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Brief Definitive Report

Molecular Cloning of the cDNA Encoding pp36, a Tyrosine-phosphorylated Adaptor Protein Selectively Expressed by T Cells and Natural Killer Cells

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Summary

Activation of T and natural killer (NK) cells leads to the tyrosine phosphorylation of pp36 and to its association with several signaling molecules, including phospholipase Cγ-1 and Grb2. Microsequencing of peptides derived from purified rat pp36 protein led to the cloning, in rat and man, of cDNA encoding a T- and NK cell–specific protein with several putative Src homology 2 domain–binding motifs. A rabbit antiserum directed against a peptide sequence from the cloned rat molecule recognized tyrosine phosphorylated pp36 from pervanadate-treated rat thymocytes. When expressed in 293T human fibroblast cells and tyrosine-phosphorylated, pp36 associated with phospholipase Cγ-1 and Grb2. Studies with GST–Grb2 fusion proteins demonstrated that the association was specific for the Src homology 2 domain of Grb-2. Molecular cloning of the gene encoding pp36 should facilitate studies examining the role of this adaptor protein in proximal signaling events during T and NK cell activation.

Tyrosine phosphorylation is a prominent mechanism for the regulation of protein–protein interactions during signal transduction (1–3). Stimulation of the TCR induces the tyrosine phosphorylation of a variety of cellular proteins, including a protein that migrates with an apparent M_r of 36 kD on SDS-PAGE and, therefore, has been designated pp36. After TCR perturbation, pp36 coimmunoprecipitates with Grb-2 and phospholipase Cγ-1 (PLCγ-1) (4–7). This interaction likely involves binding of tyrosine-phosphorylated sequences of pp36 to the Src homology (SH) 2 domains of Grb-2 and PLCγ-1, since fusion proteins containing the SH2 domains of these signaling molecules precipitate pp36 from the lysates of activated T cells.

It has been proposed that pp36 is an adaptor protein that bridges proximal TCR signals to the activation of phosphatidylinositol turnover, Ras, and, perhaps, other signaling pathways. Support for this notion has come from studies of a chimeric molecule containing the Grb-2 SH2 domain and the CD45 tyrosine phosphatase domain (8). Expression of this chimera in Jurkat T cells led to the selective dephosphorylation of pp36 and the abrogation of TCR-mediated activation of phospholipase C.

Studies of pp36 have not been limited to T lymphocytes. Although apparently not found in B cells (9), pp36 is present in NK cells, a separate lineage of lymphocytes closely related to the T cells. Perturbation of the low affinity Fc receptor (CD16) or the addition of target cells leads to the appearance of tyrosine phosphorylated pp36 and triggers its association with Grb2 and PLCγ-1 (10, 11). Interestingly, engagement of killer inhibitory receptors (KIRs) disrupts the interaction of PLCγ-1 with pp36 and blocks target cell–induced activation of phospholipase C. In vitro, pp36 serves as a substrate for the KIR-associated tyrosine phosphatase, SHP-1 (PTP-1C), suggesting that KIR engagement prevents pp36–PLCγ-1 interactions by inducing the dephosphorylation of pp36 (11).

To facilitate functional studies of pp36, we have isolated and sequenced cDNAs corresponding to rat and human pp36. The deduced amino acid sequences reveal potential binding motifs for the SH2 domains of Grb-2 and PLCγ-1 consistent with the proposed adaptor functions of pp36.

Materials and Methods

Cells and Reagents. Thymocytes isolated from F344 rats were used for initial purification of pp36, and thymocytes from PVG rats were used for precipitation studies. The human 293T fibroblast cell line, a gift from Dr. L.L. Lanier (DNAX, Palo Alto,

CA), was maintained in RPMI 1640 with 10% FCS, penicillin, and streptomycin. Cells were washed and resuspended in RPMI 1640 with 2% FCS before use. Tyrosine phosphorylation was induced with the tyrosine phosphatase inhibitor pervanadate (12) by incubation with 0.01% H_2O_2 and 100 μM sodium orthovanadate for 5 min at 37°C. Mouse mAbs to Grb2 (clone 81) and PLCγ-1 (clone 10) were from Transduction Laboratories (Lexington, KY); to hemagglutinin (HA) epitope from Babco (16B12; Richmond, CA); and to phosphotyrosine from Upstate Biotechnology Inc. (4G10; Lake Placid, NY). Polyclonal Abs were generated against two peptides corresponding to residues 35–49 (ASYDSASTESLYPRS) and 96–110 (RMPSSRQNSDDANSV) of rat pp36. They were coupled to KLH with glutaraldehyde and affinity purified on columns with immobilized peptides, as previously described (13). As secondary Abs we used horseradish peroxidase–conjugated goat anti–mouse IgG (115-035-100; Jackson Immunoresearch Labs., West Grove, PA) and horseradish peroxidase–conjugated goat anti–rabbit IgG (111-035-144; Jackson Immunoresearch Labs.). Glutathione-*S*-transferase (GST) fusion proteins with SH2 (sc-4035 AC) and N-SH3 (sc-4034 AC) domains of Grb2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Purification of pp36 Protein and Peptide Sequencing. Tyrosine-phosphorylated pp36 was purified at 4°C from lysates of 3×10^{10} pervanadate-treated thymocytes by sequential affinity chromatography. Lysates were loaded on a column of maltose binding protein–PLCγ-1 SH2 domain fusion protein coupled to amylose resin. After extensive washing, the fusion protein and bound proteins were eluted with 10 mM free maltose and applied to a second column of antiphosphotyrosine mAb (4G10) coupled to agarose. The antiphosphotyrosine column was washed and then eluted with 100 mM phenyl phosphate. Eluted proteins were resolved by SDS-PAGE. A 36-kD band was identified by Coomassie blue staining, excised, and subjected to trypsin digestion, HPLC, and microsequencing (Beckman Center, Protein and Nucleic Acid Facility, Stanford University Medical Center, Stanford, CA).

cDNA Cloning and Sequencing. The oligopeptide sequences obtained from rat pp36 were analyzed for matches in the GenBank dbest database using the tBLASTN program (14). Two contiguous overlapping human expressed sequence tags (ESTs; 15), accession numbers AA355655 and AA380490, gave a full match with the PDLLPIPR peptide corresponding to residues 79–86 of the rat sequence (see Fig. 1). Based on this information, we amplified a 276-bp DNA from a Jurkat T cell library (No. 938200; Stratagene, La Jolla, CA) with two synthetic primers (5′-CTCACACGGTTGCCCCCTGGCCAC-3′ and 5′-CAGTGCTAATGGCCGGGTTGCTGT-3′) and Taq DNA polymerase. The amplified product was then used to probe a rat cDNA library that we generated from a subset of IL-2–activated NK cells from PVG rats. A cDNA synthesis and gold cloning kit from Stratagene that inserted cDNA into the ZAP Express phage vector in a sense orientation (EcoRI–XhoI) was used. 5×10^5 plaques were screened by Southern blotting. 10 positive clones were isolated, converted into pBK-CMV phagemids, and end sequenced. One clone, 15-1, sequenced on both strands, contained an open reading frame for a 241-amino acid protein, which was confirmed in three independent clones. The human pp36 sequence was identified using human thymus Marathon RACE-ready cDNA (Clontech, Palo Alto, CA). Two primers were synthesized based on the human EST contig described above, 5′-CCAACAGTGTGGCGAGCTACGAGAACG-3′ and 5′-GTCAGGAAGCACCACCAGGTAG-3′, and used to amplify the 3′ and 5′ parts of the human pp36 cDNA with the Advantage cDNA PCR kit (Clontech). Sequencing of representative clones allowed us to deduce the open reading frame for a 233-amino acid protein, as presented in Fig. 1. Confirmation of this sequence was obtained by PCR amplification of the entire pp36-coding sequence with two additional primers (5′-GTCCGGTCCTCACCCCATCTTCATCTG-3′ and 5′-CACGCAGGCCTTTATTCTATTACACAG-3′). DNA sequencing was performed by Medigene sequencing service (Martiensried, Germany). Sequences were assembled and analyzed with the Wisconsin Genetics Computer Group program package (Madison, WI; reference 16).

Northern Blot Analysis. Total cellular RNA was extracted with the guanidinium isothiocyanate/cesium chloride method, resolved by formaldehyde agarose gel electrophoresis, and transferred to nylon membranes (17). Hybridization with radiolabeled cDNA (a PstI fragment from clone 15-1, containing most of the open reading frame of rat pp36) was performed overnight at 42°C in a formamide-based hybridization solution. Before autoradiography, final wash of blots was with 0.1 × SSC, 0.1% SDS for 2× 30 min at 50°C.

Generation of Expression Construct and Transient Transfection of 293T. The open reading frame of rat pp36 was amplified by PCR with two primers introducing ClaI restriction sites (5′-AATCGATATGGAAGCAGACGCCTTGAGCC-3′, and 5′-AATCGATTCAGTTAAGTTCCTGCAGGTTC-3′). The amplified products were ligated into the pZERO-blunt vector (Invitrogen Corp., Carlsbad, CA), isolated, and subcloned in a modified pEF-BOS vector with an in-frame HA epitope. The orientation of inserts was determined using restriction enzyme digestion and sequencing. 293T cells were transiently transfected with two clones containing rat pp36 cDNA in sense (+) and antisense (−) orientations, using lipofectamine (GIBCO BRL, Gaithersburg, MD). The cells were analyzed 36–48 h after transfection.

Immunoprecipitation and Western Blotting. Cells ($2.5–10 \times 10^7$/ml) were solubilized for 30 min on ice with 2× lysis buffer (2% NP-40, 20 mM Tris, pH 7.5, 300 mM NaCl, 20% glycerol, 4 mM EDTA, 2 mM PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 2 mM sodium orthovanadate, 20 mM sodium fluoride, and 20 mM sodium pyrophosphate). 1–5 μg antibody and 25 μl of protein A/G plus agarose (SC-2003, Santa Cruz Biotechnology), or 10 μg of agarose-coupled GST fusion proteins, were used to precipitate from 5×10^7 thymocytes or 10^7 293T cells. Precipitates were washed three to four times with buffer containing 0.1–1% NP-40, resolved by 10% SDS-PAGE under reducing conditions, and transferred by semidry electroblotting to polyvinylidene difluoride membranes. Blots were blocked with 5% dry milk (2% BSA was added for antiphosphotyrosine blots) in PBS-T (PBS/0.1% Tween 20), before incubation with the indicated Ab. After extensive washings with PBS-T, blots were incubated with either horseradish peroxidase HRP–conjugated goat anti–mouse or goat anti–rabbit IgG, washed, and developed with enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Results

cDNA Cloning of Rat and Human pp36. pp36 protein was purified from pervanadate-treated rat thymocytes by sequential affinity chromatography using columns composed of maltose binding protein–PLCγ-1 SH2 domain fusion protein and an antiphosphotyrosine mAb, followed by SDS-PAGE. Three oligopeptide sequences were obtained from microsequencing of tryptic peptides derived from the purified protein. Database searching with these sequences resulted in a full match, for one of them (PDLLPIPR, cor-

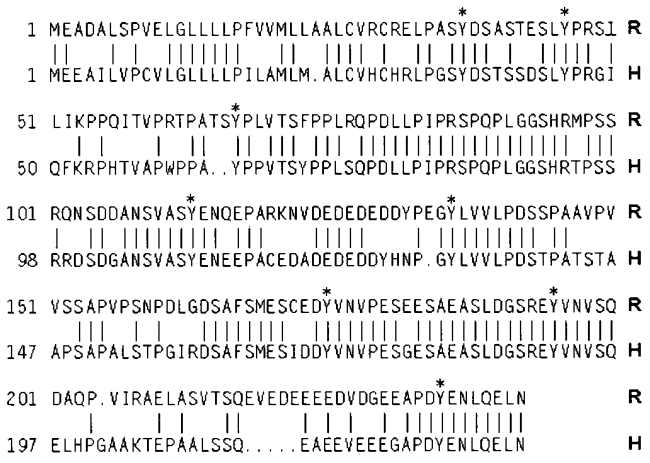

Figure 1. Deduced amino acid sequence of rat (*R*) and human (*H*) pp36. Asterisks indicate tyrosine residues conserved between rat and human. The oligopeptide sequences obtained from the purified rat pp36 protein were: ILIKPPQITVPR (amino acids 50–61); PATSYPL (amino acids 63–69); and PDLLPIPR (amino acids 79–86). The cDNA sequences are available from EMBL/GenBank/DDBJ under accession numbers AJ223280 and AJ001184.

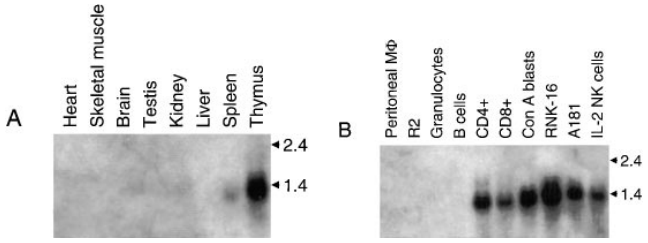

Figure 2. Expression of pp36 transcripts is limited to T and NK cells. (*A*) Northern blot analysis using a rat pp36 probe of total RNA from different rat tissues revealed a 1.4-kb transcript in thymus and spleen, but not liver, kidney, testis, brain, muscle, or heart. (*B*) The probe also detected a 1.4-kb transcript in freshly isolated $CD4^+$ and $CD8^+$ T cells, Con A lymphoblasts, IL-2–activated NK cells, and in two different NK cell lines (RNK16 and A181) but not in B cells, granulocytes, peritoneal macrophages, or a macrophage line (R2). Probing for β-actin confirmed equal loading of RNA (20 μg/lane; data not shown).

responding to amino acid 76–83 of the human sequence, Fig. 1) with two contiguous human expressed sequence tags. Based on this information, we amplified a 276-bp fragment from a Jurkat T cell library and used this fragment for homology screening of a rat NK cell cDNA library. Several individual positive clones were isolated, and one of them, 15-1, was sequenced completely on both strands. The predicted sequence of the full-length rat cDNA has 241 amino acids and contains all three of the oligopeptide sequences obtained by protein purification (Fig. 1). A human cDNA was isolated from a human thymus library by 5′ and 3′ rapid amplification of cDNA ends (RACE). Human pp36 encodes a somewhat shorter protein of 233 amino acids and displays 67% amino acid identity with the rat molecule. Although both rat and human pp36 have a high content of acidic amino acids (20 and 17%, respectively), as well as many prolines (~12%) and serines (~12%), there were relatively few basic residues (~5%). Database searching revealed no closely homologous proteins or known signaling motifs.

Transcripts for the Cloned cDNA Are Selectively Expressed by T Cells and NK Cells. Northern blot analysis with the rat cDNA probe revealed a 1.4-kb transcript expressed at high levels in thymus and at lower levels in spleen. No signal was detected in the other tissues investigated (Fig. 2 *A*). Analysis of different leukocyte populations revealed abundant message in $CD4^+$ and $CD8^+$ T cells, Con A–activated

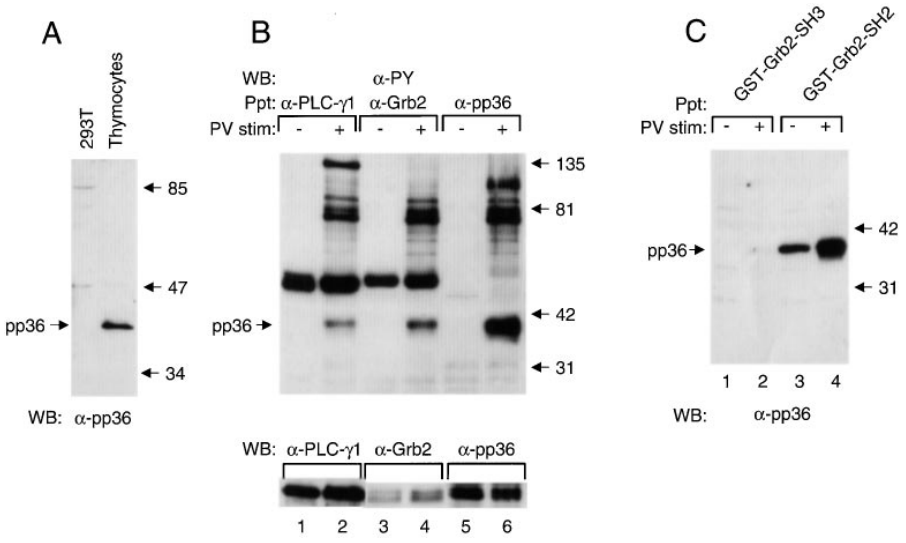

Figure 3. A rabbit antiserum to a peptide from the cloned pp36 sequence recognizes a tyrosine-phosphorylated protein of apparent M_r 36 kD that associates with a Grb2-SH2 domain fusion protein. (*A*) Whole cell lysates of 293T fibroblasts cells and unstimulated rat thymocytes were resolved by SDS-PAGE, transferred to PVDF, and analyzed by immunoblotting with an affinity-purified rabbit antiserum made to a peptide from the cloned rat pp36 sequence. The antiserum detected a protein of apparent M_r 36 kD in thymocytes but not in 293T cells. (*B*) Lysates from pervanadate-treated (+) or unstimulated (−) rat thymocytes were subjected to immunoprecipitation with anti–PLCγ-1, anti-Grb2, and the anti-pp36 rabbit antiserum. After resolution by SDS-PAGE and transfer to PVDF, the precipitates were analyzed by immunoblotting with an mAb to phosphotyrosine. In the lower panel, parallel blots were probed with anti–PLCγ-1 (146 kD; lanes *1* and *2*), anti-Grb2 (24 kD; lanes *3* and *4*), and anti-pp36 (36 kD; lanes *5* and *6*). (*C*) Lysates from pervanadate-treated (+) and unstimulated (−) rat thymocytes were subjected to precipitation with Grb2 SH2 domain–GST and Grb2 SH3 domain–GST fusion proteins. The precipitated proteins were analyzed by immunoblotting with the anti-pp36 rabbit antiserum.

lymphoblasts, IL-2–activated NK cells, and two different NK lines (RNK-16 and A181), but not in B cells, granulocytes, peritoneal macrophages, or the macrophage cell line, R2.

The Cloned cDNA Encodes a Protein that Migrates with an Apparent M_r of 36 kD on SDS-PAGE and Associates with Grb-2 and PLCγ-1 when Tyrosine-phosphorylated. The calculated molecular weight of the deduced amino acid sequence of the cloned cDNAs was ~26 kD, considerably less than the apparent M_r of pp36 based on its migration in SDS-PAGE. To confirm that the cloned cDNAs corresponded to pp36, we generated a rabbit antiserum to a peptide from the predicted amino acid sequence of the rat molecule. When affinity purified and used for immunoblot analysis, the antiserum detected a 36-kD protein in a whole cell lysate of rat thymocytes, but not of a 293T human fibroblast line (Fig. 3 *A*). Identical results were obtained using a rabbit antiserum to a second peptide (data not shown). Treatment of rat thymocytes with pervanadate induced the tyrosine phosphorylation of the 36-kD protein immunoprecipitated by the antiserum, and this protein comigrated on SDS-PAGE with pp36 that coimmunoprecipitated with Grb2 and PLCγ-1 (Fig. 3 *B*). To investigate further the interactions between the 36-kD protein and Grb2, we used GST fusion proteins containing either SH2 or SH3 domains of Grb2. The GST-Grb2-SH2, but not GST-Grb2-SH3, fusion protein precipitated the 36-kD protein detected by the antipeptide antiserum (Fig. 3 *C*). Although some 36-kD protein bound GST-Grb2-SH2 in lysates of unstimulated thymocytes, treatment of the cells with pervanadate led to a substantial increase in the amount of precipitated 36-kD protein.

Studies with an HA-tagged version of the cloned rat cDNA provided additional evidence for its identity with pp36. The rat cDNA was cloned into the pEF-BOS vector containing an HA epitope, and the construct was used to transiently transfect 293T cells. Cells transfected with the sense (+), but not antisense (−), orientation of the cloned cDNA expressed a 40-kD, HA-tagged protein that could be immunoprecipitated by either an anti-HA mAb or by the antipeptide antiserum (Fig. 4 *A*). After treatment of the transfected cells with pervanadate, the HA-tagged protein coimmunoprecipitated with Grb2 and PLCγ-1 (Fig. 4 *B*).

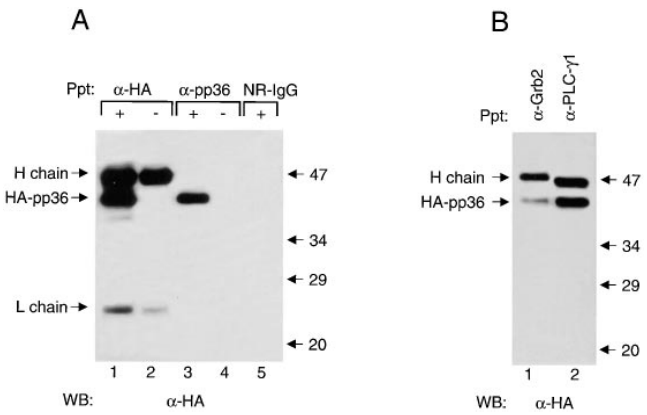

Figure 4. HA-pp36 associates with PLCγ-1 and Grb2 in pervanadate-treated 293T cells. (*A*) 293T cells were transiently transfected with either HA-pp36 sense (+) or antisense (−) expression constructs. Lysates from the transfected cells were subjected to immunoprecipitation with anti-HA mAb, the anti-pp36 rabbit antiserum, or normal rabbit (*NR*) IgG, and the immunoprecipitates were analyzed by immunoblotting with an mAb to HA. The heavy (*H*) and light (*L*) chains of the precipitating HA mAb, revealed by the second step goat anti–mouse IgG antiserum, are indicated. (*B*) 293T cells were transfected with the HA-pp36 sense expression construct, treated with pervanadate, and solubilized. Immunoprecipitates with anti-Grb2 and anti–PLCγ-1 were analyzed by immunoblotting with anti-HA mAb.

Discussion

Here we report the cDNA cloning of the rat and human genes encoding pp36, a previously identified adaptor molecule involved in early signaling events during T and NK cell activation. The cloned molecule exhibits the known properties of pp36: expression in T lymphocytes, inducible tyrosine phosphorylation, binding to the SH2 domains of Grb-2 and PLCγ-1, and an apparent M_r of 36 kD on SDS-PAGE. Although the calculated molecular weight of the cloned molecules was ~26 kD, antisera directed against peptides from the predicted rat amino acid sequence recognized a 36-kD protein on immunoblot analysis. Furthermore, the cloned molecule migrated with an apparent M_r of 40 kD on SDS-PAGE when expressed as a fusion protein with a short HA peptide. A discrepancy between molecular weight and migration on SDS-PAGE also has been noted for another Grb2 binding protein, SLP-76, and has been attributed to the high content of negatively charged residues in SLP-76 (18). Because acidic residues account for ~20 and 17% of the amino acids of rat and human pp36, respectively, a similar mechanism may explain the migration of pp36 on SDS-PAGE.

pp36 was recognized on the basis of its tyrosine phosphorylation after T and NK cell activation and by its ability to interact with Grb-2 and PLCγ-1 by means of their SH2 domains. Eight tyrosine residues are conserved in the rat and human sequences. Four of these (Y113, Y175, Y195, and Y234 in the rat sequence) are contained in amino acid sequences identified as potential binding sites for Grb2 SH2 domains (YxNx), and one (Y136 in the rat sequence) is contained in a putative PLCγ-1 SH2 binding site (19). The presence of these sequence motifs suggests that the interactions of pp36 with Grb-2 and PLCγ-1 are probably direct.

pp36 has been reported to be membrane associated and thus may serve to localize Grb-2 and PLCγ-1 to the plasma membrane during activation (4, 5). The pp36 cDNAs lack leader sequences. However, in the NH_2-terminal part of both rat and human pp36, beginning with A5 and A4, respectively, there is a stretch of ~20 hydrophobic residues with a predicted α-helical structure. This hydrophobic region is followed by basic residues (R29 and R31 in the rat; H28, H30, R31 in human) and therefore may represent a transmembrane domain. Surprisingly, this region contains an acidic residue (E10) in the rat, but not in the human, sequence. The potential tyrosine phosphorylation sites and

SH2 domain binding motifs are all located on the COOH-terminal side of the hydrophobic region, indicating that, if the NH_2-terminal hydrophobic region is a transmembrane domain, pp36 would have only three to four extracellular amino acids and a relatively large cytoplasmic region of 205 (human) to 212 (rat) amino acids.

pp36 appears to be involved in the activation of the phospholipase C pathway in T cells and NK cells and thus in the responses of these cells to antigen and target cells (8, 11). The cloning of cDNA encoding pp36 and the development of serologic reagents should facilitate the further dissection of the role of pp36 in T and NK cell activation.

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Note added in proof. Zhang et al. recently reported the cloning of human and mouse pp36. *Cell*. 92:83.

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