methods

able for quantification of PtdIns(3,4,5)P_3 in primary cells is limited (8). Most of the existing methods are time-consuming and expensive and require specific equipment and expertise.

Here we have investigated whether a specific phosphoinositide (PI) binding domain could enable the development of a novel assay to quantitate PtdIns(3,4,5)P_3 in cell extracts as an alternative to the use of radioactive tracers or microscopy-based assays. Divecha and colleagues (9) provided evidence that lipid overlay assays using the PH domain of phospholipase Cδ_1 could be used to quantify PtdIns(4,5)P_2 purified from cell extracts using neomycin beads. The PH domain of the general receptor for phosphoinositides-1 (GRP1) specifically binds PtdIns(3,4,5)P_3 (10) and has been used as a probe to quantify PtdIns(3,4,5)P_3, mainly using microscopy-based assays (11–13). Here we have developed a novel assay for PtdIns(3,4,5)P_3 and have used it to measure transient changes in PtdIns(3,4,5)P_3 abundance in human neutrophils and the impact on PtdIns(3,4,5)P_3 accumulation in neutrophils from mice expressing the DASAA-mutated PI3Kg. This novel assay is rapid and sensitive and could be used to design high-throughput assays for PtdIns(3,4,5)P_3 and possibly other low-abundance inositol lipids using alternative PI-specific domains.

MATERIALS AND METHODS

Tumor necrosis factor a (TNFa) was from R & D; N-formyl-methionyl-leucyl-phenylalanine (fMLP) and HBSS were from Sigma. Neomycin beads were a generous gift of Dr. Robin Irvine (Cambridge University).

Construction of expression vectors

The GFP-GRP1 PH domain fusion, initially cloned into EGFP C1 vector (11), was subcloned into pFbac A. This construct allows expression of the GFP-GRP1 PH domain in the context of an N-terminal 6 3 histidine tag. A mutant version of the GRP1 PH domain unable to interact with phosphoinositides was obtained by site-directed mutagenesis, changing K273 to A. The sequences of the wild-type and mutant constructs were verified by sequencing. Baculovirus DNA was obtained by transfecting DH10 BAC cells with the above constructs according to the Invitrogen protocols.

The PH domain of dual adaptor for phosphotyrosine and 3-phosphoinositide 1 (DAPP1) fused to GFP (14) was cloned into a modified version of pQE30 and sequenced. The new construct allowed expression of a 63 histidine-tagged version of the GFP-DAPP1 PH domain in BL-21DE3PlysS cells.

Recombinant protein purification

Sf9 cells were transfected using Insectin (Invitrogen) liposomes with linearized baculo gold DNA (BD Biosciences) and the relevant baculovirus transfer vectors. The recombinant baculoviruses were plaque purified and amplified. After infection, cells were harvested into ice-cold 0.41% KCl, 2.66% sucrose, 20 mM $MgCl_2$, and 8 mM NaH_2PO_4 (pH 6.2, 25°C) containing 1 mM di-isopropylfluorophosphate. The cells were then washed and frozen in liquid N_2 and stored at −80°C.

The GFP-fused PH domains were purified using a metal-ion chelation column (Talon, Clontech). Cell pellets were thawed and sonicated into 0.1 M NaCl and 50 mM sodium phosphate (pH 8.0, 4°C), 10 mM Tris-HCl (pH 8.0, 4° C), 1 mM $MgCl_2$, and antiproteases (10 mg/ml each of pepstatin A, aprotinin, leupeptin, antipain, and bestatin and 0.1 mM PMSF). A 120,000 g cytosolic fraction was supplemented with Tween 20 and betaine (0.05%, w/v, and 1%, respectively) and loaded onto Talon resin, which was subsequently washed sequentially with 20 column volumes each of buffers A, B, C, and D. Buffer A contained 50 mM sodium phosphate (pH 8.0, 4°C), 10 mM Tris-HCl (pH 8.0, 4° C), 0.15 M NaCl, 1% betaine, and 0.5% Tween 20 (w/v). Buffer B contained buffer A plus Triton X-100 (1%, w/v). Buffer C contained buffer A but was at pH 7.1 and 4°C. Buffer D contained buffer A but was at pH 7.5 and contained 0.02% Tween 20 (w/v), ethylene glycol (0.05%, v/v), and 1 mM $MgCl_2$. The Talon resin was then washed with 8 column volumes of buffer E, which contained buffer D supplemented with 10 mM imidazole (pH 7.5) and buffer F, which contained buffer D supplemented with 70 mM imidazole (pH 7.5, final concentration). Typically, 1 ml fractions were immediately collected and supplemented with 1 mM DTT and 1 mM EGTA (final concentrations). This yielded 3 mg of recombinant protein per liter of Sf9 culture.

The DAPP1 PH domain fused to a 6 3 histidine and GFP was expressed in BL-21DE3PlysS after induction with IPTG (0.1 mM) for 16 h at 25 °C and purified with Talon resin as described above.

Preparation of radiolabeled PtdIns(3,4,5)P_3

Radiolabeled PtdIns(3,4,5)P_3 was prepared as described previously (15). Briefly, phosphatidylserine (PtdS, 250 mM) and PtdIns(4,5)P_2 (50 mM) were dried and resuspended by sonication in a buffer containing 0.1 M NaCl, 20 mM HEPES, and 1 mM EGTA. Conversion of PtdIns(4,5)P_2 to radiolabeled PtdIns(3,4,5)P_3 was achieved by mixing the PtdS-PtdIns(4,5)P_2 liposomes with [g^{32}P]ATP and recombinant p110g (5) in a buffer containing 0.1 M NaCl, 1 mM EGTA, 1 mM DTT, 2 mM $MgCl_2$, and 20 mM HEPES for 20 min at 30°C. The reaction was terminated by addition of chloroform-methanol (2:1), and the lipid fraction containing the radiolabeled PtdIns(3,4,5)P_3 was subsequently extracted.

Preparation of neutrophils

Human neutrophils (purity $ 95% by cytospin) were isolated from the peripheral blood of healthy volunteers by centrifugation over plasma/Percoll gradients (16). The blood was centrifuged at 316 g for 20 min to pellet erythrocytes and leukocytes. The supernatant was collected and centrifuged at 1,912 g for 30 min, with the resultant supernatant yielding platelet-poor plasma (PPP). Dextran was added to the pelleted erythrocytes and leukocytes [2.5 ml of 6% dextran (Amersham Biosciences)], and saline (Baxters Healthcare) was added so that the volume was equal to the original volume of blood. The erythrocytes were left to sediment at room temperature until a clean interface between the erythrocytes and the leukocyte-rich plasma could be seen. The leukocyte-rich plasma was centrifuged at 316 g for 6 min, and the pelleted leukocytes were resuspended in PPP and loaded onto a discontinuous 42%/51% percoll gradient (Amersham Biosciences) (percoll diluted 9:1 with saline to give a 90% solution, and further dilutions made in PPP), which was centrifuged at 262 g for 10 min. Neutrophils were harvested from the 42%/51% interface, diluted into the remaining PPP, and centrifuged at 316 g for 6 min. The cells were then centrifuged and washed twice in HBSS.

Murine bone marrow-derived neutrophils were prepared essentially as described previously (17). Briefly, murine bone mar-

row was dispersed in HBSS (without Ca^{2+} and Mg^{2+}) with 0.25% fatty acid-free BSA (HBSS/BSA) and centrifuged (1,256 g, 30 min, room temperature) over discontinuous gradients comprised of 81, 62, and 55% Percoll in HBSS. Mature neutrophils were obtained from the 55%/62% interface (purity 75–85% by

PtdIns(3,4,5)P_3 was added to a total lipid extract from 1.10^6 human neutrophils. [^{32}P]PtdIns(3,4,5)P_3 was reproducibly recovered to 70% of the total input (Fig. 1), indicating that these beads can be used to enrich PtdIns(3,4,5)P_3 from a cellular extract.

We chose to use GRP1-PH domain as a probe to detect PtdIns(3,4,5)P_3 on blots because it had been reported to be highly specific for PtdIns(3,4,5)P_3 (10). The recombinant protein was assembled in the context of both a 6 3 histidine tag and a GFP tag. The 63 histidine tag allowed purification of the recombinant protein after expression in Sf9 cells. The purified protein was subsequently used in a protein-lipid overlay-based procedure (20) to detect PtdIns(3,4,5)P_3 immobilized on a nitrocellulose filter. This probe was, in turn, decorated with anti-GFP antibody followed by an anti-IgG coupled to HRP. The relative amount of PtdIns(3,4,5)P_3 was then estimated by direct visualization of the light emitted in a chemiluminescence assay using a CCD camera. Consistent with data published by others (20), the GRP1 reporter showed high specificity for PtdIns(3,4,5)P_3 over PtdIns(3,4)P_2 and PtdIns(4,5)P_2 when used in our protein-lipid overlay assay compared with a GFP-DAPP1 PH domain construct used under the same conditions (Fig. 2). As expected, mutation of lysine K273 in the GRP1 PH domain, previously shown to be critical for PtdIns(3,4,5)P_3 binding (21), attenuated the interaction of the probe with PtdIns(3,4,5)P_3 in a protein-lipid overlay assay (Fig. 3A), thus providing further evidence for the specificity of the GRP1 probe.

Use of neomycin beads, as described by Shacht (18), allows purification of all polyphosphoinositides from a complex lipid mixture. Because PIs purified using neomycin are likely to be composed mainly of PtdIns(4,5)P_2, we tested the ability of our assay to quantitate PtdIns(3,4,5)P_3 in the presence of an excess of PtdIns(4,5)P_2. In human neutrophils, we have previously shown that the level of PtdIns(4,5)P_2 is 10 (stimulated cells) to 100 (unstimulated cells) times higher than PtdIns(3,4,5)P_3 (22). We show that our assay is linear, with quantities of PtdIns(3,4,5)P_3 up to 20 pmol when mixed with 100 pmol of PtdIns(4,5)P_2

Fig. 3. Specificity and sensitivity of the GRP1 PH domain probe and its use for detection of PtdIns(3,4,5)P_3 in a PtdIns(3,4,5)P_3/PtdIns(4,5)P_2 mixture. A: 0 to 2 pmol of PtdIns(3,4,5)P_3 were mixed with a constant amount of PtdIns(4,5)P_2 (100 pmol), dried under vacuum, resuspended in 3 ml of chloroform-methanol-HCl (12 mM; 200:100:1), spotted onto nitrocellulose membranes, and analyzed by a protein-lipid overlay procedure with GFP-(K273A)-GRP1(mutant) or GFP-GRP1 (WT) PH domain as the primary probe (both probes used at the same concentration of 0.5 mg/ml^{-1} in TBS containing 2% BSA and 0.05% Tween 20). B: 0, 1, 5, 10, and 20 pmol of PtdIns(3,4,5)P_3 were mixed with 100 pmol of PtdIns(4,5)P_2, dried under vacuum, resuspended in 3 ml of chloroform-methanol-HCl (12 mM; 200:100:1), spotted onto nitrocellulose membranes, analyzed by a protein-lipid overlay procedure using the GFP-GRP1 PH domain as the primary probe, and subjected to analysis by densitometry. Data are means ± SD (n = 3).

(Fig. 3A). Furthermore, we were able to detect as little as 0.2 pmol of PtdIns(3,4,5)P_3 mixed in 100 pmol of PtdIns (4,5)P_2 (Fig. 3B).

PI3Ks contribute to the control of numerous neutrophil functions. Using ^{32}P-PI radiolabeling of neutrophils, we had shown previously that human neutrophils transiently synthesize PtdIns(3,4,5)P_3 when challenged with agonists such as bacterially derived fMLP (17). Consistent with this previous report, we showed that the GRP1 probe allowed detection of PtdIns(3,4,5)P_3 accumulation in human neutrophils stimulated with fMLP (Fig. 4). Increased PtdIns (3,4,5)P_3 synthesis was detected using the wild-type probe but not the point-mutated probe (Fig. 4). Furthermore, the increase in PtdIns(3,4,5)P_3 detected by the GRP 1 probe was abolished when cells were preincubated with wortmannin, a potent inhibitor of PI3K, further supporting the specificity of our PtdIns(3,4,5)P_3 assay.

We also measured the increase in PtdIns(3,4,5)P_3 in fMLP-stimulated TNFa-primed human neutrophils over time. We observed a transient biphasic increase in PtdIns (3,4,5)P_3 level peaking at 10 s and 60 s after addition of fMLP (Fig. 5). These results were consistent with those obtained previously using metabolic labeling of cells (17), presented as a dotted line for reference in Fig. 5. As shown in Fig. 2, the GRP1 PH domain binds to PtdIns(3,4,5)P_3 and, to a much lesser extent, to PtdIns(3,4)P_2. The similarity between the kinetics of PtdIns(3,4,5)P_3 accumulation measured by two different techniques (Fig. 5) further suggested that the protein-lipid overlay assay faithfully reported PtdIns(3,4,5)P_3 levels from the complex PI mixture purified from the cells with neomycin beads because of their clear difference compared with the slower monophasic accumulation of PtdIns(3,4)P_2 reported in the radiolabeling studies (17).

To extend our findings, we used our assay to measure PtdIns(3,4,5)P_3 accumulation in bone marrow-derived neutrophils from wild-type mice and mice carrying a homozygous knock-in of PI3Kg mutated in its Ras binding site ('DASAA') (5) after stimulation with fMLP. As expected, fMLP increased PtdIns(3,4,5)P_3 levels by over 4-fold in wild-type neutrophils. In neutrophils derived from mutant mice, PtdIns(3,4,5)P_3 accumulation was

Fig. 4. GFP-GRP1 can be used to quantitate PtdIns(3,4,5)P_3 in lipid extracts from human neutrophils. A: Human neutrophils (1.10^6) were primed with tumor necrosis factor a (TNFa; 200 U/ml^{-1}), preincubated for 5 min (+) or not (−) with wortmannin (100 nM) and stimulated (+) or not (−) with N-formyl-methionyl-leucyl-phenylalanine (fMLP) (100 nM) for 10 s. Total lipids were extracted, and phosphatidylinositols (PIs) were purified using neomycin beads and spotted onto nitrocellulose membranes for protein-lipid overlay using the GFP-GRP1 PH domain or GFP-(K273A)-GRP1 as primary probes. B: Data were analyzed by densitometry and are presented as means ± SD (n = 3).

Fig. 5. Transient and bi-phasic accumulation of PtdIns $(3,4,5)P_3$ in TNFa-primed, fMLP-stimulated human neutrophils. A: Human neutrophils (1.10^6) were primed with TNFa (200 U/ml $^{-1}$) and stimulated with fMLP (100 nM) for 0, 6, 10, 20, 60, and 120 s. Total lipids were extracted, and PIs were purified using neomycin beads and spotted onto nitrocellulose filters for protein-lipid overlay using the GFP-GRP1 PH domain or GFP-(K273A)-GRP1 as primary probes. B: Data for the GFP-GRP1 probe were analyzed by densitometry and are presented as means ± SD (n = 3). The dotted line indicates the relative PtdIns$(3,4,5)P_3$ levels as measured by radiolabeling and previously published (17).

attenuated. Our present study shows that fMLP-stimulated accumulation of the mass of PtdIns$(3,4,5)P_3$ [as opposed to the ^{32}P content described in (5)] is markedly reduced in neutrophils prepared from p110 $g^{DASAA/DASAA}$ mice as compared with their wild-type controls (Fig. 6). Both Gbg and Ras contribute to activation of PI3Kg and PtdIns $(3,4,5)P_3$ accumulation in mouse neutrophils (5). Consistent with the ability of Gbg to activate PI3Kg independently of Ras, we observed that in neutrophils prepared from p110$g^{DASAA/DASAA}$ mice, the level of PtdIns$(3,4,5)P_3$ accumulation was reduced but not completely abolished (Fig. 6).

Fig. 6. Reduced accumulation of PtdIns$(3,4,5)P_3$ in neutrophils isolated from p110g $^{DASAA/DASAA}$ mice. A: Neutrophils prepared from wild-type mice (WT) and p110$g^{DASAA/DASAA}$ mice (DASAA) (2.10^6) were stimulated with fMLP (10 mM) for 10 s. Total lipids were extracted, and PIs were purified using neomycin beads and spotted onto nitrocellulose filters for protein-lipid overlay using GFP-GRP1 PH or GFP-(K273A)-GRP1 (no data are shown for the mutant probe) as the primary probes. PIs purified from 2.10^6 wild-type murine neutrophils were used as a control (ctrl). B: Data were analyzed by densitometry and are presented as means ± SD (n = 3).

CONCLUSIONS

The data presented in this report validate a novel assay for PtdIns(3,4,5)P_3 in cell extracts. The assay is simple, rapid, highly sensitive, and specific for PtdIns(3,4,5)P_3,