Src Family Kinases Mediate Receptor-stimulated, Phosphoinositide 3-Kinase-dependent, Tyrosine Phosphorylation of Dual Adaptor for Phosphotyrosine and 3-Phosphoinositides-1 in Endothelial and B Cell Lines*

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DAPP-1 (dual-adaptor for phosphotyrosine and 3-phosphoinositides-1) is a broadly distributed pleckstrin homology (PH) and Src homology 2 domain containing protein that can bind phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) and can be phosphorylated on tyrosine 139 and internalised in response to activation of type I phosphoinositide 3-kinases (PI3K). Tyrosine phosphorylation of DAPP-1 appears important for appropriate intracellular targeting and creates a potential binding site for Src homology 2 domain-containing proteins. In endothelial cells overexpressing wild-type platelet-derived growth factor β (PDGFβ) receptors, which express Bmx and Src as their major Btk (Bruton's tyrosine kinase) family and Src family tyrosine kinases, respectively, PDGF can stimulate PI3K-dependent tyrosine phosphorylation of DAPP-1. Transient overexpression of Src most effectively, compared with Bmx and Syk, augments basal and PDGF-stimulated tyrosine phosphorylation of DAPP-1, whereas overexpression of dominant-negative Src, but not dominant-negative Bmx, inhibits PDGF-stimulated phosphorylation of DAPP-1. Cells expressing mutant PDGFβ(Y579F/Y581F) receptors (which fail to bind and activate Src-type kinases) fail to tyrosine phosphorylate DAPP-1 in response to PDGF. We show that in DT40 chicken B cell lines, antibody stimulation leads to PI3K-dependent tyrosine phosphorylation of DAPP-1 that is lost in Lyn- or Syk-deficient cell lines but not Btk-deficient cell lines. PI3K-dependent activation of PKB is only lost in Syk-deficient lines. Finally, in vitro we find lipid-modified Src to be the most effective DAPP-1 tyrosine kinase (versus Syk, Lyn, Btk, and Bmx); phosphorylation of DAPP-1 but not Src autophosphorylation is stimulated ~10-fold by PtdIns(3,4,5)P3 (IC50 = 150 nM) and phosphatidylinositol 3,4-bisphosphate but not by their nonbiological diastereoisomers and depends on PH domain mediated binding of DAPP-1 to PtdIns(3,4,5)P3-containing membranes. We conclude that Src family kinases are responsible for tyrosine phosphorylation of DAPP-1 in vivo and that PI3K regulation is at the level of PH domain-mediated translocation of DAPP-1 to PI3K products in the membrane.

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Receptor mediated activation of type I phosphoinositide 3-kinases (PI3Ks)1 (1) leads to enhanced 3-phosphorylation of phosphatidylinositol (4,5)-bisphosphate and accumulation of PtdIns(3,4,5)P3 in the plasma membrane (2). This rise in PtdIns(3,4,5)P3, which is transient as a result of receptor inactivation and the action of PtdIns(3,4,5)P3 3- and 5-phosphomonoesterases (2, 3), acts as a signal driving recruitment of proteins capable of binding tightly and selectively to the lipid (4).

The large majority of proteins capable of binding PtdIns(3,4,5)P3 do so via pleckstrin homology (PH) domains (5). PH domains form a large and diverse family that are capable of binding a variety of ligands; a subfamily that can be recognized by their possession of characteristically distributed basic residues can bind to the most polar 3-phosphorylated lipids: phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2) and/or PtdIns(3,4,5)P3 (5). These PtdIns(3,4,5)P3-binding proteins are effectors in PI3K signaling pathways that transduce lipid signals into changes in protein kinase activity (6) or Rac (7) or ADP ribosylation factor (ARF) (8) guanine nucleotide exchange activity.

DAPP-1 is a PH domain- and Src homology 2 (SH2) domain-containing, PtdIns(3,4,5)P3-binding protein that was independently identified by a number of laboratories (9–12). DAPP-1 translocates to the cell surface in response to receptor-driven accumulation of PtdIns(3,4,5)P3 (11, 12). DAPP-1 translocation is dependent on its PH domain, and the PH domain alone will translocate. Interestingly, DAPP-1 also becomes tyrosine phosphorylated on residue Tyr139 in a PI3K- and translocation-dependent manner (12, 13). This phosphorylation can be only partially mimicked by membrane targeting of DAPP-1, with a dual myristoylation/palmitylation consensus from the Src-type kinase Yes, or by expression of constitutively active type I PI3Ks that also cause membrane translocation of DAPP-1 (12). The implication of these results is that other events, possibly, for example, PI3K-independent activation of the relevant tyrosine kinase, are required to deliver a full tyrosine phosphorylation response. Thus, although PI3K-dependent translocation is essential for this event, it is only sufficient to drive partial tyrosine phosphorylation. The Src family selective protein kinase inhibitor PP2 and transient overexpression of Src-type kinases have been used to implicate Src-type kinases in phosphorylation of Tyr139 in vivo (13), although, importantly, the identity of the Src family member(s) responsible for this re-
sponse in any particular receptor-stimulated context remains to be defined. Subsequent to the translocation of DAPP-1 to the plasma membrane, it becomes internalized via a dynamin-depen-
dent mechanism, like that used to internalize cell surface receptors and is delivered to the endosomal membrane system (12). It is not yet clear how DAPP-1 exits the endosomal sys-
tem. Internalization is not absolutely dependent on phospho-
rylation of Tyr\(^{139}\), however, in endothelial cells expressing PDGF\(\beta\) (Y579F/Y581F) receptors, which fail to activate Src-
type kinases, PDGF fails to direct normal targeting of DAPP-1 and DAPP-1-containing endosomes are fewer and remain nearer to their site of formation at the cell surface (12). The specific failure in Src signaling that underlies this event is not clear; however, the fact that DAPP-1 (Y139F) constructs display a similar pattern of behavior to wild-type DAPP-1 in PDGF\(\beta\) (Y579F/Y581F) receptor-expressing endothelial cells suggests this is a specific result of failing to phosphorylate DAPP-1 rather than a general deficiency in Src signaling.

The clear potential importance of a tyrosine phosphate resis-
tue on DAPP-1 for forming SH2 domain-mediated interactions with other molecules and the increasing appreciation of the importance of tyrosine phosphorylation in receptor endocytosis and vesicle targeting lead us to attempt to define the cellular kinases responsible for phosphorylation of tyrosine 139 in DAPP-1 in endothelial and B cell lines.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs—**DAPP-1 constructs were inserted into pCMV3 vec-
tors for mammalian expression, pGEX 4Ti vectors for bacterial expres-
sion, and pACOG1 vectors for SF9 expression, as described previously (14). Baculoviruses encoding GST-Lyn, GST-Syk, and GST-Btk were generously provided by Dr. Chan (Howard Hughes Medical Institute, Saint Louis, MO). Hs-cSrc was inserted into pCMV3 without NH\(_2\)-terminal tagging but incorporating a COOH-terminal EE tag (EE-FMPME) via a polymerase chain reaction primer; Hs-cSrc was also generously provided by Dr. Chan (Howard Hughes Medical Institute, Saint Louis, MO). Hs-cSrc was inserted into pCMV3 without NH\(_2\)-terminal tagging but incorporating a COOH-terminal EE tag (EE-FMPME) via a polymerase chain reaction primer; Hs-cSrc was also inserted into pACOG1 incorporating an NH\(_2\)-terminal EE tag that pre-
vents correct post-translational lipid modification. Bmx was obtained from Dr. Ekman (Heartman Institute, Helsinki, Finland) and inserted into pCMV3 in frame with a NH\(_2\)-terminal EE tag. Site-directed mu-
tant and COOH-terminal EE tag-expressing Bmx were created by standard reverse-genet-ic procedures; all constructs were sequenced prior to use.

**Production of Recombinant Proteins—**GST-DAPP-1 and its R184A, R61M, and Y139F point mutants were expressed in *Esherichia coli* BL-21 s, purified via their GST tags, cleaved with thrombin while still bound to the glutathione beads or eluted with glutathione, concent-
trated, and buffer exchanged by gel filtration into 1x phosphate-buff-
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tau-
RESULTS

We have utilized DT40 chicken B cell lines that express only three of the classical, nonreceptor, ligand-regulated tyrosine kinases: Lyn, Btk, and Syk. Clonal cell lines in which each of these kinases have been deleted genetically have been isolated (17, 20). We have shown that antibody stimulation of wild-type DT40 cells leads to translocation and internalization of DAPP-1 (12). Fig. 1 shows that in response to antibody stimulation, transiently expressed EE-DAPP-1 becomes tyrosine phosphorylated, and PKB becomes phosphorylated on serine 473 in parallel. Both antibody-stimulated phosphorylation of PKB and DAPP-1 were inhibited by the PI3K inhibitor wortmannin (data not shown). This antibody-stimulated tyrosine phosphorylation of DAPP-1 was lost in B cell lines that lacked Syk and Lyn, whereas antibody-stimulated phosphorylation of serine 473 in PKB was only lost in Syk-deficient cells (Fig. 1). To compare the ability of Syk, Lyn, and Btk to directly phosphorylate DAPP-1 in vivo, Sf9 cells were infected with baculoviruses encoding GST-fusions of one of the three kinases and/or EE-DAPP-1 in the presence or absence of PI3K components and Gβγ subunits that stimulate PtdIns(3,4,5)P3 accumulation in Sf9 cells (data not shown). After 1.5 days of infection the cells were lysed, and relative expression of DAPP-1 and the three kinases and the extents of tyrosine phosphorylation of DAPP-1 were determined (Fig. 2). Lyn kinase tyrosine phosphorylated DAPP-1 approximately six times more effectively than similarly expressed Syk kinase. Elevation of PtdIns(3,4,5)P3 levels significantly stimulated Btk and Lyn but not Syk-mediated phosphorylation of DAPP-1 by relatively small extents. In the context of other work showing, firstly, that PI3K activity is sufficient and necessary for receptor-stimulated phosphorylation of serine 473 in PKB (21, 22) and, secondly, that in the signaling cascades downstream of antibody receptors Syk may be required for activation of type I PI3Ks (23–25), these data would suggest that the dependence of antibody-stimulated DAPP-1 phosphorylation on Syk is a manifestation of the requirement for activation of PI3Ks, and the dependence on Lyn may be a result of the fact that it is directly responsible for phosphorylation of DAPP-1.

PDGFβ (Y579F/Y581F) receptors have been shown to fail to bind and activate Src family tyrosine kinases (16). We have demonstrated that PAE cells expressing these receptors show substantially altered PDGF-induced DAPP-1 accumulation in the endosome compartment (12). We have assessed tyrosine phosphorylation of DAPP-1 in response to PDGF in clonal cell lines expressing similar numbers of wild-type PDGFβ receptors, PDGFβ (Y579F/Y581F) receptors, and PDGFβ (Y1021F) receptors (Y1021 is the phospholipase Cγ-binding site; Fig. 3). Tyrosine phosphorylation of DAPP-1 was reduced to less than 5% of control in the PDGFβ (Y579F/Y581F) receptor-expressing cell line. This result suggests that the substantial reduction in DAPP-1-endosome targeting we have observed in PDGFβ (Y579F/Y581F) receptor-expressing cells could be due to the fact DAPP-1 is not tyrosine phosphorylated and that a Src family kinase-dependent pathway controls phosphorylation of DAPP-1.

The PAE cells we have used to demonstrate translocation and internalization of DAPP-1 do not express detectable Lyn, Yes, Fyn, Btk, or Syk kinases; rather they express Src and Bmx (a more broadly expressed Btk family kinase; data not shown). This means the Src family kinase that binds to the PDGFβ
receptor in PAE cells is probably Src. We transiently expressed Src, Syk, and Bmx kinases in endothelial cells expressing the wild-type PDGFβ receptor. We found co-expression of Src but not Bmx or Syk with DAPP-1 dramatically inhibited our ability to recover DAPP-1 from cell lysates. This occurred even when cells were lysed into solutions containing 1% Triton X-100, 0.5% sodium cholate, and 0.1% SDS or hot SDS sample buffer, and we assume that this reflects a reduction in DAPP-1 expression (data not shown). To allow us to determine whether we could compare the tyrosine phosphorylation of DAPP-1 in cells with substantially different levels of DAPP-1, we transfected PAE cells with different amounts of DAPP-1 and estimated their extent of tyrosine phosphorylation in response to PDGF by immunoblotting. We found that the amount of phosphotyrosine on DAPP-1 was apparently a linear function of the amount of DAPP-1 (Fig. 3). Transient expression of Syk had no effect on PDGF-stimulated tyrosine phosphorylation of DAPP-1 but to a lesser extent than for Src (Fig. 4). Both Bmx and Src driven enhancement of PDGF-stimulated tyrosine phosphorylation of DAPP-1 was lost in PAE cells transiently expressing DAPP-1 (Y139F) constructs (data not shown). These results suggest that Bmx and Src can drive tyrosine phosphorylation of DAPP-1 in endothelial cells and can be regulated by cell surface receptors that control DAPP-1 tyrosine phosphorylation.

Mouse Src (K295R/Y527F) has been shown to act as a dominant-negative construct (26); we identified the equivalent sites in human Src and prepared Src (K298R/Y530F). Similarly we prepared Bmx (E42K/K445R), which has been shown to act as a dominant-negative construct (27). We found that transiently co-expressed dominant-negative Src but not Bmx blocked PDGF-stimulated tyrosine phosphorylation of DAPP-1 (Fig. 5); although the dominant-negative Bmx could inhibit Bmx augmentation of PDGF-stimulated tyrosine phosphorylation of DAPP-1. These data are consistent with the above work and implicate Src kinase-dependent signaling in the pathway by which PDGF stimulates tyrosine phosphorylation of DAPP-1.

To establish the relative abilities of Src, Syk, and Btk family kinases to directly phosphorylate DAPP-1, we purified the kinases from appropriately infected or transfected SF9 or Cos-7 cells. A variety of purified wild-type, Y139F, R61M (SH2 domain point mutant), and R184A (PH domain point mutant) DAPP-1 preparations (EE-tagged from Sf9 cells; GST-tagged or bacterially derived) were used as potential substrates in the presence of mixed phospholipid vesicles containing low molar percentages of various phosphoinositides and [γ-32P]ATP. These results showed that the presence of an NH2-terminal tag on DAPP-1 or the source of DAPP-1 (i.e. SF9 cell or bacterially derived) did not influence its ability to serve as a substrate for various protein-tyrosine kinases (data not shown). Src was the most effective of the kinases tested at phosphorylating tyrosine 139 in DAPP-1 (Fig. 6). This result was confirmed in an experiment comparing phosphorylation of DAPP-1 by Syk, Src, Bmx, and Btk that was conducted in the presence of 75 μM ATP, suggesting that these differences do not reflect differences in the kinases Kcat values for ATP (data not shown); this observation is also supported by the similarity of these data to that describing the relative abilities of these kinases to phosphorylate DAPP-1 in vivo (Figs. 2 and 4). The ability of Src to phosphorylate DAPP-1 depended on the kinase being post-translationally lipid-modified. COOH terminus EE-tagged Src was purified from SF9 cells via a Triton X-114 detergent partition procedure and EE-directed immunoprecipitation; this protein was substantially more active as a DAPP-1 kinase than non-lipid-modified, NH2 terminus EE-tagged Src (Fig. 6). Lipid-modified Src associated with the phospholipid vesicles in the kinase assays in a manner that was not affected by the presence of PtdIns(3,4,5)P3 (results of experiments using sucrose-loaded phospholipid vesicles and...
immunoblotting; not shown). The presence of PtdIns(3,4,5)P3 stimulated phosphorylation of tyrosine 139 in DAPP-1 by 8–10-fold, depending on the assay, but not of Src itself (Fig. 7). These results suggest that the PtdIns(3,4,5)P3 has no effect on Src kinase activity but acts by influencing DAPP-1. This view is supported by a number of additional observations. The phosphoinositide specificity (PtdIns(3,4,5)P3 and phosphatidylinositol 3,4-bisphosphate are the most effective lipids; Fig. 8A and data not shown) and sensitivity (IC50 for PtdIns(3,4,5)P3 of 150 nM; Fig. 8B) of this effect is consistent with the phosphoinositide binding characteristics of the PH domain of DAPP-1 (10). Furthermore, the presence of PtdIns(3,4,5)P3 has no effect on phosphorylation of DAPP-1 R184A (PH domain point mutant that does not bind phosphoinositides (12)) by Src (Fig. 7). Assays with Btk family kinases revealed that although they were less effective than Src as DAPP-1 kinases, they supported greater fold activations with PtdIns(3,4,5)P3 (Fig. 6 and data not shown), indicating that the Btk family kinases themselves may be regulated by PtdIns(3,4,5)P3. This effect was probably at the level of the distribution of the kinases within the assays, i.e. in the presence of PtdIns(3,4,5)P3, the Btk family kinase also associated with the lipid vesicles (PtdIns(3,4,5)P3 had no effect on Btk family kinase autophosphorylation; not shown); this is consistent with the well documented affinity of the PH domain of Btk for this lipid (28).

We attempted to address the issue of whether receptor activation of Src might influence its ability to phosphorylate DAPP-1; this is in the context of our observations that membrane targeting of DAPP-1 with a Src family targeting motif and co-expression of constitutively active p110γ with an identical targeting motif both caused relatively small activations of DAPP-1 tyrosine phosphorylation compared with receptor ac-
The extent of 32P incorporation into the indicated proteins (all thrombin-cleaved GST-constructs at 1 μM final concentration) in the presence of base lipids with or without PtdIns(3,4,5)P3 (10 μM) were carried out with [γ-32P]ATP as described under “Experimental Procedures.” The upper panel is an autoradiograph showing the extent of 32P incorporation into the indicated DAPP-1 protein and the level of 32P Src autophosphorylation in each assay; the lower panel is a Coomassie-stained gel illustrating the equivalent amounts of each of the different DAPP-1 proteins used in the assays. WT, wild type.

**DISCUSSION**

We present a number of lines of evidence that in vivo, Src-type tyrosine kinases are responsible for the phosphorylation of tyrosine 139 in DAPP-1. In B cells, loss of Syk or Lyn abolished antibody-stimulated phosphorylation of tyrosine 139 in DAPP-1, but loss of Syk also abolished phosphorylation of serine 473 in PKB. Other data suggest that PI3Ks are sufficient and necessary for phosphorylation of serine 473 in PKB (21, 22) and furthermore that Syk is a primary target of the activated antibody receptor and is capable of stimulating type I PI3Ks (23–25). Hence our observation, and that of others, that antibody-stimulated phosphorylation of serine 473 in PKB is lost in Syk-deficient cells, suggests that the dependence of antibody-stimulated tyrosine phosphorylation of DAPP-1 on Syk is a consequence of a requirement for activation of type I PI3Ks. The dependence on Lyn is possibly a result of the fact that it phosphorylates tyrosine 139 in DAPP-1. This is supported by our data showing heterologous Lyn phosphorylates heterologous DAPP-1 in Sf9 cells substantially more effectively than Syk and in vitro data indicating the same.

In PAE cells transient expression of Src substantially augmented PDGF-stimulated phosphorylation of DAPP-1, suggesting that the heterologous Src could be regulated by the relevant cellular pathways. Further, dominant-negative Src blocked PDGF-stimulated phosphorylation of DAPP-1, and PDGFβ receptors unable to bind and activate Src specifically failed to stimulate tyrosine phosphorylation of DAPP-1. In the context of the data describing Src-mediated phosphorylation of DAPP-1 in vitro and other work described above, these results strongly suggest Src is activated directly by the PDGFβ receptor and phosphorylates tyrosine 139 in DAPP-1. However, this is insufficient to deliver maximal phosphorylation of DAPP-1. PI3K activity is also required. Our data derived from in vitro studies of Src-mediated phosphorylation of DAPP-1 show that PtdIns(3,4,5)P3 stimulates phosphorylation by acting on the PH domain of DAPP-1. In the context of previous data establishing that DAPP-1 binds to similar PtdIns(3,4,5)P3-containing phospholipid monolayers with similar specificity and affinity (10),2 this is very likely a result of PtdIns(3,4,5)P3 driving the binding of DAPP-1 to the Src-containing phospholipid vesicles in our assays.

There is, however, an interesting difference between what we observe of Src phosphorylation of DAPP-1 in vitro and PDGF-stimulated phosphorylation in vivo. Phosphorylation is very substantially reduced in vivo with DAPP-1 (R61M) constructs (12), but in vitro this construct is phosphorylated like the wild-type protein. We originally speculated that this might be a result of an interaction between DAPP-1 and its upstream

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2 L. R. Stephens, K. E. Anderson, P. T. Hawkins, and K. Davidson, unpublished observations.
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kinase. Our in vitro data indicate this is not the case. However, an interaction with a third protein(s) that acts effectively as a scaffold co-localizing DAPP-1 via its SH2 domain and Src would reconcile these results. This possibility would also explain why Btk family kinases may not be as effective in vivo as their in vitro activities imply (see below) and also point to a deficiency in our in vitro assays that underlies the reason why the scale of activation of DAPP-1 phosphorylation we observe is well below what we detect in vivo. The suggestion that a scaffold protein may be involved in assembling Src and DAPP-1 is supported by the precedent set by the associations found in other, analogous, signaling complexes between Syk, BLNK, and phospholipase Cγ.

Previous workers studying Src family kinase-mediated phosphorylation of DAPP-1 in vitro failed to detect significant regulation by PtdIns(3,4,5)P3 (13). The lipid modification status of the tyrosine kinases was not defined, and hence their results may reflect the fact that the Src kinases were not co-localized with the PtdIns(3,4,5)P3 in the assays.

Although Btk appears to phosphorylate DAPP-1 relatively efficiently both in Sf9 cells and in vitro, we find no evidence that in either B cells or PAE cells Btk family kinases phosphorylate DAPP-1. Initially we supposed that this might be a result of the distinct subcellular localizations of Btk and Src family kinases; however, we found that fusing a Src-type membrane-targeting consensus to the NH2 terminus of Bmx did not significantly augment its ability to phosphorylate DAPP-1 when transiently expressed in PAE cells (data not shown). The most likely explanation for this is that other targeting mechanisms (e.g. based on tyrosine-phosphate-SH2 domain interactions) are important. Finding potential protein binding partners for DAPP-1 may better define the mechanism of activation of DAPP-1 by Src family kinases and, importantly, may also provide clues as to the physiological functions of this protein.

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