A Novel Interaction between the Juxtamembrane Region of the p55 Tumor Necrosis Factor Receptor and Phosphatidylinositol-4-phosphate 5-Kinase*

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Tumor necrosis factor-α (TNF-α)1 initiates its proliferative, differentiative, or cytotoxic actions on mammalian cells by binding to two transmembrane molecules, the p55 and p75 TNF receptors (1–3). The p55 receptor is responsible for many of the biological effects of TNF, including programmed cell death, cell differentiation, and cell proliferation (4–6). Am major of the biological effects of TNF, including programmed cell death, cell differentiation, and cell proliferation (4–6). A major step in understanding the mechanism of the p55 receptor has been the identification of the interacting protein, TRADD, which accounts for signals leading to apoptosis and increased gene expression through NF-kB-mediated events (7). Likewise, the p75 receptor is capable of signal transduction through the association of ring finger proteins, such as TRAF1 and TRAF2, with the cytoplasmic domain of p75 (8, 9).

The interaction of TRADD with the p55 TNF receptor has revealed the importance of protein-protein interactions via a region of homology called the “death domain.” This sequence has been found in a variety of transmembrane and cytosolic molecules and is usually localized at the C-terminal region of each protein. The functional significance of this domain has been demonstrated in studies with the p55 TNF receptor and the Fas antigen, which contain similar functional death domain sequences (10). The binding of p55 receptors to TRADD, a cytoplasmic protein containing a death domain, and the binding of the Fas antigen to FADD, an analogous protein, have been localized to an 80-amino acid region at the C terminus of both receptors. While overexpression of TRADD or FADD in heterologous cells leads to cell death (7, 11), deletions or mutations in the death domains abolish the ability of these molecules to participate in the initiation of apoptosis (6, 12).

A link to the interleukin converting enzyme/ced-3 protease family was made recently with the identification of a cysteine protease, FLICE/MACH, which interacts with the death effector domain of FADD (13, 14). The connection of the Fas antigen and the p55 TNF receptor with a member of the interleukin converting enzyme protease family provides a mechanism for how cell death signals are initiated by ligand-receptor interactions.

While cell death can be instigated by TNF-α and represents the major signaling function of the Fas antigen, TNF-α can participate in many other diverse activities, including the synthesis of proinflammatory mediators and cell proliferation and differentiation (15), neuroprotection (16), and synaptic transmission (17). The striking interactions of the C-terminal half of the intracellular domain of the p55 receptor raise the issue of the functional significance of the juxtamembrane region of the p55 receptor. This region is ~100 amino acids in length, is rich in proline residues, and is not required for TNF-mediated cellular cytotoxicity. Here we report the interaction of the juxtamembrane region of the p55 TNF receptor with phosphatidylinositol-4-phosphate 5-kinase (PIP5K), an enzyme that produces phosphatidylinositol 4,5-bisphosphate (PIP2). PIP5K is a significant enzymatic activity to be linked to TNF signaling since its product, PIP2, is a critical second messenger intermediate that also has many direct modulatory effects. Moreover, this study has identified a new member of the PIP5K family, PIP5KIIβ. The association of the p55 receptor with PIP5K

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The abbreviations used are: TNF-α, tumor necrosis factor-α; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PCR, polymerase chain reaction; GST, glutathione S-transferase; MBP, maltose-binding protein; kb, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; c11, clone 11.
indicates how diverse functions are encoded in the TNF receptor structure to generate multiple signal transduction events after ligand binding.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa, U373, and MCF7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. In experiments using TNF, the cells were transfected to serum-free medium when an 80% confluency was reached. The cells were serum-starved for 12–24 h before TNF was added at a final concentration of 100 ng/ml. Cells (10–cm dishes) were harvested at appropriate time intervals and washed three times in cold phosphate-buffered saline (0.14 M NaCl, 0.0025 M KCl, 0.0025 M NaH2PO4, pH 7.4) and lysed in 500 μl of cold lysis buffer (20 mM Tris, pH 7.5, 25 mM β-glycerophosphate, 137 mM NaCl, 100 mM EDTA, 1% Triton X-100, 2 mM sodium pyrophosphate, 100 μM sodium vanadate, 0.5 mM LiCl, 1 μg/ml leupeptin, 2 μg/ml aprotinin, and 25 μM phenylmethylsulfonyl fluoride). The cell debris was pelleted after a 20-min incubation on ice. The lysates were stored frozen at -70°C.

**Construction of Yeast Expression Vectors**—The yeast expression vector pSD0.4 containing the lexA DNA-binding domain (18), was used for these studies. All recombinant clones were generated by PCR using primers containing a 5′-NotI linker and a 3′-SphiI linker. The bait used in the primary screening protocol was Y1 (residues 204–280 of the human p55). Other regions of the human p55 receptor used were Y2 (residues 278–364), Y3 (residues 324–426), Y4 (residues 204–364), and Y5 (residues 204–426). Other constructs used the cytoplasmic domains of the Fas antigen, p75 TNF receptor, and p75 neurotrophin receptor. The primers for the baits were as follows: Y1, 5′-primer (5′-ATTTTGCGGCCGCGTATGTGGCACTACGCG-3′) and 3′-primer (5′-CTAATACGTTCAGGCGCGGACTTTGCGGTGC-3′); Y2, 5′-primer (5′-ATTGGCGCCGGTGTTCGCGCTCCCGAGAAG-3′) and 3′-primer (5′-CTAACGTCACCCCTGTCGACTCCT-3′); Y3, 5′-primer (5′-ATTTTGCGGCCGCTGATGCAAGACTCAG-3′) and 3′-primer (5′-CTAACGTTCACCCCTGTCGACTCCT-3′); Y4, 5′-primer (5′-ATTGGCGCCGCGTATGTGGCACTACGCG-3′) and 3′-primer (5′-CTAACGTCACCCCTGTCGACTCCT-3′); Y5, 5′-primer (5′-ATTTTGCGGCCGCGTATGTGGCACTACGCG-3′) and 3′-primer (5′-CTAACGTCACCCCTGTCGACTCCT-3′). Amplification reactions were carried out as described above using 30 cycles of 94°C for 1 min, a melting temperature of 56°C for 1 min, and an extension temperature of 72°C for 1 min. The PCR fragments were ligated with BamHI and SalI and ligated into pGStag (22). Large-scale GST fusion proteins expressed from the various constructs were purified according to published procedures (23).

For making GST and MBP fusion proteins of clone 11 (c11), the yeast library plasmid corresponding to c11 was used as a template to amplify the library insert. In both cases, the 5′-AAAGATGCTGGGTGGGAGATTTGCGGCAGCG-3′ and the 3′-primer (5′-TATCTAAGCTCTTCAGTGGCACTACGCG-3′) had a BamHI linker incorporated in the 5′-primer. The fragment was PCR-amplified as described above using the conditions described for the p75 TNF receptor. Digestion with BamHI and SalI and ligation separately into pGStag and pMAL-c2 (New England Biolabs Inc.). The positive clones were tested for their ability to yield fusion proteins of the expected sizes after isopropyl-1-thio-β-D-galactopyranoside induction (0.5 mM). The GST fusion protein was purified as described (23), and the MBP fusion protein was purified as described by protocol provided by New England Biolabs Inc.

**Generation of Anti-GST-c11 and Anti-MBP-c11 Antibodies**—The GST-c11 and MBP-c11 fusion proteins were denatured in SDS and used as immunogens to generate polyclonal antibodies (Bocono Farms).

**Isolation of PIP5KIIβ cDNA**—In a data base search, the PIP5KIIβ sequence (GenBankTM/EMBL accession number U14957) matched the HBFP52 (GenBankTM/EMBL accession number T07883) expressed sequence tag from the human infant brain cDNA library with 70.5% identity. This 5.7-kb clone was obtained and sequenced (Sequenase Version 2.0, U. S. Biochemical Corp.). A 2.0-kb EcoRI fragment corresponding largely to the 3′-untranslated region of this clone was used to screen 8 × 105 plaques from a human fetal brain AD2 cDNA library (CLONTECH). The plaques were transferred to nitrocellulose filters and hybridized with the probe at 42°C in 5× SSPE, 5× Denhardt’s solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA. These filters were washed at high stringency (65°C, 0.1× SSC and 0.01% SDS) for 20 min. Eleven positive clones were obtained, plaque-purified, and converted to their respective phagemids. Upon sequencing, two clones were found to extend the sequence of HBFP52 in both the 5′- and 3′-directions. Additional sequencing of the 5′-region was done on an Applied Biosystems Model 373 sequencer at the University of Wisconsin Biotechnology Center. The DNA sequences were represented with 1.2-kb open reading frame of clone 11 (c11), which lies entirely within the 3′-untranslated region of PIP5KIIβ. The DNA fragment was gel-purified following restriction enzyme digestion and labeled by random priming with [α-32P]dATP and Klenow DNA polymerase. Hybridization and washing were performed as outlined by the manufacturer, and the membrane was exposed to film overnight.

**Expression of PIP5KIIβ in E. coli**—The 1.2-kb open reading frame of PIP5KIIβ was amplified by PCR (30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min following by an extension temperature of 72°C for 10 min) using native Fnu polymerase (Stratagene) and the following primers: 5′-GCCGCCGGCGGATGTATGTGGCACTACGCG-3′ (forward) and 5′-GGCTGAAGGTATCGTGGCAGACTACGCAAGTTG-3′ (reverse). The forward primer was designed to anneal over the start codon (in boldface) and to introduce a BamHI site (underlined) just upstream of the start site of the cDNA. The reverse primer was designed to introduce a stop codon (in boldface) and to introduce an XhoI site (underlined) just downstream of the termination codon. These PCR products were cloned into the BamHI and XhoI sites of pBluescript SK(-) (Stratagene). The entire insert was sequenced to confirm that no errors had been introduced by PCR. The BamHI/XhoI fragment was then subcloned into pET28b (Novagen) for the purpose of expressing a histidine fusion protein.
version of PIP5KIIβ (His-PIP5KIIβ). E. coli strain BL21(DE3) was transformed with either pET28b containing the PIP5KIIβ open reading frame or just pET28b. For the initial expression, these strains were grown at 37 °C to A660 = 0.6, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside, and incubated at 37 °C for an additional 3 h. The cells were pelleted and resuspended in 50 mM Tris-HCl, pH 8.0, and 2 mM EDTA. Next, 4000 units of Ready-lyse solution (Epicentre Technologies Corp.) was added to the resuspended cells, and Triton X-100 was added to a final concentration of 0.1%. After incubating the cells at 30 °C for 15 min, they were sonicated at 70 watts twice for 10 s. These lysates were centrifuged at 12,000 × g for 15 min at 4 °C, and the supernatant was decanted. The pellet was dissolved in 1 × SDS-PAGE sample buffer, while an aliquot of the supernatant was mixed with an equal volume of 2 × sample buffer. All samples were boiled for 10 min and analyzed by SDS-PAGE in the presence of 250 mM β-mercaptoethanol.

His-PIP5KIIβ was purified by Ni²⁺-chelate chromatography. The strain containing the His-PIP5KIIβ open reading frame was grown at 30 °C to A660 = 0.6, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside, and incubated at 30 °C for an additional 3 h. The cells were centrifuged and resuspended in 8 ml of 1 × binding buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 5 mM imidazole). After sonication with three 20-s pulses at 70 watts, the extract was centrifuged at −39,000 × g for 20 min at 4 °C and passed through a 0.45-μm syringe filter. An aliquot of this extract was set aside, and the rest was purified on a 2.5-ml gravity-packed column of His-Bind resin (Novagen). Bound proteins were eluted in three 5-ml fractions. Each of these fractions was dialyzed overnight at 4 °C against 2.0 liters of saline.

**Kinase Activity Assays**—The supernatants of the crude fraction of His-PIP5KIIβ and of partially purified His-PIP5KIIβ were assayed for phosphatidylinositol-4-phosphate 5-kinase activity as described previously (25) with some modifications. Kinase activity was assayed in 50-μl reactions done for 20 min at room temperature in a final concentration of 50 mM Tris-HCl, pH 7.6, 0.5 mM EGTA, 10 mM MgCl₂, 160 μM phosphatidylinositol 4-phosphate (Sigma), 50 μM ATP, and 10 μM of γ-[32P]ATP. The lipids were extracted, and the labeled products were separated by thin layer chromatography and detected by autoradiography.

**Western Blotting**—Proteins were transferred to nitrocellulose (Micron Separations, Inc.) following SDS-PAGE. Antibodies were incubated with the membrane for 1 h at room temperature following blocking with 5% powdered milk in phosphate-buffered saline containing 0.2% Tween 20. The primary antibody was raised against the MBP-c11 fusion protein and was detected by chemiluminescence using a horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc.) and LumiGLO substrates (Kierkegaard and Perry Laboratories, Inc.).

**In Vitro Binding**—GST fusion proteins containing the cytoplasmic, the juxtamembrane, or the death domain of the p55 fusion protein were purified on glutathione-Sepharose beads, and 100 μl of the fusion protein on the glutathione beads was incubated with 3 μg of purified MBP-c11. The reaction volume was brought up to 500 μl with 50 mM Tris, pH 7.5, 200 mM NaCl, 20 mM EDTA, and 0.1% Nonidet P-40. After incubation at 4 °C for 1 h, the slurry was washed three times with the same buffer. The slurry was then boiled in SDS-PAGE buffer, and the samples were loaded on a 10% SDS-polyacrylamide gel and electrophoresed at 100 V until the dye front reached the bottom of the gel. The gel was then transferred onto an Immobilon-P transfer membrane (Millipore Corp.), and immunoblotting was carried out using chemiluminescence. The primary antibody was the rabbit anti-MBP antibody (New England Biolabs Inc.) used at a 1:1000 dilution. The secondary antibody was a peroxidase-conjugated goat anti-rabbit IgG (Sigma) used at a 1:15,000 dilution.

**Immunoprecipitation**—Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Near confluency, the cells were washed three times with phosphate-buffered saline, scraped, and centrifuged. The cells were lysed in 500 μl of radioimmuno precipitation assay buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton, and 0.4% SDS) supplemented with protease inhibitors. Lysates were incubated on ice for 30 min; the cell debris was removed by centrifugation; and an aliquot of the lysate was used to determine protein concentration using the Bio-Rad reagent. Lysates (4 mg) were subjected to immunoprecipitation in a final volume of 500 μl of radioimmuno precipitation assay buffer. Antibodies against PIP5K (protein A-purified) that were coupled to Affi-Gel-10 beads (Bio-Rad) were added to the lysate, and the mixture was incubated at 4 °C for 1 h. In separate reactions, lysates were incubated either with 100 μl of protein A-purified anti-p55 antibodies (Genzyme Corp.) coupled to Affi- Gel-10 beads or with non-specific goat serum. The immunoprecipitate was washed three times in radioimmuno precipitation assay buffer and boiled for 30 s in SDS-PAGE buffer. The sample was subjected to electrophoresis on a 10% SDS-polyacrylamide gel. Chemiluminescence was carried out using the ECL procedure (Amersham Corp.).

**RESULTS**

The p55 TNF receptor is a transmembrane protein with four extracellular cysteine-rich repeats and an intracellular domain of 222 amino acids. While the sequences responsible for mediating cytotoxicity (6) and aggregation of the p55 receptor (26) have been localized to the death domain in the C-terminal half of the cytoplasmic tail, the functional significance of the juxtamembrane region has not been fully defined.

To identify cellular proteins that bound specifically to the juxtamembrane domain of the p55 TNF receptor, a recombinant LexA fusion protein was generated that contained amino acids 204–280 (Y1), beginning at the end of the transmembrane domain and extending to the middle of the cytoplasmic domain. Y1 did not contain sequences representing the death domain of p55. The Y1 domain was then used as the bait in the yeast two-hybrid screen.

A mouse cerebellar cDNA library was used to screen for proteins that interacted specifically with the Y1 region. The library was engineered in a yeast expression vector containing VP16 as the activation domain (18). Colonies were obtained following cotransformation of the S260 yeast strain with the Y1-LexA construct, and the cDNA library colonies were analyzed by β-galactosidase activity measurements. Of 1.5 × 10⁷
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cotransformants, 20 cDNAs were found to be positive using β-galactosidase activity as a measure of the activation of the Y1-LexA construct. After tests for specificity and DNA sequence analysis were conducted, one positive clone of 0.7 kb in size (c11) was pursued for further analysis.

The yeast two-hybrid assay was further used to test the specificity of the interaction. For this purpose, four other LexA fusion proteins containing different regions of p55 were generated. These proteins are schematically represented in Fig. 1. Each construct (called Y2, Y3, Y4, and Y5) encoded different segments of the intracellular domain of p55, including the juxtamembrane region, the C-terminal death domain (Y3), or both regions (Y4, Y5). The interaction of c11 with the p55 receptor was found to be confined to sequences representing the juxtamembrane region. Cotransformation of yeast strain S260 with each of these constructs and the plasmid harboring c11 rescued from the initial screening indicates that p55 constructs containing the death domain (Y3) or LexA alone did not yield any β-galactosidase activity, whereas other constructs containing p55 juxtamembrane sequences (Y1, Y4, and Y5) gave positive β-galactosidase activity (Fig. 1).

This analysis was also extended to the cytoplasmic regions of the p75 TNF receptor, the Fas antigen, and the p75 neurotrophin receptor. No interactions were detected between c11 and LexA constructs containing these receptor sequences (Fig. 1). In support of this observation is the fact that there is little similarity in sequence between these family members in the juxtamembrane region. Therefore, the yeast two-hybrid results indicate that the protein expressed from the c11 cDNA specifically associates with the juxtamembrane domain of the p55 TNF receptor.

The cDNA Encodes Phosphatidylinositol-4-phosphate 5-Kinase—Sequence analysis revealed that the mouse c11 cDNA clone encoded a protein that was highly homologous to human

![Fig. 2–continued](image-url)
PIP5KII cloned previously (25). The c11 cDNA sequence showed 77% (protein level: 83%) identity to the published sequence of PIP5K cloned from a human placental cDNA library (Fig. 2B). The PIP5K enzymes have been defined as types I and II on the basis of their elution from a phosphocellulose ion exchange column. These kinases differ in size, kinetic properties, and differential sensitivity to heparin, spermine, and phosphatidic acid (27, 28). The placental PIP5K sequence, encoded by a 4.1-kb mRNA, may now be identified as the type II isoform (25), which was previously isolated as a 53-kDa enzyme (28, 29).

A search of the sequence data bases with the DNA sequence of PIP5KIIα revealed a putatively transcribed sequence that was highly identical to the query sequence. This clone, HF-BEP52, was obtained, sequenced, and used to screen a human fetal brain λDR2 cDNA library. Several different cDNAs were obtained, and the composite sequences from these partial cDNAs predicted an open reading frame of 1248 base pairs, encoding a 416-amino acid protein with a calculated molecular mass of 47,378 Da (Fig. 2A). Based on this composite sequence, the full-length open reading frame was amplified by PCR from the human fetal brain λDR2 cDNA library. The open reading frame was found to be 77.8% identical to PIP5KIIα at both the nucleotide and protein levels.

Based on this similarity, this new clone has been designated as PIP5KIIβ. The murine clone c11 isolated in the yeast two-hybrid screen is 90.3% identical to PIP5KIIβ at the nucleotide level and 99.5% identical at the amino acid level. Because of the level of identity, the c11 cDNA corresponds to a newly identified isoform of PIP5K, referred to as PIP5KIIβ. A comparison of the amino acid sequences of PIP5KIIα, PIP5KIIβ, and c11 appears in Fig. 2B.

Using a probe representing the 3′-untranslated region of HF-BEP52, a discrete 6.3-kb mRNA, distinct from the α-isoform 4.1-kb mRNA (25), was detected (Fig. 3). Identical results were obtained by hybridization with the c11 cDNA (data not shown). Hence, PIP5KIIβ is encoded by a 6.3-kb mRNA, while PIP5KIIα is represented by a 4.1-kb mRNA. Northern blot analysis also revealed that a probe derived from the 5′-coding region of PIP5KIIα also detected the 6.3-kb transcript at lower stringency (data not shown). This observation was expected, considering the degree of identity between the two isoforms.

Abundant levels of messages were found in heart, placenta, kidney, and pancreas, whereas lung and liver displayed lower

**FIG. 3. Human multiple tissue Northern blot.** A human multiple tissue Northern blot with 2 μg of poly(A)+ RNA/lane was hybridized with a 32P-labeled 1.7-kb KpnI fragment derived from the 3′-untranslated region of HF-BEP52 (PIP5KIIβ) according to the manufacturer's instructions (CLONTECH).

**FIG. 4. Expression, purification, detection, and kinase activity of recombinant PIP5KIIβ.** A, PIP5KIIβ expressed in E. coli as a hexahistidine fusion protein and purified from cell lysates by Ni2+–charged chromatography. The samples were separated by SDS-PAGE and stained with Coomassie Blue. Lane 1, E. coli lysate from an induced strain containing the empty vector; lane 2, E. coli lysate from an induced strain containing His-PIP5KIIβ cDNA; lane 3, affinity-purified recombinant hexahistidine fusion of PIP5KIIβ (5 μg). B, Western blot with antibodies raised against the MBP-c11 fusion protein. The lanes are the same described for A, except that lane 3 (His-PIP5KIIβ) contained 4 ng of protein. C, phosphatidylinositol-4-phosphate 5-kinase activity of recombinant His-PIP5KIIβ. Lane 1, affinity-purified His-PIP5KIIβ; lane 2, E. coli lysate from an induced strain harboring the empty vector; lane 3, recombinant His-PIP5KIIα.
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levels. Although both the 4.1- and 6.3-kb transcripts are highly expressed in brain, a major difference is that skeletal muscle is more enriched for β isoform mRNAs than the α isoform.

Enzymatic Activity—To verify the enzymatic activity of PIP5KIIβ, a cDNA containing the coding region of PIP5KIIβ was expressed as a hexahistidine fusion protein (Fig. 4A). The recombinant His-PIP5KIIβ protein was partially purified by Ni²⁺ affinity chromatography and found to be ~51 kDa, as measured by SDS-PAGE (Fig. 4B). This molecular mass is ~3 kDa larger than the native enzyme due to the hexahistidine tag and linker sequence, which would make the size of the wild-type protein 47 kDa. This is consistent with the calculated molecular mass of 47.4 kDa. The purified His-PIP5KIIβ protein exhibited phosphatidylinositol-4-phosphate 5-kinase activity (Fig. 4C). As a control, lysates from cells containing the empty vector lacked kinase activity (Fig. 4C), whereas lysates expressing PIP5KIIβ displayed enzymatic activity (data not shown).

In Vitro Interaction—To verify the interaction of PIP5KIIβ with the p55 receptor in vitro, two separate approaches were taken. In the first case, the truncated PIP5KIIβ cDNA (c11) isolated from the yeast two-hybrid screen was fused in frame with the coding sequences for MBP. The resulting fusion protein was expressed and purified from E. coli. To test for binding in vitro, the purified MBP-PIP5KIIβ fusion protein (Fig. 5A, right panel, lane 4) was incubated with glutathione-Sepharose beads and GST fusion proteins representing different regions of the p55 TNF receptor. After incubation, the reaction was extensively washed, separated by SDS-PAGE, and analyzed by Western blotting using antibodies against MBP. These antibodies do not cross-react with the GST protein alone, PIP5K, or p55 receptor proteins (data not shown). The PIP5KIIβ fusion protein interacted with GST fusion proteins containing the juxtamembrane region (residues 204–337) and the entire cytoplasmic region (residues 204–426) of the p55 receptor (Fig. 5A). The full-length p55 cytoplasmic fusion protein was somewhat less reactive. However, the fusion protein containing the p55 death domain did not exhibit any binding to MBP-PIP5KIIβ. This provides independent evidence that the interaction between P5KIIβ and p55 TNF receptor is restricted to the juxtamembrane region.

In a separate approach, an analogous experiment was undertaken with recombinant His-PIP5KIIβ or His-PIP5KIIα proteins (Fig. 5B, lanes 5 and 6). The immunoblot indicates that PIP5KIIβ binds to the cytoplasmic region, and not to the death domain. No interaction was detected between p55 cytoplasmic sequences and PIP5KIIβ. Taken together, these results suggest that the juxtamembrane region of the p55 TNF receptor interacts specifically with P5KIIβ.

Co-immunoprecipitation—To determine if the p55 TNF receptor interacts with PIP5KIIβ in vivo, extracts of two different cell lines, MCF7 and HeLa cells, were prepared and immunoprecipitated either with antibodies against the p55 receptor or with antibodies directed against MBP-c11 coupled to Affi-Gel-10 beads. Following immunoprecipitation with anti-p55 antibodies, Western blot analysis using anti-PIP5KIIβ (c11) antibodies was carried out. A 47-kDa protein, representing PIP5KIIβ, was detected after immunoprecipitation with anti-p55 antibodies, indicating that the p55 TNF receptor directly associated with PIP5KIIβ in HeLa and MCF7 cells (Fig. 6A). Further immunoblotting verified that this 47-kDa isoform was expressed in the cell lines (Fig. 6B). Mock immunoprecipitation reactions with nonspecific goat serum (Fig. 6C) indicated the co-immunoprecipitation of P5K and p55 was specific. These results indicate that PIP5KIIβ is associated with the p55 TNF receptor in two TNF-responsive cell lines.

**Fig. 5. Binding of p55 TNF receptor cytoplasmic domains to PIP5K.** A, the flow chart indicates the experimental scheme (left panel). GST fusion proteins containing the cytoplasmic region (residues 204–426) (right panel, lane 1), the juxtamembrane region (residues 204–337) (lane 2), or the death domain region (residues 340–426) (lane 3) of the p55 TNF receptor were purified on glutathione-Sepharose beads and incubated with the maltose-binding fusion protein of clone c11 (PIP5KIIβ). Lane 4, input PIP5KIIβ protein. The beads were washed, and the products were run on an SDS-polyacrylamide gel followed by an immunoblot with anti-MBP-c11 antibodies. B, hexahistidine-tagged PIP5KIIα or PIP5KIIβ expressed and purified from E. coli was incubated with the GST fusion proteins containing either the death domain or the cytoplasmic domain of the p55 TNF receptor on glutathione beads. The reaction was washed, and the products were separated by SDS-PAGE and immunoblotted with anti-MBP-c11 antibodies. The amounts of P5KIIβ (lane 5) and P5KIIα (lane 6) used are indicated.

**Activation of PIP5K**—PIP5K is a pivotal enzyme in phosphoinositide metabolism since it gives rise to PIP₂, the parent molecule for the production of 1,2-diacylglycerol, inositol 1,4,5-trisphosphate, and phosphatidylidyinositol 3,4,5-trisphosphate. These lipid second messengers are involved in mitogenic responses to polypeptide growth factors and G proteins through phospholipase Cβ (30, 31). To investigate whether the enzymatic activity of PIP5K is relevant to TNF-mediated signaling, the activity of PIP5K was determined in cell lysates of TNF responsive cells, HeLa, MCF7, and U373.

Cells were treated with 100 ng/ml TNF-α, and lysates were prepared as described under “Experimental Procedures.” To determine the activity of the β isoform, cell lysates were first immunodepleted of the P5KIIα isoform using antibody sc-1330 (Santa Cruz Biotechnology, Inc.) raised against the N-terminal peptide of P5KIIα. Western blot analysis indicated that the immunodepleted lysates contained the β isoform (data not shown). Using phosphatidylinositol 4-phosphate as a substrate in the presence of [γ-32P]ATP, the activity of PIP5K was measured (Fig. 7). After 30 min of TNF treatment, a significant increase in the level of PIP₃ was detected. The ligand-dependent activation of P5KIIβ in TNF-responsive cells demonstrates that one potential signaling mechanism for TNF-α may be in the induction of the phosphatidylinositol pathway.

**DISCUSSION**

Multiple signaling pathways have been characterized for TNF-α, a prominent cytokine produced by macrophages. Since
The binding of PIP2 to pleckstrin homology domains is thought to influence localization of pleckstrin homology domain-containing proteins to the membrane. PIP2 can be further hydrolyzed to inositol 1,4,5-trisphosphate, giving rise to increased PIP2 production through the association of PIP5K and phosphatidylinositol 4-kinase. One potential function of these lipid kinases is to regulate the activities of these phosphoinositide kinases, phosphatidylinositol 4-kinase and PIP5K. In the case of the EGF receptor, PIP5K activity is associated with the EGF receptor (36). Treatment with EGF increases the activities of these phosphoinositide kinases, phosphatidylinositol 4-kinase and PIP5K. One potential function of this association may be to aid the mitogenic responses to EGF stimulation (37).

A mitogenic role for PIP5K is also supported by evidence showing that monoclonal antibodies against PIP2 block cell proliferation in response to platelet-derived growth factor and bombesin (38) and are growth inhibitory for S. cerevisiae (39). Moreover, PIP5K activity has been directly linked to proliferation and malignancy (40). The increase in PIP5K activity observed following TNF treatment suggests that the known mitogenic effects of TNF upon cells (41) may be facilitated by increased PIP2 production through the association of PIP5K with the p55 receptor.

How can the same receptor system give rise to such divergent biological responses such as cell death and cell proliferation? Through its interaction with the p55 and p75 receptors, TNF mediates divergent responses ranging from inflammatory, cytotoxic, and metabolic functions, it is likely that many second messengers are responsible for TNF-dependent signaling. These may include increased tyrosine phosphorylation, production of ceramide from the hydrolysis of sphingomyelin, activation of the mitogen-activated kinase cascade and p38 stress-activated kinase, production of arachidonic acid, and activation of protein kinase C and phosphatidylcholine-specific phospholipase C (3, 15). The mechanism of TNF in initiating cellular responses such as cell death has been clarified by the identification of receptor adaptor proteins, such as TRADD, which are required for apoptosis signal transduction. The binding of the p55 receptor to TRADD occurs via domains located at the C terminus of both proteins, which can lead to the recruitment of FLICE/MACH proteases (13, 14).

Here we have identified a distinctive protein interaction between the juxtamembrane region of the p55 TNF receptor and a phosphatidylinositol lipid kinase, PIP5KIIß. This is the first identified enzymatic activity directly associated with the TNF receptor. The PIP5K enzyme is responsible for the phosphorylation of phosphatidylinositol 4-phosphate, giving rise to PIP2. PIP2 can be further hydrolyzed to inositol 1,4,5-trisphosphate and 1,2-diacylglycerol by phospholipase C, or it can be phosphorylated by phosphatidylinositol 3-kinases to generate another lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. The interaction of the TNF receptor with PIP5K therefore reveals a potential link to several other lipid second messenger systems and cellular activities, including vesicular transport, endocytosis, and lysosomal function (32).

In addition to the activation of protein kinase C by diacylglycerol and the mobilization of intracellular calcium by inositol 1,4,5-trisphosphate, phosphoinositide metabolites contribute to other cellular activities that may be relevant to TNF signaling. The binding of PIP2 to pleckstrin homology domains may influence localization of pleckstrin homology domain-containing proteins to the membrane. PIP2 binds to cytoskeletal proteins such as gelsolin and profilin that can influence cytoskeletal remodeling (33). Also, clathrin coat-associated proteins, such as AP-2, bind avidly to inositol polyphosphates (34). In the case of AP-2, the binding of inositol phosphate blocks its clathrin coat assembly properties.

Other growth factor receptor systems have been linked to PIP5K activities. EGF stimulation of A431 cells results in an increase in the activity of lipid kinases associated with the cytoskeleton (35). Indeed, co-immunoprecipitation experiments using antibodies against the EGF receptor indicate that phosphatidylinositol 4-kinase and PIP5K activities are directly associated with the EGF receptor. Interestingly, these lipid kinases also interact in the juxtamembrane region of the EGF receptor near the ATP-binding site of the catalytic tyrosine kinase domain (36). Treatment with EGF increases the activities of these phosphoinositide kinases, phosphatidylinositol 4-kinase and PIP5K. One potential function of this association may be to aid the mitogenic responses to EGF stimulation (37).
TNF can potentially generate second messengers, such as ceramide (42) and proinflammatory metabolites (15). The activity of PIP5K versus other signaling activities may determine whether cells respond to TNF by cell proliferation or apoptosis. Discrete actions by TNF may be dictated by the presence or absence of cellular proteins, such as TRADD or PIP5K. Other proteins, such as FADD, RIP, and TRAF2, can be recruited as a result of these initial interactions with TRADD (43, 44). When multiple proteins are present, the affinity of binding with the receptor will undoubtedly dictate the choice of the signaling pathway. It is important to note that the binding of PIP5KIIβ to the p55 receptor was not observed with other family members, such as Fas and the p75 neurotrophin receptor, and that PIP5KIIβ interacted preferentially over PIP5KIIα (Fig. 5C). These interactions would be expected to take place only in specific cell types, thereby providing the diversity in signaling potential by this cytokine family.

Additionally, phospholipase Cγ (45, 46) and phosphatidylinositol 3-kinase (47) contain intrinsic SH3 domains or are nositol 3-kinase (47) contain intrinsic SH3 domains or are

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