Annexin II Is a Major Component of Fusogenic Endosomal Vesicles

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Abstract. We have used an in vitro assay to follow the proteins transferred from a donor to an acceptor upon fusion of early endosomes. The acceptor was a purified early endosomal fraction immunoisolated on beads and the donor was a metabolically-labeled early endosomal fraction in suspension. In the assay, both fractions were mixed in the presence of unlabeled cytosol, and then the beads were retrieved and washed. The donor proteins transferred to the acceptor were identified by two-dimensional gel electrophoresis and autoradiography. Approximately 50 major proteins were transferred and this transfer fulfilled all criteria established for endosome fusion in vitro. However, only a small subset of proteins was efficiently transferred, if donor endosomes were briefly sonicated

to generate small (0.1 μm diam) vesicles before the assay. These include two acidic membrane proteins, and three alkaline peripheral proteins exposed on the cytoplasmic face of the membrane. Partial sequencing and Western blotting indicated that one of the latter components is annexin II, a protein known to mediate membrane–membrane interactions. Immunogold labeling of cryosections confirmed that annexin II is present on early endosomes in vivo. These data demonstrate that annexin II, together with the other four proteins we have identified, is a major component of fusogenic endosomal vesicles, suggesting that these proteins are involved in the binding and/or fusion process.

THE pathways of membrane traffic are now well established, as are some of the mechanisms involved in sorting and transport of proteins between different compartments. Recent advances have come predominantly from genetic analysis in yeast (Schekman, 1992) and from the in vitro reconstitution of individual steps of the biosynthetic or the endocytic pathway (Balch, 1989; Goda and Pfeffer, 1989; Gruenberg and Howell, 1989; Rothman and Orci, 1992). In mammalian cells, several proteins required for membrane transport have been identified and characterized (Gruenberg and Clague, 1992), including NSF and associated proteins (Rothman and Orci, 1992), coat proteins (Pearse and Robinson, 1990; Serafini et al., 1991; Duden et al., 1991) and regulatory components belonging to the GTP-binding protein super-family (Bourne et al., 1990; Balch, 1992).

The mechanisms which mediate first the formation of close contacts between membranes and then fusion itself are still unclear. Studies on fusion of secretory granules or enveloped viruses have both led to the view that a pore or a collar may be formed at the site of contact via the oligomer-

ization of specific proteins (Almers, 1990; Bentz et al., 1990). Lipid flow would then be initiated along the extended hydrophobic surfaces of these proteins. The hemagglutinin of influenza virus envelope contains an amphipathic α-helix, which becomes exposed upon fusion (Wiley and Skehel, 1987); a similar motif has also been found in other viral fusion proteins and in a protein implicated in sperm-egg fusion (Blobel et al., 1992). Until now, this domain has not been found in any of the proteins that have been implicated in intracellular membrane transport. However, the protein complex consisting of NSF and associated proteins, which are required for intra-Golgi transport, is proposed to regulate intracellular fusions, although its precise role is not known (Rothman and Orci, 1992).

In this paper, we modified our cell-free fusion assay (Gruenberg and Howell, 1986, 1987; Gruenberg et al., 1989) to identify proteins that may mediate binding/fusion of early endosomal membranes. This analysis was possible because early endosomes display a striking tendency to undergo homotypic fusion with each other in vitro (Davey et al., 1985; Gruenberg and Howell, 1986, 1987; Braell, 1987; Diaz et al., 1988; Woodman and Warren, 1988; Gruenberg et al., 1989). This process is highly specific (Gruenberg et al., 1989; Bomsel et al., 1990) and microtubule-independent (Bomsel et al., 1990). It is regulated by phosphorylations (Tuomikoski et al., 1989; Thomas et al., 1992; Woodman

et al., 1992), N-ethyl-maleimide-sensitive factor (NSF)[1] (Diaz et al., 1989) and GTP-binding proteins (Gorvel et al., 1991; Colombo et al., 1992; Lenhard et al., 1992). This high and specific fusion activity in vitro may indicate that, in vivo, early endosomes, like other cellular organelles, are organized in a dynamic network connected by fusion/fission events (Gruenberg and Howell, 1989). Recent in vivo observations, in fact, show that endosomes can be organized in an extensive and dynamic tubular network (Hopkins et al., 1990), which may be part of the early endosome (Tooze and Hollinshead, 1991).

Our approach was to study the transfer of early endosomal proteins from a donor to an acceptor membrane upon fusion. As acceptor, we used an early endosomal fraction immobilized on a solid support by immunoisolation (Gruenberg and Howell, 1986, 1987; Gruenberg et al., 1989). The donor was either a metabolically-labeled, early endosomal fraction in suspension, or the same fraction which had been briefly sonicated to generate small vesicles. The proteins transferred in the fusion assay were then identified by high resolution two-dimensional gel electrophoresis (Bravo, 1984; Celis et al., 1990) and autoradiography. Essentially all early endosomal proteins were transferred from an intact donor ($\approx$50 major species), as expected for a homotypic fusion. The small GTP-binding protein rab5, which localizes to early endosome (Chavrier et al., 1990; Chavrier et al., 1991) and regulates early endosome fusion in vitro (Gorvel et al., 1991) and endocytosis in vivo (Bucci et al., 1992), was identified amongst the transferred proteins.

The number of transferred proteins could be significantly reduced when the donor was briefly sonicated before the assay. More importantly, only five proteins were then transferred with high efficiency (two membrane proteins and three peripheral proteins). One of the latter components was found to be annexin II, which we could detect on the surface of early endosomes in vivo. Proteins of the annexin family, in particular annexin II (see, Burgoyne, 1988; Gerke, 1989a), have been previously implicated in membrane–membrane interactions, although their physiological functions are still debated (see, Südhof et al., 1982; Crompton et al., 1988; Burgoyne and Geisow, 1989; Zaks and Creutz, 1990a; Pollard et al., 1990a; Lin et al., 1992). Our data suggest that annexin II, together with the other four proteins we have identified, mediates membrane–membrane interactions between early endosomes in the assay.

Materials and Methods

Cells and Viruses

Monolayers of BHK cells were grown and maintained as described (Gruenberg et al., 1989). For each experiment, 6 × 10-cm Petri dishes were plated 16 h before use. Cells were metabolically labeled with 0.2 mCi/dish [35S]Met for 16 h in medium containing low Met (1.5 mg/liter). To depolymerize microtubules, cells were preincubated with 10 μM nocodazole for 1 h at 37°C and nocodazole remained present in all incubations (Gruenberg et al., 1989). HRP was internalized by fluid phase endocytosis as described in Gruenberg et al. (1989) and Gorvel et al. (1991). Vesicular stomatitis virus (VSV) was produced as previously described (Gruenberg and Howell, 1985). All manipulations of the cells were at 4°C, except when indicated.

G-Protein Implantation and Internalization into Early Endosomes

We have previously described our protocol to introduce the transmembrane spike glycoprotein G of vesicular stomatitis virus (VSV-G) into the plasma membrane by low pH-mediated fusion of the viral envelope with the plasma membrane (Gruenberg and Howell, 1985, 1986, 1987; Gruenberg et al., 1989). Briefly, we added 50 μg VSV to 1.3 × 10^{7} cells (one 10-cm dish); 8 μg total VSV were fused with the plasma membrane, corresponding to a density of $\approx$70 VSV-G molecules/μm^2 membrane surface area. Upon warming the cells up to 37°C for 5 min, the VSV-G molecules were rapidly internalized into early endosomes, essentially as a synchronous wave (Gruenberg and Howell, 1986, 1987; Gruenberg et al., 1989). We used six dishes in a typical experiment.

Fractionation of Endosomes on a Flotation Gradient

After homogenization of the cells and preparation of a post-nuclear supernatant (PNS) (Gruenberg et al., 1989; Gorvel et al., 1991), the first fractionation step was the same flotation gradient we used previously (Gorvel et al., 1991). Briefly, the PNS was brought to 40.6% sucrose and 0.5 mM EDTA, loaded at the bottom of an SW 60 tube, sequentially overlaid with 1.5 ml 16% sucrose in D$_2$O, 3 mM imidazole pH 7.4 containing 0.5 mM EDTA, then 1.0 ml 10% sucrose in D$_2$O, 3 mM imidazole pH 7.4 containing 0.5 mM EDTA and finally 0.5 ml homogenization buffer (250 mM sucrose, 3 mM imidazole pH 7.4). The gradient was run for 60 min at 35,000 rpm using a SW 60 rotor. Early endosomes were collected at the 16–10% sucrose interface. The fraction was then split into four equal aliquots and immediately processed for immunoisolation. Protein determination in the fractions was as described by Bradford (1976).

Immunoisolation

We have previously described our protocol to immunoisolate endosomal fractions, using the cytoplasmic domain of VSV-G as antigen (Gruenberg and Howell, 1985, 1986, 1987; Gruenberg et al., 1989). To analyze the protein composition of the acceptor, the fractions were prepared from metabolically-labeled cells. The endosomal fractions recovered from the flotation gradient were used as input for subsequent immunoisolation. The immuno-adsorbent was prepared by binding $\approx$5 μg of a monoclonal antibody against a cytoplasmic epitope of VSV-G (P5D4; Kreis, 1986) to 1 mg anti-mouse polyacrylamide beads, as described (reviewed in Howell et al., 1989). Approximately 20 μg protein of the fraction recovered from the gradient were mixed with 1 mg immuno-adsorbent in 1 ml PBS-containing 5 mg/ml BSA (PBS-BSA), and rotated end-over-end for 2 h at 4°C. The beads with bound cellular materials were then recovered by centrifugation at 2500 g for 5 min, and washed once with PBS-BSA and once with PBS. The beads were processed for two-dimensional gel electrophoresis and the gels analyzed by autoradiography.

In the cell-free assay, the acceptor fraction was prepared with the same protocol, but the cells were not metabolically labeled. The immunoisolated fraction was then immediately added to the assay, as described below.

Quantification of Endocytic Vesicle Fusion In Vitro

We have quantified the occurrence of early endosome fusion in vitro with the cell-free assay we have established (Gruenberg et al., 1989; Tuomikoski et al., 1989; Bomsel et al., 1990; Gorvel et al., 1991). The assay uses two early endosomal fractions prepared after separate internalization of avidin and biotinylated HRP (bHRP) into two cell populations. (a) bHRP present in the fluid phase was cointernalized with the implanted VSV-G protein for 5 min at 37°C. The cells were homogenized and fractionated using the gradient (see above). Early endosomes were collected from the 16–10% interface and immunoisolated as above. After washing once with PBS-BSA, 1 mg beads with bound bHRP-labeled fraction was resuspended in 50 μl homogenization buffer and directly used in the cell-free assay. (b) Avidin was prebound at 4°C to cell surface biotinylated proteins under conditions where it retained free biotin binding sites, providing a membrane-attached marker in the fusion assay (as described in Gorvel et al., 1991). The complex avidin-biotinylated protein was then internalized into early endosomes for 5 min at 37°C and the avidin remaining at the cell surface was quenched with excess biotinylated insulin. Avidin-labeled early endosomes were then fractionated using the flotation gradient, and one 50-μl aliquot (15 μg protein) was directly used in the fusion assay. In some experiments, avidin-labeled early endosomal elements were fragmented by sonication for 1 × 7 s on ice with a probe-type sonicator. In the fusion assay, the two fractions were combined in the presence of 50 μl cytosol (5 mg/ml protein), adjusted

to 12.5 mM Hepes (pH 7.4), 1.5 mM MgOAc, 3 mM imidazole (pH 7.4), 1 mM DTT, 50 mM KOAc, and complemented with 8 μl of an ATP-regenerating system (1:1:1 mixture of 100 mM ATP brought to pH 7.0 with KOH, 800 mM creatine phosphate, and 4 mg/ml creatine phosphokinase) and 8 μl of 1 mg/ml biotin-insulin stock solution as a quenching agent. To deplete ATP, 1.6 μl of apyrase (1,200 U/ml) replaced the ATP-regenerating system. The assay as well as the quantification of the avidin-bHRP complex formed during fusion were carried out as described (Gruenberg et al., 1989; Bomsel et al., 1990; Gorvel et al., 1991).

Transfer of Proteins during Fusion of Endocytic Vesicle In Vitro

To identify the proteins transferred between early endosomal elements upon fusion in vitro, essentially the same assay was used. One early endosomal fraction was freshly prepared from unlabeled cells after internalization of VSV-G for 5 min at 37°C and fractionation using the flotation gradient. The early endosomes were then immunoisolated from the 16–10% interface and resuspended in 50 μl homogenization buffer. In the transfer assay, this fraction is referred to as acceptor fraction. The second fraction, referred to as donor, was prepared from cells metabolically labeled with [35S]Met and was directly collected from the 16–10% interface of the gradient. The fusion assay was carried out as above. To identify the proteins transferred during fusion, the immuno-adsorbent plus bound material was washed and then processed for two-dimensional gel electrophoresis. The gels were analyzed by autoradiography. In some experiments, the donor was fragmented by a brief sonication step (1 × 7 s) before the transfer assay. To identify the membrane-associated proteins of the sonicated donor, the fraction was then centrifuged at 150,000 g for 45 min and then pellets and supernatants were processed for two-dimensional gel analysis. Extraction in Triton X-114 was as described by Bordier (1981).

Two-dimensional Gel Electrophoresis

A combination of IEF and SDS-PAGE was used to resolve proteins in two dimensions as described by Bravo (1984). The samples were solubilized in 9.8 M urea, 4% w/v NP40, 2% v/v ampholines pH 7–9 and 100 mM DTT. The tube gels used for the IEF gels were 25 cm long and 2.5 mm in internal diameter. IEF gels were run at 1,200 V for 17 h. The pH gradient was linear between pH = 4.5 and 7.4. The second dimension resolving gels were 15% (wt/vol) acrylamide, 0.075% (wt/vol) N-N' methylene-bis-acrylamide and the stacking gels were 5% (wt/vol) acrylamide, 0.25% (wt/vol) N-N' methylene-bis-acrylamide. After electrophoresis the gels were fixed and prepared for autoradiography using Entensify (Dupont, New England Nuclear Research Products, Boston, MA).

Sequencing

The spot corresponding to protein b was excised from six two-dimensional gels, minced, electroeluted in one dimension and analyzed as previously described (Kurzchalia et al., 1992). Briefly, the protein was digested with trypsin and the resulting peptides extracted from the gel slice were separated by reverse phase HPLC. As a control, we used spots cut out from nonrelevant regions of the same two-dimensional gels. The elution profiles of the specific and control digests were compared to select specific peptides, which were analyzed by Edman degradation using a sequencer (model 477A, Applied Biosystems, Inc., Foster City, CA).

Electron Microscopy

To provide an electron-dense marker of early endosomes, the cells were incubated for 5 min at 37°C in the presence of 5 nm BSA-gold (Ludwig et al., 1991). The cells were then washed in ice-cold PBS, and processed for cryosections and immunogold labeling as described by Griffiths et al. (1984).

Results

Our initial goal was to reconstitute in vitro the transfer of proteins that occurs upon fusion of a donor with an acceptor endosome, and then to use this assay to identify proteins that may be involved in the process. We used early endosomes immunoisolated on a solid support as an acceptor fraction and metabolically-labeled early endosomes in suspension as a donor fraction. In the assay, fusion would result in the

transfer of metabolically-labeled proteins from the donor to the unlabeled acceptor. The acceptor can then be retrieved, washed and analyzed by two-dimensional gel electrophoresis and autoradiography, to identify donor proteins that have been transferred.

Preparation of the Acceptor Fraction

As antigen for immunoisolation of the acceptor, we used the cytoplasmic domain of the spike VSV-G. The G-protein was first implanted into the plasma membrane by low pH-mediated fusion of the viral envelope with the plasma membrane (White et al., 1980; Gruenberg and Howell, 1985) and then internalized into early endosomes by incubation for 5 min at 37°C (Gruenberg and Howell, 1986, 1987; Gruenberg et al., 1989). The cells were homogenized and a PNS was prepared. The PNS was then fractionated using a step flotation gradient, which separates early endosomes containing the small GTP-binding protein rab5, from the plasma membrane and from late endosomes containing rab7 and the cation-independent mannose-6-phosphate receptor (Gorvel et al., 1991). The early endosomal fraction recovered from the gradient was enriched ≈13-fold. As a second step, early endosomes containing the VSV-G protein were immunoisolated using beads with a bound antibody recognizing the VSV-G cytoplasmic domain (Gruenberg and Howell, 1985, 1986, 1987; Gruenberg et al., 1989; Howell et al., 1989; Thomas et al., 1992). Early endosomes could be prepared with a yield of 12% and an enrichment of 73× over the homogenate, using HRP cointernalized with the G-protein as a marker of the early endosome content.

Purified Acceptor Fraction Fuses with Donor Membranes In Vitro

Using our cell-free assay (Gorvel et al., 1991), we then investigated whether the purified early endosomal acceptor retained its fusion activity in vitro. In these experiments, the lumenal content of acceptor early endosomes was labeled with bHRP instead of HRP. The donor fraction was an early endosomal fraction recovered from the flotation gradient (enrichment ≈13×, see above) and contained endosomes labeled with tetravalent avidin attached to the lumenal side of the endosomal membrane via proteins originally biotinylated at the cell surface (Gorvel et al., 1991). If fusion occurred upon incubation of acceptor and donor membranes in the assay, a complex was formed between avidin and bHRP. The immunoisolated fraction was then retrieved by centrifugation and, after washing, the avidin-bHRP complex was immunoprecipitated with anti-avidin antibody and the enzymatic activity of bHRP was quantified (see Gorvel et al., 1991 and references therein). Table I shows that fusion occurred with a high efficiency (≈60%). In agreement with our previous studies, fusion was inhibited by low concentrations of GTPγS (Table I), low temperatures, 1 mM N-ethylmaleimide (NEM) or in the absence of ATP or cytosol (not shown).

Protein Transfer during Early Endosome Fusion In Vitro

Since fusion activity was high after purification, the acceptor and donor fractions were then used to study the transfer of proteins during fusion. Both fractions were prepared as above, except that avidin and bHRP were omitted and that

Table I. Fusion Efficiency of Early Endosomal Fractions before and after Fragmentation

	OD bHRP	Efficiency
		%
(A) Total amount	0.206 ± 0.004	100
(B) Fusion with intact EE	0.126 ± 0.005	61 ± 3
+GTPγS	0.002 ± 0.003	1 ± 2
(C) Fusion with fragmented EE	0.042 ± 0.006	20 ± 3
+GTPγS	0.001 ± 0.002	1 ± 1

Fusion was measured by the formation of a complex between avidin and bHRP, which had been separately internalized into the early endosomes (EE) of two cell populations. After internalization, avidin was bound to the lumenal side of the endosomal membrane via biotin, whereas bHRP distributed within the endosomal content. The bHRP-labeled fraction was prepared using a gradient followed by immunoisolation (acceptor) and the avidin-labeled fraction was recovered from the gradient (donor). (A) The efficiency of avidin-bHRP complex formation during fusion is indicated as a percentage of the total amount of complex formed in the presence of detergent (Gruenberg et al., 1989). The complex was then immunoprecipitated with anti-avidin antibodies and the activity of bHRP quantified (OD bHRP). (B) Fusion with intact early endosomes. Subcellular fractions prepared from avidin- and bHRP-labeled cells were combined in the fusion assay in the presence of 5 mg/ml cytosol and ATP and incubated for 45 min at 37°C. When indicated 10 μM GTPγS was present. (C) Fragmented early endosomes. As in B, but after brief sonication of the avidin-labeled fraction.

the donor was prepared from cells which had been labeled with [35S]Met for 16 h. In the cell-free assay, acceptor and donor were mixed in the presence of unlabeled cytosol, the donor membranes being thus, the only 35S-labeled component in the reaction. At the end of the experiment, the beads were retrieved and washed, and the bound cellular materials were analyzed by high resolution two-dimensional gel electrophoresis (Bravo, 1984; Celis et al., 1990) and autoradiography.

Fig. 1 A shows the pattern of 35S-labeled donor proteins transferred to the unlabeled acceptor in the assay. This transfer fulfilled the different criteria established for the fusion of early endosomes in vitro. Transfer was abolished at 4°C, in the absence of ATP or cytosol and in the presence of 1 mM NEM (not shown) or 10 μM GTPγS (Fig. 1 B) (see Gruenberg and Howell, 1989; Mayorga et al., 1989). Transfer was also abolished with antibodies against the small GTP binding protein rab5 (not shown), as expected for early endosome fusion (Gorvel et al., 1991). As an additional control, we used a metabolically-labeled late endosome fraction recovered from our gradient (Gorvel et al., 1991) as donor. No transfer was then detected (not shown), in agreement with our previous findings that early and late endosomes do not directly fuse with each other (Gruenberg et al., 1989; Bomsel et al., 1990; Gorvel et al., 1991).

As shown in Fig. 1 A, ~50 major proteins were transferred in the assay, which distributed over the entire pH range during isoelectric focusing in the first dimension and over apparent mobilities from 14 to 200 kD after electrophoresis in the second dimension. In this and in the following experiments, several proteins that were detected in the controls (Figs. 1 B, 2 B, and 4 B) appeared somewhat enriched in the specific fractions (Figs. 1 A, 2 A, and 4 A), including actin (*small star*) and a few heat-shock proteins, identified by their typical mobilities (Celis et al., 1991). The significance of this observation is not clear. However, these proteins, like all proteins detected in the control experiments, were disregarded in our analysis. Long thin arrows and letters in Fig. 1 A indi-
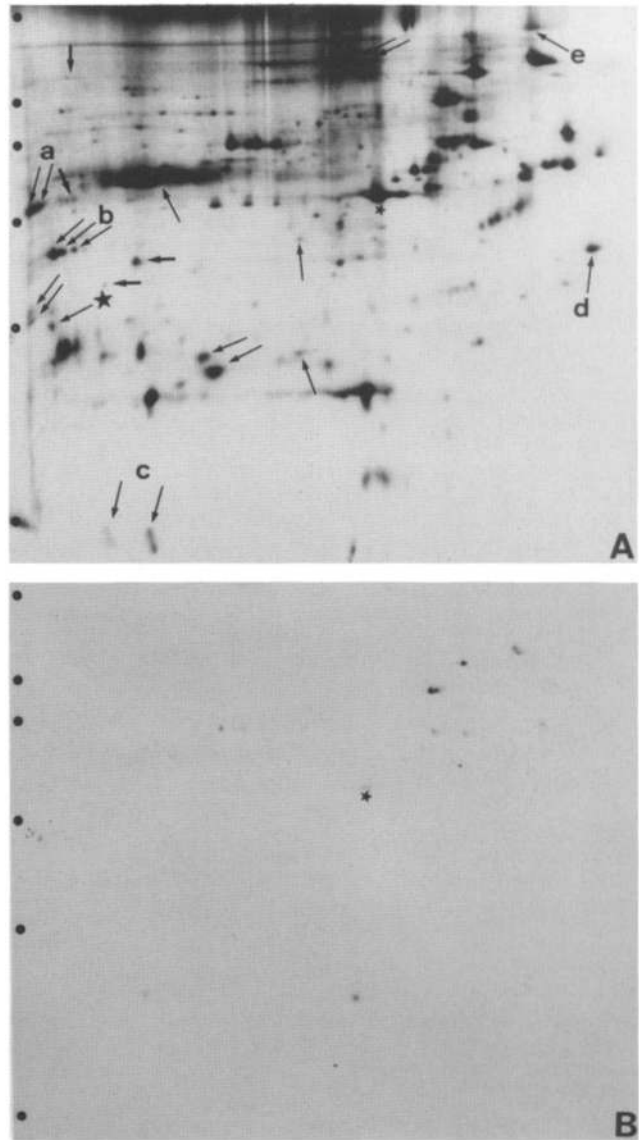

Figure 1. Identification of early endosomal proteins transferred from donor to acceptor after fusion. (A) The acceptor early endosomal fraction was prepared by immunoisolation from unlabeled cells, and the donor early endosomal fraction was prepared from cells metabolically labeled with [35S]Met using a flotation gradient. In the assay, both fractions were mixed in the presence of unlabeled cytosol and ATP, and then incubated for 45 min at 37°C. At the end of the assay, the fraction bound to the solid support was retrieved, washed and analyzed by isoelectric focusing in the first dimension (direction of electrophoresis from left to right) followed by SDS-PAGE in the second dimension (direction of electrophoresis from top to bottom). The gels were then analyzed by autoradiography. The arrows point at typical examples of transferred proteins. The position of rab5 is indicated by a large star next to the corresponding arrow. The small star shows the position of actin, identified by its mobility on two-dimensional gels. The molecular weight markers are indicated by solid dots (14.3, 30, 46, 69, 97, and 200 kD). (B) Same as A, but in the presence of 10 μM GTPγS.

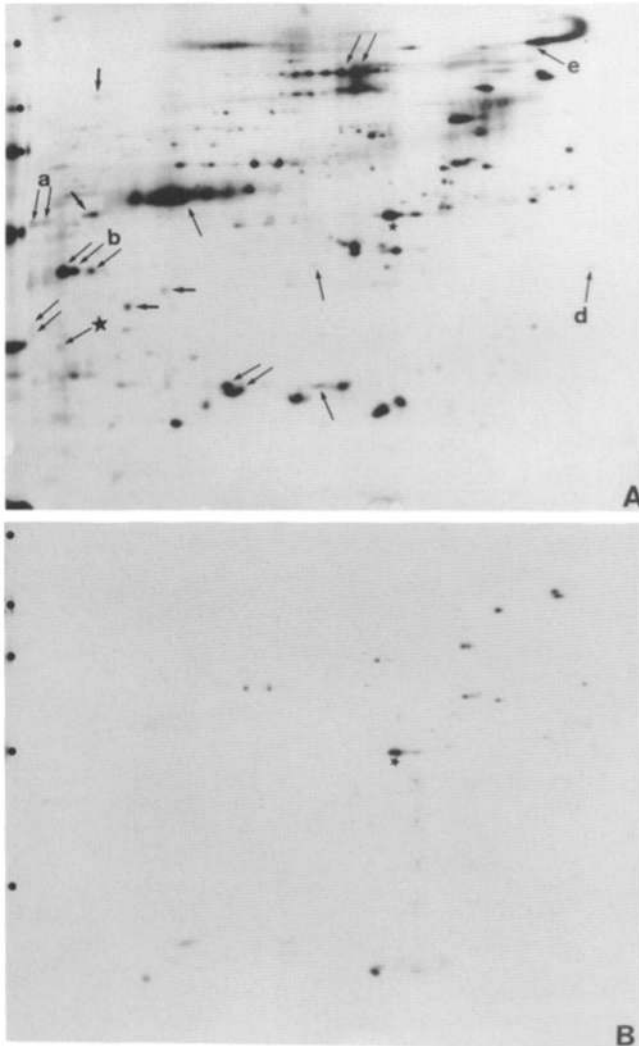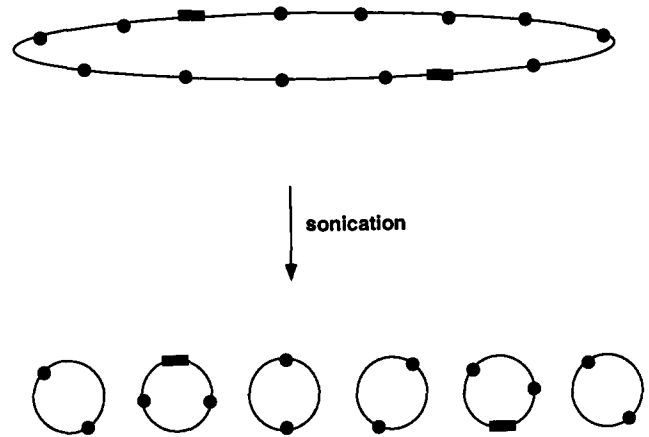Figure 3. Outline of donor sonication. The sonication of a donor early endosomal element is outlined. Some early endosomal components (*solid boxes*) may not be very abundant or present in specialized regions of the membrane, in contrast to others (*solid circles*). When small vesicles are generated by sonication, the former components may be present in a subpopulation of vesicles, but not the latter ones.

Figure 2. Protein composition of the early endosome acceptor fraction. (*A*) The acceptor early endosomal fraction was prepared by immunoisolation as in Fig. 1 (except that the cells had been metabolically-labeled with [³⁵S]Met) and then analyzed in two-dimensional gels followed by autoradiography. Directions of electrophoresis and labeling of typical proteins are as in Fig. 1. (*B*) Same as *A*, but the specific antibody was omitted during immunoisolation.

cate typical examples of transferred proteins, which will be discussed below. Because a large collection of donor proteins were transferred in the assay, we compared them with the protein pattern of the acceptor fraction itself.

Comparison with the Protein Composition of the Acceptor

The acceptor fraction was prepared as above from cells which had been labeled with [³⁵S]Met for 16 h, and then analyzed in two-dimensional gels (Fig. 2 *A*). Approximately 50 major proteins could be identified which were absent from the control, and their pattern closely resembled that obtained after transfer (compare Fig. 1 *A* with Fig. 2 *A*). Some differences could be observed when comparing autoradiograms from several experiments. In the experiment shown in Figs. 1 *A* and 2 *A*, a group of acidic proteins (40–50 kD) were more abundant after transfer (Fig. 1 *A*) than after direct im-

munoisolation (Fig. 2 *A*), whereas a few high molecular weight proteins exhibited the opposite distribution. The donor proteins transferred in vitro (Fig. 1 *A*) were, however, essentially identical to those of the acceptor (Fig. 2 *A*).

It is difficult to compare the patterns we obtained with the two-dimensional endosomal patterns obtained by other groups (Baenziger and Fiete, 1986; Schmid et al., 1988; Beaumelle et al., 1990). The conditions used to prepare the fractions, to label the cellular materials, and to run the isoelectric focusing gels were different. However, proteins with similar mobilities can be identified, particularly in the low molecular weight region (see Beaumelle et al., 1990).

We could identify the small GTP-binding protein rab5 among both the acceptor proteins (Fig. 2 *A, large star*) and the donor proteins that are transferred (Fig. 1 *A, large star*), in good agreement with the fact that rab5 localizes to early endosomes (Chavrier et al., 1990, 1991) and regulates early endosome fusion in vitro (Gorvel et al., 1991) and endocytosis in vivo (Bucci et al., 1992). To localize rab5 in the gels, [³⁵S]Met-labeled acceptor membranes were prepared from cells overexpressing rab5. The gels were then blotted onto nitrocellulose and the overexpressed rab5 was identified after [³²P]GTP overlay (Gorvel, J.-P., M. J. Clague, L. Huber, P. Chavrier, O. Steele-Mortimer, and J. Gruenberg, submitted for publication). The rab5 protein migrated to an alkaline position (pI ≈ 9.0) relative to other rab proteins (L. Huber and K. Simons, EMBL, unpublished observation). These experiments indicate that acceptor and donor membranes have essentially the same protein composition and that both contain rab5.

Protein Transfer From Sonicated Donor Vesicles

We reasoned that molecules mediating endosome–endosome interactions may be heterogeneously distributed in the plane of the membrane (see outline Fig. 3). If donor endosomes were fragmented into small vesicles, these proteins would be present in a sub-population of vesicles, which would remain fusion competent. In our assay, we would then expect these proteins to be more efficiently transferred (if they are re-
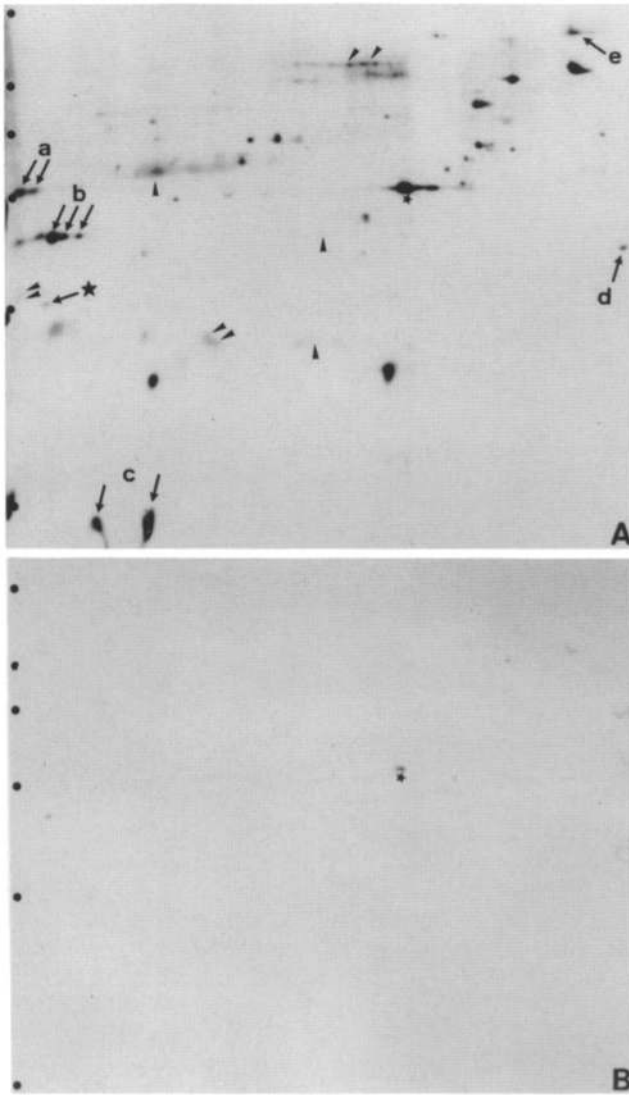
Figure 4. Identification of proteins transferred after fragmentation of the early endosomal donor fraction. (*A*) The transfer assay was carried out in parallel from the same early endosomal fractions as in Fig. 1, except that in this experiment the metabolically-labeled donor fraction was fragmented by sonication for 7 s before the transfer assay. The sample was then analyzed as in Fig. 1 and the autoradiogram was exposed for the same length of time. Only a subset of early endosomal proteins was transferred after fragmentation. Letters indicate early endosomal proteins transferred with the same efficiency from sonicated or intact donors (compare with Fig. 1). Rab5 is indicated by a star. Arrowheads point at proteins transferred with a reduced efficiency. Both arrowheads and arrows point at the proteins indicated by long arrows in Figs. 1 and 2. Star (actin) is as in Figs. 1 and 2. (*B*) Same as *A*, but in the presence of 10 μM GTPγS.

quired for the process), than other endosomal proteins. To fragment the donor, we used a single 7-s sonication pulse. An analysis by electron microscopy and flow cytometry showed that a homogeneous population of small vesicles of $\approx$100 nm diam had been generated (not shown). Fusion activity measured as above with membrane-bound markers was retained after sonication, albeit with a reduced efficiency (Table I), possibly indicating that only a subpopulation of avidin-labeled vesicles remained fusogenic (see below).
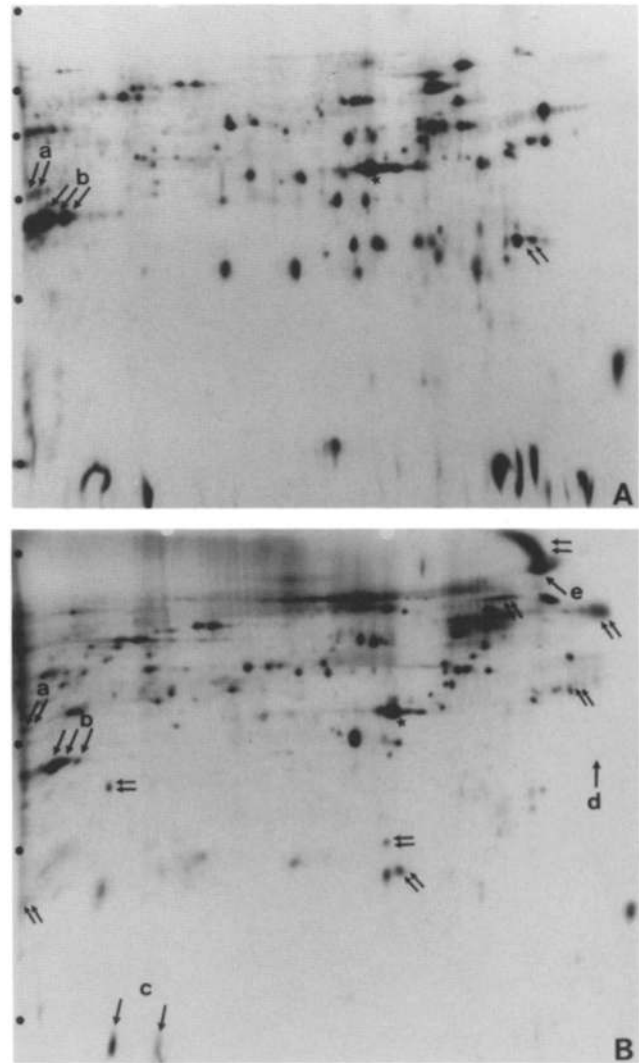
Figure 5. Soluble and membrane-associated proteins of the sonicated donor fraction. The donor fraction was prepared from metabolically-labeled cells (as in Fig. 1), briefly sonicated (as in Fig. 4), centrifuged at 150,000 g for 45 min. Pellets and supernatants were then analyzed in two-dimensional gels. Letters indicate the same proteins as in Figs. 1, 2, and 4. Whereas proteins *c*, *d*, and *e* were only membrane-associated, a fraction of proteins *a* and *b* were released after sonication. Proteins not transferred after sonication are indicated by small double arrows. Metabolically-labeled rab5 could only be identified in immunoisolated fractions or after transfer (*large star* in Figs. 1, 2, and 4), but not in the complete donor fraction (Figs. 5 and 6). (*A*) Supernatant (soluble proteins). (*B*) Pellet (membrane-associated proteins).

As shown in Fig. 4 *A*, protein transfer occurred after sonication, and retained all characteristics of endosome fusion, including GTPγS-sensitivity (Fig. 4 *B*). The number of transferred proteins was significantly reduced, as predicted (compare with Fig. 1 *A*), and each one of them, including rab5, was identified in the acceptor (Fig. 2 *A*) and in the fused donor (Fig. 1 *A*). These data indicate that fusogenic donor vesicles generated by sonication contain a defined subset of proteins.

Protein Transfer In Vitro Is Selective

We first investigated whether the number of transferred pro-

teins was reduced after sonication, because lumenal proteins were lost. In the absence of sonication, both internalized HRP, a fluid phase marker, and ^{35}S-labeled proteins were only associated with the pellet after high speed centrifugation of endosomal fractions recovered from the gradient. When the same experiment was repeated after sonication, >95% of internalized HRP was found in the supernatant, but only a relatively small fraction (30%) of the ^{35}S-labeled proteins (data not shown).

We then analyzed in two-dimensional gels the high speed pellets and supernatants obtained after centrifugation of the sonicated ^{35}S-labeled donor. As shown in Fig. 5, the majority of the donor proteins which remained associated with vesicles after sonication were clearly not transferred to the acceptor membranes in the assay (examples are indicated by *small double arrows* in Fig. 5 *B*). (The fraction containing the donor exhibits a protein pattern distinct from that of the immunoisolated acceptor, because the former is enriched 15× in early endosomes and the latter 73×.) To extend these observations, intact ^{35}S-labeled donors were extracted in Triton X-114 (Bordier, 1981). Detergent and aqueous phases were then analyzed in two-dimensional gels. Many putative transmembrane proteins present in the detergent phase were not detected amongst the proteins transferred after sonication (*small double arrows* in Fig. 6). As expected, high speed pellets obtained after sonication (Fig. 5 *B*) and Triton X-114 extracts (Fig. 6 *B*) did not exhibit identical protein compositions (see below and Table II), although overall patterns were comparable (particularly for proteins with a molecular weight ≤30 kD).

These experiments demonstrate that many membrane-associated proteins of the sonicated donor were not transferred in the assay. Thus, protein transfer after sonication was not caused by random fusion of donor membranes but occurred in a selective manner. The proteins which are then transferred include both membrane and peripheral proteins, and are presumably present within a subpopulation of donor vesicles which remain fusogenic in the assay.

A Subset of Proteins Is Transferred with High Efficiency after Sonication

Our prediction was that proteins involved in membrane-membrane interactions should be transferred with high efficiency from a sonicated donor. In fact, a small group of five proteins was not only efficiently transferred, when compared to other proteins, but this efficiency was essentially identical to that observed if the donor was intact (compare Fig. 4 *A* with Fig. 1 *A*; all proteins that could be detected in any of the controls were disregarded). These five proteins are indicated by letters (*a* to *e*) in Figs. 1, 2, 4, and 6, whereas examples of proteins transferred with lower efficiency are indicated by arrowheads in Fig. 4 *A*. Proteins a, b and c migrate as a series of spots, possibly reflecting post-translational modifications. Rab5 itself may not belong to this small group, although it is transferred with a relatively high efficiency.

The state of membrane-association of these five proteins is summarized in Table II. They include two acidic putative membrane proteins of ≈40 kD (*d*) and ≈200 kD (*e*), and three groups of alkaline peripheral proteins of ≈48 (*a*), 38 (*b*), and 14 kD (*c*). The latter proteins (*a–c*) are likely to be exposed on the cytoplasmic face of endosomes, because they
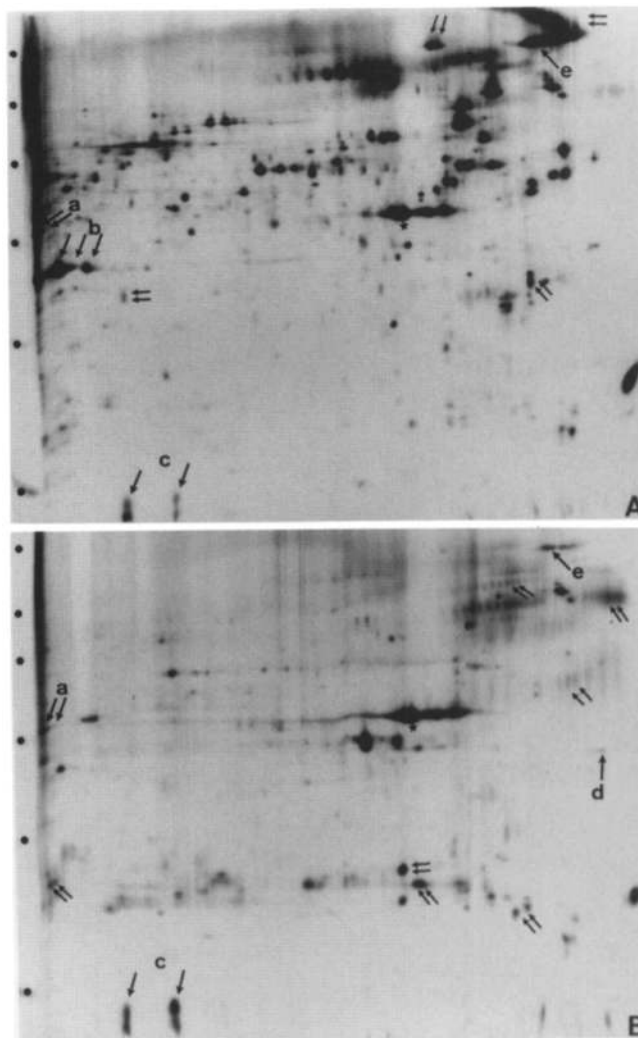

Figure 6. Triton X-114 extraction of the sonicated donor fraction. The donor fraction was prepared from metabolically-labeled cells as described in the legend of Fig. 1, extracted in Triton X-114 (Bordier, 1981), and analyzed in two-dimensional gels. Letters are as in Fig. 4. Protein *b* and *d* partitioned exclusively into the water and the detergent phase, respectively. In contrast, proteins *a*, *c*, and *e* were found in both phases. Proteins not transferred after sonication are indicated by small double arrows. As in Fig. 5, metabolically-labeled rab5 could not be identified in the complete donor fraction. However, rab5 is associated to the cytoplasmic face of endosomes via addition of an aliphatic chain and partitions into the detergent phase of Triton X-114 (Gorvel et al., 1991). (*A*) Aqueous phase. (*B*) Detergent phase.

could be released by salt treatment (not shown). Because these proteins are efficiently transferred from a sonicated donor (transfer efficiency is identical both with and without sonication) in contrast to most other donor proteins, we believe that they are strong candidates for being components that mediate the interactions between endosomal membranes in the assay.

Annexin II Belongs to the Subset of Proteins Transferred with High Efficiency In Vitro

The spot corresponding to protein b in two-dimensional gels of donor membranes was excised from the gel and digested with protease, and the resulting peptides were separated by

Table II. Proteins Transferred with the Same Efficiency with or without Sonication of the Donor

Protein	MW	IP	Sonication	Triton X-114	pH 11
a	48	alk	m/s	m/s	s
b	38	alk	m/s	s	s
c	14	alk	m	m/s	s
d	40	ac	m	m	m
e	200	ac	m	m/s	m

The table summarizes the properties of the five proteins transferred with the same efficiency with or without sonication. They are indicated by the same letters as in the figures. Fig. 5 shows the high speed pellet (B) and supernatant (A) after sonication of the donor; Fig. 6 shows the detergent (B) and water phase (A) after Triton X-114 extraction of the donor (pH 11 treatment of the donor was carried out in 0.1 N NaOH for 30 min on ice, not shown). Proteins a to c migrated as a few spots with the same apparent molecular weight, possibly reflecting post-translational modifications. MW, approximate molecular weight; IP, isoelectric point; alk, alkaline; ac, acidic; m, membrane-associated (or detergent-associated for Triton X-114); s, soluble; m/s, both membrane-associated and soluble.

HPLC and sequenced. The entire process was repeated, to limit the possibility of an error, and the characteristic peptides are indicated in Fig. 7. Both rounds resulted in the identification of a single protein, annexin II, when searching the SWISSPROT database with the FASTA (GCG package) and SCRUTINEER (Sibbald and Argos, 1990) programs. Protein b and annexin II also share the same apparent molecular weight. Moreover, the predicted isoelectric point of annexin II, obtained using the PEPTIDESORT program (GCG package), is in good agreement with that of protein b.

We could unambiguously establish that protein b is annexin II, and not another member of the annexin family, because both peptides indicated in Fig. 7 correspond to motives completely conserved in chick, mouse, bovine, and human annexin II proteins (amino acids 233–240 and 251–260; see Gerke et al., 1991, for annexin II sequence comparison), but not in other annexins. Finally, to demonstrate that protein b is an annexin, we used an antibody against a conserved annexin motif (cp2; Gerke, 1989b) to blot our two-dimensional gels. As shown in Fig. 8, the antibody only recognized protein b. We, therefore, conclude that protein b is annexin II, or a very closely related protein, because both share common sequences, as well as physical and immunological characteristics.

Annexin II Localizes to Early Endosomal Membranes In Vivo

Having established that protein b was annexin II, we then studied its subcellular distribution in vivo after immunogold-labeling on cryosections. We used a rabbit polyclonal antibody raised against purified annexin II, which only recognized annexin II on Western blots (not shown). As previously reported (see Gerke, 1989a), annexin II was present on the plasma membrane (Figs. 9 and 10). The gold particles were often distributed unevenly: while some areas were heavily labeled, others were essentially devoid of label (compare the two adjacent areas indicated by *large arrows* in Fig. 9 B). Small but significant amounts of gold were associated with clathrin-coated pits and vesicles (Fig. 9, C–E). In contrast, caveolae-like structures were usually unlabeled (Figs. 9, A–B and 10 A).

We then investigated whether annexin II was also present in early endosomes. These were unambiguously identified in

233	240
...S Y S	P Y D M L...

251	260
...L E N A F L N L V Q...	

Figure 7. Sequences of protein b peptides. The amino acid sequences of two peptides obtained after two separate rounds of proteolytic digestion of protein b are indicated. Each peptide corresponds to a stretch of amino acids, which is completely conserved in the sequences of bovine, human, mouse, and chick annexin II, and absent from the sequence of other members of the annexin family in the same species. The first and last amino acids of each peptide is numbered after the sequence in these species.

cryosections after the internalization of 5 nm BSA-gold from the medium for 5 min at 37°C, as previously described (Ludwig et al., 1991). Annexin II was found to be abundant on the surface of early endosomes containing 5 nm BSA gold (Figs. 9 B, and 10, A–C), although labeling of the plasma membrane was even higher (see Fig. 9 B and 10, A and B). In early endosomes, tubular or cisternal regions of the membrane appeared to be preferentially labeled, whereas little, if any, labeling was associated with the vesicular regions (see Fig. 10, A and B). These observations demonstrate that the protein we have identified with our in vitro assay as an early endosomal component presumably involved in membrane-membrane interactions, indeed localizes to early endosomes in vivo.

Discussion

Recent studies on the mechanisms of membrane transport have revealed the existence of complex regulatory mechanisms, including GTP-dependent switches mediated by members of the GTP-binding protein super-family as well as post-translational modifications (Gruenberg and Clague, 1992; Schekman, 1992). However, relatively little is known about proteins that may facilitate membrane-membrane interactions and/or catalyze the fusion step itself. In yeast, the small GTP-binding proteins Sec4p (Goud et al., 1988) and Yptlp (Oka et al., 1991; Rexach and Schekman, 1991; Segev, 1991) may be required for vesicle targeting at two different steps of secretory membrane transport. During intra-Golgi transport in mammalian cells, coat proteins are presumably involved in the docking of Golgi vesicles (see Orci et al., 1989) and a complex formed by NSF and its associated proteins is necessary for fusion (Rothman and Orci, 1992). NSF, and its homologue Sec18p in yeast, is also required in other steps of membrane transport. Finally, proteins which may contribute to the docking/fusion of synaptic vesicles with the plasma membrane have been identified (Thomas and Betz, 1990; Petrenko et al., 1991; Bennett et al., 1992).

In the present study, our goal was to identify proteins that may mediate interactions occurring between early endosomal membranes. Our approach was to use an in vitro assay that measures the transfer of proteins from a metabolically-labeled donor to an unlabeled acceptor immunoisolated on beads. In the assay, both fractions are mixed in the presence of unlabeled cytosol, the donor fraction thus being the only labeled component. If fusion occurs, metabolically-labeled proteins, originally present in donor endosomes, become components of acceptor endosomes. The beads can be retrieved and washed, and the transferred proteins analyzed by high resolution two-dimensional gels and autoradiography.

Western blot with anti-annexin antibody
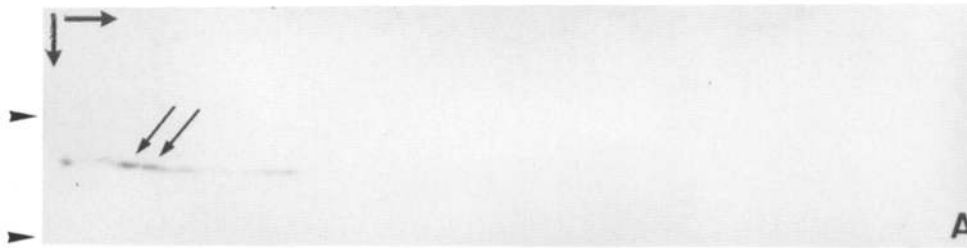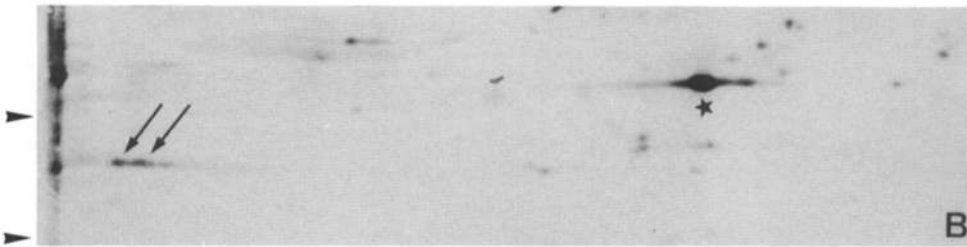

Autoradiogram of the nitrocellulose sheet

Figure 8. Western blotting of protein b with anti-annexin antibodies. PNSs prepared from unlabeled cells were mixed with donor fractions prepared from metabolically-labeled cells (see Fig. 1). The mixture was analyzed in two-dimensional gels and transferred to nitrocellulose. The part of the gel containing protein b was blotted with anti-annexin antibodies (cp2, Gerke, 1989*b*). The nitrocellulose sheet was then exposed onto x-ray film to reveal the position of protein b (*arrow*). *A* shows the blot and *B* the autoradiogram.

This transfer assay fulfills the different criteria that have been previously established for the fusion of early endosomes. It is inhibited by antibodies against the small GTP-binding protein rab5 (Gorvel et al., 1991). In addition, no transfer is detected between early and late endosomes, in agreement with the fact that these two compartments do not directly fuse with each other in vitro (Gruenberg et al., 1989; Bomsel et al., 1990; Gorvel et al., 1991).

Early Endosomal Proteins are Transferred upon Fusion

We observed that the donor proteins which are transferred upon fusion and the proteins of the immunopurified acceptor exhibit essentially the same, relatively complex composition ($\approx$50 major species). Both contain the small GTP-binding protein rab5, consistent with its localization to early endosomes (Chavrier et al., 1990, 1991; Gorvel et al., 1991). Because the same proteins are detected by either method (indirect donor-to-acceptor transfer or direct analysis of the acceptor), we believe that the significance of both observations is considerably strengthened and that these proteins are likely to be bona fide constituents of early endosomes. Moreover, this protein pattern is significantly different from that of purified late endosomes, which are enriched in the small GTP-binding protein rab7, the mannose-6-phosphate receptor and lysosomal membrane glycoproteins (Gorvel et al., 1991; F. Aniento, N. Emans, and J. Gruenberg, manuscript in preparation). These observations strongly suggest that acceptor and donor compartments in the assay share the same protein composition (see Gruenberg et al., 1989).

A Subset of Five Early Endosomal Proteins Are Transferred with High Efficiency

It is highly unlikely that the majority of the early endosomal proteins detected in the autoradiograms are involved in membrane–membrane interactions. When sonicating the donor into small (0.1 μm diam) vesicles before the assay, the number of transferred proteins is significantly decreased. These proteins can all be identified amongst early endosomal

proteins, and their transfer retains all criteria established for early endosome fusion in vitro. Moreover, these proteins only represent a small fraction of the total donor membrane-associated proteins, ruling out the possibility that random fusion events occurred after sonication.

Most strikingly, only a small set of five proteins is then transferred with an efficiency identical to that observed with an intact donor (Table II). These proteins include two putative *trans*-membrane proteins and three groups of peripheral membrane proteins. Salt extractions suggest that the latter proteins are exposed on the cytoplasmic face of early endosomes. Rab5 itself may not belong to this subset, although it is transferred with a relatively high efficiency after sonication. Further studies are clearly required to elucidate the role of rab5 in early endosome recognition/fusion in vitro (Gorvel et al., 1991) and to determine whether rab5 interacts, perhaps transiently, with one of the five proteins we have identified. Because these five proteins are selectively transferred with high efficiency from sonicated donor vesicles, we believe that they are strong candidates for being components of the mechanism controlling membrane-membrane interactions.

Annexin II May Mediate Endosome Membrane Interactions

Partial sequencing of protein b (one of the five proteins we have identified; Figs. 1, 2, and 4) reveals that it is annexin II, or a very closely related protein. In addition, both proteins share physical and immunological characteristics, as well as biochemical properties. Protein b, which behaves as a peripheral membrane protein, remains membrane associated during both fractionation and in vitro fusion, and annexin II is known to be very tightly associated to membranes at physiological concentrations of Ca^{++} (Powell and Glenney, 1987; Drust and Creutz, 1988; Blackwood and Ernst, 1990). Annexin II also forms a heterotetramer (calpactin I) consisting of two annexin II molecules and two light chains of 11 kD (Gerke and Weber, 1985). In our assay, we
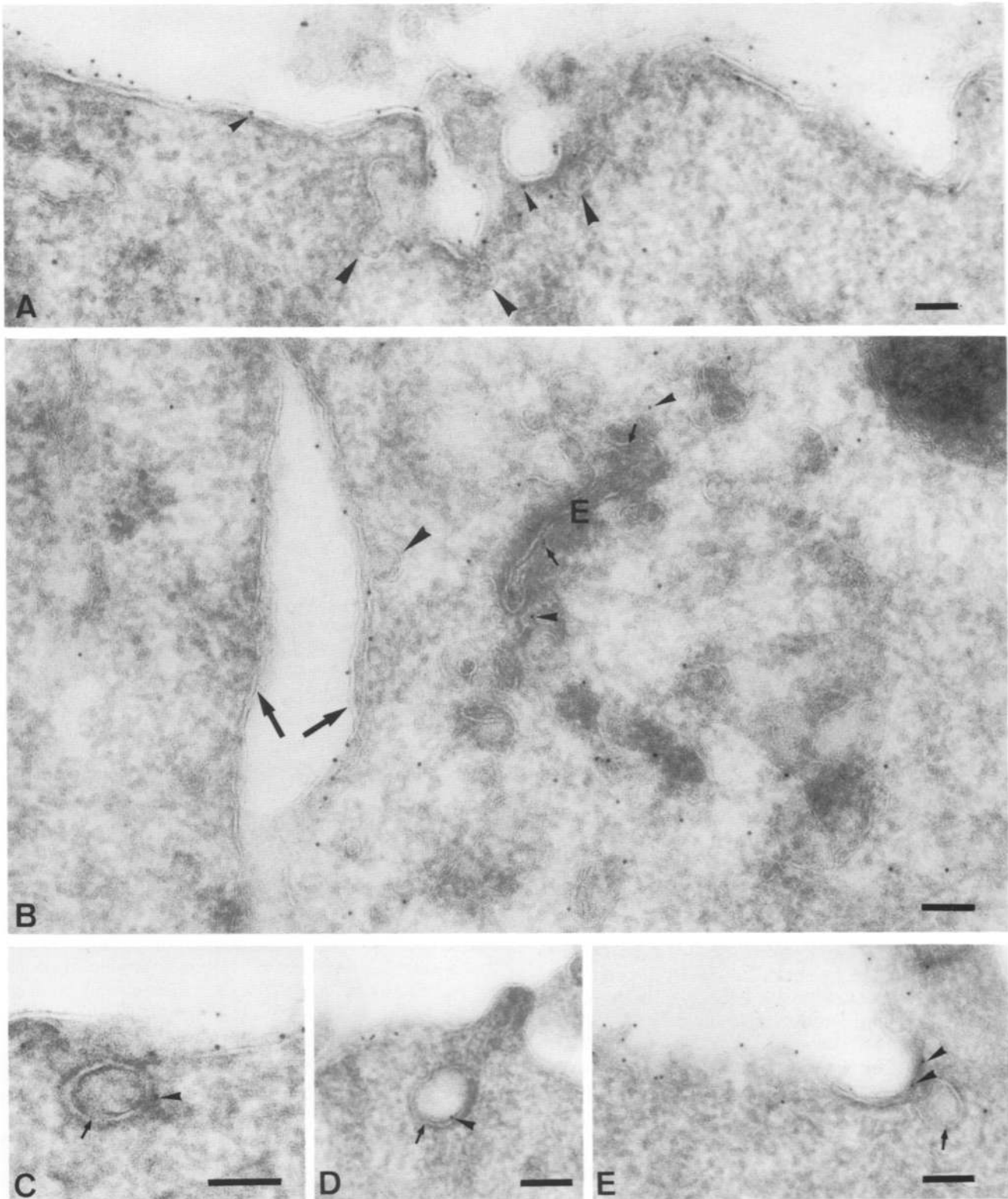
Figure 9. Localization of annexin II to the plasma membrane. Annexin II (*small arrowheads*) was localized to the plasma membrane after immunogold labeling of cryosections with a polyclonal antibody raised against purified annexin II followed by 9 nm gold coupled to protein A. Clathrin-coated pits (*C–E, small arrows*) were labeled with anti-annexin II antibodies, in contrast to caveolae-like structures (*A and B, large arrowheads*). Plasma membrane labeling was often unevenly distributed (compare areas indicated by large arrows in *B*). In *B*, early endosomes (*E*) were identified after incubation of the cells for 5 min at 37°C in the presence of 5 nm BSA gold (*small arrows*). Bar, 0.1 μm.
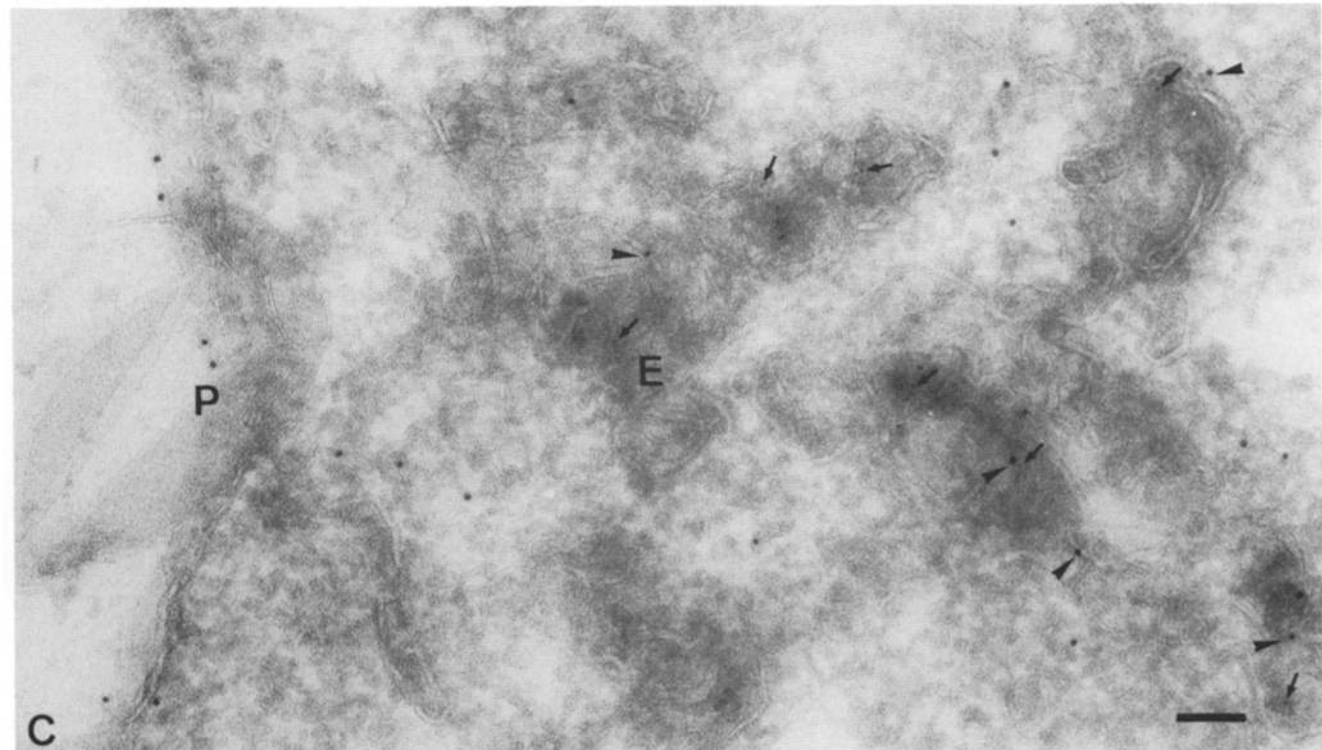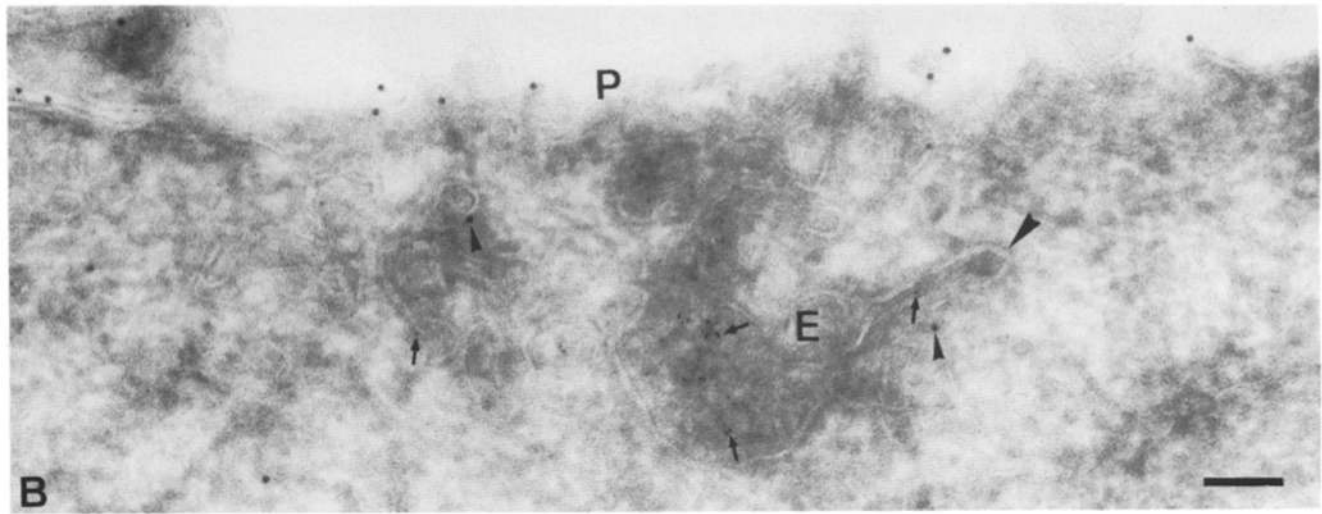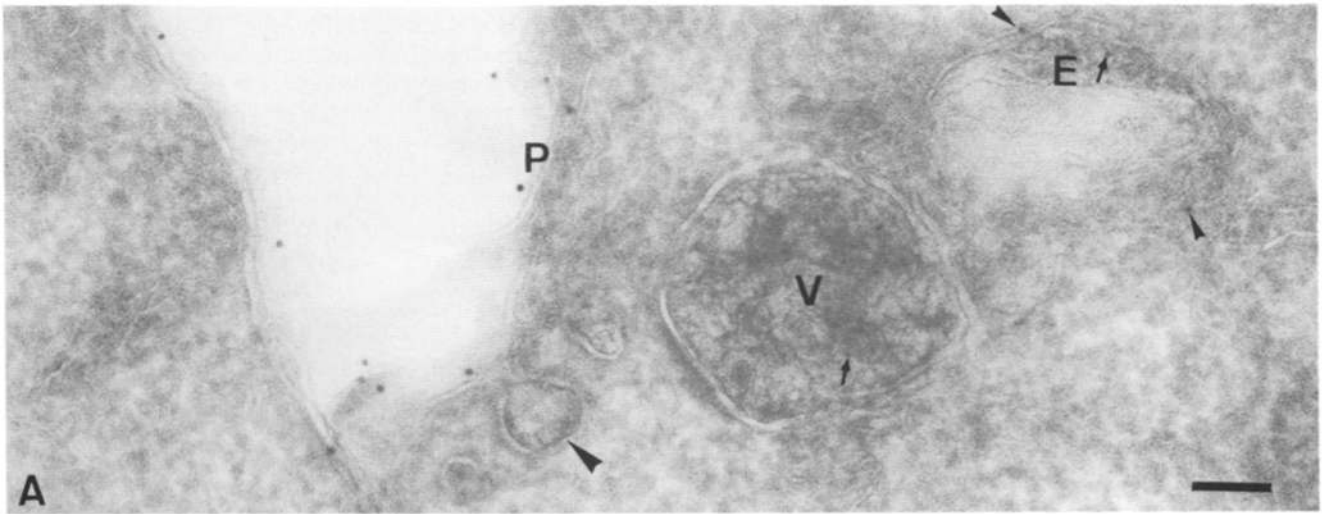
1366

Figure 10. Localization of annexin II to early endosomes. Labeling of annexin II (*small arrowheads*) on cryosections was as in Fig. 9. To identify early endosomes, the cells were incubated for 5 min at 37°C in the presence of 5 nm BSA gold (*small arrows*), as in Fig. 9 *B*. Annexin II was present on cisternal and tubular membranes of early endosomes (*E*), whereas little, if any, labeling was detected on vesicular regions (for example, *V* in *A*). In *B*, a clathrin-coated region of the early endosome is indicated by a large arrowhead. (*P*) Plasma membrane. (*Large arrowhead in A*) Unlabeled caveolae-like structure. Bar, 0.1 μm.

do not know whether monomeric or heterotetrameric annexin II is involved. However, protein c, which is transferred with high efficiency, exhibits a molecular weight in the range expected for the light chain, and, as the light chain, behaves as a peripheral protein.

The role of Ca^{++} itself in the endosome recognition/fusion process is far from clear. Ca^{++} chelators do not affect endosome fusion (Diaz et al., 1988; Wessling-Resnick and Braell, 1990). Under these conditions, however, annexin II remains associated to endosomal membranes (unpublished observation). In fact, extremely low free Ca^{++} concentrations ($<10^{-8}$ M) are needed for the association of the annexin II-light chain complex to phospholipid vesicles (Powell and Glenney, 1987). Chelators may, therefore, not suffice to cause the release of the membrane-associated annexin molecules. Annexin II may also remain membrane associated via interactions with other components of the endosomal membranes, possibly including one of the proteins we have identified, or via post-translational modifications. It is well established that annexin II can be phosphorylated, in particular by the p60^{src} kinase (Glenney, 1987; Gerke, 1989a), and recently p60^{c-src} has been localized to endosomal membranes (Kaplan et al., 1992).

Annexin II has been previously implicated in membrane-membrane interactions. The protein causes phospholipid vesicles and chromaffin granules to aggregate and to fuse (Drust and Creutz, 1988; Blackwood and Ernst, 1990). In addition, annexin II seems to be involved in the interactions of chromaffin granules with the plasma membrane (Ali et al., 1989), a process dependent on phosphorylation via protein kinase C (Sarafian et al., 1991). The precise function of annexin II is, however, not clear. From these data and from our observations, it is tempting to speculate that annexin II may be required for the formation of tight interactions between endosomal membranes, a step preceding the occurrence of fusion. Annexin II may also be directly involved in the fusion process itself, possibly in conjunction with other factors including NSF (Diaz et al., 1989; Rothman and Orci, 1992).

The studies mentioned above suggest that annexin II is involved in the fusion of secretory vesicles with the plasma membrane, whereas our work suggests that the same molecule, or a closely related species, is involved in early endosome–early endosome interactions. The subcellular distribution of annexin II which we have observed (plasma membrane and endosomes), is consistent with the participation of the protein in both pathways. However, we also observed some immunogold labeling of late endosomes and tubular membranes in the vicinity of the Golgi complex, presumably the TGN (not shown). Because our antibody only recognized annexin II on Western blots, these observations may suggest that annexin II is also involved in other pathways. Alternatively, our polyclonal antibody may recognize, albeit with a reduced efficiency, some epitopes common to other members of the annexin family. Several lines of evidence, in fact, suggest that other annexins are also involved in membrane traffic. Annexin VII (synexin) has been implicated in membrane interactions (Creutz et al., 1978) and can promote liposome fusion (Pollard et al., 1990a and b) as can annexin I (Blackwood and Ernst, 1990; Oshry et al., 1991). In addition, annexin VII (synexin), annexin IV and annexin VI (p67) can all cause chromaffin granule aggregation in

vitro (Zaks and Creutz, 1990b). Finally, the latter protein has also been recently implicated in the formation of coated vesicles at the plasma membrane (Lin et al., 1992). It will clearly be important to establish to what extent annexin proteins may have specific functions in different steps of membrane transport. Our future studies will attempt to elucidate the role of annexin II, and the other four proteins we have identified, in the process of early endosome–early endosome interactions.

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