Biochem. J. (1988) 255, 999–1005 (Printed in Great Britain)

A 115 kDa calmodulin-binding protein is located in rat liver endosome fractions

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The distribution of calmodulin-binding polypeptides in various rat liver subcellular fractions was investigated. Plasma-membrane, endosome, Golgi and lysosome fractions were prepared by established procedures. The calmodulin-binding polypeptides present in the subcellular fractions were identified by using an overlay technique after transfer from gels to nitrocellulose sheets. Distinctive populations of calmodulin-binding polypeptides were present in all the fractions examined except lysosomes. A major 115 kDa calmodulin-binding polypeptide of pI 4.3 was located to the endosome subfractions, and it emerges as a candidate endosome-specific protein. Partitioning of endosome fractions between aqueous and Triton X-114 phases indicated that the calmodulin-binding polypeptide was hydrophobic. Major calmodulin-binding polypeptides of 140 and 240 kDa and minor polypeptides of 40–60 kDa were present in plasma membranes. The distribution of calmodulin in the various endosome and plasma-membrane fractions was also analysed, and the results indicated that the amounts were high compared with those in the cytosol.

INTRODUCTION

The membranes comprising the endocytic compartment and the cell surface of animal cells are functionally inter-related (Wileman *et al.*, 1985). Analyses of membrane vesicles originating from the endocytic compartment (endosomes) have shown that, although these are derived from a functionally distinctive intracellular organelle, they do resemble the plasma membrane in some respects. Endosomes contain plasma-membrane receptors and ectoenzymes, e.g. 5′-nucleotidase, and this is usually interpreted as reflecting the internalization and recycling of proteins between these two membrane networks (Quintart *et al.*, 1983; Evans & Flint, 1985; Baenziger & Fiete, 1986; Mueller & Hubbard, 1986; Mullock *et al.*, 1987; Belcher *et al.*, 1987). In contrast with those proteins that migrate between the plasma membrane and the membranes constituting the endocytic compartment, other proteins are restricted to one membrane compartment; for example, adenylate cyclase activity stimulated by neurotransmitters is mainly resident in plasma membranes (Hadjiivanova *et al.*, 1984). Since many independent functions are now credited to the endocytic compartment, including a role in the control of the uptake, pH-induced dissociation and sorting of ligand–receptor complexes, a search for proteins mainly resident in endosome membranes appears justified.

The isolation by subcellular fractionation and the characterization of endosome fractions has allowed comparisons of the major polypeptides to be made with those present in plasma membranes. These approaches have indicated some biochemical similarities in liver (Evans & Hardison, 1985; Baenziger & Fiete, 1986; Belcher *et al.*, 1987) and cultured cells (Beardmore *et al.*, 1987; Marsh *et al.*, 1987). In the present studies we have investigated the distribution of calmodulin-binding proteins in liver endosomes and compared these with the proteins present in plasma membrane and other liver subcellular fractions. Calmodulin is an ubiquitous Ca^{2+}-binding protein that regulates several Ca^{2+}-dependent processes (Adelstein, 1982). In the context of endocytosis, several Ca^{2+}–calmodulin-dependent protein kinases, including many that phosphorylate cytoskeletal proteins, have been described (Chou & Rebhun, 1986; Payne *et al.*, 1983). Calmodulin inhibits Ca^{2+}/phospholipid-dependent phosphorylation, suggesting a regulatory role in intracellular membrane transport (Albert *et al.*, 1984). These properties have prompted the present studies that have led to the identification of a major calmodulin-binding protein in liver endosome fractions.

EXPERIMENTAL

Materials

Calmodulin was purified from bovine brain by the method of Guerini *et al.* (1984) and iodinated with the Bolton and Hunter reagent (The Radiochemical Centre, Amersham, Bucks., U.K.). Ampholines were obtained from LKB, Stockholm, Sweden. Other materials were from Sigma or BDH.

Preparation of rat liver endosomes and other subcellular fractions

Endosomes were prepared from rat livers perfused with ice-cold 0.9% NaCl. Livers (60–70 g) were homogenized in 0.25 M-sucrose with a loose-fitting Dounce homogenizer, filtered (nylon gauze, 50–100 mesh), and re-homogenized with a tight-fitting Dounce homogenizer (clearance 0.07 mm). The homogenate was centrifuged at 3000 rev./min for 10 min (Sorvall SS-34 rotor) and the pellet washed twice in 0.25 M-sucrose by centrifugation under the same conditions. The combined supernatants were centrifuged at 33000 *g* for 8 min at speed in a Beckman type 30 rotor, and the final

supernatant (approx. 180 ml) was collected, with care not to disperse the sediment, and applied to 12 sucrose gradients [1 ml of 70% (w/v), 5 ml of 43%, and a continuous gradient formed from 7.5 ml of 15% and 7.5 ml of 40%]. After centrifugation (Beckman SW28 rotor) for $3\frac{1}{2}$ h at 100000 *g*, 40 0.8 ml fractions were collected and those within the density range 1.070–1.125 g/ml pooled. After dilution with water, the pooled fractions were loaded on to gradients constructed by mixing 6 ml of 27.6% (w/v) Nycodenz dissolved in 8% sucrose with 6 ml of 13.8% Nycodenz dissolved in water; these rested on cushions of 1 ml of 70% sucrose. After centrifugation for 16 h at 100000 *g* (Beckman SW28 rotor), gradients were unloaded as 30 0.5 ml fractions. 'Early' endosomes corresponded to fractions equilibrating in the Nycodenz gradient within the refractive-index range 1.365–1.375 (peak density of endocytosed ligands 2–4 min after uptake by liver was 1.115 g/ml), and 'late' endosomes corresponded to those within the refractive index range 1.355–1.364 (peak density of endocytosed ligands 10–15 min after uptake was 1.090 g/ml). A further endosome fraction (DR), shown to contain large amounts of insulin- and asialoglycoprotein-binding sites, was collected at the interface of the Nycodenz gradient and the sucrose cushion (Evans & Flint, 1985). Fractions were diluted with 10 mM-Tris/HCl, pH 7.4, pelleted by centrifugation at 100000 *g* for 30 min and stored suspended in 10 mM-Tris/HCl, pH 7.4, at −70 °C.

Liver plasma-membrane subfractions originating from the sinusoidal, lateral and canalicular domains were isolated and characterized as described by Wisher & Evans (1975). Lysosomes were isolated by the method of Wattiaux *et al.* (1978); acid phosphatase specific activity was increased 20-fold over that of the homogenate. A Golgi intermediate subfraction depleted of endosome contamination was isolated as described previously (Evans, 1985); the galactosyltransferase specific activity was increased 82-fold relative to the homogenate. Cytosol was obtained by homogenization of 0.5 g of liver (1:10, v/w) in 124 mM-borate/1 mM-EGTA. Homogenates were centrifuged at 105000 *g* for 1 h, and the supernatants obtained were heated for 5 min at 90 °C, rapidly cooled in an ice bath and re-centrifuged at 50000 *g* for 30 min (Soriano *et al.*, 1985). The resulting supernatants were stored at −40 °C to await calmodulin assays. The protein content of the fractions was determined as described by Lowry *et al.* (1951).

Metabolic labelling of endosomes

Two rats were injected via the tail vein with 5 mCi of [^{35}S]methionine (The Radiochemical Centre) in 2 ml of phosphate-buffered saline, pH 7.4 (Evans & Flint, 1985). After 3 h, the rats were killed, livers excised and endosome fractions prepared.

Two-phase extraction of membrane proteins

Endosomes were solubilized at 0 °C in 10 mM-Tris/HCl (pH 7.4)/150 mM-NaCl/2% Triton X-114 (precondensed) at 1 mg of protein/ml, and partitioning of proteins into aqueous and detergent phases was performed (Bordier, 1981). Proteins partitioning into the detergent phase were analysed by two-dimensional electrophoresis.

One- and two-dimensional gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out in 8%-acrylamide resolving gels (Laemmli, 1970). Two-dimensional gels were carried out as described by O'Farrell (1975), with the following modifications: endosomes and plasma membranes (200 μg) were solubilized in 2% SDS/0.5 mM-$MgCl_2$/50 mM-Tris/HCl, pH 6.8, by boiling (5 min) and the samples diluted with 2 vol. of freshly made 9.5 M-urea/2% Ampholines (pH 3.5–10 and 5–7) / 5% 2-mercaptoethanol / 8% Nonidet P-40. Isoelectrofocusing was carried out in tube gels at 300 V for 20 h, followed by 400 V for 1.5 h. The rod gels were then equilibrated for 2 h at room temperature in 0.063 M-Tris/HCl (pH 6.8)/2.3% SDS/5% 2-mercaptoethanol/10% glycerol and then electrophoresed in 8%-polyacrylamide gels. Gels were stained with Coomassie Blue. With [^{35}S]methionine-labelled membranes, gels were exposed for fluorography (Bonner & Laskey, 1974).

Calmodulin-overlay technique

Proteins were transferred to nitrocellulose sheets by electrophoresis at 30 V for 3 h (one-dimensional gels) or 20 V for 16 h (two-dimensional gels), and incubated overnight in phosphate-buffered saline, pH 7.4, containing 2% defatted milk at 4 °C. The sheets were incubated for 4 h at room temperature in phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin and ^{125}I-calmodulin and 0.5 mM-$CaCl_2$ or 2 mM-EGTA (potassium salt) (Bachs & Carafoli, 1987). Sheets were washed for 3 × 10 min in the same buffer but without calmodulin, and finally once in phosphate-buffered saline, pH 7.4. Dried nitrocellulose sheets were exposed to Kodak AR-5 films.

Measurement of calmodulin in subcellular fractions

Portions of endosome and plasma-membrane subfractions suspended in 0.25 M-sucrose were diluted in 3 vol. of 80 mM-Tris/HCl (pH 7.8)/80 mM-imidazole/5 mM-$MgCl_2$/0.2 mM-$CaCl_2$. Cell cytosol was analysed without dilution. Samples were heated to 95 °C for 3 min and rapidly cooled on ice. After centrifugation at 10000 *g* for 10 min, the calmodulin in the supernatant was measured by its activation of a calmodulin-deficient brain cyclic nucleotide phosphodiesterase (Sharma & Wang, 1978).

RESULTS

Polypeptide composition of endosomes and other subcellular fractions

The polypeptide compositions of the liver subcellular fractions were analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 1). The comparison by one-dimensional electrophoresis of the 'early', 'late' and receptor-enriched endosome fractions with sinusoidal, canalicular and lateral plasma-membrane fractions indicated that, although the overall polypeptide profiles were different, there were many polypeptides with similar electrophoretic mobilities. A more detailed comparison of an 'early' endosome fraction with blood-sinusoidal plasma membranes was carried out by two-dimensional electrophoresis (Fig. 2) and indicated that specific polypeptides could be assigned to one fraction or the

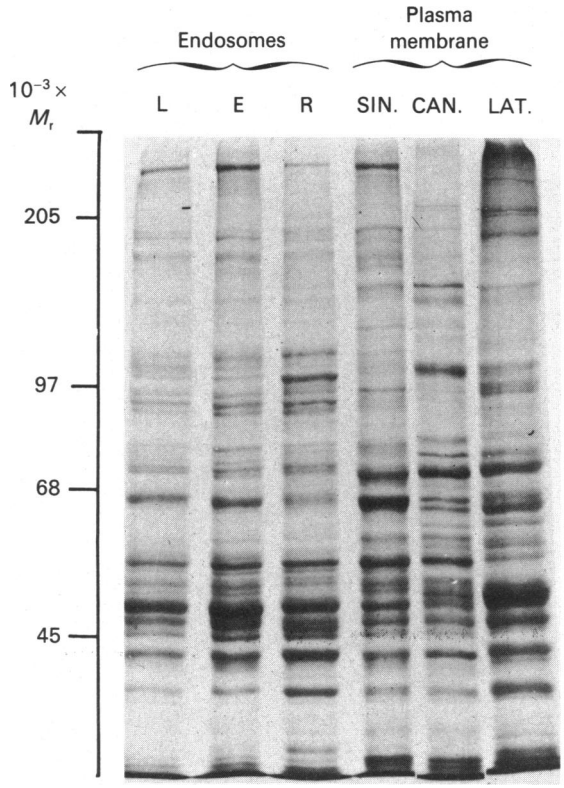

Fig. 1. SDS/polyacrylamide-gel electrophoresis of liver endosome and plasma-membrane subfractions

'Late' (L), 'early' (E) and receptor-enriched (R) endosome fractions collected from the Nycodenz density gradients and plasma-membrane subfractions originating mainly from the sinusoidal (SIN.), canalicular (CAN.) and lateral (LAT.) domains were prepared as described in the text. Equal amounts of protein (approx. 100 μg) were applied to each lane, and gels were stained with Coomassie Blue.

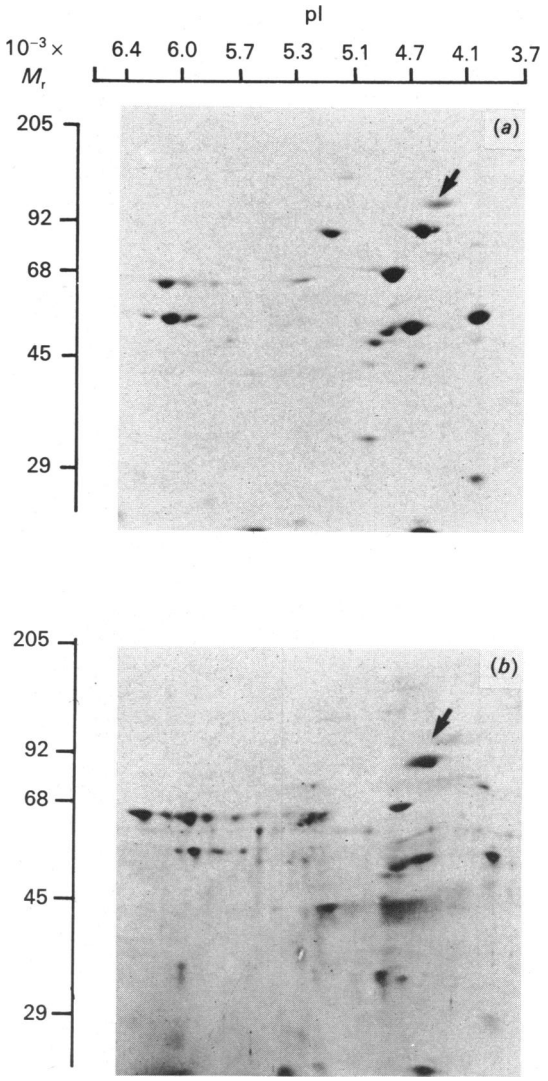

Fig. 2. Two-dimensional analysis of polypeptides of 'early' endosomes (*a*) and sinusoidal plasma membranes (*b*)

Fractions (200 μg of protein) were analysed by isoelectric focusing, followed by SDS/polyacrylamide-gel electrophoresis. Gels were stained with Coomassie Blue. The position of the 115 kDa polypeptide is indicated by the arrow.

other. [A 'late' endosome fraction yielded a similar profile (not shown).] Of especial note was a 115 kDa polypeptide of pI 4.3 present in endosomes, but only detected in low amounts in the sinusoidal plasma membranes. This polypeptide was also identified by fluorography of ^{35}S-labelled endosomal polypeptides resolved in two dimensions (Fig. 3*a*). When the endosomal proteins were partitioned between aqueous and Triton X-114 phases, a procedure in which a number of hydrophobic proteins enter the detergent-rich phase, leaving hydrophilic proteins in the detergent-poor phase (Bordier, 1981), it was apparent that the 115 kDa pI-4.3 polypeptide was recovered in the detergent phase (Fig. 3*b*), indicating its hydrophobic nature.

Calmodulin-binding polypeptides

By use of the calmodulin-overlay technique, the polypeptides that bound specifically ^{125}I-calmodulin were examined after transfer by electrophoresis from polyacrylamide gels to nitrocellulose sheets. Fig. 4 compares the calmodulin-binding polypeptides of several rat liver subcellular fractions. In the endosome subfractions, and especially in the 'early' and 'late' endosomes, there were two calmodulin-binding polypeptides, of 140 and 115 kDa; although the 140 kDa polypeptide was also prominent in plasma membranes and Golgi membranes, the 115 kDa polypeptide was present in very low amounts in the plasma-membrane subfractions and Golgi membranes, and it was absent from lysosomes. On the other hand, a 240 kDa polypeptide associated with all three plasma-membrane fractions and Golgi membranes was hardly detected in the endosome fractions. Plasma-membrane fractions contained a more extensive range of calmodulin-binding polypeptides, especially in the 40-60 kDa range. No calmodulin-binding polypeptides were identified in lysosomes. In the presence of EGTA, no calmodulin was bound by any of the fractions.

Analysis of the calmodulin-binding polypeptides by electrophoresis in two dimensions (Fig. 5) showed that the major calmodulin-binding polypeptide, of 115 kDa and pI 4.3, was present in 'early' and 'late' endosomes; a calmodulin-binding polypeptide of pI 4.3 was detected in lower amounts in the sinusoidal plasma-membrane fraction. Two major calmodulin-binding polypeptides,

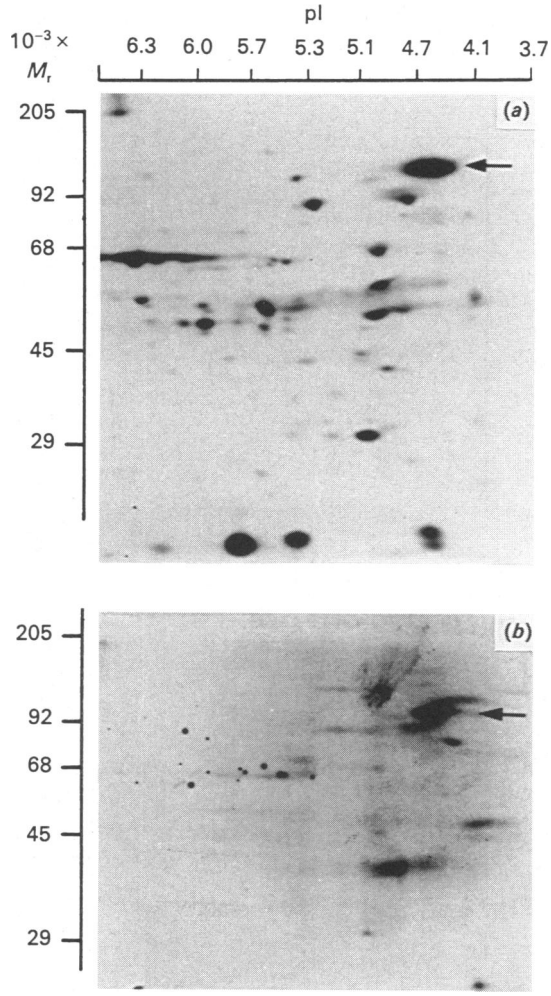

Fig. 3. Two-dimensional analysis of [^{35}S]methionine-labelled endosomal proteins

Membranes were isolated from rats injected with [^{35}S]-methionine 3 h previously. The two-phase aqueous-detergent partitioning of the endosomes was carried out as described in the Experimental section. (*a*) 'Early' endosome fraction; (*b*) Triton X-114-phase extract. Arrows point to 115 kDa polypeptide of pI 4.3.

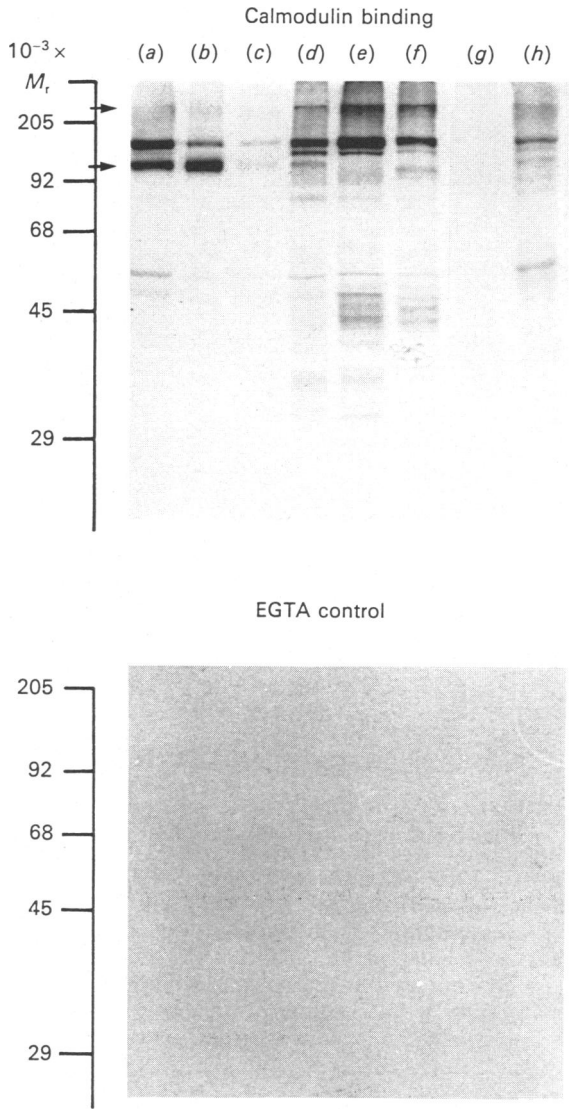

Fig. 4. Identification of ^{125}I-calmodulin-binding polypeptides of rat liver subcellular fractions

The following fractions were separated by SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose sheets, and overlaid with ^{125}I-calmodulin. Lanes: (*a*), (*b*) and (*c*), 'late', 'early' and receptor-enriched endosome subfractions; (*d*), (*e*) and (*f*), plasma-membrane subfractions derived from sinusoidal, canalicular and lateral domains; (*g*), lysosomes; (*h*), Golgi membranes. Control gels were overlaid in the presence of 2 mM-EGTA. The same amount of protein (100 μg) was loaded on to each channel. Arrows point to 240 kDa polypeptide of plasma-membrane and Golgi fractions and to the 115 kDa polypeptide of the endosome fractions.

of 140 and 240 kDa, identified when the plasma-membrane subfractions were separated in one dimension, could not be focused within the pH range used. However, when a wider isoelectric-focusing range was used, the 140 kDa calmodulin-binding polypeptide was located in the basic region of the gels, with pI < 6.6 (results not shown).

Determination of calmodulin in endosomes and plasma membranes

The contents of calmodulin in the three endosome subfractions and sinusoidal and lateral plasma membranes were measured. Table 1 shows that the amounts of calmodulin present in 'early' and 'late' endosomes and in sinusoidal plasma membranes were high relative to that in the cytosol. The receptor-enriched endosome fraction had much higher contents of calmodulin, with the amounts being similar to those measured in lateral plasma membranes.

DISCUSSION

'Early' and 'late' endosome fractions were prepared from rat liver homogenates according to the position in gradients of vesicles containing radiolabelled ligands prepared at two time intervals after their uptake by liver (Evans & Flint, 1985). A third and heavier endosome subfraction examined had high contents of asialoglycoprotein and insulin receptors (Evans & Flint, 1985). The ligand-containing, time-defined, 'early' and

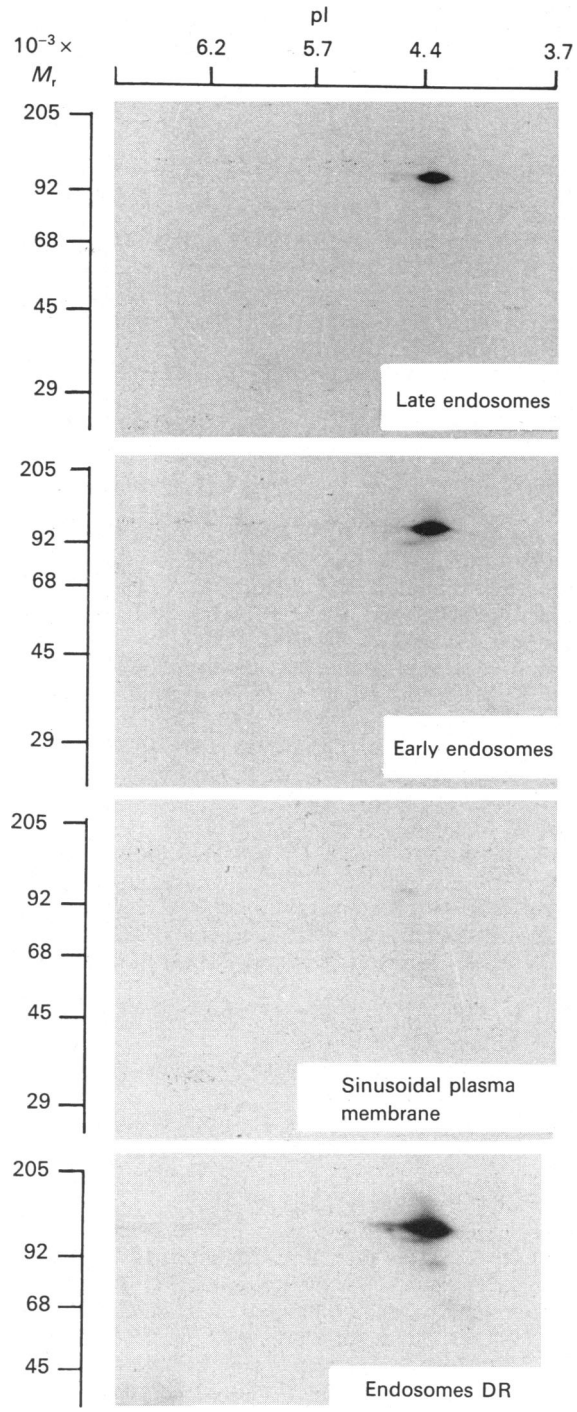

Fig. 5. Two-dimensional polyacrylamide-gel analysis of ^{125}I-calmodulin-binding polypeptides of endosomes and sinusoidal plasma membranes

The same amount of protein (200 μg) was used for each fraction, and all three fractions were analysed in an identical fashion and exposed for autoradiography for 4 days, except for endosome fraction DR, which was exposed for 21 days.

'late' endosome vesicles were shown to be functional, being capable of acidifying in the presence of MgATP (Saermark *et al.*, 1985). The endosome fractions resembled in enzymic and chemical composition those prepared from liver homogenates by others (Quintart *et al.*, 1983; Baenziger & Fiete, 1986). Endosomes prepared by a density-perturbation technique (Quintart *et al.*, 1984), although less amenable to compositional analysis owing to extensive cross-linking of the proteins (Ajioka & Kaplan, 1987), were of a generally similar enzymic composition.

Table 1. Calmodulin amounts in various endosome and plasma-membrane fractions and in cell cytosol

Conditions used to isolate the fractions and the methods used for the assay of calmodulin are described in the Experimental section. The calmodulin concentrations are expressed as ng/mg of protein in the fractions. Each value is the mean ± S.D. for three determinations.

Fraction	Calmodulin
Endosomes ('late')	2.23 ± 0.13
Endosomes ('early')	2.66 ± 0.05
Endosomes (receptor-enriched)	4.03 ± 0.90
Plasma membranes (sinusoidal)	2.00 ± 0.06
Plasma membranes (lateral)	3.60 ± 0.27
Cytosol	0.50 ± 0.10

Calmodulin is an important Ca^{2+}-binding protein in non-muscle cells. It is involved in the regulation of many Ca^{2+}-dependent processes such as secretion, cell proliferation, actin–myosin interactions, microtubule organization etc. Binding of Ca^{2+} to calmodulin induces a change that results in the exposure of a hydrophobic surface on the calmodulin that permits its modulation by target proteins (Cheung, 1980). Many calmodulin-binding proteins have now been described, including the spectrin-like proteins associated with the cytoskeleton (Levine & Villard, 1981; Repatsky *et al.*, 1982), the desmocalmins (Tsukita & Tsukita, 1985), and proteins present in intestinal brush borders that attach the cytoskeleton to the cytoplasmic face of the plasma membrane (Palfrey *et al.*, 1982). Some calmodulin-binding proteins are located intracellularly, and their presence in the nuclear matrix has been demonstrated (Bachs & Carafoli, 1987). Calmodulin-binding proteins have also been shown to be present in the plasma membrane of many eukaryotic cells (Palfrey *et al.*, 1982; Burgess *et al.*, 1984; Klee *et al.*, 1984), including liver plasma membranes (Gloor & Gazzotti, 1986; Gazzotti *et al.*, 1985).

In the present work, we have surveyed the distribution of calmodulin-binding polypeptides in various liver subcellular fractions and have identified a calmodulin-binding polypeptide that was located primarily in endosome fractions. This 115 kDa polypeptide was shown by isoelectric focusing to have pI 4.3, and it was more acidic than the calmodulin-binding polypeptides of liver plasma-membrane subfractions that proved difficult to resolve in the pH-focusing gradients adopted. The 115 kDa polypeptide in endosomes is unlikely to be the soluble calmodulin-binding protein identified by Palfrey *et al.* (1982) in a variety of vertebrate tissues, since the present protein behaves as a hydrophobic integral membrane protein, as demonstrated by its partitioning into a Triton X-114-enriched phase. The calmodulin-binding polypeptide was absent from lysosomal and bile-

canalicular membranes. This calmodulin-binding polypeptide is probably an endosome-specific protein. It can be distinguished further from a family of liver glycoproteins of similar molecular mass located at the bile canalicular membrane (Meier, 1987; Petell *et al.*, 1987), in lysosomes (Reggio *et al.*, 1984), and a 110 kDa transformation-sensitive plasma-membrane glycoprotein with pI 3.5, identified in liver and hepatoma by Becker *et al.* (1985).

The calmodulin-binding polypeptides present in fractions originating from the blood-sinusoidal, lateral and canalicular domains of the plasma membrane of the hepatocyte were of a similar molecular mass to those described by Gloor & Gazzotti (1986). The 240 kDa calmodulin-binding polypeptide was postulated to act as an actin-binding protein in the intercellular junctions located at the lateral domain of the plasma membrane (Amrein-Gloor & Gazzotti, 1986). This calmodulin-binding polypeptide was mainly resident in the plasma membrane, for it was hardly detected in endosomes. The low amounts of the 115 kDa calmodulin-binding polypeptide detected in sinusoidal plasma membranes can be explained by their contamination by endosomes, for the polypeptide was not detected in lateral and bile-canalicular plasma membranes. The same explanation also applies to its presence in low amounts in Golgi membranes, for these have been shown to be contaminated by endosomal membranes (Kay *et al.*, 1984). The trafficking of the 115 kDa polypeptide from the Golgi apparatus directly to the endocytic compartment may occur, and this can also explain its presence in low amounts in Golgi fractions. Lysosomes contained very low amounts of calmodulin-binding proteins.

Finally, it was observed that a third endosome subfraction (D-R), inferred to be enriched in receptors (Evans & Flint, 1985), had an extremely high content of calmodulin in comparison with the other membrane fractions or the cytosol. The calmodulin content was similar to that of plasma membrane originating from the lateral domain, where calmodulin-binding proteins are associated with cytoskeletal components attached to intercellular junctions. Perhaps, in addition to the postulated role of these endosome vesicles in shuttling receptors from the endocytic component to the plasma membrane, they may also function in the storing of Ca^{2+} and/or calmodulin. Indeed, a subcellular fraction called 'calciosomes' has been postulated to fulfil this role (Volpe *et al.*, 1987).

The precise functions of the calmodulin-binding proteins, and especially the 115 kDa polypeptide now identified in endosomes, remain to be studied. Proteins that regulate intracellular Ca^{2+} concentrations have been implicated in many cellular processes, including secretion, proliferation and motility. In the endocytic compartment, calmodulin-binding proteins may play an indirect role in transmembrane signalling by their influence on protein kinases (Albert *et al.*, 1984). Ion fluxes across undefined intracellular membranes (endoplasmic reticulum, endosomes?) have been implicated as a major aftermath of the binding of various ligands to receptors at the plasma membrane (Berridge, 1987). Calmodulin regulates actin–myosin interactions; it may thus influence intracellular membrane transport, involving, for example, the functional interaction by budding and fusion of 'early' and 'late' endosomes and lysosomes.

C. E. thanks the Wellcome Trust for support. We thank Mr. P. Tabona for technical help, and Mrs. Lydia Pearson for preparing the manuscript.

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Received 8 April 1988/15 June 1988; accepted 23 June 1988