Identification of cytoskeleton-associated proteins in isolated rat liver endosomes

Albert POL, David ORTEGA and Carlos ENRICH[1]

Departamento de Biologia Celular, Facultad de Medicina, Universidad de Barcelona, Casanova 143, 08036 Barcelona, Spain

The polypeptides of three highly purified endosomal fractions isolated from the livers of oestradiol-treated rats were analysed by Western blotting, and the amount and distribution of intrinsic and cytoskeletal-associated proteins were quantified and studied. The 'late' endosomes [multivesicular bodies (MVBs)] had the lowest content of cytoskeletal-associated proteins, the most significant being the presence of 25% of the total dynein found in endosomes. The 'early' endosome [compartment of uncoupling receptors and ligands (CURL)] fraction contained kinesin (40% of the total in endosomes), dynein (23%), actin (15%) and tubulin (10%). The receptor-recycling compartment (RRC), also demonstrated to be involved in transcytosis, contained the largest number and enrichment of cytoskeletal proteins: actin (84% of the total in endosomes), α-actinin (90%), dynein (52%), tubulin (91%) and kinesin (45%). We also analysed and compared the presence of different endosomal markers such as Rab4, Rab5 and cellubrevin (vesicle soluble NSF attachment protein receptor) in CURL (41%, 15% and 60%) and in RRC (44%, 75% and 30% respectively). Finally, the expression of annexins I, II, IV and VI was studied: annexin I was equally distributed between MVBs and CURL; annexin II was highly enriched in RRC (95%), annexin IV was equally distributed between CURL and RRC, and annexin VI was enriched in CURL (57%). The results indicate that isolated rat liver endosomes contain all the required molecular machinery for the achievement of their role in intracellular trafficking.

INTRODUCTION

Endosomes are complex heterogeneous organelles within the eukaryotic cells involved in the transport and sorting of a great variety of molecules, including membrane receptors, ligands, metobolites and other extracellular macromolecules internalized during endocytosis.

Although arising from different ports of entry (clathrin-coated and non-clathrin-coated pits), most internalized molecules converge on the 'early' endosomes where the dissociation of receptors and ligands and sorting occur. Then transport to lysosomes through the 'late' endosomes, and recycling and transcytosis (in epithelial cells), is mediated by a series of protein mediators (of vesicle formation) – docking and fusion – and is a dynamic process that requires co-ordinated interactions between endosomes and cytoskeleton [1–3].

The cytoskeleton has an important role in the transport of endosomes. Treatment of cells with cytoskeletal inhibitors such as nocodazole or colchicine inhibits different pathways of transport (delivery to lysosomes or transcytosis) [3]. It is becoming clearer that actin cytoskeleton is also very important for the early steps in endosome motility and distribution, and that GTP-binding proteins (Rho subfamily) mediate the link between the endosomes and the cytoskeleton [4].

We recently performed a comprehensive analysis, by two-dimentional PAGE, of the major receptors and ligands that follow the main intracellular routes in the hepatocytes [5]. Here we analyse the composition of cytoskeletal-associated proteins in the three different morphological and kinetically isolated endosomal fractions from rat liver: the compartment of uncoupling receptors and ligands (CURL), the multivesicular bodies (MVBs) and the receptor-recycling compartment (RRC).

Cytoskeletal proteins such as actin, tubulin and α-actinin and the motor proteins dynein and kinesin were found to be unevenly distributed between the three endosomal fractions. The presence and the distribution of annexins I, II, IV and VI were also studied. The annexins are the most abundant proteins that bind reversibly to phospholipids at micromolar Ca^{2+} ion concentrations (Ca^{2+}-dependent phospholipid-binding proteins) [6–8]. They constitute a significant proportion of total cellular protein (approx. 1%) and have been highly conserved through evolution [9,10]. Several studies have reported a variety of cellular functions and it is now becoming clear that annexins are also involved in several steps of the endocytic pathway and in connection with the cytoskeleton [11–16].

It was also demonstrated that cytosolic factors are involved in vesicle/tubule formation and in regulating intracellular trafficking. In fact, calmodulin and calmodulin-binding proteins have been demonstrated to be important regulators of these processes [17–21]. Using the potent calmodulin antagonist W13, we demonstrated that calmodulin activity is required for transcytosis and the recycling of endocytosed transferrin receptor to the basolateral plasma membrane in polarized Madin–Darby canine kidney (MDCK) cells [21]. Thus calmodulin and calmodulin-binding proteins might function as modulators of endosome–cytoskeleton interactions. A number of actin-binding proteins that govern different steps of polymerization and organization of actin network are also calmodulin-binding proteins: α-actinin, caldesmon, α-fodrin, myosin I, adducin and myristoylated alanine-rich C kinase substrate are examples of such an integrated complex network.

In this study we demonstrate that isolated rat liver endosomes contain all the necessary molecular machinery to provide the expected interactions and movement throughout the different

Abbreviations used: CURL, compartment of uncoupling receptors and ligands; ECL, enhanced chemiluminescence; LDL, low-density lipoprotein; MDCK, Madin–Darby canine kidney; MVB, multivesicular body; RRC, receptor-recycling compartment; TGN, *trans*-Golgi network.

[1] To whom correspondence should be addressed.

stations of the hepatic endocytic compartment. Their distribution between early endosomes and the recycling/transcytotic compartment is in agreement with the expected dynamic functioning.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats weighing 200–250 g were kept under a controlled lighting schedule with a 12 h dark period. All animals received humane care in compliance with institutional guidelines. Food and water were available *ad libitum*.

Before the isolation of endosomes, animals were treated with 17-α-ethynyl oestradiol (1 mg/ml in propylene glycol) for 3 days to induce the expression of low-density lipoprotein (LDL) receptors [22].

Antibodies

Table 1 shows all the antibodies used and their sources.

Isolation of endosomes and plasma membrane fractions from rat liver

After 3 days of treatment with 17-α-ethynyl oestradiol, rats were anaesthetized with diethyl ether, and human LDL [23] (5 mg of protein) was injected into the femoral vein. The method used for the isolation of three endosomal fractions from rat liver has been described in detail by Belcher et al. [24] and Jäckle et al. [25] and also briefly in our previous paper [5].

Electron microscopy

Pellets from isolated endosome fractions were fixed in 2.5% (w/v) glutaraldehyde/1% (w/v) paraformaldehyde/0.1 M cacodylate buffer (pH 7.4) and prepared for electron microscopy [26].

Gel electrophoresis and Western blotting

Two-dimensional PAGE was performed by the slightly modified method of O'Farrell et al. [27] described in detail by Enrich et al. [28] with a Miniprotean II 2-D cell from Bio-Rad Laboratories. SDS/PAGE of proteins (2 μg per channel) was performed in 10% (w/v) polyacrylamide as described by Laemmli [29]. Polypeptides were then silver-stained using the method of Blum et al. [30] or transferred electrophoretically at 70 V for 90 min at 4 °C to Immobilon-P transfer membranes (Millipore). Antigens were identified by using different antibodies (see Table 1) diluted in PBS containing 0.5% powdered skimmed milk; the reaction product was detected by the enhanced chemiluminescence (ECL) system (Amersham). For the detection of calmodulin the SDS/PAGE sample buffer contained 2 mM EGTA.

Image analysis of Western blots and band quantification were performed with a Bio-Image system. The intensities shown in Table 2 were calculated densitometrically with the Bio-Image (Millipore) program that reads and calculates the band intensity and the band area. The results are expressed as percentages of every band (one-dimensional gels) compared with the total intensity (the addition of intensities of the three fractions MVBs, CURL and RRC); the intensity of MVB, for example, is given as the intensity of MVB divided by total intensity. Only bands in the same gel were compared, but the final percentage shown corresponds to the mean of at least three different experiments.

To test the validity of the quantitative analysis of Western blots by densitometry, different amounts of calmodulin as standard antigen, isolated by the method described by Guerini et al. [31], were subjected to SDS/PAGE [12% (w/v) gel] and quantified after ECL Western blot and exposure to Hyperfilm (Amersham, Little Chalfont, Bucks, U.K.) (Figure 1).

For two-dimensional gel protein identification, a gel for silver staining and a gel for Western blot were always run in parallel. The protein content of the samples was measured by the method of Bradford [32] with BSA as standard.

RESULTS

Recently, using highly purified endosome fractions from rat liver, we identified the major receptors, ligands and the proteins secreted into the bile [5]. Here, using the same endocytic fractions and a set of specific antibodies (Table 1), we study the presence and distribution of structural proteins. The following groups of proteins were considered: (1) the endosomal markers Rab4, Rab5 and cellubrevin; (2) annexins I, II, IV and VI; (3) α-actinin, α-fodrin and caldesmon (actin and actin-binding proteins); (4) tubulin and the 'motor' proteins dynein and kinesin; and (5) calmodulin and calmodulin-dependent protein kinase II.

Table 1 Antibodies used in this work

Target	Host	Specificity	Source	Code
Rab5	Rabbit	Polyclonal, affinity-purified	Santa Cruz	sc-309
Rab4	Rabbit	Polyclonal, affinity-purified	Santa Cruz	sc-312
Cellubrevin	Rabbit	Polyclonal, affinity-purified	J. Blasi (Barcelona)	–
Annexin I	Mouse	Monoclonal	ICN	69-154
Annexin II	Mouse	Monoclonal	Transduction Laboratories	A14020
Annexin IV	Rabbit	Polyclonal, affinity-purified	V. Gerke (Göttingen)	–
Annexin VI	Rabbit	Polyclonal, affinity-purified	S. Jäckle (Hamburg)	–
Actin	Mouse	Monoclonal	ICN	69-100
α-Actinin	Mouse	Monoclonal	Sigma	A-5044
α-Fodrin	Mouse	Monoclonal	ICN	69-327
Caldesmon	Mouse	Monoclonal	Sigma	C-0297
β-Tubulin	Mouse	Monoclonal	Boehringer Mannheim	1111 876
Kinesin	Mouse	Monoclonal	Sigma	K-1005
Dynein intermediate chain	Mouse	Monoclonal	Sigma	D-5167
Calmodulin	Mouse	Monoclonal	UBI	05-173
Calmodulin-dependent protein kinase II	Mouse	Monoclonal	Boehringer Mannheim	1481 703
Ca^{2+}-ATPase	Mouse	Monoclonal	Sigma	A-7952

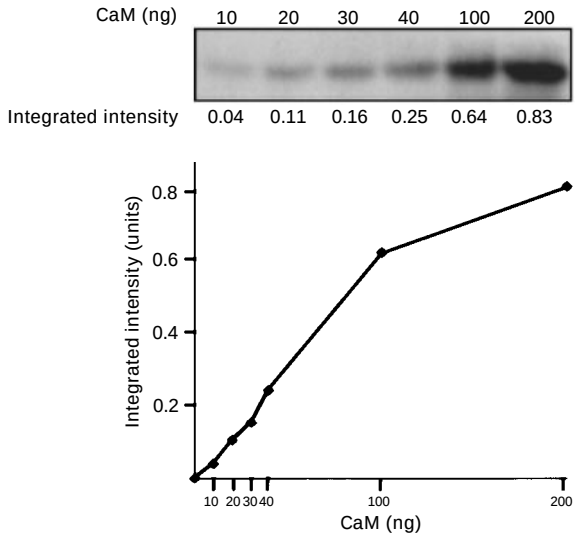

Figure 1 Quantitative analysis of calmodulin by ECL Western blot

Abbreviation: CaM, calmodulin.

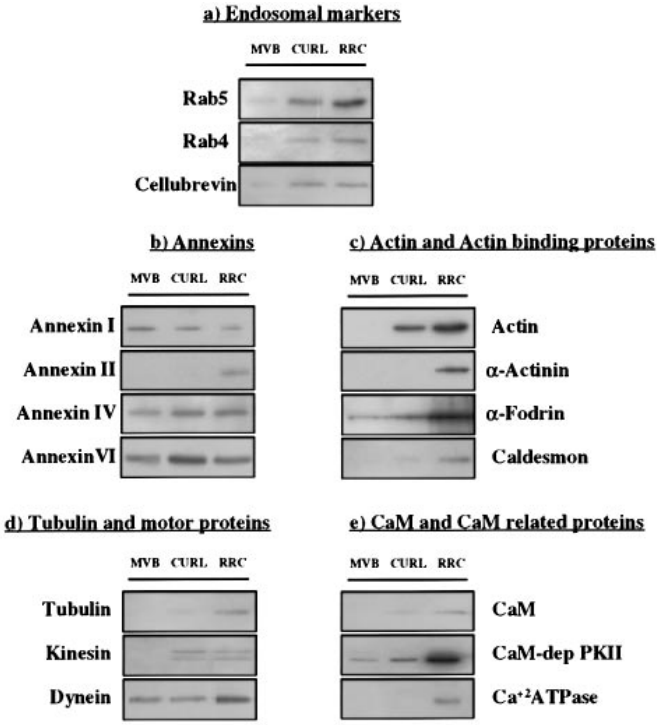

Figure 2 Western blotting analysis of rat liver endosomal proteins

Protein (2 μg) from the isolated endosome fractions was loaded and bands were quantified after ECL staining (see legend to Table 2). Abbreviation: CaM, calmodulin.

Table 2 Densitometric quantification of Western blots

Image analysis of Western blots and band quantification were performed with a Bio-Image (Millipore) program imaging system [5] that reads and calculates the band intensity and the band area. The results are expressed as a percentage of every band (one-dimensional gels) compared with the total intensity (the addition of intensities of the three fractions MVB, CURL and RRC), as described in the Experimental section. Only the bands of the same gel were compared, but the final percentages corresponded to the means of at least three different experiments. Abbreviation: n.d., not detected.

	Protein distribution (%)		
Protein	MVB	CURL	RRC
Endosomal markers			
Rab5	10	15	75
Rab4	15	41	44
Cellubrevin	15	45	40
Annexins			
Annexin I	38	43	19
Annexin II	n.d.	5	95
Annexin IV	12	43	45
Annexin VI	17	57	26
Actin and actin-binding proteins			
Actin	1	15	84
α-Actinin	n.d.	9	91
α-Fodrin	9	26	65
Caldesmon	n.d.	4	96
Tubulin and motor proteins			
β-Tubulin	n.d.	10	90
Kinesin	5	40	45
Dynein	25	23	52
Calmodulin and calmodulin-related proteins			
Calmodulin	–	35	65
Calmodulin-dependent protein kinase II	8	19	73
Ca^{2+}-ATPase	nd	4	96

CURL: the early 'sorting' endocytic compartment

Kinetically defined as the 'early' endosomal fraction from rat liver, CURL has been found enriched in LDL, transferrin and polymeric IgA at short times (7.5 min) after internalization [5,19,24,25,33]. Here we show that the early endocytic 'markers' Rab4 and Rab5 are located in CURL. Indeed, Rab4 was demonstrated to be an early endosomal GTPase that controls the function or formation of the endosomes involved in recycling [34] and Rab5 is required for early endosome fusion *in vitro* and regulates transport between the plasma membrane and early endosomes [35].

In addition, the presence of cellubrevin, a vesicle-soluble NSF attachment protein receptor protein, in the 'early' and recycling compartments was demonstrated in the present study with an affinity-purified antibody. To our knowledge this is the first time that cellubrevin has been identified in isolated endosomal fractions from rat liver; it is similarly distributed between 'early' (CURL, 45%) and recycling (RRC, 40%) endosomes in agreement with the studies of Daro et al. [36] in non-polarized cells. Figure 2 and Table 2 show CURL and RRC containing similar amount of Rab4 and cellubrevin, whereas Rab5 was present in greater quantities in RRC (see below).

Furthermore the presence of annexins I, IV and VI was observed (43%, 43% and 57% respectively). Finally, 40% of the total kinesin found in endosomes was located in CURL.

Figure 3 shows silver-stained two-dimensional gels and the corresponding Western blots of gels in which cellubrevin and annexin VI were identified.

MVB: 'late' endosomes

MVBs have been demonstrated to be enriched in proteins in the pathway of degradation such as epidermal growth protein, α_2-macroglobulin and haptoglobin or in mannose 6-phosphate receptor [5,33]. This is the most homogeneous fraction (from the morphological point of view) and MVBs are considered to be pre-

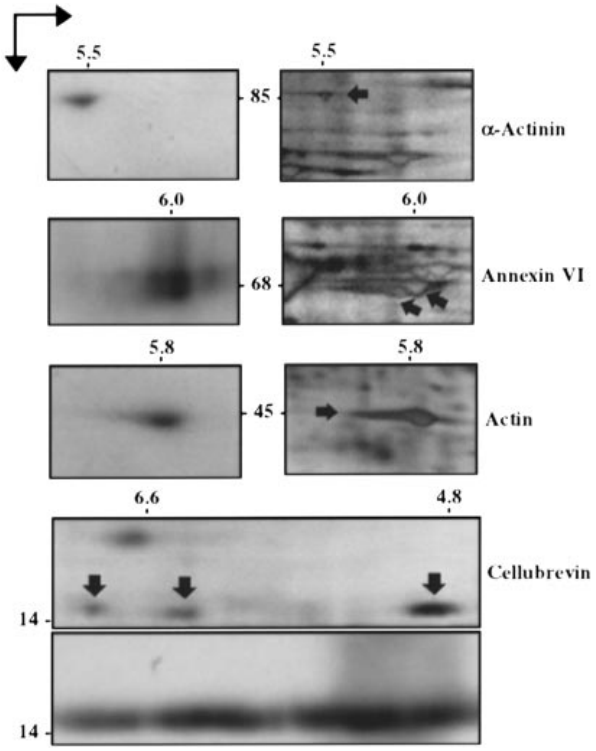

Figure 3 Identification of endosomal proteins by two-dimensional gel electrophoresis

Silver-stained two-dimensional gels (three right panels and top panel of bottom pair) and corresponding Western blot gels (three left panels and bottom panel of bottom pair) show the positions of the indicated proteins: annexin VI, actin, α-actinin and cellubrevin. Identification of proteins was performed by running parallel two-dimensional gels and subsequent Western blotting analysis.

lysosomal structures. Figure 2 and Table 2 show that annexin I (38 %) is the only protein in which the MVBs are enriched.

This fraction is poor in cytoskeletal or cytoskeletal-associated proteins and only dynein was present (25 %). This is in agreement with the observation made by Oda et al. [37] that cytoplasmic dynein was tightly bound to a population of ligand-containing vesicles in the lysosomal pathway.

RRC: the recycling/transcytotic compartment

RRC contains 84 % of the actin and 90 % of the tubulin in endosomes but also 91 % of the α-actinin, 45 % of the kinesin and 52 % of the dynein (Figure 2 and Table 2). Actin and α-actinin are major proteins in this fraction and were identified in two-dimensional gels (Figure 3).

Two calmodulin-binding and actin-binding proteins, caldesmon (96 %) and fodrin (65 %), were also detected in this RRC endocytic fraction (Figure 2 and Table 2) and two annexins, annexin II (95 %) and annexin IV (43 %). Finally, RRC contains Rab4 (44 %), Rab5 (75 %) cellubrevin (40 %), calmodulin (65 %) and calmodulin-dependent protein kinase II (73 %).

DISCUSSION

Vesicular transport between organelles and membranes is controlled by the cytoskeleton. In non-polarized cells, actin filaments are involved in the uptake of ligands, in their delivery to the degradative compartment, and facilitate the recycling of transferrin from the perinuclear region to the plasma membrane. Microtubules are required for the maintenance of distribution of the late endosomal compartment [38]. Interactions between plasma membrane and cytoskeleton are governed by RhoD protein, actin and Ca^{2+}-binding proteins (e.g. annexins) that control Ca^{2+} homoeostasis and in turn regulate the cytoskeleton dynamics as well as organelle fusion.

One major problem in the integrated understanding of the intracellular trafficking is that the biochemistry of different endosomal compartments is largely unresolved. Using highly purified endocytic fractions isolated from rat liver we have undertaken a comprehensive molecular analysis of endosomal constituents and demonstrate that the three endosomal fractions are endowed with the required molecular machinery to allow the transport events in the cell.

Organization of the hepatic endocytic compartment

CURL, the 'central sorting station'

Figure 4 shows a postulated sequence of events leading to the formation of MVB and RRC fractions from a proposed 'central sorting station' (CURL). Three different types of structure can be observed attached to CURL. First, there are vesicular structures (square) with a diameter between 45 and 60 nm; these vesicles, also observed in RRC, might represent early RRC structures and can therefore be considered as fusion events at the CURL (square). A second population of vesicles 100–150 nm in diameter that differ in their content (more electron-dense and lipoprotein-like in content) (white circle in Figure 4 and high magnification in the top left panel) are considered as fission (budding) events towards the 'late' pathways. Thirdly, tubular extensions from the vesicular body (dotted white circle in Figure 4) could be the origin of tubules found in RRC and described by Geuze et al. [39] as structures involved in the recycling of receptors. In addition, the ring-like structures observed in the RRC fractions could be formed by following the sequence depicted in the bottom panels of Figure 4, starting from tubular extensions and progressing through the arc-shaped structures with terminal swellings (lower panels, from dotted white circle).

The right panel of Figure 4 shows an MVB; two possibilities have been considered for its formation. One is through a maturation process, where the vesicular body of CURL will mature and finally become an MVB; the second is by fusion of vesicles originating in CURL. Electron-dense vesicles (white circle, in Figure 4) budding from CURL will fuse and subsequently form a late endosome (MVB). One has to consider that material from the *trans*-Golgi network will enlarge and also contribute to the formation of MVBs (the diameter of a CURL is approx. 300 nm, whereas the diameter of a mature MVB is approx. 500 nm).

For 'early-sorting' endosomes, the situations in non-polarized and polarized cells are different. In hepatocytes, as in MDCK or CaCo-2 cells, different populations of endosomes, namely basolateral and apical endosomes, have been described, which adds a further complication to the general understanding of the organization and dynamics of the endocytic compartment. Annexin VI defines an apical CURL compartment (D. Ortega and C. Enrich, unpublished work). Whereas cellubrevin and Rab4 might control the sorting events, annexins I, IV and VI might be involved in the regulation of Ca^{2+} homoeostasis that in turn will implicate endosome fusion. Thus CURL is biochemically and structurally an intermediate compartment between early RRC (Rab4 and

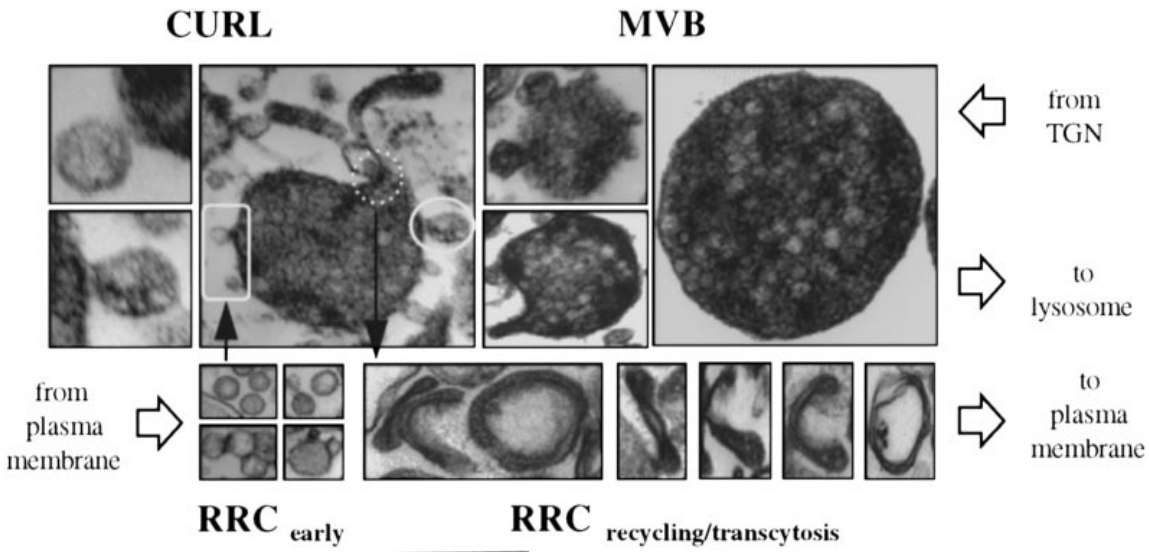

Figure 4 Proposed model for the origin of RRC and MVBs from CURL in isolated rat liver endocytic fractions

Abbreviation: TGN, *trans*-Golgi network.

cellubrevin) and late MVB (epidermal growth factor receptor and annexin I) events.

Although hepatocytes and MDCK cells share several general aspects of intracellular trafficking, the generation of cell polarity, and especially the transport of newly synthesized apical membrane proteins, is different [40].

RRC, the dynamic endocytic structures

RRC contains structures of the recycling and transcytotic compartments of hepatocyte. Recycling is a default process; experimental evidence that recycling of transmembrane proteins is not dependent on a signal sequence comes from studies with fluorescent lipid analogues [41]. In epithelial polarized cells such as hepatocytes, three different recycling pathways have to be considered: (1) peripheral recycling through early endosomes and recycling structures, in the subsinusoidal region (for example the LDL receptor), (2) deep (late) recycling down to the perinuclear (pericanalicular) region (for example the ASGP receptor) and (3) less known, but plausible, is the recycling of apical membrane proteins throughout the apical recycling compartment (in MDCK cells [42]). Rab4 is one of the few proteins that have been directly involved in the control of recycling in non-polarized cells [34]. Also we have demonstrated that recycling is a calmodulin-dependent process [21].

Recycling and transcytotic events take place in regions of the cytoplasm where cytoskeleton is highly organized (i.e. the cortical cytoskeleton and the actin-rich cytoskeleton surrounding the bile canaliculus). RRC structures must interact and pass through this cortex. Several proteins have now been identified as having a regulatory role in these 'early' peripheral steps (indicating an interaction between the plasma membrane and the cytoskeleton): RhoD protein [4], gelsolin, Myr 4 and calmodulin [43].

In this study we demonstrate that RRC is the endosomal fraction with the highest amount of calmodulin, cytoskeleton and cytoskeleton-associated proteins. Although the analysis by two-dimensional PAGE revealed a great similarity with CURL [5], this fraction showed a different quantitative and qualitative pattern of cytoskeletal and cytoskeletal-associated proteins.

MVB, the static pre-lysosomal compartment

In the hepatocyte, MVBs are located mainly in the pericanalicular region. This region is shared by apical 'early' endosomes, by transcytotic structures and, as discussed previously, by potential apical recycling endosomes. MVBs in non-polarized cells are concentrated in the central zone, the minus end, guided by dynein to the microtubule-organizing centre. Dynein (25%) is the only cytoskeleton-related protein in which this late endocytic compartment is enriched. Oda et al. [37] have shown that dynein, but not kinesin, is involved in the movement of endocytic vesicles along the microtubules during the sorting of ligand from receptor.

Finally, it was demonstrated that annexin I was involved in the inward vesiculation of MVBs induced by the phosphorylation of epidermal growth factor receptor and in the formation of these structures [15]. In the present work 38% of the total annexin I was found in MVBs.

Thus this paper contributes two main additions to the general knowledge of hepatic endocytic compartment. First, regarding the identification of cytoskeleton-associated proteins in highly purified endocytic fractions (molecular characterization of endosomes), we demonstrate that isolated endosomal fractions, despite undergoing a subcellular fractionation process, not only show morphological preservation but also contain key components of molecular machinery for fusion and transport [44]. Secondly, we suggest a potential role for calmodulin and annexins as general modulators of dynamics in the endocytic compartment, and especially in the complex recycling compartment.

We thank Dr. Joan Blasi (Universitat de Barcelona, Barcelona, Spain) for the gift of affinity-purified anti-cellubrevin antibody; the personnel of 'Servies Cientific i Tècnics de la Universitat de Barcelona', for the electron microscope facilities; and Dr. S. Jäckle (University of Hamburg, Hamburg, Germany) for providing human LDL. This work was supported by FISS 95/0479. A. P. is a recipient of a FISS grant.

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Received 27 February 1997/23 May 1997; accepted 3 July 1997