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The goal of this project is to determine the role of the Src Homology 2 (SH2) domain protein, Grb7, in tyrosine kinase signal transduction. Grb7 is overexpressed in approximately 20% of breast cancers in combination with the HER2 receptor tyrosine kinase. Our initial goals of this project were to determine the role of Grb7 overexpression on breast cancer cell behavior as well as to identify proteins that interact with Grb7. Our initial studies have not indicated a specific phenotype induced by Grb7 overexpression or specific proteins that can associate with Grb7. In order to determine a function for Grb7, we are currently generating a mouse deficient in Grb7. In addition we have initiated a new project to study other SH2 domain proteins that may play a role in tyrosine kinases signal transduction in breast cancer. We describe our studies with the protein Shc and the identification of a new domain within Shc called the phosphotyrosine interaction/phosphotyrosine binding domain. We demonstrate that overexpression of this domain alone can block cell transformation mediated by certain oncogenes.

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Ben Mayhew 1/29/99

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INTRODUCTION

Our initial project goal for this grant was to determine a function for the Grb7 protein. Grb7 is amplified and overexpressed in breast cancer where it is bound to the HER2 protein (1). We have described the close similarity of Grb7 to a gene in *C. Elegans* called *mig-10* invloved in neuronal migration (2). In our initial years of the grant we had searched for the effects of Grb7 overexpression on the phenotype of either breast epithelial cells or kidney epithelial cells. Unfortunately, Grb7 overexpression has not resulted in a clear phenotype in these cells. Thus, the function of Grb7 continues to be uncertain. We have also proposed in the Statement of Work to search for proteins that interact with Grb7. In this year's report we describe additional studies searching for Grb7 interacting proteins. We plan to generate a Grb7 knockout mouse and in this report we provide the initial genomic cloning of Grb7 as well as construction of targeting vector that will be used to generate embryonic stem cells where Grb7 is targeted.

As was discussed in last years report, the work on Grb7 overexpression and interacting proteins has encountered difficulties. The reviewer of last year's report wondered if in view of these difficulties we might attempt to tackle additional topics. We also realized the difficulties and decided to explore additional topics related to Grb7 signaling. In our initial studies we found that Grb7 not only bound to HER2 but also to another tyrosine phosphorylated proteinsin breast cancer cells called Shc (1). Shc is an SH2 domain protein that binds to HER2 and other growth factor receptors (3). Once tyrosine phosphorylated Shc binds to Grb2 as well as Grb7 (1,4). This binding to Grb2 has been shown to be important for Ras activation and cell growth. Recently we identified a new domain in Shc that binds growth factor receptors and is called the phosphotyrosine interaction (PI) or phosphotyrosine binding (PTB) domain (5,6). This report will detail our studies on the role of Shc and the PI/PTB domains in cell transformation induced by the polyoma Middle T Antigen.

BODY

Methods

I. Grb7 Binding studies. To further search for Grb7 binding proteins, we examined epithelial cells for proteins that would co-immunoprecipitate with Grb7. We used MDCK cells because of the difficulties in establishing cell lines from nontransformed breast cancer cells. We used two labeling approaches to look for co-immunoprecipitating proteins; ^{32}P-Orthophosphate and ^{35}S-Methionine.

A. Orthophosphate labeling of MDCK cells

Confluent 10 cm dishes of wild type MDCK cells or MDCK cells transfected with myc tagged Grb7 were used for orthophosphate labeling. Cells were washed two times in phosphate free warm media. Media for this experiment was phosphate free Minimum Essential Media with 25 mM HEPES and 10% dialyzed Fetal Bovine Serum (FBS). After washing, phosphate free media containing 1mCi/ml ^{32}P, was added to each plate and the cells were incubated at 37^{0}C, 5% CO_2 for 3 hours. Cells were then washed twice with ice cold PBS and

lysed with 500 µl 1% Triton X-100 lysis buffer with protease and phosphatase inhibitors (7). After 30 minutes on ice, lysates were collected and spun 30 minutes, 4^0C, 15,000xg. Supernatants were harvested and 20 µg of antibody was added (anti-myc monoclonal 9E10; affinity purified anti-Grb7 polyclonal #222; 1,8) and incubated overnight at 4^0C. Protein A beads were added to the immunoprecipitation and incubated an additional 90 minutes at 4^0C. Protein A beads were pelleted, the supernatants removed, and the beads were washed six times in cold HNTG (150 mM NaCl, 0.1% Triton-X100, 10% glycerol, 50 mM HEPES, pH 7.5). 50 µl sample buffer was added to each condition, heated 10 min. at 90^0C and loaded onto a 10% SDS-PAGE gel. The gel was dried and exposed to film.

B. ^{35}S-Methionine labeling of MDCK cells

Subconfluent plates of MDCK cells stably expressing myc tagged Grb7 or Grb7 containing a CAAX box (see last year's report) were washed twice with phosphate buffered saline (PBS) and starved for one hour with methionine free Dulbeccos Modified Eagle Medium with 3% FBS. After one hour, fresh methionine free media containing 300 µCi ^{35}S-Methionine/plate was added and the cells were incubated overnight at 37^0C. Cells were washed two times in cold PBS and lysed in 400 µl HNTG with protease inhibitors. Cells were incubated 10 minutes on ice in lysis buffer before harvesting and then spun 15 minutes 15,000xg, and the supernatants collected. The supernatants were precleared with protein A sepharose beads for 1 hour at 4^0C on a nutator. Then 20 µgs of antibody (monoclonal anti-myc or polyclonal anti-Grb7 #222) was added. Immunoprecipitations were incubated at 4^0C overnight. Protein A beads were added and incubated an additional hour. The beads were pelleted and washed 3 times in cold lysis buffer, heated in sample buffer and loaded onto a 12% SDS-PAGE gel. After separation the gel was soaked in Amplify (Amersham) for 30 minutes. The gel was dried and exposed to film.

II. Genomic mapping of Grb 7.

A. Isolation and sequencing of mouse genomic clones.

To isolate mouse genomic clones we screened a 129SVJ genomic mouse library in the phage λFixII (Stratagene) with a polymerase chain reaction (PCR) probe from Grb7 (nucleotides 40-813). The PCR probe was labelled with ^{32}P-dCTP using a random primed labelling kit (Boehringer-Mannheim). The library was plated using E. coli XL1 Blue per manufacturer's instructions. Library screening was performed at 42^0C in 50% formamide as described (9). Positive plaques were picked and subjected to secondary and tertiary screening to obtain a pure phage. Phage DNA was purified using the Qiagen column lambda protocol per manufacturer's instruction. The phage DNA insert was removed by digestion with NotI and subcloned into Bluescript IIKS. Bluescript containing the Grb7 genomic clone was partially sequenced using Sequenase version II (US Biochemical Corp.) and the sequencing core at The University of Michigan. Primers used for sequencing were the same ones used to sequence the Grb7 cDNA as previously described (9).

B. Southern Blotting

Southern blotting was performed on Bluescript Grb7 clones and confirmed using genomic DNA from 129SVJ mice as well as original phage clones isolated from the 129SVJ library. The DNA was digested overnight with restriction enzyme at 37°C. An additional aliquot of the enzyme was added in the morning

and the DNA was digested for an additional 60 minutes. The DNA was separated on a 0.7% agarose gel, depurinated for 30 minutes, rinsed and denatured two times for 30 minutes and then neutralized three times as described (10). The DNA was transferred to Nytran overnight in 20X SSC and UV cross linked using 1800 joules. The membrane was air dried and then prehybridized for 1-2 hours at 42°C in 50% formamide, 6X SSC, 5X Denhardts, 1% SDS and 1 mg/ml salmon sperm DNA. The probe was then added, (2 million cpm/ml) in the prehyb buffer and hybridized overnight at 42°. The blots were washed at 0.1X SSC and 0.1% SDS at 42° and then exposed to film.

Genomic DNA was prepared from SVJ129 mouse liver. Liver was homogenized in TNES (10 mM Tris, 400 mM NaCl, 100 mM EDTA, 0.6% SDS), then 50 μg proteinase K was added and the sample was incubated overnight at 57°C. One ml of saturated sodium chloride was then added and vortexed. The sample was centrifuged at 2500 RPM for 15 minutes. The supernatant was collected and 2 volumes of 100% ethanol was added. DNA was spooled with a pasteur pipette, washed in 70% ethanol and dried. It was then redissolved in TE for subsequent digestion.

III. Role of the Shc PI/PTB domain in cell transformation.

More details on these studies can be found in reference 11 included in the appendix.

A. DNA constructs and expression vector

Using murine p52 Shc cDNA as a template, p52 ShcF198V was created by site-directed mutagenesis using the appropriate oligonucleotides and standard PCR (10). The Shc deletion mutants, Shc PI/PTB (Shc 1-238) and Shc PI/PTB+Y239/Y240 (Shc 1-260) were generated by PCR using standard techniques (10). Using the EcoR1 site, all constructs were then subcloned into pBABE-myc vector. pBABE-myc was generated by cloning a 92-bp insert encoding the myc-epitope tag amino acid sequence MEQKLISEEDLLEGSPGILD (8) into a retroviral expression vector pBABE, containing a puromycin resistance marker (12). The myc epitope is preceded by a Kozak sequence and followed by sites allowing cloning of cDNAs. All constructs were sequenced.

B. Generation of Retrovirus

Helper-free infectious retrovirus was produced by transiently transfecting the various wildtype and mutant pBABE p52 Shc constructs into the retroviral packaging cell line, Bosc 293, using Ca_2PO_4 precipitation (12,13). Resultant virus was used to infect NIH-3T3 cells stably transformed by MT. Infected cells were then placed under puromycin selection (2 μg/ml) for 10-14 days. Resistant clones were selected and expression of the appropriate construct was confirmed by immunoblotting with anti-myc or anti-Shc antibody. Pooled transformants were used in all experiments.

C. Cell Culture

NIH 3T3 cells stably transformed by MT were a gift from Dr. Sarah Courtneidge and have been described elsewhere (14). These cells were grown in Dulbecco's modified Eagle's medium with 10% calf serum, 50 U/ml penicillin, 50 ug/ml streptomycin and 0.5 mg/ml geneticin. The soft assay was carried out in standard six well tissue culture plates. Approximately 3 x 10^4 cells were seeded into an upper layer containing MEM 0.36% agar (Difco, Detroit, MI) supplemented with 10% calf serum. The colonies were photographed (20X magnification) after fourteen days.

Results and Discussion

Failure to detect Grb7 interaction with other proteins.

As of last year's report we had not completed studies searching for proteins that coimmunoprecipitated with Grb7. This was completed this year using epithelial cells transfected with various Grb7 constructs. MDCK cells were used for these studies as a model system due to the difficulty in generating non transformed breast epithelia overexpressing Grb7. Using wild type MDCK cells or MDCK cells that overexpressed myc tagged Grb7 we immunoprecipitated with myc antibodies or affinity purified anti-Grb7 antibodies and looked for proteins that were bound to Grb7 in ^{32}P-Orthophosphate labeled MDCK cells. Our criteria was that a true Grb7 binding protein would be coimmunoprecipitated with both anti-myc and anti-Grb7 antibodies in cells overexpressing myc-tagged Grb7. Similarly the coimmunoprecipitating protein should be more clearly seen in cells overexpressing Grb7 versus wild type MDCK cells. While we were easily able to detect Grb7 in these experiments, we were not able to detect any clear coimmunoprecipitating proteins using the criteria described above (Fig. 1). Similar results were found for ^{35}S-Methionine labeled cells in repeated experiments (Fig. 2). This result, in combination with other results we have previously reported, indicate that our initial goal of detecting proteins that interact with Grb7 was likely to be unsuccessful.

As this point we were faced with a dilemma. To complete our Statement of Work we needed either to find a phenotype in Grb7 overexpressing cells or identify proteins that interact with Grb7. However neither of these goals have been realized. However we were still very interested in determining the basic biological function of Grb7 and hope that we can then relate it to breast cancer. We decided not to pursue other protein-protein interaction approaches such as the yeast-two hybrid system because it would be very difficult to determine which proteins were true or false binding partners. This was because we had no assay to assess the activity of these binding partners and no proteins appeared to coimmunoprecipitate with Grb7. It was with this in mind that we turned to the Grb7 knockout approach detailed below. We strongly feel that this approach is much more likely to reveal the function of Grb7 than any other studies we could undertake at this point. We realize that the studies outlined below are a deviation from the initial Statement of Work but felt this was necessary to make meaningful scientific progress.

Mapping of Grb7 genomic DNA and generation of a targeting vector.

Several steps are required to generate a Grb7 knockout mouse. The first step was to determine the genomic organization of Grb7 in order to determine which exons could be deleted. We initially needed to isolate Grb7 genomic DNA from a mouse genomic library. After screening 1 x 10^6 plaques we isolated three phages that contained genomic DNA containing Grb7. The largest insert was 10 Kb which we excised from the phage DNA and subcloned into the Bluescript IIKS vector. This made the insert easier to sequence, restriction map and manipulate. Using primers from the original cDNA sequencing (9) we were able to map the first seven exon/intron boundaries and locate the start site ATG (see below Fig. 5). Restriction mapping of the Grb7 genomic insert in Bluescript gave the results shown in Fig. 3. Only one EcoR1 site was found which by

sequencing was found to be the EcoR1 site in the Grb7 cDNA just upstream of the stop codon at 1970. These results indicated that this 10 Kb genomic DNA contained the full length cDNA of Grb7 and would be suitable for generating a targeting vector. We prepared specific exon probes using a PCR based approach that contained Exon 1 alone or Exon 2, 3 and 4 combined. The Grb7 genomic DNA in Bluescript was cut with several restriction enzymes and probed with Exon 1 and Exon 2, 3 and 4 probes (Fig. 4). These types of studies allowed us to obtain a partial map of the Grb7 gene and the location of specific restriction sites (Fig. 5). The mapping was confirmed by southern blotting of mouse liver genomic DNA (not shown).

We prepared a targeting vector from this Grb7 genomic DNA to eliminate almost all the Grb7 coding sequence (Fig. 5). We used the targeting vector pPNT (15) which contains a *neo* gene for positive selection and Herpes Simplex Virus thymidine kinase (HSV-tk) gene for negative selection. We used a 2 Kb BamH1 fragment from the 3' prime end of Grb7 and cloned that downstream of the *neo* gene into the BamH1 site of pPNT. We took a 4 Kb NotI-NcoI fragment from the 5' end of the Grb7 gene and cloned that into the Not 1 site upstream of the *neo* gene. The NcoI site was converted to a NotI site by blunted ending with Klenow fragment and adding a NotI linker. In the our targeting vector, the NotI-NcoI fragment contained sequence upstream of the Grb7 cDNA as well as Exon 1 and a small piece of Exon 2. The BamH1 fragment contains a genomic sequence downstream of the Grb7 cDNA and contains no exons (Fig. 5). When our targeting vector undergoes homologous recombination with the endogenous Grb7 gene, it will remove almost all exons of Grb7. We will now proceed with the next steps in generating Grb7 knockout mice by electroporation of the pPNT-Grb7 plasmid into mouse ES cells and the selection of clones that have undergone homologous recombination.

Role of the Shc PI/PTB domain in cell transformation.

In view of the problems with Grb7, we realized that we could not continue to put all our efforts onto this protein. Thus, we began to use some of the money from the breast cancer grant to focus on another project that had relevance to tyrosine kinase signaling, breast cancer and cell transformation. These studies focused on Shc, another SH2 domain protein that signals downstream of HER2 and other receptor tyrosine kinases. Previous work in our lab had shown that Shc has PI/PTB domain in addition to an SH2 domain (5,6). After growth factor stimulation, Shc becomes tyrosine phosphorylated. We had shown that in breast cancer cells, tyrosine phosphorylated Shc can bind Grb2 as well as Grb7 (1). The goal of this specific project was to look at the role of the Shc PI/PTB domain in cell transformation. We used the polyoma Middle T antigen (MT) as a model of cell transformation by activated growth factor receptors (16). MT acts like an activated receptor tyrosine kinase once it complexes with Src family kinases at the cell surface. Once bound to these kinases it becomes tyrosine phosphorylated and binds a number of downstream signaling molecules including Shc. Middle T antigen has in the past proved to be a good model for breast cancer when expressed in the breast using the mouse mammary tumor virus promoter (17). Overexpression of middle T antigen in the breast leads to tumors with widespread metastases particularly in the lung. For this reason we pursued the role of the Shc PI/PTB domain in transformation by middle T antigen.

In this report we only present a summary of our studies on the Shc PI/PTB domain. The full story may be found in reference 11 included in the appendix. In this project we overexpressed myc tagged wild type Shc, full length Shc with a mutation in the PI/PTB (Shc F198V;18), the Shc PI/PTB domain alone or the PI/PTB domain plus Y239/Y240, tyrosine phosphorylation sites that can bind Grb2 (Fig. 6) in fibroblasts transformed by MT. Normally Shc is tyrosine phosphorylated in MT transformed cells and binds Grb2. We found that a mutation in the PI/PTB domain severely impairs the ability of full length Shc to bind to MT, to become tyrosine phosphorylated and to bind Grb2 (Fig. 7). This indicates the importance of the Shc PI/PTB domain in binding to MT and receptor tyrosine kinases. When overexpressed the Shc PI/PTB domain with Y239/240 can become tyrosine phosphorylated and bind Grb2. However the PI/PTB domain alone does not become tyrosine phosphorylated or bind Grb2 (Fig. 8). This suggested to us that overexpression of the PI/PTB domain alone might act in a dominant negative fashion to block transformation. The overexpression of the Shc PI/PTB domain with the Y239/Y240 sites can support transformation by MT (as assessed by a soft agar assay; Fig. 9) because it becomes tyrosine phosphorylated and binds Grb2 (Fig. 8). In contrast the PI/PTB domain alone blocks transformation (Fig. 9) because it does not become tyrosine phosphorylated or bind Grb2 (Fig. 8). Additionally the PI/PTB domain alone blocks the ability of endogenous Shc to bind to MT (11). In contrast while the Shc PI/PTB +Y239/Y240 also blocks the binding of endogenous Shc, it itself can couple to Grb2. This work demonstrates the importance of the PI/PTB domain in transformation by Shc. It also indicates that the PI/PTB domain could be a target for the development of novel therapeutic agents that would block the binding of Shc to activated tyrosine kinases.

CONCLUSIONS

Our results examining the overexpression of Grb7 have been unproductive in delineating a role for Grb7 in cell signaling. Further attempts to define proteins that bind to Grb7 have not been successful using a variety of techniques including expressing cloning as well as coimmunoprecipitation. Accordingly, we have decided to change directions and develop a Grb7 knockout mouse for the reasons described previously. We have made substantial progress in the last year in mapping the Grb7 genomic organization and developing a targeting vector. Our goal in the next year will be to generate ES cells with one copy of Grb7 deleted and to generate chimeric mice carrying these ES cells in their germline.

We also used our resources for the examination of other proteins crucial for cell transformation such as the Shc protein. Our work has identified a new domain known as the PI/PTB domain. We showed the crucial role of the Shc PI/PTB domain in cell transformation by MT antigen, a good model for receptor tyrosine kinase signaling. Our studies indicate that the PI/PTB domain might be a good target for the development of anti-cancer agents. Recently we have identified a new PI/PTB domain protein in epithelial cells that we call X11γ (19). We wish to examine the localization and function of X11γ in breast epithelium. Very interestingly, X11γ appears to be related to the Lin-10 gene, a gene that is very important in localizing growth factor receptors in epithelial cells (S. Kim,

personal communication). Thus, we will be looking at the localization of X11γ in breast cancer and its binding to other proteins. In this manner we will extend our studies on PI/PTB domain specifically to breast epithelia.

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APPENDIX

Figure Legends

Figure 1. Lack of Grb7 coimmunoprecipitating proteins in ^{32}P-Orthophosphate labeled cells. Wild-type MDCK cells or MDCK cells overexpressing a myc tag Grb7 were labeled with ^{32}P-Orthophosphate as indicated in the Methods Section. After labeling the cells were lysed in 1% Triton X-100 lysis buffer and immunoprecipitated either with 9E10 anti-myc monoclonal antibody or anti-Grb7 antibody (affinity purified #222). After immunoprecipitation overnight, protein A beads were added for 1 hour at 4^0C. The beads were then washed, incubated with sample buffer and run on an SDS-gel. The gel was then dried and exposed for autoradiography.

Figure 2. Immunoprecipitation of Grb7 from ^{35}S-Methionine labeled MDCK cells. Wild-type MDCK or MDCK cells containing myc tag Grb7 or CAAX box Grb7 were labeled with ^{35}S-Methionine, immunoprecipitated and run on an SDS-gel as described in Methods and in Figure 1. The CAAX box Grb7 contains Grb7 with the Ras CAAX box added at its C-terminus as well as a myc tag (see last year's report). Although many bands are seen in this gel, there is no clear coimmunoprecipitating protein seen more distinctly in Grb7 overexpressing cells and with both anti-myc and anti-Grb7 antibodies.

Figure 3. Restriction digest of a 10 kilobase insert containing Grb7 genomic DNA. Grb7 genomic DNA was excised from λFIXII and was subcloned into Bluescript IIKS using NotI. This Bluescript plasmid was then cut with a variety of restriction enzymes as indicated and run on a 0.7% agarose gel. The agarose gel was then stained with ethidium bromide and photographed under UV light.

Figure 4. Southern blotting of the Grb7 genomic DNA The Bluescript DNA containing the 10 Kb Grb7 genomic insert was digested as in Fig. 3. Southern blotting was performed as described in the Methods Sections. Two probes were utilized, an Exon 1 probe or a probe containing Exons 2,3 and 4. Using this approach we were able to generate a map of the Grb7 genomic DNA as indicated in Figure 5. One tenth the DNA used in Figure 3 was used for southern blotting.

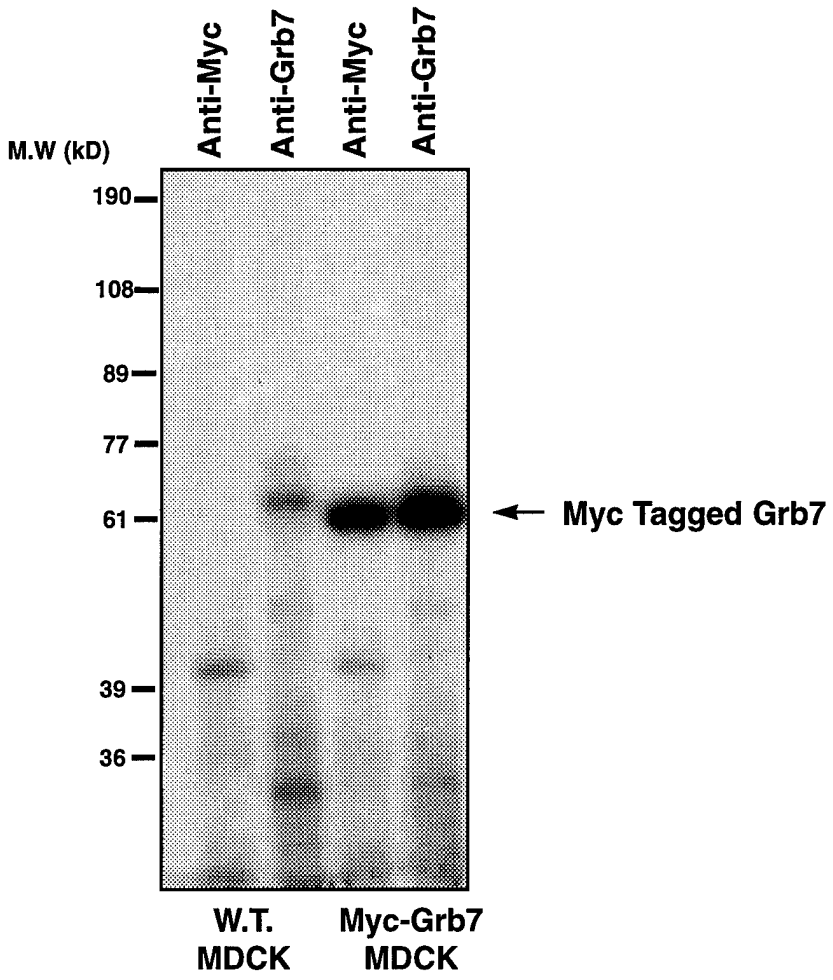
Figure 5. Genomic organization of the Grb7 gene. Using a combination of sequencing and Southern blotting as shown in Figure 4, we were able to construct this map of the Grb7 genomic sequence (upper figure). We mapped the first seven exons on the Grb7 gene but we did not map the remaining ones as it was not crucial for generation of the targeting vector. The genomic map was then used to generate the targeting vector in pPNT (lower figure). A 4 Kb piece from the 5' end of Grb7 was put upstream of the *neo* gene by cloning a NotI-NcoI fragment into a NotI site on the vector using linkers. A BamHI fragment from the 3' end of the Grb7 gene was put downstream of the *neo* gene. With the exception of Exon 1 and a small fraction of Exon 2, this targeting vector will replace all remaining exons with the *neo* gene.

Figure 6. Schematic diagram depicting Shc constructs used in these studies. The proteins indicated were expressed in MT transformed NIH 3T3 cells as indicated in the methods section. F198 is an amino acid residue crucial for the binding of the PI/PTB domain to phosphopeptides which, when mutated to valine (F198V), abrogates this binding ability (18). Y239/Y240 and Y317 are the major Shc tyrosine phosphorylation sites (20). These constructs were cloned into pBABE-myc, a retroviral expression vector which allowed an inframe amino-terminal myc tag (12). Helper-free retrovirus was produced as described in the methods section and used to infect NIH 3T3 cells stably transformed by MT. Clonal cell lines were selected using puromycin (2 μg/ml, Sigma) as the selectable marker. Abbreviations: PI/PTB, phosphotyrosine interaction/phosphotyrosine binding domain; SH2, src homology 2 domain; CH1, collagen homology 1 domain

Figure 7 p52 Shc carrying the F198V mutation is weakly phosphorylated and does not bind Grb2 or MT. NIH 3T3 cells stably transformed by MT and expressing either p52 Shc or p52 ShcF198V were lysed and immunoprecipitated with the anti-myc antibody (A, B, and C) or anti-MT antibody (D). Immune complexes were then separated by SDS-PAGE and transferred to nitrocellulose. The membrane was then probed with anti-phosphotyrosine antibody (A), anti-Grb2 antibody (B), and anti-Shc antibody (D). In the case of immunoprecipitation with the anti-myc antibody, membranes were stripped and reprobed with the anti-myc antibody to ensure equal and efficient immunoprecipitation (C). The dark band running beneath myc-tagged p52 Shc in this and other figures represents the immunoglobulin heavy chain and possibly tyrosine phosphorylated MT.

Figure 8. Differential binding and tyrosine phosphorylation of Shc PI/PTB and Shc PI/PTB+Y239/Y240. NIH 3T3 cells stably transformed by MT and expressing either wildtype p52 Shc, Shc PI/PTB or Shc PI/PTB+Y239/Y240 were lysed and immunoprecipitated with the anti-myc antibody (A, B). Immune complexes were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was then probed with anti-phosphotyrosine antibody (A), and anti-Grb2 antibody (B). Cell lysates were separated by SDS-PAGE, transferred onto nitrocellulose and immunoblotted with the anti-myc antibody to verify protein expression (C).

Figure 9. Shc PI/PTB domain inhibits MT induced transformation. NIH 3T3 cells stably transformed by MT expressing either vector alone (A), Shc PI/PTB (B), Shc PI/PTB+Y239/Y240 (C), or Shc PI/PTB F198V (D) were seeded in soft agar at a density of 3×10^4 per well of a standard six well dish and supplemented every five days with 10% calf serum. Cell growth was assessed every two days and colonies were photographed (20 X magnification) after fourteen days.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Anti-Grb7
Anti-Myc
Anti-Grb7
M.W (kD)
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108
89
77
61
39
36
Myc Tagged Grb7
W.T.
MDCK
Myc-Grb7
MDCK

Figure 1

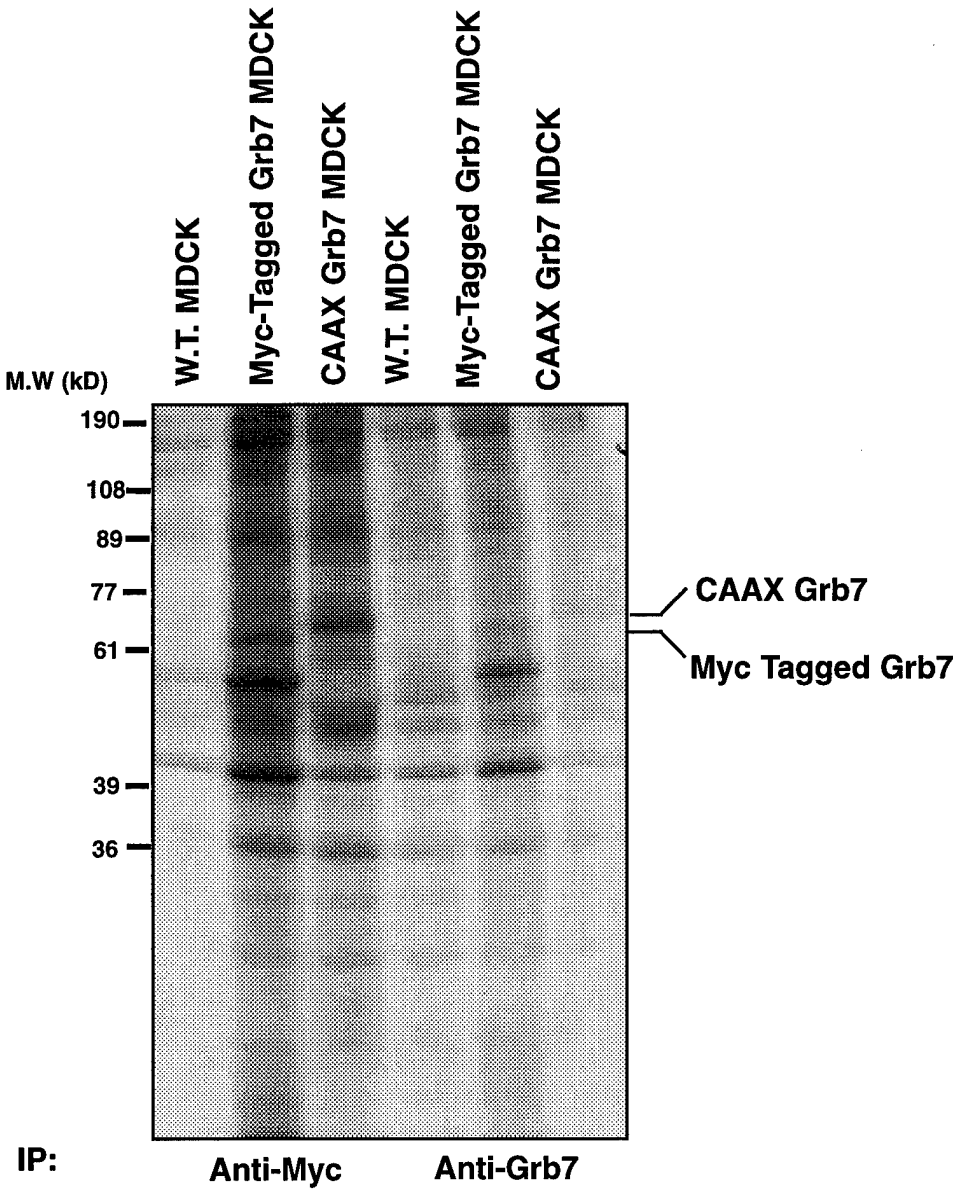

W.T. MDCK
Myc-Tagged Grb7 MDCK
CAAX Grb7 MDCK
W.T. MDCK
Myc-Tagged Grb7 MDCK
CAAX Grb7 MDCK
M.W (kD)
190
108
89
77
61
39
36
CAAX Grb7
Myc Tagged Grb7
IP:
Anti-Myc
Anti-Grb7

Figure 2

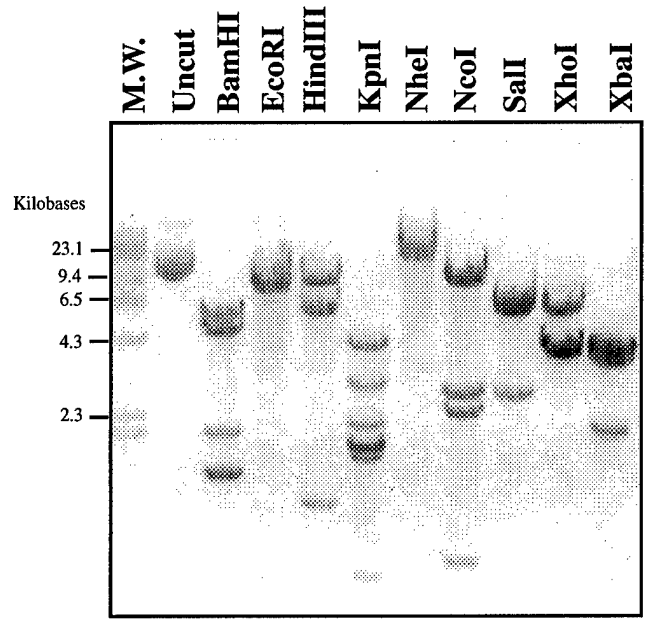


Figure 3

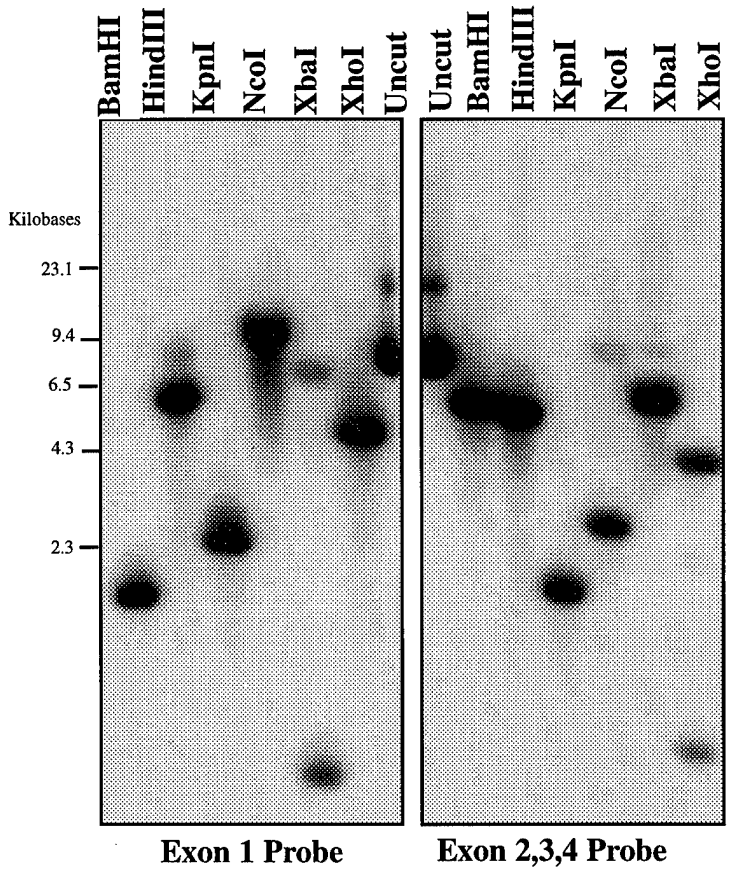


Figure 4

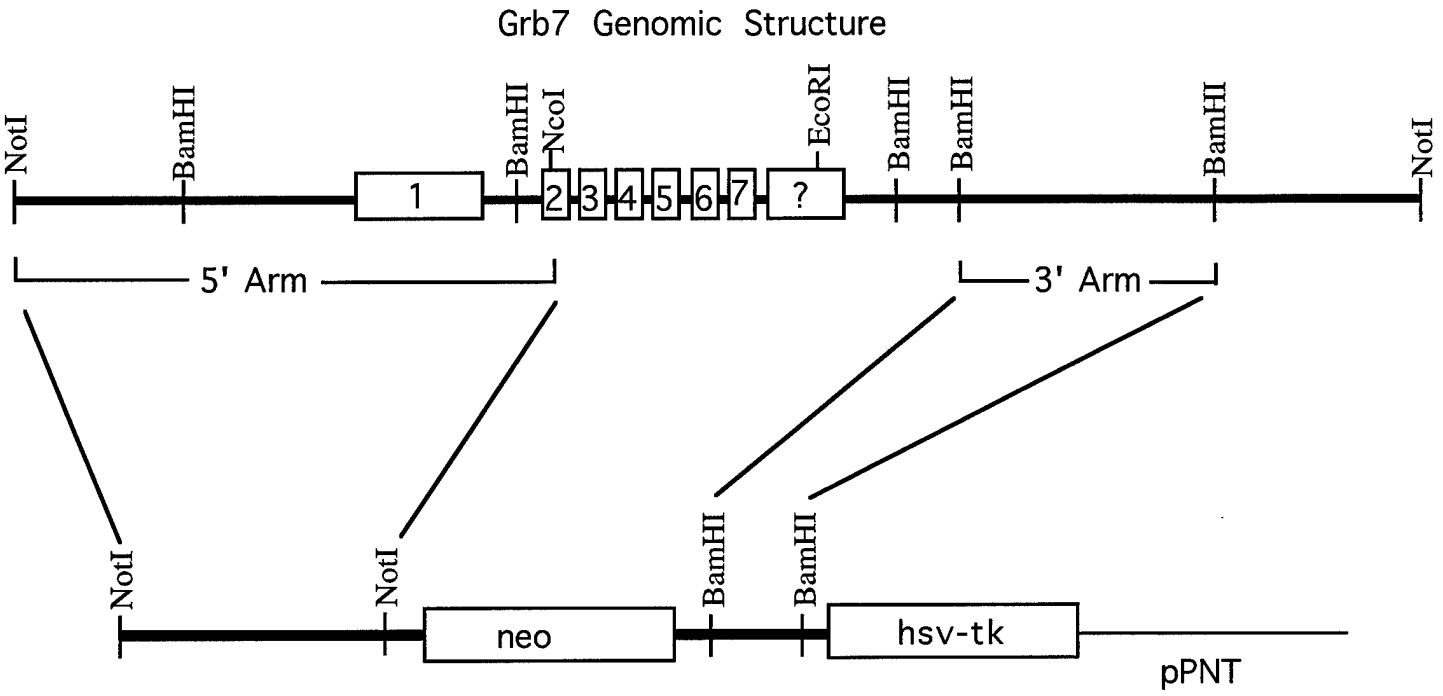


Figure 5

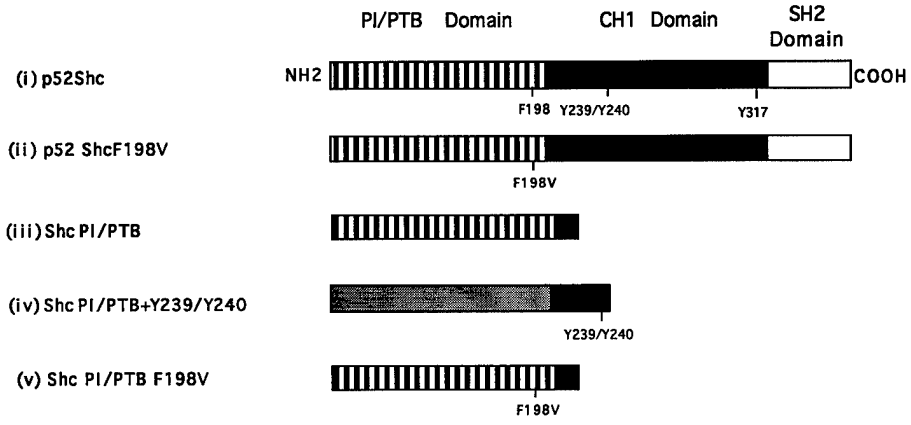
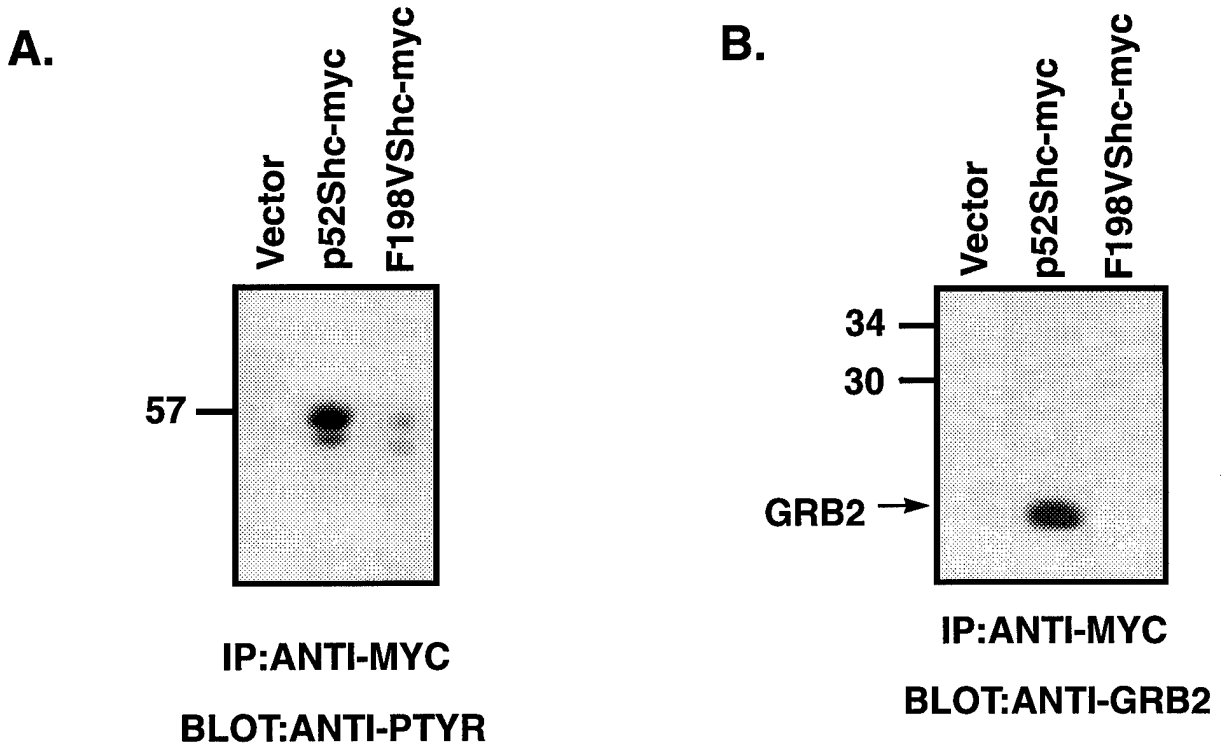


Figure 6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F198VShc-myc
34
30
GRB2
IP:ANTI-MYC
BLOT:ANTI-GRB2
C.
Vector
p52Shc-myc
F198VShc-myc
57
IP:ANTI-MYC
BLOT:ANTI-MYC
D.
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F198VShc-myc
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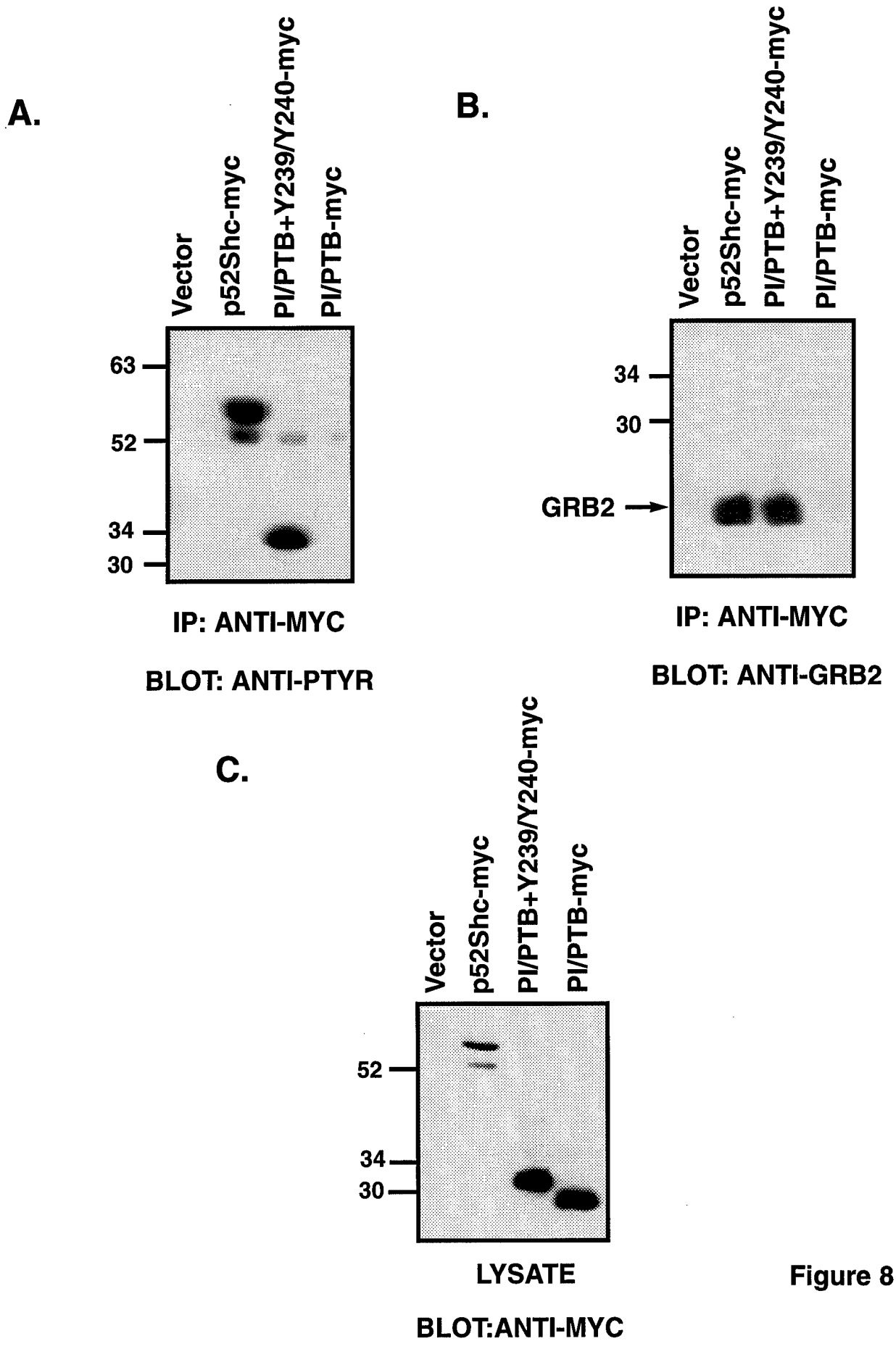
57
IP:ANTI- MT
BLOT:ANTI-SHC

Figure 7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BLOT: ANTI-PTYR
B.
Vector
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PI/PTB+Y239/Y240-myc
PI/PTB-myc
34
30
GRB2
IP: ANTI-MYC
BLOT: ANTI-GRB2
C.
Vector
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PI/PTB+Y239/Y240-myc
PI/PTB-myc
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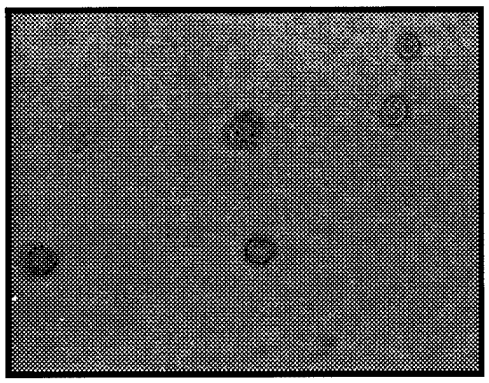
34
30
LYSATE
BLOT:ANTI-MYC

Figure 8



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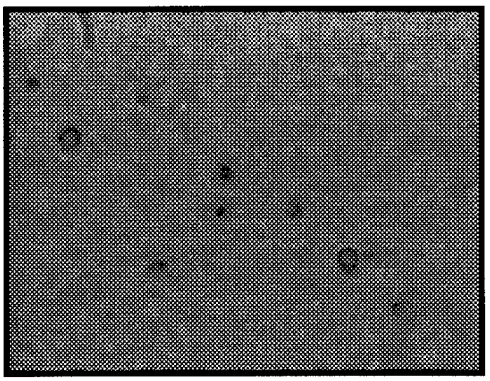

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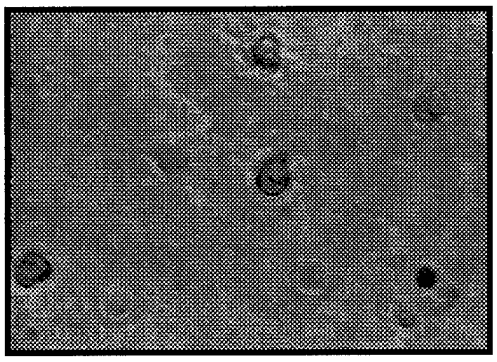
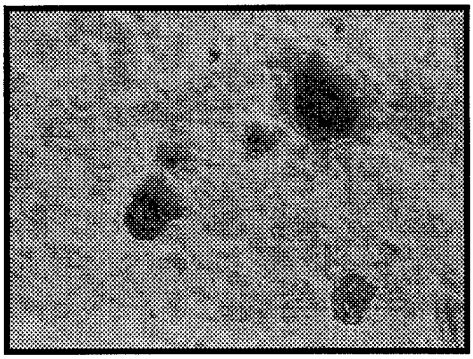


C. Shc PI/PTB+
Y239/Y240



D. Shc PI/PTB
F198V

Figure 9

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The Role of the Shc Phosphotyrosine Interaction/Phosphotyrosine Binding Domain and Tyrosine Phosphorylation Sites in Polyoma Middle T Antigen-mediated Cell Transformation*

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The phosphotyrosine interaction (PI)/phosphotyrosine binding (PTB) domain of Shc binds specific tyrosine-phosphorylated motifs found on activated growth factor receptors and proteins such as polyoma virus middle T antigen (MT). Phenylalanine 198 (Phe^{198}) has been identified as a crucial residue involved in the interaction of the Shc PI/PTB with phosphopeptides. In NIH 3T3 cells expressing MT, p52 Shc carrying the F198V mutation is weakly phosphorylated and does not bind MT or Grb2. Overexpression of the PI/PTB domain alone as Shc amino acids 1–238 acted in a dominant interfering fashion blocking MT-induced transformation. However, expression of a slightly longer construct, Shc 1–260, which encompasses Tyr^{239}/Tyr^{240}, a novel Shc tyrosine phosphorylation site, did not block transformation. This was found to be due to the ability of Shc 1–260 to become tyrosine-phosphorylated and bind Grb2. Furthermore, full-length Shc in which Tyr^{239}/Tyr^{240} had been mutated to phenylalanine did not become tyrosine-phosphorylated or bind Grb2 but did inhibit colony formation in soft agar. Conversely, p52 Shc carrying a mutation in the other tyrosine phosphorylation site, Tyr^{317}, became heavily tyrosine-phosphorylated, bound Grb2, and gave rise to colonies in soft agar.

Shc is a ubiquitously expressed adaptor protein that exists in three different isoforms, p46 Shc, p52 Shc, and p66 Shc, which differ only in the extent of their amino-terminal sequences. The p46 and p52 isoforms arise by alternate translational start sites, whereas the p66 form is generated by alternate splicing (1). Shc becomes tyrosine-phosphorylated after cell stimulation with a wide variety of growth factors and cytokines (1–5). It is also phosphorylated in cells expressing activated nonreceptor tyrosine kinases (6, 7) and other tyrosine-phosphorylated proteins (8, 9). p52 Shc contains an amino-terminal PI[1]/PTB domain (10–13), a central collagen homology domain (CH1), and a carboxyl-terminal SH2 domain (1). The CH1 domain contains what was previously thought to be the principal Shc tyrosine phosphorylation site, Tyr^{317} (1), and the more recently identified tyrosine phosphorylation sites, Tyr^{239}/Tyr^{240} (14, 15). Shc overexpression results in transformation of fibroblasts (1) as well as differentiation of PC12 cells and is implicated in activating Ras via its association with Grb2/Sos (16).

Shc has a unique ability to interact with tyrosine-phosphorylated proteins bearing the sequence ΨXNPXpY (9, 17, 18), a sequence that is present in many Shc-associated proteins. This interaction is unusual for SH2 domains that usually select specificity based on amino acids carboxyl-terminal to the phosphotyrosine (19, 20). This contradiction was resolved when it was determined that Shc bound to the ΨXNPXpY motif through the amino-terminal PI/PTB domain rather than through its carboxyl-terminal SH2 domain (21–26). The PI/PTB domain represents a novel protein binding domain that has been identified in other proteins (27) suggesting a general role for this domain in protein-protein interactions and signal transduction.

Random mutagenesis of the Shc PI/PTB identified phenylalanine residue 198 of the Shc PI/PTB domain as essential for binding to the ΨXNPXpY motif. Mutation of this residue to valine abrogated both the ability of the Shc PI/PTB domain to bind to the activated epidermal growth factor receptor (28) and the ability of p52 Shc to undergo phosphorylation by the insulin receptor (29). The importance of this residue in mediating Shc PI/PTB domain binding was confirmed by a recent structural analysis of the Shc PI/PTB domain which revealed the importance of the phenylalanine 198 in directly contacting the ΨXNPXpY motif by interacting with the hydrophobic amino acid at position −5 and the asparagine at −3 relative to the phosphotyrosine (30). It is interesting to note that phenylalanine 198 is conserved in the majority of other PI/PTB domain-containing proteins identified to date (28). Studies by other groups have indicated that in the case of the Shc PI/PTB domain, arginine 175, which is also evolutionarily conserved, is important for ligand binding and is involved in phosphotyrosine recognition (24, 30).

Middle tumor antigen (MT), the principal transforming protein of polyoma virus, has no intrinsic enzyme activity and exerts its transforming effect on cells by associating with and

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[1] The abbreviations used are: PI, phosphotyrosine interaction; PTB, phosphotyrosine binding; CH, collagen homology; SH2, Src homology 2; Ψ, hydrophobic residue; pY, phosphotyrosine; MT, middle T antigen; PAGE, polyacrylamide gel electrophoresis.

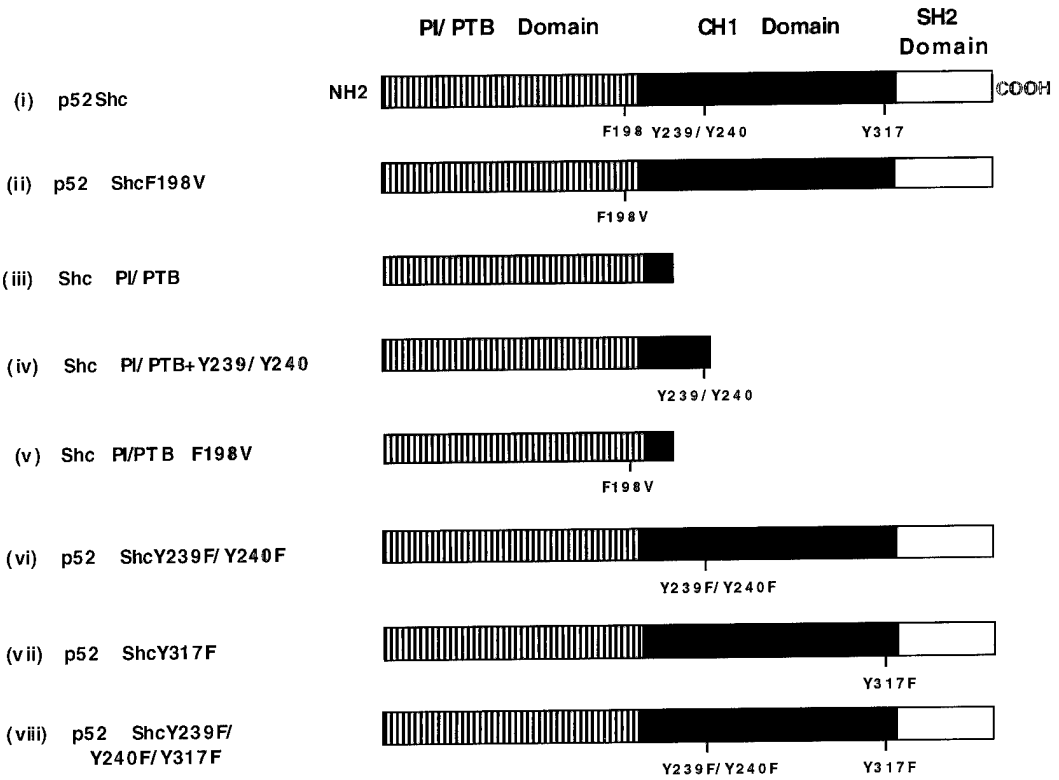

FIG. 1. **Schematic diagram depicting Shc constructs used in these studies.** Phe^{198} is an amino acid residue crucial for the binding of the PI/PTB domain to phosphopeptides which, when mutated to valine (F198V), abrogates this binding ability. Tyr^{239}/Tyr^{240} and Tyr^{317} are the major Shc tyrosine phosphorylation sites. These constructs were cloned into pBabe-Myc, a retroviral expression vector that allowed an in-frame amino-terminal Myc tag. Helper-free retrovirus was produced as described under "Experimental Procedures" and used to infect NIH 3T3 cells stably transformed by MT. Clonal cell lines were selected using puromycin (2 μg/ml, Sigma) as the selectable marker.

modulating the activities of various cellular proteins involved in cell proliferation such as c-Src, c-Fyn and c-Yes (32–34). Genetic analysis has revealed that the tetrameric sequence NPTY is an essential requirement for MT-mediated transformation (35, 36). This NPTY motif is required for the interaction between MT and Shc resulting in Shc tyrosine phosphorylation and association with Grb2/Sos, leading to Ras activation and cellular growth (9). Initially, the SH2 domain of Shc was implicated in this interaction (8). However, more recent data tend to suggest that the Shc PI/PTB domain is involved (22). In this study, our goal was to determine the exact role of the Shc PI/PTB domain in MT-mediated signal transduction.

EXPERIMENTAL PROCEDURES

DNA Constructs and Expression Vector—Using murine p52 Shc cDNA as a template, p52 ShcF198V was created by site-directed mutagenesis using the appropriate oligonucleotides and standard polymerase chain reaction (37). The Shc deletion mutants Shc PI/PTB (Shc 1–238) and Shc PI/PTB+Y239/Y240 (Shc 1–260) were generated by polymerase chain reaction using standard techniques (37). Shc tyrosine mutant constructs p52 ShcY239F/Y240F, p52 ShcY317F, and p52 ShcY239F/Y240F/Y317F were generated using the Transformer Mutagenesis Kit (CLONTECH, Palo Alto, CA). Using the *Eco*RI site, all constructs were then subcloned into pBabe-Myc vector. pBabe-Myc was generated by cloning a 92-base pair insert encoding the Myc epitope tag amino acid sequence MEQKLISEEDLLEGSPGILD (38, 39) into a retroviral expression vector pBabe, containing a puromycin resistance marker (40). The Myc epitope is preceded by a Kozak sequence and followed by sites allowing cloning of cDNAs. All constructs were sequenced with Sequenase version 2.0 (U. S. Biochemical Corp.).

Generation of Retrovirus—Helper-free infectious retrovirus was produced by transiently transfecting the various wild type and mutant pBabe p52 Shc constructs into the retroviral packaging cell line, Bosc 293, using Ca_2PO_4 precipitation (40–43). The resultant virus was used to infect NIH 3T3 cells stably transformed by MT (40, 44). Infected cells were then placed under puromycin selection (2 μg/ml) for 10–14 days. Resistant clones were selected, and expression of the appropriate construct was confirmed by immunoblotting with anti-Myc or anti-Shc antibody. Pooled transformants were used in all experiments. Attempts to obtain stable cells lines expressing the minimum Shc PI/PTB domain as Shc 1–209 were unsuccessful.

Antibodies—Anti-Myc monoclonal antibody 9E10 directed against a peptide with the sequence MEQKLISEEDLN was used for immunoprecipitation and immunoblotting and monoclonal anti-MT (PAb 762) antibody was used for immunoprecipitation. Polyclonal anti-Shc antibody used for immunoprecipitation was a gift from Dr. Ivan Dikic and Dr. J. Schlessinger (NYU Medical Center, New York), and the anti-Shc antibody used for immunoblotting was purchased from Transduction Laboratories (Lexington, KY). Anti-phosphotyrosine antibodies were prepared as described previously (45, 46). Anti-Grb2 antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA).

Cell Culture—NIH 3T3 cells stably transformed by MT were a gift from Dr. Sarah Courtneidge (Sugen, Redwood City, CA) and have been described elsewhere (47). These cells were grown in Dulbecco's modified Eagle's medium with 10% calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.5 mg/ml Geneticin.

Cell Lysis, Immunoprecipitation, and Western Blotting—Confluent dishes of cells expressing the various Shc proteins were washed with ice-cold phosphate-buffered saline and lysed in 1% Triton X-100 lysis buffer containing protease and phosphatase inhibitors (45). Lysate protein content was normalized using the Bio-Rad protein assay. Cell lysates were incubated for 2 h at 4 °C with the appropriate antibody that had been covalently bound to protein A- Sepharose beads (48). The beads were then washed three times in HNTG (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100), boiled in 1 × sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose. Immunoblotting was performed as described (45, 46). Blots were visualized using chemiluminescence (NEN Life Science Products).

Soft Agar Assay—This assay was carried out in standard six-well tissue culture plates. Approximately 3×10^4 cells were seeded into an upper layer containing modified Eagle's medium, 0.36% agar (Difco) supplemented with 10% calf serum. The colonies were photographed (magnification × 20) after 14 days.

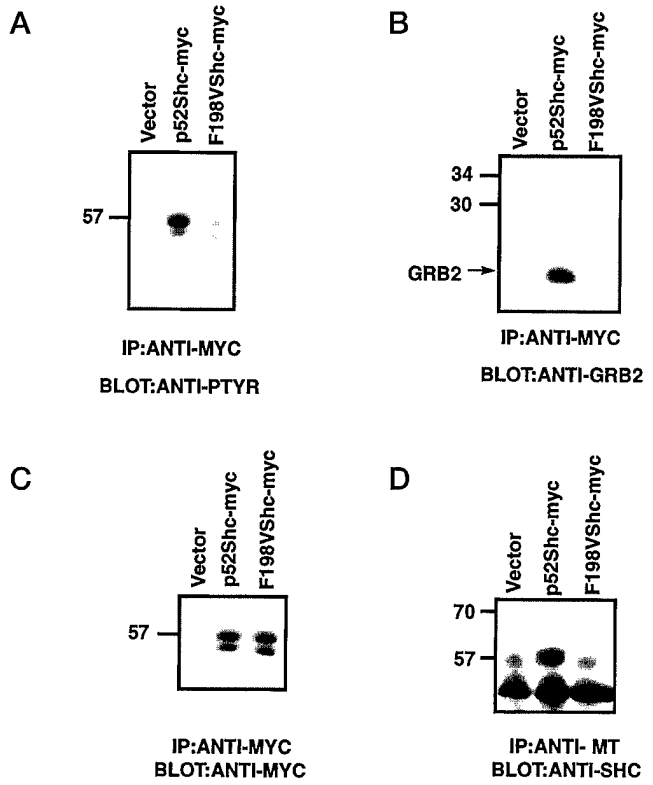

FIG. 2. **p52 Shc carrying the F198V mutation is weakly phosphorylated and does not bind Grb2 or MT.** NIH 3T3 cells stably transformed by MT and expressing either p52 Shc or p52 ShcF198V were lysed and immunoprecipitated with the anti-Myc antibody (*panels A, B,* and *C*) or anti-MT antibody (*panel D*). Immune complexes were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was then probed with anti-phosphotyrosine antibody (*panel A*), anti-Grb2 antibody (*panel B*), and anti-Shc antibody (*panel D*). In the case of immunoprecipitation with the anti-Myc antibody, membranes were stripped and reprobed with the anti-Myc antibody to ensure equal and efficient immunoprecipitation (*panel C*). The *dark band* running beneath Myc-tagged p52 Shc in this and other figures represents the immunoglobulin heavy chain and possibly tyrosine-phosphorylated MT.

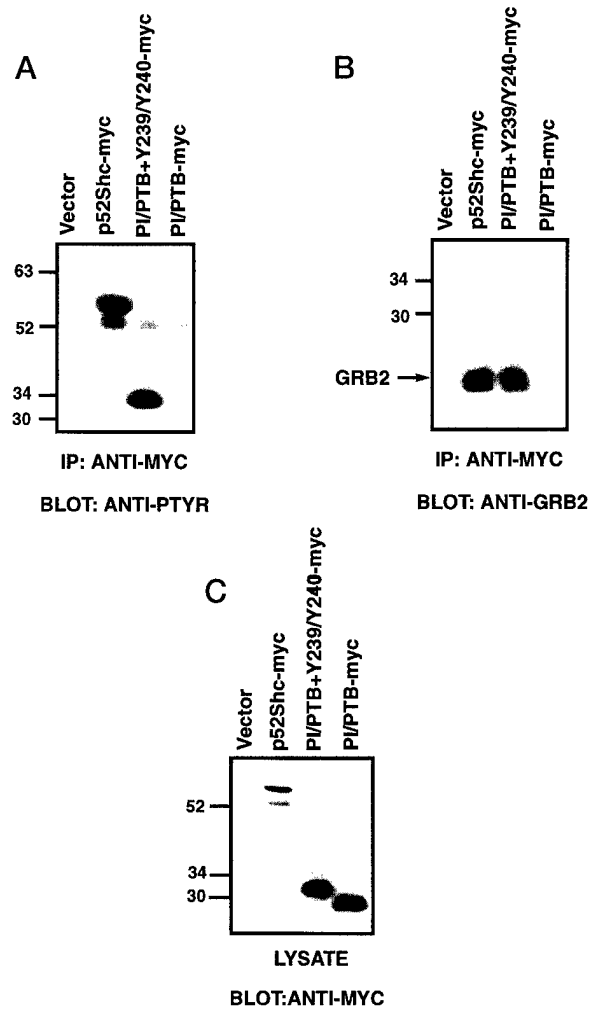

FIG. 3. **Differential binding and tyrosine phosphorylation of the Shc PI/PTB and Shc PI/PTB+Y239/Y240.** NIH 3T3 cells stably transformed by MT and expressing wild type p52 Shc, the Shc PI/PTB, or Shc PI/PTB+Y239/Y240 were lysed and immunoprecipitated with the anti-Myc antibody (*panels A* and *B*). Immune complexes were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was then probed with anti-phosphotyrosine antibody (*panel A*) and anti-Grb2 antibody (*panel B*). Cell lysates were separated by SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with the anti-Myc antibody to verify protein expression (*panel C*).

RESULTS

The Shc PI/PTB Domain Is Crucial for Shc Phosphorylation in MT-transformed Cells—To determine the importance of the Shc PI/PTB domain in MT signal transduction we undertook a comparative analysis of Shc tyrosine phosphorylation and Grb2 binding using wild type p52 Shc and p52 ShcF198V tagged with the Myc epitope (Fig. 1). High titer p52 Shc and p52 ShcF198V retroviruses were generated and used to infect NIH 3T3 cells stably transformed by MT. Clonal cell lines were developed and maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Cell lines were selected which had equal expression of transfected Shc wild type and mutant proteins as determined by immunoblotting. To determine the tyrosine phosphorylation status of wild type *versus* mutant Shc, cell lysates expressing either p52 Shc or p52 ShcF198V were immunoprecipitated with the anti-Myc antibody, resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with anti-phosphotyrosine antibody and anti-Grb2 antibody. Wild type p52 Shc became heavily tyrosine-phosphorylated and bound to Grb2, whereas p52 ShcF198V failed to become tyrosine-phosphorylated and did not bind Grb2 (Fig. 2, *A* and *B*). In both cases, membranes were stripped and reprobed with the anti-Myc antibody to ensure equal and efficient imunoprecipitation (Fig. 2*C*). To determine the ability of wild type and mutant Shc to bind MT, cells lysates expressing the respective proteins were lysed and immunoprecipitated with monoclonal anti-MT antibody. Immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the anti-Shc antibody. Wild type Shc coimmunoprecipitated with MT, whereas p52 Shc carrying the F198V mutation did not (Fig. 2*D*).

Shc 1–260 Becomes Tyrosine-phosphorylated and Binds to Grb2—The CH1 domain of Shc apparently provides a scaffold for further protein-protein interactions. Tyr^{317} is contained within the pYVNV consensus binding motif for the Grb2 SH2 domain (19, 20) and has traditionally been regarded as the major tyrosine phosphorylation and Grb2 binding site of Shc (1). More recent data have identified Tyr^{239}/Tyr^{240} (which are located in the CH1 domain just downstream of the Shc PI/PTB domain) as two novel and major tyrosine phosphorylation sites of Shc (14, 15). It is of interest to note that CH1 residues homologous to ShcY239 and ShcY240 are present in *Drosophila* Shc, whereas ShcY317 is absent (49). The fact that Tyr^{239}/Tyr^{240} are highly conserved in evolution suggests that the phosphorylation of these residues is of fundamental importance. In an effort to develop a dominant interfering Shc PI/PTB domain to study the role of the Shc PI/PTB domain in

TABLE I
Colony size of Shc transformants in soft agar

Quantitation of colony size from the soft agar assays displayed in Figs. 5 and 7 is shown. MT-expressing cells were infected with the Shc constructs, and pooled transformants were plated in soft agar as described under "Experimental Procedures." Results are from one soft agar plating but are representative of three separate experiments.

Infection	Mean colony size
	$\mu M \pm S.E.$
Vector	120 ± 8.2
p52 Shc	142 ± 9.7
p52 ShcY239F/Y240F	48 ± 3.9[a]
p52 ShcY317F	113 ± 8.8
p52 ShcY239F/Y240F/Y317F	47 ± 5.3[a]
Shc PI/PTB	38 ± 3.9[a]
Shc PI/PTB+Y239/Y240	115 ± 5.9
Shc PI/PTB F198V	115 ± 5.3

[a] $p < 0.001$ *versus* vector control in unpaired Student's t test.

MT-mediated signal transduction, we overexpressed Shc 1–238 (Shc PI/PTB) and Shc 1–260 (Shc PI/PTB+Y239/Y240) tagged with the Myc epitope. Shc PI/PTB and Shc PI/PTB+Y239/Y240 retroviruses were generated and used to infect NIH 3T3 cells stably transformed by MT which were then placed under puromycin (2 μg/ml) selection.

Clones expressing the desired protein were selected for further analysis. The Shc PI/PTB domain alone did not become tyrosine-phosphorylated (Fig. 3*A*) and did not bind to Grb2 (Fig. 3*B*). In contrast, the Shc PI/PTB+Y239/Y240 became heavily tyrosine-phosphorylated (Fig. 3*A*) and bound to Grb2 (Fig. 3*B*). In both cases, immunoblots were stripped and reprobed with the anti-Myc antibody to ensure equal and efficient immunoprecipitation (Fig. 3*C*). These findings suggest that Tyr^{239}/Tyr^{240} is an important Shc tyrosine phosphorylation site and a Grb2 binding site in MT-transformed fibroblasts.

Overexpression of Either the Shc PI/PTB or Shc PI/PTB+Y239/Y240 Inhibits the Binding of Endogenous Shc to MT and Grb2—To analyze further the role of the Shc PI/PTB domain in MT-mediated signal transduction, we investigated the ability of the Shc PI/PTB domain alone and the Shc PI/PTB+Y239/Y240 to interfere with endogenous p52 Shc signaling in MT-transformed NIH 3T3. Lysates from NIH 3T3 cells transformed by MT expressing either the Shc PI/PTB alone or Shc PI/PTB+Y239/Y240 were immunoprecipitated with anti-MT antibody (Fig. 4, *A* and *B*) or anti-Shc antibody (Fig. 4*C*). Immune complexes were separated by SDS-PAGE and immunoblotted with anti-Shc antibody (Fig. 4*A*), anti-phosphotyrosine antibody (Fig. 4*B*), and anti-Grb2 antibody (Fig. 4*C*). The anti-Shc antibody is directed against the Shc SH2 domain and does not immunoprecipitate the Shc PI/PTB proteins. Overexpression of the Shc PI/PTB domain alone or Shc PI/PTB+Y239/Y240 inhibited the binding of endogenous p52 Shc to MT (Fig. 4, *A* and *B*) and Grb2 (Fig. 4*C*). These data suggest that the Shc PI/PTB domain is directly involved in interaction with MT. When overexpressed in cells transformed by MT, it displaces endogenous Shc from MT and reduces the binding of endogenous Shc to Grb2.

Differential Effects of the Shc PI/PTB versus Shc PI/PTB+Y239/Y240 on Growth of MT-transformed Cells in Soft Agar—In an attempt to investigate the dominant interfering effect of the Shc PI/PTB domain on MT-induced transformation, we investigated the effect of overexpression of either the Shc PI/PTB or Shc PI/PTB+Y239/Y240 on the behavior in soft agar of NIH 3T3 cells stably transformed by MT. Cells were seeded at a density of 3×10^4/well in a six-well dish and supplemented every 5 days with 10% calf serum. Colonies were photographed after 14 days. Overexpression of the Shc PI/PTB domain acted in a dominant interfering fashion to inhibit MT-induced transformation (Fig. 5*B*). Overexpression of the Shc PI/PTB+Y239/Y240 did not inhibit transformation (Fig. 5*C*), presumably because of the ability of the Shc PI/PTB+Y239/Y240 domain to become tyrosine-phosphorylated and bind Grb2 (Fig. 3). Shc PI/PTB+Y239/Y240 overexpression resulted in colonies similar in size to those seen in uninfected cells (Fig. 5*A*). The inhibitory effect of the Shc PI/PTB domain on colony formation in soft agar was abrogated by the introduction of the F198V mutation (Fig. 5*D*, Table I).

Tyr^{239}/Tyr^{240} Are Crucial for Shc Binding to Grb2 in MT-transformed Cells—In an effort to determine the relative significance of the two Shc tyrosine phosphorylation sites, Tyr^{317}

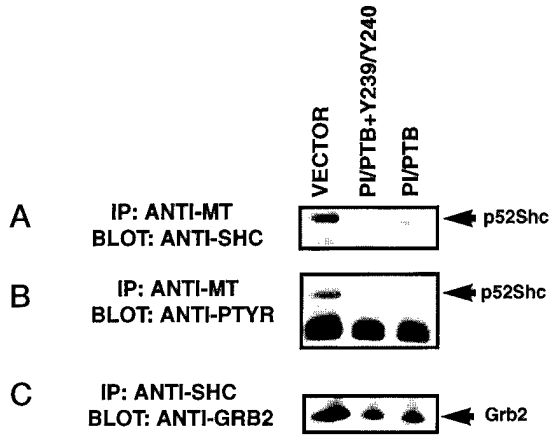

FIG. 4. **Overexpression of either Shc PI/PTB or Shc PI/PTB+Y239/Y240 in NIH 3T3 cells stably transformed by MT inhibits the binding of endogenous Shc to MT and Grb2.** NIH 3T3 cells stably transformed by MT and expressing either wild type Shc PI/PTB or Shc PI/PTB+Y239/Y240 were lysed and immunoprecipitated with anti-MT antibody (*panels A* and *B*) or anti-Shc antibody (*panel C*). Immune complexes were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was then probed with anti-Shc antibody (*panel A*), anti-phosphotyrosine antibody (*panel B*), and anti-Grb2 antibody (*panel C*).

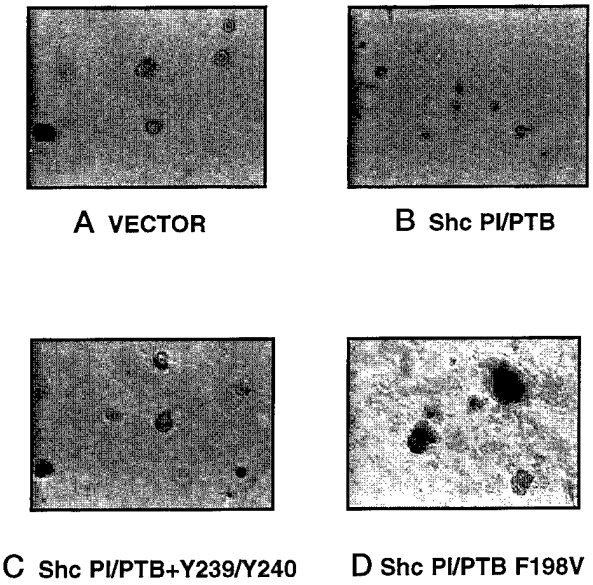

FIG. 5. **The Shc PI/PTB domain inhibits MT antigen-induced transformation.** NIH 3T3 cells stably transformed by MT expressing vector alone (*panel A*), the Shc PI/PTB (*panel B*), Shc PI/PTB+Y239/Y240 (*panel C*), or Shc PI/PTB F198V (*panel D*) were seeded in soft agar at a density of 3×10^4/well of a standard six-well dish and supplemented every 5 days with 10% calf serum. Cell growth was assessed every 2 days, and colonies were photographed (magnification × 20) after 14 days.

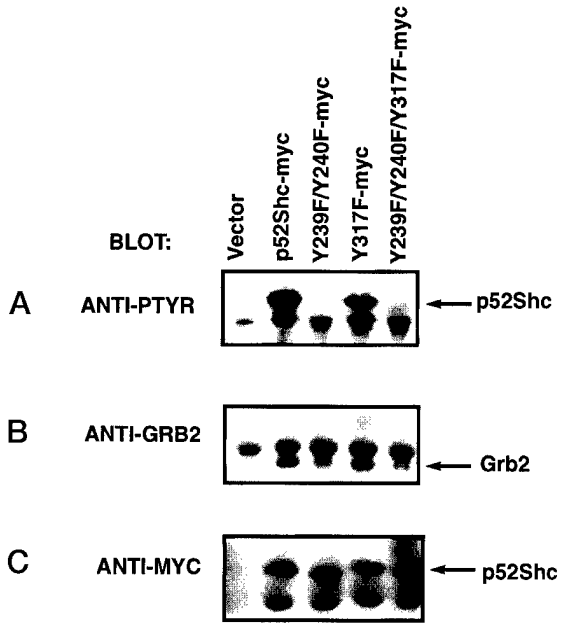

FIG. 6. **p52 Shc carrying the Y239F/Y240F mutation does not become tyrosine-phosphorylated and does not bind Grb2.** NIH 3T3 cells stably transformed by MT and expressing wild type p52 Shc, p52 ShcY239F/Y240F, p52 ShcY317F, or p52 ShcY239F/Y240F/Y317F were lysed and immunoprecipitated with the anti-Myc antibody. Immune complexes were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was then probed with anti-phosphotyrosine antibody (*panel A*), anti-Grb2 antibody (*panel B*), and anti-Myc antibody (*panel C*) to ensure equal and efficient immunoprecipitation.

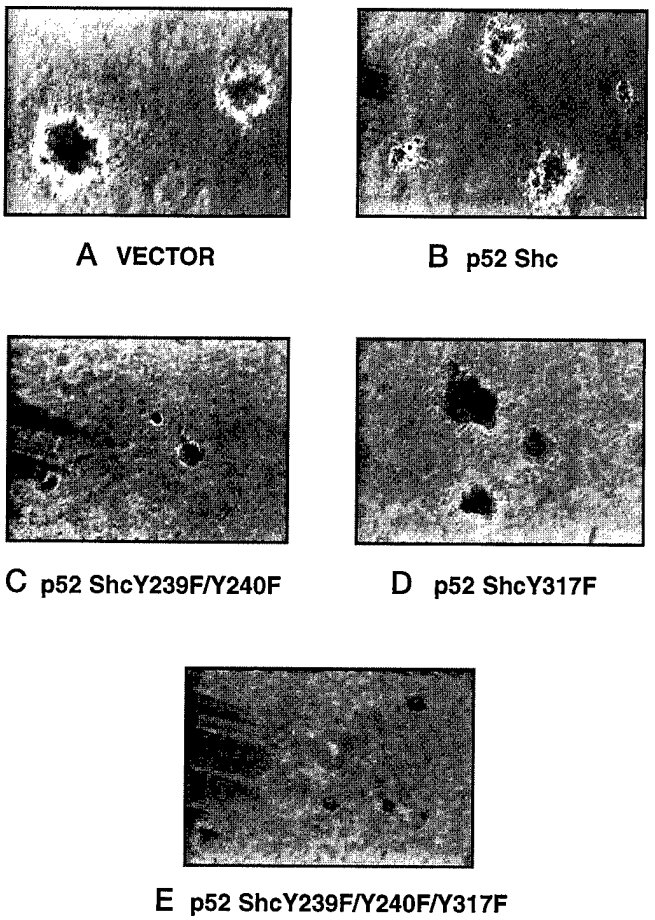

FIG. 7. **p52 ShcY239F/Y240F inhibits MT-induced transformation in soft agar.** NIH 3T3 cells stably transformed by MT expressing vector alone (*panel A*), wild type p52 Shc (*panel B*), p52 ShcY239F/Y240F (*panel C*), p52 ShcY317F (*panel D*), or p52 ShcY239F/Y240F/Y317F (*panel E*) were seeded in soft agar at a density of 3×10^4/well of a standard six-well dish and supplemented every 5 days with 10% calf serum. Cell growth was assessed every 2 days, and colonies were photographed (magnification × 20) after 14 days.

and the recently identified Tyr^{239}/Tyr^{240}, in MT signal transduction, the appropriate tyrosine residues were mutated to phenylalanine and incorporated into full-length p52 Shc. The resultant Myc epitope-tagged Shc constructs, p52 ShcY239F/Y240F, p52 ShcY317F, and p52 ShcY239F/Y240F/Y317F, were used to generate high titer retrovirus that was then used to infect NIH 3T3 cells stably transformed by MT. Cells were placed under puromycin selection (2 μg/ml) and screened for protein expression. Confluent dishes of cells were lysed and lysates subjected to immunoprecipitation with anti-Myc antibody. Immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine antibody (Fig. 6*A*), anti-Grb2 antibody (Fig. 6*B*), and anti-Myc antibody (Fig. 6*C*) to ensure equal and efficient immunoprecipitation. p52 ShcY317F behaved in a fashion similar to that of wild type p52 Shc, becoming heavily tyrosine-phosphorylated (Fig. 6*A*) and binding to Grb2 (Fig. 6*B*). In contrast, p52 ShcY239F/Y240F failed to become tyrosine-phosphorylated (Fig. 6*A*) and did not bind to Grb2 (Fig. 6*B*). p52 ShcY239F/Y240F/Y317F did not become tyrosine-phosphorylated (Fig. 6*A*) and did not bind to Grb2 (Fig. 6*B*). These data suggest that Y239F/Y240F is an important tyrosine phosphorylation site of p52 Shc and is essential for Grb2 binding and downstream signaling in the MT-mediated signal transduction.

Differential Effects of p52 ShcY239F/Y240F versus p52 ShcY317F on Growth of MT-transformed Cells in Soft Agar—To determine the effect of the Shc tyrosine mutations on cellular growth, we investigated the growth in soft agar of NIH 3T3 cells stably transformed by MT and expressing wild type p52 Shc, p52 ShcY239F/Y240F, p52 ShcY317F, or p52 Shc Y239F/Y240F/Y317F. Cells were seeded in soft agar and supplemented with 10% calf serum. Growth was assessed every 2 days and colonies photographed after 14 days. Cells overexpressing p52 Shc formed colonies in soft agar similar in size to those seen in cells infected with vector alone (Fig. 7, *A* and *B*). Cells overexpressing p52 ShcY317F also formed colonies similar in size to those seen with wild type p52 Shc (Fig. 7*D*). However, cells expressing p52 ShcY239F/Y240F either formed very small colonies or failed to form colonies at all (Fig. 7*C*). Cells expressing p52 Shc carrying all three tyrosine mutations also failed to form colonies in soft agar (Fig. 7*E*, Table I). These data suggest that the Tyr^{239}/Tyr^{240} tyrosine phosphorylation site of p52 Shc is crucial for signaling in the MT-transformed cells, and mutation of these residues to phenylalanine results in growth inhibition.

DISCUSSION

It is now well established that the Shc PI/PTB domain is a protein module that can mediate the formation of protein complexes via its recognition of specific phosphotyrosine-containing motifs (10, 21, 22, 27, 50). Amino acid residue Phe^{198} of the Shc PI/PTB domain has been identified as a crucial residue for binding of the Shc PI/PTB domain, and its mutation to valine abrogated the ability of p52 Shc to bind activated epidermal growth factor receptor (28) and inhibited Shc phosphorylation by the insulin receptor (29). The importance of this residue has been confirmed by structural analysis of the Shc PI/PTB domain complexed with the TrkA phosphopeptide, which revealed that Phe^{198} is important for establishing contact with

the hydrophobic residue at the −5 position and the asparagine at the −3 position relative to the phosphotyrosine (30).

We have analyzed the role of the Shc PI/PTB domain in MT-mediated signal transduction. By using the retroviral expression system, we have generated high titer retrovirus that was then used to infect NIH 3T3 cells stably transformed by MT. We have demonstrated that p52 Shc carrying the F198V mutation was poorly phosphorylated and failed to bind to Grb2 or MT, whereas wild type p52 Shc became heavily tyrosine-phosphorylated and coimmunoprecipitated with both Grb2 and MT. This was presumably the result of the nullifying effect of the F198V mutation on the binding ability of the Shc PI/PTB domain rendering p52 ShcF198V unable to bind to MT and therefore inhibiting its tyrosine phosphorylation and association with Grb2. When we overexpressed the Shc PI/PTB domain alone in MT-transformed, cells we found that it did not become tyrosine-phosphorylated, nor was it able to bind to Grb2. It did, however, inhibit the phosphorylation and binding of endogenous Shc to MT and Grb2. When we overexpressed the Shc PI/PTB+Y239/Y240, a construct encompassing the adjacent, recently identified Shc tyrosine phosphorylation site Tyr^{239}/Tyr^{240} (14, 15), we found that this protein became heavily tyrosine-phosphorylated and formed a complex with Grb2 in MT-transformed cells. To determine the effect on transformation *in vivo*, we used MT-transformed cells expressing either Shc PI/PTB or ShcPI/PTB+Y239/Y240 in a soft agar assay. Interestingly, we found that overexpression of the Shc PI/PTB alone acted in a dominant interfering fashion, inhibiting MT-induced transformation. However, the ShcPI/PTB+Y239/Y240 did not inhibit MT-mediated transformation. This was because of the ability of Shc PI/PTB+Y239/Y240 to become tyrosine-phosphorylated and bind Grb2. In additional studies using p52 Shc in which Tyr^{239}/Tyr^{240} and Tyr^{317} were mutated to phenylalanine either separately or together, we found that p52 ShcY239F/Y240F failed to become tyrosine-phosphorylated, did not bind to Grb2, and inhibited MT-mediated transformation on soft agar. In contrast, p52 ShcY317F behaved in a fashion similar to that of wild type p52 Shc, becoming heavily tyrosine-phosphorylated, binding to Grb2, and forming colonies in soft agar similar to those seen in uninfected cells. Taken together, these data confirm the importance of the Shc PI/PTB domain in MT-mediated signal transduction and, in addition, suggest an important role for Tyr^{239}/Tyr^{240} in Shc tyrosine phosphorylation and Grb2 binding.

Shc is an important adaptor protein responsible for linking many activated proteins to the Ras pathway via Grb2/Sos. The presence in Shc of both a PI/PTB domain and an SH2 domain presumably gives Shc the ability to interact with a large number of tyrosine-phosphorylated proteins in many different systems. Although we detect a very important role for the Shc PI/PTB domain in MT-induced transformation, we could detect little role for the SH2 domain in this system. A Shc construct containing residues 1–260 (Shc PI/PTB+Y239/Y240) transformed cells nearly as well as wild type p52 Shc, even though it lacked the SH2 domain. The presence of two Grb2 binding sites in mammalian Shc proteins adds to the complexity of this signaling molecule. It is possible that in some systems, such as the MT system, Tyr^{239}/Tyr^{240} may have the role as the major tyrosine phosphorylation and Grb2 binding site, whereas in other systems, Tyr^{317} is the main player (51, 52). It is also possible that in some systems, both may play a role. It is of great interest that Tyr^{239}/Tyr^{240} are conserved among the Shc family members (11, 31), suggesting a conservation of function. In contrast to Tyr^{317}, which is absent in *Drosophila* Shc, Tyr^{239}/Tyr^{240} is conserved in *Drosophila* Shc (49), suggesting that the function of Tyr^{239}/Tyr^{240} was established early in evolution and may be conserved between *Drosophila* and man, whereas the function of Tyr^{317} may have evolved more recently.

In conclusion, our data demonstrate that the Shc PI/PTB domain interacts with tyrosine-phosphorylated MT. This interaction is essential for the tyrosine phosphorylation of Shc which occurs predominantly at Tyr^{239}/Tyr^{240}. This phosphorylation is crucial for Shc binding to Grb2 and MT-induced transformation.

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