# p84, a New Gβγ-Activated Regulatory Subunit of the Type IB Phosphoinositide 3-Kinase p110γ

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## Summary

**A variety of genetic and inhibitor studies have shown that phosphoinositide 3-kinase γ (PI3Kγ) plays an essential role in a number of physiological responses, including neutrophil chemotaxis, mast cell degranulation, and cardiac function [1–6]. PI3Kγ is currently thought to be composed of a p110γ catalytic subunit and a single regulatory subunit, p101. The binding of p110γ to p101 dramatically increases the activation of the complex by Gβγ subunits and, hence, is thought to be critical for the coupling of PI3Kγ to G protein coupled receptors [7–9]. Here, we characterize a new regulatory subunit for PI3Kγ. p84 is present in human, mouse, chicken, frog, and fugu genomes and is located beside the p101 locus. It is broadly expressed in cells of the murine immune system. Both recombinant and endogenous p84 bind p110γ specifically and with high affinity. Binding of p84 to p110γ substantially increases the ability of Gβγ to stimulate phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)$P_3$) production both in vitro and in vivo. However, the p84/p110γ heterodimer is approximately 4-fold less sensitive to Gβγs than p101/p110γ. Endogenous murine p84 expression is substantially reduced in the absence of p110γ expression. We conclude that p110γ has two potential regulatory subunits in vivo, p84 and p101.**

## Results and Discussion

### Cloning and Expression of Mouse p84

The program BLAST was used to search for sequence similarity to the mouse p101 sequence in the mouse EST nucleotide sequences present in the EMBL database. Significant hits for sequences, other than the true p101, were used to scan the Ensembl database (release 6.33b.1) with the program SSAHA. We identified a genomic region stretched over 50 kb of the chromosome 11 just downstream of the p101 gene. This gene has been highly conserved during evolution as it is found in fugu, chicken, mouse, and human; and interestingly, in all cases, it is immediately downstream of the p101 locus (Figure 1A and Figure S1 available with this article online). We found evidence of a single gene related similarly to both p84 and p101 in the ciona genome.

Although we can not exclude the possibility that there is a second related gene in ciona, it appears that a gene duplication event occurred subsequent to the divergence of the vertebrates. The coding sequence of the murine gene is arranged into 20 exons, and the identified amino acid sequence shared 30% identity and 37% similarity with p101, mainly in the C- and N-terminal ends (see Figure S2). The full mouse protein sequence was 752 amino acids long with an estimated molecular mass of 84,276 Da. Thus far, we have failed to identify any recognized domains within its structure.

We have studied p84 mRNA expression by analyzing human multiple-tissue Northern blots with a probe generated from 525 bp of the human coding sequence. The Northern blots revealed a band of approximately 4 Kb, which was a little higher than the 3.2 Kb expected size of full-length p84 mRNA (see Figure S3). These blots showed that the mRNA was expressed mainly in leukocytes, bone marrow, spleen, and lymph node and less in fetal liver, placenta, and small intestine. Another band of about 2 Kb was found in heart and skeletal muscle, revealing a possible splice variant (see Figure S3). By screening EST databases, we found three ESTs that suggest possible mouse variants. One would form either a very short peptide or an N-terminal truncation of about 30 amino acids (identified EST by201034). The other two change the reading frame and lead to premature termination after 500 to 600 amino acids (55–65 KDa, bb556657 and bb617125).

The same immune tissue blots were also hybridised with p101- or p110γ-directed probes (see Figure S3). The p101 mRNAs were expressed at high level in leukocytes and much less in spleen, lymph node, thymus, and bone marrow. The p110γ expression was similar in all immune tissues screened. These results show that p84, like p101 and p110γ, is mostly expressed in cells from the haematopoeitic compartment.

We then performed Western blots within mouse immune tissues with a rabbit anti-p84 antiserum raised against full-length, recombinant, Sf9-derived, (EE)-tagged p84 (murine sequence). The antiserum was affinity purified with full-length, recombinant, bacterially-derived, (HIS)-tagged p84 (murine sequence). Immunoblots with this antibody preparation decorated a protein with an apparent size of approximately 84 KDa, which was found expressed at similar levels in murine macrophages, bone marrow, and neutrophil cytosolic fractions. Its level was lower in the cytosolic fractions prepared from thymus and lymph node. Interestingly, the level of p84 in cytosolic fractions derived from neutrophils from p110γ knockout (p110γ$^{-/-}$) mice was reduced to about 10% of that in wild-type mouse neutrophils (Figure 1B).

Reprobing the same blots with an affinity-purified, sheep, anti-p101 antiserum revealed that p101 was expressed at the same level in thymus, lymph node, neutrophils, and bone marrow. Its expression was reduced in macrophages and neutrophils from p110γ$^{-/-}$ mice. Similarly, reprobing these filters with a rabbit anti-p110γ antiserum revealed a doublet, of approximately 120

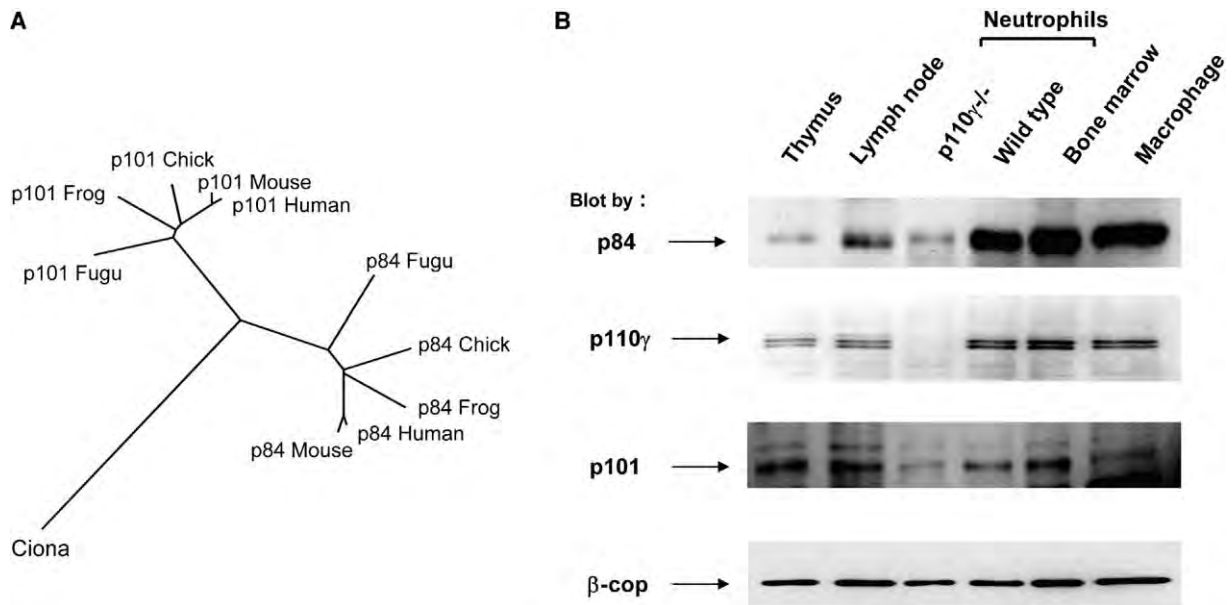*Correspondence: len.stephens@bbsrc.ac.uk

**A**



**B**



Figure 1. Characterization of p84

(A) Radial tree showing the phylogenetic relationship of p84 and p101. This was created from the CLUSTALX alignment with the PHYLIP programs, PROTDIST, FITCH, and DRAWTREE. A single sequence was found in the current Ciona database. Sequence identity could not determine with which of the paralogs it was homologous.

(B) Tissue distribution of p84 within the murine immune system. Western blots were performed on cytosol fractions of different mouse immune tissues. The same immune blots were used for immunoblotting with anti-p84, anti-p101, anti-p110γ, and anti-β-cop antibodies (loading control). Similar results were obtained in one further experiment.

KDa, that was equivalently expressed in neutrophils, bone marrow, and macrophages and less in thymus and lymph node (Figure 1B).

These results demonstrate that the expression of both p84 and p101 is dependent on p110γ. They also highlight the problems in interpreting the molecular origins of the interesting phenotype of p110γ$^{-/-}$ mice as these animals are also substantially deficient in p101 and p84. The recent development of p110γ kinase-dead, knockin (PI3K$^{KD/KD}$) mice, which should not have altered p101 nor p84 expression, has revealed that many features of the p110γ$^{-/-}$ phenotype are the result of loss of p110γ catalytic activity, and hence, the consequences of partial deficiencies in p101 and p84 must be small or they substantially manifest via p110γ. However, analysis of PI3K$^{KD/KD}$ mice has shown they do not present the same aspects of the cardiac phenotype of p110γ$^{-/-}$ mice. Evidence was presented that shows that this discrepancy may result from p110γ acting as a scaffold for a complex containing phosphodiesterase 3B (PDE3B) [10]. Our results indicate it is also possible these p110γ:PDE3B interactions could be via p84 and/or p101.

**Regulation of p110γ by p84 In Vitro**
To address questions about the effect of p84 on the kinase activity of p110γ, we cloned it into a mammalian-expression vector downstream of, and in frame with, an (EE) epitope tag and cotransfected it into COS-7 cells with (Myc)-p110γ (Figure 2). We used (EE)-p101 as a positive control. The transfected cells were lysed and the lysates immunoprecipitated with anti-(EE) monoclonal antibody covalently coupled to protein G-Sephar-

ose beads. After immunoprecipitation, (Myc)-p110γ was only recovered from the beads when coexpressed either with p84 or p101, demonstrating a specific, tight interaction between p84 and p110γ. Immunoblotting with an anti-(EE) antibody revealed p101 and p84 were similarly expressed in these experiments. The anti-(EE) immunoprecipitates prepared in these experiments were eluted and assayed for PI3K activity; only when p110γ was associated with p84 could we detect Gβγ-stimulated PI3K activity (Figure 2B).

To more fully characterize the catalytic properties of the p84/p110γ heterodimer, we prepared parallel preparations of (EE)-p84/p110γ and (EE)-p101/p110γ by anti-(EE) immunoprecipitation from lysates of relevantly infected Sf9 cells. The protein preparations were substantially pure showing that interactions between p84 and p110γ were direct and the near stoichiometric recovery of p110γ confirmed the high affinity of their interaction. PI3K assays were performed on these proteins and revealed p84/p110γ heterodimers, although substantially activated by Gβ$_1$γ$_2$, were approximately four times less sensitive to Gβ$_1$γ$_2$ than p101/p110γ (Figure 2C).

**Regulation of p110γ by p84 In Vivo**
To investigate the regulation of p84/p110γ in vivo, we cotransfected COS-7 cells with various combinations of expression vectors encoding for (EE)-p84, (Myc)-p110γ, (EE)-p101, (EE)-Gβ$_1$, and (Myc)-Gγ$_2$. After labeling the cells with [$^{32}$P]-Pi, phosphoinositides were extracted, deacylated, and separated by HPLC (Figure 3A). These experiments showed the presence of p84 augmented PtdIns(3,4)$P_2$ and PtdIns(3,4,5)$P_3$ accumu-
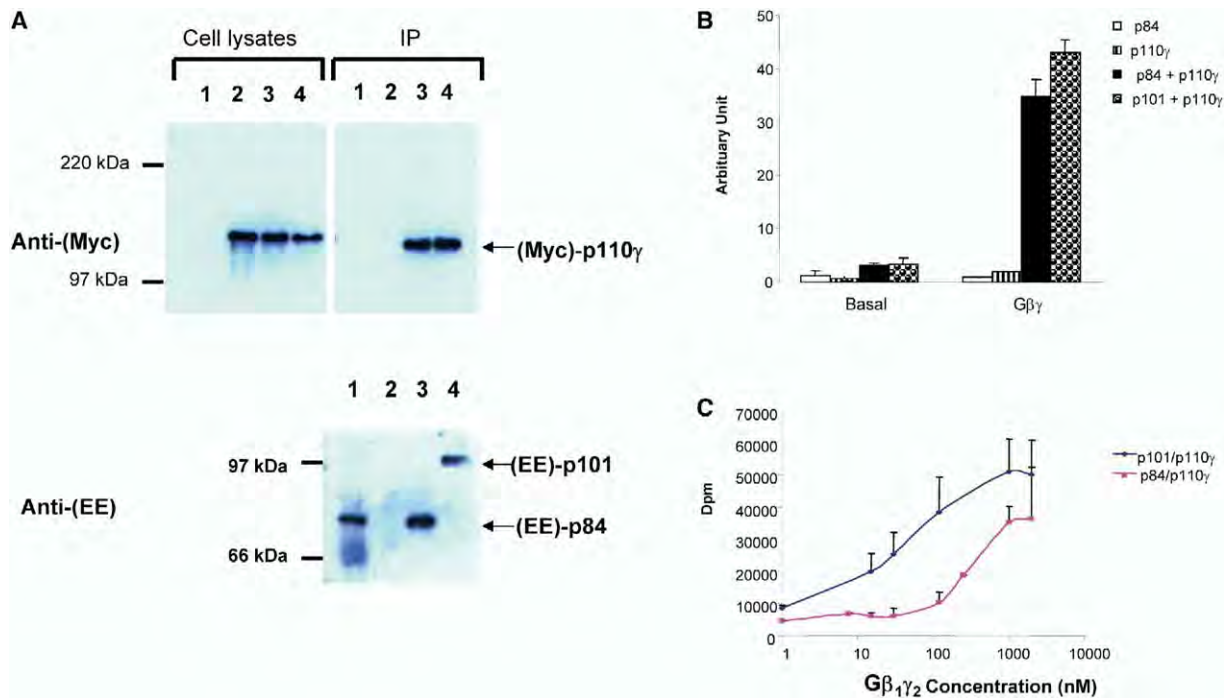
Figure 2. p84 Binds to and Activates p110γ In Vitro

(A) Interaction of p84 and p110γ in vitro. (EE)-p84 and (Myc)-p110γ were transfected individually or together into COS-7 cells. p101 and p110γ were used as a positive control. Lane 1, (EE)-p84; lane 2, (Myc)-p110γ; lane 3, (EE)-p84 + (Myc)-p110γ; lane 4, (EE)-p101 + (Myc)-p110γ. The cells lysates were then immunoprecipitated with anti-(EE) beads. Samples of the total input protein (cell lysates) and the pellets resulting from the immunoprecipitation were immunoblotted either with anti-(Myc) (top) or anti-(EE) antibody preparations (bottom).

(B) PI3K assays performed on proteins eluted from the anti-(EE)-immunoprecipitates. The immunoprecipitated and eluted protein fractions were incubated with liposomes containing PtdIns(4,5)$P_2$ in the presence or absence of Gβγ and [γ³²P]-ATP. [³²P]-labeled phosphoinositide products were extracted, deacylated, and resolved on TLC plates. The data shown here are a representative experiment of three and are presented as the means (n = 3, ± SE) from a densitometric analysis of primary autoradiographs.

(C) Characterization of Gβγ activation of p84/p110γ. The PI3K activity of p84/p110γ or p101/p110γ was determined in the presence of a range of Gβ$_1$γ$_2$ concentrations. The data presented are means Dpms (n = 3 ± SE).

lation in the presence of p110γ and Gβ$_1$γ$_2$. The extent of augmentation by p84 was significantly less than that with p101. Western blots were performed to assess the quantity of the (EE)-proteins expressed in the experiments and revealed a similar expression of (EE)-p84 and (EE)-p101 (Figure 3B).

We further tested whether endogenous p84 could interact with endogenous p110γ in mouse neutrophils. Using anti-p84 antiserum, we immunoprecipitated the endogenous p84 from neutrophil cytosolic fractions and performed kinase assays with liposomes containing PtdIns(4,5)$P_2$ and [γ³²P]-ATP (Figure 4A). For controls, we performed immunoprecipitations with a preimmune antiserum (from the animal that generated the anti-p84 antibody) and an anti-p101 antiserum and also used cytosolic fractions from the neutrophils of p110γ⁻/⁻ mice.

We could specifically immunoprecipitate PI3K activity from murine neutrophil cytosol fractions with anti-p84-antiserum (Figure 4B). Parallel immunoprecipitations from matched cytosolic fractions, prepared from p110γ⁻/⁻ murine neutrophils, failed to specifically immunoprecipitate any PI3K activity (i.e., the activity detected was the same as the preimmune control). This suggests endogenous p84 is associated with a PI3K activity that is due to p110γ. Examination of the relative extents of immunoprecipitation of p84, p101, and p110γ

and comparison of the PI3K activity that (via their depletion from the cytosolic fractions) appeared in these immunoprecipitates suggests the major p110γ adaptor in murine neutrophils is p101.

**Conclusions**

We previously cloned and characterized p101, the hitherto only known regulatory subunit of p110γ [7]. Here, we present data describing a second regulatory subunit of p110γ, p84. Both regulatory subunits are mainly expressed in cells of haematopoietic origin. Based on its primary structure p84 has low homology with p101. Indeed, only 30% identity was found between the two proteins, mainly in the N- and C-terminal ends. However, previous work in our laboratory showed that these two areas were important for the interaction of p101 with p110γ and that the N terminus of p101 was absolutely required for the Gβγ activation of p110γ [11].

In this report, we have demonstrated that p84 can bind to p110γ in vitro and in vivo and by doing so substantially augmented the activation of p110γ by Gβγs. At present, we can only speculate about the possible significance of there being two potential regulatory subunits for p110γ. The fact that both p84 and p101 have been faithfully conserved and yet can be found expressed in the same cell types suggests that they contribute significantly different properties to p110γ
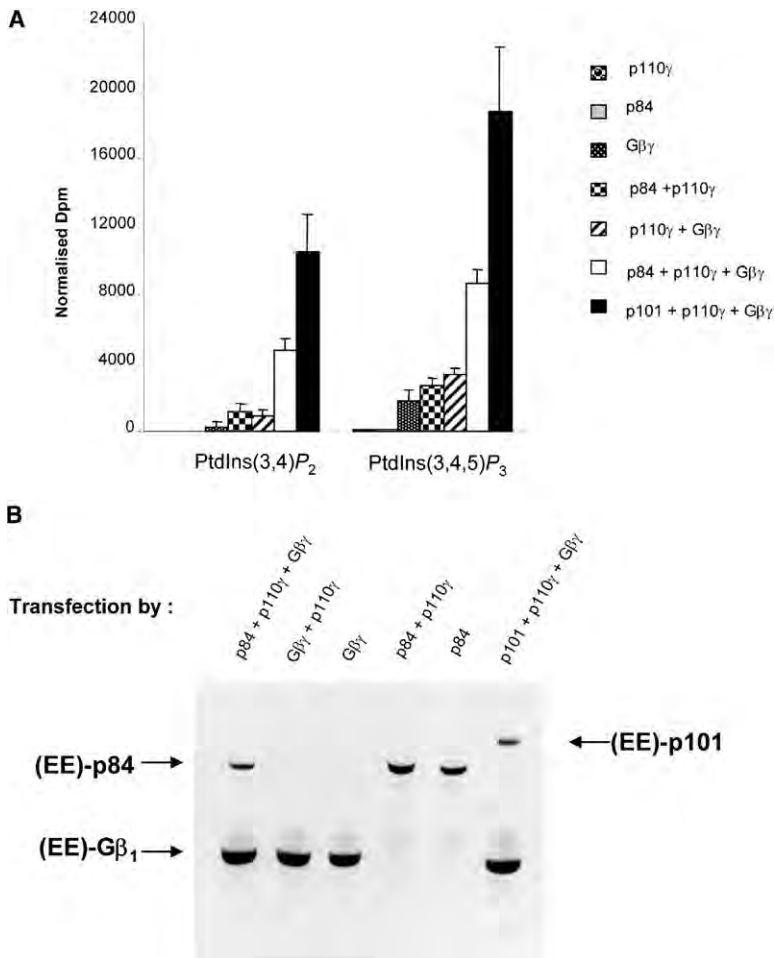
**A**



**B**

Transfection by :

(EE)-p84 →

(EE)-Gβ₁ →

→ (EE)-p101

**Figure 3. p84 Binds to p110γ In Vivo**

(A) In vivo activation of (EE)-p84/(Myc)-p110γ by Gβγ. COS-7 cells were transfected with expression vectors containing the indicated cDNAs. 40 hr posttransfection, the cells were serum starved for 6 hr then labeled with [$^{32}$P]-Pi for 90 min. Phosphoinositides were extracted, deacylated, and resolved by HPLC and quantified by scintillation counting. The data presented are means (n = 3 or 4, ± range, pooled from two independent experiments) and were normalised via the [$^{32}$P]-PtdIns(4)$P$ produced during the assay. (B) Western blots of total cell lysates (from replicate dishes of cells to those which were [$^{32}$P]-Pi-labeled) with anti-(EE) antibodies, showing expression levels of (EE)-p84, (EE)-p101, and (EE)-Gβ₁.

heterodimers. Precedent in the signaling field indicates this sort of diversity underpins additional specific signaling connections. For example, by providing a different membrane-targeting address, PtdIns(3,4,5)$P_3$ might be produced in a distinct compartment; changed membrane association could also enable different kinetics of activation of PtdIns(3,4,5)$P_3$ accumulation, or alternatively, a different pattern of sensitivity to Gβγ species might enable coupling to other receptors.

Because PI3Kγ is also regulated by Ras-GTPases, further investigations should assess the effect of p84 on p110γ activity in the presence of GTP-Ras [12]. It also remains to be determined which of the many important physiological roles of p110γ depend on p101 and which on p84.

**Supplemental Data**
Supplemental Data include three figures and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/6/566/DC1/.

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**A**



**B**
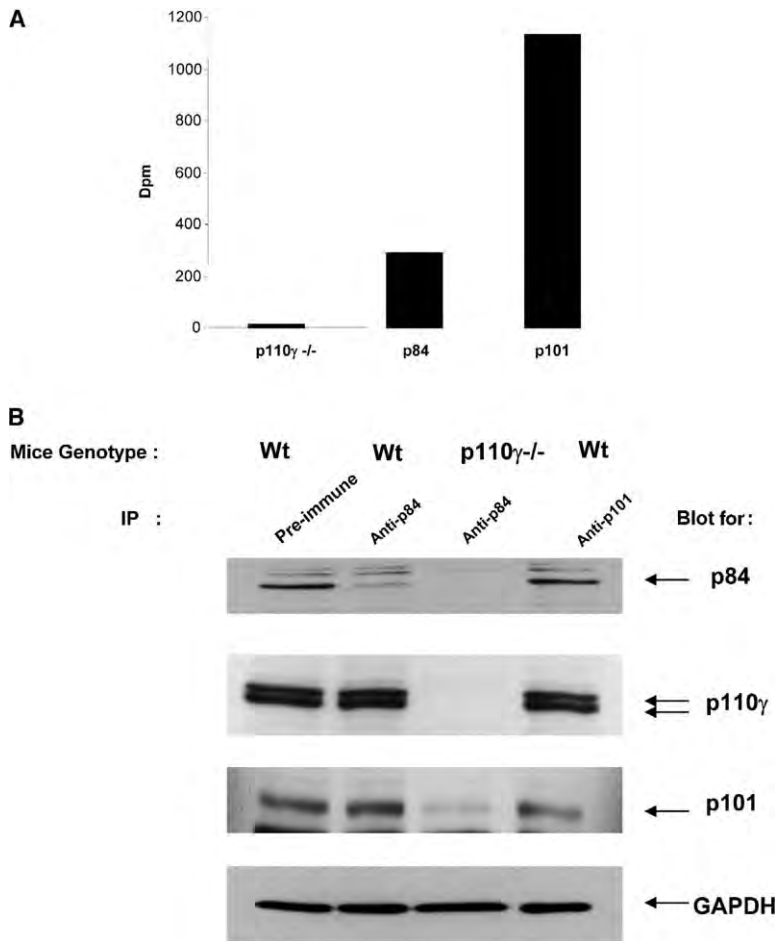


Figure 4. p84 Activates p110γ In Vivo

(A) Neutrophil cytosolic fractions from either wild-type (WT) or p110γ-deficient mice (p110γ$^{-/-}$) were immunoprecipitated with anti-p84 polyclonal antibody. As controls, the relevant preimmune serum or an anti-p101 polyclonal serum were used in parallel. The immunoprecipitates were subjected to PI3K assays with liposomes containing PtdIns(4,5)$P_2$ in the absence of Gβγs. The assays were stopped, extracted, deacylated, and resolved by HPLC and [$^{32}$P]-PtdIns(3,4,5)$P_3$ quantified by scintillation counting. Nonspecific "PI3K" activity, defined by parallel preimmune controls (227 dpm), was subtracted from the data presented in the figure. The data shown are from a representative experiment of two.

(B) Western Blots were performed on the supernatants of the immunoprecipitates described in (A) as indicated. Similar results were obtained in one further experiment.

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