Activation of phosphatidylinositol 3-kinase by concanavalin A through dual signalling pathways, G-protein-coupled and phosphotyrosine-related, and an essential role of the G-protein-coupled signals for the lectin-induced respiratory burst in human monocytic THP-1 cells

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Stimulation of monocytic THP-1 cells by a lectin, concanavalin A (Con A), resulted in protein-tyrosine phosphorylation and association of some of the thus phosphorylated proteins with the 85 kDa regulatory subunit of PtdIns 3-kinase. Both actions of Con A were not inhibited by wortmannin, a PtdIns 3-kinase inhibitor, or by prior exposure of cells to pertussis toxin which uncouples certain G-proteins from receptors. The binding of PtdIns 3-kinase to the tyrosine-phosphorylated proteins increased upon Con A stimulation; there was a marked increase in the enzymic activity in the anti-phosphotyrosine immunoprecipitates from Con A-treated cells. The increase was abolished by wortmannin but not affected by pertussis toxin. The incorporation of ^{32}P into PtdIns P_3 also increased during incubation of [^{32}P]P $_i$ -prelabelled cells with Con A, reflecting activation of whole-cell PtdIns 3-kinase which could not be accounted for solely by the increase in the phosphotyrosine-bound enzyme activity from the following aspects: (1) different concentration

dependencies for Con A; and (2) almost total susceptibility of the incorporation to pertussis toxin. This notion appears to be supported by different time courses between increases in PtdIns P_3 production and the phosphotyrosine-bound activity. The susceptibility to the toxin may reflect involvement of the toxin-sensitive G-proteins. In contrast, insulin-induced increases in PtdIns P_3 production, as well as increases in phosphotyrosine-bound PtdIns 3-kinase activity, were blocked by wortmannin, but never affected by prior exposure of cells to pertussis toxin, excluding a possible involvement of G-proteins in the insulin-induced activation. Con-A-induced O_2^- production was almost inhibited by either pertussis toxin or wortmannin. These results suggest that oligomerization of cell-surface glycoproteins with Con A gives rise to activation of G-protein(s) and certain tyrosine kinase(s), both of which were responsible for PtdIns 3-kinase activation; the G-protein-mediated activation led to the respiratory burst.

INTRODUCTION

PtdIns 3-kinase was initially characterized as a lipid kinase that associates with activated tyrosine kinases, such as growth factor receptors and oncogene products (for review, see [1–3]). The mammalian enzyme is a heterodimer of an 85 kDa regulatory subunit (p85 α , β) and a 110 kDa catalytic subunit (p110 α , β). The p85 subunit possesses two SH2 (Src homology 2) domains located in its C-terminal half, and an N-terminal SH3 domain. Phosphotyrosine residues within the consensus sequence YXXM bind either of the p85 SH2 domains, thereby activating the catalytic function of the associated p110 subunit. The binding to both of the SH2 domains caused the full activation of PtdIns 3-kinase [4]. The motif, YXXM, is found in the intracellular domain of protein tyrosine kinase (PTK)-type receptors (e.g. the platelet-derived growth factor β receptor) and at several positions in their intracellular substrates such as the insulin receptor substrate-1 (IRS-1) and polyoma middle T antigen, and in cytosolic receptor-linked PTKs, such as pp60^{c-src}. Such phosphotyrosine-linked activation of intracellular PtdIns 3-kinase is reflected in increases in the enzymic activity immunoprecipitable with anti-phosphotyrosine antibodies.

The PtdIns 3-kinase activity has also been found to increase when G-protein-linked receptors were stimulated in neutrophils [5,6] or platelets [7]. For instance, incubation of neutrophils with formylmethionyl-leucylphenylalanine (fMLP), a typical

G-protein-coupled receptor agonist in this cell type, brought about significant generation of PtdIns P_3 during the incubation. This reflects activation of PtdIns 3-kinase in the whole cells [6,8], without a significant increase in the PtdIns 3-kinase activity in the anti-phosphotyrosine immunoprecipitates prepared from the same cells [6]. The fMLP-induced generation of PtdIns P_3 was totally blocked by prior exposure of cells to pertussis toxin (PTX) [8]. In this case, therefore, receptor-mediated activation of PtdIns 3-kinase appears to take place via the toxin-sensitive G-protein rather than protein-tyrosine phosphorylation.

PtdIns 3-kinase that can be activated by the addition of the G-protein $\beta\gamma$ -subunit ($G\beta\gamma$) has recently been reported to occur in human neutrophils, myeloid-derived U937 cells and platelets [9–11]. A new isoform of the enzyme distinct from the already cloned p85–p110 heterodimer was found to be the entity directly activated by $G\beta\gamma$ in neutrophils or platelets [9,11], although the enzymic activity of the latter heterodimer was raised by the binding to $G\beta\gamma$ as well as to phosphotyrosine [10]. PTX-susceptible G_i or G_o is much more abundant than unsusceptible G-proteins such as G_s and G_q in most cell types, where $G\beta\gamma$ involved in PtdIns 3-kinase activation appears to originate from the abundant toxin-susceptible G-proteins, making it reasonable to suppose that activation of PtdIns 3-kinase via G-protein-coupled receptors is readily blocked by the PTX treatment of cells [12].

It remains to be fully understood as to the relative roles of G-

Abbreviations used: Con A, concanavalin A; fMLP, formylmethionyl-leucylphenylalanine; $G\beta\gamma$, G-protein $\beta\gamma$ -subunit; IRS-1, insulin receptor substrate-1; NP-40, Nonidet P-40; PTK, protein tyrosine kinase; PTX, pertussis toxin; SH2, Src homology 2.

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protein-coupled and phosphotyrosine-linked mechanisms in the receptor-triggered activation of PtdIns 3-kinase, as well as the kinase-mediated eventual cellular responses. As a clue to addressing this problem, we have sought cell types in which membrane receptor stimulation leads to activation of PtdIns 3-kinase via G-proteins as well as increases in phosphotyrosine-bound enzymic activity. This paper describes human monocytic THP-1 cells equipped with the receptors for concanavalin A (Con A) which provide a useful model system suitable for this purpose. Con A, the jack bean lectin [13], mimics the actions of antigens to induce polyclonal activation of lymphocytes as a result of its specific tetravalent binding to D-glucopyranoside or D-mannopyranoside, thereby oligomerizing the cell-surface glycoproteins and activating intracellular PTKs [14–16]. In accordance with previous reports that the Con A receptor is coupled to G_i in human neutrophils [17], Con-A-induced generation of PtdIns P_3 was blocked by PTX treatment of THP-1 cells. We also show that the G-protein-coupled activation of PtdIns 3-kinase is responsible for the Con-A-induced respiratory burst in THP-1 cells.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources. Wortmannin and PTX were kindly donated by Drs. Y. Matsuda (Kyowa Hakko Kogyo Co.) and M. Tamura (Kaken Seiyaku Co.), respectively. The stock solution (10 mM) of wortmannin was prepared in DMSO, stored at -20°C in the dark, and diluted with appropriate buffer immediately before use. Polyclonal antibody (αp85) against the 85 kDa subunit of PtdIns 3-kinase was prepared as described elsewhere [18]. Monoclonal anti-phosphotyrosine antibody (PY-20) and rabbit polyclonal anti-phosphotyrosine antibody were purchased from Zymed (San Francisco, CA, U.S.A.). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, ^{125}I -Protein G and $^{32}\text{P}\text{P}_i$ were from Dupont/NEN. Protein G-Sepharose was from Pharmacia. Phospholipids, Con A (type VI), dibutyl cAMP, cytochrome *c* and BSA (fatty acid-free) were from Sigma. All other reagents from commercial sources were of analytical grade.

Cells

The human monocytic cell line THP-1 obtained from the American Type Culture Collection was maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal-calf serum, penicillin (100 units/ml), streptomycin (100 mg/ml) and 20 mM Hepes (pH 7.2). The cells had been differentiated into reticulo-lytes by culture with 0.5 mM dibutyl cAMP for 72 h before use. The culture medium was supplemented with 100 ng/ml of PTX for the last 4 h for the purpose of preparing the toxin-treated cells. Under ordinary conditions, the cultured cells were treated with wortmannin or Con A as follows. The cultured cells were washed twice and suspended in Krebs–Ringer–Hepes medium (134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 2.5 mM CaCl_2 , 5 mM glucose, 0.2% BSA, 20 mM Hepes, pH 7.4). Aliquots (250 μl) of the cell suspension were incubated at 37°C first for 10 min with or without wortmannin, and then further incubated for the indicated times with the addition of Con A.

Analysis of tyrosine-phosphorylated proteins

THP-1 cells (10^7 cells/0.5 ml) incubated with or without Con A were lysed in an ice-cold buffer containing 25 mM Tris/HCl (pH 7.5), 1 mM Na_3VO_4 , 30 mM NaF, 50 units/ml of aprotinin, 1 mM PMSF, 5 mM EDTA, 1% Nonidet P-40 (NP-40), 1 mM

dithiothreitol, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 20 μM leupeptin and 1% BSA. The lysate is henceforth referred to, for brevity, as ‘NP-40 extract’. The NP-40 extract was incubated at 4°C first with appropriate antibodies for more than 1 h, and then with the addition of Protein G-Sepharose for 1 h or a longer period of time. After repeated washings, immunoprecipitates were heated at 100°C for 3 min in 30 μl of the sample buffer consisting of 1% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.02% Bromophenol Blue and 62.5 mM Tris (pH 6.8). Solubilized peptides were then separated by SDS/PAGE on a 7.5% polyacrylamide slab gel and transferred electrophoretically to a nitrocellulose membrane at 2 mA/cm 2 for 40 min. After blocking in 3% (w/v) fatty acid-free BSA, the blot was incubated with appropriate antibody, washed and then incubated with ^{125}I -Protein G. Following repeated washings, associated radioactivity was visualized using a Fuji BAS2000 bioimaging analyser.

Assay of PtdIns 3-kinase activity in intact cells by ^{32}P -labelling of the cellular phospholipids

The PtdIns P_3 production in cells was measured essentially according to the method described by Traynor-Kaplan et al. [5]. THP-1 cells were suspended at a density of 2×10^7 cells/ml in the labelling medium consisting of 10 mM Hepes (pH 7.4), 136 mM NaCl, 4.9 mM KCl and 5.5 mM glucose. After incubation at 37°C for 1 h with the addition of $^{32}\text{P}\text{P}_i$ (150 $\mu\text{Ci}/\text{ml}$), the cells were subjected to the ordinary incubation (see above) with or without wortmannin followed by stimulation with 2 mg/ml of Con A for 15–40 s, unless otherwise stated, in Krebs–Ringer–Hepes medium. The incubation was stopped by the addition of 1.55 ml of chloroform/methanol/8% HClO_4 (50:100:5, by vol.) followed by vigorous stirring. The mixture was further supplemented with 0.5 ml of chloroform and 0.5 ml of 8% HClO_4 to separate the organic phase, which was then washed with chloroform-saturated 1% HClO_4 before being dried. The lipids were dissolved in 20 μl of chloroform/methanol (90:10, v/v) to be spotted on a TLC plate (Silica Gel 60, Merck) which had been impregnated with potassium oxalate by developing with a solvent system containing 1.2% potassium oxalate in methanol/water (2:3, v/v) and pre-activated by heating at 110°C for 20 min before spotting. The plate, after being developed in chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14, by vol.), was dried and visualized for radioactivities with a Fuji BAS2000 bioimaging analyser.

Assay of PtdIns 3-kinase activities of anti-phosphotyrosine or anti-(PtdIns 3-kinase) immunoprecipitates

The anti-phosphotyrosine (PY-20) or anti-p85 immunoprecipitates were assayed for their PtdIns 3-kinase activities. The NP-40 extract of THP-1 cells was precleared with preimmune IgG and Protein G-Sepharose at 4°C for 1 h, and subjected to immunoprecipitation with these antibodies. The immunoprecipitates were washed twice with the buffer consisting of 40 mM Tris/HCl (pH 7.4), 5 mM EDTA and 1% NP-40, twice with the same buffer supplemented with 0.5 M LiCl, and three times with the buffer supplemented with 100 mM NaCl. Aliquots (2×10^6 cells equivalent) of immunoprecipitates were suspended in 0.1 ml of the reaction mixture consisting of 40 mM Tris/HCl (pH 7.4), 0.5 mM EGTA, 0.2 mM PtdIns, 0.2 mM phosphatidylserine, 5 mM MgCl_2 and 0.1 μM (1 μCi) $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The reaction was allowed to proceed at 37°C for 15 min before termination by the addition of 20 μl of 8% HClO_4 and 0.45 ml of chloroform/methanol (1:2, v/v). After vigorous stirring, the mixture was added to 0.15 ml of chloroform and 0.15 ml of 8% HClO_4 to separate the organic phase, which was washed with chloroform-

saturated 0.5 M NaCl containing 1% HClO₄ and then evaporated to dryness. The extract was dissolved in 40 μ l of chloroform/methanol (9:1, v/v) to be spotted on a silica gel plate (Silica Gel 60, Merck). The plate was developed in chloroform/methanol/28% NH₄OH/water (70:100:25:15, by vol.), dried and visualized for radioactivities in the PtdIns3P fraction with a Fuji BAS2000 bioimaging analyser.

Superoxide anion generation

Superoxide generation was determined by measuring the reduction of cytochrome *c*. THP-1 cells (1×10^6 cells/0.5 ml) were first incubated in Krebs–Ringer–Hepes medium at 37 °C for 10 min with or without wortmannin and then with Con A together with 80 μ M cytochrome *c* for an additional 10 min. The reaction with Con A was stopped by adding 0.5 ml of the same ice-cold medium which was supplemented with 300 units/ml of superoxide dismutase and 10 mM EGTA instead of CaCl₂. The difference-absorption (550–540 nm) of the reaction mixture was determined.

RESULTS

Con A-induced protein-tyrosine phosphorylation

The dibutyl cAMP-differentiated THP-1 cells were incubated with Con A and lysed with NP-40. The phosphotyrosine-

Figure 1 Con-A-induced protein-tyrosine phosphorylation in a wortmannin- and PTX-insensitive manner in THP-1 cells

The differentiated THP-1 cells were incubated with [as indicated by 'Con A' in (a) or + in (b)] or without ['Control' in (a) or – in (b)] 2 mg/ml of Con A for various periods of time as indicated at the bottom of the panel (a) or for 4 min (b). Prior exposure of cells to 1 μ M wortmannin or 100 ng/ml of PTX (as shown by + in b) was achieved as described in the Materials and methods section. After incubation, the cells were lysed to prepare the NP-40 extract (see the Materials and methods section), which was subjected to immunoprecipitation with anti-phosphotyrosine antibodies (PY-20). Immunoprecipitated proteins were then separated by SDS/PAGE and their phosphotyrosine contents were analysed by immunoblotting with polyclonal rabbit anti-phosphotyrosine antibodies. Mobility of the molecular-mass standards is indicated in kDa. The data are from an experiment representative of more than three.

Figure 2 Con A-induced activation of PtdIns 3-kinase in THP-1 cells as measured by two different methods of analysis

THP-1 cells were incubated with (●) or without (○) 2 mg/ml of Con A for various lengths of time. (a) The anti-phosphotyrosine (PY20) immunoprecipitates prepared from the NP-40 extract of the incubated cells were assayed for their PtdIns 3-kinase (PI3K) activity with the addition of PtdIns and [γ -³²P]ATP as substrates. (b) Generation of PtdInsP₃, the product of PtdIns 3-kinase, during the incubation was measured with cells that had been labelled with [³²P]P_i before the addition of Con A. Phospholipids were extracted and processed as described in the Materials and methods section to be determined for the radioactivities in the PtdIns3P and PtdInsP₃ fractions, respectively, in (a) and (b). The ³²P content, quantified by an image analyser, is plotted in arbitrary units as a function of incubation time. Similar results were obtained in three separate experiments.

containing proteins in the lysate were immunoprecipitated with monoclonal anti-phosphotyrosine antibody (PY20), and the phosphotyrosine content was determined by immunoblotting with polyclonal anti-phosphotyrosine antibody (Figure 1a). Con A caused protein-tyrosine phosphorylation, as had been reported previously for lymphocytes [14–16]. Con A-induced phosphorylation was observed within 15 s, reached the maximum level at 1 min, and then rapidly declined. The tyrosine-phosphorylated proteins we could identify were Lyn, a Src family kinase, and GAP (Ras-p21 GTPase-activating protein)-associated p62 (results not shown).

The Con-A-induced protein-tyrosine phosphorylation was not affected significantly by prior treatment of cells with PTX, the procedure that inhibits G_i activation in response to receptor stimulation [19] (Figure 1b). The tyrosine phosphorylation, not mediated by the G-protein, may be upstream, or independent, of PtdIns 3-kinase, as revealed by failure of wortmannin, the specific enzyme inhibitor [8,12,20], to affect the Con A-induced protein-tyrosine phosphorylation (Figure 1b) in agreement with a previous report with other but similar cell types [21].

Con A-induced activation of PtdIns 3-kinase

The PtdIns 3-kinase activity was measured by two different methods of analysis. The lipid kinase activity associated with the PY-20 immunoprecipitates prepared from the Con-A-stimulated cells displayed a time course of somewhat transient increase (Figure 2a). The enzyme activity increased almost linearly from 15 s to 1 min after the Con A stimulation. The 1-min peak value of PtdIns 3-kinase activity was followed by a rapid return towards the initial level within 8 min. This time course for the

Figure 3 Con-A-induced association of the tyrosine-phosphorylated proteins with the 85 kDa subunit of PtdIns 3-kinase in a PTX- or wortmannin-insensitive manner

THP-1 cells that had been cultured with (+) or without (-) PTX for 4 h, or further with (+) or without (-) 1 μ M wortmannin (Wort.) for 10 min were subjected to 4-min stimulation with 2 mg/ml of Con A (Con A +). Proteins in the NP-40 extract were immunoprecipitated (I.P.) with anti-(PtdIns 3-kinase) antibodies (α p85) or anti-phosphotyrosine antibodies (PY20), and these immunoprecipitates were separated by SDS/PAGE to be further analysed by immunoblotting (I.B.) with PY20 or α p85, respectively. Mobility of the molecular-mass standards is indicated in kDa. The data are from an experiment representative of more than three.

Con A-induced increase in the PtdIns 3-kinase activity present in the anti-phosphotyrosine immunoprecipitates (Figure 2a) was almost coincident with that for the protein-tyrosine phosphorylation under the same conditions (Figure 1a).

For the purpose of estimating whole-cell PtdIns 3-kinase activity, the cells were first labelled with [32 P]P_i to determine generation of PtdInsP₃, a product of the enzyme, in intact cells (Figure 2b). The labelled cells were stimulated with Con A before separation of the cellular phospholipids by TLC and analysis of radioactivities associated with PtdInsP₃ by an imaging analyser. The labelled PtdInsP₃ production increased to the maximal level within 15 s, the earliest time of observation, which was followed by a very slow decline; a significant level of 32 P-labelled PtdInsP₃ was maintained during the entire period of the 8-min incubation (Figure 2b).

Apparently different time courses between panels (a) and (b) in Figure 2 may suggest that Con A-induced activation of PtdIns 3-kinase in whole THP-1 cells (Figure 2b) could not be accounted for solely by increases in the phosphotyrosine-bound enzymic activity (Figure 2a).

Con-A-induced binding of PtdIns 3-kinase to tyrosine-phosphorylated proteins was not inhibited by wortmannin, although activity of the bound enzyme was inhibited

Tyrosine-phosphorylated proteins that are associated with the 85 kDa regulatory subunit of PtdIns 3-kinase were immunoprecipitated from the NP-40 extract with anti-p85. The immunoprecipitates were then analysed for the phosphotyrosine content by immunoblotting with anti-phosphotyrosine antibodies (Figure 3). Stimulation of cells with Con A resulted in

Figure 4 Failure of the PTX treatment of cells to inhibit Con-A-induced increases in PtdIns 3-kinase activity in the anti-phosphotyrosine immunoprecipitates

(a) THP-1 cells treated with (+) or without (-) 1 μ M wortmannin (Wort.) for 10 min were incubated with 2 mg/ml of Con A (+). In (b), the cells cultured with PTX or not ('Control') were incubated with increasing concentrations of Con A. (' ∞ ' indicates 'without Con A'). The time of incubation with Con A was 3 min. The anti-phosphotyrosine (PY20) immunoprecipitate prepared from the NP-40 extract was assayed for PtdIns 3-kinase activity with [γ - 32 P]ATP and PtdIns as substrates. Autoradiograms of the TLC plates used for the separation of lipid products are shown with the positions of PtdIns3P indicated by arrows. The data are from an experiment representative of more than three.

association of several tyrosine-phosphorylated proteins with PtdIns 3-kinase, marked association being observed for 115 kDa and 95 kDa peptides. No tyrosine-phosphorylation was detectable at the position of p85. Probably, p85 itself was not phosphorylated when cells were stimulated with Con A. When NP-40 extracts were immunoprecipitated with anti-phosphotyrosine antibodies, p85 was detected in the precipitates from the Con-A-stimulated cells (Figure 3), indicating that p85 made a complex with tyrosine-phosphorylated protein(s) upon cell stimulation by the lectin.

Wortmannin was without effect on the tyrosine phosphorylation of the p85-associated peptides or the amount of p85 bound to the tyrosine-phosphorylated proteins (Figure 3), despite complete inhibition of PtdIns 3-kinase activity in the anti-phosphotyrosine immunoprecipitates (Figure 4a). The results indicate that the inhibitor directly affected the catalytic activity of the enzyme. The interaction between PtdIns 3-kinase and tyrosine-phosphorylated peptides was unchanged also by prior treatment of the cells with PTX as shown in Figure 3. The phosphotyrosine-associated PtdIns 3-kinase activity was dependent on the concentration of Con A, with the maximum stimulation being observed at 0.1 μ M (Figure 4b). The PY20-immunoprecipitable PtdIns 3-kinase activities were essentially insensitive to PTX treatment of cells (Figure 4b). Thus, association of phosphotyrosine with PtdIns 3-kinase was not inhibited by wortmannin, which is an inhibitor of the enzyme, and PTX-sensitive G-proteins are unlikely to mediate any pathway responsible for the increase in phosphotyrosine-bound PtdIns 3-kinase activity in THP-1 cells.

Figure 5 Inhibition by PTX or wortmannin of the PtdIns 3-kinase activity measured as incorporation of ^{32}P into PtdIns P_3 during incubation of cells with Con A

^{32}P -labelled THP-1 cells were stimulated by incubation with increasing concentrations (a and b) or 2 mg/ml (c) of Con A for 15 s to measure PtdIns P_3 production due to PtdIns 3-kinase activation. Before stimulation, the cells had been exposed to 100 ng/ml of PTX ['PTX' in (a) and ● in (b)] or vehicle ['Control' in (a) and ○ in (b)] for 4 h or to increasing concentrations of wortmannin for 10 min (c). Autoradiograms of the TLC plates used for the separation of lipid products are shown in (a) with the position of PtdIns P_3 indicated by an arrow. The ^{32}P content of the PtdIns P_3 fraction in (a), quantified by an image analyser, is plotted in an arbitrary unit in (b). The autoradiogram for (c) is not shown. Similar results were obtained in three separate experiments.

Inhibition of Con A-induced activation of intact-cell PtdIns 3-kinase by prior treatment of cells with PTX

The increase by Con A of the PtdIns 3-kinase activity in intact cells (i.e. the enzymic activity measured as ^{32}P incorporation into PtdIns P_3 during intact-cell preparation; see Figure 2b) was dependent on the concentration of the lectin (Figures 5a and 5b). The concentrations of Con A required for activation of the intact-cell PtdIns 3-kinase were much higher than those required for increases in the PtdIns 3-kinase activity associated with phosphotyrosine (compare Figures 5a and 5b with 4b). The Con A-induced activation of intact-cell PtdIns 3-kinase was mostly abolished by prior treatment of cells with PTX (Figures 5a and 5b). Thus, PTX-sensitive PtdIns 3-kinase activation by Con A observed in the intact-cell preparation was not reflected in the increase in phosphotyrosine-bound PtdIns 3-kinase activity in the lectin-treated cells. Wortmannin also inhibited the intact-cell PtdIns 3-kinase activity in a dose-dependent manner with the IC_{50} value (the concentration causing 50% inhibition) around 50 nM (Figure 5c), which was the same as that previously observed for the inhibition of tyrosine-bound PtdIns 3-kinase activity [20].

Figure 6 Insulin-induced activation of phosphotyrosine-bound PtdIns 3-kinase and PtdIns P_3 production in cells in a PTX-insensitive manner

THP-1 cells cultured with ['PTX' in (a) and ● in (b)] or without ['Control' in (a) and ○ in (b)] 100 ng/ml of PTX were incubated with increasing concentrations of insulin for 1 min. (a) The NP-40 extract prepared from the incubated cells was subjected to immunoprecipitation with anti-phosphotyrosine antibody (PY20); the immunoprecipitate was assayed for PtdIns 3-kinase activity with PtdIns and [γ - ^{32}P]ATP as substrates, and the positions of the product, PtdIns P_3 , on a TLC plate is indicated by an arrow. (b) The cells had been labelled with [^{32}P]P_i before incubation with insulin and production of ^{32}P -labelled PtdIns P_3 was determined as PtdIns 3-kinase activity. Results are expressed as percentages of the control (without insulin). Similar results were obtained in three separate experiments.

Activation by insulin of both intact-cell and phosphotyrosine-bound PtdIns 3-kinase activities in a PTX-resistant manner

The insulin receptor belongs to the family of transmembrane growth factor receptors equipped with ligand-activated PTK activity [22,23]. The mechanism by which the PtdIns 3-kinase activity increases in insulin-treated cells has been well documented: IRS-1, whose phosphorylation on tyrosine in the YXXM motif after insulin receptor stimulation, activates PtdIns 3-kinase as a result of the association with the 85 kDa regulatory subunit of the kinase (see [24] for review). Stimulation of THP-1 cells with insulin, just like stimulation by Con A, brought about both activation of intact-cell PtdIns 3-kinase and an increase in the phosphotyrosine-bound enzyme (Figure 6). In the case of insulin-induced activation of the enzyme, however, increases in

Figure 7 Inhibition of Con-A-induced O_2^- generation by PTX and wortmannin in THP-1 cells

Superoxide anion production during 10-min incubation with Con A was determined as described in the Materials and methods section. (a) The cells had been cultured with (●) or without (○) 100 ng/ml PTX before the incubation with the indicated concentrations of Con A. (b) The cells that had been treated with the indicated concentrations of wortmannin for 10 min were incubated with (●) or without (○) 2 mg/ml Con A. Similar results were obtained in three separate experiments.

neither intact-cell activity nor phosphotyrosine-bound activity were susceptible to the PTX treatment of cells (Figure 6). The concentrations of insulin required for PtdIns 3-kinase activation were not significantly different between the increases in phosphotyrosine-bound activity (Figure 6a) and in PtdIns P_3 production (Figure 6b). Insulin-induced activation of PtdIns 3-kinase appears to reflect solely the increase in the phosphotyrosine-bound enzyme without mediation of PTX-susceptible G-proteins.

Involvement of G-protein-linked PtdIns 3-kinase in Con A-induced respiratory burst

One of the final responses of THP-1 cells to Con A was O_2^- generation (Figure 7). The dependency on Con A concentrations was almost the same between the PtdIns P_3 production (Figure 5b) and the O_2^- generation (Figure 7a). Also, Con A-induced O_2^- generation was inhibited by wortmannin in just the same concentration range as for the inhibition of the lectin-induced PtdIns P_3 production (compare Figure 7b with 5c). Thus, PtdIns 3-kinase activation is very likely to mediate the Con A-induced respiratory burst in intact THP-1 cells. An intriguing observation was that Con A-induced respiratory burst was mostly, if not totally, inhibited by prior treatment of cells with PTX. Thus, the activation of PtdIns 3-kinase via PTX-sensitive G-proteins, but not the increase in phosphotyrosine-associated enzymic activity, is responsible for the superoxide-generating response of THP-1 cells to Con A.

DISCUSSION

Cellular proteins phosphorylated on tyrosines and bound to PtdIns 3-kinase upon Con A stimulation of cells

Con A, a plant lectin, gave rise to rapid phosphorylation of several proteins on tyrosines when it was added to the incubation medium bathing human monocytic THP-1 cells that had been differentiated into reticulocytes (Figure 1). The phosphotyrosine residues thus formed on the protein molecules must be responsible for the Con A-induced increase in PtdIns 3-kinase that binds to

tyrosine-phosphorylated proteins via the 85 kDa regulatory subunit, because p85 formed an immunocomplex with anti-phosphotyrosine antibodies in Con A-treated cells (Figure 3), and because the increase of the phosphotyrosine-bound enzyme activity displayed a time course similar to protein-tyrosine phosphorylation following the addition of the lectin into the cell suspension (Figure 2a). Thus, the ligand engagement of the Con A receptors on the surface of THP-1 cells appears to activate non-receptor-type tyrosine kinases, e.g. those belonging to the Src superfamily, as have been reported for activation of PtdIns 3-kinase via receptors likewise having no intrinsic tyrosine kinase such as Fc γ [25,26], T-cell [27–32] and B-cell [33–35] receptors and receptors for interleukins 2 [36], 3 [37], 4 [38,39], and 7 [40], erythropoietin [41,42] and granulocyte macrophage colony-stimulating factor [37,43]. In fact, Con A has recently been reported to activate a non-receptor PTK, p72^{src}, resulting in multiple protein phosphorylation in peripheral blood leucocytes [15,16]. In the present study, however, p56^{l^{ym}}, another src family kinase, rather than p72^{src} was tyrosine-phosphorylated (results not shown), probably because of the use of different cell types and experimental conditions.

The major tyrosine-phosphorylated proteins that bound to the p85 regulatory subunit of PtdIns 3-kinase upon Con-A stimulation of cells were those with molecular masses of 115 and 95 kDa (Figure 3). We have previously recognized the same molecular-mass 115 kDa protein that was tyrosine-phosphorylated and tightly bound to p85 upon stimulation of the antigen Fc γ receptor in the human monocytic cell line U937, and hypothesized that this tyrosine-phosphorylated protein would be an activator of PtdIns 3-kinase [21]. Since Con A mimics antigen-induced stimulation of cells in some respects [13], tyrosine-phosphorylation of the same 115 kDa protein would be likely to contribute to the activation of PtdIns 3-kinase when cells were stimulated with Con A as well. Recent papers have shown that the 115–120 kDa product of proto-oncogene *c-cbl* is tyrosine-phosphorylated upon stimulation of T-cell receptors [44,45], Fc γ receptors [46] or receptors for erythropoietin and granulocyte-macrophage colony-stimulating factor [47]. *c-Cbl* thus tyrosine-phosphorylated binds p85, thereby activating associated PtdIns 3-kinase [45,48]. Non-receptor-type tyrosine kinases, such as Fyn, Lck and Syk [44,46], as well as adaptor proteins (e.g. Ash/Grb2 [44,45,47]), appear to bind to *c-Cbl* in these cells. Further studies are now in progress to address whether or not the 115 kDa protein that was tyrosine-phosphorylated and bound p85 upon stimulation of THP-1 cells by Con A is identical with *c-Cbl*.

In any event, Con A caused phosphorylation of cellular proteins on tyrosines and their association with p85 without mediation of PTX-sensitive G-proteins in THP-1 cells (Figures 1 and 3).

Dual mechanisms for activation of PtdIns 3-kinase following Con A stimulation of cells

Changes of cellular PtdIns 3-kinase activity can be followed by two assay methods; one depends on changes in the PtdIns 3-kinase activity that can be immunoprecipitated with anti-phosphotyrosine antibodies after cell disruption, while the other is based on the measurement of PtdIns 3-kinase products, PtdIns P_3 , from 32 P-labelled intact cells. The latter intact-cell assay should afford a much more accurate measure of the enzyme activity than the former cell-free immune complex assay [49]. In the case of Con A stimulation of THP-1 cells, the cell-free immune complex assay does not reflect accurately the extent

of PtdIns 3-kinase activation in intact cells, as judged by the following criteria.

First, the concentrations of Con A required for PtdIns P_3 production in intact cells (Figure 5b) were much higher than those estimated by the immune complex assay (Figure 4b). Secondly, Con A-induced activation of PtdIns 3-kinase in intact cells was mostly abolished by prior treatment of cells with PTX (Figures 5a and 5b), which was without effect on the lectin-induced increases in the phosphotyrosine-associated PtdIns 3-kinase (Figure 4b). Moreover, time courses for changes following Con A addition appeared to be somewhat different between production of PtdIns P_3 in intact cells and the enzymic activity associated with anti-phosphotyrosine immunoprecipitates from cytosol (Figure 2). In a preliminary experiment achieved under conditions similar to Figure 2(b), we added wortmannin to the incubation medium at 30 s after the start of the 32 P-labelled cell incubation with Con A. Wortmannin thus added caused a rapid decrease of 32 P-labelled PtdIns P_3 from the peak value (results not shown). It is likely, therefore, that the 8-min durable plateau of 32 P-labelled PtdIns P_3 in Figure 2(b) is a reflection of the continuous generation of PtdIns P_3 , rather than stability of the once-formed product, during the incubation time. We are then led to the conclusion that stimulation of THP-1 cells with Con A gives rise to activation of PtdIns 3-kinase via PTX-susceptible G-proteins in addition to the activation by the canonical or conventional phosphotyrosine-linked mechanism. A similar discrepancy between these two assays has been observed when efficacies of PtdIns 3-kinase activation were compared between multiple cytokines in haematopoietic cells [49].

It would be reasonable to assume that the increase in PtdIns 3-kinase activity measured as the increased product formation in intact 32 P-labelled cells should consist of all the enzymic activities that have increased by multiple mechanisms including phosphotyrosine-linked and G-protein-mediated ones. It is rather surprising, therefore, that the increase in 'all the enzymic activities' assayed in Con A-treated intact cells was mostly abolished by PTX treatment of cells, despite failure of the toxin treatment to attenuate the increases in phosphotyrosine-associated PtdIns 3-kinase that should form a considerable fraction of the enzyme activation. Conceivably, proteins phosphorylated by Con A on tyrosines bind PtdIns 3-kinase without enormous activation of the enzymic activity itself; the increase in phosphotyrosine-bound PtdIns 3-kinase may result in enzyme activation due to its recruitment to the intracellular loci where more substrate molecules are available. Such a manner of 'local' activation might not contribute significantly to the increase in the PtdIns P_3 formation in the whole-cell assay. It would be possible also to assume that assay of phosphotyrosine-bound PtdIns 3-kinase is so sensitive as to reveal minor changes that could not be detected by a less sensitive whole-cell assay of the enzyme.

In sharp contrast to Con A, insulin activated PtdIns 3-kinase in intact THP-1 cells solely as a result of increases in the phosphotyrosine-associated enzymic activity, as revealed by the same concentration dependency and the same PTX insusceptibility observed upon application of either of these two assay methods (Figure 6). Association of phosphotyrosines (e.g. those on IRS-1 or IRS-2) with PtdIns 3-kinase appears to be the only mechanism by which insulin activates the enzyme in THP-1 cells.

Essential role of G-protein-mediated activation of PtdIns 3-kinase in the Con A-induced respiratory burst

The $\beta\gamma$ -subunits of G-proteins ($G\beta\gamma$) must be involved in intracellular signalling that is blocked by prior treatment of cells

with PTX [12]. Probably, PtdIns 3-kinase is directly activated by $G\beta\gamma$ that is released from the toxin-sensitive G_i upon Con A engagement of cell-surface glycoproteins in THP-1 cells. A surprising and intriguing finding was that the PTX treatment of cells almost abolished the Con A-induced respiratory burst (Figure 7) as well as the PtdIns 3-kinase activation in intact cells (Figure 5). It is very likely, therefore, that Con A-induced and PTX-insensitive increases in phosphotyrosine-bound PtdIns 3-kinase activity do not play any significant role as the intracellular signal leading to the respiratory burst. An essential role of G-protein-mediated activation of PtdIns 3-kinase, apart from the phosphotyrosine-linked activation, as the signal for the respiratory burst is further supported by the following findings. First, virtually the same concentration range of Con A was required to cause either of these two PTX-sensitive responses; i.e. the respiratory burst (Figure 7a) and PtdIns P_3 production in intact cells (Figures 5a and 5b). Much lower concentrations of the lectin were enough to induce the toxin-insensitive increases in phosphotyrosine-bound PtdIns 3-kinase (Figure 4b). Secondly, similar concentrations of wortmannin inhibited both of these two PTX-sensitive responses (Figures 5c and 7b). Thirdly, the fMLP-induced respiratory burst in THP-1 cells was well correlated with activation of PtdIns 3-kinase measured by the intact-cell assay with respect to the concentrations of the chemotactic peptide required, but was not accompanied by a detectable increase in phosphotyrosine-bound PtdIns 3-kinase (results not shown; however, see refs. [6] and [8] for similar effects of fMLP on myeloid-derived cells and neutrophils).

Hence, it would be possible to presume that PtdIns 3-kinase activated via G-proteins could trigger its own downstream signals, eventually leading to the respiratory burst, distinct from those initiated by phosphotyrosine-bound PtdIns 3-kinase. Neither detectable production of PtdIns P_3 nor significant generation of superoxide anion was brought about upon IgG engagement of Fc γ receptors in THP-1 cells, despite enormous increases in protein-tyrosine phosphorylation with the associated PtdIns 3-kinase activity (results not shown). The engagement of Fc γ receptors led to phagocytosis via increases in phosphotyrosine-bound PtdIns 3-kinase without detectable production of PtdIns P_3 in intact U937 cells [21]. It is tempting to speculate that phosphotyrosine-linked activation of PtdIns 3-kinase is responsible for phagocytosis, whereas G-protein-mediated activation of the same enzyme gives rise to the respiratory burst. The speculation is in accordance with recent publications reporting the existence of a new isoform of PtdIns 3-kinase directly activated by $G\beta\gamma$ but not activated by phosphotyrosines [9,11], although the possibility cannot be excluded that the same molecule of PtdIns 3-kinase is activated by both activators ([10]; T. Okada, O. Hazeki, M. Ui and T. Katuda, unpublished work).

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