**Specific role of phosphoinositide 3-kinase p110α in the regulation of phagocytosis and pinocytosis in macrophages**

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PI3K (phosphoinositide 3-kinase) α has been implicated in phagocytosis and fluid-phase pinocytosis in macrophages. The subtype-specific role of PI3K in these processes is poorly understood. To elucidate this issue, we made Raw 264.7 cells (a mouse leukaemic monocye–macrophage cell line) deficient in each of the class-I PI3K catalytic subunits: p110α, p110β, p110δ and p110γ. Among these cells, only the p110α-deficient cells exhibited lower phagocytosis of opsonized and non-opsonized zymosan. The p110α-deficient cells also showed the impaired phagocytosis of IgG-opsonized erythrocytes and the impaired fluid-phase pinocytosis of dextran (molecular mass of 40 kDa). Receptor-mediated pinocytosis of Dil (1,1-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate)-labelled acetylated low-density lipoprotein and fluid-phase pinocytosis of Lucifer Yellow (molecular mass of 500 Da) were resistant to p110α depletion. None of these processes were impaired in cells lacking p110β, p110δ or p110γ, but were susceptible to a pan-PI3K inhibitor wortmannin. In cells deficient in the enzymes catalysing PtdIns(3,4,5)P3 breakdown [PTEN (phosphatase and tensin homologue deleted on chromosome 10) or SHIP-1 (Src-homology-2-domain-containing inositol phosphatase-1)], uptake of IgG-opsonized particles was enhanced. These results indicated that phagocytosis and fluid-phase pinocytosis of larger molecules are dependent on the lipid kinase activity of p110α, whereas pinocytosis via clathrin-coated and small non-coated vesicles may depend on subtypes of PI3Ks other than class I.

**Key words:** Fcγ receptor (FcγR), macrophage, phagocytosis, phosphoinositide 3-kinase α (PI3Kα), pinocytosis, zymosan.

**INTRODUCTION**

Phagocytosis and pinocytosis have substantial roles in the entry of pathogens into host cells, innate and adaptive immunity and apoptosis [1–3]. The processes also play a critical part in foam-cell formation of macrophages to cause development of atherosclerotic plaques [4]. The mechanism of their regulation therefore attracts the attention of various medical specialties.

Phagocytosis is a process by which solid particles are taken up by professional phagocytes such as macrophages, neutrophils and dendritic cells. Pinocytosis occurs in all cells and functions to engulf fluid and solutes. The process of phagocytosis involves specific cell-surface receptors. One group of the receptors, including mannose receptors and TLRs (Toll-like receptors), interact directly with pathogens by recognizing the conserved motifs of micro-organisms [5,6]. Another group of receptors recognize opsonins, host molecules (e.g. antibodies and complement components) that interact with the surface molecules of invaders [7,8]. Fluid-phase pinocytosis occurs by at least four basic mechanisms, namely macrophagocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis and clathrin- and caveolin-independent endocytosis [9].

Mammalian PI3Ks (phosphoinositide 3-kinases) can be grouped into three major classes (I, II and III) on the basis of their primary sequences, mechanism of regulation and substrate specificities [10]. Of the class-I PI3Ks, class-IA subtypes are heterodimers comprising a catalytic subunit (p110) and a regulatory subunit (p85) [10]. These subtypes are thought to be the major in vivo source of PtdIns(3,4,5)P3, that is produced upon activation of receptors possessing protein-tyrosine kinase activity or receptors coupling to Src-type protein-tyrosine kinases [10]. In mammals, there are multiple isoforms of class-IA PI3K [10]. Different genes encode class-IA catalytic subunits (referred to as p110α, p110β and p110δ), whereas two genes encode the associating regulatory subunits (referred to as p85α and p85β). Class I includes another member, PI3Kγ, which is mainly expressed in haematopoietic cells. This subtype consists of a catalytic subunit (p110γ) and a regulatory subunit (p101) and is classified as class IB [11,12]. PI3Kγ can be activated by the βγ subunits of G-proteins and thus mediate the signal from G-protein-coupled receptors [13,14]. Class-II PI3Ks are 170–210 kDa proteins with an in vitro substrate specificity restricted to PtdIns and PtdIns4P [15]. Class-III PI3Ks are homologues of the baker’s yeast (Saccharomyces cerevisiae) Vps34p (vacuolar protein sorting protein 34) and exclusively phosphorylate PtdIns [16].

PI3K has been implicated in the regulation of phagocytosis, pinocytosis, membrane ruffling, cytoskeletal organization and intracellular membrane traffic [1,2]. The involvement of PI3K in phagocytosis is well characterized, particularly by examining the FcγR (Fcγ receptor)-mediated process in macrophages. PtdIns(3,4,5)P3 has been shown to localize in the phagocytic cup at the early stage of phagocytosis before closure of the phagosomal vacuole [17–19]. The accumulation of PtdIns(3,4,5)P3 is transient, followed by the accumulation of PtdIns3P concomitant with

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**Abbreviations used:** Dil, 1,1-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate; DTT, dithiothreitol; EA, IgG-coated SRBC; ERK, extracellular-signal-regulated kinase; FCS, fetal-calf serum; FcγR, Fcγ receptor; LDL, low-density lipoprotein; LPS, lipopolysaccharide; pAkt(Ser473), Akt/protein kinase B phosphorylated on Ser473; pERK1/2(Thr202/Tyr204), ERK 1/2 phosphorylated on Thr202 and Tyr204; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SHIP, SH2 (Src homology 2)-domain-containing inositol phosphatase; shRNA, short hairpin RNA; SRBC, sheep red blood cell; TLR, Toll-like receptor.

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the sealing of the vacuoles [18,20]. In macropinocytosis, the PtdIns(3,4,5)P3 concentration has been shown to increase dramatically in the macropinocytic cup, and PI3K is suggested to function in the closure of ruffles to form macropinosomes, as in the case of phagosome formation from the phagocytic cup [1,21]. Receptor-mediated pinocytosis of soluble ligands through clathrin-coated vesicles has been shown to be less sensitive to PI3K inhibitors than macropinocytosis, indicating that the roles of PI3K in the different types of pinocytosis may not be the same [21].

Recent studies have suggested that class-I PI3K triggers initiation of endocytosis [2], but the subtype of class-I PI3K responsible has not been elucidated. To examine this issue, we prepared cells deficient in each of the class-I PI3K subtypes and determined their abilities to take up different sizes of particles and solutes. We show that the phagocytosis of particles and the pinocytosis of larger molecules are critically dependent on the p110α subtype of class-I PI3K. We also show that receptor-mediated pinocytosis and micropinocytosis of smaller molecules are not controlled by the class-I subtypes of PI3K.

EXPERIMENTAL

Materials
The sources of materials were as follows: ATP, LPS (lipopolysaccharide) (Escherichia coli serotype 0111:B4), FITC–dextran (molecular mass of kDa 40), Lucifer Yellow (molecular mass of 522 Da) and calycin-A were from Sigma; zymosan, FITC–zymosan, anti-zymosan IgG and Dil (1′,1′,3-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate)-labelled acetylated LDL (low-density lipoprotein) were from Molecular Probes; SRBCs (sheep red blood cells) were from Japan Lamb; wortmannin was from Kyowa Medex; RPMI 1640 medium was from Invitrogen; the protein assay kit was from Bio-Rad; cytochalasin-E and latrunculin-B were from ICN; 1% fetal calf serum (FCS) without NaHCO3, fortified with 20 mM Hepes/NaOH, 100 mM NaCl, 1 mM Na2VO4, 1 mM EDTA, 0.1% BSA, 20 mM NaF, 1 mM PMSF, 2 μM leupeptin, 20 μM p-amidino-PMSF and 1 mM DTT (dithiothreitol) were from Matreya; phosvitin was from InterCell Technologies; anti-pERK1/2 (Thr202/Tyr204) antibodies were from BD Transduction Laboratories; anti-Akt1/2, anti-p110α antibodies were from Abcam; anti-p110γ antibodies were from Santa Cruz Biotechnology; anti-SHIP-1 antibodies were from Upstate; anti-Src antibodies were from Cell Signaling Technology; anti-Akt1/2, anti-p110β and anti-p110γ antibodies were from Intercell Technologies; anti-pERK1/2 (Thr202/Tyr204) antibodies were from Santa Cruz Biotechnology; anti-SRC antibody was from Calbiochem; antibodies against pAkt(Ser473) (Akt/protein kinase B phosphorylated on Ser473) and PTEN (phosphatase and tensin homologue deleted on chromosome 10) were from Cell Signalling Technology; anti-Akt1/2, anti-p110β and anti-p110γ antibodies were from Santa Cruz Biotechnology; anti-SRC antibody was from Intercell Technologies; anti-pERK1/2(Thr202/Tyr204) [anti-ERK (extracellular-signal-regulated kinase) 1/2 phosphorylated on Thr202 and Tyr204], anti-p110α, anti-SHIP-1 [anti-(Src-homology-2-domain-containing inositol phosphatase)-1] and anti-p110β antibodies were from BD Transduction Laboratories; phosphatidylinositol and phosphatidylinositol were from Matreya; [γ-32P]ATP was from New England Nuclear; and Na243CrO4 was from MP Biomedicals. Anti-p85α polyclonal antibody was prepared by immunizing rabbits with full-length GST (glutathione transferase)–p85α.

Cells
Female C57/BL6 mice (age 8–12 weeks) were purchased from Japan SLC. PI3Kγ−/− mice with a C57/BL6 background were kindly donated by Dr Takehiko Sasaki (Department of Pathology and Immunology, Akita University, Akita, Japan); peritoneal macrophages were harvested from these mice. Briefly, mice were injected intraperitoneally with 2 ml of 3% thioglycollate broth. After 3 days, the mice were anaesthetized with ether inhalation and the peritoneal exudate was harvested by washing the peritoneal cavity with ice-cold PBS. Cells were seeded (about 5 × 105 cells/well) in 24-well plates and allowed to adhere to dishes by placing them in an atmosphere of humidified 5% CO2 at 37°C for 1–2 h in RPMI 1640 medium supplemented with 10% (v/v) FCS (fetal calf serum). Non-adherent cells were washed off with PBS and attached cells were designated macrophages. The mouse macrophage-like cell line Raw 264.7 was maintained in RPMI 1640 medium containing 4.5 g/l glucose and 10% FCS in humidified 5% CO2 at 37°C. For the determination of phagocytosis or pinocytosis, cells were cultured in 96-well plates or 24-well plates respectively. Culture medium were aspirated off and replenished with incubation buffer (complete RPMI 1640 medium without NaHCO3, fortified with 20 mM Hepes/NaOH, pH 7.4). Activities were then determined by incubating cells at 37°C in a water bath under ambient conditions.

Animal experiments were performed with approval by the institutional ethics committee of Hiroshima University, in accordance with the Standards Relating to the Core and Management of Experimental Animals in Japan.

Western blotting
Cells were washed with PBS and lysed in 50 μl of lysis buffer containing 25 mM Tris/HCl (pH 7.4), 0.5% Nonidet P40, 150 mM NaCl, 1 mM Na2VO4, 1 mM EDTA, 0.1% BSA, 20 mM NaF, 1 mM PMSF, 2 μM leupeptin, 20 μM p-amidino-PMSF and 1 mM DTT (dithiothreitol). Cell lysates were centrifuged at 20000 g for 10 min. Protein concentrations in the resultant supernatants were determined with the Bio-Rad protein assay kit. Total cell lysates were mixed with 10 μl of 5× sample buffer [62.5 mM Tris/HCl pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.02% Bromphenol Blue] and heated at 100°C for 3 min. Proteins (100 μg/lane) were separated by SDS/PAGE. They were then transferred by electrophoresis on to PVDF membranes (Millipore). The membranes were blocked in 5% (w/v) dried skimmed milk powder and incubated with the appropriate antibodies. Associated antibodies were detected by enhanced chemiluminescence (PerkinElmer).

Phagocytosis of zymosan
FITC-labelled zymosan particles were mixed with a 4-fold excess of unlabelled zymosan to expedite counting. Particles were then opsonized (or not opsonized) with normal mouse serum or with anti-zymosan IgG at 37°C for 30 min before use. Subconfluent monolayers of Raw 264.7 cells (103/well of a 96-well plate) were treated with or without inhibitors at 37°C for 15 min, after which 5 × 105 zymosan particles were added and the mixture incubated at 37°C for a further 60 min. Phagocytosis was stopped by the addition of ice-cold PBS. Cells were then washed three times with PBS, fixed with 4% (v/v) paraformaldehyde for 10 min at room temperature (23–27°C), and finally rinsed with PBS. Fluorescence images (excitation at 488 nm, emission at 525 nm) and phase-contrast images of at least 100 macrophages from three randomly selected fields were taken using a confocal microscope. The mean numbers of ingested zymosan particles were expressed as particles/one Raw 264.7 cell.

Preparation of IgG-coated red blood cells and measurement of phagocytosis
SRBCs were labelled with 51Cr as described previously [22]. EAs (IgG-coated SRBCs) were prepared by incubating labelled cells with rabbit anti-SRBC antibody at 37°C for 10 min in 5 mM veronal buffer (pH 7.5) supplemented with 0.1% gelatin, 75 mM NaCl, 0.15 mM CaCl2, 0.5 mM MgCl2 and 10 mM EDTA, followed by incubation on ice for 15 min. EAs were washed three times with GVB [5 mM veronal (sodium barbital) buffer, pH 7.5, supplemented with 0.1% gelatin, 75 mM NaCl, 0.15 mM CaCl2, 0.5 mM MgCl2 and 10 mM EDTA, followed by incubation on ice for 15 min. EAs were washed three times with GVB [5 mM veronal (sodium barbital) buffer, pH 7.5, supplemented with 0.1% gelatin, 75 mM NaCl,
Table 1  Oligonucleotides with sequences targeting p110α, p110β, p110γ, p110δ, PTEN or SHIP-1

<table>
<thead>
<tr>
<th>Oligonucleotide target</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>p110 isoforms</td>
<td></td>
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<tr>
<td>α</td>
<td>5'-GCCCTTCTCTTGAA(X)19GGGTGCA-3'</td>
</tr>
<tr>
<td>β</td>
<td>5'-GAGCAGCGCTGTTATATG-3'</td>
</tr>
<tr>
<td>γ</td>
<td>5'-GCAATGTTGAAACAGATGAA-3'</td>
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<tr>
<td>δ</td>
<td>5'-CAGTCAAGTGCTTTGGA-3'</td>
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<tr>
<td>PTEN</td>
<td>5'-GACCAAAATTGTGATGTA-3'</td>
</tr>
<tr>
<td>SHIP-1</td>
<td>5'-GGAATGAAATGCTTGAGA-3'</td>
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0.15 mM CaCl₂ and 0.5 mM MgCl₂] and finally suspended in short-term incubation buffer. Binding and phagocytosis of EAs were measured as reported previously [22]. Monolayers of Raw 264.7 cells (2 × 10⁵ cells/well of a 24-well plate) were incubated with 11Cr-labelled EAs (2 × 10⁵ cells) at 37°C for the indicated periods of time. Monolayers were washed three times with PBS to remove unbound EAs, before brief exposure to 0.1 ml of hypo-osmotic PBS (5-fold-diluted). The radioactivity released into the supernatant during hypo-osmotic shock was measured for the amount of EAs bound on the surface of the phagocytes. Monolayers were washed a further three times with PBS and finally solubilized in 0.5% Triton X-100. The radioactivity in the solution was measured for the amount of engulfed EAs. The number of EAs was calculated from the radioactivity and expressed as incorporated or bound EAs/one Raw 264.7 cell.

Fluid-phase pinocytosis assay

FITC-labelled dextran (0.5 mg/ml), Lucifer Yellow (0.5 mg/ml) and Dil-acetylated LDL (8 μg/ml) were used to measure pinocytosis. Cells (5 × 10⁵ cells/well of a 12-well plate) were incubated in the presence or absence of inhibitors, and the labelled targets added. After further incubation for various intervals, the medium were aspirated. Cells were washed twice with 0.1% BSA/PBS, three times with PBS, and finally solubilized in 0.5% Triton X-100. The radioactivity in the solution was measured for the amount of engulfed EAs. The number of EAs was calculated from the radioactivity and expressed as incorporated or bound EAs/one Raw 264.7 cell.

RNA interference

Oligonucleotides with the sequence targeting p110α, p110β, p110γ, p110δ, PTEN or SHIP-1 were cloned into the pH1 vector downstream of the H1 RNA promoter as described in [23,24] to express siRNA (small interfering RNA) hairpins. The sequences targeted are shown in Table 1. For each of the targeted sequences, a pair of oligonucleotides were synthesized with sequences 5′-C-C(CX)₉-TTCAAGAGA(Y)₉-TTTTTGGAAA-3′ and 5′-CTAGT-TTCCAAAAY(Y)₉-TCTCTTGGAAX₉-GGGGTGCA, where (X)₉ is the coding sequence and (Y)₉ is the complementary sequence. The oligonucleotide pair were annealed and ligated downstream of the H1 RNA promoter at the PstI and XbaI sites of the pH1 vector. The vectors were transfected into Raw 264.7 cells [(5–10) × 10⁵ cells] at 250 V/950 μF (Gene Pulser II; Bio-Rad). When p110β was targeted, two shRNA (short hairpin RNA) probes with different sequences were transfected simultaneously to fully inhibit protein expression. At 24 h after transfection, puromycin (5 μg/ml) was added to the cells for selection, and the incubation continued for several days. Resistant colonies (50–100) were replated on 96-well plates, to give 0.5–1.0 cells/well, and cultured for additional weeks to obtain monoclonal PI3K-deficient cells. To determine the efficiency of gene silencing, total RNA was isolated with RNeasy (Qiagen) and mRNA was quantified by reverse transcription–PCR. Control cells were prepared in the same way with pH1 vector containing a 400 bp stuffer sequence instead of the probe sequence.

PI3K activity assay

Anti-p85 immunoprecipitates were assayed for their PI3K activities. The cell lysate was precleared with preimmune IgG and Protein G-Sepharose at 4°C for 1 h, and subjected to immunoprecipitation with anti-p85 antibody. Immunoprecipitates were washed twice with lysis buffer, twice with 40 mM Tris/HCl (pH 7.4)/1 mM DTT/0.5 M LiCl, and twice with 40 mM Tris/HCl (pH 7.4)/1 mM DTT/100 mM NaCl. Aliquots (equivalent to 2 × 10⁶ cells) of the immunoprecipitates were suspended in 0.1 ml of a reaction mixture consisting of 40 mM Tris/HCl, pH 7.4, 0.5 mM EGTA, 0.2 mM PtdIns, 0.2 mM phosphatidylserine, 5 mM MgCl₂ and 0.2 mM (1 μCi) of [γ⁻³²P]ATP. The reaction was allowed to proceed at 37°C for 15 min before termination by adding 20 μl of 8% (w/v) HClO₄ and 0.45 ml of chloroform/methanol (1:2, v/v). After vigorous stirring, 0.15 ml of chloroform and 0.15 ml of 8% HClO₄ were added to the mixture to separate the organic phase. The organic phase was washed with chloroform-saturated 0.5 M NaCl containing 1% HClO₄ and evaporated to dryness. The extract was dissolved twice in 15 μl of chloroform/methanol (9:1, v/v) to be spotted on to a silica-gel plate (Silica Gel 60; Merck). The plate was developed in chloroform/methanol/28% NH₄OH/water (70:100:25:15 by vol.), dried and visualized for radioactivity in the phosphoinositide fraction using a Fuji BAS3000 imaging analyser.

RESULTS

Involvement of class-I PI3Ks in phagocytosis

Figure 1(A) shows microscopic images of Raw 264.7 cells given IgG-opsonized zymosan. The phase-contrast image of control cells indicates that multiple particles were engulfed by one cell. Fluorescence micrographs and the merged image show that a part of the engulfed particles was labelled by fluorescence. This is because zymosan particles were given as a mixture of FITC-labelled and non-labelled forms for convenience in quantification. When cells were treated with wortmannin before zymosan addition, uptake was minimal and particles were observed at the outer surface of cells. Because the micrographs were taken after the cells had been washed repeatedly, these particles were probably recognized by cell-surface receptors, but were not engulfed by the cells. In Figure 1(B), the number of the fluorescent particles within cells was counted using merged images. Counting was corrected by the content of the labelled form in the zymosan mixture to estimate the endocytotic activity as particles per one cell. In this experiment, zymosan particles were used in non-opsonized, IgG-opsonized or C3bi [a fragment of the third component of complement (C3)]-opsonized forms. Wortmannin inhibited zymosan uptake in a dose-dependent manner, regardless of whether the particles were opsonized or not. The inhibition of phagocytosis was observed when LY294002 was used as a PI3K inhibitor with the maximal effect at 100 μM (Figure 1C).

The results of the pharmacological analysis described above agreed well with those from previous studies [22,25] and...
suggested that the lipid products of PI3Ks play a critical role in phagocytosis. It is known that class-I PI3Ks possess protein kinase activity, which is also inhibited by PI3K inhibitors [10]. We therefore next examined whether treatments that perturb the metabolism of PI3K lipid products have an effect on phagocytosis. For this purpose, we prepared Raw 264.7 cells lacking SHIP-1 or PTEN using an shRNA technique. Uptake of IgG-opsonized SRBCs by these cells was then examined.

SHIP is a 5′-phosphatase that metabolizes the in vivo product of class-IA PI3Ks, PtdIns(3,4,5)P_3, to produce PtdIns(3,4)P_2 [26]. Introduction of the shRNA probe abolished the protein expression of SHIP-1 (Figure 2A). FcγR-mediated phagocytosis of IgG-opsonized SRBCs doubled in SHIP-1-deficient cells (Figure 2B), suggesting that the increased level of PtdIns(3,4,5)P_3 enhances the activity. The increased number of FcγRs is not the mechanism of this enhancement, because the binding of IgG-opsonized SRBCs on the cell surface in these cells was unchanged (Figure 2C). This was confirmed by the finding that the uptake of non-opsonized zymosan engulfed through binding to mannose receptors and TLRs instead of FcγR was again increased in these cells (results not shown). PI3K inhibition by wortmannin decreased the phagocytosis of control and SHIP-1-deficient cells in a similar dose-dependent manner (Figure 2B), but had no effect on the cell-surface binding of SRBCs (Figure 2C).

PTEN is another enzyme metabolizing PtdIns(3,4,5)P_3 that catalyses the 3′-dephosphorylation to produce PtdIns(4,5)P_2 [27]. The knockdown of PTEN markedly increased the rate...
of FcγR-mediated phagocytosis (Figure 3B). The increase was more remarkable than that observed in SHIP-1-deficient cells (Figure 2B), probably because of the different spatial distribution of these enzymes and the presence of 5′-phosphatase isozymes [28–30]. The amount of IgG-opsonized SRBCs on the cell surface decreased slightly in PTEN-deficient cells, presumably because of the higher uptake of target particles (Figure 3C).

Specific role of p110α in phagocytosis

The results described above, together with a previous report showing the specific localization of PtdIns(3,4,5)P3 in phagocytic cups [1], suggested that the lipid product of class-I PI3K has a substantial role in FcγR-mediated phagocytosis. To determine the PI3K subtype responsible for this process, Raw 264.7 cells deficient in p110α, p110β, p110δ or p110γ were prepared. Raw 264.7 cells were transfected with an shRNA probe targeting each of the p110 catalytic subunits by electroporation. Cells were cultured in the presence of puromycin for about 2 weeks; resistant cells were cloned and expanded for use. Figure 4(A) shows the result of Western-blotting analysis of a series of p110α-depressed clones. Clones 2.4 and 2.1 showed minimum expression of p110α, and thus these clones were used in the experiments described below. The p110α-deficient cells showed normal expression of the p85 regulatory subunit of PI3K and the catalytic subunits other than p110α (Figure 4B). The p110β-, p110δ- or p110γ-deficient cells showed a specific decrease of the targeted subunit (Figure 4B).

Prepared cells were first examined for their ability to engulf IgG-opsonized (Figure 5A), C3bi-opsonized (Figure 5B) and non-opsonized (Figure 5C) zymosan particles. The phagocytosis of these particles was impaired markedly in p110α-deficient cells. Microscopic images of p110α-deficient cells given FITC-labelled IgG-opsonized zymosan showed that the particles were observed sometimes on the surface of, but merely within, the cells (Figure 5D), as seen in wortmannin-treated cells (see Figure 1A). The decreased phagocytosis in p110α-deficient cells was also observed when uptake of IgG-opsonized SRBCs was examined (Figures 5E and 5F). The phagocytosis of the particles was not decreased in cells deficient in p110β, p110δ and p110γ (Figures 5A–5C and 5F). The irrelevance of p110γ in the processes was confirmed by the experiment with macrophages from p110γ−/− mice (right panels of Figures 5A–5C). The effects of the p110α deficiency described above were observed similarly when another targeting sequence (5′-GCAACCTATGTAATGTA-3′) was used to silence p110α expression (results not shown).

Roles of p110α in macropinocytosis, micropinocytosis and receptor-mediated endocytosis

Macrophages are endocytic cells known to exhibit, in addition to the phagocytosis of solid particles, fluid-phase pinocytosis and receptor-mediated endocytosis of soluble ligands. We next examined whether these processes deteriorate in p110α-deficient

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Figure 5 Decreased phagocytosis in p110α-deficient cells

(A–C, left-hand panels) Control cells and cells deficient in p110α, p110δ, or p110γ (Δα–Δγ) were incubated with IgG-opsonized zymosan (A), serum (C3bi)-opsonized zymosan (B) or non-opsonized zymosan (C) for 60 min. The amounts of engulfed zymosan were quantified as described in Figure 1 and the amount of zymosan taken up by control cells during 60 min incubation is expressed as 100%. Each bar represents the mean ± S.D. for three independent experiments (*P < 0.05; **P < 0.01). Wort, wortmannin. (A–C, right-hand panels) Macrophages from wild-type (WT) or p110γ-knockout (p110γ−/−) mice were evaluated for phagocytosis. Results shown are the means for two separate experiments. (D) Fluorescence, phase-contrast and merged images of control and p110α-deficient cells given FITC-labelled IgG-opsonized zymosan. (E) p110α-deficient cells (○) or control cells (■) were incubated for various periods of time with the addition of EAs. After hypo-osmotic lysis of extracellular EAs, the number of EAs within the cells was determined as described in the Experimental section. (F) Control cells and cells deficient in p110α, p110γ, p110δ or p110γ were incubated for 60 min with the addition of EA. The numbers of cell-surface (binding) and intracellular (phagocytosis) EAs were determined as described in the Experimental section. The results are shown as percentages of those for control cells.

The uptake of FITC-labelled dextran (molecular mass of 40 kDa) and Lucifer Yellow (molecular mass of 500 Da) was examined because it has been shown that different mechanisms function in the pinocytosis of different ligands depending on their size [31]. Nocodazole prevented the uptake of these soluble ligands as well as that of IgG-opsonized particles, indicating the indispensable role of microtubule re-organization in these processes (Figure 6). Inhibition of actin assembly by cytochalasin-E or latrunculin-B did not decrease the pinocytosis, despite the fact that the treatment markedly impaired phagocytosis (Figure 6). The uptake mechanism of soluble ligands was therefore considered to be different from that of solid particles, in agreement with the results of previous studies [32]. In spite of this difference, FITC–dextran uptake was severely inhibited by wortmannin (Figure 7A), as was phagocytosis. FITC–dextran uptake was decreased by the depletion of p110α, but was not affected by the depletion of other subtypes (Figure 7B).

The uptake of a smaller molecule, Lucifer Yellow, was similar to that of FITC–labelled dextran in its sensitivity to nocodazole, cytochalasin-E and latrunculin-B (Figure 6). The uptake mechanism may be different from that of the larger molecule, because the volume of engulfed liquid containing Lucifer Yellow was calculated to be 1.3 μl/h per 10⁶ cells, which
Raw 264.7 cells were preincubated with nocodazole (1 μM), latrunculin-B (100 μM) cytochalasin-E (10 μM) or vehicle (control) for 15 min. IgG-opsonized (op) SRBC, IgG-opsonized zymosan, dextran or Lucifer Yellow was added and the mixture incubated for a further 60 min. The amount of each ligand taken up by control cells during 60 min incubation is expressed as 100 %. Results are means for two independent experiments.

was quite different from that containing dextran (0.1 μl/h per 10^6 cells). The uptake of Lucifer Yellow was not affected by the depletion of p110α, p110β, p110γ or p110δ (Figure 8A). Despite this apparent independence of class-IA PI3K subtypes, pinocytosis was partially inhibited by wortmannin (Figure 8B). The target molecule of wortmannin functioning in this system may be class-II or class-III PI3K.

Foam-cell formation in macrophages is an important process in the development of atherosclerotic plaques. LDL uptake in this process has been reported to occur through actin-dependent macropinocytosis mediated by Rho GTPase and PI3K signalling [4,21]. We tested p110α involvement in this process using DiI-labelled acetylated LDL. Similar to the case of Lucifer Yellow, wortmannin partially inhibited LDL uptake but the defect in p110α did not affect uptake (Figure 9).

**Lower steady-state level of PI3K activity in quiescent p110α-deficient cells**

The results detailed above suggested that p110α is the sole specific player among class-IA PI3K subtypes that regulates the phagocytosis of solid particles and the pinocytosis of larger molecules. One possible explanation for the results is that activation of every PI3K subtype is impaired in p110α-deficient cells by a non-specific effect of the shRNA probe. We therefore examined the activation of a downstream target of class I PI3K subtypes, Akt (also called protein kinase B), in response to LPS stimulation. LPS-induced Akt phosphorylation was not impaired, but markedly increased in p110α-deficient cells (Figure 10A), in agreement with our previous report [33]. The activation of ERK, which is not impaired by PI3K inhibitors in these cells (results not shown), was unaffected in p110α-deficient cells (Figure 10A), suggesting that these cells do not suffer non-specific loss of viability.

We next determined the PI3K activity in the cells deficient in each of the p110 subunits. Lysates from quiescent cells were used for immunoprecipitation with the antibody against the p85 regulatory subunit. PI3K activity associated with the precipitates was determined in a cell-free system. PI3K activity was extremely low in the p110α-deficient cells compared with the other cells (Figure 10B), indicating that basal PI3K activity in the quiescent state is mainly governed by the α-subtype. This basal activity may be critical in determining the steady-state levels of phagocytosis and macropinocytosis.

**DISCUSSION**

Four isoforms of class-I PI3K catalytic subunit, termed p110α, p110β, p110γ and p110δ, have been identified. Although p110α and p110β are widely expressed and share a high degree of sequence homology, growing evidence suggests a difference in their physiological roles. This difference is shown by the fact that targeted disruption of p110α or p110β causes death at an early embryonic stage [34]. Studies (including those with subtype-selective inhibitors) are accumulating detailed information on their functional difference [35,36]. In macrophages, we have reported that p110β has a critical role in LPS-induced activation of Akt and negative regulation of nitrite production [33]. The present study indicated the specific function of p110α in two processes...
of endocytosis: phagocytosis of solid particles and pinocytosis of larger molecules.

Biochemical studies have identified the functional difference between p110α and p110β. The activity of p110β is, whereas that of p110α is not, enhanced by the βγ subunits of G-proteins [24,37]. It has also been reported that p110α is more active than p110β at higher substrate concentrations, whereas the opposite is true at lower substrate concentrations, indicating that p110α has a higher K_m and V_max than p110β [38]. The authors of the latter study suggested that p110α works best in areas of high substrate density, whereas p110β would function better in areas of membranes containing low levels of substrate [38]. The difference in kinetic parameters may explain (at least in part) why p110α has a preferential role in PI3K-dependent endocytic processes, because the local level of phosphoinositides is reported to be high in membranes of phagocytic and macropinocytic cups [1,39]. The higher V_max value of p110α may also explain why the in vitro PI3K activity from p110α-deficient cells is markedly lower than...
that from other cells (Figure 10C), despite the expression of the p85 regulatory subunit being not quite different (Figure 4B). The apparent decrease in PI3K activity in p110α-deficient cells does not impair every PI3K function in the cells, because LPS-induced activation of Akt (a p110β-dependent process) did not deteriorate (Figure 10A).

Several lines of evidence have indicated the role of PtdIns(3,4,5)P3 in the FcyR-mediated process of phagocytosis [17–19]. Overexpression of the 3′-phosphatase PTEN or the 5′-phosphatase SHIP-1 is reported to inhibit phagocytosis [40–43]. Macrophages from SHIP-1 knockout mice exhibit increased uptake of IgG-opsonized SRBC [41]. In the present study, we confirmed the physiological role of lipid phosphatases (Figures 2 and 3). The enhancement in phagocytosis (fold increase above that seen in control cells) by specific knockdown was greater in PTEN-deficient cells than in SHIP-1-deficient cells. One possible explanation for the difference is that PTEN is a predominant catalyst of PtdIns(3,4,5)P3, breakdown in Raw 264.7 cells. The difference may be best explained by their different roles in the process of phagocytosis; recent studies have demonstrated that these enzymes show temporally and spatially different behaviour in phagosomes [28]. The existence of isoforms such as SHIP-2 may also explain the minor effect of SHIP-1 deficiency [30].

The p85 regulatory subunit has been shown to be recruited to the phagocytic cups during FcyR-mediated phagocytosis [28]. This suggests a role for class-IA PI3Ks in increasing the local concentration of PtdIns(3,4,5)P3 at the site where an opsonized particle is taken up. In the present study, we showed that one subtype of class-IA PI3K (p110α), not class-IB PI3K p110γ, which is expressed in haematopoietic cells specifically and abundantly, is a critical determinant of FcyR-mediated phagocytosis. We also showed that the uptake of non-opsonized zymosan particles mediated by TLRs and mannose receptors was impaired in p110α-deficient cells (Figure 5). One possible function of p110α in these processes is the stimulation of extension of actin-rich pseudopodia along the engulfed particles. This possibility may not be the case, because PI3K inhibitors have been reported not to influence this process [21]. The action of p110α on processes other than pseudopodia extension is supported by the present study; the pinocytosis of dextran molecules, which was not affected by the inhibitors of actin assembly (Figure 6), was also impaired in p110α-deficient cells.

During the course of the present study, Lee et al. [44] reported that p110α is required for the uptake of IgG-opsonized and non-opsonized zymosan in PMA-differentiated THP-1 cells, although the roles of other isoforms were not examined. The result is in good agreement with the present study with Raw 264.7 cells, but was in sharp contrast with the result obtained for bone-marrow-derived mouse macrophages [45]. The latter study indicated that microinjection of an inhibitory antibody against p110β (and to a lesser extent an anti-p110α antibody) impaired, whereas anti-p110α antibody had no effect on, FcγR-mediated phagocytosis. Distinct roles of PI3K isoforms in different cell types have been observed in several cell functions. For example, p110γ is a main player in insulin signalling in several cell lines, whereas this is not true in other cells in which p110β and p110δ show redundant functions [46]. Colony-stimulating-factor-1-induced proliferation of bone-marrow macrophages is critically dependent on p110δ, but that of murine macrophage BAC1.2F5 cells is dependent on p110α [47]. One simple explanation for the difference is that PI3K isoforms have redundant functions, but their relative expression level is different between cells. This may not be true in the latter case at least, because inhibition of p110δ attenuates the PI3K-dependent chemotaxis in both cell lines [47]. In Raw 264.7 cells, LPS-induced activation of Akt is not impaired in p110α-deficient cells (Figure 10A). It is of interest to consider that the kinetic parameters of PI3K isoforms are differently influenced by many factors in cells (e.g. composition of membrane phospholipids, presence of co-signals).

Engulfment of soluble ligands is known to occur through multiple mechanisms, including macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, and clathrin- and caveolin-independent endocytosis. The role of phosphoinositides in the regulation of these processes is incompletely understood. We observed that a pan-PI3K inhibitor wortmannin inhibits, although partly, the uptake of Lucifer Yellow (Figure 8B) and acetylated LDL (Figure 9), in agreement with previous studies [21]. Cells lacking class-IA PI3Ks engulfed these ligands normally (Figures 8A and 9). Because wortmannin is known to inhibit a broad array of enzymes, its effects on cells is not necessarily due to changes in phosphoinositide turnover. The possibility that PI3Ks other than class-I subtypes are operating in these processes cannot be excluded. Alternatively, deletion of a single subtype may not have an effect because of the functional redundancy of class-IA subtypes in these types of endocytosis.

In the present study we have shown that shRNA-based knockdown of class-IA PI3K is a useful approach to examine its roles in endocytosis. Further study targeting the subtypes of class-II PI3K, class-III PI3K and the regulatory subunits of class-IA PI3K may help us to understand the mechanisms of p110α-independent processes of endocytosis and to identify the target of wortmannin in the processes.

**AUTHOR CONTRIBUTION**

Namiko Tamura and Kaoru Hazeki designed the experiments, performed most of the experiments and wrote the manuscript. Natsumi Okazaki prepared p110α-knockdown cells. Yukiko Kametani assisted in the pinocytosis assay. Hiromi Murakami prepared SHIP-1 knockdown cells and performed the experiments described in Figure 2. Yuki Takaba prepared PTEN-knockdown cells and performed the experiments described in Figure 3. Yuki Ishikawa assisted in the pinocytosis assay. Kyomi Nigorikawa discussed the manuscript with us. Osamu Hazeki designed experiments, critically analysed the data and wrote the manuscript.

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