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Wortmannin binds specifically to 1-phosphatidylinositol 3-kinase while inhibiting guanine nucleotide-binding protein-coupled receptor signaling in neutrophil leukocytes

(PI 3-kinase/signal transduction)

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ABSTRACT **Wortmannin (WT) and its derivative 17-hydroxywortmannin (HWT) inhibit at nanomolar concentrations superoxide formation and exocytosis in neutrophils stimulated with chemotactic agonists. Treatment of neutrophils with radiolabeled [^{3}H]HWT resulted in specific and saturable binding that paralleled the inhibition of the respiratory burst. Both half-maximal binding and half-maximal inhibition were observed at 5 nM, and >90% of maximal binding and inhibition was observed at 20 nM HWT. Fluorography of subcellular fractions that were separated on NaDodSO$_4$/PAGE showed that [^{3}H]HWT binds covalently to a 110-kDa cytosolic protein. The WT-binding protein was purified from human neutrophils and bovine brain homogenates by column chromatography. The pure protein was eluted from gel filtration columns with an apparent molecular mass of 200 kDa and showed a heterodimeric structure on Coomassie-stained NaDodSO$_4$/PAGE. In addition to the 110 kDa wortmannin binding protein an equally intense band was seen migrating at 85 kDa. This band was identified on Western blots as p85α, the regulatory subunit of phosphatidylinositol (PI) 3-kinase (ATP:1-phosphatidyl-1D-*myo*-inositol 3-phosphotransferase, EC 2.7.1.137). The purified protein contained PI 3-kinase activity that was enriched >20,000-fold from human neutrophil cytosol during preparation. The data impose a key role for PI 3-kinase-mediated signal transduction through guanine nucleotide-binding protein-coupled receptors and suggest that 3-phosphorylated inositol phospholipids are important second messengers for immediate responses in neutrophils. Furthermore, the results show that WT is a powerful and selective tool to study the function of PI 3-kinase.**

Activation of phagocytes is essential for antimicrobial defense. Neutrophils migrate to the site of infection and eliminate the microorganisms by phagocytosis combined with the release of hydrolytic enzymes from storage organelles and the production of superoxide by a membrane-bound NADPH oxidase (1, 2). Chemotaxis, exocytosis, and the respiratory burst are induced by a variety of agonists, including bacterially derived chemotactic formyl-peptides and chemokines through specific seven-transmembrane-domain receptors (3). The receptors are coupled to the inhibitory (G$_i$) subclass of heterotrimeric guanine nucleotide-binding proteins (G proteins), which are inhibited by ADP-ribosylation with *Bordetella pertussis* toxin (4). Downstream of the G protein, two signal-transduction sequences are necessary for stimulation of the respiratory burst (5); one includes G protein $\beta\gamma$-subunit-mediated activation of phospholipase Cβ_2 (6, 7) and further downstream protein kinase C-dependent phosphorylations, while the other is characterized by its exquisite sensitivity to the fungal metabolite wortmannin (WT) (8). Detailed studies of the activation of the NADPH oxidase in neutrophils indicate that the WT-sensitive signal-transduction pathway is rapidly activated (9), transient in nature, and calcium-independent (5). However, so far no distinct enzyme activity has been associated with this pathway.

Following stimulation with fMet-Leu-Phe, neutrophils rapidly phosphorylate phosphatidylinositol (PI) 4,5-bisphosphate at the 3-OH position to form phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (10). The synthesis is transient and correlates with the activation of the respiratory burst (11). Treatment of neutrophils with *B. pertussis* toxin inhibits agonist-stimulated PIP_3 synthesis, suggesting that inhibitory G proteins are involved in the activation of the PI 3-kinase (ATP:1-phosphatidyl-1D-*myo*-inositol 3-phosphotransferase, EC 2.7.1.137) (12, 13). Recently it was shown that WT inhibits PIP_3 formation in neutrophils (14) and CHO cells (15). Activation of PI 3-kinase by growth factor receptors is well characterized (16). The regulatory subunit p85 binds via its src-homology (SH2) domains to the autophosphorylated cytoplasmic domains of the receptors (17–19), thereby translocating and activating the 110-kDa catalytic subunit (20). The mechanism of activation of PI 3-kinase by G protein-coupled receptors is less well characterized (21).

The sterol-like WT and its chemically derived analogue 17-hydroxy wortmannin (HWT) are potent inhibitors of several phagocyte responses (4, 5, 8, 14, 22–25). WT contains a potential site for nucleophilic substitution by primary amines and sulfhydryl groups. Two lines of evidence suggest the inhibitory effect is caused by covalent binding of WT to a target molecule. Inhibition by WT is irreversible (5), and the inhibitory activity is abrogated by chemical modifications that impair nucleophilic additions (8). Controlled chemical reduction of the keto group at position 17 (26) was used to generate the tritium-labeled derivative, 17-[^{3}H]hydroxy-WT ([^{3}H]HWT). In neutrophils, [^{3}H]HWT inhibited the respiratory burst and selectively labeled a cytosolic protein of 110-kDa. By using the specific binding of [^{3}H]HWT as the assay, a heterodimeric protein was purified from neutrophil cytosol that copurified with PI 3-kinase activity.

MATERIALS AND METHODS

Chemicals. Custom-prepared [^{3}H]HWT (17.7 Ci/mmol; 1 Ci = 37 GBq) and [^{32}P]ATP (3000 Ci/mmol) were from Amersham. WT and HWT were gifts from T. Payne (Sandoz). Monoclonal antibodies (mAbs) against p85α and p85β (mAbs U13 and T4; ref. 27) and a polyclonal rabbit anti-p110

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Abbreviations: WT, wortmannin; HWT, 17-hydroxy-WT; PI, phosphatidylinositol; PIP_3, PI 3,4,5-trisphosphate; G protein, guanine nucleotide-binding protein; HTP, hydroxylapatite column; mAb, monoclonal antibody.

antiserum (28) were provided by M. D. Waterfield (Ludwig Institute, London). Dulbecco's phosphate-buffered saline (PBS) was from Amimed (Basel), and EN^{3}HANCE was from New England Nuclear.

Buffers. Buffer A contained 10 mM Tris chloride (pH 7.6), 0.5 mM EDTA, 0.5 mM EGTA, 0.01% NaN_3, 0.1% 2-mercaptoethanol, and 10% (vol/vol) ethylene glycol. Buffer B contained 10 mM Tris chloride (pH 8), 0.5 mM EDTA, 0.5 mM EGTA, 0.01% NaN_3, 0.1% 2-mercaptoethanol, and 10% ethylene glycol. Buffer C contained 10 mM potassium Pipes (pH 6.8), 0.5 mM EDTA, 0.5 mM EGTA, 0.01% NaN_3, and 10% ethylene glycol. Buffer D contained 10 mM Tris chloride (pH 8), 0.5 mM EDTA, 0.5 mM EGTA, 0.01% NaN_3, and 10% ethylene glycol. Buffer E contained 10 mM Tris chloride (pH 8), 200 mM NaCl, 0.5 mM EDTA, and 10% ethylene glycol.

Respiratory Burst and Cell Labeling. Neutrophils (5) (10^7 cells per ml of PBS) were prewarmed at 37°C (5 min), and the incubation was continued for 10 min in the absence or presence of HWT. From the suspension, 3×10^6 cells were removed to measure superoxide formation. Cytochrome *c* reduction was continuously measured following stimulation with 100 nM fMet-Leu-Phe as described (29). The remaining cells were precipitated with ice-cold CCl_3COOH (final concentration, 10%). After 20 min, pellets were recovered by centrifugation and washed three times with cold acetone. The pellets were resuspended in 50 μl of 2% $NaDodSO_4$/25 mM Tris/192 mM glycine and boiled for 5 min. Aliquots were used for liquid scintillation counting (Beckman) and separation on reducing $NaDodSO_4$/PAGE. Radiolabeled proteins were visualized on the gels by fluorography using EN^{3}HANCE on Kodak X-OMAT-AR film. Nonspecific binding was measured in the presence of 1000-fold excess of unlabeled HWT.

Binding Assay. Cell homogenates and column fractions were diluted with PBS (200–500 μl), warmed up to 37°C for 5 min, and incubated for 10 min in the absence and presence of [^{3}H]HWT. Incubations were terminated with ice-cold CCl_3COOH, and the precipitates were washed three times with cold acetone. Pellets were processed for $NaDodSO_4$/PAGE and scintillation counting as described above. During some preparations, one-fifth of the pools (usually obtained after the initial steps of separation) were labeled with 20 nM [^{3}H]HWT for 10 min at 37°C. The incubations were quenched by dilution with ice-cold column buffer, and the samples were immediately applied to the next chromatography step to remove unbound HWT.

Protein Purification. Neutrophils (5) (2×10^{10}) were treated with 2.5 mM diisopropyl fluorophosphate for 20 min at room temperature (30), cooled to 4°C, and disrupted (10^8 cells per ml) by nitrogen cavitation (31). Cytosol was prepared from the postnuclear supernatant by centrifugation at 100,000 × *g* for 1 hr. Proteins were precipitated between 20% and 40% ammonium sulfate saturation at pH 7.6, resuspended in buffer A, and applied to a hydroxylapatite (HTP) column. Proteins were eluted with a linear gradient (100–500 mM) of potassium phosphate in buffer A. Selected fractions were pooled, diluted with buffer B, loaded on a Mono Q HR 5/5 column (FPLC system, Pharmacia), and eluted with 0–400 mM NaCl. Pooled fractions from the Mono Q column were diluted with buffer C, applied to a Mini S column (SMART System, Pharmacia), and eluted with 0–500 mM NaCl. Fractions with HWT binding activity were pooled, diluted with buffer D, loaded on a Mini Q column (SMART System), and eluted with 0–500 mM NaCl. Peak fractions expressing HWT binding activity (50 μl) were subjected to gel filtration on Superdex S-200 PC 3.2/30 (SMART System) in buffer E. Calf brain homogenates were prepared as described (32), absorbed on DE-52 (Whatman), and eluted with 150 mM NaCl. The recovered proteins were subjected to ammonium sulfate fractionation and column chromatography as described for neutrophil cytosol, except that the Mini Q step was omitted.

Kinase Assay. PI 3-kinase activity was measured as described (14, 33). Briefly, protein samples were mixed with PI (200 μM) and phosphatidylserine (300 μM) in 20 mM Hepes, pH 7.4/5 mM $MgCl_2$ and warmed to 37°C. Phosphorylation was initiated by the addition of [^{32}P]ATP (10 μM, 10 μCi). After 15 min of incubation, the reactions were terminated with 40 μl of 1 M HCl. Lipids were extracted with $CHCl_3$/CH_3OH, 1:1 (vol/vol), and separated by TLC with $CHCl_3$/CH_3OH/H_2O/NH_4OH, 45:35:8.5:1.5 (vol/vol). Radioactive lipids were quantified with a PhosphorImager (Molecular Dynamics), scraped, and deacylated (34). Glycerophosphoinositol phosphates were separated by HPLC on a Partisphere Sax column (14).

Protein Detection, Electrophoresis, and Western Blotting. Protein contents were measured with a micro-BCA (bicinchoninic acid) protein assay reagent (Pierce) with bovine serum albumin as standard. $NaDodSO_4$/PAGE was performed as described by Laemmli (35) with 6% polyacrylamide containing 2% methylenebisacrylamide. Western blots were performed as described (36) in the presence of 20% (vol/vol) methanol. Peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (Amersham) was used as the second antibody and was detected by enhanced chemiluminescence (ECL, Amersham) on Kodak X-OMAT-AR film.

RESULTS

HWT Binding and Inhibition of the Respiratory Burst. Stimulation of neutrophils with the chemotactic peptide fMet-Leu-Phe induces the rapid formation of superoxide. Preincubation of the cells with 0–20 nM HWT caused a dose-dependent inhibition (Fig. 1*A*) of the respiratory burst. The same incubations were used to measure the concentration-dependent binding of [^{3}H]HWT to CCl_3COOH-precipitable protein (Fig. 1*A*). Binding of [^{3}H]HWT was complete within 10 min (not shown) and was prevented in the presence of a 1000-fold excess of nonradioactive HWT, suggesting a specific interaction of [^{3}H]HWT with a target

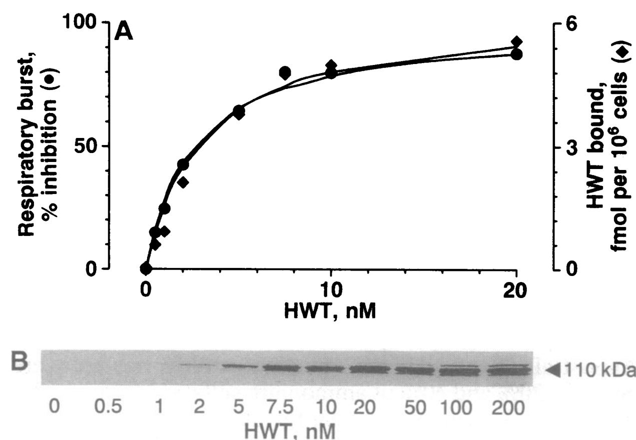

FIG. 1. Effect of [^{3}H]HWT on human neutrophils. (*A*) Human neutrophils (10^7 cells per ml) were incubated with the indicated concentrations of [^{3}H]HWT for 10 min at 37°C. Aliquots of the suspensions (300 μl) were diluted with warm buffer containing cytochrome *c* and transferred to the spectrophotometer, and the respiratory burst (●) was stimulated with 100 nM fMet-Leu-Phe. From the same incubations, 7×10^6 cells were precipitated with CCl_3COOH. Specific binding (◆) was determined by counting 5×10^6 cell equivalents and subtracting nonspecific binding. Results are from a single experiment of three independent determinations. (*B*) Fluorography of $NaDodSO_4$/PAGE of human neutrophils incubated with the indicated concentration of [^{3}H]HWT for 10 min at 37°C (10^6 cells per lane, 2-week exposure).

Table 1. Purification of PI 3-kinase from human neutrophils

Fraction	Protein, mg total	Protein, mg/ml	Specific activity, nmol of *P* per mg of protein	Yield, %	Fold purification
Cytosol	495	4.2	0.007	100	1
$(NH_4)_2SO_4$	109	3.4	0.019	60	2.9
HTP	37.4	1.7	0.062	66	9.5
Mono Q	7.2	1.2	0.42	86	64.6
Mini S	0.07	0.31	13.38	27	2,060
Mini Q	0.012	0.40	35.7	12	5,500
S-200	0.0043	0.032	140	17	21,500

protein. Both, the inhibition of the respiratory burst and the binding reached 50% and 90% of maximum at [^{3}H]HWT concentrations of 5 and 20 nM, respectively (Fig. 1*A*). The close correlation between specific binding and the inhibitory effect indicates that HWT targets an essential component of the neutrophil activation pathway. Binding analysis yielded ≈5000 HWT binding sites per neutrophil. Total lysates from neutrophils that were incubated with 0–200 nM [^{3}H]HWT were resolved on $NaDodSO_4$/PAGE. Fluorography of radiolabeled proteins (Fig. 1*B*) showed that at concentrations up to 20 nM, [^{3}H]HWT bound covalently to a single protein with an apparent molecular mass of 110-kDa. With increasing concentrations, two additional proteins with slightly higher and lower molecular mass became labeled as well. At high concentrations (>200 nM) and prolonged incubation times, HWT tended to bind nonspecifically to several proteins (data not shown). In the presence of a 1000-fold excess of nonradioactive HWT, labeling was prevented, and no radioactive bands were detected by fluorography (same exposure time, data not shown). Densitometric analysis of the 110-kDa band revealed a similar concentration dependence of the binding as shown in Fig. 1*A*, where CCl_3COOH precipitation and scintillation counting were used, indicating that the 110-kDa protein is a specific target for the selective covalent binding of [^{3}H]HWT. The low abundancy of the HWT binding protein on the one hand and the strong inhibitory ability on the respiratory burst suggest that HWT binds and inhibits the activity of a key component of neutrophil signaling.

Purification of the 110-kDa Protein. Fractionation of lysates obtained by nitrogen cavitation (31) of [^{3}H]HWT-treated neutrophils revealed that >90% of the 110-kDa HWT-binding protein resides in the cytosol. Therefore, neutrophil cytosol was tested for its ability to specifically bind [^{3}H]HWT. Incubations of cytosol in the presence of 0–20 nM [^{3}H]HWT revealed binding to CCl_3COOH-precipitable protein that exhibited similar kinetics as in intact cells. $NaDodSO_4$/PAGE analysis proved covalent binding of HWT to a 110-kDa protein (data not shown). The *in vitro* binding properties were adopted to follow the 110-kDa protein during the purification.

Cytosol from 2×10^{10} human neutrophils was prepared by nitrogen cavitation and precipitated with ammonium sulfate. Almost all of the [^{3}H]HWT-binding activity was recovered between 20% and 40% of ammonium sulfate saturation. This fraction was dissolved in buffer A and applied to HTP chromatography. The 110-kDa protein bound tightly to the column, allowing the bulk of proteins to be preeluted with 100 mM potassium phosphate. The [^{3}H]HWT-binding activity was recovered at about 200 mM potassium phosphate by elution with a linear gradient and pooled. About 20% of this pool was incubated with 20 nM [^{3}H]HWT for 10 min and then diluted 1:5 with cold buffer to reduce the potassium phosphate concentration. The material was immediately loaded on a Mono Q column, and the excess of [^{3}H]HWT was removed by washing with column buffer. The unlabeled material from the pool was similarly diluted and loaded to the same column. A sharp peak of [^{3}H]HWT-labeled protein was eluted around 200 mM NaCl. Incubation of the fractions with [^{3}H]HWT revealed that the bulk of binding activity was coeluted with the prelabeled protein, indicating that binding of HWT does not alter the elution properties of the 110-kDa protein. Identical chromatographic behavior of the labeled protein and the protein labeled after column chromatography was observed during the following steps of purification. Repeated ion-exchange chromatography on high-resolution matrices (Mini S and Mini Q) resulted in sharp elution profiles with high protein recovery (Table 1). During final gel filtration on Superdex S-200, the labeled protein comigrated as a symmetric peak with an apparent molecular mass of 200 kDa with a protein that showed high affinity for [^{3}H]HWT in a binding assay. The peak fraction of the S-200 gel filtration was analyzed on $NaDodSO_4$/PAGE (Fig. 2*B*) and revealed a heterodimeric structure of the purified protein. The two proteins that were present in the peak fraction migrated with an apparent molecular mass of 110 and 85 kDa, respectively, and stained equally well with Coomassie blue (Fig. 2), suggesting that they form a stable 1:1 complex in solution. The protein composition of each purification step is shown in Fig. 2*B* and in parallel binding of [^{3}H]HWT to the same fractions (fluorograph in Fig. 2*A*). Due to the short exposure of the gel (20 hr), the 110-kDa protein was only visualized after the second step of purification. Longer exposure of the same gel (7 days) demonstrated also the presence of the protein in the cytosol and ammonium sulfate fractions (not shown). The weak fluorographic signal of the first steps of

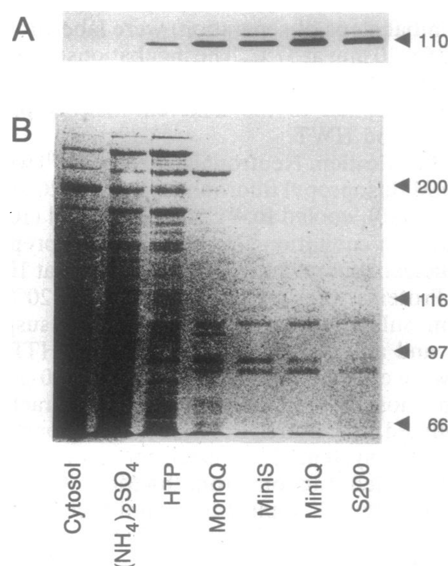

FIG. 2. Purification of the 110-kDa HWT-binding protein. Protein in aliquots from the purification steps [cytosol, $(NH_4)_2SO_4$, and HTP, 21 μg; Mono Q, 10 μg; Mini S, 1.6 μg; Mini Q, 1.2 μg; and S-200, 0.65 μg] was labeled with [^{3}H]HWT for 10 min at 37°C, precipitated with CCl_3COOH, and separated on $NaDodSO_4$/PAGE. (*A*) Fluorograph (20-hr exposure). (*B*) Coomassie blue stain. Molecular size markers are in kilodaltons on the right.

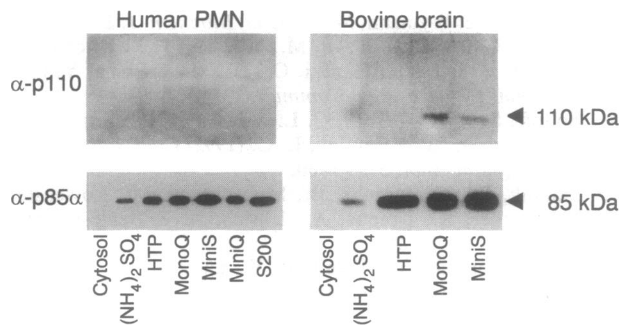

FIG. 3. Western blots. Aliquots from the purification steps of human neutrophil HWT-binding protein (*Left*) and of calf brain HWT-binding protein (*Right*) were resolved on $NaDodSO_4$/PAGE, transferred to polyvinylidene difluoride membranes, and probed with a polyclonal antiserum (α-p110) (*Upper*) or with a mAb (α-p85α) (*Lower*). Immunoreactive bands were detected with enhanced chemiluminescence. PMN, polymorphonuclear leukocytes.

purification is in good agreement with the low abundancy of the protein in neutrophil cytosol.

Immunochemical Analysis. The heterodimeric structure and the characteristic molecular mass of the purified protein prompted an investigation into whether the [^{3}H]HWT binding activity is associated with the p85/p110 PI 3-kinase. A mAb, U13, generated against bovine p85α, the regulatory subunit of PI 3-kinase, was used to screen the preparation on Western blots. $NaDodSO_4$/PAGE analysis of the purification shows that p85α copurified through all chromatography steps with the 110-kDa HWT-binding protein (Fig. 3). The crude and the purified material did not react with a mAb directed against bovine p85β (mAb T4), suggesting that the 110-kDa HWT-binding protein specifically associates with p85α (data not shown). Western blots of the same fractions with two different preparations of polyclonal rabbit sera generated against the C terminus of bovine p110 (28) failed to detect the 110-kDa HWT-binding protein from human neutrophils (Fig. 3). However, when the HWT-binding protein was purified by a similar procedure from calf brain, the 110-kDa protein was recognized by the antibodies, suggesting structural differences between the human and the bovine proteins (Fig. 3).

PI 3-Kinase. The immunochemical recognition of p85α and the molecular mass of the purified material suggested that the HWT-binding protein purified from human neutrophils is homologous to the bovine brain PI 3-kinase. Measurements of lipid kinase activity in the different pools obtained during the purification revealed that a PI 3-kinase activity copurified with the HWT-binding protein. On TLC [^{32}P]phosphorylated lipids comigrated with a PI monophosphate standard. To determine the specificity of the kinase activity for the inositol 3-OH position, phosphorylated lipids were scraped from the TLC plates and deacylated (34). Ion-exchange chromatography of the deacylation products is illustrated in Fig. 4. The elution profile shows that the initial fractions contained marginal inositol 4-OH kinase activity, which was completely absent in the final pure preparation. The main kinase activity, however, was selective for the 3-OH position and phosphorylated equally well PI 4,5-bisphosphate to the 3,4,5-trisphosphate PIP_3, suggesting that the HWT-binding protein is a PI 3-kinase. Accumulation of PI 3-kinase activity during a typical preparation is summarized in the purification table (Table 1). From the cytosol of human neutrophils, the specific activity of PI 3-kinase was purified >20,000-fold, which correlated with the increase in specific binding of [^{3}H]HWT to the 110-kDa protein (Fig. 2).

DISCUSSION

Former studies on the inhibition of neutrophil responses by WT revealed a transient, calcium-independent signal-

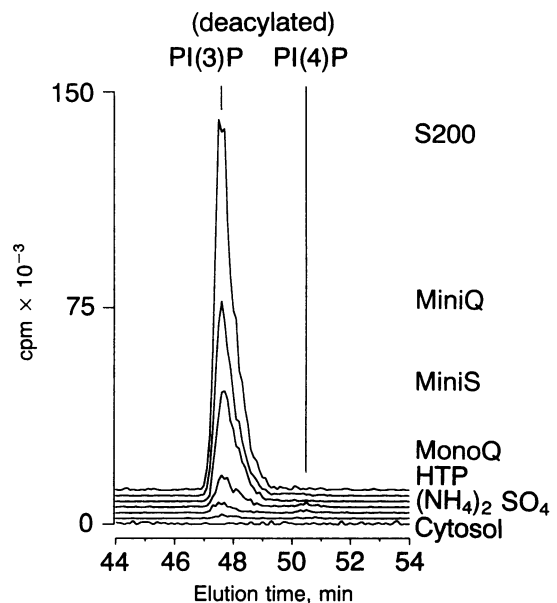

FIG. 4. Phosphorylation of PI by the HWT-binding protein. PI was incubated for 15 min at 37°C in the presence of [γ-^{32}P]ATP with fractions from the purification steps (see Table 1): cytosol, 1 mg; $(NH_4)_2SO_4$, 0.2 mg; HTP, 0.1 mg; Mono Q, 50 μg; Mini S, 4.5 μg; Mini Q, 4 μg; and S-200, 1.5 μg. Phosphorylated lipids were separated on TLC, and the deacylated glycerophosphoinositol phosphates were resolved on HPLC. Retention times of glycerophosphoinositol phosphate standards are indicated as deacylated PI 3-monophosphate (PI(3)*P*) and deacylated PI 4-monophosphate (PI(4)*P*).

transduction pathway, that is necessary for eliciting the respiratory burst and exocytosis (5). The present results show that WT at nanomolar concentrations selectively targets PI 3-kinase. Thus, the kinase represents an essential element of the signal-transduction pathway initiated by stimulation of G protein-coupled receptors. The low number of WT-binding sites found in neutrophils confirms the view that the target protein is a key enzyme of the signal-transduction cascade.

In neutrophils, binding of HWT to the 110-kDa protein was saturated at 20 nM and paralleled the inhibition of the respiratory burst and formation of PIP_3 (14). On the fluorographs of lysates, additional proteins with similar electrophoretic mobility became apparent when the cells were tested with >5–10 nM [^{3}H]HWT. The binding to these proteins was saturated only with 3- to 4-fold higher concentrations than those for p110. Further investigations will establish whether the proteins represent isoforms of PI 3-kinase. It has been reported (37) that WT inhibits myosin light chain kinase. However, these effects were obtained at WT concentrations of 0.3 to 1 μM—i.e., about 100-fold higher than those shown here to affect PI 3-kinase. When using polyclonal antibodies against nonmuscle myosin light chain kinase (38), no binding of WT to this protein was detected under conditions that inhibit the respiratory burst of the neutrophils by >90%, indicating that the myosin light chain kinase is not a relevant target for WT in neutrophils.

The 110-kDa WT-binding activity from neutrophils copurifies with p85α and PI 3-kinase, indicating that it represents the human homolog of the structurally characterized bovine p85/p110 PI 3-kinase (27, 28). The regulatory p85α subunits of bovine and human PI 3-kinase show >96% amino acid sequence identity (17, 27). Accordingly, by using mAb U13 (27, 39), p85α was readily detected on Western blots of the purified proteins from both species (Fig. 3). The related protein p85β, which shows 62% identity to p85α (27), was not

detected in our PI 3-kinase preparations from neutrophils or bovine brain (data not shown), in agreement with previous observations that p110 specifically associates with p85α (40). Polyclonal antibodies directed against a 17-amino acid C-terminal domain of the 110-kDa (catalytic) subunit of PI 3-kinase from bovine brain (28) did not recognize the human protein on Western blots nor did they precipitate the protein from neutrophil lysates. These findings may indicate some structural diversity between the human and the bovine proteins. On the other hand, the similar migration on $NaDodSO_4$/PAGE, the identical chromatographic behavior, and the heterodimeric structure of the PI 3-kinase suggest a high structural similarity between the bovine and human enzyme.

PI 3-kinase was originally detected in immunoprecipitates of activated tyrosine kinases (41, 42), and its function has been related mainly to growth control (16, 43). In neutrophils a rapid rise of PIP_3 formation is observed upon G protein-coupled receptor stimulation, which returns to baseline within a few minutes (10, 44). This peak is followed by a slower, more protracted phase of PIP_3 formation that reaches only about 10% of the rapid initial level and that is not temporally related to the respiratory burst. The initial, rapid stimulation of PI 3-kinase appears to be independent of tyrosine phosphorylation(s), whereas the second wave of PI 3-kinase activity is associated with tyrosine kinase activation (21, 45). Inhibition by WT (14) and its transient formation in chemoattractant-stimulated neutrophils suggest that PIP_3 may have a second messenger function in processes that are triggered via G protein-coupled receptors (10, 13, 44, 45). Therefore, it is conceivable that PIP_3 generation in neutrophils is primarily associated with vesicle transport and membrane remodeling, processes that are necessary for exocytosis and assembly of the NADPH oxidase at the plasma membrane (1). However, the mode of activation of PI 3-kinase and the actual role of PIP_3 in signal transduction remain to be established.

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