-6-phosphate receptor, the receptor was mixed with adaptors in buffer B prior to dialysis into buffer A as in previous reconstitution experiments (Pearse, 1985) before application to the BP2 matrix. Parallel control experiments were always performed which lacked the competing receptor or receptor tail.

Bovine liver mannose-6-phosphate receptor was purified as previously described (Brown and Farquhar, 1984; Pearse, 1985).

Poly Ig receptor cytoplasmic region was a kind gift from Dr Keith Mostov of the Whitehead Institute.

The 8 residue peptide corresponding to the influenza HA tail and its sister peptide with a tyrosine substituted for a cysteine at position 4 were synthesized by J.Holder and O.Nguyen, using the continuous flow Fmoc-polyamide technique (Cameron *et al.*, 1988). The blocking acetamidomethyl on the sulphydryl groups of the cysteine residues was removed by treatment with a 7-fold molar excess of mercuric acetate in dilute acetic acid at pH 4.0 at a concentration of 0.35 mM peptide. The mercury was removed by bubbling H_2S through the solution in the presence of mercaptoethanol. The precipitate was removed, the supernatant concentrated on a freeze drier, and the peptide purified by gel filtration on G10 Sephadex in 10% acetic acid and 0.1% β-mercaptoethanol. The peptide containing fractions were pooled and freeze dried and stored at −20°C (Veber *et al.*, 1972).

Peptide and receptor concentrations were estimated by amino acid analysis.

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