Biochemical and Biophysical Research Communications **273,** 294–301 (2000)
doi:10.1006/bbrc.2000.2887, available online at http://www.idealibrary.com on IDEAL®

# Characterization of the Tyrosine Kinase Tnk1 and Its Binding with Phospholipase C-γ1

Donna M. Felschow, Curt I. Civin, and Gerard T. Hoehn[1]
*Johns Hopkins Oncology Center, Bunting–Blaustein Cancer Research Building, 1650 Orleans Street, Baltimore, Maryland 21231*

Received May 14, 2000

**Tnk1 is a nonreceptor tyrosine kinase cloned from CD34+/Lin−/CD38− hematopoietic stem/progenitor cells. The cDNA predicts a 72-kDa protein containing an $NH_2$-terminal kinase, a Src Homology 3 (SH3) domain, and a proline-rich (PR) tail. We generated rabbit antiserum to a GST–Tnk1(SH3) fusion protein. Affinity-purified anti-Tnk1 antibodies specifically recognized a 72-kDa protein in Tnk1-transfected COS-1 cells and cells which express Tnk1 mRNA. Western blot analysis indicated that Tnk1 is expressed in fetal blood cells, but not in any other hematopoietic tissues examined. Tnk1 immunoprecipitated from cell lysates possessed kinase activity and was tyrosine phosphorylated. In binding experiments with a panel of GST-fusion constructs, only GST–PLC-γ1(SH3) interacted with *in vitro* translated Tnk1. GST–protein precipitations from cell lysates confirmed that GST–PLC-γ1(SH3) associated with endogenously expressed Tnk1. Conversely, GST–Tnk1(PR) protein constructs complexed with endogenously expressed PLC-γ1. The association of Tnk1 with PLC-γ1 suggests a role for Tnk1 in phospholipid signal transduction.** © 2000 Academic Press

***Key Words:*** **Tnk1; tyrosine kinase; Ack; SH3 domain; PLC-γ1.**

Through modular domains such as Src homology 2 and Src homology 3 (SH2 and SH3) domains and proline-rich (PR) motifs, nonreceptor tyrosine kinases bind to specific target proteins and convey signals which lead to a plethora of cellular functions such as proliferation, secretion, cytoskeletal assembly, adhesion, or differentiation (1–3). Signaling cascades orchestrated by tyrosine kinases are essential to regulate hematopoiesis (4). We used degenerate PCR to clone a novel nonreceptor tyrosine kinase gene, Tnk1, from a population of human umbilical cord blood enriched $CD34^+/Lin^-/CD38^-$ hematopoietic stem/progenitor cells (5). Tnk1 cDNA predicts a 72-kDa polypeptide, containing an $NH_2$-terminal kinase region followed by an SH3 domain and a PR motif near the COOH-terminus. mRNA expression patterns suggested that Tnk1 is involved in signaling pathways during fetal development and in select adult tissues (5).

Tnk1 is most homologous to the Ack family of nonreceptor tyrosine kinases, displaying approximately 60% similarity throughout the length of the protein (5–7). Tnk1 and Ack are also related to FAK and Pyk2, nonreceptor tyrosine kinases involved in signal transduction pathways initiated by cell adhesion to the extracellular matrix (8–11). Ack tyrosine kinases associate with the GTP-bound form of Cdc42, a Rho family GTP-binding protein (6, 7). Binding of Ack to Cdc42 inhibits the intrinsic GTPase activity of Cdc42, and increases tyrosine phosphorylation of Ack (6). Ack1 has also been shown to phosphorylate the Rho-family nucleotide exchange factor, Dbl, following cotransfection of Ack1 and wild-type Cdc42 (12). Although the physiological role of Ack is unknown, recent evidence suggests that Ack is involved in signal transduction pathways initiated by growth factor stimulation, β1-integrin mediated cell adhesion, and has been implicated in cell spreading by metastatic melanoma cells (7, 13–15). Stimulation of Ack1-transfected COS-1 cells with EGF enhanced the interaction of Ack1 with Grb2, and SH3 containing adaptor protein important in Ras signaling pathways (12). This association was shown to be mediated by an Grb2 SH3 domain and a proline-rich region of Ack.

Binding of SH3 domains to PR motifs is highly selective and is contingent upon the amino acid sequence

Abbreviations used: SH2, Src homology region 2; SH3, Src homology region 3; PR, proline-rich region; GST, glutathione *S*-Transferase; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; PLC-γ1, phospholipase C-γ1; HRP, horseradish peroxidase; IPTG, isopropylthio-β-D-galactoside; PBS, phosphate-buffered saline; EDTA, ethylenedinitrilotetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline; TBST, TBS with Tween 20; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MNC, mononuclear cells; PB, peripheral blood.

[1] To whom correspondence should be addressed. Fax: (410) 955-8897. E-mail: gthoehn@jhmi.edu.

0006-291X/00 $35.00

Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

surrounding the core PxxP motif (16–20). PR regions which contain the motif PxxPxK/R (where P is a conserved proline, x is any amino acid, and K/R is a conserved lysine or arginine residue), are termed class II SH3-binding consensus sites (18, 20). Several known signaling proteins, including Grb2, CrkII, Nck, src family kinases, and PLC-γ1 interact via their SH3 domains to proteins with this consensus sequence (18, 20, 21). Within its PR region, Tnk1 contains the sequence PPGLPPRP, which meets the criteria of a class II SH3-binding consensus sequence.

To begin to characterize Tnk1, we generated rabbit polyclonal antibodies directed against a GST–Tnk1(SH3) fusion protein. In this paper, we utilized this affinity-purified anti-Tnk1 antibody preparation to evaluate Tnk1 protein expression in various cell lines and human blood cell populations, to determine its subcellular localization and to demonstrate its catalytic activity. Furthermore, using GST-constructs to several different SH3 containing signaling molecules, we show that the SH3 domain of PLC-γ1 specifically associates with the PR region of Tnk1.

## MATERIALS AND METHODS

*Cell lines.* Dr. J. Hilton graciously provided the Igrove ovarian carcinoma cell line, and Dr. R. Casero the LNCaP, DU145, and PC3 prostate carcinoma lines (Johns Hopkins Oncology Center, MD). All other cell lines were acquired from the American Type Culture Collection (ATCC, Arlington, VA).

*Commercial antibodies.* PY20 monoclonal anti-phosphotyrosine antibody was purchased from Transduction Laboratories (Lexington, KY), anti-PLC-γ1 antibody cocktail from Upstate Biotechnology (Lake Placid, NY), rabbit anti-mouse IgG from Sigma (St. Louis, MO), horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG from Amersham Life Sciences (Arlington Heights, IL).

*Preparation of antibodies against Tnk1.* Hyperimmune rabbit antiserum was generated against the above bacterially expressed GST–Tnk1(SH3) by HRP, Inc. (Denver, PA). Bacterially expressed GST or GST–Tnk1(SH3) fusion protein was covalently coupled to activated CH Sepharose (Pharmacia Biotech, Piscattaway, NJ), at 1 mg protein/ml Sepharose resin following manufacturer's instructions. Rabbit antiserum was first depleted of antibodies against GST by four passages over a GST–Sepharose column. The GST-absorbed antiserum was affinity purified on a GST–Tnk1(SH3)–Sepharose column, then anti-Tnk1 antibodies eluted with 0.2 M glycine, pH 2.8, and neutralized with 2 M Tris–HCl, pH 8.0 (22). The eluted, affinity-purified, anti-Tnk1 antibody preparation was dialyzed against 1 × PBS pH 7.4 containing 0.02% $NaN_3$ overnight at 4°C, and concentrated using a Centriprep device (Amicon, Beverly, MA).

*Cell extracts.* Near-confluent adherent cells were lysed directly on tissue culture plates by addition of Hepes lysis buffer [1% NP-40, 25 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 100 mM NaF, 10% glycerol, 2 mM $Na_2VO_3$, 50 μg/ml aprotinin, 5 μg/ml antipain, 2 μg/ml leupeptin, and 1 mM PMSF (Sigma)]. Suspension cells were pelleted by centrifugation (300*g* for 5 min), washed twice in ice-cold PBS, and resuspended in 1 ml of Hepes lysis buffer. Lysates were chilled on ice for 10 min., and cleared by centrifugation (16,000*g*, 20 min, 4°C). Total protein from the recovered supernatant was measured by BCA assay (Pierce, Rockford, IL). Mononuclear cells (MNC) from human hematopoietic tissues were purified by Ficoll–Hypaque density centrifugation (5), washed 2× in RPMI medium, and then lysed directly in 1 ml of Hepes lysis buffer. Frozen tissues from an aborted fetus (8th week), were thawed, disaggregated by dounce homogenization, and lysed in 1 ml of Hepes lysis buffer.

*Immunoprecipitation.* Whole-cell extracts (1 mg/ml) were precleared by incubation with rabbit anti-mouse IgG (5 μg IgG per milliliter of cell extract) for 4 h at 4°C, precipitated with 100 μl of protein A–Sepharose beads (Pharmacia) for 1 h, and then centrifuged (16,000*g*, 4 min, 4°C). Antibody (1 μg) was added to the recovered precleared supernatant. After incubation overnight at 4°C, protein A–Sepharose beads (100 μl) were added for 1 h at 4°C. Beads were washed twice with TBS-T and twice with TBS, resuspended in 2× Laemmli sample buffer (50 μl), boiled, separated by SDS–PAGE, then transferred to Immobilon-P (Pharmacia).

*Kinase assay.* Immunoprecipitated complexes were washed twice with TBS-T and twice with kinase buffer (10 mM Hepes pH 7.4, containing 150 mM NaCl, 0.5 M $MnCl_2$, 0.5 M $MgCl_2$, 1 mM dithiothreitol, 0.1% NP-40, 50 μg/ml aprotinin and 2 μg/ml leupeptin). Fresh kinase buffer (50 μl) was added to each pellet, and the samples were incubated with 10 μCi [$\gamma$-$^{32}$P]ATP (Amersham) at 30°C for 30 min. The reaction was stopped by the addition of 2× Laemmli sample buffer and separated by SDS–PAGE.

*Subcellular fractionation.* Subcellular fractionation of cell extracts was performed by the protocol of Thom *et al.* (23).

*GST fusion proteins.* GST-fusion proteins of Tnk1, CrkII, and Nck were generated by cloning RT–PCR products from Igrove ovarian carcinoma cDNA in-frame into the *Bam*HI and *Eco*RI cloning sites of the pGEX-3X vector (Pharmacia, Piscattaway, NJ). The primers and the conditions for PCR were as follows: Tnk1 (SH3): forward 5′-GAGCGGATCCGTTGTGTGGAGGGATGCCACAGAAC-3′, reverse 5′-GAGCGAATTCACAGAACTGACTCCAGACTCCTGG-3′; Tnk1 (PR): forward 5′-GCGCAGGATCCTGAAAGGCATTTCCAGGAGTCTG-3′, reverse 5′-GCGCAGAATTCTCCTCTGCAACTCAGGATCGG-3′; CrkII: forward 5′-GCGCAGGATCCACTTCGACTCGGAGGAGCGG-3′, reverse 5′-GCGCAGAATTCGTTCCCATCTGTCAGCAAAACTG-3′; and Nck: forward 5′-GCGCAGGATCCAACAAGAGTTGGACATCAAGAAGAATG-3′, reverse 5′-GCGCAGAATTCCACGCTGCCCAATGCAGTAG-3′.

Parameters for PCR of Tnk1(SH3) and Tnk1(PR) were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, 35 cycles, followed by 1 cycle for 10 min at 72°C. For CrkII and Nck amplification, conditions were 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min, 35 cycles, followed by 1 cycle for 10 min at 72°C. PCR products were purified, digested and ligated to *Bam*HI/*Eco*RI digested pGEX-3X vector, and transformed into DH5α *Escherichia coli.* All constructs were sequence verified. Fusion proteins were purified after IPTG (0.1 mM) induction following the method of Smith and Johnson (24). The GST-fusion proteins, GST–Grb2, GST–PLC-γ1(SH3), and GST–PLC-γ1(SH2), were purchased from Upstate Biotechnology (Lake Placid, NY).

*GST precipitation.* Whole-cell lysates (1 mg/ml) were precleared by incubation with 5 μg GST (4 h, 4°C, rotating) and precipitated with 50 μl glutathione–Sepharose 4B beads (Pharmacia) for 1 h at 4°C. GST-fusion proteins (1–2 μg) were added to the recovered supernatant and incubated overnight at 4°C. Glutathione–Sepharose 4B beads (50 μl) were added to the protein complexes for 1 h at 4°C, then washed twice with TBS-T and twice with TBS. Laemmli sample buffer (2×) was added and SDS–PAGE/Western blot analysis performed.

*In vitro translation of Tnk1.* Expression constructs [pCI-Neo, pCI-Tnk1, or pCI-Tnk1(PR)] were *in vitro* translated using the TnT coupled reticulocyte system (Promega, Madison, WI) following manufacturer's instructions.

*Transfection of COS-1 cells.* COS-1 cells were transfected using Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) and 2 μg of plasmid DNA following the recommendations of the manufacturer.

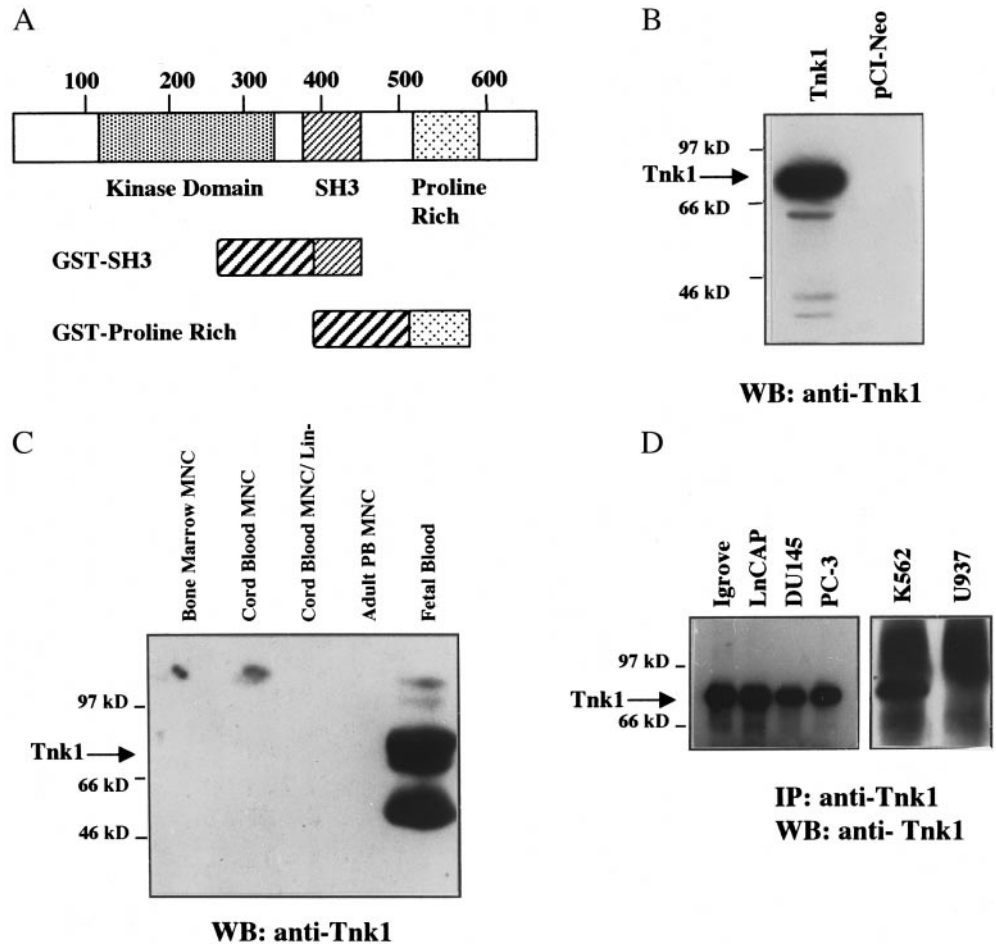


**FIG. 1.** Tnk1 protein expression. (A) A protein map of Tnk1 shows positions of the kinase domain (aa 120–331), the SH3 domain and the PR region. GST-fusion proteins of partial Tnk1 sequences spanning the SH3 domain (aa 387–503) and the PR (aa 493–589) region are indicated. (B) Western blot analysis using anti-Tnk1 antibodies of COS-1 cells transfected with either full-length Tnk1 or vector (pCI-Neo) alone. (C) Lysates (50 $\mu$g) from the indicated sources of human hematopoietic tissue were examined for Tnk1 expression by Western blot analysis. MNC is mononuclear cells; PB is peripheral blood. (D) Immunoprecipitation of Tnk1 from human cell lines. Affinity purified anti-Tnk1 antibodies were used to immunoprecipitate Tnk1 from the indicated human solid tumor (Igrove, LNCaP, DU145, and PC 3) or leukemia (K562 and U937) cell lines. Immunoprecipitates were assayed by Western blot analysis using anti-Tnk1 antibodies.

Forty-eight hours following transfection, cells were trypsinized and plated in media containing G418 (1 mg/ml) (Life Technologies, Inc.). G418 resistant colonies were picked and assayed for Tnk1 expression by Western blot analysis.

## RESULTS

*Anti-Tnk1 antibodies recognize Tnk1 protein.* We generated rabbit antiserum to a bacterial expressed GST–Tnk1(SH3) fusion protein which included amino acids 387–503 of the predicted Tnk1 protein (Fig. 1A). The GST-adsorbed, affinity-purified rabbit anti-Tnk1 antibody preparation detected GST–Tnk1(SH3), but did not detect GST or GST–Tnk1(PR) (amino acids 493–589 of the predicted Tnk1 protein; data not shown). This affinity-purified anti-Tnk1 antibody preparation immunoblotted a 72-kDa protein band (the predicted molecular mass of the Tnk1 protein) from COS-1 cells transfected with Tnk1 cDNA, but not from COS-1 cells transfected with vector alone (Fig. 1B), confirming the specificity of this anti-Tnk1 antibody.

*Expression of Tnk1 protein in human hematopoietic tissues.* Since Tnk1 was originally cloned from CD34+/Lin−/CD38− umbilical cord blood cells, we examined several human sources of blood cells for Tnk1 protein expression by Western blot analysis. Tnk1 expression was detected only in fetal blood cell samples (Fig. 1C). Cross-reactive bands (60 and 80 kDa) are also observed. No Tnk1 protein expression was detected in mononuclear cells from lineage antigen-depleted umbilical cord blood cells, adult bone marrow and peripheral blood, nor in neonatal umbilical cord blood cells.

*Expression of Tnk1 protein in human cell lines.* Extracts from leukemia cell lines K562 and U937, as well as from several different solid tumor lines including,

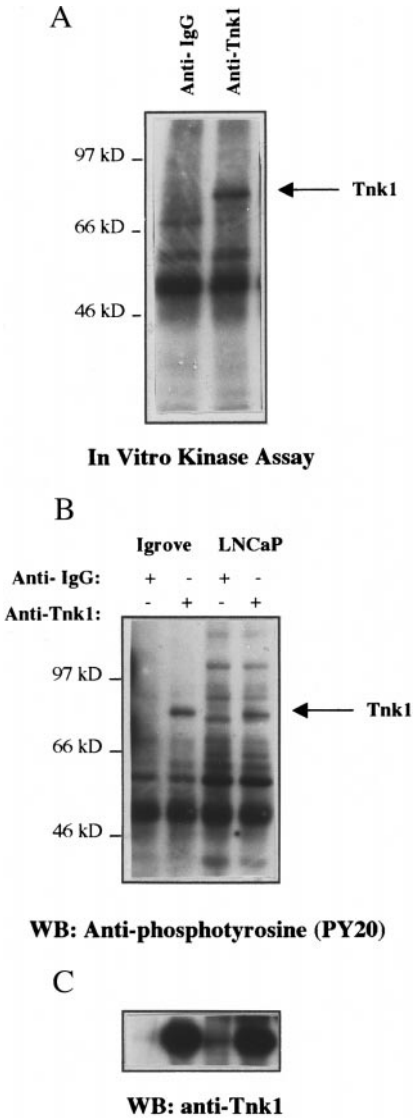


**FIG. 2.** Tnk1 has kinase activity and is tyrosine phosphorylated under basal conditions. (A) Tnk1 has autokinase activity. Immunoprecipitated Tnk1 or rabbit anti-mouse IgG–protein complexes from Igrove cell lysates were assayed for *in vitro* autokinase activity and examined by SDS–PAGE. (B) Tnk1 is tyrosine phosphorylated. Western Blot analysis with PY20 anti-phosphotyrosine monoclonal antibody of Igrove or LNCaP cells immunoprecipitated with either anti-Tnk1 antibodies or rabbit anti-mouse IgG. (C) To verify that the 72-kDa phosphotyrosine protein was Tnk1, the blot from B was stripped with 0.2 M glycine, pH 2.8, and reprobed with anti-Tnk1 antibody.

DU145, LNCaP, and PC3 prostate carcinoma cells, and Igrove ovarian carcinoma cells, were examined for Tnk1 protein expression (Fig. 1D). Western blot analyses of Tnk1 immunoprecipitates revealed Tnk1 expression in K562, Igrove, LNCaP, DU145, and PC3 cells, but not in U937 cells.

*Tnk1 has kinase activity and is tyrosine phosphorylated under basal conditions.* *In vitro* kinase assays were performed using extracts obtained from untreated Igrove ovarian carcinoma cells. Extracts immunoprecipitated with anti-Tnk1 antibody displayed autophosphokinase activity (Fig. 2A), while no activity was observed with control rabbit IgG antibody. We next examined the tyrosine phosphorylation status of Tnk1 from Igrove or LNCaP cell lysates under basal conditions. Without prior stimulation, tyrosine phosphorylation of Tnk1 protein was detected by anti-phosphotyrosine antibody PY20. No other specific phosphorylated proteins coimmunoprecipitated with anti-Tnk1 antibodies (Fig. 2B). We confirmed that this band was Tnk1 by stripping the blot in Fig. 2B and reprobing with anti-Tnk1 antibody (Fig. 2C).

*Tnk1 localizes to the cell membrane.* Whole-cell lysates from Igrove cells were fractionated by sucrose gradient centrifugation, and fractions containing equivalent amounts of protein were probed for Tnk1 protein by immunoblotting. Although Tnk1 can be detected in the cytosol, there is an enrichment of Tnk1 in the membrane fraction (Fig. 3).

*Tnk1 associates with PLC-γ1 in vitro.* We investigated whether the PR motif within the COOH-terminus of Tnk1 is involved in binding of SH3-containing signal transduction proteins. The PR region of Tnk1 contains the sequence, pPGLPPRPp, which conforms to the class II SH3-binding consensus sequence (18, 20). GST fusion proteins were tested for their ability to precipitate *in vitro* translated full-length Tnk1 protein or a construct containing the Tnk1-PR region. As shown in Fig. 4A, GST–PLC-γ1(SH3) fusion protein precipitated full-length Tnk1

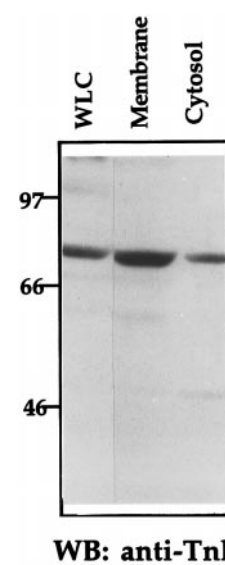


**FIG. 3.** Tnk1 is enriched in membrane fractions. Lysates from Igrove cells were subfractionated on a 38% sucrose cushion and equal amounts of protein from the indicated fractions were electrophoresed on an SDS–PAGE gel, transferred and immunoblotted with anti-Tnk1 antibodies. WLC is whole lysed cell extract.

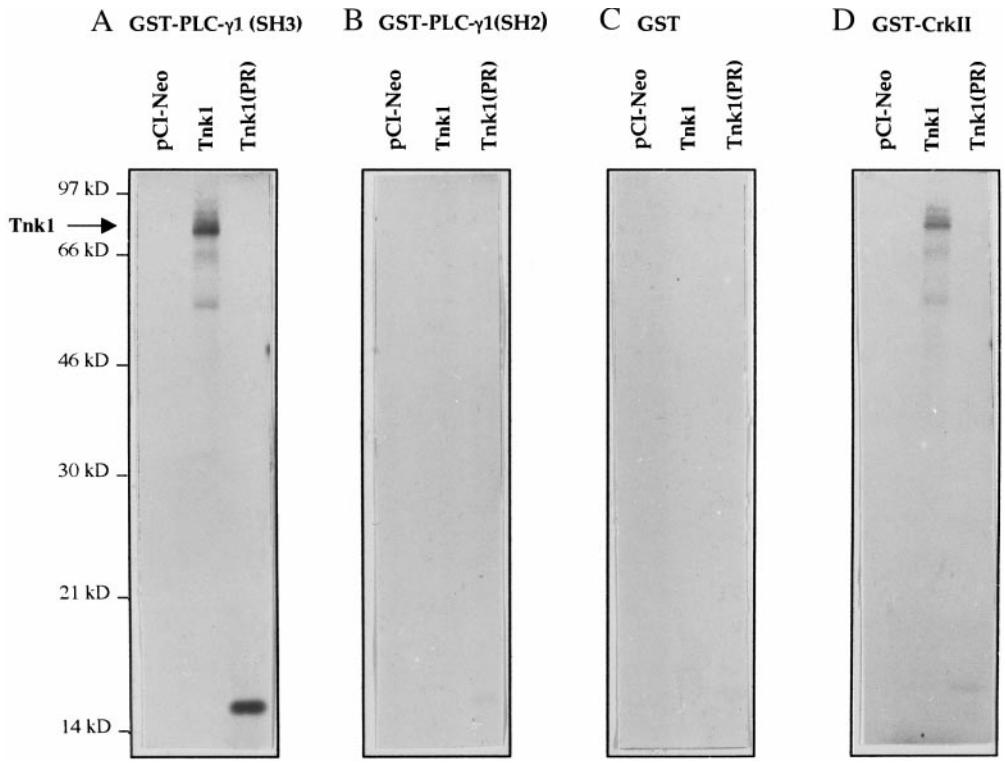


**FIG. 4.** *In vitro* translated Tnk1 associates with GST–PLC-γ1(SH3). Full-length pCI-Tnk1, pCI-Tnk1(PR), or pCI-neo were *in vitro* translated in the presence of [$^{35}$S]methionine. Equal volume aliquots were incubated with the various GST proteins, then precipitated with glutathione beads. Precipitating proteins were electrophoresed on an SDS–PAGE gel, transferred to Immobilon-P and autoradiographed. (A) PLC-γ1(SH3), (B) PLC-γ1(SH2) (C) GST, or (D) GST–CrkII. GST–PLC-γ1(SH3) precipitates full-length Tnk1 and Tnk(PR), whereas GST–PLC-γ1(SH2) and the GST control do not.

and Tnk1-PR. However, neither a GST–PLC-γ1(SH2) fusion protein nor GST protein alone interacted with full-length Tnk1 or Tnk1-PR (Figs. 4B and 4C). GST–CrkII bound only very weakly to full-length Tnk1 and Tnk1-PR (Fig. 4D).

To confirm the interaction of PLC-γ1 with Tnk1, GST-fusion proteins of PLC-γ1, CrkII, Grb2, and Nck were used to precipitate endogenously expressed Tnk1 from Igrove cell lysates. Only the PLC-γ1(SH3) GST-fusion protein precipitated endogenously expressed Tnk1 (Fig. 5A). All other fusion proteins, including CrkII, were unable to precipitate Tnk1 from whole cell lysates. In the complementary experiment, a GST–Tnk1(PR) fusion protein, which spanned the PR region of Tnk1 (aa 493–589; Fig. 1A), was used to precipitate endogenously expressed PLC-γ1 from Igrove cell lysates. GST–Tnk1(PR) specifically precipitated PLC-γ1 (Fig. 5B), whereas GST, GST–CrkII, and GST–Nck did not. GST–Grb2 also precipitated PLC-γ1, as reported previously (25). Similar results were obtained with lysates from LNCaP cells (data not shown).

## DISCUSSION

We characterized the expression of Tnk1 in cell lines and tissues, determined its subcellular localization and assessed its association with several known signaling proteins. A major tool in this study was the rabbit antiserum raised against a bacterially expressed GST–Tnk1(SH3) fusion protein (aa 387–503). The resulting affinity purified anti-Tnk1 antibodies detected a protein of the predicted molecular mass from cells transfected with Tnk1 cDNA, and specifically immunoprecipitated and immunoblotted a 72-kDa protein from cell lines which endogenously express Tnk1 mRNA, but not from cells known to be negative for Tnk1 mRNA [Fig. 1; (5)]. These results confirm the specificity of this affinity-purified anti-Tnk1 antibody preparation.

Interestingly, we detected Tnk1 protein expression in samples of fetal blood cells, but were unable to detect expression in several other sources of hematopoietic tissues (Fig. 1C). Expression of Tnk1 in human fetal blood suggests that Tnk1 is expressed in developmentally early blood cells, and is consistent with the fact that it was originally cloned from CD34+/Lin−/CD38− umbilical cord blood cells, an enriched source of human hematopoietic stem/progenitor cells (5). Correspondingly, Tnk1 mRNA is readily detected in murine yolk sac, Day 3 embryonic stem cells, and throughout embryogenesis, but not in mature, murine blood cell populations (G. T. Hoehn, manuscript in preparation).

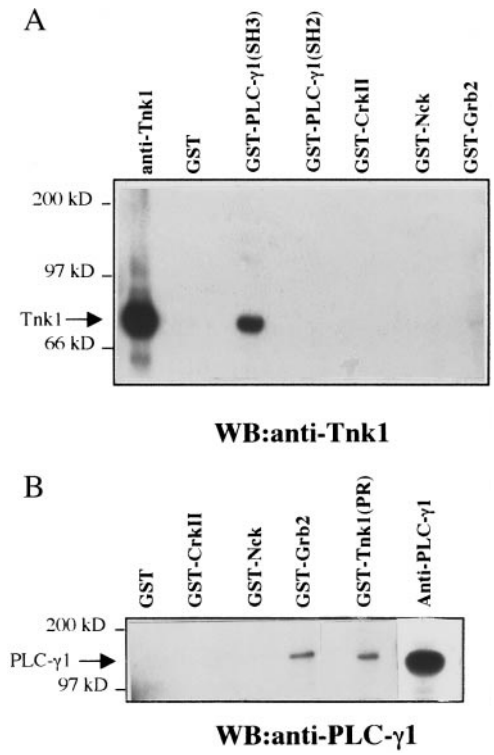


**FIG. 5.** Tnk1 associates with PLC-γ1 from Igrove whole-cell lysates. (A) GST–PLC-γ1(SH3) precipitates Tnk1 from Igrove whole cell lysates. Whole-cell lysates from Igrove cells were incubated with the indicated GST-fusion constructs, or immunoprecipitated with anti-Tnk1 antibody. Precipitating proteins were electrophoresed on an SDS–PAGE gel, transferred to Immobilon-P, and immunoblotted with anti-Tnk1 antibodies. (B) GST–Tnk1(PR) precipitates PLC-γ1 from Igrove whole cell lysates. In the converse experiment, lysates from Igrove cells were incubated with the indicated GST-fusion constructs and precipitated proteins were electrophoresed on an SDS–PAGE gel, transferred to Immobilon-P and immunoblotted with anti-PLC-γ1.

Tnk1 expressed sequence tags (ESTs) have been identified in human fetal heart and lung, pregnant uterus, normal colon and prostate, and several cancerous tissues, including adenocarcinomas of the stomach and pancreas, prostate and colon carcinomas, and squamous cell carcinoma of the tongue (dbEST, NCBI). Taken together, this expression pattern suggests that Tnk1 may be important in certain normal tissues and cancers and during early fetal development.

Under basal conditions, Tnk1 possessed autophosphorylation activity and was tyrosine phosphorylated (Fig. 2). In an effort to stimulate Tnk1 activity, we treated cell lines which endogenously express Tnk1 with known inducers of the Ack tyrosine kinases (6, 7, 12, 13). These included treatments such as temperature downshift, hyperosmotic shock, cell attachment, and stimulation with EGF and bradykinin. None of these conditions, nor treatment with other factors known to activate tyrosine kinases (e.g., IL-3, IL-6, phorbol myristate acetate, lysophophatidic acid, DNA damage by UV irradiation) enhanced Tnk1 auto- or transphosphorylation kinase activity in any of the cell lines tested. There were also no observable increases in Tnk1 tyrosine phosphorylation (data not shown). This suggests that Tnk1 activity is strictly regulated and activated only by a specific set of conditions which we have not yet identified.

Since we have been unable to find conditions which activate Tnk1, we examined Tnk1 signaling by investigating the binding of several SH3 containing proteins to the proline rich region of Tnk1. This region of Tnk1 contains the class II SH3-binding consensus sequence, P<u>P</u>GL<u>P</u>P<u>R</u>P, which has previously been shown to mediate binding of SH3 containing signaling molecules. Of the different SH3-containing proteins that we examined, only PLC-γ1 specifically associated with Tnk1. The SH3 domain of PLC-γ1 specifically bound both full-length Tnk1 and Tnk1(PR) *in vitro* translated constructs, as well as endogenously expressed Tnk1 from Igrove cells (Figs. 4 and 5), but did not bind to other GST-fusion constructs including the SH2 domain of PLC-γ1. Moreover, GST–Tnk1(PR) precipitated endogenously expressed PLC-γ1 from Igrove whole cell lysates, confirming the association of Tnk1 with PLC-γ1.

The finding that the PR tail of Tnk1 specifically associates with the SH3 domain of PLC-γ1 may implicate Tnk1 in phospholipid-mediated signaling pathways. When activated, PLC-γ1 hydrolyzes phosphatidylinositol 4,5 bisphosphate ($PIP_2$) to generate the second messengers inositol 1,4,5-triphosphate ($IP_3$) and diacyl glycerol (DAG) (26–28). Tyrosine phosphorylation of PLC-γ1 is induced by numerous growth factors and cytokines (29, 30) and has been shown to be involved in diverse signaling pathways regulating mitogenesis, secretion, and cytoskeletal assembly (31, 32). PLC-γ1 also appears to be critical during development, since mice lacking PLC-γ1 die at embryonic day nine (33). Following stimulation with EGF, PLC-γ1 translocates via its SH3 domain to the cell membrane or to sites on the actin cytoskeleton (34, 35), although the mechanism by which this occurs is not fully elucidated. We showed by subcellular fractionation (Fig. 3) that Tnk1 is greatly enriched in membrane fractions. Additionally, immunofluorescence of Igrove cells with anti-Tnk1 antibodies demonstrates that Tnk1 localizes to the cell membrane (data not shown). Taken together with our GST binding studies, these data suggest that Tnk1 and PLC-γ1 may be a part of a multi-protein signaling complex at the plasma membrane.

It is not known how Tnk1 binding affects PLC-γ1 function. Tnk1 might phosphorylate PLC-γ1, leading to PLC-γ1 activations. Currently, we are attempting to detect *in vitro* phosphorylation of PLC-γ1 by Tnk1 following treatment with known activators of PLC-γ1. It is also possible that PLC-γ1 is not a physiological substrate for Tnk1, but promotes the recruitment of PLC-γ1 to the membrane. In this way, Tnk1 would be

similar to FAK, which binds to PLC-γ1, but does not directly phosphorylate it (36). In response to integrin activation, FAK recruits PLC-γ1 to sites of cell matrix adhesion, thereby stimulating PLC-γ1 activity. Alternatively, it is possible that Tnk1 acts in downstream signaling pathways following activation of PLC-γ1. The results from these studies warrant a closer examination of the interaction of Tnk1 with PLC-γ1 *in vivo.* Currently, we are pursuing coimmunoprecipitation studies and conditions which activate Tnk1 in order to elucidate its role in PLC-γ1 signaling pathways.

## ACKNOWLEDGMENTS

This study was supported in part by a research grant from Gryphon Pharmaceuticals Inc. Gryphon Pharmaceuticals, Inc., provided partial funding for the study described in this paper. Under an agreement between Gryphon Pharmaceuticals, Inc., and the Johns Hopkins University, the University and Dr. Civin will receive a share of sales royalty. The University and Dr. Civin also own Gryphon Pharmaceuticals stock, the sale of which is subject to certain restrictions under University policy. Dr. Civin also serves on the Board of Directors and on the Scientific Advisory Board of Gryphon. The terms of this arrangement are being managed by the University in accordance with its conflict of interest policies.

## REFERENCES

1. Bolen, J. B. (1993) Nonreceptor tyrosine kinases. *Oncogene* **8,** 2025–2031.
2. Pawson, T., and Schlessinger, J. S. (1993) SH2 and SH3 domains. *Curr. Biol.* **3,** 434–442.
3. Pawson, T. (1995) Protein modules and signalling networks. *Nature* **373,** 573–580.
4. Ihle, J. N., Witthuhn, B. A., Quelle, F. W., Yamamoto, K., and Silvennoinen, O. (1995) Signalling through the hematopoietic cytokine receptors. *Annu. Rev. Immunol.* **13,** 369–398.
5. Hoehn, G. T., Stokland, T., Amin, S., Ramirez, M., Hawkins, A. L., Griffin, C. A., Small, D., and Civin, C. I. (1996) Tnk1: A novel intracellular tyrosine kinase gene isolated from human umbilical cord blood CD34+/Lin−/CD38− stem/progenitor cells. *Oncogene* **12,** 903–913.
6. Manser, E., Leung, T., Salihuddin, H., Tan, L., and Lim, L. (1993) A non-receptor tyrosine kinase that inhibits the GTPase activity of p21cdc42. *Nature* **363,** 364–367.
7. Yang, W., and Cerione, R. A. (1997) Cloning and characterization of a novel Cdc42-associated tyrosine kinase. *J. Biol. Chem.* **272,** 24819–24824.
8. Guan, J.-L., and Shalloway, D. (1992) Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature* **358,** 690–692.
9. Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M., and Juliano, R. L. (1992) Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *J. Biol. Chem.* **267,** 23439–23442.
10. Li, J., Avraham, H., Rogers, R. A., Raja, S., and Avraham, S. (1996) Characterization of RAFTK, a novel focal adhesion kinase, and its integrin-dependent phosphorylation and activation in megakaryocytes. *Blood* **8,** 417–4288.
11. Astier, A., Avraham, H., Manie, S. N., Groopman, J., Canty, T., Avraham, S., and Freedman, A. S. (1997) The related adhesion focal tyrosine kinase is tyrosine-phosphorylated after β1-integrin stimulation in B cells and binds to p130$^{cas}$. *J. Biol. Chem.* **272,** 228–232.
12. Kato, J., Kaziro, Y., and Satoh, T. (2000) Activation of the guanine nucleotide exchange factor Dbl following Ack1-dependent tyrosine phosphorylation. *Biochem. Biophys. Res. Commun.* **268,** 141–147, doi:10.1006/bbrc.2000.2106.
13. Satoh, T., Kato, J., Nishida, K., and Kaziro, Y. (1996) Tyrosine phosphorylation of ACK in response to temperature shift-down, hyperosmotic shock, and epidermal growth factor stimulation. *FEBS Lett.* **386,** 230–234.
14. Yang, W., Lin, Q., Guan, J-L., and Cerione, R. A. (1999) Activation of the Cdc42-associated tyrosine kinase-2 (ACK-2) by cell adhesion via integrin β1. *J. Biol. Chem.* **274,** 524–8530.
15. Eisenmann, K. M., McCarthy, J. B., Simpson, M. A., Keely, P., Guan, J-L., Tachibana, K., Lim, L., Manser, Furcht, L. T., and Iida, J. (1999) Melanoma chondrotin sulphate proteoglycan regulates cell spreading through Cdc42, Ack-1 and p130$^{cas}$. *Nat. Cell Biol.* **1,** 507–513.
16. Posern, G., Zheng, J., Knudsen, B., Kardinal, C., Muller, K., Voss, J., Shishido, T., Cowburn, D., Cheng, G., Wang, B., Kruh, G. D., Burrell, S. K., Jacobson, C., Lenz, D., Zamborelli, T., Adermann, K., Hanafusa, H., and Feller, S. M. (1998) Development of highly selective SH3 binding peptides for Crk and CRKL which disrupt Crk-complexes with DOCK180, SoS, and C3G. *Oncogene* **16,** 1903–1912.
17. Sparks, A. B., Rider, J. E., Hoffman, N., Fowkles, D. M., Quilliam, L. A., and Kay, B. K. (1996) Distinct ligand preferences of Src homology 3 domains from Src, Yes, Abl, Cortactin, p53bp2, PLCγ, Crk, and Grb2. *Proc. Natl. Acad. Sci. USA* **93,** 1540–1544.
18. Alexandropoulos, K., Cheng, G., and Baltimore, D. (1995) Proline-rich sequences that bind to Src homology 3 domains with individual specificities. *Proc. Natl. Acad. Sci. USA* **92,** 3110–3114.
19. Nguyen, J. T., Turck, C. W., Cohen, F. E., Zuckerman, R. N., and Lim, W. A. (1998) Exploiting the basis of proline recognition by SH3 and WW domains: Design of N-substituted inhibitors. *Science* **282,** 2088–2092.
20. Feng, S., Chen, J. K., Yu, H., Simon, J. A., and Schreiber, S. L. (1994) Two binding orientations for peptides to the Src SH3 domain: Development of a general model for SH3–ligand interactions. *Science* **266,** 1241–1247.
21. Knudsen, B. S., Zheng, J., Feller, S. M., Mayer, J. P., Burrell, S. K., Cowburn, D., and Hanafusa, H. (1995) Affinity and specificity requirements for the first Src homology 3 domain of the Crk proteins. *EMBO J.* **14,** 2191–2198.
22. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. Thom, D., Powell, A. J., Lloyd, C. W., and Rees, D. A. (1977) Rapid isolation of plasma membranes in high yield from cultured fibroblasts. *Biochem. J.* **168,** 187–194.
24. Smith, D. B., and Johnson, K. S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene* **67,** 31–40.
25. Pei, Z., Maloney, J., Yang, L., and Williamson, J. R. (1997) A new function for phospholipase C-γ1: Coupling to the adaptor protein GRB2. *Arch. Biochem. Biophys.* **345,** 103–110.
26. Cockcroft, S., and Thomas, G. M. (1992) Inositol–lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. *Biochem. J.* **288,** 1–14.
27. Berridge, M. J. (1993) Inositol triphosphate and calcium signalling. *Nature* **361,** 315–325.
28. Liscovitch, M., and Cantley, L. C. (1994) Lipid second messengers. *Cell* **77,** 329–334.
29. Noh, D. Y., Shin, S. H., and Rhee, S. G. (1995) Phosphoinositide-

specific phospholipase C and mitogenic signalling. *Biochim. Biophys. Acta* **1242,** 99–114.

30. Rhee, S. G., and Bae, Y. S. (1997) Regulation of phosphoinositide-specific phospholipase C isozymes. *J. Biol. Chem.* **272,** 15045–15048.
31. Cantley, L., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, R., Kapellar, R., and Solotoff, S. (1991) Oncogenes and signal transduction. *Cell* **64,** 281–302.
32. Bae, Y. S., Cantley, L. G., Chen, C-S., Kim, S-R., Kwon, K-S., and Rhee, S. G. (1998) Activation of phospholipase C-γ by phosphatidylinositol 3,4,5-triphosphate. *J. Biol. Chem.* **273,** 4465–4469.
33. Ji, Q. S., Winnier, G. E., Niswender, K. D., Horstman, D., Wisdom, R., Magnuson, M. A. M., and Carpenter, G. (1997) Essential role of the tyrosine kinase substrate phospholipase C-gamma1 in mammalian growth and development. *Proc. Natl. Acad. Sci. USA* **94,** 2999–3003.
34. Todderud, G., Wahl, M. I., Rhee, S. G., and Carpenter, G. (1990) Stimulation of phospholipase C-γ1 membrane association by epidermal growth factor. *Science* **249,** 296–299.
35. Bar-Sagi, D., Rotin, D., Batzer, A., Mandiyan, V., and Schlessinger, J. (1993) SH3 domains direct cellular localization of signaling molecules. *Cell* **74,** 83–91.
36. Zhang, X., Chattpadhyay, A., Ji, Q-S., Owen, J. D., Ruest, P. J., Carpenter, G., and Hanks, S. K. (1999) Focal adhesion kinase promotes phospholipase C-γ1 activity. *Proc. Natl. Acad. Sci. USA* **96,** 9021–9026.