Regulation of clathrin-dependent endocytosis by diacylglycerol kinase δ: importance of kinase activity and binding to AP2α

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INTRODUCTION

Among the various types of endocytic machinery on the plasma membrane, clathrin-dependent endocytosis is a major pathway by which hormones, receptors and numerous other signalling factors are internalized. Endocytosis requires the recruitment of specific molecules to the membrane pits that bud into the cytoplasm; in particular, endocytosis through clathrin-coated pits involves the assembly of clathrin and AP-2 (adaptor protein 2) complex on the membrane. The process of endocytosis begins with the invagination of the coated pit from the membrane, which is followed by the scission of the pit to the vesicle, the release of clathrin and AP-2 complex from the vesicle and then the reconstitution of new coated pits [1]. The AP-2 complex is a key component for the vesicle formation that mediates the binding of receptors to clathrin or other accessory proteins. The AP-2 complex consists of four subunits: two large subunits (α- and β2-adaptin), a medium subunit (μ2) and a small subunit (σ2). The μ2 subunit particularly recognizes a residue of either tyrosine (YXXΦ, where Y is tyrosine, X is any residue and Φ is a hydrophobic residue) or dileucine (D/EXXLL) of the endocytosis motifs in the cytosolic domains of receptors [2]. On the other hand, the appendage (ear) domain in α- and β2-adaptin binds to clathrin or several endocytic proteins such as dynamin and amphiphysin [3], indicating that the AP-2 complex acts as an adaptor protein to connect the receptors and various accessory proteins around pits.

The lipid composition of the membrane is important for endocytosis. In particular, PtdIns(4,5)P2 is known to be the most important for the regulation of the endocytosis in several ways. PtdIns(4,5)P2 plays a role in assembling AP-2 complex or other accessory proteins such as dynamin [4], synaptojanin [5] and AP180 [6] to the membrane surface. It is also known that PtdIns(4,5)P2 or acidic phospholipids bind to dynamin and affect its activity, a GTPase that pinches off the invaginated vesicle, suggesting that PtdIns(4,5)P2 controls the occurrence of pits and the rate of pit formation [4]. In addition, cholesterol also has an ability to gather PtdIns(4,5)P2 and stabilize PtdIns(4,5)P2 as lipid domain, because acute cholesterol depletion inhibits the invagination of the coated pits [7]. This evidence highlights the importance of the enzyme, which modifies the lipid metabolism in endocytosis. For example, synaptojanin, which dephosphorylates PtdIns(4,5)P2 and produces PtdIns(4)P, plays an important role in synaptic vesicle regeneration by promoting the uncoating process of the clathrin-coated vesicles [8]. Phosphatidylinositol kinases (PIPKI/phosphatidylinositol-4-phosphate 5-kinase) [9,10]; PIP3K (phosphatidylinositol-4-phosphate 3-kinase) [11,12] and PLD (phospholipase D) [13], which cleave the head group from phosphatidylcholine to produce PA (phosphatidic acid), are also known to regulate endocytosis.

To understand the further details of the regulatory mechanism of endocytosis, genome-wide RNAi (RNA interference) screening of kinases has been performed and the effect of RNAi on endocytosis was analysed [14]. It was revealed that a large number

Abbreviations used: AP-2, adaptor protein 2; ARF, ADP-ribosylation factor; Cy3, indocarbocyanine; DAG, diacylglycerol; DGK, diacylglycerol kinase; EGF, epidermal growth factor; GFP, green fluorescent protein; GST, glutathione transferase; PA, phosphatidic acid; PD, pleckstrin homology; PIP3K, phosphatidylinositol-4-phosphate 3-kinase; PKC, protein kinase C; PLD, phospholipase D; RNAi, RNA interference; SAM, sterile α motif; siRNA, small interfering RNA.

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of kinases regulate the endocytosis: kinases related to the JNK (c-Jun N-terminal kinase)/MAPK (mitogen-activated protein kinase) cascades, the TOR (target of rapamycin) pathway and the integrin-signalling cascade. Among these kinases, knockdown of DGK (diacylglycerol kinase), a lipid kinase, reduced uptake through clathrin-dependent endocytosis.

DGK phosphorylates DAG (diacylglycerol) and converts it into PA. DGK consists of ten isoenzymes (α, β, γ, δ, ε, ζ, κ, η, θ and i), classed on the basis of their primary structures. All isoenzymes contain two or three cysteine-rich regions which are homologous with the C1α and C1b domain of PKC (protein kinase C) in the N-terminal half and also a catalytic region in the C-terminal half. The unique structural features among these DGK isoenzymes suggest their different enzymatic regulation and distinct functions in lipid metabolism [15]. In general, it is known that DGK acts as an indirect terminator of PKC signalling by suppressing the amount of DAG which activates PKC [16–18]. DGKδ contains a PH (pleckstrin homology) domain, in addition to the cysteine-rich region in the N-terminal half and SAM (sterile α motif) at C-terminus [19], and this DGK isozyme was shown to reduce the internalization through the clathrin-dependent endocytosis in RNAi [14]. Although the activation mechanism of DGKδ has not been fully elucidated, there is an increasing number of reports showing the existence of multiple activation pathways of DGKδ. Briefly, PKC-dependent phosphorylation of the PH domain in DGKδ [20] and its oligomerization through the SAM domain [21] regulate the enzymatic properties, especially the localization of DGKδ at the cellular level. It was also revealed that PtdIns(4,5)P2 promotes the activation of DGKδ [19]. DGKδ is involved in the traffic from the endoplasmic reticulum to the Golgi [22], and an experiment using knockout mice revealed that role in the EGF (epidermal growth factor) receptor signalling pathway [23]. However, little is known concerning DGK function in endocytosis. In the present study, we investigated the molecular regulatory mechanism of DGKδ in clathrin-dependent endocytosis.

MATERIALS AND METHODS

Cell culture

COS7 cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) (Nacalai Tesque), and HeLa cells in minimal essential medium (Gibco BRL) at 37°C in a humidified atmosphere containing 5% CO2. Both media contained 25 mM glucose, were buffered with 44 mM NaHCO3 and were supplemented with 10% (v/v) foetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). The foetal bovine serum used was not heat-inactivated.

Plasmid and protein expression

The cDNA of human DGKδ2 in pBR was kindly donated by Dr Hideo Kanoh [19,24]. DGKδ2 and its domains (N-terminus, amino acids 1–320; catalytic domain, amino acids 321–920; C-terminus, amino acids 921–1214) were subcloned in pFLAG-CMV10 vector (Sigma). Point mutations were introduced into DGKδ2 at the catalytic site (G381D; kinase-negative) and the AP2α-binding site (F369A/F372A, F748A and F369A/F372A/F748A) using the QuikChange® II XL site-directed mutagenesis kit (Stratagene). Mouse AP2α ear domain (amino acids 707–937) was subcloned into pGEX6p-1 vector or pFLAG-CMV10 vector, and the point mutation was introduced at Trp480. GST (glutathione transferase)–AP2α ear fusion protein was expressed and purified as described elsewhere [3]. BL21 plLys cells were transformed with expression plasmids for the production of recombinant protein. Expression of GST–AP2α ear domain was induced by 0.1 mM IPTG (isopropyl β-D-thiogalactoside) at 25°C overnight. The cells were then harvested and lysed in buffer containing 20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose, 20 µg/ml leupeptin and 1 mM PMSF. GST-fusion protein was purified on glutathione–Sepharose 4B resin (GE Healthcare).

RNAi

Cells were grown in a six-well plate, and transfected with dsRNA (double-stranded RNA) using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer’s instructions. RNA oligonucleotides Control 2, siRNA1 (ID #1418), targeting the non-coding region in DGKδ2, and siRNA2 (ID #1323), targeting the coding region in DGKδ2, were obtained from Ambion. Confinement of knockdown of DGKδ was analysed by Western blotting with DGKδ antiserum which was kindly donated by Dr Hideo Kanoh [19].

Internalization of Alexa Fluor® 488–transferrin and Alexa Fluor® 488–EGF

For the endocytosis assay, cells were grown in six-well plates and treated with 10 pM siRNA (small interfering RNA) as described above. After 3 days, cells were transferred to 24-well plates, washed with Ringer’s solution (135 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM Hepes and 10 mM glucose, pH 7.3) and serum-starved in the same medium for 30 min at 37°C. Cells were then put on ice, and the same medium was added, but containing human Alexa Fluor® 488–transferrin (5 µg/ml dye/protein), before incubating for 30 min on ice. Human transferrin (Sigma) was labelled with Alexa Fluor® 488 carboxylic acid, succinimidyl ester (Invitrogen) according to the manufacturer’s instructions. Labelled transferrin was separated from free Alexa Fluor® 488 on a PD10 column (GE Healthcare). For the EGF internalization assay, cells starved for 2 h were incubated with Alexa Fluor® 488–EGF (10 ng/ml) for 1 h on ice. Cells were then moved to a 37°C water bath for various periods to allow transferrin and EGF internalization. To measure internalized ligand, surface-bound ligand was stripped using ice-cold 135 mM NaCl, 5.4 mM KCl, 0.4 mM MgCl2, 1.8 mM CaCl2, 20 mM Mes and 1 mM glucose (pH 5.0) for 10 min, followed by a wash with Ringer’s solution. To analyse the amount of internalized transferrin, the cells were lysed, then moved to a 96-well black plate, and intensity was measured by fluorescence photon counter (LB 940, Biotek, USA).

Immunoprecipitation and GST pull-down

FLAG–DGKδ2, AP-2–GFP (green fluorescent protein) and GFP were expressed in COS7 cells. Cells were lysed 48 h after electroporation in homogenization buffer (150 mM NaCl, 10 mM Hepes, pH 7.3, 2 mM dithiothreitol, 0.05% Triton X-100 1 mM PMSF and 20 µg/ml leupeptin). After centrifugation at 20000 g for 30 min, the supernatants from each cell were mixed and immunoprecipitations were performed at 3 h using anti-GFP antibody (GE Healthcare), then anti-GFP antibody was precipitated further using Protein G–Sepharose (GE Healthcare) or anti-FLAG M2–agarose resin (Sigma). For GST pull-down, GST-fusion proteins were incubated with glutathione–Sepharose beads (GE Healthcare) for 3 h. Cell lysate and 7.5 µg of GST-fusion protein bound to glutathione–Sepharose beads was incubated for 1 h at 4°C. The bound proteins were washed three times with homogenization buffer. Purified rabbit anti-DGKδ antibody for co-immunoprecipitation assay was kindly donated by Dr Hideo Kanoh [19].
Figure 1  Effect of siRNA of DGKδ on transferrin internalization

(A) HeLa cells and (B) COS7 cells were transfected with control, siRNA1 or siRNA2 targeting to DGKδ siRNA oligonucleotides, and the internalization of fluorescently labelled transferrin was measured for 5, 10 and 20 min after incubation at 37°C (see the Materials and methods section). Results are means ± S.D. for three experiments. Upper panels show the expression level of DGKδ assessed by Western blotting with DGKδ antisera. HeLa cells expressed both type 1 (DGKδ1) and type 2 (DGKδ2) DGKδ, whereas COS7 cells expressed only type 2 DGKδ (B). Immunoblots against clathrin heavy chain (Clathrin H) and β-actin are shown as control.

Cell imaging

Cells were transfected using FuGENE6™ (Roche Molecular Biochemicals) 24–48 h before fixation. Cells were fixed for 20 min in 4% (w/v) formaldehyde at room temperature (25°C), permeabilized using 0.05% (v/v) Triton X-100 and blocked by 10% (v/v) sheep serum in PBS at 4°C overnight. AP2α or clathrin staining was performed using mouse monoclonal anti-AP2α (BD Bioscience) or anti-clathrin (BD Bioscience) antibody respectively, and following this by biotinylated anti-mouse antibody (Vector Laboratory). Biotin was detected by Alexa Fluor® 488-conjugated streptavidin (Sigma). Flag staining was performed using rabbit polyclonal anti-FLAG antibody (Cell Signaling Technology), followed by Cy3 (indocarbocyanine)-conjugated anti-rabbit antibody (Invitrogen). Confocal images were obtained using a Zeiss 510 microscope with standard filter for FITC/Cy3-type fluorophores (488 nm excitation, emission at 505–530 nm; 543 nm excitation, emission at 560–600 nm).

DGK assay

FLAG–DGKδ2 and its mutants were transfected into COS7 cells by electroporation. After 48 h, cells were harvested and lysed in homogenization buffer (150 mM NaCl, 10 mM Hepes, pH 7.3, 0.5% Triton X-100, 1 mM PMSF and 20 µg/ml leupeptin). After centrifugation at 20000 g for 30 min, supernatants from each of the cells expressing FLAG–DGKδ2 were immunoprecipitated by anti-FLAG M2–agarose resin at 4°C for 3 h. Immunoblots were extended to DGKδ1, whereas COS7 cells expressed only type 2 DGKδ.

RESULTS

Inhibitions of endocytosis by DGK knockdown

Previously, genome-wide siRNA screening revealed that many kinases are associated with endocytosis. Among all subtypes of DGK (α, β, γ, δ, ε, ζ, η, θ and ι) examined, only DGKδ was shown to relate to clathrin-dependent endocytosis [14]. To study the role of DGKδ in the regulation of clathrin-dependent endocytosis, the expression of endogenous DGKδ was inhibited by siRNA in HeLa (Figure 1A) and COS7 (Figure 1B) cells, and then transferrin internalization assays were carried out in these cells. DGKδ has alternative splicing variants, type 1 (DGKδ1) and type 2 (DGKδ2), with calculated molecular masses of 130 and 134 kDa respectively. In DGKδδ2, the N-terminal 52 residues containing proline-rich and glutamate/aspartate-rich sequences were extended to DGKδ1. Although the expression of DGKδ1 was restricted to ovary and spleen, DGKδ2 was widely expressed in several tissues and cell lines [24]. As shown in Figure 1, HeLa cells expressed both type 1 and 2 DGKδ, and COS7 cells expressed only type 2 DGKδ. We used siRNAs against DGKδ that specifically knocked down both type 1 and 2 DGKδ in these cells. Under these conditions, the uptake of transferrin was reduced in DGKδ knockdown cells (Figure 1).

Co-localization of DGKδ2 with clathrin-coated pits

To elucidate the functional role of DGKδ in clathrin-dependent endocytosis, we examined the subcellular localization of DGKδ in endocytosis. In COS7 cells expressing FLAG-tagged DGKδ2, the localization of DGKδ2 was compared with that of clathrin-coated pits or AP2α visualized by immunostaining. DGKδ2 showed dot-like localization in COS7 cells, and DGKδ2 was partially co-localized with clathrin or AP2α in COS7 cells,
suggested the interaction between DGKδ and these proteins that regulate endocytosis (Figure 2).

**Interaction between DGKδ2 and AP2α**

To examine the molecular interaction of DGKδ with the proteins that regulate clathrin-dependent endocytosis, we surveyed the motif sequence that could bind to the endocytosis-related proteins within DGKδ. For endocytosis, it is known that connections between coated pits and protein are achieved by low-affinity interaction [25]. Both clathrin and AP2α act as important proteins which assemble several accessory proteins to the pits. For example, amphiphysin [26], HIP1 (huntingtin-interacting protein 1) [27], stonin [28] and dynamin [29] each have a binding motif for clathrin or AP2α and progress the endocytosis. The binding motif to clathrin or AP2α in these accessory proteins is fully characterized. The most prevalent binding motif that facilitates the association with clathrin is based upon the consensus L(L/I)(D/E/N)(L/F)(D/E) motif, which is called a clathrin-binding box [30]. This motif sequence, however, was not found in DGKδ. In the case of AP2α, the tripeptide DP(F/W), the FX(N/D/S)X(F/L) motif (where X represents any amino acid), the FXFXX(F/L) motif and the WXFX motif have been identified as possible binding sites of the ear domain in AP2α [31,32]. DGKδ2 contains two AP2α-binding sequences, DP(F/W) and FX(N/D/S)X(F/L), suggesting the possibility of an interaction between DGKδ2 and the ear domain in AP2α. We therefore examined the binding between AP2α and DGKδ2 by immunoprecipitation. COS7 cells transfected with FLAG–DGKδ2 and AP2α were prepared and homogenized. These two kinds of homogenates were mixed for 1 h and used for immunoprecipitation. As shown in Figure 3, DGKδ2 was co-immunoprecipitated with GFP–AP2α, and GFP–AP2α was co-immunoprecipitated with FLAG–DGKδ2, indicating the interaction between AP2α and DGKδ2. In addition, it is known that several endocytic accessory proteins bind to the ear domain in the C-terminus of AP2α, and the N-terminus of AP2α acts as an engaging site in the AP-2 complex [1], strongly suggesting that DGKδ is a member of the AP-2 complex through the binding to its ear domain. To examine the interaction between DGKδ2 and the ear domain in AP2α, pull-down and co-immunoprecipitation experiments were performed. The AP2α ear domain was expressed and purified as a GST-fusion protein from *Escherichia coli* and incubated with COS7 cell lysates overexpressing FLAG–DGKδ2. DGKδ2 binds to GST–AP2α ear domain, but not to GST (Figure 4A). The immunoprecipitation assay shown in Figure 4B also confirmed the binding of the AP2α ear domain and DGKδ2. Binding between endogenous DGKδ2 and tagged AP2α ear domain was detected by pull-down assay using GST–AP2α ear domain (Figure 4C) and by immunoprecipitation assay using FLAG–AP2α ear domain expressed in COS7 cells (Figure 4D). In addition, we confirmed the endogenous interaction between DGKδ and AP2α by immunoprecipitation of endogenous complex of AP2α and DGKδ (Figure 4E). To determine further the binding site within DGKδ2 to the AP2α ear domain, DGKδ2 was fragmented into three domains including N-terminal, catalytic and C-terminal domains, and these domains were used for the pull-down assay. Figure 4F shows that the catalytic domain binds mainly to AP2α ear domain, although the C-terminus including the SAM domain is slightly associated with AP2α ear domain. The AP2α ear domain contained two subdomains, the platform subdomain and the β-sandwich subdomain, which have been characterized as binding motifs. The platform subdomain was shown to be the binding site for DXF-like motifs (DPF, FXDXF and FXXFXXL motifs), and the β-sandwich subdomain is the
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Importance of kinase activity of DGKδ and its binding to AP2α for endocytosis

We measured kinase activities of DGKδ mutants having one (F748A), two (F369A/F372A) or three (F369A/F372A/F748A) mutations (Figure 7A). Lysates of COS7 cells expressing FLAG–DGKδ series were precipitated using anti-FLAG M2–agarose resin, and then the activities of precipitated DGKδs were measured by the DGK assay (see the Materials and methods section). Figure 7(A) shows that the F369A/F372A mutant has significantly lower kinase activity than the wild-type, but the F748A mutant shows kinase activity similar to that of wild-type DGKδ. Mutation of both Phe369 and Phe372 may cause the reduction of kinase activity, as these residues are located close to the ATP binding site of DGKδ. It is noteworthy that the F748A mutant having high kinase activity failed to bind to AP2α and F369/372A, with no activity possible.

For further insight into the role of DGKδ in clathrin-dependent endocytosis, we measured the transferrin uptake in COS7 cells expressing wild-type or mutations of DGKδ under knockdown of endogenous DGKδ. Figure 7(B) shows that none of the mutants, as well as kinase-negative DGKδ, could completely compensate for the uptake of transferrin inhibited by siRNA treatment, whereas overexpression of wild-type DGKδ completely recovered the transferrin uptake. Finally, we measured regulated clathrin-dependent endocytic events by monitoring EGF internalization (Figure 8A). Internalization of EGF was also inhibited by the knockdown of DGKδ and was compensated for by wild-type, but not by F748A, DGKδ (Figure 8B).

DISCUSSION

In the present study, we provide evidence that DGKδ is involved in clathrin-dependent endocytosis through its binding to AP2α. In addition, DGK activity is necessary for the regulation of the endocytic process. We studied the role of DGKδ in clathrin-dependent endocytosis by the measurement of transferrin uptake. Transferrin uptake was reduced by the knockdown of DGKδ (Figure 1). Because the intracellular localization of DGKδ overlapped with those of AP2α and clathrin (Figure 2), we predicted the possible regulation of clathrin-dependent endocytosis by DGKδ and its interaction with some endocytosis-regulatory proteins. As a result, DGKδ was found to contain DXF-type binding motifs, and DGKδ binding to AP2α was detected by immunoprecipitation (Figure 3). DGKδ interacted with the platform subdomain in AP2α ear domain via F369DTFRIL and D746PF sequences in the catalytic domain of DGKδ. Each interaction by these motif sequences was reported to be low affinity [3], but tandem arrangement of these motifs may enhance the binding and could be important for strong binding and physiological regulation.
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Figure 4 Interaction of DGKδ2 with the ear domain of AP2α

(A) GST proteins were pre-bound to glutathione–Sepharose and then the lysates from COS7 cells expressing FLAG–DGKδ2 were applied and incubated for 1 h at 4°C. The Sepharose beads were washed three times, treated with SDS sample buffer and used for Western blot analysis. (B) Purified FLAG–DGKδ2 using anti-FLAG M2–agarose was incubated with recombinant GST–AP2α ear domain. The Sepharose beads were washed three times, treated with SDS sample buffer and used for Western blot analysis. (C) GST protein (10 µg) was incubated with COS7 cells lysates from a 10 cm² dish. Endogenous DGKδ specifically bound to the beads were analysed by Western blot with anti-DGKδ antibody. Blotting for anti-(clathrin heavy chain) antibody is shown as the positive control. (D) FLAG–AP2 ear domain was overexpressed in COS7 cells and precipitated with anti-FLAG M2–agarose. Co-precipitated DGKδ was detected by Western blotting with anti-DGKδ antibody. (E) DGKδ was precipitated from COS7 cells using the purified anti-DGKδ antibody. Endogenous AP2α bound to DGKδ were analysed by Western blotting with anti-AP2α antibody. (F) The upper scheme shows the fragmented DGKδ2 in three domains. Lysates from COS7 cells expressing each DGKδ2 fragment, N-terminal, catalytic and C-terminal, were incubated with the GST–AP2α ear domain bound to glutathione–Sepharose. The Sepharose beads were washed three times, treated with SDS sample buffer and used for Western blot analysis. Molecular masses are indicated in kDa.

I.P., immunoprecipitation; W.B., Western blot.

Pelkmans et al. [14] performed genome-wide RNAi screening by monitoring vesicular stomatitis virus uptake and suggested that DGKδ positively regulated clathrin-dependent endocytosis. We hypothesized that DGKδ has a specific regulatory role for endocytosis, because only DGKδ affected the endocytosis among multiple subtypes of DGKs. We knocked down the expression of DGKδ and examined its effects on clathrin-dependent endocytosis by measuring uptake of transferrin. Transferrin uptake was reduced by 30–40% after DGKδ knockdown. These slight reductions of transferrin uptake may be caused by the compensation by the other subtype of DGKs, because transferrin uptake was strongly inhibited by the treatment with DGK inhibitor (R59949), an inhibitor for class I-specific DGK [33], although DGKδ belongs to class II [15] (results not shown). We also examined the effects of overexpression of DGKδ on transferrin uptake, but we could not detect the enhancement of transferrin uptake (results not shown).

Many reports have proposed the roles of the AP-2-binding sequences found in many accessory proteins of endocytosis [30]. In addition to binding to AP-2, binding to the lipids of accessory proteins are also known to be important for regulation of endocytosis [1]. In the case of DGKδ, DGKδ contains two C1 domains and PH domains in the N-terminus, suggesting their interaction with DAG and negatively charged phospholipids such as PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ respectively. Previous reports showed that PtdIns(4,5)P₂ induced the activation of DGKδ in a kinase assay in vitro [19], and DGKδ1 was collected in the microsome fraction by subcellular fractionation [22]. These results suggest that DGKδ can bind to lipids to associate with the membrane. Moreover, AP-2 binding and lipid binding of DGKδ may work cooperatively for the interaction with the coated pits and regulation of the endocytosis.

We next examined the sites responsible for AP2α binding in the catalytic domain of DGKδ2, and demonstrated that the
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Figure 5 Interaction of DGKδ with the platform subdomain in the AP2α ear domain

GST, GST–AP2α ear domain or GST–AP2α ear domain (W840A) was pre-bound to glutathione–Sepharose. Lysates from COS7 cells expressing FLAG–DGKδ or FLAG-tagged catalytic domain of DGKδ were incubated with GST proteins for 1 h at 4°C. The Sepharose beads were washed three times, treated with SDS sample buffer and used for Western blot (W.B.) analysis. Molecular masses are indicated in kDa.

mutation of phenylalanine at positions 368, 372 and 746 of DGKδ to alanine weakened the binding to AP2α (Figure 6). However, slight, but significant, association was observed between F367A/F372A/F748A catalytic domain and AP2α ear domain. These results suggest that phenylalanine residues at residues 368, 372 and 764 of DGKδ are primarily important for the DGKδ–AP2α binding, but other binding sites besides Phe368, Phe372 and Phe748 may exist within the catalytic domain of DGKδ. Unfortunately, however, we could find other AP2α-binding motifs in the catalytic domain (Figure 6). In addition to the catalytic domain, as shown in Figure 4(D), the C-terminal domain was slightly associated with the AP2α ear domain and contained one binding motif at F945ESKL. But the F945A mutation failed to alter the binding, suggesting that these motifs were not the binding site.

Figure 7 Roles of DGKδ/AP2α binding and DGKδ activity in transferrin internalization

(A) FLAG–DGKδ series (wild-type, F369A/F372A, F748A, F369A/F742A/F748A and kinase-negative) were purified using anti-FLAG M2–agarose resin from lysates of COS7 cells expressing FLAG–DGKδ series. Purified proteins were used for in vitro DGK assay (upper panel) and the amount of proteins was measured by CBB (Coomassie Brilliant Blue) staining (lower panel). The DGK activity results are means ± S.D. (n = 4). (B) The siRNA oligonucleotides for DGKδ (siRNA2) or control oligonucleotides were co-transfected with empty vector or FLAG–DGKδ series (wild-type, kinase-negative, F369A/F372A, F748A and F369A/F742A/F748A) in COS7 cells. Expression levels of FLAG–DGKδ series were studied by Western blotting (W.B.) (inset). Transfection efficiency was approx. 80–90% of cells. The internalization of fluorescently labelled transferrin was measured for 10 min after incubation at 37°C and results are means ± S.D. (n = 7). Molecular masses are indicated in kDa. cont., control; Ori, origin.
and the internalization of fluorescently labelled EGF was measured for 3, 5 and 10 min after incubation at 37°C. The siRNA oligonucleotides for control and DGKδ (siRNA2) were co-transfected with empty vector or FLAG–DGKδ2 series (wild-type or F748A) in COS7 cells. The internalization of fluorescently labelled EGF was measured for 5 min after incubation at 37°C. Results are means ± S.D. (n < 3), cont., control.

Figure 8 Role of DGKδ2/AP2ζ binding in EGF internalization
(A) COS7 cells were transfected with siRNA oligonucleotides for control and DGKδ (siRNA2) and the internalization of fluorescently labelled EGF was measured for 3, 5 and 10 min after incubation at 37°C. (B) The siRNA oligonucleotides for control and DGKδ (siRNA2) were co-transfected with empty vector or FLAG–DGKδ2 series (wild-type or F748A) in COS7 cells. The internalization of fluorescently labelled EGF was measured for 5 min after incubation at 37°C. Results are means ± S.D. (n < 3), cont., control.

site (results not shown). As shown in Figure 7(B), F368A/F372A and F748A mutants and the kinase-negative mutant of DGKδ2 could not recover the transferrin uptake, indicating that the lipid kinase activity of DGKδ, as well as the binding between DGKδ2 and AP2ζ, are necessary for DGKδ-mediated regulation of endocytosis. Furthermore, we examined the functional roles of DGKδ in the internalization of EGF, which is regulated in clathrin-dependent endocytosis. Knockdown of DGKδ also suppressed internalization of EGF. Wild-type DGKδ completely compensated for the EGF uptake, although the uptake was not compensated for by the overexpression of the F748A mutant that lacks AP2ζ binding without alteration of kinase activity (Figure 8).

It has been reported that lipids and lipid-modifying enzymes are important for endocytosis [34]. Studies have demonstrated the involvement of PLD, which cleaves the headgroup from phosphatidylycerol and then produces PA in endocytosis and membrane traffic [13,35–37]. Although the details of the mechanism of endocytosis regulation by PLD are still controversial, PA could act as a second messenger by activating specific enzymes, such as PIP5K [38], which leads to the production of PtdIns(4,5)P₂. PA is also produced from the phosphorylation of DAG by DGK, so that DGK may indirectly activate PIP5K and then control the endocytosis by the regulation of PtdIns(4,5)P₂ production. On the other hand, DAG is a well-known second messenger which activates several proteins, for example, PKC, RasGRP, chimaerins and Unc-13 [39]. The GAP (GTPase-activating protein) activity of ARF (ADP-ribosylation factor) is also regulated by DAG [40]. In addition, ARF is well known not only as the regulator of vesicle budding, but also as the regulator of clathrin-dependent endocytosis [41,42]. This evidence suggests that the DAG level controlled by DGKδ regulates clathrin-dependent endocytosis.

In conclusion, the kinase activity of DGKδ and its interaction with AP2ζ are important for the regulation of clathrin-dependent endocytosis, suggesting that DGK-mediated lipid conversion from DAG into PA at clathrin-coated pits could be the key role for endocytosis, although the activation mechanism of DGKδ remains to be clarified. Furthermore, it has been reported recently that DGKs interact physically with β-arrestin, which is essential for G-protein-coupled receptor internalization from clathrin-coated pits, and that these interactions are necessary for conversion of DAG into PA after agonist stimulation [43], suggesting that DGK may be deeply engaged in clathrin-dependent endocytosis in several aspects.

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