A Dominant-negative Clathrin Mutant Differentially Affects Trafficking of Molecules with Distinct Sorting Motifs in the Class II Major Histocompatibility Complex (MHC) Pathway

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Abstract. The role of clathrin in intracellular sorting was investigated by expression of a dominant-negative mutant form of clathrin, termed the hub fragment. Hub inhibition of clathrin-mediated membrane transport was established by demonstrating a block of transferrin internalization and an alteration in the intracellular distribution of the cation-independent mannose-6-phosphate receptor. Hubs had no effect on uptake of FITC-dextran, adaptor distribution, organelle integrity in the secretory pathway, or cell surface expression of constitutively secreted molecules. Hub expression blocked lysosomal delivery of chimeric molecules containing either the tyrosine-based sorting signal of H2M or the dileucine-based sorting signal of CD3γ, confirming a role for clathrin-coated vesicles (CCVs) in recognizing these signals and sorting them to the endocytic pathway. Hub expression was then used to probe the role of CCVs in targeting native molecules bearing these sorting signals in the context of HLA–DM and the invariant chain (I chain) complexed to HLA–DR. The distribution of these molecules was differentially affected. Accumulation of hubs before expression of the DM dimer blocked DM export from the TGN, whereas hubs had no effect on direct targeting of the DR–I chain complex from the TGN to the endocytic pathway. However, concurrent expression of hubs, such that hubs were building to inhibitory concentrations during DM or DR–I chain expression, caused cell surface accumulation of both complexes. These observations suggest that both DM and DR–I chain are directly transported to the endocytic pathway from the TGN, DM in CCVs, and DR–I chain independent of CCVs. Subsequently, both complexes can appear at the cell surface from where they are both internalized by CCVs. Differential packaging in CCVs in the TGN, mediated by tyrosine- and dileucine-based sorting signals, could be a mechanism for functional segregation of DM from DR–I chain until their intended rendezvous in late endocytic compartments.

CLATHRIN-COATED vesicles (CCVs)[1] can mediate selective budding of receptors and ligands from one cellular membrane for transport to another (Brodsky, 1988; Pley and Parham, 1993; Schmid, 1997). This is achieved by self-assembly of clathrin into a polyhedral membrane coat that incorporates the adaptor protein (AP)1 or AP2 molecules that bind receptors at the TGN and plasma membrane (PM), respectively. There is strong evidence that CCV formation is responsible for receptor-mediated endocytosis at the PM and for the sorting of lysosomal hydrolases in the TGN. However, there is an increasing need for the precise definition of the role of CCVs in other intracellular pathways given the proliferation of specialized sorting pathways, novel receptor sorting motifs, and the recent discovery of a novel adaptor molecule, AP3, that recognizes conventional CCV sorting motifs but is apparently not a component of CCVs (Dell'Angelica et al., 1997a,b; Simpson et al., 1997). Furthermore, there are cellular sites to which clathrin has been localized, such as maturing secretory granules (Tooze and Tooze, 1986; Dittié et al., 1996) and endosomes (Stoorvogel et al.,

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1. *Abbreviations used in this paper*: AP, adaptor protein; CCV, clathrin-coated vesicle; CI-M6PR, cation-independent mannose-6-phosphate receptor; CMV, cytomegalovirus; COP, coat protein; FITC-Tfn, fluorescein-conjugated transferrin; I chain, invariant chain; IL, interleukin; LC, light chain; LRSC, lissamine rhodamine; PM, plasma membrane; TfnR, transferrin; TfnR, transferrin receptor.

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1996), where the function of clathrin is not clearly understood. Here we establish a system to probe clathrin's intracellular function in mammalian cells by transfecting cells with a dominant-negative mutant of clathrin. We then apply this approach to defining the role of CCVs in the specialized sorting pathways that control antigen presentation by class II molecules of the major histocompatibility complex (MHC).

Class II MHC molecules are targeted to the endocytic pathway where they bind antigenic peptides that are then displayed on the cell surface. This so-called "antigen presentation" by class II MHC molecules activates helper T cells that stimulate B cells to produce antibody. Two of the accessory molecules required for functional antigen presentation by class II MHC molecules bear sorting motifs that suggest a role for CCVs in their intracellular targeting, but sorting steps at which CCVs are involved in this process have yet to be completely defined. Endocytic localization of the invariant chain (I chain), which is responsible for transport of the class II molecule itself, depends on a dileucine-containing sequence (Bakke and Dobberstein, 1990; Lotteau et al., 1990; Pieters et al., 1993; Odorizzi et al., 1994), whereas localization of the HLA–DM molecule, which is a catalyst for peptide loading, depends on a tyrosine-containing sequence (Lindstedt et al., 1995; Marks et al., 1995; Ohno et al., 1995; Copier et al., 1996). These sequences conform to two of the general consensus motifs associated with binding to adaptor components of CCVs (Johnson and Kornfeld, 1992; Letourneur and Klausner, 1992; Marks et al., 1997). In vitro studies show that sequences with the tyrosine-containing motif interact with the $\mu 1$ or $\mu 2$ subunits of the AP1 or AP2 adaptors (Ohno et al., 1995, 1996; Rapoport et al., 1997), and there is evidence that the dileucine motif can mediate binding of intact AP1 and AP2 molecules to receptor tails (Heilker et al., 1996; Salamero et al., 1996; Dietrich et al., 1997). However, the intracellular locations where CCVs actually sequester receptors with the different motifs and perform their sorting function are more specific than these binding studies indicate. Here we establish where CCVs play a role in targeting the I chain with associated class II molecules and HLA–DM to the endocytic pathway, defining the actual intracellular steps where these motifs contribute to clathrin-mediated sorting and where CCVs play a role in antigen presentation.

There is considerable successful precedent for using mutants to analyze clathrin function. Clathrin-deficient mutants of *Saccharomyces cerevisiae* (Lemmon et al., 1991; Seeger and Payne, 1992), *Dictyostelium discoideum* (Niswonger and O'Halloran, 1997), and *Drosophila melanogaster* (Bazinet et al., 1993) have phenotypes that confirm roles for CCVs in endocytosis, retention of TGN proteins, and sorting of lysosomal hydrolases and reveal roles in spermatogenesis and cytokinesis. Mutation of the GTPase dynamin, which plays a specific role in the scission of CCVs during receptor-mediated endocytosis, has been effectively applied to dissecting clathrin's role at the PM in *Drosophila* and mammalian cells (Herskovits et al., 1993; van der Bliek et al., 1993; Damke et al., 1995; Wang et al., 1997). To design a dominant-negative mutant of clathrin that would enable analysis of clathrin-mediated transport throughout the cell, we took advantage of our understanding of the domain structure of clathrin. Clathrin is a triskelion-shaped molecule composed of three heavy chains (192-kD) and three light chains (25–29 kD) of which there are two types, LCa and LCb, in mammalian cells (for review see Brodsky, 1988). Using a bacterial expression system, we have previously demonstrated that the carboxy-terminal third of the clathrin heavy chain trimerizes and folds to reproduce the central portion of the triskelion, forming the hub fragment (Liu et al., 1995). The hub molecules bind clathrin light chains and can self-assemble with the same kinetics as intact clathrin but they polymerize into an open-ended lattice instead of a closed polyhedron. The potential ability of hubs to interact with endogenous clathrin heavy and light chains and the fact that they assemble into nonfunctional structures, made the hub fragment a good candidate for a dominant-negative mutant that could disrupt clathrin-dependent functions. Here we demonstrate that expression of clathrin hub fragments by transfection of mammalian cells perturbs receptor-mediated endocytosis, lysosomal targeting, and clathrin-mediated intracellular sorting in the TGN, without pleiotropic effects on other intracellular transport pathways. Expression of hub fragments was found to inhibit the intracellular targeting of HLA–DM molecules to the endocytic pathway. In contrast, the direct targeting of I chain to the endocytic pathway was unaffected in the presence of hubs. The surface levels of both molecules, however, were elevated when clathrin-mediated endocytosis was blocked in hub-transfected cells. Thus, CCVs participate in the differential sorting of the class II molecule/I chain complex and HLA–DM. Because the sorting of these complexes is known to be controlled by either dileucine- or tyrosine-based motifs, these findings also suggest that whereas CCVs sort both motifs at the PM, the dileucine-based motif is sorted independently of CCVs in the TGN.

Materials and Methods

Antibodies

Antibodies obtained from commercial sources were anti-T7 epitope tag mAb and its biotinylated version (subtype IgG$_{2b}$; Novagen, Inc., Madison, WI), anti–β-coat protein (COP) mAb M3A5 (Sigma Chemical Co., St. Louis, MO), anti-CD63 mAb (Immunotech Inc., Westbrook, ME). FITC- and rhodamine-conjugated goat anti–rabbit and FITC-conjugated goat anti–mouse Igs were from Organon Teknika Corp. (West Chester, PA). Lissamine rhodamine (LRSC)-conjugated goat anti–mouse Ig and TRITC-conjugated streptavidin were purchased from Jackson ImmunoResearch Laboratories, Inc., (West Grove, PA). FITC-conjugated goat anti–mouse IgG$_1$ and IgG$_{2a}$–specific secondary antibodies were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Antibodies produced in the laboratory, using standard methods were anti-LC (anti-clathrin light chain polyclonal rabbit antiserum recognizing the consensus sequence in all LCs, residues 23–40; Acton and Brodsky, 1990), X32 mAb (anticlathrin heavy chain, which does not react with the clathrin heavy chain hub region; Blank and Brodsky, 1986), AP.6 mAb (anti–α chain of the AP2 adaptor; Chin et al., 1989), KESL (polyclonal rabbit antiserum, recognizing the carboxy-terminal residues in the lumenal domain of human I chain), and W6/32 mAb (anti-MHC class I [HLA-A, -B, and -C]; Barnstable et al., 1978). Antibodies obtained as gifts from colleagues were 100/3 mAb (anti–γ chain of the AP1 adaptor, gift from E. Ungewickell, Washington University, St. Louis, MO; Ahle et al., 1988), anti-ER (rabbit polyclonal antiserum, gift from D. Louvard, Institut Curie, Paris, France; Louvard et al., 1982), anti-Tac (rabbit polyclonal antiserum, gift from W. Greene, University of California, San Francisco, CA), anti-DM (rabbit polyclonal antiserum, gift from D. Zaller, Merck Research Laboratories, Rahway, NJ; Sloan et al., 1995), PIN.1.1 mAb (anti-I chain cytoplasmic do-

main, gift from P. Cresswell, Yale University, New Haven, CT; Roche et al., 1991) and anti–cation-independent mannose-6-phosphate receptor (CI-M6PR, polyclonal rabbit antiserum; gift from L. Traub, Washington University).

Plasmid Construction

The cDNA encoding bovine clathrin heavy chain residues 1073–1675 (Liu et al., 1995) was first cloned into the BamHI and HindIII sites of the vector pET23d (Novagen, Inc.) after the T7 gene 10-leader peptide sequence. The T7Hub cDNA fragment was then cut out and inserted between the HindIII and XhoI sites of the vector pCDM8 (Invitrogen, Carlsbad, CA) under the cytomegalovirus (CMV) promoter to generate pCDM8T7Hub for transfection. HLA–DM α and β subunit cDNAs were obtained from the XhoI fragments of pCDM8DMα and pCDM8DMβ (Kelly et al., 1991; Marks et al., 1995), respectively, and then subcloned into the vector pMTΔ302 containing the modified human metallothionein IIA promoter (gift from S. Haskill, University of North Carolina, Chapel Hill, NC; Makarov et al., 1994) to produce pMTΔ302DMα and pMTΔ302DMβ. The DMβHSSΔ construct, lacking the carboxy-terminal 16 residues of the 23-residue cytoplasmic domain that eliminates the DMβ internalization motif, was in pCDM8.1 and its expression was regulatable by cadmium when coexpressed with pMTΔ302DMα. I chain p33 (Ip33) cDNA was derived from pCDM8Ip33 (gift from P. Roche, National Institutes of Health, Bethesda, MD; Roche et al., 1992) and inserted into pMTΔ302 to generate plasmid pMTΔ302Ip33. Detailed cloning procedures will be provided upon request. Tac (interleukin [IL]-2 receptor α subunit) chimeric constructs TTM.GSTYΔ (mouse H2M cytoplasmic tail fused to the lumenal and transmembrane domain of Tac; Marks et al., 1995) and Tac-DKQTLL (the T cell receptor CD3γ dileucine sequence DKQTLL attached to the carboxyl terminus of Tac, also called TTγt3-t2; Letourneur and Klausner, 1992), both in the vector pCDM8.1 (Invitrogen), were used as described previously (Marks et al., 1996, 1997). Plasmids containing MHC class II DR α and β chain cDNA, pCDM8–DRA, and pCDM8–DRB (pCDM8–DRB1*0101) were obtained from E. Long, National Institutes of Health; Long et al., 1994.

Cell Culture and Transfection

HeLa 229 cells (American Type Culture Collection, Rockville, MD) were maintained in DME supplemented with 10% fetal bovine serum. Cells were plated the day before transfection and then transfected by the standard calcium phosphate method at 30–50% confluency. Typically, 7.2 μg per 60-mm plate or 0.7 μg per coverslip of T7Hub plasmid was used for transfection. 12 h later the transfection mixture was removed and then replaced with fresh medium. Cells were generally assayed at 40–48 h after transfection. To induce expression of transfected genes under the metallothionein promoter, 10 μM CdCl$_2$ was added to the culture 20–24 h before analysis.

Endocytosis Assay

For transferrin (Tfn) uptake, pCDM8T7Hub–transfected HeLa cells were changed to transferrin uptake buffer PBS, with 10 mM Tris-HCl, 10 mM Pipes, 5 mM glucose, 1 mg/ml BSA, 0.1 g/L MgCl$_2$· 6 H$_2$O, 0.1 g/L CaCl$_2$· 2 H$_2$O, pH 7.4) for 3 h at 37°C. Fluorescein-conjugated human Tfn (FITC-Tfn; Molecular Probes, Eugene, OR) at 50 μg/ml in the uptake buffer was then added to the cells for 10 min at 37°C, after which the medium was removed and then the cells were washed three times with ice-cold PBS and processed for indirect immunofluorescent microscopy. To measure bulk fluid-phase endocytosis, 1 mg/ml lysine-fixable FITC-dextran (70K; Molecular Probes) was added to the cells for 3 h at 37°C in PBS buffer (with Ca^{2+} and Mg^{2+}).

Immunoprecipitation and Immunoblotting

Transfected cells on 60-mm plates were harvested and then lysed in cell lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1 mM EDTA, pH 7.4) plus protease inhibitors. For immunoprecipitation of T7Hub from transfected cells, 5 μl of 1 mg/ml purified anti-T7 mAb was added to the cell lysate for at least 1 h at 4°C, followed by 50 μl of protein G–Sepharose slurry for another hour (Pharmacia Diagnostics AB, Uppsala, Sweden). The unbound fractions were removed and then the precipitates were washed three times with lysis buffer at 4°C. Both supernatants and immunoprecipitates were then resolved by SDS-PAGE (Laemmli, 1970), trans-

ferred to nitrocellulose, and then analyzed by immunoblotting (Towbin et al., 1979). Antibody binding was detected with secondary antibodies conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) using the enhanced chemiluminescence substrate (Amersham Corp., Arlington Heights, IL).

Indirect Immunofluorescent Microscopy

HeLa cells plated on 12-mm coverslips were transfected as described above. 40–48 h after transfection, cells were washed three times with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, and then permeabilized with 0.04% saponin (Sigma Chemical Co.) for another 10 min. Cells were then incubated with blocking buffer (PBS, 0.02% SDS, 0.1% NP-40, 0.02% NaN$_3$ with 5% goat serum or with 0.66% gelatin and 1% BSA when labeling with anti–CI-M6PR) for 30 min, followed by incubation with various primary and fluorochrome-conjugated secondary antibodies diluted in appropriate blocking buffer, for 1 h each. After the final wash, the samples were mounted in 0.1% p-phenylene diamine (Sigma Chemical Co.) in Fluoromount G (Fisher Scientific Co., Pittsburgh, PA) and then viewed with a Zeiss Axiophot fluorescence microscope (Thornwood, NY). For cytosol depletion, transfected cells were treated with 0.004% digitonin (Calbiochem-Novabiochem Corp., La Jolla, CA) in permeabilization buffer (12.5 mM Hepes–KOH, 50 mM Pipes, 1 mM MgSO$_4$, 4 mM EGTA, pH 7.0) for 5 min on ice before fixation. To detect the surface level of certain antigens, the transfected cells were incubated with primary antibodies in PBS (with Ca^{2+} and Mg^{2+}) on ice for 30 min, the antibody solution was removed, and then the cells were washed three times with ice-cold PBS before fixation.

Surface Biotinylation

The surface biotinylation procedure was modified from an earlier method (Bonnerot et al., 1995). Transfected HeLa cells were washed three times with ice-cold PBS and then incubated with freshly prepared 0.5 mg/ml sulfo-NHS-LC-Biotin (Pierce Chemical Co., Rockford, IL) in PBS for 15 min on ice. The reaction was quenched with 50 mM NH$_4$Cl/PBS for another 15 min. Cells were lysed and then the biotinylated proteins were isolated with avidin–agarose (Pierce Chemical Co.) in the cell lysis buffer.

Results

Expression of Hubs Affects the Intracellular Distribution of Both Clathrin Heavy and Light Chains but Not Adaptors

The hub fragment of clathrin, comprising the carboxy-terminal third of the clathrin heavy chain, can bind clathrin LCs in vitro (Liu et al., 1995) and can interact with intact clathrin (data not shown). Therefore, we predicted that expression of the hub in mammalian cells would affect CCV formation in vivo. To test whether hub would bind endogenous clathrin LCs during intracellular expression, HeLa cells were transfected with a mammalian expression vector encoding the hub fragment of bovine clathrin modified with a T7 epitope-tag at the amino terminus (T7Hub). The T7Hub protein was immunoisolated from the cell lysate, using anti-T7 epitope antibody, 48 h after transfection. Endogenous clathrin light chains, LCa and LCb, were both observed associated with the T7Hub proteins (Fig. 1 *A*, lane *4*). Hubs presumably compete with endogenous clathrin heavy chains for newly synthesized LCs, since LCs do not readily dissociate from preassembled complexes with clathrin heavy chains (Acton and Brodsky, 1990).

The localization of LCs in the presence of hubs was further examined by immunofluorescent microscopy. Punctate staining typical of CCVs was observed in untransfected cells labeled with anti-LC antibody, but in the cells expressing hubs, the LC staining was diffuse and nonvesicular, implying its cytosolic distribution (Fig. 1 *B*). The
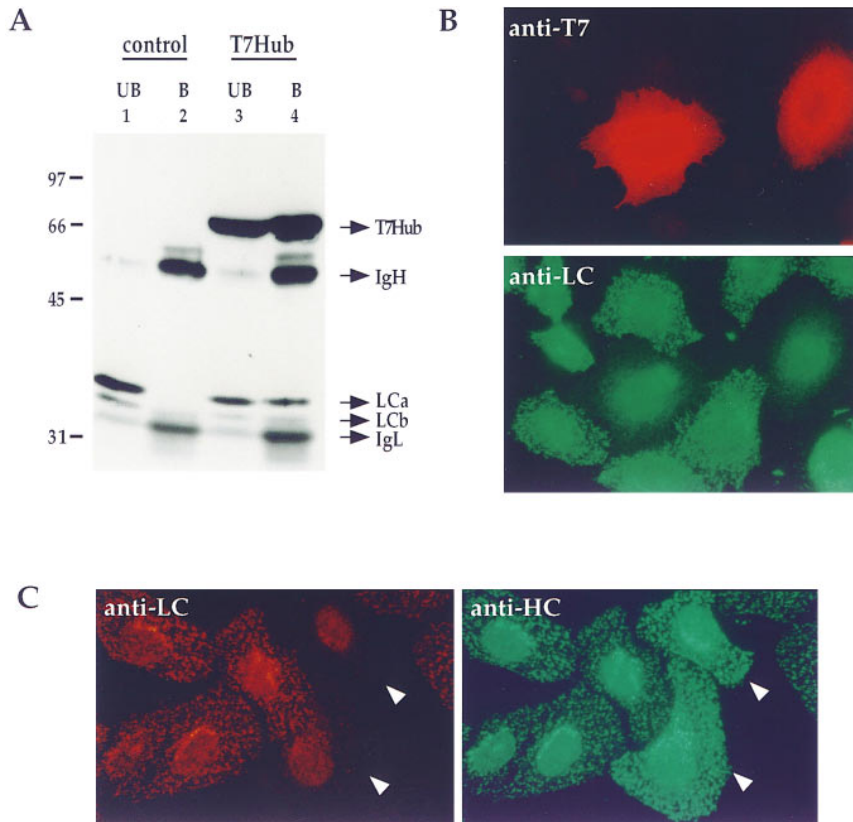
A control / T7Hub — UB B UB B (lanes 1, 2, 3, 4); 97—, 66—, 45—, 31— markers; T7Hub, IgH, LCa, LCb, IgL

B anti-T7, anti-LC

C anti-LC, anti-HC

Figure 1. Transfected hubs alter the intracellular distribution of both clathrin heavy chains and LCs. (*A*) HeLa cells were transfected with control vector (pCDM8 without the T7Hub insert, lanes *1* and *2*) or with T7Hub (pCDM8T7Hub vector, lanes *3* and *4*). 48 h after transfection, cells were lysed and then the T7Hub protein was immunoprecipitated with anti-T7 mAb bound to protein G–Sepharose. The unbound (*UB*) and bound (*B*) fractions were then analyzed by SDS-PAGE and immunoblotting. The T7Hub protein and clathrin LCs (*LCa* and *LCb*) were detected with anti-T7 mAb and anti-LC antiserum, respectively. IgH and IgL are the subunits of the mAb used for immunoprecipitation, detected by the secondary anti-immunoglobulin antibody. (*B*) HeLa cells were transfected with T7Hub. 48 h after transfection, cells were processed for immunofluorescent microscopy and stained with anti-T7 mAb and anti-LC antiserum (*anti-LC*) followed by LRSC-conjugated goat anti–mouse IgG and FITC-conjugated goat anti–rabbit IgG. (*C*) T7Hub-transfected HeLa cells were permeabilized with 0.004% digitonin to remove cytosol before fixation and thereby visualize membrane-associated clathrin. The cells were stained with anti-LC antiserum (*anti-LC*) and antiheavy chain mAb X32 (*anti-HC*) followed by rhodamine-conjugated goat anti–rabbit IgG and FITC-conjugated goat anti–mouse IgG. The T7Hub-transfected cells were identified by the loss of punctate clathrin LC staining (*arrowheads*) and are the ones with increased staining of clathrin heavy chain at the PM.

A anti-T7, AP2
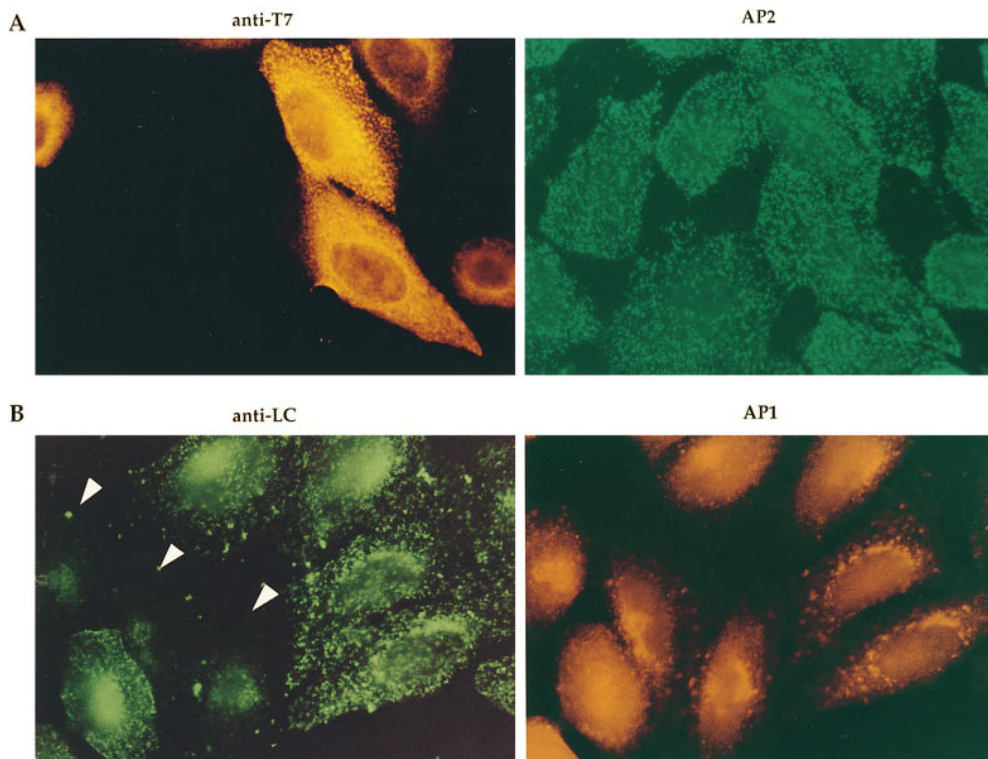
B anti-LC, AP1

Figure 2. Expression of T7Hub in transfected cells does not alter the general distribution of adaptor molecules. HeLa cells were transfected with T7Hub and after 48 h were then analyzed by immunofluorescence for the distribution of the AP1 and AP2 adaptors. (*A*) Cells stained with anti-AP2 mAb AP.6 (*AP2*) and biotinylated anti-T7 mAb followed by FITC-conjugated anti–mouse IgG$_1$–specific secondary antibody (for AP.6) and TRITC-conjugated streptavidin. (*B*) Cells stained with anti-AP1 mAb 100/3 (*AP1*) and anti-LC (*anti-LC*) antiserum followed by LRSC-conjugated goat anti–mouse IgG and FITC-conjugated goat anti–rabbit IgG. The T7Hub–transfected cells in *B* were identified by the loss of punctate clathrin LC staining (*arrowheads*).

T7Hub proteins themselves were also localized mostly in the cytoplasm (Fig. 1 B, top), though weak staining of distinct membrane-associated regions was observed after a mild detergent wash (0.004% digitonin) to remove cytosol (data not shown). On the other hand, increased levels of endogenous clathrin heavy chains were membrane-associated in hub-transfected cells (Fig. 1 C, anti-HC), as demonstrated by cell staining with a mAb recognizing an epitope on endogenous clathrin heavy chains absent from the hub fragment. In vitro studies have shown that without LCs, heavy chains no longer require low pH and high calcium for assembly, and can spontaneously assemble under physiological conditions, without a requirement for adaptors (Ungewickell and Ungewickell, 1991; Liu et al., 1995; Ybe et al., 1998). We suggest that the increased membrane recruitment of clathrin heavy chain represents endogenous heavy chains that are assembling as a result of depleting their LCs through the expression of hubs. These results establish that introduction of hub fragments into mammalian cells can directly alter the distribution and behavior of endogenous clathrin.

Binding of clathrin to cellular membranes is mediated through AP2 and AP1 adaptors (Keen, 1990; Pearse and Robinson, 1990). To establish whether the increase of clathrin at membranes in the presence of hubs caused recruitment of additional adaptors, the distribution of adaptors was assessed in hub-transfected cells. Labeling with mAbs specific for AP1 or AP2 revealed that the general distribution of adaptors was not affected by hub expression. In the T7 Hub-transfected cells, AP2 remained in peripheral punctate structures and AP1 staining was still mostly perinuclear (Fig. 2). The lack of effect on adaptor distribution suggests that the excess clathrin associated with membranes in hub-transfected cells represents hyperassembly due to altered clathrin–clathrin interactions that are adaptor independent.

Inhibition of Clathrin-dependent Functions in Hub-transfected Cells

Having demonstrated an effect of hub expression on clathrin localization and assembly at the PM, we next investigated whether intracellular expression of hubs can disrupt clathrin function. To this end, receptor-mediated endocytosis of FITC-Tfn was assayed in hub-transfected cells. When cells were allowed to endocytose FITC-Tfn continuously for 10 min, the ability of T7-Hub-expressing cells to accumulate ligand was almost completely inhibited, compared with accumulation of FITC-Tfn by untransfected cells (Fig. 3 A). In contrast, the uptake of FITC-dextran, a marker for bulk flow fluid-phase endocytosis, was comparable in hub-transfected and control cells (Fig. 3 B). These results demonstrate that T7Hub expression specifically impairs clathrin-dependent receptor-mediated endocytosis in transfected cells, but does not affect clathrin-independent fluid-phase uptake. The ability to measure hub effects on FITC-Tfn uptake made it possible to analyze the time course of hub-induced inhibition of CCV function. Significant inhibition of FITC-Tfn uptake was observed within a 20–40 h period after transfection with hubs, correlating with accumulation of approximately 15-fold excess of hubs over endogenous clathrin. This lag period for inhi-

bition is explained by the fact that cellular clathrin is abundant and the subunits turn over with half-lives of 24–48 h (Acton and Brodsky, 1990).

CCVs are known to sort M6PR in the TGN for targeting of lysosomal hydrolases to lysosomes (Kornfeld, 1992). In many hub-transfected cells, the distribution of the CI-M6PR was altered to display broadened perinuclear staining and diminished staining of peripheral endosomes, in comparison to the tight vesicular perinuclear crescent of staining observed in untransfected cells (Fig. 3 C). This phenotype suggested that hubs were inhibiting CI-M6PR sorting by CCVs in the TGN, with the result of preventing vesicular targeting and causing accumulation of nonsorted molecules in the secretory pathway. This phenotype was observed more clearly with hub effects on HLA–DM sorting in the TGN in experiments described below, in which vesicular targeting was completely inhibited. It is likely that the apparent partial effect of hubs on CI-M6PR distribution was due to the fact that a significant pool of endogenous CI-M6PR molecules were sorted before hub expression and had not yet turned over completely. Therefore, hub expression is most useful for studying the role of CCV in processes that can be measured de novo, such as the internalization of exogenous ligands or the targeting of molecules introduced into hub-expressing cells by transfection.

To ascertain that hub expression does not cause a general pleiotropic effect on organelle integrity, the ER and Golgi structures of cells transfected with T7Hub cDNA were examined by immunofluorescent staining with anti-ER antibody and a mAb against the Golgi-associated β-COP (Allan and Kreis, 1986). In cells expressing T7Hub, no change of the ER staining was observed, and β-COP remained concentrated in the apparently intact Golgi apparatus (Fig. 4, A and B). The cellular distribution of class I MHC molecules, which are efficiently transported through the secretory pathway to their site of accumulation on the PM, with no known requirement for sorting, was also not altered in cells expressing hubs (Fig. 4 C).

Expression of Hub Affects Distribution of Chimeric Proteins with Either Tyrosine or Dileucine Sorting Motifs

CCVs have been implicated in the transport of proteins containing specific sorting signals in their cytoplasmic domains. There is strong evidence from receptor mutagenesis and morphological studies that a tyrosine-based signal, with the amino acid sequence YXXØ (X is any amino acid and Ø is an amino acid with a bulky hydrophobic side chain), is responsible for sequestration of receptors in CCV at the PM (Marks et al., 1997). Yeast two-hybrid experiments with the μ subunits of AP1 and AP2 adaptors suggest that CCV might also be involved in sorting receptors with the tyrosine-based motif in the TGN (Ohno et al., 1995). A dileucine-based signal, with two consecutive leucine/isoleucine residues, also confers intracellular targeting to the endocytic pathway from the PM, and in vitro binding studies likewise implicate an interaction between this motif and both adaptors (Heilker et al., 1996; Dietrich et al., 1997). We analyzed the effect of hub expression on the localization of chimeric proteins with either a YXXØ-
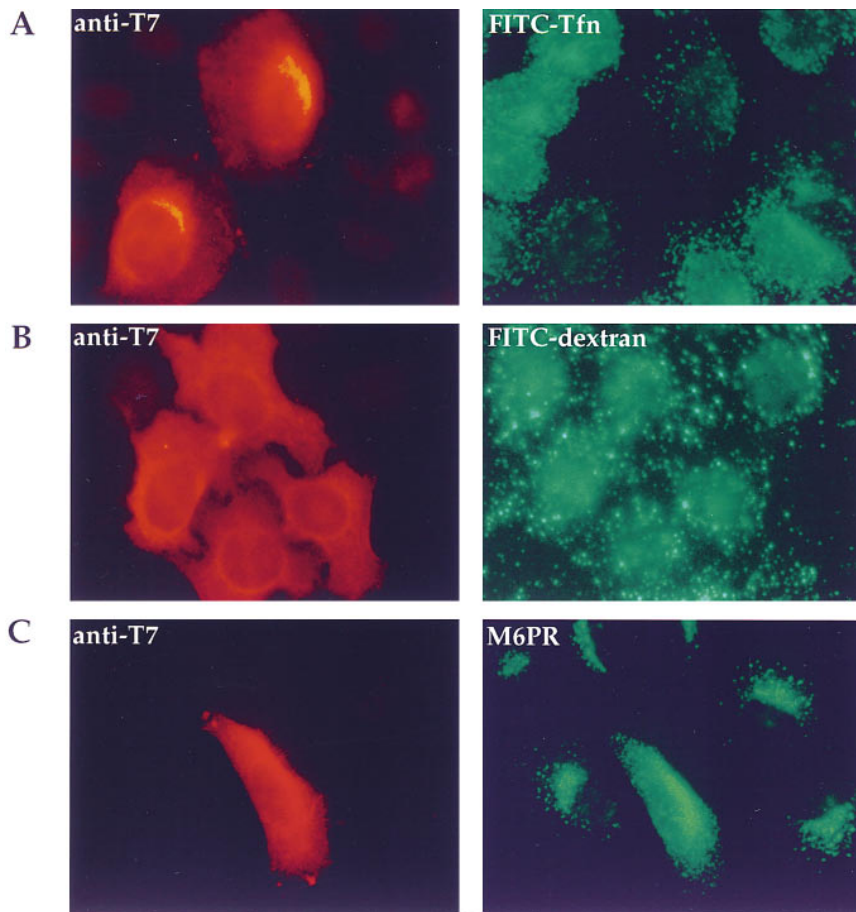
Figure 3. Expression of T7Hub in transfected cells inhibits receptor-mediated endocytosis but not bulk fluid-phase uptake and alters the intracellular distribution of the CI-M6PR. (*A*) HeLa cells were transfected with T7Hub. 48 h after transfection, cells were allowed to internalize FITC-Tfn for 10 min at 37°C, and then were processed for immunofluorescent microscopy. Hub-transfected cells were visualized by anti-T7 mAb and LRSC-conjugated goat anti–mouse IgG. (*B*) HeLa cells were transfected with T7Hub and, after 48 h, were allowed to internalize FITC-dextran at 37°C for 3 h before being processed for anti-T7 mAb staining to visualize cells with hubs. (*C*) HeLa cells were transfected with T7Hub and after 48 h were processed for immunofluorescent microscopy to visualize the CI-M6PR, with specific rabbit antiserum and FITC-conjugated goat anti–rabbit IgG, and hub-transfected cells, with anti-T7 mAb and LRSC-conjugated goat anti–mouse IgG.
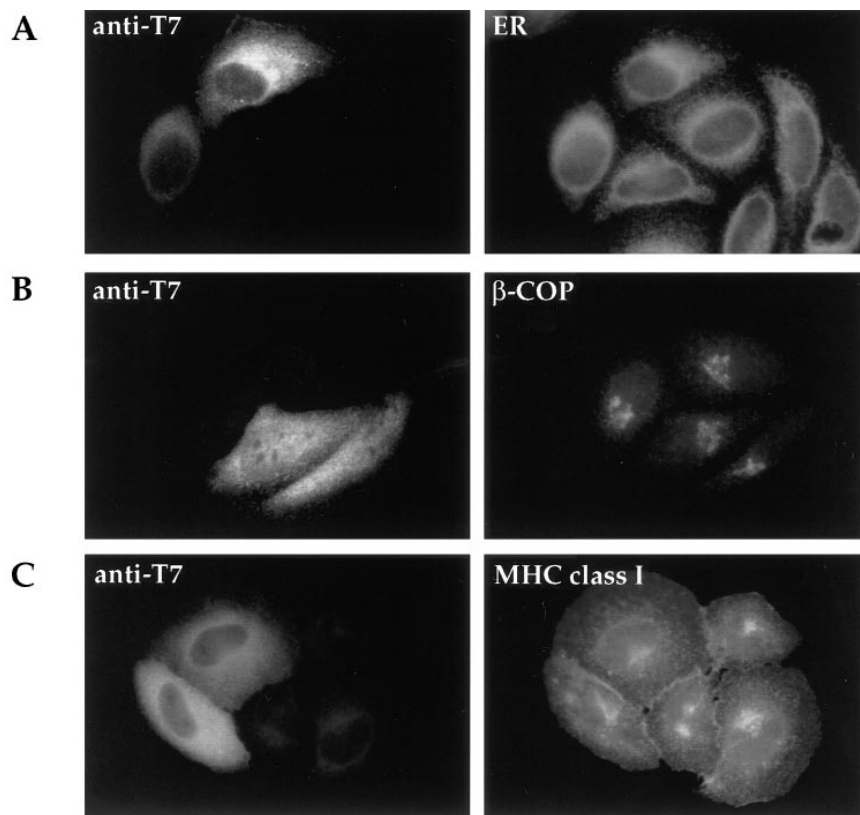

Figure 4. Hub expression does not have pleiotropic effects on organelle integrity or the constitutive secretory pathway. HeLa cells were transfected with T7Hub and, after 48 h, were stained with (*A*) anti-ER antiserum and FITC-conjugated goat anti–rabbit IgG, (*B*) anti–β-COP mAb M3A5 and FITC-conjugated goat anti–mouse IgG₁–specific secondary antibody, or (*C*) anti-MHC class I mAb W6/32 and FITC-conjugated anti–mouse IgG₂ₐ–specific secondary antibody. The T7Hub-transfected cells in each field were visualized either by (*A*) anti-T7 mAb followed by LRSC-conjugated goat anti–mouse IgG or (*B* and *C*) biotinylated anti-T7 mAb followed by TRITC-conjugated streptavidin.

internal | surface
Y | LL | Y | LL
V H | V H | V H | V H

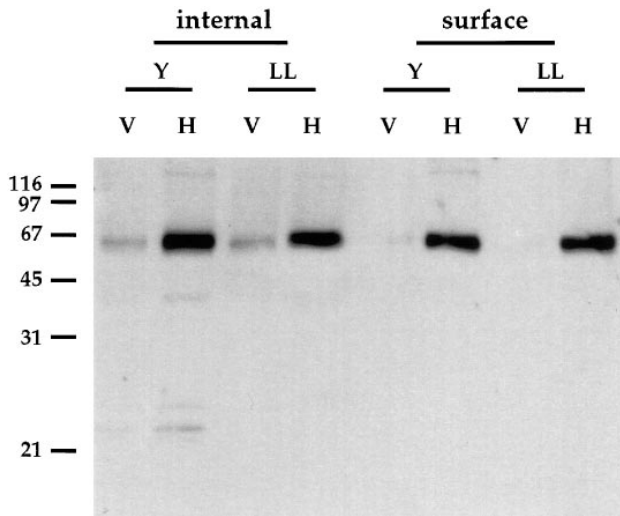116 —
97 —
67 —
45 —
31 —
21 —

Figure 5. Expression of T7Hub increases steady-state levels of cotransfected Tac chimeras bearing tyrosine- and dileucine-based sorting motifs and causes their cell surface accumulation. HeLa cells were transfected with control vector without the hub insert (*V*) or T7Hub (*H*). Each population of cells was cotransfected with vector encoding the Tac chimera with the tyrosine-based sorting signal of H2M (*Y*) or vector encoding the Tac chimera with the dileucine-based sorting motif of CD3γ (*LL*). 48 h after transfection, cells were surface biotinylated and lysates were prepared. Surface molecules were removed from the lysate with avidin–agarose and then internal molecules were left in the unbound fraction. The presence of Tac chimeras in each fraction of the lysate was established by immunoblotting with anti-Tac antiserum after SDS-PAGE. For each sample, 100% of the surface fraction and 25% of the internal fraction were analyzed.

tyrosine (TTM.GSTYΔ) or a dileucine (Tac-DKQTLL) targeting signal to investigate which intracellular sorting steps actually depend on CCV recognition of either signal. The chimeric molecules both have the extracellular domain of the IL-2 receptor α chain, designated Tac, and have cytoplasmic domains with targeting signals derived from H2M (YTPL) and CD3γ (DKQTLL), respectively. Both molecules have been previously shown to be targeted efficiently to lysosomes, where the lumenal domain is rapidly proteolyzed (Letourneur and Klausner, 1992; Marks et al., 1995). Thus, loss of immunoreactivity to Tac can be considered a measure of lysosomal delivery.

Chimeric molecules were transfected into HeLa cells with or without cotransfection of T7Hub and their steady-state levels were measured by immunoblotting with Tac antibody (Fig. 5). Surface biotinylation was also performed on the transfected cells to assess the surface distribution of the chimeras by isolating surface molecules with avidin and then immunoblotting with Tac. When either of the Tac chimeras were expressed alone in transfected cells, only low levels were detectable in total cell lysate and virtually none were detectable in the cell surface-biotinylated pool (Fig. 5), consistent with lysosomal targeting. Cotransfection with T7Hub cDNA caused dramatic increases in the cellular level of either chimera. In addition, a detectable proportion (∼20%) appeared in the cell surface-biotinylated pool (Fig. 5), a ratio that is probably an underestimate due to the inefficiency of biotinylation. Intracellular distribution of the chimeric proteins was further examined by immunofluorescent microscopy. In accordance with the biochemical analysis, the fluorescent signals from staining the Tac constructs were greatly enhanced upon cotransfec-
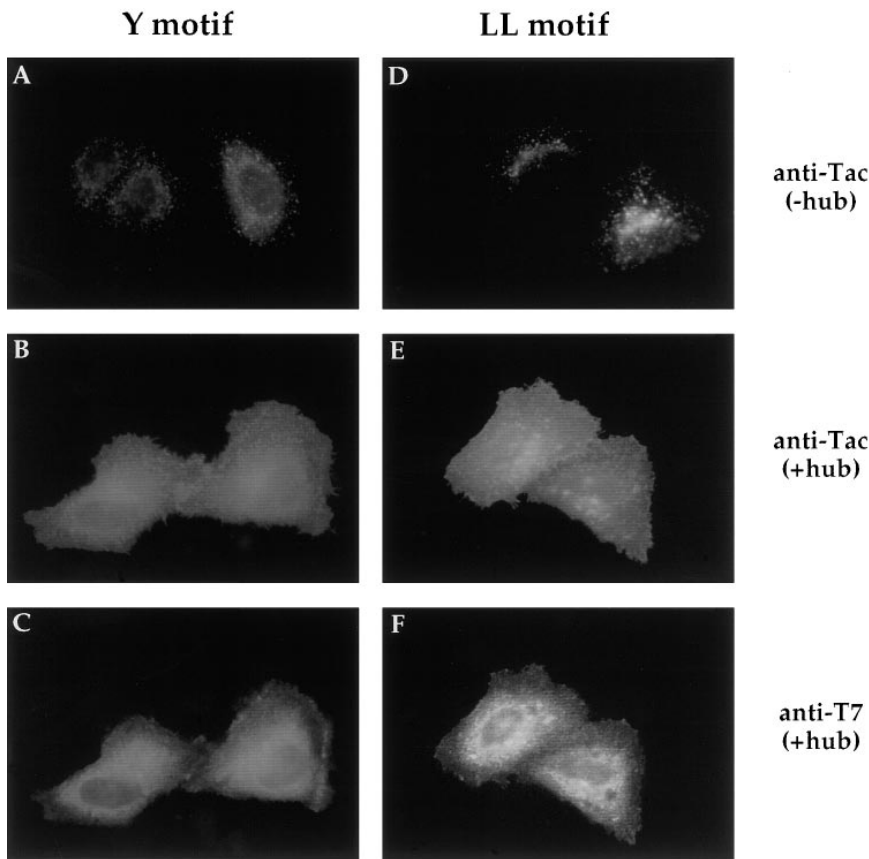
Y motif | LL motif

anti-Tac
(-hub)

anti-Tac
(+hub)

anti-T7
(+hub)

Figure 6. Expression of T7Hub results in cellular redistribution of cotransfected Tac chimeras bearing tyrosine- and dileucine-based sorting motifs. HeLa cells were transfected with (*A* and *D*) control vector (−*hub*) or (*B, C, E*, and *F*) T7Hub (+*hub*) together with (*A–C*) vector encoding the Tac chimera with a tyrosine-based sorting signal (*Y motif*) or (*D–F*) vector encoding the Tac chimera with a dileucine-based sorting signal (*LL motif*). 48 h after transfection, cells were processed for immunofluorescence and stained with (*A, B, D,* and *E*) anti-Tac antiserum followed by FITC-conjugated goat anti–rabbit IgG and (*C* and *F*) anti-T7 mAb followed by LRSC-conjugated goat anti–mouse IgG.

tion with T7Hub, compared to the generally weak staining obtained in the control cells (Fig. 6). The bulk of the chimeric proteins in hub-transfected cells also seemed to be predominantly localized on the PM (Fig. 6), whereas those in cells without hubs had a punctate intracellular distribution. These results indicate that blocking CCV function results in the mistargeting of proteins normally destined for lysosomes and because of a block in receptor-mediated endocytosis, they were accumulating on the cell surface. It is not possible to determine whether these proteins were expressed on the cell surface as part of their normal transport pathway to lysosomes or whether they were diverted to the cell surface because of missorting in the TGN. However, these results clearly show that chimeric molecules with either the tyrosine or dileucine motif rely on CCVs for their endocytosis. They also confirm that hub transfection blocks CCV function at the cell surface.

CCVs Are Involved in Both the Intracellular Targeting and Endocytosis of HLA–DM Molecules

To address whether CCVs are also involved in sorting molecules with tyrosine or dileucine signals in the TGN, we chose to investigate the effect of hub expression on the sorting of biologically active molecules bearing these signals in the class II MHC–antigen presentation pathway. The YXXØ–tyrosine signals of the HLA–DM β chain and its rodent homologue H2Mb are necessary and sufficient for their delivery to the lysosomal compartment and for interaction with the μ1 and μ2 adaptor subunits (Lindstedt et al., 1995; Marks et al., 1995; Ohno et al., 1995; Copier et al., 1996). To investigate where CCVs are actually involved in HLA–DM sorting, plasmids containing HLA–DM α and β subunit DNA sequences under the transcriptional control of the CMV promoter were transfected into HeLa cells with or without T7Hub cDNA. The majority of HLA–DM dimers was targeted to lysosome-like compartments (Marks et al., 1995) undetectable on the PM (Fig. 7 A) when expressed in the absence of hub molecules. However, a significant increase of cell surface DM staining was observed upon coexpression with the T7Hub (Fig. 7, B and C). This result indicates that CCVs are indeed responsible for the internalization of DM molecules from the cell surface. The internal staining for DM had a lysosome-like distribution, possibly because some of the DM molecules would have been targeted to intracellular compartments before hubs accumulated to an inhibitory level, as we had observed for endogenous CI-M6PRs. The route by which the DM molecules reached these compartments needed further clarification.

To determine whether HLA–DM was directly targeted from the TGN to the endocytic pathway and to establish whether CCVs are involved in DM sorting in the TGN, we developed a new expression strategy. cDNA encoding the DMα and DMβ chains were subcloned into expression vectors driven by a modified version of the inducible human metallothionein promoter that was developed to reduce basal expression levels (Makarov et al., 1994). Thus, when cells were cotransfected with these constructs and hubs were expressed from a constitutive promoter, hubs could accumulate to inhibitory levels before inducing DM expression. In the absence of cadmium, DM was barely
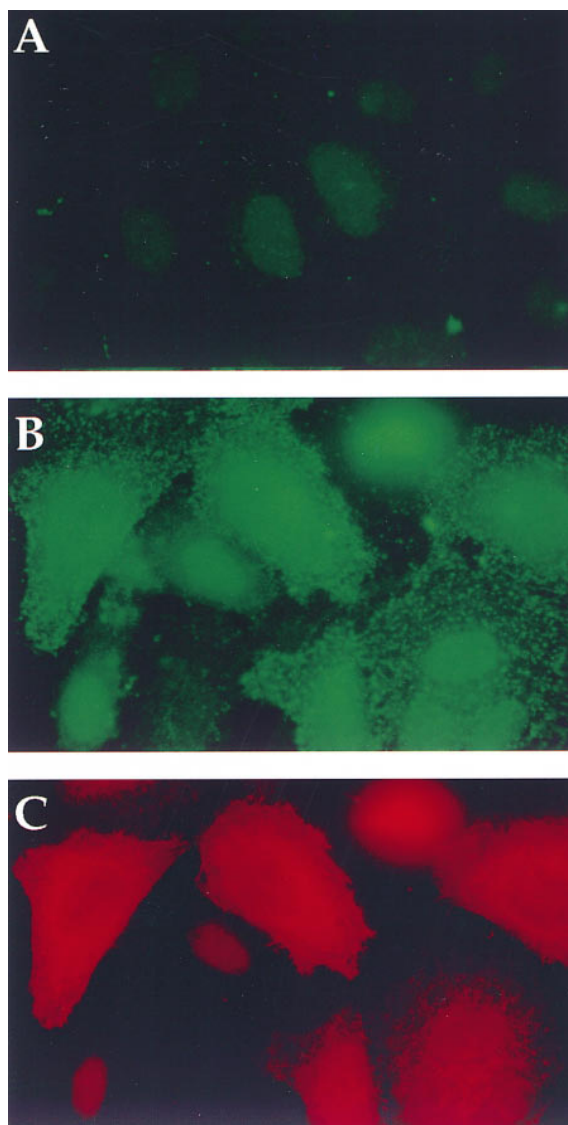

Figure 7. Hub expression results in surface accumulation of coexpressed HLA–DM molecules. HeLa cells were transfected with either (*A*) control vector (no hub) or (*B* and *C*) T7Hub and (*A–C*) cotransfected with vectors encoding the HLA–DM α/β dimer (pCDM8DMα and pCDM8DMβ). (*A* and *B*) Surface expression of DM was analyzed by incubating nonpermeabilized, live cells with anti-DM antiserum for 30 min on ice. Cells were then fixed and processed for immunofluorescent microscopy using FITC-conjugated goat anti–rabbit IgG to detect surface DM. (*C*) Cells in *B* were labeled with anti-T7 mAb followed by LRSC-conjugated goat anti–mouse IgG to identify cells with hubs.

detectable by immunofluorescence in transfected cells. DM dimers, induced by the addition of cadmium in the absence of hubs, were targeted to lysosome-like compartments and colocalized with the lysosomal marker CD63 (Fig. 8, *A* and *B*). In cells cotransfected with hubs, DM molecules induced 24 h after cotransfection had a diffuse perinuclear distribution consistent with localization to the ER and Golgi complex. This was similar to the pattern seen for endogenous CI-M6PR when its distribution was altered by hub expression (Fig. 3 *C*), further indicating that hubs inhibit sorting in the TGN, resulting in defective
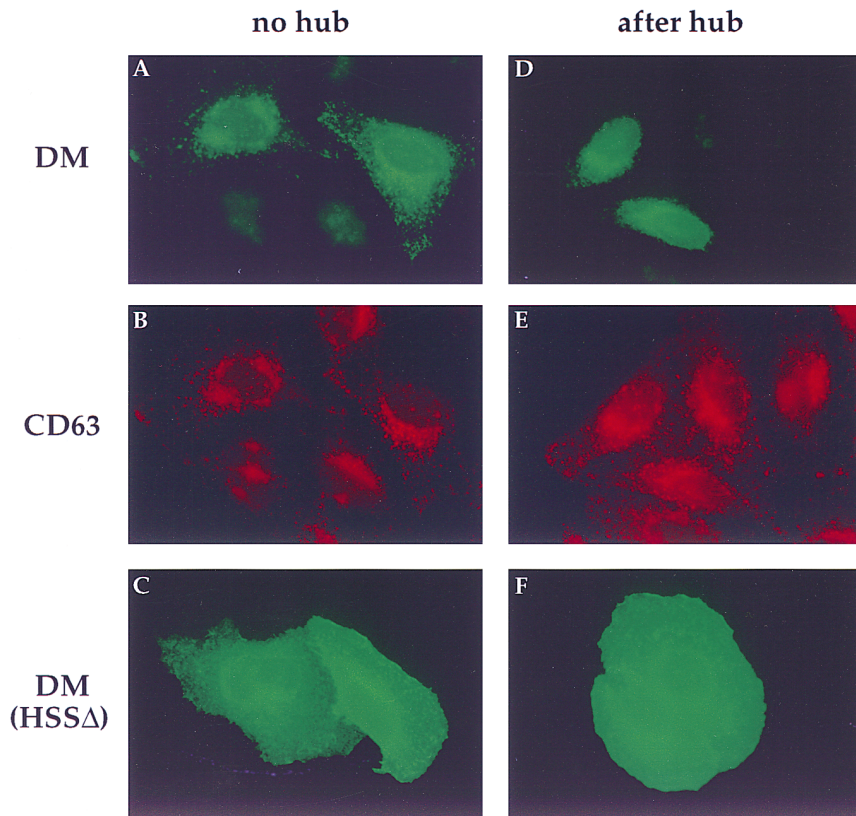
no hub   after hub

DM — A | D

CD63 — B | E

DM (HSSΔ) — C | F

Figure 8. Direct delivery of HLA–DM molecules from the TGN to lysosome-like compartments is inhibited by hubs. HeLa cells were transfected with (*A–C*) control vector or (*D–F*) T7Hub. (*A*, *B*, *D*, and *E*) were cotransfected with vectors encoding the HLA–DM α/β dimer driven by the inducible human metallothionein promoter (pMTΔ302DMα and pMTΔ302DMβ). *C* and *F* were cotransfected with a vector encoding the DMβ chain without the YTPL internalization motif (pCDM8.1.DMβ-HSSΔ) and the inducible DMα chain (pMTΔ302DMα). 24 h after transfection, expression of DM molecules was induced by exposure to 10 µM CdCl$_2$ for 20 h. Cells were fixed, permeabilized, and then processed for intracellular immunofluorescence. Colocalization of DM relative to the lysosomal marker CD63 was analyzed by double-staining cells with (*A* and *D*) anti-DM antiserum followed by FITC-conjugated goat anti–rabbit IgG and (*B* and *E*) anti-CD63 mAb followed by LRSC-conjugated goat anti–mouse IgG. Note that in *A* and *D*, DM is detected only in transfected cells that are cotransfected with control vector or hub, whereas CD63 (*B* and *E*) is detected in all cells. The surface staining of DM in comparable samples was barely detectable for both control and hub-transfected cells (data not shown). In *C* and *F* the surface expression of the DMβ chain without the YTPL internalization motif occured when expression of cotransfected DMα was induced and was detected in control or hub-transfected cells by labeling with anti-DM antiserum followed by FITC-conjugated goat anti–rabbit IgG.

targeting to vesicular structures and apparent backup in the secretory pathway. In the case of DM expressed de novo, no vesicular structures containing DM were detected, and the staining patterns for DM and CD63 were distinct in hub-transfected cells (Fig. 8, *D* and *E*). In addition, no significant level of induced DM was detected on the cell surface for cells transfected with either hub-containing or control vectors (data not shown). The hub effects on DM expression, causing accumulation in the perinuclear area, depletion from endocytic compartments, and preventing DM's transient appearance on the PM indicate that DM export from the TGN is a function of CCVs and that the targeting of DM to lysosome-like compartments is clathrin dependent. These data also suggest that CCVs sort DM directly from the TGN to the endocytic pathway before it can get to the cell surface. Thus, the endocytosis of DM in CCVs is probably a recapture mechanism used to recycle DM released from the endocytic pathway, rather than the primary route taken to reach the endocytic compartments in which it resides.

In clathrin-deficient yeast, TGN sorting is impaired and the protease Kex2p, normally sequestered in the TGN, is expressed on the cell surface (Seeger and Payne, 1992). In hub-transfected cells, inhibition of CCV function at the TGN results in a broadened perinuclear distribution of molecules that are normally targeted to endocytic vesicles. We hypothesized that these different outcomes reflect the fact that hubs cause hyperassembly of endogenous clathrin around TGN–coated pits, forming a nonfunctional struc-

ture which, via adaptors, acts as a trap for molecules that are normally sorted, and prevents their exit from the secretory pathway. This is in contrast to mutant yeast that lack functional clathrin and are permissive for cell surface export in the absence of sorting. To investigate the validity of the "trap" theory, we analyzed the effect of hub expression on the TGN sorting of DM molecules lacking their sorting signals (Fig. 8, *C* and *F*). Such molecules should be able to evade being trapped by adaptors in nonfunctional clathrin assemblies. Cells were transfected with control vector or hub and cotransfected with mutant DMβ, lacking its sorting signal, along with DMα, expressed under an inducible promoter. Since DMβ depends on DMα for export from the ER, this system results in inducible expression of mutant DM dimers (Karlsson et al., 1994; Marks et al., 1995). When induced by addition of cadmium, the mutant DM dimers were expressed on the cell surface whether or not hub was present in the transfected cells. Thus, the DM mutants were not trapped into a perinuclear distribution, indicating that hub effects depend on interaction with the DM sorting signal, and that hub inhibition of sorting in the TGN is consistent with a trapping mechanism. In addition, this provides further evidence that hubs do not inhibit the constitutive secretory pathway.

CCVs Mediate Endocytosis but Not the Intracellular Targeting of MHC Class II DR–I Chain Complex

The dileucine signal of the cytoplasmic tail of the I chain is

responsible for the targeting of the I chain complexed with class II MHC molecules to the endocytic pathway (Pieters et al., 1993; Odorizzi et al., 1994). To determine whether CCVs play a role in this targeting and to establish what steps depend on their participation, I chain–class II MHC complexes were coexpressed with hubs. For this study, a plasmid encoding the Ip33 form of the I chain, which is the most abundant form responsible for class II molecule targeting, was chosen for transfection along with plasmids encoding the α and β chain of HLA–DR, a human class II MHC molecule. In initial experiments all these cDNAs were under transcriptional control of the constitutive CMV promoter, and then cotransfected into HeLa cells, with or without cotransfection of T7Hub. First, the surface expression of these molecules was examined by immunofluorescent microscopy. I chain, coexpressed with HLA–DR, was not detectable on the surface of control cells, (Fig. 9 A) but was significantly increased on the surface of cells cotransfected with T7Hub (Fig. 9, B and C). This observation demonstrates that surface I chain–DR is normally endocytosed through CCVs and is consistent with the conclusions of a recent study of the effects of a dynamin mutant on I chain–DR distribution (Wang et al., 1997), a finding that we have confirmed independently by transfection with mutant dynamin (Marks, M., unpublished results). Hub-transfected cells were also analyzed by intracellular immunofluorescent microscopy for the distribution of I chain–DR complexes (data not shown), revealing a normal distribution in the endocytic pathway. This normal localization could be a function of targeting during hub accumulation or could be due to the fact that I chain transport to the endocytic pathway may not depend on CCVs.

To clarify the mechanism and primary destination of I chain export from the TGN, I chain cDNA was subcloned for expression driven by the modified metallothionein promoter. The inducible I chain plasmid was transfected into HeLa cells together with plasmids encoding DR subunits, with or without hubs. I chain expression was then induced after 24 h of constitutive hub expression. In cells expressing hubs, the cadmium-induced I chain still appeared localized in vesicular structures, indistinguishable from the expression pattern observed in control cells with no hubs (Fig. 10, A and B). A similar result was obtained when I chain was expressed alone without DR dimers (data not shown). Note that with or without hub expression, the intracellular I chain signal in the majority of the cells was weak due to the lysosomal degradation of the epitope recognized by the antibody used. These observations indicate that direct targeting of I chain from the TGN to the endocytic pathway is clathrin independent, consistent with an earlier immunoelectron microscopy study localizing I chain–DR complexes to budding structures in the TGN lacking clathrin or other CCV coat proteins (Glickman et al., 1996).

Discussion

A Direct Approach to Establishing the Role of Clathrin in Intracellular Transport

Clathrin's role in receptor-mediated endocytosis from the PM has been well-established through a large body of cumulative evidence. The role of CCV in sorting proteins at intracellular membranes needs to be better defined. Existing methods for analyzing the role of clathrin in intracellular transport pathways in mammalian cells are indirect or inefficient. Pharmacological methods that disrupt clathrin function at the PM such as induction of cytosol acidification, exposure to hypertonicity, or "potassium depletion" may cause pleiotropic changes in the cell, in addition to their effects on clathrin. Morphological localization of clathrin to a particular cellular location or visualization of a receptor in a coated pit or vesicle suggests the involvement of clathrin in transport, but does not demonstrate function. Microinjection of antibodies to clathrin, although informative, has technological limitations (Doxsey et al., 1987). Genetic approaches in yeast, *Drosophila*, and *Dictyostelium* have proven valuable in analyzing clathrin function (Silveira et al., 1990; Seeger and Payne, 1992; Bazinet et al., 1993; Niswonger and O'Halloran, 1997) in these organisms. Accordingly, expression of a dominant-negative mutant of dynamin in mammalian cells, reproducing the *shibire* phenotype of *Drosophila*, has been very useful for demonstrating the role of CCV in uptake from the PM (van der Bliek et al., 1993; Damke et al., 1995; Wang et al., 1997). However, this approach has been unable to investigate the role of CCV in internal sorting processes. To address this problem in mammalian cells, we transfected cells with the hub fragment of the clathrin triskelion, which we demonstrate acts as a dominant-negative mutant, specifically inhibiting clathrin function both at the PM and in intracellular sorting steps. Blocks in endocytosis were observed for uptake of endogenous TfnR and four different transfected molecules. Blocks in TGN sorting were observed for endogenous CI-M6PR and for transfected HLA–DM. We also demonstrate that hub expression has no effect on fluid-phase endocytosis or organelle integrity in the constitutive secretory pathway. In addition, hubs had no effect on constitutive secretion of class I MHC molecules or a transfected mutant DM molecule lacking its CCV sorting signal.

Previous studies from the laboratory established that the hub fragment, formed by the carboxy-terminal third of the clathrin heavy chain, trimerizes and folds to mimic the central portion of the clathrin triskelion. Here, we show that the hub fragment expressed in mammalian cells binds endogenous clathrin LCs. Accompanying this behavior is a hyperassembly of LC-free endogenous triskelions onto cellular membranes. This can be explained by the fact that LCs prevent clathrin assembly at physiological pH in the absence of adaptors (Liu et al., 1995), so triskelions without LCs could be adding onto existing clathrin lattices, without the need for adaptors. Indeed, although we observe increased membrane recruitment of endogenous clathrin heavy chain in hub-transfected cells, we see no change in the distribution of either AP1 or AP2 adaptors on cellular membranes. Therefore, we hypothesize that hubs inhibit clathrin function primarily by LC depletion and thus induce hyperassembly at the edge of existing clathrin-coated pits. Hubs themselves may also be incorporated into existing clathrin lattices, as we can detect some membrane-associated hub molecules. The outcome of this inhibition mechanism is that clathrin-coated pits are
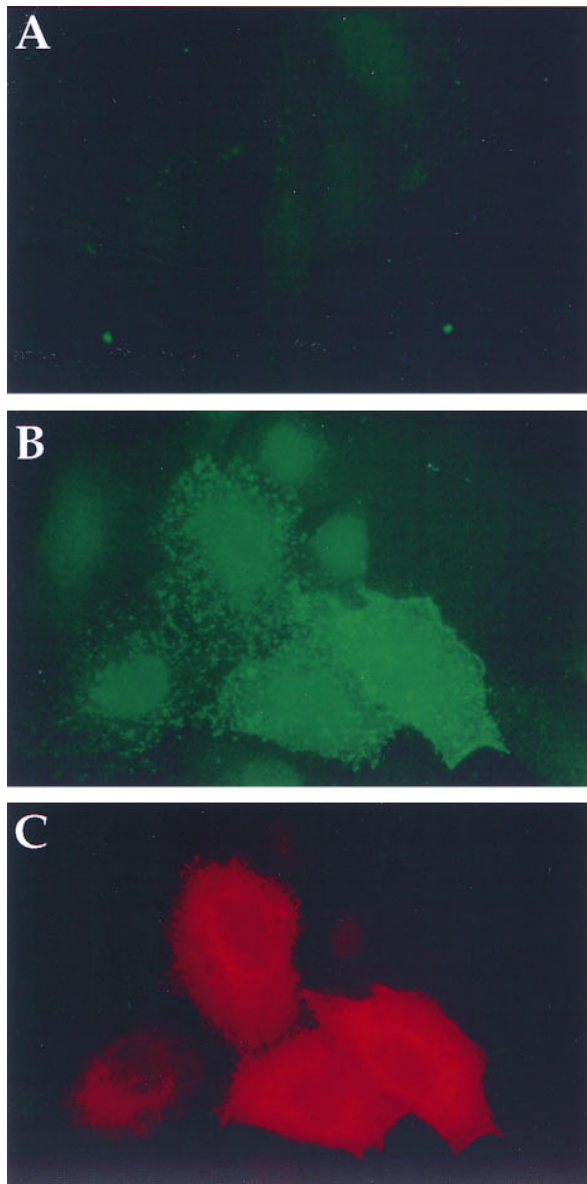
Figure 9. Hub expression results in the surface accumulation of coexpressed I chain. HeLa cells were transfected with (*A*) control vector (no hub) or (*B* and *C*) T7Hub and (*A*, *B*, and *C*) cotransfected with vectors encoding I chain and HLA–DRα and β chains (pCDM8Ip33, pCDM8DRα, pCDM8DRβ). (*A* and *B*) Surface expression of I chain was analyzed by incubating nonpermeabilized, live cells with anti-I chain antiserum KESL for 30 min on ice. Cells were then fixed and processed for immunofluorescent microscopy using FITC-conjugated goat anti–rabbit IgG to detect surface I chain. (*C*) Cells in *B* were labeled with anti-T7 mAb followed by LRSC-conjugated goat anti–mouse IgG to identify cells with hubs.

Figure 10. Direct delivery of I chain (coexpressed with DR) from the TGN to the endocytic pathway is not affected by expression of hubs. HeLa cells were transfected with (*A*) control vector (no hub) or (*B* and *C*) T7Hub and (*A*–*C*) cotransfected with vectors encoding HLA-DR α and β chains (pCDM8DRα and pCDM8DRβ) and the I chain driven by human metallothionein promoter (pMTΔ302Ip33). 24 h after transfection, I chain expression was induced by exposure to 10 μM CdCl$_2$ for 20 h. Cells were fixed, permeabilized, and then processed for immunofluorescence. The cells were stained with (*A* and *B*) anti-I chain mAb PIN.1.1, followed by FITC-conjugated goat anti–mouse IgG$_1$–specific secondary antibody and then (*C*) biotinylated anti-T7 mAb followed by TRITC-conjugated streptavidin.

frozen at the PM and TGN, trapping associated adaptors and receptors. This results in a phenotype in which CCV-mediated import or export of receptors, which are not overexpressed, is blocked, rather than the missorting of the phenotype observed in clathrin-deficient yeast mutants (Seeger and Payne, 1992). Characteristic of this phenotype is the patchy accumulation of molecules on the cell surf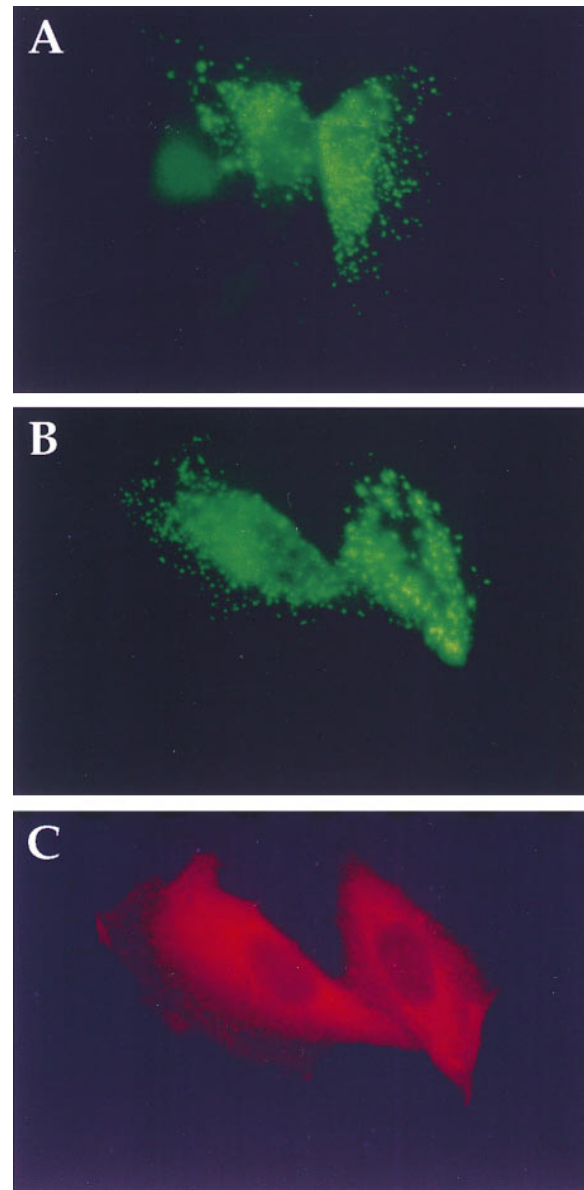ace when endocytosis is blocked as seen in Figs. 7 and 9, and the apparent backup of molecules in the secretory pathway when TGN sorting is blocked by hubs, as seen in Fig. 3 *C* for the CI-M6PR and in Fig. 8 *D* for HLA–DM.

It has not yet been possible to establish permanent transfectants with the hub construct under an inducible promoter, likely due to a long-term toxic effect of even low levels of hub expression from leaky promoters. Analy-
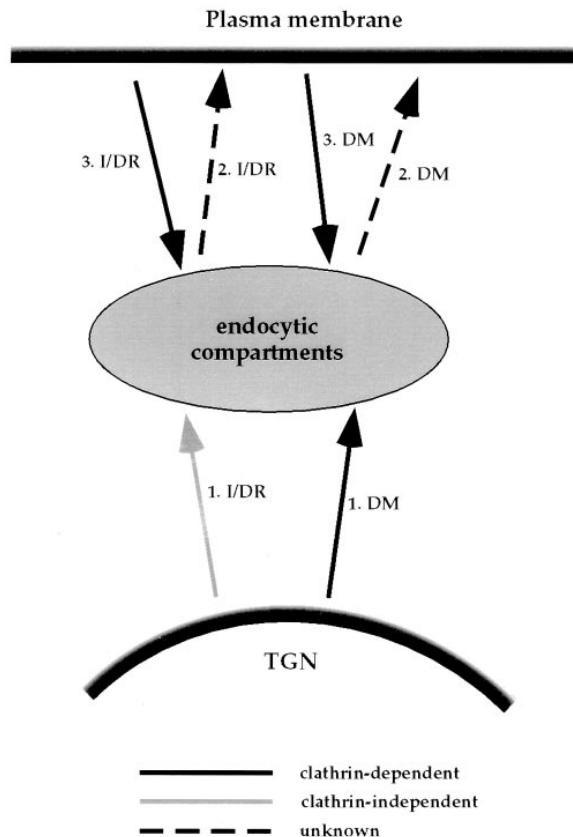
Figure 11. The role of clathrin and routes of transport in targeting I chain–class II MHC complexes and HLA–DM to the endocytic pathway. Analysis of DM and I chain–class II targeting, in the presence of hub molecules that inhibit CCV–mediated transport, suggests that both DM and I chain–DR are directly transported to the endocytic pathway from the TGN, without initial routing via the PM. In the TGN, DM is sorted in CCV and I chain–DR by a clathrin-independent mechanism. Our results also indicate that both complexes can subsequently appear on the cell surface and are then internalized by CCV, hence we propose they can follow routes *2* and *3*, as shown. Solid arrows indicate pathways determined by the data reported, with clathrin-dependence shown in black and clathrin-independent pathways depicted in gray. Dashed arrows indicate speculated pathways where the role of clathrin is unknown. The detailed rationale behind this transport scheme is presented in the Discussion.

sis of the distribution of existing pools of molecules after hub transfection showed varying degrees of effects on steady-state distribution. For example, when the distribution of TfnR was analyzed in hub-expressing cells, we observed a variable, but notable, reduction of TfnR on the cell surface (data not shown). This correlates with previous observations that the bulk of cellular transferrin receptor is endosomal (Futter et al., 1995). It also suggests that hubs are probably inhibiting clathrin-dependent recycling (Stoorvogel et al., 1996), as well as clathrin-dependent endocytosis. The distribution of endogenous CI-M6PR was also affected to varying degrees in different cells, after 24 h of hub expression.

For a more definitive application of hub expression to studying the role of clathrin in the sorting of particular molecules, we took the approach of cotransfecting cells with novel molecules to be analyzed, so that these molecules were expressed either during hub accumulation or, when the test molecule was under the control of an inducible promoter, it was expressed after hub accumulation. This approach made it possible to analyze clearly the role of clathrin in the intracellular targeting of test molecules. Cotransfection of hubs with chimeric molecules bearing cytoplasmic domains with predicted CCV recognition signals attached to the Tac extracellular domain of the IL-2 receptor α-chain, revealed that hub expression blocked the internalization of the chimeric molecules with either the tyrosine-containing signal of the H2M β chain or the dileucine signal of the CD3 γ chain. This is, in fact, the first functional demonstration of a role for clathrin in internalizing molecules with this particular YXXØ-tyrosine signal. It is also the first confirmation of the role of CCVs in uptake of molecules with the dileucine signal, which has been implicated in endocytosis. In sum, these experiments characterizing the clathrin hub expression system revealed its utility in studying the role of clathrin-dependent processes in mammalian cells.

The Role of CCVs in the Class II MHC–Molecule Antigen Presentation Pathway

Class II MHC molecules acquire peptides for presentation to helper T cells when they intersect the endocytic pathway after biosynthesis. Targeting of class II molecules to the endocytic pathway and their peptide loading depends on two important accessory molecules that have predicted CCV association signals in their cytoplasmic domains, the I chain, and HLA–DM. By analyzing the effects of hub expression on the endocytic delivery of these molecules, we can establish where CCVs are involved in sorting molecules with dileucine- or tyrosine-based CCV association signals, and where such sorting is critical for antigen presentation.

A dileucine signal in the I chain is responsible for targeting associated class II molecules to the endocytic pathway, where the I chain is degraded and its residual CLIP peptide, which occupies the class II molecule's peptide binding site, is replaced by an antigenic peptide. How the I chain–class II complex is targeted to the endocytic pathway and whether it is first transported to the PM and then internalized has not yet been established. It is clear that a substantial proportion of newly synthesized class II–I chain complexes appears on the cell surface before their processing in the endocytic pathway (Roche et al., 1993; Odorizzi et al., 1994; Wang et al., 1997) and morphological evidence indicates intact I chain can be detected in early, as well as late, endocytic compartments (Guagliardi et al., 1990; Castellino and Germain, 1995). Clathrin does not colocalize with I chain–class II complexes in the TGN (Glickman et al., 1996) but overexpression of I chain–class II complexes results in an increase in AP1 binding sites on internal cellular membranes (Salamero et al., 1996). Taken together, these results suggest that clathrin may be involved in uptake and targeting of the I chain–class II complex and could also play a role at some stage in its intracellular sorting.

A tyrosine-based signal in the β chains of HLA–DM or the mouse equivalent H2Mb (Lindstedt et al., 1995; Marks

et al., 1995; Ohno et al., 1995; Copier et al., 1996) is required for targeting these molecules to lysosome-like endocytic compartments where they interact with class II molecules (Amigorena et al., 1994; Tulp et al., 1994; West et al., 1994). DM serves as a catalyst to antigen presentation by stabilizing the empty form of class II molecules (Denzin et al., 1996; Kropshofer et al., 1997), thereby promoting the exchange of the CLIP fragment of I chain for peptides derived from exogenous internalized proteins (Denzin and Cresswell, 1995; Sherman et al., 1995; Sloan et al., 1995; Weber et al., 1996). Yeast two-hybrid interaction studies suggest a possible role for CCVs in sorting DM to the endocytic pathway at both the PM and the TGN. However, under steady-state conditions, DM cannot be detected on the surface of cells in which it is expressed (Sanderson et al., 1994).

The distribution of both I chain–class II and DM complexes is altered by hub expression, making it possible to define the steps where CCVs play a role in targeting these molecules to the endocytic pathway and the routes by which they are transported (Fig. 11). Expression of I chain–class II complexes or HLA–DM heterodimers in the presence of clathrin hubs resulted in accumulation of both complexes on the cell surface. In the absence of hubs, both complexes were undetectable on the cell surface, confirming that they are preferentially targeted to the endocytic pathway, and that their targeting mechanism was not saturated at these levels of expression. These results indicate that at some point in their transport, both I chain–class II complexes and HLA–DM access the cell surface and are internalized in CCVs. When HLA–DM or I chain–class II complexes were expressed from an inducible promoter in cells that had already accumulated hubs, HLA–DM molecules were trapped in the secretory pathway and never reached endocytic compartments, whereas endocytic targeting of the I chain–class II complexes was unaffected by hubs. Thus, CCVs are required for DM sorting in the TGN but sorting of I chain–class II complexes is clathrin independent. In addition, when HLA–DM was expressed after hub accumulation, none appeared on the cell surface and when I chain–class II complexes were expressed under the same conditions, only low levels were detectable on the cell surface. This implies both complexes are primarily targeted to the endocytic pathway before surface expression, otherwise they would have accumulated on the cell surface, since hubs block their endocytosis.

Since sorting of DM and I chain–class II complexes to the endocytic pathway has been previously shown to depend on dileucine and tyrosine-based adaptor-binding motifs, this study also identifies the pathways where these motifs function to regulate CCV association. Our findings suggest that both motifs are sorted in CCVs at the PM, but that in targeting molecules from the TGN to the endocytic pathway, CCVs transport molecules with the tyrosine-based motif, and sorting of molecules with a dileucine motif is clathrin independent. AP1 is the likely adaptor candidate for sorting DM into CCVs at this stage. It will be interesting to see whether the AP3 adaptor, which plays a role in lysosomal targeting (Cowles et al., 1997) but is apparently clathrin independent (Dell'Angelica et al., 1997b; Simpson et al., 1997) might be involved in targeting I chain to the endocytic pathway from the TGN. Whether both

DM and I chain are initially delivered from the TGN to the same endocytic compartments is not possible to determine from our data because we can only observe their final distribution. We speculate that they may have distinct endocytic destinations, with I chain–class II complexes being delivered to earlier compartments than DM. However, our data do indicate that both molecules can be released to the PM after their initial delivery to the endocytic pathway. For I chain, we suggest its export to the PM from the endocytic pathway could be a clathrin-dependent step, such as recycling from an endosome, because its PM delivery seems to be inhibited in hub-expressing cells, even though the I chain still accesses the endocytic pathway. Such a pathway would be analogous to the route of cell surface expression that has been proposed for the TfnR (Futter et al., 1995). When I chain is expressed at high levels, recruitment of AP1 to intracellular membranes is observed, suggesting that AP1 could be the functional adaptor for export of I chain to the cell surface (Salamero et al., 1996). The mechanism by which DM is released to the PM cannot be predicted from our data, but one possibility is that DM routinely escapes with peptide-loaded class II to the cell surface, and that these molecules dissociate only when they leave the acidic environment of the loading compartment. Thus, endocytosis of DM might represent a critical recapture step. Controlling the differential interaction of DM with class II MHC molecules in various cellular environments is fundamental to antigen presentation. Here, we demonstrate that CCVs play a critical role in coordinating this molecular interplay by recognizing different sorting signals at different stages of intracellular transport.

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