killed. Histological sections were immunostained as previously described[28]. The mitotic index equals the percentage of BrdU-positive nuclei. Detection of apoptotic cells using the TUNEL-technique was performed as previously described[28]. The apoptotic index is the percentage of TUNEL-positive nuclei.

Histopathological analysis. Tissue embedding in JB4 plastic and preparation of semi-thin sections was as described[10]. For immunohistochemical analysis pancreas were removed and fixed in HBS-Ca^{2+} (HEPES-buffered saline, 1 mM $CaCl_2$) containing 4% paraformaldehyde for 2 h at 4 °C[14]. Tissues were incubated overnight at 4 °C in HBS-Ca^{2+}/30% sucrose, embedded in O.C.T. compound (Tissue Tek), and frozen in liquid nitrogen. Immunostaining was as described[14]. Specific binding of primary antibodies was visualized using either the ABC Vector-horseradish peroxidase kit according to the manufacturer's recommendations or fluorescence-labelled secondary antibodies as indicated. Antibodies were monoclonal rat anti-mouse E-cadherin (Zymed; 1:150), monoclonal rat anti-mouse N-cadherin[14] (a gift from M. Takeichi, Kyoto), polyclonal guinea pig anti-insulin (DAKO, 1:150), and rabbit polyclonal serum against mouse Na/K-ATPase (a gift from W. J. Nelson, Stanford, 1:500). Biotin-conjugated donkey anti-rat and anti-rabbit IgG (1:500) and Cy3-streptavidin (1:750) were purchased from Jackson ImmunoResearch Laboratories.

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A lipid associated with the antiphospholipid syndrome regulates endosome structure and function

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Little is known about the structure and function of membrane domains in the vacuolar apparatus of animal cells. A unique feature of late endosomes, which are part of the pathway that leads to lysosomes, is that they contain a complex system of poorly characterized internal membranes in their lumen. These endosomes are therefore known as multivesicular or multilamellar organelles[1,2]. Some proteins distribute preferentially within these internal membranes, whereas others are exclusively localized to the organelle's limiting membrane[3]. The composition and function of this membrane system are poorly understood. Here we show that these internal membranes contain large amounts of a unique lipid, and thus form specialized domains within endosomes. These specialized domains are involved in sorting the multifunctional receptor[4] for insulin-like growth factor 2 and ligands bearing mannose-6-phosphate, in particular lysosomal enzymes. We also show that this unique lipid is a specific antigen for human antibodies associated with the antiphospholipid syndrome[5,6]. These antibodies may act intracellularly by altering the protein-sorting functions of endosomes.

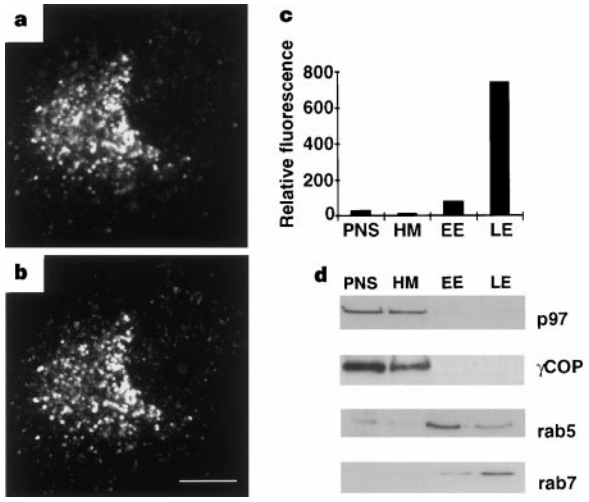

Figure 1 The 6C4 antigen is localized in late endosomes. **a**, **b**, BHK cells were fixed, permeabilized, and then double-labelled with 6C4 (**a**) and anti-rab7 (**b**) antibodies. Bar represents 10 μm. **c**, **d**, Subcellular fractions were analysed to establish the distribution of the following markers: **c**, 6C4 antigen using an ELISA assay; **d**, p97, γCOP, rab5 and rab7 by SDS-gel electrophoresis followed by western blotting. PNS, postnuclear supernatant; HM, heavy membranes; EE, early endosomes; LE, late endosomes.

We generated a monoclonal antibody (6C4), using endosomal membranes from BHK cells as antigens. The epitope recognized by 6C4 was absent from the cell surface (data not shown), and co-localized with the small GTPase rab7, a marker of late endosomes[7] (Fig. 1a, b). We did not identify any protein recognized by the 6C4 antibody. However, using an enzyme-linked immunosorbent assay (ELISA), we found that the total BHK lipid extract contained the immunoreactive material (Fig. 2d). The antigen was cellular, as no immunoreactivity was detected in the serum used to grow the cells; it was also sensitive to mild alkaline treatment (results not shown), and was therefore not a glycolipid. The antigen recognized by 6C4 co-purified with late endosomes containing rab7 (Fig. 1c, d), but not with p97 and γCOP, two proteins of the early protein-biosynthetic pathway[8–10], or with the early-endosomal marker rab5[11]. 6C4 therefore recognizes a late-endosomal lipid.

Late-endosomal fractions contained high amounts of triglycerides and cholesterol esters (Fig. 2a), and a limited subset of phospholipids (Fig. 2b), including a unique, highly hydrophobic, acidic phospholipid, which accounted for ~15% of the total phospholipids in the fractions (Table 1). This lipid migrated at the known position of lysobisphosphatidic acid (LBPA), and its deacylated form corresponded with the expected glycerylphosphorylglycerol backbone of LBPA (Fig. 2c). LBPA is not easily degraded by phospholipases[12], is structurally related to cardiolipin, and has been reported to distribute to lysosomes[13]. Purified LBPA was highly immunoreactive towards 6C4 in an ELISA assay (Fig. 2d); this immunoreactivity was confirmed by immunoblotting after chromatography (Fig. 2e). By dot blotting, 6C4 recognized only LBPA and none of the other lipids tested (Fig. 2f).

The precise subcellular distribution of LBPA was established by immunogold labelling of crysosections. As expected (Fig. 1), 6C4 labelled typical late-endosomal structures (Fig. 3b), which contained lgp120, an abundant membrane protein of BHK late

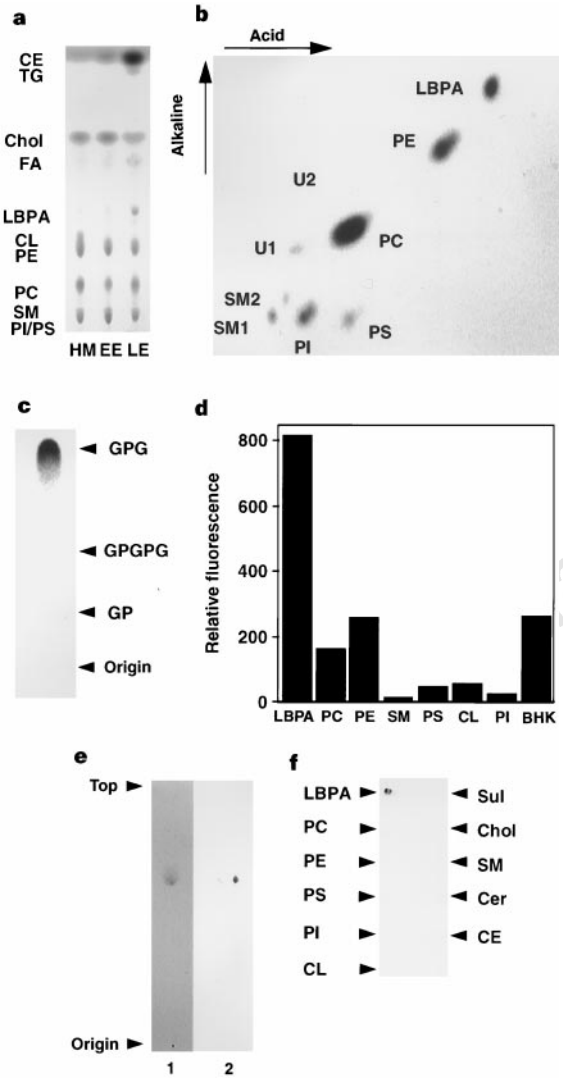

Figure 2 LBPA is enriched in late-endosome fractions and is the 6C4 antigen. **a**, Total lipid extracts (10-nmol phospholipid) from HM, EE or LE fractions (prepared as in Fig. 1c–d) were separated by TLC. CE, cholesterol ester; TG, triglyceride; Chol, cholesterol; FA, fatty acid; LBPA, lysobisphosphatidic acid; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine. **b**, ^{32}P-labelled phospholipids of the LE fraction were extracted and separated by two-dimensional TLC. U1 and U2, unidentified spots; other abbreviations as in **a**. Sphingomyelin migrates as two spots in this system. **c**, The water-soluble product generated by mild alkaline methanolysis of ^{32}P-labelled LBPA. GPG, glycerophosphorylglycerol; GPGPG, bis(glycerophosphoryl)glycerol; GP, glycerophosphate. **d**, Microtitre wells were coated with 1.5 nmol of each lipid indicated and incubated with 6C4. Amounts of bound 6C4 were then quantified using a fluorescent-detection system (relative fluorescence signals are indicated). BHK, total lipid from BHK cells; other abbreviations as in **a**. **e**, Purified LBPA was developed on silica-gel 60 HPTLC with chloroform/methanol/32% ammonia (65:35:5, v/v). Lane 1 shows LBPA itself detected by phosphate staining, and lane 2 shows the same lane immunoblotted with 6C4 and analysed by enhanced chemiluminescence (ECL). **f**, 1 n mol of each lipid shown was spotted on an HPTLC plate, and then blotted with the 6C4 antibody. Sul, sulfatides; Cer, cerebrosides; other abbreviations as in **a**.

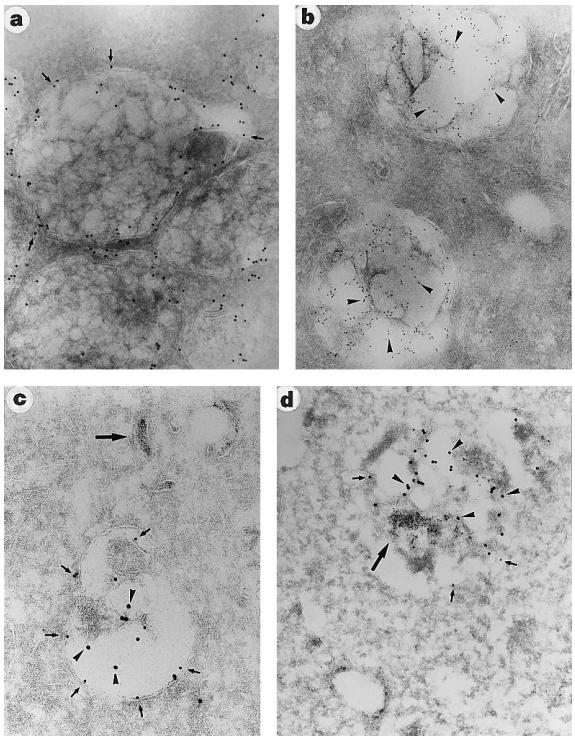

Figure 3 Electron microscopy. **a**–**b**, Cryosections were labelled with the 4A1 antibody against lgp120 (**a**, arrows) or the 6C4 antibody against LBPA (**b**, arrowheads) followed by rabbit antibodies against mouse IgG and then 10-nm (**a**) or 5-nm (**b**) protein-A gold. **c**, **d**, Early (**c**) or late (**d**) endosomes were labelled with 5-nm BSA–gold, which had been pre-internalized for 10 or 60 min at 37 °C (thick arrows). Cryosections were double-labelled with 6C4 (arrowheads) and 15-nm protein-A gold followed by 4A1 (small arrows) and 10-nm protein-A gold. Micrographs were printed lightly to reveal BSA–gold particles (thick arrows). Labelling efficiency is reduced as compared with **a**, **b**, because the linker rabbit antibodies against mouse IgG were omitted for double-labelling.

endosomes[14]. Bovine serum albumin (BSA)–gold that had been endocytosed for 60 min, but not for 10 min, also labelled 6C4-positive late endosomes (Fig. 3c, d). However, 6C4–immunogold particles were only associated with the complex system of internal membranes; these membranes are characteristic of late endosomes in animal cells. The absence of immunoreactivity on the surface of the limiting endosomal membrane was confirmed biochemically by comparing intact and disrupted late endosomes (results not shown). In contrast, lgp120 was found almost exclusively on the limiting membranes of the same endosomes (Fig. 3a, d)[3,14]. The composition of these internal endosomal membranes is therefore different from that of the limiting endosomal membrane, indicating that the internal membranes form specialized microdomains within endosomes.

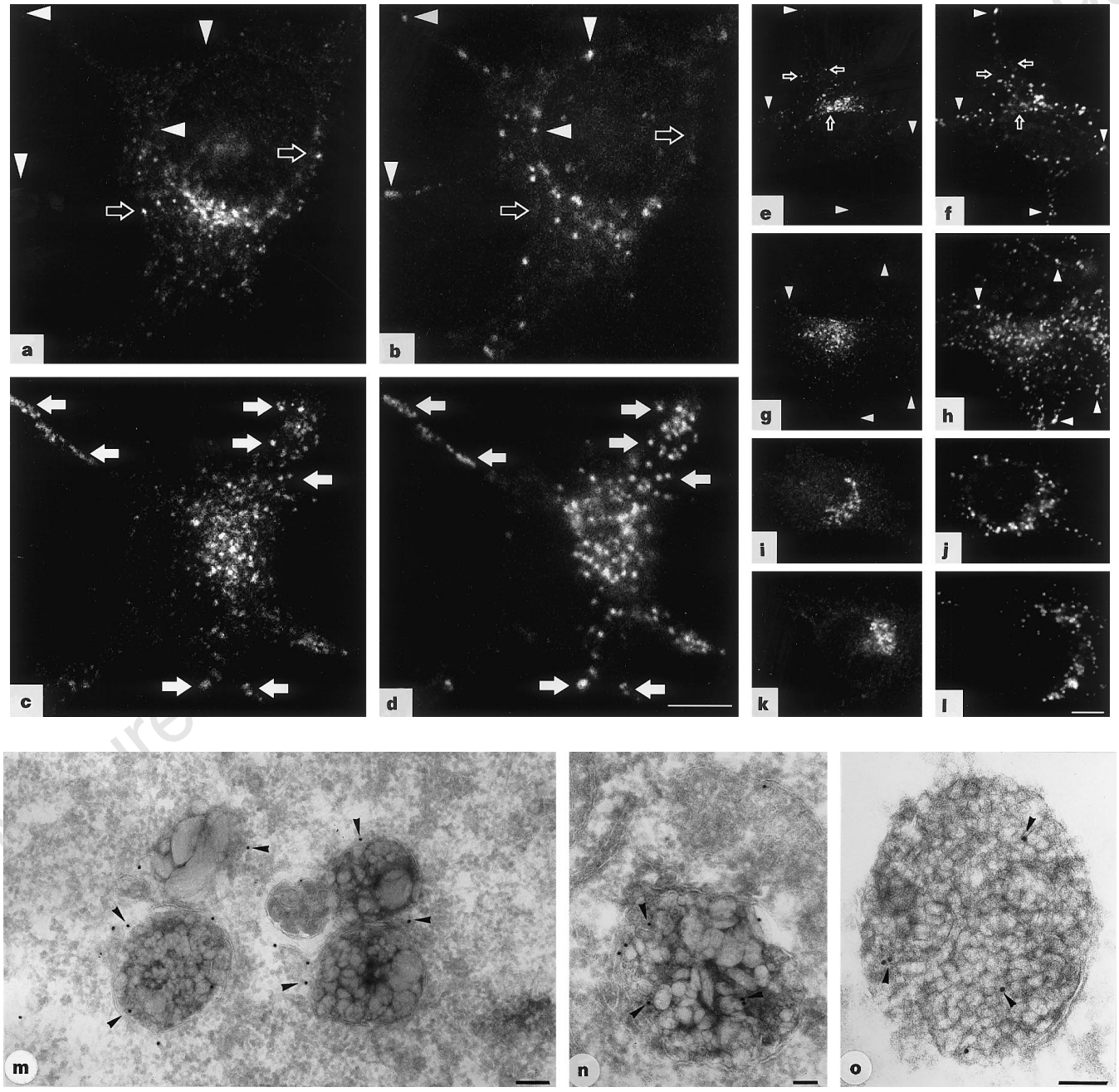

Figure 4 The ingested anti-LBPA antibody causes IGF2/MPR redistribution and alter late endosome ultrastructure. **a**, **b**, Steady-state distribution of IGF2/MPR (**a**) and LBPA (**b**) by indirect immunofluorescence microscopy. Arrows indicate IGF2/MPR-positive but LBPA-negative TGN elements; arrowheads indicate LBPA-positive but IGF2/MPR-negative late endosomes. **c**, **d**, Cells were incubated for 24 h with 50 μg ml^{-1} 6C4 (anti-LBPA) to allow endocytosis of the antibody to occur. IGF2/MPR (**c**) shows extensive co-localization (arrows) with endocytosed 6C4 antibodies (**d**). **e**, **f**, Steady-state distribution of IGF2/MPR (**e**) and lgp120 (**f**). Arrows indicate IGF2/MPR-positive but lgp120-negative TGN elements; arrowheads indicate lgp120-positive but IGF2/MPR-negative late endosomes. **g**, **h**, as for **c**, **d**, but 6C4 was replaced by 200 μg ml^{-1} 4A1. Arrowheads indicate vesicles containing endocytosed antibodies against lgp120 (**h**) but not IGF2/MPR (**g**) **i**, **j**, Steady-state distribution of TGN38 (**i**) and LBPA (**j**). **k**, **l**, Antibodies against LBPA (**l**), endocytosed as in **c**, **d**, do not cause redistribution of TGN38 (**k**). **m**–**o**, Cells were pre-incubated for 24 h with 6C4, as in **c**, **d**. Cryosections were prepared and labelled with 4A1 (anti-lgp120, **m**), or rabbit antibodies against mouse IgG (to detect internalized 6C4, **n**) or anti-IGF2/MPR antibodies (**o**) followed by 15-nm protein-A gold. Micrographs were printed lightly (as in Fig. 3c–d) to reveal gold particles. Bars in **a**–**l** represent 10 μm and in **m**–**o** represent 100 nm.

To investigate the possible role of LBPA-rich membrane domains, 6C4 was internalized by fluid-phase endocytosis from the medium. As expected, the endocytosed antibody accumulated in late endosomes after binding to its antigen (Fig. 4d). In contrast, a non-relevant antibody could not be accumulated intracellularly, presumably because it was transported to lysosomes and then degraded (results not shown). One of the important functions of late endosomes is the sorting of the multifunctional receptor (IGF2/MPR) for mannose-6-phosphate-bearing ligands and insulin-like growth factor 2 (ref. 4). IGF2/MPR delivers newly synthesized lysosomal enzymes, which carry the mannose-6-phosphate signal, from the *trans*-Golgi network (TGN) to late endosomes, and must recycle back to the TGN for re-utilization. In the BHK cells, IGF2/MPR is predominantly localized to the TGN at steady state, and does not co-localize with LBPA to any significant extent (Fig. 4a, b). However, after treatment with 6C4, but not with control antibodies (results not shown), IGF2/MPR distribution was markedly changed. Most of the receptor was then found in late endosomes, and co-localized with LBPA (Fig. 4c, d; see also Fig. 4n, o) in >70% of the 6C4-treated cells, whereas co-localization occurred in <5% of untreated cells.

We performed the same experiment with anti-lgp120 antibody; lgp120 localizes to the limiting membrane of late endosomes. The antibody also accumulated in the cells (Fig. 4h), as would be predicted because the epitope is lumenal, but IGF2/MPR trafficking was unaffected, even when using high antibody concentrations (Fig. 4j). Moreover, the distribution of TGN38, a protein which also cycles rapidly between TGN and endosomes and localizes to the TGN at steady state[15] (Fig. 4i, j), was unaffected after 6C4 treatment (Fig. 4k, l). Ingested antibodies against LBPA therefore prevent proper IGF2/MPR trafficking in late endosomes in a highly selective manner, and thus interfere with late-endosome-sorting functions.

Endocytosed antibodies did not cause general perturbations of late endosomes/lysosomes, as the acidification properties of these cellular compartments were unaffected (results not shown). However, the treatment caused the appearance of a population of abnormal, electron-dense late endosomes, filled with densely packed membranes (Fig. 4m–o); such endosomes were rarely seen in control cells (see Fig. 3c, d). These abnormal late endosomes were lgp120-positive (Fig. 4m), and contained both endocytosed 6C4 antibodies (Fig. 4n) and IGF2/MPR (Fig. 4o), as expected (Fig. 4c, d). In contrast, uptake of anti-lgp120 antibodies (see Fig. 4h, j) did not alter endosome ultrastructure, nor did they cause lgp120 redistribution to internal membranes (results not shown). The striking disorganization of the inner-membrane system caused by anti-LBPA antibodies probably prevents proper segregation of IGF2/MPR molecules, thus causing them to be trapped within late endosomes. Indeed, IGF2/MPR, in contrast to lgp120, seems to segregate preferentially to LBPA-rich internal domains (Fig. 4o)[3], although the mechanism that regulates this selective incorporation is unknown. However, our data suggest that anti-LBPA antibodies selectively alter the organization and dynamics of the complex system of internal membranes within late endosomes.

Human antiphospholipid antibodies are associated with a well described, yet poorly understood, antiphospholipid syndrome[5,6] that is partly characterized by increased risk of thrombosis, recurrent fetal loss and thrombocytopenia[16]. A current view is that various antiphospholipid antibodies exist, some being directed against phospholipids alone, others being directed against protein–phospholipid complexes, and a third group being directed against proteins such as β2-glycoprotein I (ref. 17). No antiphospholipid antigen had been localized at the cellular or subcellular level until now, and little is known about how these antibodies act. Antibodies of the antiphospholipid family that bind pure lipids in solid-phase assays are generally referred to as anti-cardiolipin, as cardiolipin is the most commonly used antigen, although these antibodies also bind phosphatidylserine and other anionic phospholipids to varying extents[5].

As shown in Fig. 5a, b, human sera from patients with antiphospholipid syndrome, but not from healthy donors (results not shown), labelled late endosomes containing LBPA, but did not label the plasma membrane (which contains phosphatidylserine) or mitochondria (which contain cardiolipin). A comparative ELISA assay showed that these sera exhibited a strong immunoreactivity

Table 1 Phospholipid composition of the fractions

	HM	EE	LE
PC	52.9 ± 1.0	47.4 ± 0.3	49.2 ± 1.4
PE	18.2 ± 3.6	23.2 ± 1.8	18.5 ± 0.4
SM	5.3 ± 0.1	9.0 ± 0.3	3.2 ± 0.3
PS	5.9 ± 0.2	8.5 ± 0.2	3.9 ± 0.5
CL	3.8 ± 0.6	0.2 ± 0.2	0.2 ± 0.1
PI	8.6 ± 0.1	8.0 ± 1.0	7.0 ± 0.4
LBPA	0.8 ± 0.4	1.3 ± 0.4	13.8 ± 0.8
U1	3.3 ± 1.8	1.4 ± 0.5	2.4 ± 0.0
U2	1.2 ± 0.2	1.0 ± 0.1	2.0 ± 0.3

Cells were labelled with ^{32}P for 20 h before the experiments, homogenized, and then fractionated by flotation on a step sucrose gradient. Lipids from each fraction were extracted and then separated (see Fig. 2b). Radioactivity for each spot was quantified, and normalized to 100% for each fraction. Data represent the mean of two independent experiments. CL, cardiolipin; EE, early endosomes; HM, heavy membranes; LE, late endosomes; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; U1 and U2, unidentified spots.

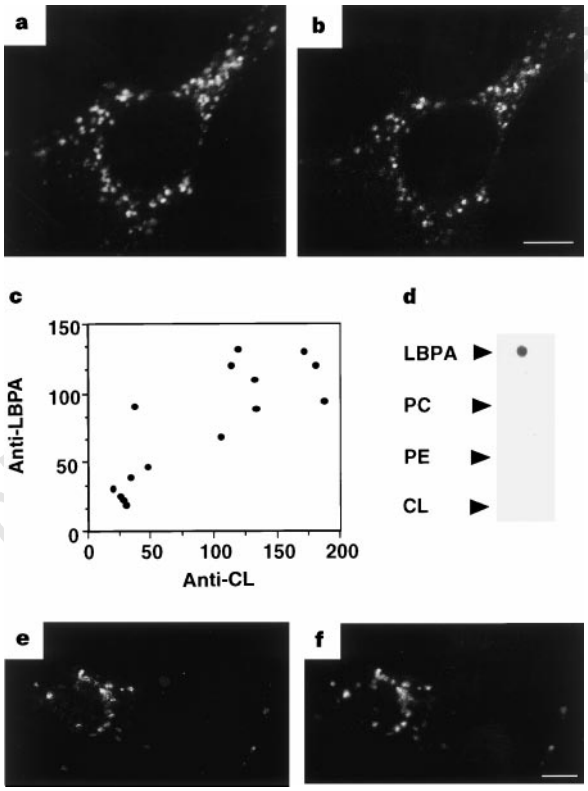

Figure 5 Sera from patients with antiphospholipid syndrome recognize LBPA. **a**, **b**, Cells were double-labelled with 6C4 (**a**) and aPL antiserum diluted 1:100 (**b**) and analysed by immunofluorescence. Three sera with a high anti-cardiolipin titer were tested (see **c**), and all labelled late endosomes containing LBPA. Bar represents 10 μm. **c**, Purified CL (cardiolipin) and LBPA were separately bound to microtitre wells, and reacted with 1:100 dilutions of different human sera. The anti-LBPA activity of each serum, quantified by ELISA, is plotted as a function of its anti-CL activity. **d**, The indicated lipids were spotted onto HPTLC plates and blotted with a 10-fold dilution of the serum used in **b**. Abbreviations as in Fig. 2. **e**–**f**, Cells were incubated for 13 h with 10% of the human serum used in **b** and processed as in Fig. 4c–d. IGF2/MPR (**e**) then co-localized with LBPA (**f**).

against LBPA that correlated well with their established immunoreactivity against cardiolipin (Fig. 5c). Recent studies suggest that cardiolipin becomes oxidized when immobilized onto plastic in ELISA reactions, and that oxidation products, rather than cardiolipin itself, are recognized by patients' antisera[18]. We therefore tested the reactivity of human sera after spotting lipids directly onto high-performance thin-layer chromatography (HPTLC) plates, and found that only LBPA was antigenic (Fig. 5d). Finally, after incubation of living cells with serum from a patient with antiphospholipid syndrome, endocytosed antibodies accumulated in late endosomes containing LBPA (results not shown). Concomitantly, IGF2/MPR was redistributed, as observed after 6C4 uptake (Fig. 4c, d), and co-localized with LBPA in late endosomes (Fig. 5e–f); human sera from healthy donors had no effects (results not shown).

We show, therefore, that inner membranes of late endosomes are highly specialized and contain high amounts of the unconventional lipid LBPA, and that these LBPA-rich membranes play an important role in proper sorting and trafficking of IGF2/MPR. Our results indicate that LBPA is one of the physiological antigens that is recognized by sera from patients with antiphospholipid syndrome, and suggest that these antibodies exert some pathological effects intracellularly by altering endosomal sorting and/or trafficking of IGF2/MPR. As IGF2/MPR is multifunctional, changes in its transport cycle may have multiple effects, including in vesicular traffic[19], lysosome biogenesis[4], cell growth[20], and angiogenesis[21]. The antiphospholipid syndrome, particularly fetal loss, may be at least partly related to the role of IGF2/MPR in endothelial cell migration and neovascularization[21,22]. □

Methods

Cells and reagents. Baby hamster kidney (BHK-21) cells were grown and maintained as described[23]. Where indicated, cells were incubated for 24 h with medium supplemented either with hybridoma supernatant containing 50 μg ml^{-1} 6C4 or 200 μg ml^{-1} 4A1 monoclonal antibody, or with 10% human serum. The 6C4 monoclonal antibody (IgG) was generated after intrasplenic injection of immuno-isolated endosomes[23]. The 4A1 monoclonal antibody (IgG) has been described[14]. Antibodies against rab5 or rab7 were raised against carboxy-terminal peptides[7]. Polyclonal antibodies against γCOP, IGF2/MPR and TGN38 were gifts from F. Wieland (Ruprecht Karl University, Heidelberg), B. Hoflack (Institut Pasteur, Lilles), and G. Banting (University of Bristol), respectively. The anti-p97 monoclonal antibody was a gift from J. Peters (Harvard University, Boston). Sera from patients with antiphospholipid syndrome were from the Haemostasis Unit, University Hospital of Geneva. LBPA was purified from BHK lipid extracts by preparative thin-layer chromatography after silica gel and DEAE column chromatography; the purified lipid migrated as a single spot on HPTLC plates, was resistant to phospholipase A_2 (ref. 12), and did not react with ninhydrin, as expected. Egg-yolk phosphatidylcholine, egg phosphatidylethanolamine, brain phosphatidylserine, bovine-liver phosphatidylinositol, bovine-heart cardiolipin, brain sphingomyelin and brain cerebrosides were from Avanti polar lipids. Bovine-brain sulfatides, cholesterol and cholesterol oleate were from Sigma. Tetraoleoyl bisphosphatidic acid was from Doosan Serdary.

Lipid analysis. Subcellular fractions were prepared using a step-flotation gradient[11,14]. Lipids were extracted and separated on silica-gel 60 HPTLC plates (Merck) by a two-step migration in the same direction, using chloroform/methanol/32% ammonia (65:35:5, v/v), then hexane/diethylether/acetic acid (16:4:2, v/v). Lipids were visualized after charring with cupric acetate. $^{32}P_i$-labelled lipids were extracted and then separated by two-dimensional chromatography[24], detected by autoradiography, and quantified using the Molecular Imager System (BioRad). Mild alkaline methanolysis of LBPA was performed as described[25]. The reference compounds were prepared from phosphatidic acid (glycerophosphate), cardiolipin (bis(glycerophosphoryl)-glycerol) and bisphosphatidic acid (glycerylphosphorylglycerol).

Immunodetection. An ELISA assay of lipids was performed as described[26]; for human sera, microtitre wells were blocked with 10% FCS instead of 3% BSA (ref. 27). TLC plates were blotted with antibodies using a modified version of the method described in ref. 28. After treatment with polyisobutylmethacrylate for 5 s, HPTLC plates were blocked for 2 h either with 20-mM HEPES at pH 7.4 and 150-mM NaCl (HEPES–NaCl) containing 3% BSA (for 6C4), or with 10% FCS in HEPES–NaCl (for human sera). After washing, the plates were incubated for 2 h with 10 μg ml^{-1} 6C4 in HEPES–NaCl containing 1% BSA, or with 10× diluted human aPL antisera in HEPES–NaCl containing 10% FCS. The plates were reincubated with peroxidase-conjugated sheep antibodies against mouse IgG for 1.5 h. Bound antibodies were detected by chemiluminescence using the ECL reagent (Amersham).

Microscopy. Immunofluorescence was performed as described[29]. To label early or late endosomes for electron microscopy, BHK cells were incubated for 10 or for 60 min, respectively, at 37 °C in the presence of 5-nm BSA–gold. Cells were then processed for immunogold labelling[30].

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