The Smaller Isoforms of Ankyrin 3 Bind to the p85 Subunit of Phosphatidylinositol 3′-Kinase and Enhance Platelet-derived Growth Factor Receptor Down-regulation*

Ashley Ignatiuk‡, Jeremy P. Quickfall‡, Andrea D. Hawrysh‡, M. Dean Chamberlain‡¶, and Deborah H. Anderson‡§

From the ‡Cancer Research Unit, Health Research Division, Saskatchewan Cancer Agency, Saskatoon, Saskatchewan S7N 4H4, Canada and the Departments of ¶Biochemistry and §Oncology, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E5, Canada

The Src homology 2 (SH2) domains of the p85 subunit of phosphatidylinositol 3′-kinase have been shown to bind to the tyrosine-phosphorylated platelet-derived growth factor receptor (PDGFR). Previously, we have demonstrated that p85 SH2 domains can also bind to the serine/threonine kinase A-Raf via a unique phosphorylation-independent interaction. In this report, we describe a new phosphotyrosine-independent p85 SH2-binding protein, ankyrin 3 (Ank3). In general, ankyrins serve a structural role by binding to both integral membrane proteins at the plasma membrane and spectrin/fodrin proteins of the cytoskeleton. However, smaller isoforms of Ank3 lack the membrane domain and are localized to late endosomes and lysosomes. We found that p85 binds directly to these smaller 120- and 105-kDa Ank3 isoforms. Both the spectrin domain and the regulatory domain of Ank3 are involved in binding to p85. At least two domains of p85 can bind to Ank3, and the interaction involving the p85 C-SH2 domain was found to be phosphotyrosine-independent. Overexpression of the 120- or 105-kDa Ank3 proteins resulted in significantly enhanced PDGFR degradation and a reduced ability to proliferate in response to PDGF. Ank3 overexpression also differentially regulated signaling pathways downstream from the PDGFR. Chloroquine, an inhibitor of lysosomal-mediated degradation pathways, blocked the ability of Ank3 to enhance PDGFR degradation. Immunofluorescence experiments demonstrated that both small Ank3 isoforms colocalized with the lysosomal-associated membrane protein and with p85 and the PDGFR. These results suggest that Ank3 plays an important role in lysosomal-mediated receptor down-regulation, likely through a p85-Ank3 interaction.

Cells transduce information across the plasma membrane, in part via the activation of transmembrane receptors such as the PDGFR. Activation of the receptor by PDGF binding induces autophosphorylation of the PDGFR on tyrosine residues and recruits a variety of cytoplasmic signaling proteins (reviewed in Refs. 1 and 2). Many of these signaling proteins contain SH2 or phosphotyrosine-binding domains that bind directly to specific phosphotyrosine residues on the receptor. These interactions allow the receptor to activate downstream signaling pathways such as the Ras-MAPK pathway and the phosphatidylinositol 3′-kinase (PI3K)-Akt pathway, important for processes that include cell proliferation and cell survival.

Down-regulation of these signal transduction pathways includes endocytosis of the activated receptor complex (3–5). In addition, signal transduction is thought to continue during this process and may even require intracellular localization for some signals to be transmitted (6–9). Receptor-mediated endocytosis involves multiple vesicle fusion events that effectively deliver the receptor signaling complex to the early endosome. This complex is then disassembled, and the receptor is either recycled back to the plasma membrane or sorted to the late endosome and lysosome for degradation (10). Receptors that fail to down-regulate remain constitutively active and have been shown to cause uncontrollable cell proliferation (11, 12). Defects in the endocytic pathway can result in cancer and other diseases as a result of these pathways remaining active for extended periods of time (13, 14).

The p85 subunit of PI3K is a PDGFR-binding protein that contains two SH2 domains for phosphotyrosine-dependent PDGFR binding, as well as a binding site for the 110-kDa catalytic subunit of PI3K, p110. The 85 protein remains associated with the PDGFR during endocytosis (15). Mutation of the PI3K-binding sites on the PDGFR disrupts PDGFR trafficking such that receptors are initially internalized but undergo recycling back to the cell membrane instead of targeting to the lysosome (16). Thus, it has been suggested that PI3K-binding sites on the PDGFR are required to divert the PDGFR to a degradative pathway. This effect is specific for PI3K-binding sites because mutant PDGFRs lacking binding sites for other signaling proteins (p120GAP, PLCγ1, and Syp) are endocytosed and degraded as wild type PDGFRs. The net effect is an increase in the number of tyrosine-phosphorylated PDGFRs at the membrane, which has been suggested to cause the sustained activation of downstream proliferative pathways (10). This has also led to the suggestion that class I PI3Ks (p85/p110) may function to regulate endocytosis and intracellular protein sorting.

We have previously carried out experiments using a phage display library to identify hexapeptide sequences capable of binding to the SH2 domains of the p85 subunit of PI3K (17). A search of the sequence data base for proteins containing these p85 SH2-binding peptides yielded several candidate p85 SH2-binding proteins including A-Raf. We subsequently demonstrated that the serine/threonine kinase A-Raf bound directly to each of the SH2 domains of p85 in a phosphorylation-independent manner (17, 18). There were four separate basic-X–basic sequences (where X indicates any amino acid) within A-Raf, each capa-
p85 of PI3K Binds Ank3 and Regulates PDGFR Degradation

Expression of Recombinant HA-Ank3—Various regions of three mouse Ank3 cDNAs (Ank3, SP7, and 1600; see Fig. 2A) were amplified by PCR using *Pfu* polymerase (Stratagene) or ELONGASE enzyme mix (Invitrogen) such that 5′-BglII and 3′-EcoRI sites were incorporated at the ends. The BglII/EcoRI-digested PCR products were subcloned into a similarly digested HA3 vector (17). Expression of proteins in mammalian cells incorporates three copies of a hemagglutinin (HA) tag (YPY-DVDPYA) at the N terminus of the Ank3 protein. The amino acids of mouse Ank3 present in each fusion protein are: BSpecReg (amino acids 778–1961; excluding insert A amino acids 834–854 and insert C amino acids 1588–1783), B (amino acids 778–975; excluding insert A), AB (amino acids 846–975), Spec (amino acids 874–1455), Reg+C (amino acids 1456–1961), Reg (amino acids 1456–1961; excluding insert C), NReg+C (amino acids 1456–1783), C+Reg (amino acids 1588–1961), C (amino acids 1588–1961), Δ12 (amino acids 790–1961, excluding inserts A and C), and Δ40 (amino acids 818–1961; excluding inserts A and C). To create the 105-kDa isoform of Ank3 (Ank105; amino acids 874–1961; excluding insert C) containing the unique six amino acids at the N terminus (MALPH5), the sequences encoding the spectrin and regulatory domains, lacking insert C were amplified by PCR from a BSpecReg template using an extra long 5′ primer encoding these amino acid residues. This insert was cloned into a mutant HA3 vector in which the unique HindIII site upstream of the multiple cloning site had been destroyed (17). To create the 120-kDa isoform of Ank3 (Ank120; amino acids 874–1961) containing insert C, the HA-Reg+C plasmid was digested with HindIII, which cuts twice within the regulatory domain, on either side of the region encoding insert C. The HA-Ank105 DNA was similarly digested with HindIII, and the small fragment within the regulatory domain lacking insert C was replaced by the corresponding HindIII fragment including insert C obtained from HA-Reg+C. The mutants were generated using the QuikChange mutagenesis method (Stratagene) according to the manufacturer’s directions. The DNA for each clone was verified by sequencing the entire coding region.

Cell Culture, Transfections, and Immunofluorescence—Expression of HA-tagged fusion proteins in COS-1 and NIH 3T3 cells was carried out as described previously (17). For PDGF stimulation time course experiments, the cells were stimulated with PDGF-BB (50 ng/ml) for the indicated times, washed, and lysed as described previously (23). Chloroquine, a tertiary amine that accumulates within and neutralizes the pH of acidic organelles, thereby inhibiting the lysosomal degradation pathway (24), was added for 2 h prior to stimulation with PDGF to a final concentration of 100 μM.

Cell proliferation was assayed using the Cell Titer 96 nonradioactive proliferation assay kit (Promega) according to the manufacturer’s instructions. The cells were serum-starved for 24 h in 0.5% fetal bovine serum-containing medium prior to plating 5000 cells/well for the assay. Different concentrations of PDGF, as indicated, were tested for 3 days. The results from three independent experiments, each with duplicate readings, were plotted using Prism software (version 4; GraphPad Software, Inc., San Diego, CA). The standard error of the mean is indicated with *error bars.*

For immunofluorescence experiments, the cells were seeded onto multi-chamber slides (VWR CA62405–176) and treated or not with PDGF as indicated. Each was fixed (4% paraformaldehyde, 30 min), permeabilized (0.05% saponin, 1% bovine serum albumin in PBS, 30 min), and incubated overnight with primary antibodies, followed by secondary antibodies (Molecular Probes: Alexa 488 green and Alexa 594 red). The coverslips were mounted (Prolong Gold antifade; Invitrogen catalog number P36930), and photos were taken using an Olympus F300

EXPERIMENTAL PROCEDURES

Pull-down Experiments, Immunoprecipitations, and Immunoblots—Pull-down experiments (22) and the glutathione S-transferase (GST)-p85 fusion proteins have been described previously (17, 18). Immunoprecipitations and immunoblotting have been described (23). Anti-Ank3 antibody was a gift from S. E. Lux (generated to amino acids 1550–1587 and 1784–1905 of the regulatory domain of Ank3 (no insert C); anti-Ank3R1 (20)). The anti-p85 antibody used for immunoprecipitation recognizes amino acids 314–724 and has been described previously (17), whereas the p85 antibody used for immunoblotting was obtained from Upstate Biotechnology (catalog number 05-217). Anti-HA (F7, sc-7392), anti-pDGFR (sc-432), anti-pPLCy1 (sc-12943, Y783), PLCy1 (sc-7290), and Shc (sc-1695) antibodies were obtained from Santa Cruz Biotechnology. Antibodies specific for pAkt (New England Biolabs catalog number 9271, S473), Akt (New England Biolabs catalog number 9272), pMAPK (Cell Signaling catalog number 9101, phospho-44/42 MAPK T202/Y204), MAPK (Transduction Laboratories M12320), and pShc (Upstate Biotechnology catalog number 07-206) were all used according to the supplier’s instructions. The results shown are typical for at least three independent experiments.
confocal microscope. Primary antibodies used for immunofluorescence were: HA (Roche Applied Science catalog number 186743, rat antibody, 1 μg/ml), lysosomal–associated membrane protein (Santa Cruz Biotechnology catalog number sc-5570, rabbit antibody, 4 μg/ml), EEA1 (Santa Cruz Biotechnology catalog number sc-6414, goat antibody, 2.5 μg/ml), p85 (affinity-purified rabbit polyclonal antibody generated toward amino acids 78–332 of bovine p85α, 0.5 μg/ml), and PDGFR (Transduction Laboratories catalog number 610113, mouse antibody, 1 μg/ml).

**ELISA-based Binding Assays**—The inserts encoding Ank105 and Ank120 were excised from the HA3 vector using BglII and EcoRI and subcloned into BamHI- and EcoRI-digested pGEX6P1 (Amersham Biosciences). Each of the GST-Ank105, GST-Ank120, and GST-p85 (full-length p85 (25)), GST-p110 (p85-binding domain of p110; amino acid residues 1–128 of mouse p110α) fusion proteins were induced, purified, and cleaved from GST using PreScission protease (Amersham Biosciences), following the manufacturer’s instructions. Any residual GST or uncleaved GST-p85 proteins were removed from the cleaved p85 samples by binding to glutathione-Sepharose beads. The final p85 preparation was determined to be free of GST contaminants using an immunoblot analysis with anti-GST antibodies (Santa Cruz Biotechnology, sc-138). Purified Ank105, Ank120, and p85 proteins that had been cleaved from GST were used in an ELISA-based binding assay as described (18), with the following changes. Ank105, Ank120, and control bovine serum albumin were bound to the wells, and increasing concentrations of p85 protein were added. Bound p85 protein was detected using an anti-p85 antibody as detailed previously (25). Unphosphorylated (DGGYMDMSKDESVDYVPML) and phosphorylated (DGpYMMDKDESVDpYVPML, where pY is phosphorylated tyrosine) peptides based on the p85 SH2-binding sites on the PDGFR were selected for the ELISA plates (Fig. 2A). The peptides (100 μM) and/or the p85-binding fragment of p110 (p110; 20 μM) were preincubated with the p85 proteins for 10 min at room temperature, prior to addition to the ELISA plate. The results from three independent experiments were averaged and plotted using Prism software. Standard error of the mean is indicated with error bars. Where no error bars are visible, the standard error is less than the size of the symbol on the graph.

**RESULTS**

**The p85 Protein Associates with the Smaller Isoforms of Ank3 in Cells**—Ank3 was identified as a potential p85-binding protein as a result of a sequence search with p85 SH2-binding hexapeptides (IARIRV, AIARIR, and LARIRS) (17). The largest isoforms of Ank3 contained four short sequence motifs similar to these hexapeptides, two in the membrane domain (KNIRIV and AIARRL), one in the spectrin domain (TKRIRV), and one in the regulatory domain (INQIRV; Fig. 1A). There are five main Ank3 isoforms of 215, 200, 170, 120, and 105 kDa. The domains present in the various Ank3 isoforms are known except for the 170-kDa isoform (Fig. 1A). To determine whether Ank3 formed a complex with p85 in cells, we used a coimmunoprecipitation and immunoblot blot analysis. Anti-p85 immunoprecipitates from lysates of untransfected PDGF-stimulated NIH 3T3 cells were resolved by SDS-PAGE and transferred onto nitrocellulose. The immunoblot was then probed with an anti-Ank3 antibody (Fig. 1B). As positive and negative controls, we carried out parallel immunoprecipitations with an anti-Ank3 antibody and a control IgG, respectively. The 170-, 120-, and 105-kDa isoforms of Ank3 were found associated with p85 but not the 215- and 200-kDa isoforms. This result suggested that p85 was associated in cells with the smaller isoforms of Ank3 that lack the N-terminal membrane domain.

**Mapping the Regions of Ank3 and p85 Involved in Binding**—To localize the region(s) of Ank3 and p85 responsible for mediating their interaction, a series of pull-down experiments were performed. Different regions of Ank3 were amplified by PCR from several Ank3 cDNA templates (Fig. 2A) (20) and expressed as HA-tagged Ank3 fusion proteins in COS-1 cells (Fig. 2, B and C). We noted that the small HA-Ank3 fusion proteins containing the acidic insert C (p1 = 4.0) (i.e. Reg+C, NReg+C, C+CReg, and C) migrated less than expected during the SDS-PAGE. This has been observed for other highly acidic proteins (26–29) and is likely a result of reduced binding of SDS to these very negatively charged proteins (29).

For the pull-down experiments, fragments of p85 were expressed in bacteria as GST-p85 fusion proteins and immobilized on glutathione-Sepharose beads. These immobilized GST-p85 fusion proteins were tested for their ability to bind to the HA-Ank3 fusion proteins in lysates of the COS-1 transfections (Fig. 2C). No binding of HA-Ank3 proteins was observed to the control GST protein or the p85 SH3-, BH-, or p110-binding domains. The largest HA-Ank3 protein tested, BSpecReg, bound to each of the p85 SH2 domains, as well as the full-length p85 protein. The HA-Ank3-BSpecReg protein also bound to a mutant p85C-SH2 protein (R649A), containing a point mutation that prevents phosphotyrosine-dependent binding (30). Thus, the interaction between the C-SH2 domain of p85 and Ank3 was phosphotyrosine-independent. Both the spectrin domain (HA-Ank3-Spec) and the regulatory domain with insert C (HA-Ank3-Reg+C) also bound well to the full-length p85 protein and showed a small amount of binding to the wild type and R649A mutant of the C-SH2 of p85 (Fig. 2C). The remaining HA-Ank3 fragments, including the regulatory domain without insert C (HA-Ank3-Reg), did not bind to p85. In some instances, fragments of HA-Ank3 bound better to the R649A mutant than the wild type p85 C-SH2 domain. Because the wild type domain can still bind
tyrosine-phosphorylated proteins within the Ank3-expressing COS-1 cell lysate, whereas the R649A mutant cannot, this may block some of the binding between the p85 C-SH2 and various HA-Ank3 proteins. These results show that there are at least two separate regions of Ank3 involved in p85 binding: the spectrin domain, and the regulatory domain with insert C. The observation that subsections of the Ank3 regulatory domain failed to bind p85 suggested that the correct folding of the Ank3 regulatory domain is likely an important factor in p85 binding. The only Ank3 fragment capable of binding to the N-SH2 domain of p85 was the HA-Ank3-BSpecReg. No subsections of Ank3-BSpecReg were able to bind to the p85 N-SH2 domain. This result also suggests that the folded structure of Ank3 may be important to correctly position residues involved in interacting with the p85 N-SH2 domain.

Next we generated HA-Ank3 fusion proteins that corresponded precisely to the 120–105 kDa (HA-Ank105) isomers including the unique six amino acids at the N terminus (Fig. 3A), as characterized previously (20). The HA-Ank120 and HA-Ank105 proteins were expressed in COS-1 cells and found to associate with p85 in cells (Fig. 3B). The HA-Ank120 and HA-Ank105 proteins also associated with full-length p85, the C-SH2 domain, and the R649A C-SH2 domain mutant of p85 in a GST-pull-down assay (Fig. 3C). In contrast, Ank120 and Ank105 did not associate with the N-SH2 domain of p85. This result was somewhat unexpected because Ank105 is very similar to the Ank3-BSpecReg, previously found to bind to the N-SH2 domain of p85 (Fig. 2C). In addition, Ank120 and Ank105 bound to the region of p85 that binds the p110 subunit of PI3K (Fig. 3C). Thus, it appears that the N-terminal region of Ank3-BSpecReg specifies binding to the p85 N-SH2 domain, whereas the distinct N-terminal region of Ank105 (and likely Ank120) allows binding to the p85 p110-binding domain.

We set out to identify the precise residues within Ank3-BSpecReg, but absent from Ank105 (and Ank120), that were required for p85 N-SH2 binding. The Ank3-BSpecReg protein contains an additional 75 amino acid sequence (Fig. 4A) including the sequence AIARRL, similar to one of the potential p85 SH2 binding hexapeptide sequences (AIARRR). We generated two N-terminal deletion mutants of Ank3-BSpecReg lacking 12 amino acid residues including the AIARRR sequence (∆12) or 40 residues (∆40). The ∆12 mutant retained binding to the p85 N-SH2 domain, whereas the ∆40 mutant did not (Fig. 4B). Thus, the AIARRR sequence of Ank3 was not required for binding. This result suggested that Ank3 amino acid residues 790–817 may contain a sequence responsible for binding to the p85 N-SH2 domain. Previous studies have shown that p85 SH2 domains can either bind to pYXXX(M/V) sequences in a phosphorylation-independent interaction (31, 32) or to basic-X-basic sequences (where basic = Arg or Lys) in a phosphorylation-dependent interaction (17, 18). Between Ank3 residues 790–817 there is one YXXV motif and one basic-X-basic motif (AIARRL) (Fig. 4A). Point mutations in these sequence motifs were generated by changing Tyr to Phe (∆12 Y791F) and changing both Lys814 and Lys816 to Ala (∆12 K814A,K816A). Mutation K814A,K816A but not Y791F was sufficient to specifically prevent Ank3 binding to the p85 N-SH2 domain (Fig. 4C). Thus, the p85 N-SH2 domain binding to Ank3 requires Lys814 and Lys816 within a basic-X-basic motif in a phosphorylation-dependent interaction.

Once it had been established that the HA-Ank3-Spec fusion protein bound to p85 and to the C-SH2 domain (Fig. 2C), it was possible to test whether the hexapeptide sequence within this domain (TKRIRV) contributed to this interaction. Mutation of these two arginine residues in the Ank3 spectrin domain to either alanine (TKAIAV) or glutamate (TKEIEV) reduced the binding to both the C-SH2 domain of p85 and the SH2 domain of p85 (Fig. 4D). These results indicated that Ank3 amino acid residues 790–817 are likely involved in binding to the p85 SH2 domains.
full-length p85 to a similar extent (Fig. 5A), suggesting that they are important in maintaining the p85-Ank3 interaction. Because these Ank3 mutants retained some ability to bind p85, additional unidentified Ank3 sequences must also contribute to p85 binding.

Similarly, the hexapeptide sequence within the regulatory domain of HA-Ank3-Reg+C (INQIRV) was mutated to (INAIAV) to determine the contribution of this motif to p85 binding (Fig. 5B). These mutations had no effect on the binding of the mutant Ank3 protein to the full-length GST-p85. The weak interaction between the C-SH2 domain of p85 and wild type, as well as mutant HA-Ank3-Reg+C (INAIYAV), was not always observed in these pull-down assays (Fig. 5B).

**Direct Binding Between Ank3 and p85 That Is Competed by Phosphopeptide**—To determine whether the interaction between the 120- and 105-kDa Ank3 isoforms and p85 were direct, each protein was expressed as a GST fusion protein, purified, and cleaved from the GST.

Liberated Ank3 proteins were bound to an ELISA plate and incubated with increasing concentrations of purified p85 in a protein binding assay (Fig. 6, A and B). The p85 protein bound directly to both Ank120 and Ank105 but not to the bovine serum albumin control protein.

We had previously found that both the p110-binding domain and the C-SH2 domain of p85 could mediate binding to Ank120 and Ank105 (Fig. 3C). Because p85 SH2 domains are also known to bind to activated PDGFRs but in a phosphotyrosine-dependent manner, we tested whether or not p85 could bind to both PDGFRs and Ank3 at the same time or whether they compete for binding to p85. For these experiments, a phosphopeptide based on the p85-binding sites on the PDGFR and the corresponding unphosphorylated peptide control were used (Fig. 6, A and B). The ability of p85 to bind to Ank120 and Ank105 was reduced, but not abolished, by the phosphopeptide but not by the unphosphorylated peptide. The remaining binding between p85 and Ank120 or Ank105 was completely blocked by the addition of a fragment of p110 corresponding to the p85-binding domain (Fig. 6, C and D). These binding experiments show that p85 and Ank120 (or Ank105) bind directly to each other and that two domains of p85 (C-SH2 and 110) mediate the interaction.

**Expression of HA-Ank120 and HA-Ank105 in Cells and Their Effects on PDGFR Down-regulation**—Our discovery that p85 binds selectively to the small 120- and 105-kDa Ank3 isoforms, previously shown to localize to late endosomes and lysosomes, coupled with the requirement for p85-binding sites on the PDGFR for receptor sorting to a degradative pathway, suggested the possibility that the p85-Ank3 interaction may play a role in targeting the PDGFR for degradation. It was not possible to generate subtle mutations in p85 or Ank120/Ank105 to disrupt this protein-protein interaction because multiple regions of both proteins appear to be involved in mediating the p85-Ank3 complex formation. Even after identifying some of these contact points, there are still additional regions of p85 and Ank120/Ank105 capable of mediating binding.

To address the function of Ank3 in PDGFR down-regulation, we generated two stable NIH 3T3 cell lines expressing HA-Ank120 or HA-Ank105 (Fig. 7). The cells were treated with PDGF for various times, and anti-HA immunoblots confirmed expression of HA-Ank120 or HA-

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**FIGURE 4.** Location of the hexapeptide sequence within Ank3 capable of binding to the p85 N-SH2 domain. A, sequence of the unique 75 amino acid residues present in HA-Ank3-BSpecReg that are absent from HA-Ank105. B, COS-1 lysates containing N-terminal deletion mutants of HA-Ank3-BSpecReg, lacking 12 residues (Δ12) or 40 residues (Δ40) were tested for their ability to bind to immobilized GST-p85 proteins containing the N-SH2 domain, the C-SH2 domain, full-length p85, or control GST protein in a pull-down analysis. C, point mutations of HA-Ank3-BSpecReg Δ12 were generated and expressed in COS-1 cells, where Tyr791 was mutated to Phe (Y791F), or both Lys814 and Lys816 were mutated to Ala (K814A, K816A). The cell lysates were used in pull-down experiments to test the ability of each Ank3 mutant to bind to the indicated GST-p85 fusion proteins.

**FIGURE 5.** Mutation of the hexapeptide sequence in the spectrin domain reduces Ank3 binding to p85. A, COS-1 lysates containing HA-Ank3-Spec wild type (TKRIRV), or mutants TKAIVV or TKEIEV were each tested for their ability to bind to p85. Pull-down experiments were carried out using the indicated GST and GST-p85 fusion proteins. B, pull-down experiments were carried out as in A, but using lysates from COS-1 cells containing HA-Ank3-Reg+C wild type (INQIRV) or mutant INAIYAV.
Ank105 in these cells (Fig. 7A, upper panels). As a loading control, these blots were also stripped and probed for the amount of p85 protein (Fig. 7A, lower panels). The amount of PDGFR protein was also analyzed using a Western blot analysis (Fig. 7B). Untransfected NIH 3T3 cells stimulated with PDGF for increasing times typically show a rapid reduction in PDGFR protein levels because of the degradation of receptor protein as part of the down-regulation mechanism. By 60–120 min the majority of the receptor has been degraded (Fig. 7C). In contrast, little or no differences were observed in the PDGF-dependent effect on major downstream receptor signaling pathways, the levels of activated Akt, MAPK, phospholipase Cγ1 (PLC), and Shc were determined as compared with the total amounts of these proteins (Fig. 7, C–F). The levels of activated PLCγ1 (Fig. 7E) and Shc (Fig. 7F) were reduced in Ank120- and Ank105-expressing cells as compared with NIH 3T3 cells and paralleled the PDGFR activation and degradation profiles (Fig. 7B). In contrast, little or no differences were observed in the PDGF-dependent activation of MAPK when phospho-MAPK levels were compared between Ank120- and Ank105-expressing cells and the parental NIH 3T3 cells (Fig. 7D). Cells expressing Ank120 or Ank105 actually showed a more sustained activation of Akt during the PDGF stimulation time course when compared with the parental NIH 3T3 cells (Fig. 7C). Therefore, downstream PDGFR signaling was differentially affected in the cells expressing Ank120 or Ank105; two pathways showed a rapid reduction in signaling (PLCγ1, Shc): one appeared unaffected (MAPK), and one showed enhanced activation (Akt).
Coimmunofluorescence experiments confirmed that HA-Ank120 and HA-Ank105 are colocalized with the lysosomal-associated membrane protein (LAMP) at late endosomes/lysosomes and increase the number of PDGFR/p85-positive vesicles. The cells were treated with PDGF for 5 min (B) or for the indicated times (C) or left untreated (A), fixed, permeabilized, and probed with the indicated primary antibodies as detailed under “Experimental Procedures.” Secondary antibodies conjugated to Alexa 488 (green) or Alexa 594 (red) were used to visualize the localization of indicated pairs of proteins. Colocalization is observed in the merged image as yellow (Both). The cells were visualized using confocal microscopy. Scale bar, 10 μm.

FIGURE 8. HA-Ank120 and HA-Ank105 colocalize with lysosomal-associated membrane protein (LAMP) at late endosomes/lysosomes and increase the number of PDGFR/p85-positive vesicles. The samples were prepared and immunofluorescence was carried out as for Fig. 8.

FIGURE 9. HA-Ank120 and HA-Ank105 colocalize with p85 (A) and PDGFR (B). The cells were visualized using confocal microscopy. Scale bar, 10 μm.

FIGURE 10. The reduced levels of PDGFR in Ank120- and Ank105-expressing cells makes them less responsive to the mitogenic effects of PDGF, and this enhanced PDGFR degradation can be blocked by inhibiting the lysosomal degradation pathway. A, the indicated cells were serum-starved for 24 h and seeded into a 96-well plate (5000 cells/well). Different concentrations of PDGF were added to duplicate wells in serial dilutions. Cell proliferation was assayed after 3 days, using the Cell Titer 96 kit as described under “Experimental Procedures.” B, cells were stimulated over the indicated times with PDGF in the presence of chloroquine (100 μM), an inhibitor of lysosomal-mediated degradation. The cell lysates (20 μg of protein/lane) were probed in an immunoblot analysis with the indicated antibodies.

Previous work has established that activated PDGFRs are degraded via a lysosomal-mediated pathway (3, 4, 24). To determine whether Ank120/Ank105 overexpression enhances activated PDGFR degradation via this lysosomal pathway, we repeated our time course of PDGF stimulation in the presence of a lysosomotropic agent, chloroquine, to inhibit lysosomal-mediated degradation (Fig. 10B). Chloroquine treat-
ment blocked the degradation of activated PDGFRs in all three cell lines, reversing the effects of Ank120/Ank105 overexpression. This suggests that Ank120/Ank105 enhances PDGFR degradation by acting on the lysosomal degradation pathway.

**DISCUSSION**

PI3K has been implicated to play multiple roles in the endocytosis and degradation of activated receptors like the PDGFR, in addition to its many important contributions in signal transduction pathways. It is now clear that phosphatidylinositol 3'-phosphate, the lipid product of class III PI3Ks p150/hVPS34, plays a important role in protein trafficking because it has a critical function in vesicle fusion events during endocytosis (33, 34). Phosphatidylinositol 3'-phosphate acts together with the active GTP-bound form of Rab5, a small monomeric GTPase, to recruit effector proteins to early endosomes. One such effector protein is the early endosomal autoantigen (EEA1), a core component required for early endosomal fusion events. Rab5 regulates fusion events such as the fusion of vesicles (containing activated receptors undergoing endocytosis) with the early/sorting endosomes (reviewed in Refs. 35 and 36).

Our laboratory has been investigating possible roles for the p85 protein during PDGFR down-regulation. Whereas Rab5 regulates vesicle fusion events during receptor-mediated endocytosis that deliver receptor signaling complexes to the early/sorting endosome, a related protein Rab4 regulates the recycling of receptors from the early endosome back to the plasma membrane. We have recently demonstrated that the BH domain of p85 has GTPase activating protein activity toward both Rab5 and Rab4 capable of stimulating their intrinsic GTPase activities ~1000-fold (25). By stimulating Rab5 and Rab4 hydrolysis of bound GTP to GDP, p85 can regulate how long each Rab protein is in its active GTP-bound state. We found that overexpression of p85 BH domain mutants reduced the rate of PDGFR degradation over a time course of PDGFR stimulation (25). Such mutants would allow Rab5-GTP and Rab4-GTP to remain in their active GTP-bound forms longer, facilitating rapid membrane fusion events that promote both endocytosis and recycling. We believe the reduction in PDGFR degradation observed in cells expressing p85 BH domain mutants is a result of a reduction in the time the PDGFR spends in the early/sorting endosome, where sorting events that target it for degradation in the lysosome are thought to take place (37, 38).

In this report, we have identified the smaller isoforms of Ank3 as new p85-binding proteins. The spectrin and regulatory domains of Ank3 contributed to p85 binding, and the interaction was partially dependent upon a basic-X-basic sequence within the spectrin domain of Ank3. The C-SH2 domain and the p110-binding domain of p85 were found to bind the 120- and 105-kDa Ank3 isoforms, via a phosphotyrosine-independent interaction. Binding was partially competed by a PDGFR-based phosphopeptide, suggesting that p85 forms separate complexes with activated tyrosine-phosphorylated receptors and Ank120/Ank105.

The fact that these 120- and 105-kDa Ank3 isoforms associate with p85 in cells and also are specifically localized to late endosomes and lysosomes (Ref. 21 and Fig. 8) prompted us to test whether these proteins play a role in the down-regulation of the PDGFR. Consistent with this hypothesis, expression of Ank120 or Ank105 resulted in reduced basal levels of the PDGFR and the extremely rapid degradation of the PDGFR (<10 min) upon PDGF stimulation, as compared with untransfected NIH 3T3 cells (>60 min). We also observed a corresponding decrease in the ability of Ank120- and Ank105-expressing cells to proliferate in response to PDGF. Moreover, inhibition of the lysosomal-mediated degradation pathway prevented the degradation of activated PDGFRs in Ank120- and Ank105-expressing cells, as well as the control NIH 3T3 cells. We were not able to test whether the p85-Ank3 interaction mediated targeting of the activated PDGFR to a degradative pathway. However, our immunofluorescence results show colocalization of Ank120 and Ank105 with both p85 and the PDGFR at intracellular locations that correspond to late endosomes/lysosomes. Together these results strongly indicate that Ank120 and Ank105 play an important role in lysosomal-mediated degradation of the activated PDGFR. It has been suggested that receptor recycling is the default process and that sorting to the late endosome and lysosome requires molecular targeting (39). Thus, we suggest that the levels of Ank120 and Ank105 in normal cells may be limiting and that overexpression of Ank120 and Ank105 could provide more targeting/sorting opportunities for activated receptors to be diverted to a degradative pathway. Even prior to PDGF stimulation, the levels of PDGFR in Ank3-expressing cells are reduced. This is consistent with Ank3 expression enhancing the degradative sorting of PDGFRs internalized during constitutive endocytosis, a well known process that cells use to sample their environment.

Ank120 and Ank105 overexpression in cells differentially affected signal transduction pathways downstream from the activated PDGFR. The PLC-γ1 and Shc proteins showed reduced activation in response to PDGF, and their activation profiles mimicked that of the activated PDGFR itself. PDGFR-mediated activation of the MAPK pathway was not significantly altered, whereas activation of the Akt pathway was actually prolonged in cells expressing Ank120 or Ank105. These results suggest that the MAPK and Akt proteins do not require sustained levels of activated PDGFRs to remain active, unlike the PLC-γ1 and Shc proteins. On the contrary, pAkt levels remained higher longer in the Ank120- or Ank105-expressing cells, suggesting that rapid degradation of the PDGFR may actually prevent this pathway from down-regulating as quickly as in the normal NIH 3T3 cells.

Because p85 is known to have an important role in the activation of the Akt pathway via its effects on PI3K activity (p85/p110), Ank120/Ank105 overexpression may have additional effects on this pathway through p85-Ank3 complex formation.

We hypothesize that PDGFR-associated p85 may assist in targeting vesicles containing activated receptors undergoing endocytosis to a degradative pathway by binding to Ank3 isoforms localized on late endosomes. We propose the following sequence of events during the endocytosis of the activated PDGFR. The p85 protein remains bound to the PDGFR in a p85 SH2 domain phosphotyrosine-dependent interaction during endocytosis of the activated receptor (15). In the early endosome, p85 associates with Rab5-GDP and/or Rab5-GTP, likely via multiple domains including its BH domain (25). The p85 protein may release the PDGFR, an interaction that represses the Rab5-GTPase-activating protein activity of p85.4 This would free up the SH2 domains of p85 to bind the smaller isoforms (120 and 105 kDa) of Ank3 on late endosomes and lysosomes (21) in a phosphorylation-independent interaction that could help to target PDGFR-containing membrane sub-domains to a degradative pathway.

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