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IgA Fc receptor (FcαR) cross-linking recruits tyrosine kinases, phosphoinositide kinases and serine/threonine kinases to glycolipid rafts

Mark L. LANG[1], Yih-Wen CHEN, Li SHEN, Hong GAO, Gillian A. LANG, Terri K. WADE and William F. WADE

Department of Microbiology and Immunology, Dartmouth Medical School, 1 Medical Center Drive, Lebanon, NH 03756, U.S.A.

The human IgA Fc receptor (FcαR, CD89) triggers several important physiological functions, including phagocytosis, NADPH oxidase activation and antigen presentation. Efforts are underway to delineate FcαR signal-transduction pathways that control these functions. In a previous study, we demonstrated that cross-linking of FcαR increased its partitioning into membrane glycolipid rafts and was accompanied by γ-chain-dependent recruitment and phosphorylation of the tyrosine kinases Lck/Yes-related novel protein tyrosine kinase (Lyn) and Bruton's tyrosine kinase (Btk). Here we have performed a more extensive characterization of signalling effectors recruited to rafts on FcαR cross-linking. We demonstrate that in addition to tyrosine kinases Lyn and Btk, FcαR cross-linking also recruits B-lymphocyte kinase (Blk) and spleen tyrosine kinase (Syk) to rafts. We show recruitment of phosphoinositide kinases, including 3-phosphoinositide 3-kinase and phospholipase Cγ2, and serine/threonine kinases such as protein kinase C (PKC) α, PKCϵ, and protein kinase B (PKB) α. This suggests that lipid rafts serve as sites for FcαR-triggered recruitment of multiple classes of signalling effectors. We further demonstrate that tyrosine kinases and PKCα have a sustained association with rafts, whereas phosphoinositide 3-kinase and its downstream effectors have a transient association with rafts. This is consistent with temporally regulated divergence of FcαR signalling pathways in rafts. Furthermore, we suggest the spatial separation of signalling effectors by transport of phosphoinositide 3-kinase, phosphoinositide-dependent kinase 1, PKBα and PKCϵ to endocytic compartments containing internalized FcαR.

Key words: CD89, PI 3-kinase, PKB, PKC, Syk.

INTRODUCTION

The human IgA Fc receptor (FcαR, CD89) is a 50–70 kDa transmembrane glycoprotein expressed primarily by myeloid cells, including neutrophils, monocytes and macrophages [1,2]. Cross-linking FcαR with aggregates of IgA, or IgA-opsonized particles, triggers a variety of cellular responses, including phagocytosis, NADPH oxidase activation and mobilization of granule enzymes in neutrophils and monocytes [3,4]. More recently, attention has turned to the role of FcαR in endocytosis of FcαR-targeted exogenous antigen and its presentation to T-cells as peptides in the context of MHC class II [5].

Despite the several functions triggered by FcαR, and the increasing understanding of those functions, this information has been obtained through studies on different cell types, including FcαR-expressing human myeloid cells, FcαR-expressing cell lines and FcαR-transfected cell lines. For these reasons, our current understanding of FcαR cell signalling is based on studies from different systems. Signalling is accomplished by FcαR by its association with the Fc receptor γ-chain to form the trimer FcαR/$\gamma\gamma$ [6]. The Fc receptor γ-chain dimer is also found in the high-affinity IgG Fc receptor I and IgE Fc receptor I (FcϵRI) receptor complexes expressed on mast cells and monocytes, respectively [7]. In the monocytic cell line U937, FcαR cross-linking results in phosphorylation of the associated Fc receptor γ-chain [8]. Following ligation, FcαR in U937 cells associates with the Src-family member tyrosine kinase p53/56 Lck/Yes-related novel protein tyrosine kinase (Lyn) [9], as well as tyrosine kinases spleen tyrosine kinase (Syk) and Bruton's tyrosine kinase (Btk) [10]. Cross-linking FcαR triggers calcium release from intracellular stores in neutrophils [11], and NADPH oxidase activity is inhibited by phosphoinositide 3-kinase (PI 3-kinase) inhibitors [12]. In U937 cells, FcαR also associates with Grb2, Shc, SHIP (SH2-containing inositol phosphatase-1), Cbl and SLP-76 (SH2-containing leukocyte protein of 76 kDa), suggesting the formation of adaptor complexes to regulate signalling [13]. In our laboratory, we demonstrated the activation of a 3-phosphoinositide-dependent kinase 1 (PDK1)/protein kinase B (PKB) α signalling pathway in FcαR-transfected A20 IIA1.6 B-cells [14]. Parent A20 IIA1.6 cells do not express Fc receptors or the γ-chain. Transfected A20 cells are therefore used as a model for dissecting the role of the γ-chain in FcαR function [5,6].

We demonstrated previously by confocal microscopy that cross-linked FcαR caps and co-localizes with the membrane raft marker GM-1. Furthermore, we observed recruitment of Lyn and the Tec family kinase Btk to rafts and their accompanying tyrosine phosphorylation [15]. During this time, researchers have started to examine the biochemical functions and biophysical properties of immune receptors in rafts. Examples include, but are not limited to, FcϵRI, T-cell antigen receptor, CD28 and B-cell antigen receptor [16–18]. Membrane rafts are plasma-membrane microdomains that are enriched in cholesterol and glycosphingolipids, and characterized by Triton X-100 insolubility [19,20]. Although typically thought of as small plasma-membrane domains, debate continues about the size and dynamics of rafts [21,22]. These microdomains are enriched in signalling molecules including Src-family tyrosine kinases and classical G-proteins, and are hypothesized to be specialized 'platforms' for initiation of signal transduction [19,20]. To date,

Abbreviations used: FcαR, IgA Fc receptor; FcϵRI, IgE Fc receptor I; Blk, B-lymphocyte kinase; Btk, Bruton's tyrosine kinase; Lyn, Lck/Yes-related novel protein tyrosine kinase; Syk, spleen tyrosine kinase; DAG, diacylglycerol; PI 3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; cPKC, classical PKC; nPKC, novel PKC; PKB, protein kinase B; PLC, phospholipase C; PDK1, 3-phosphoinositide-dependent kinase 1; mAb, monoclonal antibody; HRP, horseradish peroxidase; DAM, donkey anti-mouse IgM.

[1] To whom correspondence should be addressed (e-mail Mark.L.Lang@Dartmouth.edu).

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studies of signalling in rafts triggered by B-cell antigen receptor and FcεRI have provided some basic information [18,23]. However, other Fc receptors, including IgG Fc receptor and FcαR, have received little attention. Given that signalling by FcαR is crucial to its many physiological functions, it is necessary for us to understand the signal-transduction events triggered by FcαR in membrane rafts.

In this study, we have isolated rafts prior to and following FcαR cross-linking. We have identified a number of signalling effectors recruited to membrane rafts upon FcαR cross-linking. These include not only tyrosine kinases, but phosphoinositide kinases and serine/threonine kinases that have not previously been linked to FcαR signalling. As suggested by the studies of Wilson and co-workers [24], in the FcεRI system different tyrosine kinases have different temporal associations with rafts. Quantitative analysis of our immunoblots has revealed differences in temporal association of not only tyrosine kinases but phosphoinositide kinases and serine/threonine kinases. We demonstrate that B-lymphocyte kinase (Blk), Syk and protein kinase C (PKC) α have a sustained association with rafts, whereas PI 3-kinase, PDK1 and PKCε have a transient association with rafts. This is consistent with temporally regulated divergence of FcαR signalling pathways in rafts. Furthermore, our data suggest that the timed removal of selected signalling effectors from rafts results in a spatial re-organization of signalling effectors.

MATERIALS AND METHODS

Antibodies

Anti-FcαR monoclonal antibody (mAb; My43, mouse IgM) was produced in this laboratory by a method described previously [25]. Commercial anti-FcαR mAb (mouse IgG1) was purchased from Serotec (Birmingham, U.K.). Agarose-conjugated anti-phosphotyrosine (AC-PY20), anti-PY20, anti-Syk, anti-phospholipase Cγ2 (anti-PLCγ2), anti-PKCα and anti-PKCε polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-γ-chain, anti-Blk, anti-p85 and anti-PKBα antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 was purchased from Southern Biotech (Birmingham, AL, U.S.A.). Unlabelled donkey anti-mouse IgM (DAM), HRP-conjugated goat anti-rabbit IgG and HRP-conjugated rabbit anti-sheep IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, U.S.A.).

FcαR/γ-chain constructs

The pCAV vector containing the human FcαR cDNA was a gift from Dr C. Maliszewski (Immunex Corp., Seattle, WA, U.S.A.) [26]. A sub-line of the A20 B-cell line (IIA1.6), which is Fc-receptor-negative [27], was co-transfected with pCAV/FcαR cDNA and pNUT/γ-chain cDNA constructs by electroporation using a Bio-Rad electroporator (Bio-Rad, Richmond, CA, U.S.A.) at 250 V and 960 μF. The pCAV vector allows selection using G418, and the pNUT vector allows selection using methotrexate [28]. Alternatively, cells were transfected with plasmid encoding FcαR alone for use as non-signalling controls.

B-cell transfectants and culture

Transfectants expressing FcαR and γ-chain were cultured in RPMI medium supplemented with 10% fetal bovine serum, 40 μg/ml gentamycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 2 μM methotrexate. Cells expressing FcαR but no γ-chain were cultured similarly, except methotrexate was substituted by 1.15 mM (0.8 mg/ml) G418. Levels of FcαR cell-surface expression were monitored routinely using the My43 mAb by flow cytometry with a Becton Dickinson FACScan.

Isolation of detergent-insoluble membrane domains

Isolation of rafts was performed by the method of Fra and co-workers [29] with modifications as described previously [15]. For FcαR cross-linking, cells were chilled to 4 °C and incubated with My43 followed by F(ab)′2 DAM μ-chain before lysis. After washing, cells were warmed to 37 °C and the reactions stopped by addition of ice-cold PBS and transfer of tubes to an ice/water bath. We then pelleted 1×10^8 cells and resuspended them in 1 ml of ice-cold detergent lysis buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 40 mM Na_3VO_4, 200 mM NaF and 0.05% Triton X-100; all from Sigma, St Louis, MO, U.S.A.) and Complete™ protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN, U.S.A.), followed by 30 min of further incubation before ultracentrifugation. Samples were then adjusted to 1.5 M sucrose in 20 mM Tris, pH 7.5. Samples (3 ml) were added to 13 ml ultracentrifuge tubes, and then overlaid with 7 ml of 1.2 M sucrose, followed by a 3 ml layer of 0.15 M sucrose. Samples were centrifuged at 38000 rev./min [178000 rcf (relative centrifugal force)] in a Beckman SW41 rotor for 18 h at 4 °C. Fractions (1 ml) were withdrawn from top to bottom using a pipettor and stored at −20 °C until required. Triton X-100-insoluble fractions at the 1.2 M sucrose/0.15 M sucrose interface contained raft proteins. Fractions below the 1.2 M sucrose layer are described as non-raft.

Inhibitors

Piceatannol, an agent that preferentially inhibits Syk, and wortmannin, the PI 3-kinase-selective inhibitor, were purchased from Calbiochem (La Jolla, CA, U.S.A.). Stock solutions of inhibitors in DMSO were made 1000-fold stronger than the final concentration required in experiments. Piceatannol was added to a final concentration of 20 μM, and wortmannin to a final concentration of 0.1 μM. Piceatannol inhibits antibody secretion in B-cells with an IC_{50} value of approx. 10 μM [30], whereas wortmannin inhibits *N*-formylmethionyl-leucylphenylalanine-triggered NADPH oxidase activation in neutrophils with an IC_{50} value of approx. 10 nM [31]. The concentrations of inhibitors used in this study were typical for cell-based assays. To control for the carrier, DMSO, 1 μl/ml DMSO was added where appropriate. Cells were treated with or without inhibitors as appropriate for 1 h at 37 °C before chilling to 4 °C and ligating, then cross-linking FcαR. Inhibitors were present throughout all washing and incubation steps, as well as the 37 °C incubation following cross-linking.

Immunoprecipitation

For immunoprecipitation of tyrosine-phosphorylated proteins, the AC-PY20 antibody was incubated overnight at 4 °C with 30 μg of raft protein in 1.0% (v/v) Nonidet P40, then washed three times in 10 mM Tris/2 mM Na_3VO_4/1.0% Nonidet P40 (pH 7.0) before washing once in 10 mM Tris, pH 7.6. Immunoprecipitated proteins were immunoblotted as described below.

SDS/PAGE and immunoblotting

Raft fractions were adjusted to equal protein concentrations and equal amounts of protein resolved by SDS/PAGE electrophoresis under reducing conditions. Proteins were transferred to

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nitrocellulose membranes and equal transfer confirmed by Ponceau Red staining (results not shown). Membranes were incubated for 1 h at room temperature in 5.0 % (w/v) non-fat dry milk and 0.5 % (v/v) Tween 20 in PBS, then incubated with antibodies to signalling effectors (0.2, 0.5 or 1.0 μg/ml as appropriate) overnight at 4 °C. Membranes were washed six times for 5 min in PBS and transferred to tubes containing 3.0 % non-fat dry milk/0.05 % Tween 20 in PBS. HRP-conjugated secondary antibodies were added at a 1/10000 dilution (0.1 μg/ml) and incubated for 2 h at room temperature. Membranes were then washed six times for 5 min in PBS. Membrane-bound HRP-conjugated antibodies were then detected by ECL (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

Quantification of ECL

Photographic films for chemiluminescence applications (Kodak X-OMAT AR, Rochester, NY, U.S.A.) were scanned at a resolution of 300 pixels/in (1 in = 2.54 cm) and analysed using Adobe Photoshop 5.0™ software (Mountain View, CA, U.S.A.). Quantification of signals was achieved by determination of the integral mean pixel intensity [(mean pixel intensity of band − mean pixel intensity of background) × area]. For each experiment, the maximum signal was assigned as 100 %, and all other bands on the blot expressed as a percentage of the maximum signal. This method allows us to analyse the retention patterns of signalling effectors in rafts, but does not directly compare their levels. Quantification of at least two films from independent experiments was performed for each protein analysed. Data are expressed as the mean signal ± S.E.M. (when $n = 3$–4) or mean ± range of intensities (when $n = 2$).

RESULTS

FcαR levels in membrane rafts increase following cross-linking

Rafts were biochemically isolated by extraction in Triton X-100 followed by sucrose-density-gradient centifugation prior to and following FcαR cross-linking and incubation at 37 °C. Equal amounts of raft-associated protein were then resolved by SDS/PAGE under reducing conditions and transferred to nitrocellulose before immunoblotting for FcαR (Figure 1a). FcαR appears as a broad 50–70 kDa band consistent with its variable glycosylation status. FcαR is detectable in rafts from cells treated with DAM only and held at 4 °C (Figure 1a). In cells where FcαR had been ligated with anti-FcαR mAb (My43), then cross-linked with DAM, a substantial increase in the level of FcαR in raft fractions was observed after warming cells to 37 °C for 30 s or 2 min. After 5 min, FcαR had decreased to baseline levels. Interestingly, there appeared to be a preferential increase in higher-molecular-mass forms of FcαR, suggesting that more highly glycosylated FcαR may be more easily retained in rafts following cross-linking. Confirmation that the 50–70 kDa band reactive with anti-FcαR antibody was actually FcαR was obtained by immunoblotting of FcαR-expressing U937 cell lysates and untransfected FcαR-negative A20 IIA1.6 cells (results not shown).

Increased levels of cross-linked FcαR in rafts are accompanied by an increase in the level of the γ-chain homodimer and its tyrosine phosphorylation (Figure 1b and [15]). Following cross-linking of FcαR, we observed the appearance of higher-molecular-mass proteins reactive with the anti-γ-chain mAb that represent tyrosine-phosphorylated γ-chain [8]. The level of γ-chain was observed to correspond to the level of FcαR in the rafts (Figure 1c), consistent with the tight association of FcαR and γ-chain [6].

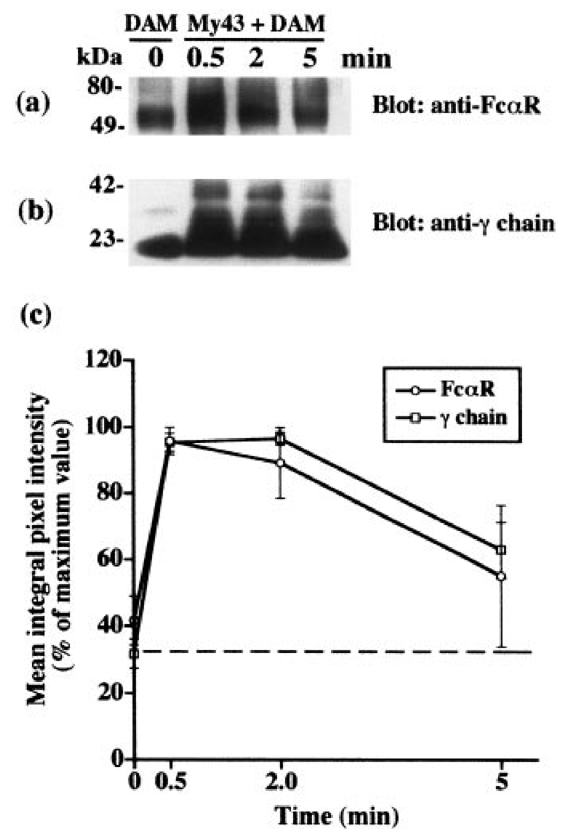

Figure 1 FcαR levels in membrane rafts increase following cross-linking

Membrane rafts were isolated from cells following treatment with DAM only, and following cross-linking of FcαR-ligated anti-FcαR mAb (My43 + DAM). DAM-treated cells were held at 4 °C while My43 + DAM cells were incubated at 37 °C for the times indicated. (**a**) Equal amounts of protein from membrane-raft fractions were resolved by SDS/PAGE before transferring to nitrocellulose and immunoblotting for FcαR. Data shown are representative of two independent experiments. (**b**) Raft fractions were resolved by SDS/PAGE before immunoblotting for the Fc receptor γ-chain. Immunoblots for γ-chain were performed for every raft preparation. Data shown are representative of four independent experiments. (**c**) Densitometry was performed on immunoblots using Adobe Photoshop 5.0 as described in the Materials and methods section. Graph shows the mean levels of FcαR-associated signal (○, $n = 2$) and γ-chain-associated signal (□, $n = 4$).

FcαR cross-linking triggers tyrosine phosphorylation of tyrosine kinases in rafts

The PY20 anti-phosphotyrosine antibody was used to immunoprecipitate tyrosine-phosphorylated proteins from rafts before immunoblotting for tyrosine-phosphorylated proteins (Figure 2a). We observed a low level of tyrosine phosphorylation in unstimulated cells (DAM only) that increased with time on FcαR cross-linking (My43 + DAM). Proteins were detected predominantly in the 50–90 kDa range, consistent with the recruitment of Lyn (53/56 kDa) and Btk (77 kDa), as well as Blk (55 kDa) and Syk (67 kDa in murine cells [32]). Transfectants expressing FcαR but no γ-chain were used as a control for the cross-linking conditions in these studies (Figure 2a). We observed a very small increase in the levels of tyrosine phosphorylation in rafts following FcαR cross-linking in γ-chain-negative transfectants, although in these cells FcαR does partition into rafts [15]. We immunoblotted rafts from FcαR- and γ-chain-transfected cells. We detected an increase in the levels of tyrosine-phosphorylated Blk following FcαR cross-linking within 30 s, which persisted

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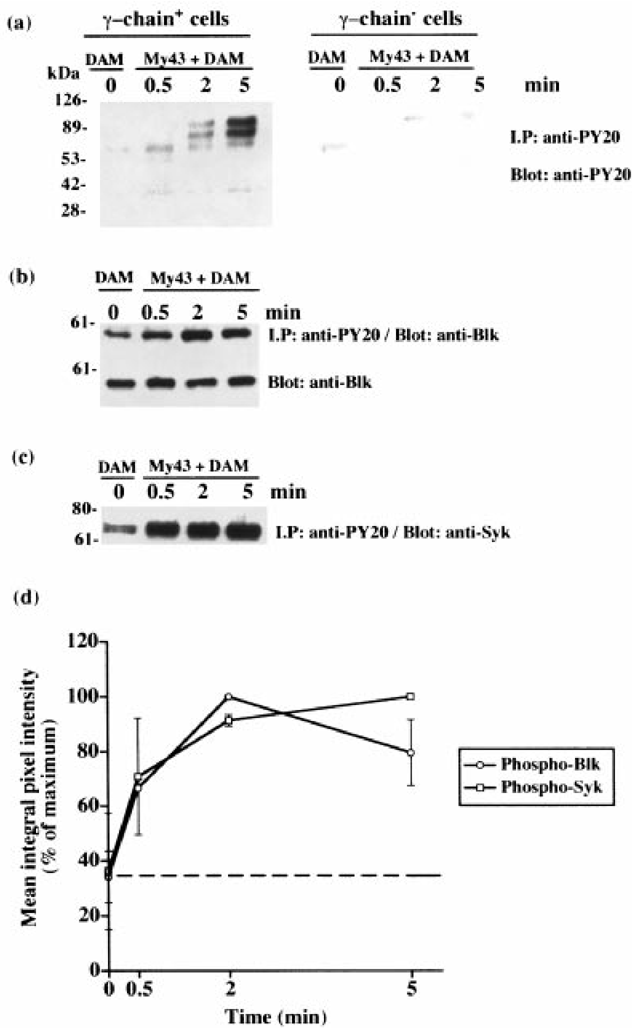

Figure 2 FcαR cross-linking triggers tyrosine phosphorylation of tyrosine kinases in rafts

Tyrosine-phosphorylated proteins were immunoprecipitated (I. P.) from equal amounts of raft protein prior to (DAM) and following (MY43 + DAM) FcαR cross-linking. (**a**) Samples were immunoblotted for tyrosine-phosphorylated proteins in FcαR/γ-chain transfectants (left-hand panel) and control γ-chain-negative transfectants (right-hand panel). (**b**) After immunoprecipitation and resolution by SDS/PAGE, tyrosine-phosphorylated proteins from FcαR/γ-chain transfectants were immunoblotted for tyrosine-phosphorylated Blk (top panel). Whole-raft fractions were also immunoblotted for total Blk (lower panel). (**c**) After immunoprecipitation and resolution by SDS/PAGE, tyrosine-phosphorylated proteins were immunoblotted for tyrosine-phosphorylated Syk. Data are representative of two independent experiments for PY20 and Blk, and three independent experiments for Syk. In (**d**), mean levels of phospho-Blk (○, $n = 2$) and phospho-Syk (□, $n = 3$) were analysed as described in the Materials and methods section and plotted against time.

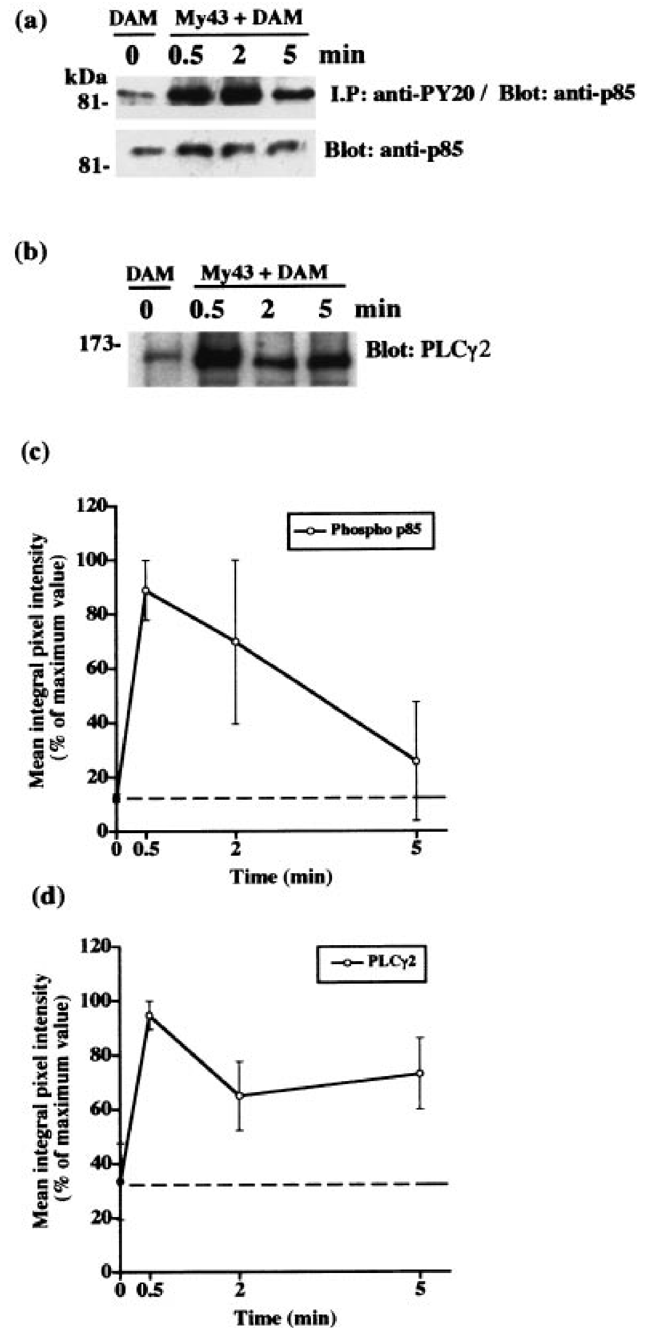

Figure 3 FcαR cross-linking triggers recruitment of phosphoinositide kinases to rafts

(**a**) Tyrosine-phosphorylated proteins were immunoprecipitated (I.P.) from equal amounts of raft protein prior to (DAM) and following (My43 + DAM) FcαR cross-linking. Samples were then resolved by SDS/PAGE and immunoblotted for tyrosine-phosphorylated p85 subunit of PI 3-kinase (top panel). Whole-raft fractions were also immunoblotted for total p85 (lower panel). (**b**) Raft fractions were immunoblotted for PLCγ2 prior to and following FcαR cross-linking. Data are representative of two independent experiments for (**a**) and four independent experiments for (**b**). (**c**, **d**) Mean levels of (**c**) p85 and (**d**) PLCγ2 were plotted against time.

over a 5 min duration (Figure 2b). The level of total Blk in rafts remained constant, consistent with observations that Src-family kinases are localized constitutively in rafts (reviewed in [33]). It is established that γ-chain is phosphorylated by Lyn in response to FcεRI receptor cross-linking and that this allows phosphorylated γ-chain to bind the SH2 domains of Syk [34]. We therefore immunoprecipitated tyrosine-phosphorylated proteins from rafts and immunoblotted for Syk (Figure 2c). We observed an increase in the level of tyrosine-phosphorylated Syk in rafts following FcαR cross-linking. We did not observe any increase in levels of tyrosine-phosphorylated Blk, or Syk in γ-chain-negative transfectants following FcαR cross-linking (results not shown). We previously reported similar findings for Lyn and Btk [15]. Interestingly, in the present studies, elevated levels of tyrosine-phosphorylated Blk and Syk were present after 5 min, when

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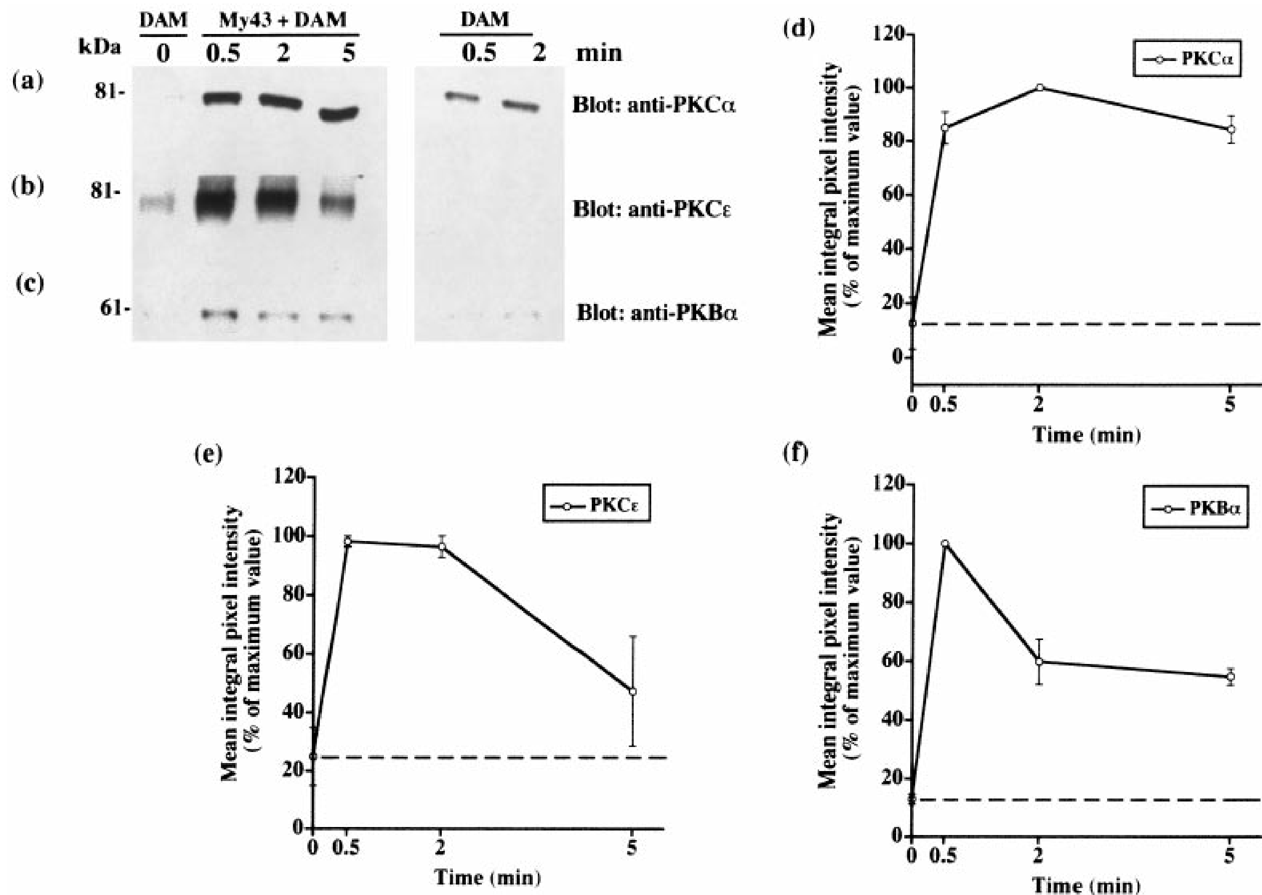

Figure 4 FcαR cross-linking triggers recruitment of serine/threonine kinases to rafts

Equal amounts of protein from membrane-raft fractions were resolved by SDS/PAGE and immunoblotted for the serine/threonine kinases PKC and PKBα. (**a**) Shows the Ca^{2+}-dependent PKCα isoform and (**b**) shows the Ca^{2+}-independent PKCε isoform. (**c**) Shows the PI 3-kinase-dependent kinase PKBα. Data shown are representative of two similar independent experiments. Mean levels of (**d**) PKCα, (**e**) PKCε and (**f**) PKBα were plotted against time.

FcαR levels had returned to those observed before cross-linking (Figure 2d). This suggests that signalling continues as FcαR is lost from the rafts.

FcαR cross-linking triggers recruitment of phosphoinositide kinases to rafts

A favoured hypothesis is that lipid rafts provide an environment rich in substrates for phosphoinositide kinases [19,20]. Membrane rafts are enriched in critical lipid species such as PtdIns(4,5)P_2, the major substrate for phosphoinositide kinases including PI 3-kinase and PLC [35]. The activity of phosphoinositide kinases may be regulated depending on whether the enzyme concerned is raft-localized or not. Using specific inhibitors, we have shown previously that MHC class II presentation of FcαR-targeted antigen is dependent on PI 3-kinase activation [14]. It was therefore feasible that FcαR cross-linking would cause recruitment of PI 3-kinase to membrane rafts. Protein p85 is the the regulatory subunit of PI 3-kinase (reviewed in [36]). We observed that, on FcαR cross-linking, there was a transient increase in the level of p85 (Figure 3a, lower panel). The level of p85 increased within 30 s and diminished over the 5 min time course. Immunoprecipitation of tyrosine-phosphorylated proteins from the same samples before immunoblotting for p85 revealed that tyrosine phosphorylation of p85 had the same pattern as the level of total p85 in the rafts (Figure 3a, top panel). This suggests that phosphorylation and regulation of p85 occurs on entry into rafts, which is consistent with the presence of kinases thought to regulate PI 3-kinase (Syk), or kinases that are activated as a result of PI 3-kinase activity (Btk). An alternative explanation is that immunoprecipitation of tyrosine-phosphorylated proteins from rafts caused an enrichment of tyrosine-kinase-associated PI 3-kinase, giving the appearance of p85 phosphorylation. However, tyrosine phosphorylation of p85 has been shown to increase PI 3-kinase activity in COS cells by removal of the inhibitory effect of p85 on the catalytic subunit p110 [37]. The observation that the level of tyrosine phosphorylation decreases as p85 leaves the rafts suggests that its activity may be decreased after removal from the rafts.

We examined raft fractions for the phosphoinositide kinase PLC, which triggers release of Ca^{2+} from intracellular stores by production of Ins(1,4,5)P_3 from the PtdIns(4,5)P_2 substrate, as well as PKC activation by the production of Ca^{2+} and diacylglycerol (DAG; reviewed in [38]). PLCγ2 was detected primarily in the non-raft fraction (results not shown). On FcαR cross-linking, increased levels of PLCγ2 were detected in rafts following incubation at 37 °C for 30 s (Figure 3b). This was followed by a sustained, albeit lower, level of PLCγ2 in the rafts for 5 min. In

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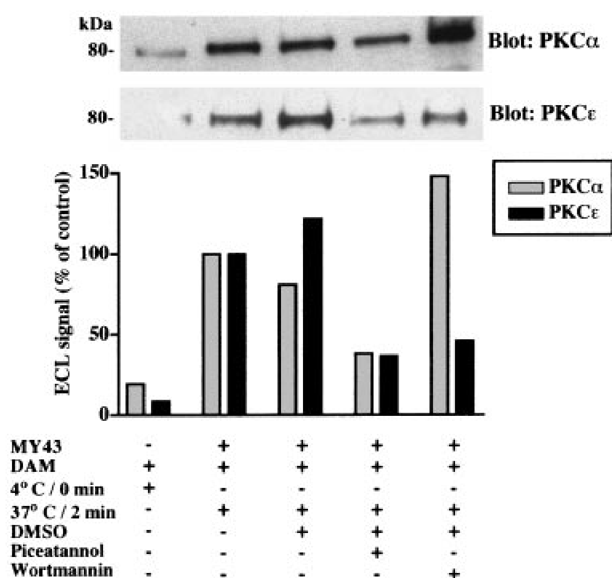

Figure 5 FcαR cross-linking triggers divergent signalling in rafts

Rafts were isolated prior to (DAM) and following (My43 + DAM) FcαR cross-linking and incubation at 37 °C. Cells were either untreated or treated with DMSO (1 μl/ml), piceatannol (20 μM) or wortmannin (0.1 μM). Equal amounts of protein were resolved by SDS/PAGE and immunoblotted for PKCα (top panel) or PKCε (middle panel). Immunoblots were quantificated by densitometry and levels of PKCα or PKCε normalized to levels of Blk in the samples. Data are expressed graphically as the percentage of total ECL signal relative to control samples (taken as FcαR cross-linkage and incubation at 37 °C for 2 min). Lanes on immunoblots correspond to bars on the graph and key.

contrast, all of the detectable PLCγ1 was observed in the non-raft fraction, and there was no observed recruitment to rafts on FcαR cross-linking (results not shown).

Quantification of immunoblots revealed that increased levels of p85 following FcαR cross-linking and incubation at 37 °C were transient, returning to basal levels between 2 and 5 min (Figure 3c). In contrast, although PLCγ2 levels reached a maximum at 30 s, increased levels of PLCγ2 (compared with 0 min) were sustained over 5 min (Figure 3d).

FcαR cross-linking triggers recruitment of serine/threonine kinases to rafts

Given the presence of PLCγ2 and PI 3-kinase in the rafts, and our prior observations that FcαR cross-linking triggers activation of a PI 3-kinase/PDK1/PKBα signalling pathway, we analysed raft fractions for the presence of serine/threonine kinases before and following FcαR cross-linking (Figure 4). We immunoblotted rafts for both Ca^{2+}-dependent and Ca^{2+}-independent isoforms of PKC, a major class of serine/threonine kinase (reviewed in [38]). We observed, upon cross-linking FcαR, an increase in the levels of the Ca^{2+}-dependent isoform, PKCα (Figure 4a), and the Ca^{2+}-independent isoform, PKCε (Figure 4b). These data show for the first time the recruitment to rafts and thus potential for activation of Ca^{2+}-dependent and Ca^{2+}-independent isoforms of PKC by FcαR. Our previous confocal microscopy studies demonstrated that capped cross-linked FcαR co-localized with PDK1 and PKBα, suggesting, but not directly demonstrating, that FcαR could recruit PKBα to membrane rafts [14]. We did not detect PKBα prior to FcαR cross-linking and incubation at 37 °C. However, after 30 s we observed the appearance of PKBα that then decreased (by approx. 50 %) over 5 min (Figure 4c), showing that cross-linked FcαR recruits PKBα to the rafts. Interestingly, the elevated level of PKCα in the rafts following FcαR cross-linking persisted longer than that of PKCε or PKBα (Figures 4d–4f). Although DAG is available to both C1 lipid-binding domains of PKCα and PKCε isoforms, differences may exist in the binding of the respective C2 domains of PKCα and PKCε to other membrane lipids, such as phosphatidic acid [35].

FcαR cross-linking triggers divergent signalling in rafts

Our analysis of immunoblots revealed that PI 3-kinase and its effectors had a more transient association with rafts than other signalling effectors. These observations are consistent with the activation of divergent signalling events in rafts, namely PLCγ2/PKCα and PI 3-kinase/PDK1/Ca^{2+}-independent serine/threonine kinases. Signalling inhibitors were therefore used to allow us to obtain evidence supporting our hypothesis. We treated cells with the Syk inhibitor piceatannol or the PI 3-kinase inhibitor wortmannin prior to FcαR cross-linking and raft isolation. We observed that inhibition of Syk prevented recruitment of PKCα to rafts, but inhibition of PI 3-kinase resulted in an enhancement of PKCα recruitment (Figure 5). We also observed that inhibition of Syk or PI 3-kinase prevented recruitment of PKCε to rafts (Figure 5). The enhancement of PKCα recruitment in response to PI 3-kinase inhibition may occur because prevention of PKCε recruitment leaves more DAG-binding sites in rafts for PKCα. At the concentrations used, piceatannol also has an inhibitory effect on PKC [39]. Thus caution must be applied in the interpretation of these data. In this context, piceatannol can be regarded as an inhibitor that cannot differentiate between Ca^{2+}-dependent PKC isoforms and Ca^{2+}-independent PKC isoforms, wheras wortmannin is able to distinguish between them. In DMSO controls there was no effect on recruitment of either PKCα or PKCε. Quantification of the immunoblots was performed (Figure 5). For quantification, signals were normalized to Blk levels, which do not change on signalling (see Figure 2b). Determination of the effect of inhibitors on PKBα recruitment was attempted, but the weak signal from the immunoblots made assessment unreliable. Attempts were also made to use inhibitors of PLCγ2, but these were disruptive to membrane rafts, preventing their isolation.

DISCUSSION

Our aim in the present study was to determine which signalling effectors were recruited to membrane rafts following FcαR cross-linking, and whether any of these effectors corresponded to those we had identified previously as being localized to the endocytic pathway following FcαR internalization [14]. Partitioning of FcαR into rafts was indeed accompanied by recruitment of multiple signalling effectors, but moreover, different effectors were observed to reside in the rafts for different lengths of time after initial recruitment. A distinct pattern of temporal localization of effectors emerged in these studies. Syk, Blk and PKCα had a sustained association ($>$ 5 min) with rafts following FcαR cross-linking and incubation at 37 °C (Figure 6a, left-hand panel). In contrast, FcαR, γ-chain, PI 3-kinase and PKCε all had a transient association (2–5 min) with rafts (Figure 6a, middle panel). In two instances (PLCγ2 and PKBα) the pattern of association in rafts was intermediate between the sustained and transient patterns (Figure 6a, right-hand panel). This intermediate pattern is perhaps attributable to greater inter-experiment variablity in the PLCγ2 signals. The partial retention (approx. 50 %) of PKBα in rafts is consistent with observations

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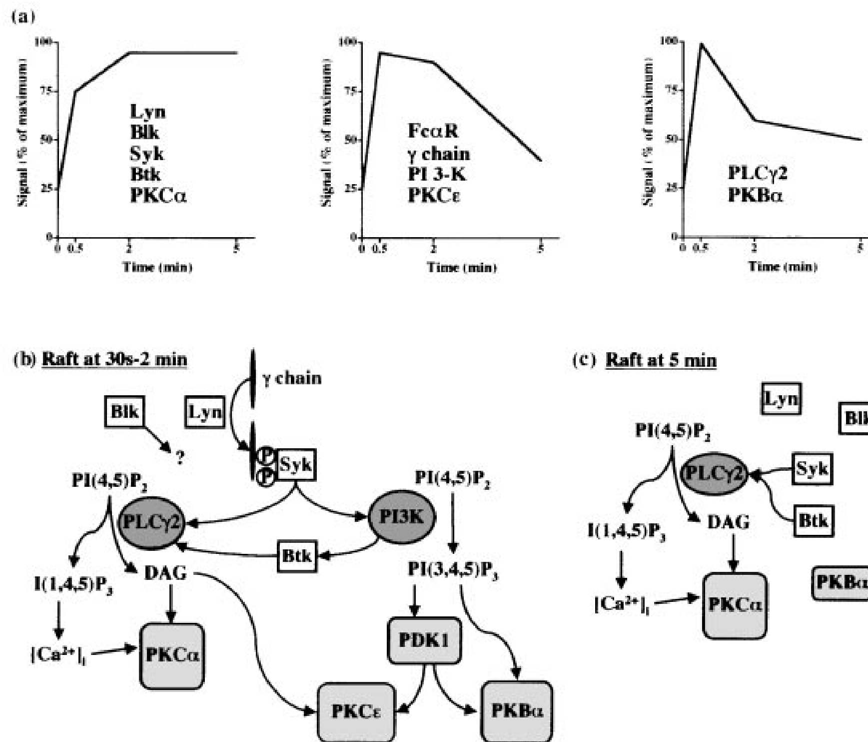

Figure 6 FcαR cross-linking triggers divergent signalling in rafts

(**a**) In this model, increased association of Lyn, Blk, Syk, Btk and PKCα (left-hand panel) with rafts is sustained beyond the duration of FcαR/γ-chain increases ($>$ 5 min). PI 3-kinase and PKCε (middle panel) have a transient increase in association with rafts, returning to baseline levels within 2–5 min. PLCγ2 and PKBα (right-hand panel) have an association with rafts that is intermediate between the sustained and transient responses. (**b**) After 30 s–2 min of FcαR cross-linking, several signal-transduction effectors are localized to rafts, consistent with divergent signalling. PI3K, PI 3-kinase. (**c**) By 5 min, FcαR/γ-chain complexes and PI 3-kinase and its effectors have been removed from rafts.

that some PKBα is retained at the membrane during its translocation to endosomes [14]. These data suggested a potential divergence of signalling events resulting from the temporal regulation of signalling effector association with rafts (Figures 6b and 6c). As we will discuss, this divergence may be amplified by a spatial re-organization of signalling effectors. Selected signalling effectors (PDK1 and PKBα) are removed from rafts to other subcellular compartments [14]. This is consistent with the reported functions of PI 3-kinase and its effectors in subcellular compartments, including early endosomes [40,41].

Differential exclusion from, or specific distribution of, tyrosine kinases in rafts following receptor cross-linking have been demonstrated recently in a related Fc receptor system. Wilson and co-workers [24], using electron microscopy, showed that following cross-linking and incubation at 37 °C for 2 min of FcεRI (also γ-chain-associated), small FcεRI- and Lyn-containing rafts in resting cells clustered to form larger domains containing Syk. Following cross-linking, Lyn was shown to be at the periphery of those FcεRI- and Syk-containing domains [24]. In a following study, the ligation of FcεRI for 5 min at 37 °C with a mAb that causes dimerization of the FcεRI$\alpha\beta\gamma_2$ tetramer resulted in association of the FcεRI with Lyn but not Syk. On cross-linking of the FcεRI-bound mAb and incubation at 37 °C for 5 min, Lyn was dissociated from FcεRI, and levels of FcεRI-associated Syk increased dramatically [42]. Data presented herein complement these studies. We observe continued phosphorylation of Syk (5 min) after Lyn phosphorylation has returned to baseline levels [15], at a time when Syk is predicted to have a greater association with FcαR than Lyn. The differential kinetics of dissociation of other signalling effectors from rafts may be enhanced by spatial regulation, as seems to be the case with Lyn and Syk. PLCγ2/PKCα-mediated and PI 3-kinase/PDK1/PKBα/PKCε-mediated signalling events may be another example of separation of signalling events, where temporal regulation and spatial reorganization occurs in rafts (Figure 6c).

To further investigate these ideas, we used signalling inhibitors to block Syk- or PI 3-kinase-dependent events. Inhibition of Syk blocked recruitment to rafts of both the classical (Ca^{2+}-dependent) PKC (cPKC; PKCα) and of the novel (Ca^{2+}-independent) PKC (nPKC; PKCε), which is expected given inhibitory effects on PKC at the concentrations used [39]. Inhibition of PI 3-kinase blocked recruitment of PKCε, but not PKCα, consistent with the need for PDK1-dependent phosphorylation of nPKC isotypes for activation [43]. Our data suggest activation of separable signalling events. PI 3-kinase-dependent recruitment of PKCε and PKBα via PDK1 activation, along with the lack of inhibition of PKCα recruitment, suggests the PI 3-kinase-independent activation of PKCα signalling events. Although there seem to be temporal differences with regard to dissociation of certain signalling effectors from rafts, existing literature on FcεRI and other systems reports that PLCγ2/PKCα signalling and PI 3-kinase/PDK1/PKBα/PKCε signalling events do interact. For example, the nPKCs as well as the cPKCs are recruited to membranes by DAG [produced by the action of PLCγ2 on PtdIns(4,5)P_2] [44]. Indeed, we observed recruitment of both cPKC (PKCα) and nPKC (PKCε) isoforms to rafts. This is consistent with PLCγ2 playing a role in recruitment of PI 3-kinase-dependent effectors to rafts (DAG-mediated recruitment

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of PKCϵ; Figure 6b). Btk can potentiate Ca^{2+} signalling, in part by increasing the duration of PLCγ activity [45]. We have shown, in a previous study, sustained recruitment of Btk to rafts following FcαR cross-linking [5]. Btk is activated through binding of its pleckstrin homology domains to the PI 3-kinase product PtdIns(3,4,5)P_3 [45]. In the FcαR system, this suggests that PI 3-kinase could influence PLCγ2/PKCα signalling through Btk (Figure 6b). Furthermore, PtdIns(3,4,5)P_3 can enhance PLCγ2 activity, allowing PI 3-kinase to potentiate PLCγ2/PKCα activation directly [46]. Thus there are at least two likely points of interaction between elements of FcαR signalling events that we have identified (Figure 6b). As regards divergence of signalling, the interactions between the PLCγ2/PKCα and PI 3-kinase/PDK1/PKBα/PKCϵ 'arms' of the signalling pathway may occur at the level of initial (0–30 s) recruitment and activation. Therefore, the temporally regulated re-organization of signalling elements (2–5 min) may occur after these events (Figure 6c).

The potential significance of our findings is apparent when we consider the signal-transduction effectors PDK1 and PKBα in the context of endosomal trafficking. Endocytosis of FcαR and γ-chain occurs following FcαR cross-linking [47], followed by transport (15–30 min) of the complex to specialized endocytic vesicles (MHC class II peptide-loading compartment) that are essential for processing and presentation of FcαR-targeted antigen [5,14]. PDK1 and PKBα are also translocated to MHC class II peptide-loading-compartment vesicles along with cross-linked FcαR [14]. The transport of PDK1 and PKBα require signalling by the FcαR-associated γ-chain. The requirement for PI 3-kinase activation in FcαR-mediated antigen presentation suggests that PDK1 and PKBα could also be involved in regulating the endocytic pathway [14]. PtdIns(3,4,5)P_3 is essential for recruitment to membranes and activation of PDK1, PKBα and PKCϵ [48,49]. We observed the production of PtdIns(3,4,5)P_3 at the plasma membrane proximal to clustered cross-linked FcαR (30 s–2 min), followed by the appearance of PtdIns(3,4,5)P_3 (5 min) in endocytic vesicles containing FcαR (M. L. Lang and W. F. Wade, unpublished work). The presence of PtdIns(3,4,5)P_3 and PDK1/PKBα along with FcαR antigen, internalized from rafts to the endocytic pathway, may be a means to maintain signalling through PI 3-kinase at multiple intracellular locations, as well as to mediate transport of signalling complexes to different intracellular locations. This postulated internalization and continued signalling of effectors is not unique to FcαR. Ligation of the platelet-derived growth factor receptor in fibroblasts results in its endocytosis to compartments containing DAPP1 (dual adaptor for phosphotyrosine and 3-phosphoinositides 1), a PI 3-kinase effector [50]. A recent review also cites several examples of continued signalling by plasma-membrane receptors following endocytosis [51].

Interestingly, PI 3-kinase is crucial to the formation of EEA1 (early endosomal antigen 1)- and Rab5-containing fusion complexes on endocytic vesicles which are thought to direct the trafficking and fusion of early endosomes [39,44]. There is an emerging viewpoint in cell biology that PI 3-kinase and its downstream effectors are important for vesicle trafficking [40,41]. Signalling events triggered by receptors directly regulate trafficking of those receptors [5,52,53]. We have shown that some signalling effectors recruited to rafts by FcαR are translocated to vesicles to facilitate FcαR transport through the endocytic pathway [15]. These events may be initiated by the recruitment of multiple kinases to membrane rafts that are then re-organized according to function, resulting in a distinct temporal and spatial organization of signalling effectors. It is now apparent that in studying the activity of signalling effectors, consideration will have to be given to their subcellular localization. Our future efforts will be directed at further identification and delineation of FcαR-triggered signalling pathways in rafts, and understanding how their regulation is important for the trafficking of FcαR to endosomal compartments which process antigen.

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