Endocytosis of FcγRI is regulated by two distinct signalling pathways

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Abstract Aggregation by immune complexes of receptors specific for the Fc region of IgG results in their internalisation and disposal by trafficking to lysosomes. We show here that internalisation of FcγRI by IFN-γ treated U937 cells following receptor aggregation by cross-linking antibodies requires the activation of two distinct signalling pathways. The pathways were functionally dissected in streptolysin-O-permeabilised cells by capitalising on their relative dependence on active GTP binding proteins. One pathway required the presence of GTP-γS or active βγ subunits, the other did not. Use of inhibitors revealed that the βγ-independent pathway required activation of PI 3-kinases and was PKC-independent In contrast, the βγ-dependent pathway involved activation of phospholipase C-β and PKC, but was PI 3-kinase-independent. Both these pathways were found to be active in intact cells and are likely to determine receptor trafficking following internalisation.

Key words: FcγRI; Endocytosis; PI 3-kinase; βγ subunit; Streptolysin-O; PKC

1. Introduction

Receptors (FcγRs) specific for the constant region (Fc) of immunoglobulin G (IgG) are expressed on the surface of many cells of the immune system and play a pivotal role linking the cellular and humoral arms of the immune response to antigen [1]. On myeloid cells, aggregation of these receptors triggers a number of different effector functions including internalisation of immune complexes by endocytosis and engulfment of opsonised particles through the process of phagocytosis.

The human high affinity IgG receptor, FcγRI, is an integral type I membrane protein [2] constitutively expressed on monocytes and macrophage cell types. The cytoplasmic tail of FcγRI contains no obvious signalling motif, however, it associates non-covalently with the γ-chain which enables the recruitment of tyrosine kinases, such as syk [3], through their src-homology domains. These tyrosine phosphorylation events have been shown to be involved in activation of calcium transients [4] and post-internalisation vesicular trafficking of FcγRI to lysosomes [5,6]. FcγRI has also been shown to activate phosphoinositide 3-kinases (PI 3-kinases) of the IA (p85-dependent) and IB (G-protein βγ subunit-dependent) classes [7]. However, although PI 3-kinases have been implicated in regulating the internalisation and post-endocytic trafficking of various tyrosine kinase linked receptors, little is known of the signal transduction mechanisms underlying FcγRI endocytosis. In this study we have used streptolysin-O-permeabilised cells to define the signal transduction pathways mediating the endocytosis of FcγRI following aggregation with a cross-linking antibody. We report that two distinct pathways are responsible for this. One is a tyrosine kinase/PI 3-kinase-dependent step which does not require the activity of PKC or phospholipase C, and the other is a phospholipase C-β- and PKC-dependent mechanism that proceeds to internalise FcγRI in the absence of PI 3-kinase activity. Both of these pathways need to be fully active in order to allow efficient internalisation of immune complexes by FcγRI.

2. Materials and methods

2.1. Materials and cells

All chemicals and antibodies were purchased from Sigma, unless otherwise stated. Tissue culture reagents were purchased from Life Technologies Ltd. All experiments employed the human histiocytic lymphoma cell line, U937, and these were cultured in RPMI supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, 10 IU/ml penicillin and 10 mg/ml streptomycin. U937 cells were stimulated with IFN-γ (200 ng/ml) for 18 h prior to experimentation.

2.2. Internalisation of ^{125}I-IgG by streptolysin-O-permeabilised cells

A suspension of cells was loaded with ^{125}I-IgG in 500 μl PBS (pH 7.4) containing 1% BSA on ice for 30 min to occupy cell surface FcγRI, as described previously [8]. Unbound ^{125}I-IgG was removed by washing with 50 ml PBS at 4°C and the cells resuspended in glutamate buffer (137 mM Na-glutamate, 2 mM $MgCl_2$, 1 mg/ml BSA and 20 mM Na-PIPES, pH 6.8).

30 μl of ice-cold cell permeabilisation buffer (glutamate buffer containing 66 U/ml streptolysin-O (Sigma S-5265), 3 mM Mg-ATP, 1 mM GDP and with the free calcium concentration held at 50–100 nM by the use of a 3 mM EGTA buffer system) was placed into 4 ml polystyrene tubes. Where appropriate 48 μg/ml of cross-linking antibody (goat anti-human (Fab-specific); Sigma I-5260), 100 μM GTP-γS, 1 μM βγ subunits (these were purified from bovine brain according to [9]) and were a kind gift from Dr Len Stephens, Babraham Institute, Babraham Hall, Cambridge CB2 4AT, UK), 0.37 mM genistein (Calbiochem; 345834), 50 nM wortmannin, 1 μM calphostin C (Calbiochem; 208725) and 10 μM of the phospholipase C inhibitor, U-73122 (Calbiochem; 662035) or its inactive analogue U-73343 (Calbiochem; 662041) were included in the incubation. In order to maintain the protein in solution, the βγ preparation contains the mild detergent, octyl-β-D-glucopyranoside (OG). The final concentration of OG in the permeabilised cells was 0.03% and this was found not to affect the internalisation of ^{125}I-IgG either basally or in response to cross-linking antibody with or without GTP-γS.

30 μl of ice-cold ^{125}I-IgG-pre-labelled cell suspension was added to the permeabilisation mixture (final assay volume of 60 μl). The cells were then warmed to 37°C for the indicated times to allow internalisation and then returned to 4°C.

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Non-internalised ^{125}I-IgG was then removed by two washes in ice-cold PBS (pH 2.5) and the cell-associated radioactivity determined. Data are expressed as a percentage of total initial cell-associated counts and presented as the mean ± S.E.M. from at least three separate experiments (as indicated in the appropriate figure legends).

2.3. Internalisation of ^{125}I-IgG by intact cells

30 μl of RPMI containing 20 mM HEPES and 1% FCS (RPMIHF) was placed into 4 ml polystyrene tubes with or without 48 μg/ml cross-linking antibody (Sigma I-5260) and 50 nM wortmannin and 1 μM calphostin C where appropriate. 30 μl of ice-cold ^{125}I-IgG preloaded cells suspended in RPMIHF were added (to a final volume of 60 μl as for streptolysin-O-permeabilised cells. Cells were warmed to 37°C for the indicated times to allow internalisation and then returned to 4°C. Internalised ^{125}I-IgG was then determined as for streptolysin-O-permeabilised cells.

3. Results

3.1. Internalisation of FcγRI in intact U937 cells

IFN-γ-primed U937 cells were loaded with ^{125}I-hIgG$_1$ at 4°C in order to tag the cell surface pool of FcγRI. Internalisation of the receptor:ligand complex was then determined following transfer of the cells to 37°C. Fig. 1 shows that as previously reported [8], addition of an antibody to aggregate FcγRI, resulted in rapid internalisation of FcγRI such that at least 80% of the receptor became internalised within 15 min. We have previously reported that inhibition of PI 3-kinases with wortmannin causes a limited reduction in FcγRI internalisation following aggregation [10]. Consistent with this, Fig. 1 shows that treatment of the cells with 50 nM wortmannin caused a small inhibition of receptor internalisation, particularly evident at early time points. However, the bulk of FcγRI internalisation was resistant to inhibition by wortmannin and, thus, may be controlled by another cell signalling pathway. The activity of PKC has been implicated in the internalisation of a number of receptors. We therefore investigated the effect

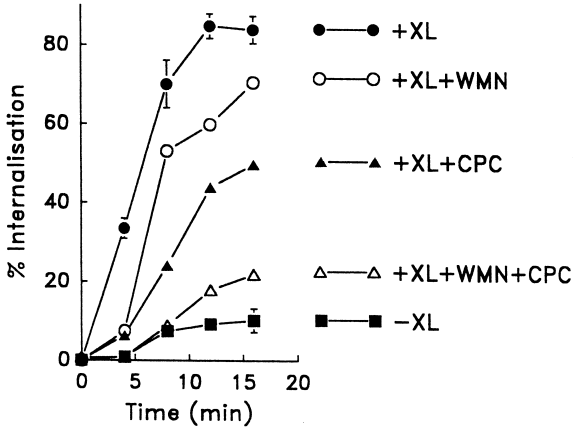

Fig. 1. Intact U937 cells require PI 3-kinase and PKC activities for efficient internalisation of aggregated FcγRI. IFN-γ treated U937 cells were incubated in the presence of 50 nM wortmannin (○), 1 μM calphostin C (▲), a combination of both reagents (△) or remained untreated (●,■) for 10 min at 37°C. Cells were then cooled to 4°C and labelled with ^{125}I-IgG$_1$. Following removal of unbound label, the cells were warmed to 37°C in the presence (●,▲,△,○) and absence (■) of goat anti-human cross-linking antibody. Non-acid releasable counts were determined at the time points indicated and expressed as a percentage of total initial cell associated counts (values are mean ± S.E.M. $n = 6$).

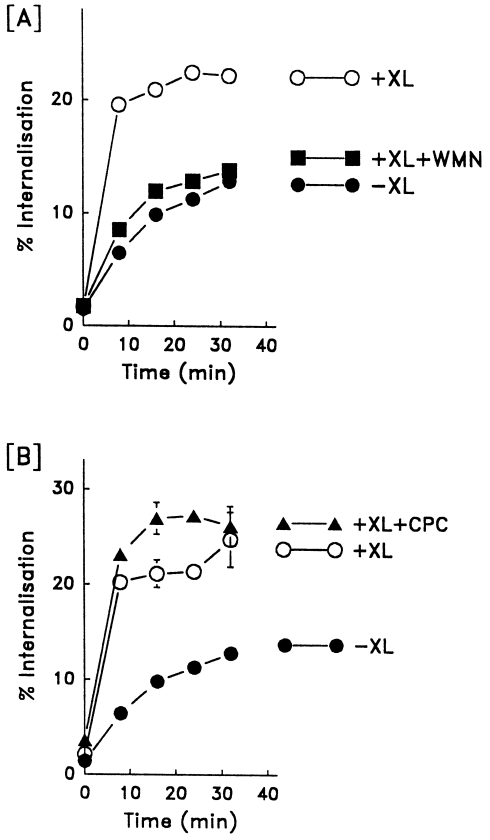

Fig. 2. Wortmannin sensitivity of FcγRI internalisation in streptolysin-O-permeabilised cells. A: Cells were incubated in the absence (○,●) and presence (■) of 50 nM wortmannin for 10 min at 37°C, cooled to 4°C and labelled with ^{125}I-IgG$_1$ as for Fig. 1. The cells were then warmed to 37°C in permeabilisation buffer in the absence (●) and presence (○,■) of goat anti-human cross-linking antibody. Internalisation was determined as for Fig. 1 (values are mean ± S.E.M. $n = 6$). B: Cells were treated as for A except that 1 μM calphostin C (▲) was used in place of wortmannin.

of calphostin C, an effector site inhibitor of PKC, on FcγRI internalisation. Calphostin C at 1 μM, inhibited the internalisation of FcγRI by approximately 50%; a more potent inhibition than wortmannin, but still not complete (Fig. 1). However, a profound inhibition of FcγRI internalisation was observed following treatment of the cells with a combination of wortmannin and calphostin C (Fig. 1). The inhibitory effects of wortmannin and calphostin C are roughly additive, indicating that FcγRI may be internalised by distinct PI 3-kinase and PKC-dependent mechanisms.

3.2. In streptolysin-O-permeabilised cells FcγRI internalisation is PI 3-kinase-dependent

In order to further dissect the cell signalling pathways involved in FcγRI internalisation we employed a cell permeabilisation approach. Streptolysin-O produces large lesions in the plasma membrane, whilst internal membranes, such as endosomes remain intact. Fig. 2A shows that following streptolysin-O permeabilisation, cross-linking of FcγRI was able to induce receptor internalisation by approximately two-fold. This was abolished by wortmannin (Fig. 2A), but was resistant to inhibition with calphostin C (Fig. 2B). This indicates the presence of a PI 3-kinase-dependent pathway responsible

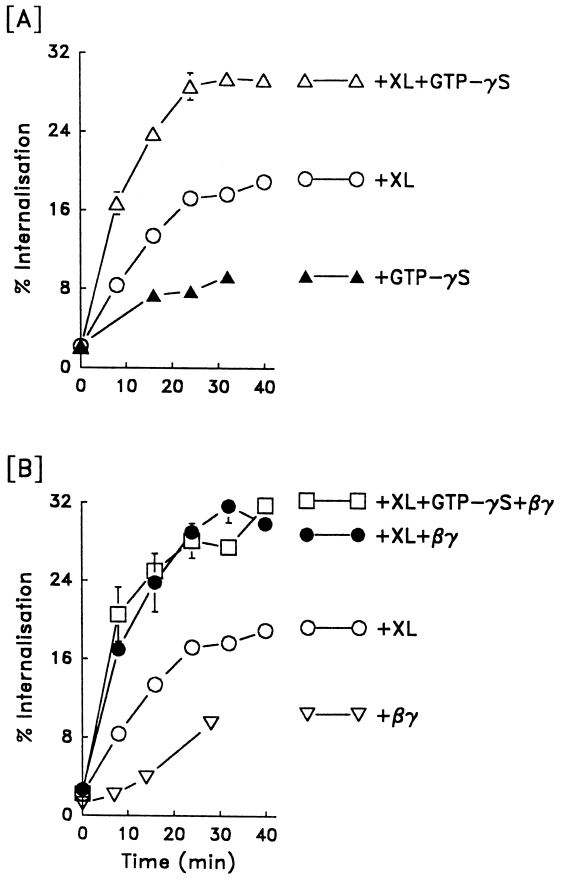

Fig. 3. βγ-dependent FcγRI internalisation. A: IFN-γ treated U937 cells were cooled to 4°C and labelled with ^{125}I-IgG$_1$. Following removal of unbound label, the cells were warmed to 37°C in permeabilisation buffer in the absence (○) and presence (▲,△) of 100 μM GTP-γS and the presence (○,△) and absence (▲) of goat anti-human cross-linking antibody. Internalisation was determined as for Fig. 1 (values are mean ± S.E.M. $n = 6$). B: Cells were treated as for A, except that cells were permeabilised in the absence (○) and presence (□,▽,●) of 1 μM purified brain βγ subunits and the presence (○,□,●) and absence (▽) of goat anti-human cross-linking antibody. A series was also included to investigate the combined presence of 100 μM GTP-γS, 1 μM βγ subunits and cross-linking antibody (□).

for internalisation of FcγRI which can operate in the absence of PKC activity.

3.3. G-protein βγ-subunits are capable of stimulating FcγRI internalisation

As both small GTPases and heterotrimeric G-proteins have been shown to be involved in regulation of endocytosis [11], we tested the ability of GTP-γS to modulate FcγRI internalisation. Fig. 3A shows that GTP-γS was able to augment internalisation of aggregated FcγRI by approximately two-fold. GTP-γS can act on heterotrimeric G-proteins to liberate βγ subunits from the heterotrimer and βγ subunits have previously been shown to regulate receptor-mediated endocytosis [12]. We investigated this possibility by determining the effect of introduction of purified brain βγ subunits into streptolysin-O-permeabilised cells following receptor cross-linking. Fig. 3Bshows that an identical result was obtained when GTP-γS was replaced with βγ subunits. Moreover, co-addition of the two reagents produced no further increase in internalisation over that achieved by GTP-γS or βγ subunits added alone. This indicates that GTPγS achieves its effects on FcγRI internalisation via the mobilisation of βγ subunits.

3.4. G-protein βγ subunit-dependent internalisation requires activity of PKC, but not PI 3-kinase

βγ subunits are known to directly activate phospholipase C-β which will then result in activation of PKC via the formation of diacylglycerol. The data in Fig. 1 suggest that PKC is involved in FcγRI internalisation. We therefore tested the ability of calphostin C to inhibit GTP-γS/βγ-induced FcγRI internalisation. Fig. 4 shows that calphostin C abolished both the GTP-γS (Fig. 4A) and βγ (Fig. 4B)-dependent internalisation of FcγRI. Additionally the phospholipase C inhibitor, U73122, had very similar effects to calphostin C on GTP-γS-dependent events, whereas its inactive isomer, U73343, was ineffective in this regard (Fig. 5B).

Wortmannin, which was able to completely inhibit the βγ-independent component of internalisation (Fig. 2A), had no effect on GTP-γS- (Fig. 4A) nor βγ- (Fig. 4B) dependent

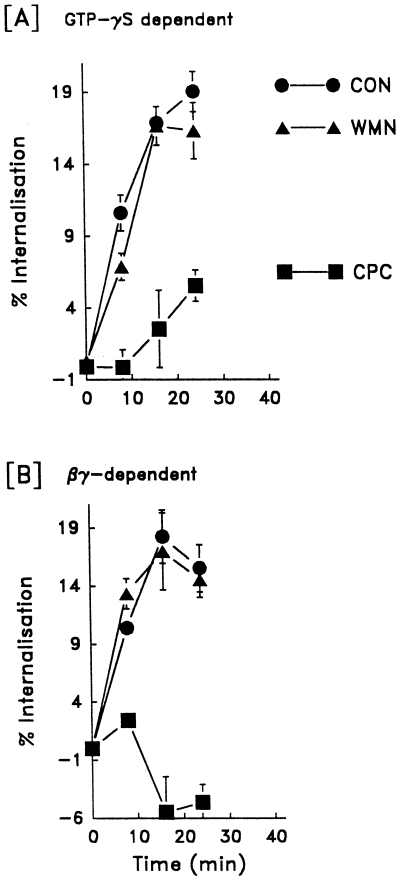

Fig. 4. Calphostin C sensitivity of βγ-dependent FcγRI internalisation. IFN-γ treated U937 cells were incubated in the presence of 50 nM wortmannin (▲), 1 μM calphostin C (■), or remained untreated (●) for 10 min at 37°C. Cells were then cooled to 4°C and labelled with ^{125}I-IgG$_1$. Following removal of unbound label, the cells were warmed to 37°C in permeabilisation buffer in the presence of goat anti-human cross-linking antibody with 100 μM GTP-γS (A) or 1 μM βγ subunits (B). Internalisation was determined as for Fig. 1 and then plotted as the value obtained in the presence of GTP-γS (A) or βγ subunits (B) with the corresponding values obtained in the absence of these reagents subtracted (values are mean ± S.E.M. $n = 8$).

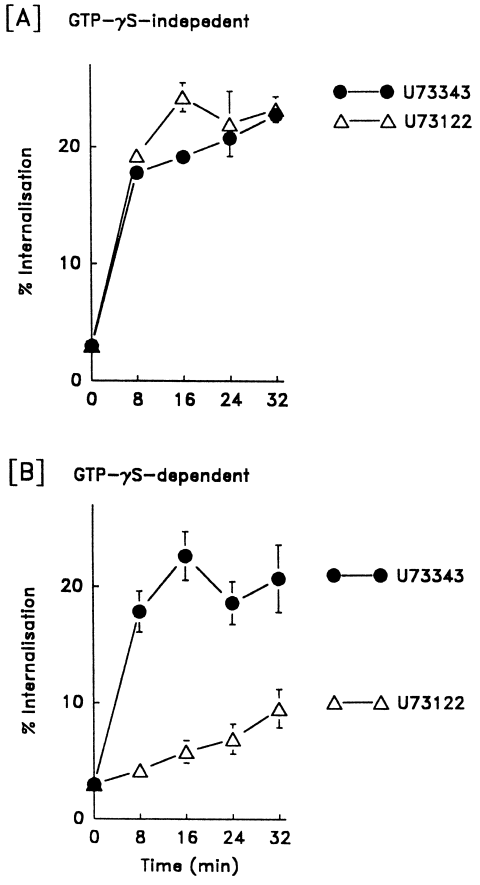

Fig. 5. Phospholipase C activity is essential for GTP-γS-dependent FcγRI internalisation. Cells were treated with the phospholipase C inhibitor, U73122 (△), or its inactive analogue, U73343 (●) at 10 μM for 5 min, cooled to 4°C and labelled with ^{125}I-IgG$_1$. The cells were then warmed to 37°C in permeabilisation buffer containing goat anti-human cross-linking antibody in the absence (A) and presence (B) of 100 μM GTP-γS. GTP-γS-independent (A) and -dependent (B) components of internalisation were determined as for Fig. 4 (values are mean ± S.E.M. $n = 4$).

FcγRI internalisation. This indicates that FcγRI may be internalised by a phospholipase C-β and PKC-dependent pathway that does not require the activity of PI 3-kinases.

4. Discussion

In this study we have shown that internalisation of FcγRI by IFN-γ-treated U937 cells following receptor aggregation requires the activation of two distinct pathways. The pathways were functionally dissected using streptolysin-O-permeabilised cells by capitalising on their relative dependence on active GTP binding proteins. One pathway required the presence of GTP-γS or active βγ subunits, the other did not. Use of inhibitors revealed that the βγ-independent pathway required activation of PI 3-kinases and was PKC-independent. By contrast, the βγ-dependent pathway involved activation of PKC and was PI 3-kinase-independent. Both these pathways were found to be active in intact cells, with the PKC-dependent pathway predominating.

The data in Fig. 1 and previous work [10] suggest that a proportion of surface FcγRI needs the activity of PI 3-kinase in order to be internalised. Use of streptolysin-O-permeabilised cells reveals that this is likely owing to the presence of a subpopulation of receptor that is completely dependent on PI 3-kinase activity for internalisation, rather than a partial effect of the inhibitor on a single pathway. We have shown previously that cross-linking of FcγRI results in the stimulation of p85-dependent PI 3-kinase activity [7] and a recent study indicates that this may be downstream of the recruitment of the tyrosine kinase, Syk to Fcγ receptors [13]. Previous work utilising a dominant negative Δp85 shows that the limited effects of wortmannin on FcγRI internalisation are likely to be via a p85-dependent PI 3-kinase [10].

It has been suggested that PI 3-kinases can modulate endocytosis via the regulation of GTPase activity or GTP exchange rates of small GTPases involved in clathrin coated pit formation, for instance dynamin and rab5 [14]. In general, however, reports on the sensitivity of clathrin-dependent endocytosis to wortmannin are not consistent. It is possible, therefore, that p85-dependent FcγRI internalisation is not via a clathrin-dependent process and it seems agreed in the literature that macropinocytic fluid-phase endocytosis is acutely sensitive to PI 3-kinase inhibition [14]. This suggests that wortmannin-sensitive FcγRI internalisation represents a subpopulation of receptors amenable to internalisation by macropinocytic fluid-phase endocytosis, a process that has been shown to be essential for efficient antigen processing and presentation in macrophage-like cells [15].

The present study indicates that, although a proportion of FcγRI is internalised via a PI 3-kinase-dependent mechanism, the majority of it follows a PKC-dependent route into the cell. The data from the streptolysin-O-permeabilised cells show clearly that this mechanism is completely dependent on the activity of phospholipase C-β and PKC and completely independent of a wortmannin-sensitive PI 3-kinase. PKC has been shown to be required for endocytosis under certain circumstances [16] and, as with PI 3-kinase, the mechanism for this is unclear. However, dynamin is known to act as a PKC substrate and phosphorylation by PKC is known to regulate the GTPase activity of dynamin [17]. The sensitivity to calphostin C, combined with the fact that internalisation proceeds at a relatively low intracellular calcium concentration (50–100 nM free calcium ions in the streptolysin-O permeabilisation buffer), suggest that βγ-dependent FcγRI internalisation is mediated via a novel PKC. Of these, PKCs δ and ε are known to be recruited to membranes following cross-linking of FcγRI in IFN-γ-treated U937 cells [18] and recently it has been shown that activation of Fcγ receptors activates PKC-δ in a PI 3-kinase-independent fashion [13]. This make PKC-δ a prime candidate for the PKC isoform mediating FcγRI endocytosis. Streptolysin-O-permeabilised cells will be ideal for elucidation of the PKC responsible for receptor internalisation by allowing the introduction of isoform-specific inhibitors of PKCs, such as PKC-pseudosubstrate peptides and recombinant proteins corresponding to the V0 regions of novel PKCs.

In conclusion, this study shows that FcγRI may be internalised in response to the activation of two independent signalling pathways; one requiring PI 3-kinase and the other involving G-protein βγ-subunit activation of phospholipase C-β and thence PKC. Clearly having two such independent internalisation mechanisms operating on the same receptor in the same cell will have the potential to allow for fine-tuning of immune complex internalisation and possibly prescribing their intracellular trafficking following internalisation.

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