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The goal of this project was to determine the role of the Src homology 2 domain protein, Grb7, in receptor tyrosine kinase signal transduction. Grb7 is overexpressed in approximately twenty percent of breast cancers coordinately with the HER2 receptor tyrosine kinase. Grb7 binds to HER2 but the role of Grb7 in HER2 signaling is unknown. We have been unable to detect a phenotype in breast or kidney epithelial cells that overexpress Grb7. Grb7 deficient mice have also been generated as another approach to analyze Grb7 function. No obvious phenotypic abnormality has been observed in these Grb7 deficient mice including no deficits in development or fertility. Thus despite performing studies where Grb7 is overexpressed or Grb7 expression is eliminated, the true biologic function of this protein remains elusive. In a separate set of studies we have analyzed another HER2 binding protein called Shc. We have demonstrated a crucial role for the Shc phosphotyrosine binding domain in Shc mediated cell transformation.

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Ben Mazzeo 7/16/2000

PI - Signature Date

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INTRODUCTION

Receptor tyrosine kinases are known to play an important role in several types of human cancers. The receptor tyrosine kinase HER2 is felt to play an especially important role in breast cancer. It is a member of the Epidermal Growth Factor Receptor (EGFR) family of receptor tyrosine kinases that includes EGFR, HER3 and HER4. HER2 is overexpressed in approximately 20% of breast tumors and portends a poorer prognosis in patients with lymph node metastasis (1). In comedo type, ductal carcinoma in situ, HER2 overexpression occurs in greater than 80% of tumors indicating it is likely to be an initiating event (2). Effective new treatments for breast cancer have been directed at the HER2 receptor utilizing a monoclonal antibody (3). Once activated, the receptor phosphorylates itself as well as substrate proteins on tyrosine residues. Autophosphorylation of the receptors allows the receptor to bind a variety of SH2 domain proteins involved in signal transduction. Understanding signal transduction events initiated by HER2 may have important implications for breast cancer therapy.

The initial focus of this project was to study one of the proteins that binds to activated HER2, Grb7. Grb7 is one of the Grb family of SH2 domain proteins that was isolated by screening bacterial expression libraries with the autophosphorylated EGFR (4). It maps close to HER2 on human chromosome 17 and our group determined that it was coamplified and overexpressed with HER2 in breast cancer cell lines and a small subset of primary breast cancers (5). Grb7 is closely related to another SH2 domain protein that we have isolated and cloned called Grb10 (6). These proteins both have SH2 domains and a conserved central domain of 300 amino acids that we have termed the GM domain because it is found in Grbs and the *C. elegans* gene, Mig-10 (7, 8). The central region of the GM domain contains a pleckstrin homology (PH) domain (9). The PH domains have been shown to be involved in lipid and to a lesser extent protein interaction. The Mig-10 gene is required for embryonic migration of a sub population of neuronal cells. However unlike Grb7, Grb10 and a third related protein Grb14, Mig-10 does not have an SH2 domain. The GM domain also contains a very conserved 100 amino acid region just amino-terminal to the PH domain (10). Thus Grb7 and Grb10 are primarily composed of an SH2 domain and a GM domain. We know that the SH2 domain allows

Grb7 to interact with HER2 and other tyrosine phosphorylated proteins. We hypothesized that the GM domain was involved in protein/protein interaction and that this interaction was crucial for Grb7 and Grb10 signaling. The goal of our research was to determine the role of Grb7 in HER2 signal transduction in normal and pathologic states.

Another protein we examined during the course of our studies was Shc. Shc is an SH2 domain that signals downstream of HER2 (11). Shc becomes tyrosine phosphorylated after binding to growth factor receptors and then binds Grb2 (12). The binding to Grb2 through Sos leads to Ras activation (13). Activation of Ras is known to be crucial for receptor tyrosine kinases to signal growth. In studies funded by this grant we made unique observations into the mechanism by which Shc couples to growth factor receptors such as HER2.

BODY

Our work during the grant period could be divided into four major areas. These were:

1) Effects of Grb7 overexpression on epithelial cell function.
2) Search for binding partners for the Grb7 GM domain.
3) Generation of Grb7 knockout mice.
4) Characterization of the Shc PI/PTB domain and its role in cellular transformation.

1) Effects of Grb7 overexpression on epithelial cell function.

Our first step in trying to delineate the role of Grb7 in breast cancer was to overexpress Grb7 in the breast tumors cell line, MCF-7, and look for alterations in cellular behavior. This cell line expresses little or no endogenous Grb7. We transfected MCF-7 cells with a sense and antisense Grb7 construct using the PMJ-30 mammalian expression vector. The transfection was performed using aminoglycoside aminotransferase (neo) as a selectable marker. After selection in 400 μg/ml geneticin, cell lines were cloned and Grb7 expression determined by immunoblotting. Of the initial seven clones isolated, three had significant levels of expression. Once we had obtained these cells, our next goal was to co-express the EGFR/HER2 chimera with Grb7 in the MCF-7 cells. This chimera can be

activated by EGF and allows us to activate the HER2 kinase. To accomplish this task we took the MCF-7 Grb7 F4 cell line and transfected in the EGFR/HER2 construct using hygromycin B phosphotransferase as a selectable marker. We selected for cell lines that were both geneticin and hygromycin resistant. Unfortunately we were only able to obtain one cell line that expressed both Grb7 and EGFR/HER2 despite screening several clones. In this cell line we were able to confirm our previous results that the EGFR/HER2 chimera was present in Grb7 immunoprecipitates. In parallel we also produced cells that expressed only EGFR/HER2 chimera without Grb7. At this point we tested the four different types of MCF-7 cell lines we had generated; MCF-7 parental; MCF-7 with Grb7, MCF-7 with EGFR/HER2 and MCF-7 with EGFR/HER2 and Grb7. We examined the phosphotyrosine content of these different cell lines after activation with EGF (Fig. 1). It can be seen that those cells that overexpress EGFR/HER2 have increased tyrosine phosphorylation of Shc but Map kinase tyrosine phosphorylation is increased in all cell lines treated with EGF. The expression of Grb7 has no apparent effect on the pattern of phosphorylation and Grb7 itself does not appear to be tyrosine phosphorylated. We assayed the growth of these cells in soft agar in the absence and presence of EGF. We found that cells expressing the EGFR/HER2 construct formed larger colonies in soft agar but there was no effect of Grb7 on soft agar growth with or without EGFR/HER2. Another assay we performed was migration of cells in collagen gels. Again we did see an effect of EGFR/HER2 expression to increase migration in response to EGF but no effect was seen with Grb7 either alone or cotransfected with EGFR/HER2.

It seemed possible that because MCF-7 cells are already transformed, it was difficult to detect an effect of Grb7. Accordingly, we attempted to repeat these studies in MCF-10A cells. These mammary cells are not transformed and require the presence of growth factors such as EGF or IGF-1 to grow. We have found little or no endogenous Grb7 expression in these MCF-10A cells. Unfortunately we were unable to obtain MCF-10A cell lines that stably overexpressed Grb7. This was despite using the same techniques we had used to transfect MCF-7 cells. However we still felt it was important to overexpress Grb7 in normal epithelial cells and turned to the MDCK cell line. MDCK is a cell line from the dog renal epithelium that normally expresses Grb7. These cells represent a good experimental system because they form branching epithelial structures in collagen gels in response to Hepatocyte Growth Factor (HGF). Breast epithelial cells display a similar response to HGF and both MDCK and mammary epithelial cells migrate (scatter) in response to HGF (14, 15).

We used two different Grb7 constructs for the transfection of MDCK cells. The first was Grb7 with a myc tag at the amino-terminus. The second construct also had a

myc tag but in addition had a CAAX box to localize Grb7 to the plasma membrane. The CAAX box would mimic the effect of Grb7 binding to growth factor receptors by localizing Grb7 to the plasma membrane. By placing a CAAX box on Grb7 we could target the protein to the plasma membrane and possibly generate an activated form of Grb7 (Fig. 2). The addition of a CAAX box has resulted in the activation of several other signaling proteins (15, 16).

With these cell lines we have evaluated the effects of Grb7 and CAAX-Grb7 overexpression on behavior of MDCK cells. As was found with MCF-7 cells, neither Grb7 nor CAAX-Grb7 had an effect on cell growth. The cells had a normal epithelial appearance and no gross differences in transport ability could be detected as assessed by dome formation (transport of fluid under the cells causing them to form domes). We next analyzed the cells for scattering ability (migration) in response to HGF. Cells were plated at 25,000 cells per well of a six well plate and stimulated with 50 units/ml of HGF for 18 hours. We found no effect of Grb7 overexpression on the ability of cells to scatter in response to HGF or to form tubules (Fig. 3).

These series were very complete examining wild type and possibly activated forms of Grb7 in several different cell lines. It was unfortunate that despite several years were never able to identify a phenotype in epithelial cells that overexpressed Grb7.

2) Search for binding partners for the Grb7 GM domain.

As outlined in the initial Statement of Work, Specific Aim #2, studies were planned in the first year to identify the specific residues on HER2 that bound Grb7. Work from other studies have revealed the binding sites for Grb7 on tyrosine phosphorylated proteins other than HER2 (17, 18). One of these studies examined the binding to SH-PTP2, a tyrosine phosphatase while the other examined binding to Platelet Derived Growth Factor Receptor (in collaboration with our group). These studies both identify the sequence phosphotyrosine-X-asparagine as the binding site for Grb7. This agreed with our initial speculations on the binding site on HER2 and indicates that the tyrosine at residue 1139 on HER2 is likely to be the binding site for the Grb7 SH2 domain. This was confirmed by work from another group (19).

As outlined in the original Specific Aim #3 in the Statement of Work, we attempted to identify proteins that bound to the GM domain (central region) of Grb7 and Grb10. The Grb7 and Grb10 GM domains are 55% identical and likely to perform a similar function. We have screened a NIH 3T3 bacterial expression library with the GM domain of Grb10 because our best expression library at that time was from NIH 3T3 cells, where

Grb10, but not Grb7, is expressed. The central domain of the protein was expressed as glutathione-s-transferase (GST) fusion protein that contains a protein kinase A phosphorylation site. This fusion protein can be radioactively labeled with protein kinase A and γ^{32}P-ATP (20). After screening 800,000 plaques with this Grb10 probe, one positive clone was identified and purified. Sequencing revealed this clone to represent a partial sequence of the protein Nopp140, a serine rich highly phosphorylated protein that may be involved in nuclear import (21). Antibodies to this protein were obtained from Dr. U.T. Meier and attempts were made to detect Nopp140 in Grb7 or Grb10 immunoprecipitates. However no interaction could be demonstrated between Grb7 or Grb10 and Nopp140 in living cells. Accordingly, the biological significance of this interaction is still open to question and may be an artifact of the cloning procedure.

During the course of these studies a potential clue as to the role of the GM domain was published. Published sequence alignment suggested that Grb7 might have some similarity to proteins that interact with Ras (10, 22). The region of similarity coincided exactly with the one hundred amino acids of the GM domain amino-terminal to the PH domain that we had previously identified. Ras is a small G-protein that is involved in many signaling pathways including the activation of the protein kinase Raf, the activation of other G-proteins such as Ral and possibly modulation of Phosphatidylinositol-3 Kinase activity. The mechanism by which Ras controls these activities is by interacting with other signaling proteins in the GTP but not GDP bound state. The best understood is the interaction of GTP bound Ras with the amino-terminus of Raf (23). This serves to bring Raf to the plasma membrane where Raf is activated. Ras also binds to Ral-GDS, a protein that activates the small G-protein Ral (24). Grb7, Grb10 and Grb14 all have similarity to the Ras binding motif in Ral GDS and another Ras binding protein known as Canoe (22). Accordingly we examined if Ras can bind to the GM domain of Grb7. We expressed Ras in bacteria as a GST fusion protein and purified the protein on glutathione agarose. We then released Ras from the GST fusion using thrombin. The cleaved Ras was loaded with GDP or GTP by incubating the G protein in EDTA and then loading either guanyl nucleotide in the presence of a high concentration of magnesium. We then added GST-Raf or GST-Grb7 on beads to GTP or GDP loaded Ras and looked for precipitation of Ras by Raf or Grb7. We detected binding of GTP but not GDP Ras to Raf as has been previously published but we found no binding of Grb7 to Ras (Fig. 4). We were also not able to detect binding to Rac or Rho GTPase proteins.

Finally we performed studies looking for proteins that coimmunoprecipitate with Grb7 using cell lysates radiolabeled with ^{35}S-methionine or ^{32}P-orthophosphate. 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. 5). Similar results were found for ^{35}S-Methionine labeled cells in repeated experiments (not shown). 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.

3) Generation of Grb7 knockout mice.

Grb7 was initially cloned from an embryo library and found to be highly expressed in the liver and kidney of a six week old mouse (4). We decided to further explore the expression of Grb7 in these two situations to begin to better understand the role of Grb7 in normal biology. We were able to determine the localization of Grb7 in the adult and embryonic kidney using RT-PCR. We also detected significant levels of Grb7 expression in lung and liver epithelia (25). These studies laid the groundwork to generate a mouse deficient in Grb7.

The first step in generating a mouse deficient in Grb7 was to analyze the genomic organization of Grb7 in order to determine which exons could be deleted. We initially isolated 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 (Fig. 6). This made the insert easier to sequence, restriction map and manipulate. Using primers from the original cDNA sequencing (4) we were able to map the first seven exon/intron boundaries and locate the start site ATG. 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 (Fig. 6). 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 exons 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. These types of studies allowed us to obtain a partial map of the Grb7 gene and the location of specific restriction sites (Fig. 6). The mapping was confirmed by southern blotting of mouse liver genomic DNA.

We prepared a targeting vector from this Grb7 genomic DNA to eliminate almost all the Grb7 coding sequence (Fig. 6). We used the targeting vector pPNT (26) that 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 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. When our targeting vector undergoes homologous recombination with the endogenous Grb7 gene, it will remove almost all exons of Grb7.

The pPNT-Grb7 plasmid was electroporated into mouse ES cells and after selection in gancyclovir and geneticin, 200 colonies were selected and grown in 96 well plates. From these 200, 150 colonies proliferated such that they could be analyzed by Southern blot. To analyze these clones, DNA from the 96 well plates was prepared and cut with HindIII. We utilized Probe B (Fig. 6) to test the DNA from the electroporated ES cells. Probe B contains sequence that should be present within both the targeted allele as well as the wild type allele. However Grb7 targeted alleles would lose two internal HindIII sites and yield a 15.3 kb band while the wild type allele is 10 kb. Unfortunately, none of these initial 150 cells had homologous recombination and we proceeded to recheck our targeting vector to ensure it was correct. After further sequencing we confirmed that the targeting vector was correct and proceeded to electroporated 4.4×10^7 ES cells with the Grb7 knockout construct. After isolating 500 clones we were able to screen 480 with analysis by Southern blot. Using this approach we were able to obtain three homologous recombinants.

Three positive clones were obtained, 2E5, 4B5 and 5D1 (Fig. 7). These clones were expanded and two different probes were used to confirm the positive clones. An exon 1 probe confirmed correct targeting of the 5' end of the recombinant allele. The exon 1 probe showed a 15.3 kb recombinant band and an 8 kb wild type band with a HindIII digest of genomic DNA. The 3' probe showed a 15.3 kb recombinant band and a 10 kb

wild type band with HindIII digestion, showing correct targeting of the 3' arm of the Grb7 knock out vector. An exon 3 probe to Grb7 confirmed the absence of that region in the homologous recombinant clones. The wild type 8kb band is present in all clones and the 15.3 kb band is absent in all clones. The clones were also scored for chromosome counts, with clones having 40 chromosomes being selected for blastocyst injection.

Two clones containing the proper chromosome counts, 4B5 and 2E5, were expanded for injection into C57BL/6 blastocysts. Sixty-five blastocysts were injected for each clone. The ES cells are agouti and X:Y and thus some of the resulting animals should be partially agouti and males should predominate over females. For clone 4B, 36 pups were born of which 20 survived. Eleven of these pups were completely black indicating they were not populated by the targeted ES cells. Of the 9 chimeric mice, 5 were male and between 10% and 90% agouti. For the 2E clone, 29 pups survived of which 6 were black. Of the remaining 23, 17 were males and had between 20% and 95% agouti coat color.

Agouti males were grown to 6 weeks and 4 of the 4B and 10 of the 2E chimeric males were mated with C57BL/6 females as described in the methods. From this breeding, agouti offspring should arise indicating that the targeted ES have been incorporated into the male germ line. From these chimeric males, male and female agouti mice were obtained that were heterozygote for Grb7. These heterozygote female and males were then crossed to obtain mice deficient in Grb7 (Fig. 8). In the first ten litters of these crosses, we obtained 18 homozygote knockout mice (-/-), 31 heterozygote (+/-), and 21 homozygote wild type (+/+). This gives the near the expected ratio of 1:2:1 indicating that the homozygote mice are likely to be viable. We found that Grb7 homozygote knockout mice did not express Grb7 (Fig. 8B). We mated homozygote knockout male and female mice and found them to be fertile. The females were able to lactate suggesting normal breast development. We have aged homozygote knockout and wild type mice to one year and to date we have seen no signs of pathophysiology. We have done gross anatomical analysis at 6 weeks and there was no difference between wild type and knockout mice. We are now subjecting knockout mice to anatomical analysis at 1 year. To date we have not detected a phenotypic abnormality in Grb7 knockout mice.

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

In view of the consistently negative results we obtained with Grb7, we realized that we could not continue to put all our efforts into this protein. Thus, we used some of the funding 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 PTB domain in addition to an SH2 domain (27, 28). 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 (5). The goal of this specific project was to look at the role of the Shc PTB domain in cell transformation. We used the polyoma Middle T antigen (MT) as a model of cell transformation by activated growth factor receptors (29). 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 (30). 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.

A summary of our studies on the Shc PTB domain is summarized below and a published reference included in the appendix includes the relevant figures (31). In these studies we overexpressed myc tagged wild type Shc, full length Shc with a mutation in the PTB (Shc F198V}, the Shc PTB domain alone or the PTB domain plus Y239/Y240, tyrosine phosphorylation sites that can bind Grb2 in fibroblasts transformed by MT. Normally Shc is tyrosine phosphorylated in MT transformed cells and binds Grb2. We found that a mutation in the PTB domain severely impairs the ability of full length Shc to bind to MT, to become tyrosine phosphorylated and to bind Grb2. This indicates the importance of the Shc PTB domain in binding to MT and receptor tyrosine kinases. When overexpressed the Shc PTB domain with Y239/240 can become tyrosine phosphorylated and bind Grb2. However the PTB domain alone does not become tyrosine phosphorylated or bind Grb2. This suggested to us that overexpression of the PTB domain alone might act in a dominant negative fashion to block transformation. The overexpression of the Shc PTB domain with the Y239/Y240 sites can support transformation by MT because it becomes tyrosine phosphorylated and binds Grb2. In contrast the PTB domain alone blocks transformation because it does not become tyrosine phosphorylated or bind Grb2. Additionally the PTB domain alone blocks the ability of endogenous Shc to bind to MT. 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 PTB domain in transformation by Shc.

Methods

I. Grb7 transfections. Grb7 transfections were performed as described in previous publications (5).

Grb7 binding studies. We used two labeling approaches to look for co-immunoprecipitating proteins, ^{32}P-Orthophosphate and ^{35}S-Methionine.

Orthophosphate labeling of MDCK cell. 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 (32). After 30 minutes on ice, lysates were collected and spun 30 minutes, 4^{0}C, 15,000xg. Supernatants were harvested and 20 μg of antibody was added (anti-myc monoclonal 9E10; affinity purified anti-Grb7 polyclonal #222; (5, 33) and incubated overnight at 4^{0}C. Protein A beads were added to the immunoprecipitation and incubated an additional 90 minutes at 4^{0}C. 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^{0}C 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 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^{0}C. 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^{0}C on a nutator. Then 20 μgs of antibody (monoclonal anti-myc or polyclonal anti-Grb7 #222) was added. Immunoprecipitations were incubated at 4^{0}C 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 labeled with ^{32}P-dCTP using a random primed labeling kit (Boehringer-Mannheim). The library was plated using E. coli XL1 Blue per manufacturer's instructions. Library screening was performed at 42^{0}C in 50% formamide as described (4). 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 (4).

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 (34). 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 prehybridization 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.

Identification of Grb7 targeted ES cells. The Grb7 targeting vector (pPNTGrb7) was electroporated into mouse Embryonic Stem (ES) cells. Pluripotent ES cells were electroporated using a BioRad electroporator set at 250 mF and 0.3 Kv with an average time constant of 0.3. The pPNTGrb7 used for electroporation was linearized with HindIII and used at a concentration of 20ug pPNTGrb7/0.8 x 10^7 cells. After electroporation, the cells were plated onto neoR Mouse Embryo Fibroblasts (MEF) feeder cells and selected in 300 μg/ml G418 and 0.2 μM Gancyclovir. Colonies were grown for 7-10 days in selection media. Neomycin resistant and Gancyclovir insensitive colonies were isolated and individual clones plated onto 96-well plates. Only clones exhibiting pluripotent morphology were isolated for further culture. Clones were expanded in 96-well plates and frozen stocks and DNA were made from each clone. DNA was harvested from 96-well plates of very densely cultured ES cells. Media was removed, cells rinsed twice with PBS, and lysed overnight at 60°C. Lysis buffer contained 10mM Tris pH 7.5, 10mM EDTA, 10mM NaCl, 0.5% Sarcosyl and 1 mg/ml proteinase K. After lysis, DNA was precipitated using 75mM NaCl/Ethanol at room temperature. The wells were rinsed twice with 70% Ethanol and air dried for 1 hour. DNA was resuspended in Tris EDTA, incubated overnight at 37°C and used for digestion for Southern analysis. DNA was analyzed by Southern blot for homologous recombination using an external probe for Grb 7. Correctly targeted clones were thawed, expanded and rechecked by Southern blotting.

Breeding of Grb7 knockout mice. The Transgenic Animal Models Core at the University of Michigan performed the blastocyst injections using the targeted ES cells and subsequent implantations into pseudopregnant females. Chimeras were generated for two cell lines. Two female mice were placed with a chimeric male for two weeks and then replaced with two new females. In this manner, six females in total were mated with each chimeric male. The litters were examined for offspring with agouti coat colors. The non agouti mice were euthanized while the agouti offspring examined for the presence of the targeted allele by Southern blotting. Using these techniques were able to obtain several heterozygote male and female mice which were then mated using standard techniques to obtain Grb7 heterozygote and homozygote knockout mice.

Role of the Shc PI/PTB domain in cell transformation. Methods for these studies can be found in reference (31) included in the Appendix.

CONCLUSIONS

During the funding period we have addressed many aspects of Grb7 function. Unfortunately even after these extensive studies we have not been able to determine the exact function of the protein. We utilized several approaches to identify Grb7 function. The first set of experiments examined the effects of overexpression of Grb7 in a variety of breast cancer and other epithelial cell lines. We assayed the cells for abnormalities in growth as Grb7 is an important binding target for growth factor receptors. We also examined the role of Grb7 in migration as our previous results suggested an important role for the Grb7 homologue of *C. elegans* in cell migration. Unfortunately in tissue culture studies, Grb7 overexpression did not alter either of these cellular activities. Accordingly we performed studies to eliminate Grb7 from mice. We generated embryonic stem cells deficient in Grb7 and injected these into mouse blastocysts. From these injections, chimeric males were generated which when crossed with wild type females lead to the generation of heterozygote offspring with one wild type and one mutant copy of Grb7. These heterozygote mice were bred and this lead to the generation of homozygote Grb7 knockout mice. To date, after one year of aging, these mice have demonstrated no phenotypic abnormalities. They have displayed normal fecundity and rearing abilities. We are currently examined the aged mice for additional problems but to date have seen no obvious phenotypic difference with wild type mice. Obviously after two years of efforts this has been a disappointing result and points to the elusive function of this protein. One likely possibility is that two other genes Grb10 and Grb14 can compensate for Grb7 function in the mouse. Future work, crossing our mice with mice deficient for Grb10 and Grb14, will be important for determining if this is the case. In addition to these overexpression and underexpression studies, we have also looked for proteins that may bind to Grb7. Although we identified proteins such as Nopp140 as potential binding partners further studies could not confirm that these were true physiologic partners of Grb7. It has been suggested that Grb7 might interact with Ras family members (10) but extensive studies from our group could not confirm this.

Funding from this grant was also utilized to support other studies in our laboratory in which positive results were obtained. We demonstrated the crucial role that the PTB domain of Shc plays in cellular transformation. Shc is a protein that binds to activated growth factor receptors and plays an important role in directing signals through the Ras pathway. Our studies with the PTB domain suggest new possibilities for drugs that might inhibits its interaction with growth factor receptors and slow tumor growth.

In summary our studies have focused on two growth factor receptor binding proteins Grb7 and Shc. Studies with dominant negative Shc shows its crucial role in positive growth signaling. Although Grb7 is overexpressed in many breast cancers it does not seems to play a major role in growth promotion and its role in cancer development is unclear. It has been reported that a truncated form of the protein may play an important role in esophageal cancer (35) but our studies of overexpression and underexpression have not lead to a clearer understanding of its physiologic role.

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APPENDIX

Personnel Supported During Grant Period

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James Ooi (Graduate Student/NYU)

Chia-Jen Liu (Research Assistant)

Kevin Patrie (Post Doctoral Fellow)
Samuel Straight (Post Doctoral Fellow)

David Karnak (Rotation Graduate Student)
E.J. Brace (Rotation Graduate Student)
Stephen Juris (Rotation Graduate Student)

Rajani Koimatter (Undergraduate Student)

Publications

Blaikie, P.A., E. Fournier, S.M. Dilworth, D. Birnbaum, J.P. Borg, and B. Margolis. 1997. The role of the Shc phosphotyrosine interaction/phosphotyrosine binding domain and tyrosine phosphorylation sites in polyoma middle T antigen-mediated cell transformation. *J.Biol.Chem.* 272:20671-20677.

Figure Legends

Figure 1. Stimulation of tyrosine phosphorylation by EGF in MCF-7 cells transfected with EGFR/HER2, Grb7 or both. MCF-7 cells, either parental or stable transfectants were stimulated with 250 ng/ml EGF for 5 minutes. Lysates were prepared, separated by SDS-PAGE, transferred to nitrocellulose and blotted with anti-phosphotyrosine antibodies. The major tyrosine phosphorylated proteins have been identified as p52 Shc and p42/p44 Map kinases.

Figure 2. Expression of Myc-Grb7 and CAAX-Grb7 in MDCK cells. MDCK cells were transfected with Myc-Grb7 or CAAX-Grb7 in combination with a neomycin resistance plasmid. Clones were selected under G418 and tested for Grb7 expression. Parental MDCK cells and clones expressing Myc-Grb7 or CAAX Grb7 were then grown to confluence and lysed by douncing in hypotonic lysis buffer without detergent. A low speed spin was used to remove unlysed cells and nuclei and the resulting supernatant was spun at 100,000xg in an ultracentrifuge. The supernatant (S100) and the pellet (P100) from this spin, were separated on a SDS-gel, transferred to nitrocellulose and blotted with anti-Grb7 antibodies. The P100 represents the membrane fraction and the S100 the cytosolic fraction. Three times as much membrane was loaded in this experiment so the amount of Grb7 in the membrane is overestimated in this figure. Nonetheless it is clear that all the CAAX Grb7 is in the membrane. The various forms of Grb7 are indicated on the right of the panel. Myc Grb-7 runs larger than Grb7 due to the addition of the myc epitope and CAAX-Grb7 is even larger due to the further addition of the H-Ras CAAX box and polybasic region.

Figure 3. Grb7 does not affect cell migration in response to HGF. Wild type MDCK or MDCK cells transfected with CAAX-Grb7 (MDCK-CAAX) were plated at 25,000 cells per well of a six well plate. The cells were treated with or without 50 units/ml of HGF for 18 hours. After 18 hours cells were photographed.

Figure 4. Lack of binding of Ras to Grb7. A region in the Grb7 GM domain has similarities to domains that bind the small G-protein Ras. To test this, bacterially expressed Ras was loaded with GTP or GDP. The GTP or GDP Ras was added to GST, GST-Grb7 (full length), GST-Grb7-GM domain or GST-Raf. After binding for 90 minutes in the

cold room, the samples were washed, run on a 15% SDS-gel, transferred to nitrocellulose and blotted with monoclonal anti-Ras antibodies. GST-Raf acted as a positive control and as expected bound GTP but not GDP Ras. There was however no binding to either of the Grb7 constructs or GST alone. The band at the top of the blot in each lane is a protein that nonspecifically interacts with the Ras antibody.

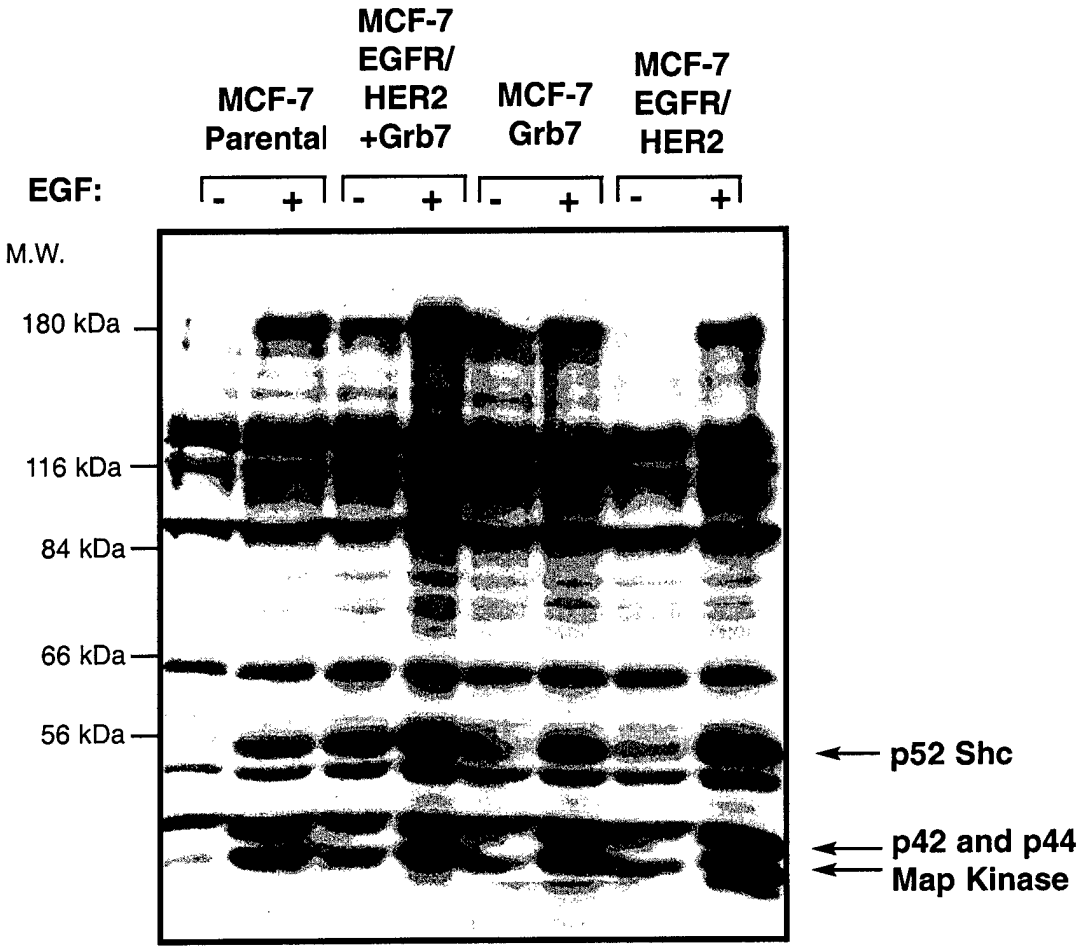
Figure 5. Lack of Grb7 coimmunoprecipitating proteins in ^{32}P-Orthophosphate labeled cells. Wild-type MDCK cells or MDCK cells overexpressing a myc tag Grb7 were labeled with ^{32}P-Orthophosphate as indicated in the Methods section. After labeling, the cells were lysed in 1% Triton X-100 lysis buffer 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 6. The Grb7 targeted allele. The wild type Grb7 genomic organization is displayed in the top schematic. The targeting vector pPNTGrb7 recombines with the wild type Grb7 allele via its 5' and 3' arms. This targeting vector was electroporated into mouse ES cells to create the targeted Grb7 allele shown at the bottom of the figure. The resulting correctly targeted allele contains the thymidine kinase gene (hsv-tk) making it resistant to gangyclovir but will contain the Neo gene making it resistant to geneticin. The targeted Grb7 allele will be missing internal HindIII sites leading to different sized fragments when HindIII digested DNA is hybridized with Probe A or Probe B in Southern blotting (see Fig. 7).

Figure 7. Southern blotting of properly targeted ES cells. After selection and growth in 96 well plates, genomic DNA from ES cells was prepared and cut with HindIII. The DNA was separated on an agarose gel, transferred to nylon membrane and subjected to Southern blotting. After examining 500 clones, three clones were found to contain a targeted allele. These three clones, 2E5, 4B5, and 5D1, are compared to wild type ES

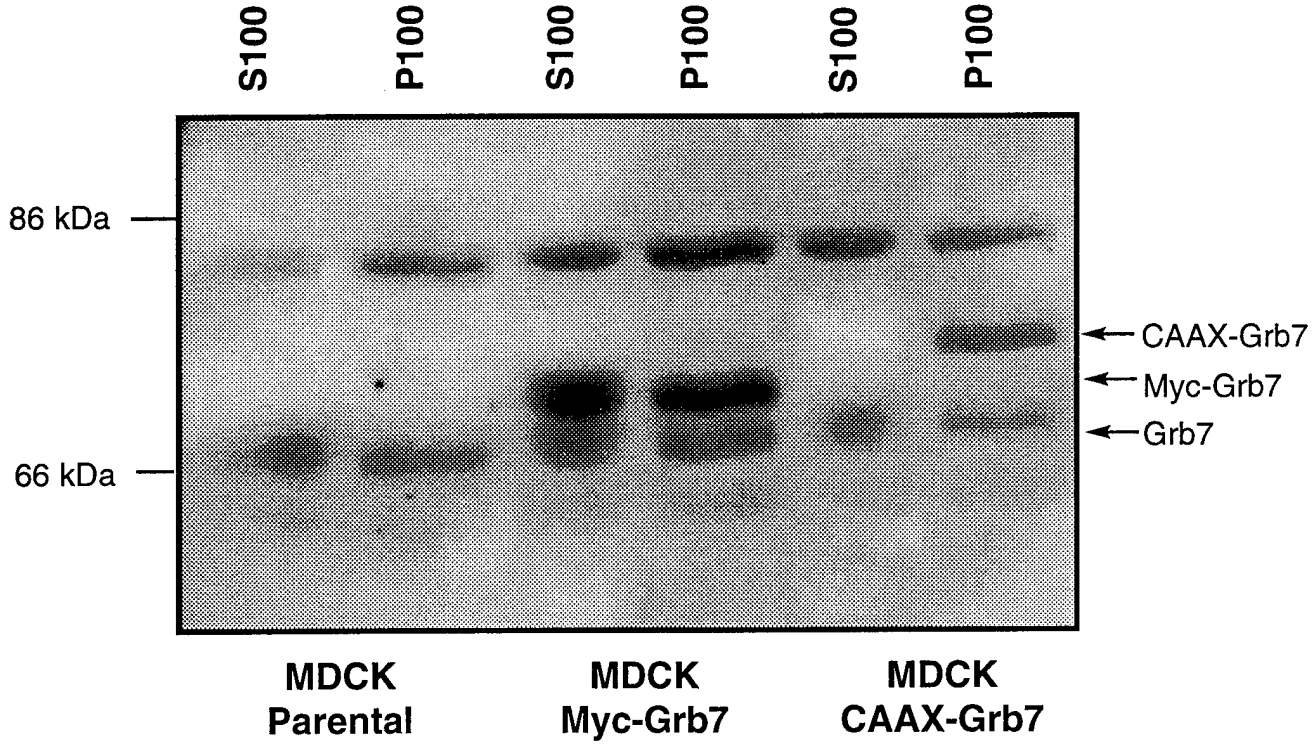
cells and mouse embryo fibroblasts (MEF). The DNA from these cell lines were probed with Probe A, Probe B or a probe from exon 3 (Fig. 6). The equal intensity of the wild type and targeted allele confirms the heterozygote genotype of these cells. Using either Probe A or Probe B, the larger Grb7 targeted allele was detected in HindIII digested ES cell DNA. Wild type ES cells or MEFs did not contain the targeted allele. As a control we probed the DNA with a probe from exon 3 in Grb7 that should be absent in the targeted allele. As expected this exon is present in the wild type allele but removed from the Grb7 targeted allele.

Figure 8. Analysis of Grb7 knockout mice. **A.** Heterozygote chimeric males were generated by blastocyst injection with 2E5 or 4B5 cell lines (see text). These chimeric males were mated with wild type females to generate Grb7 heterozygote male and female mice. Genomic DNA was prepared from the offspring of these matings and cut with HindIII. The DNA was separated on an agarose gel, transferred to nylon membrane and subjected to Southern blotting with Probe B (Fig. 6). Mice with only the larger band such as 356 and 358 are homozygous Grb7 knockout mice, mice with both bands are heterozygous, while mice with just the lower band such as 360 are wild type for Grb7. **B.** Kidney and livers from homozygous knockout and wild type mice were homogenized in 1% Triton lysis buffer. The far left panels are from kidney, the next two panels from liver and the far right panel from SKBR3, a breast cancer cell line that overexpresses Grb7. Two hundred micrograms of protein was separated by SDS Page, transferred to nitrocellulose and blotted with anti-Grb7 antibodies. The wild type mice but not the Grb7 knockout mice express Grb7.



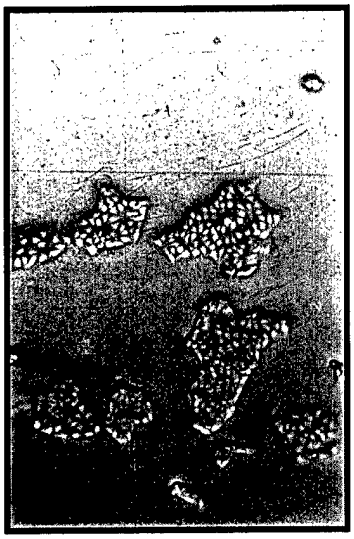
Blot: anti-PTyr

Figure 1



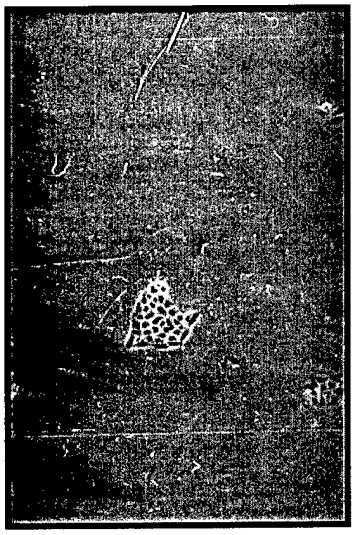
Blot: Anti-Grb7

Figure 2

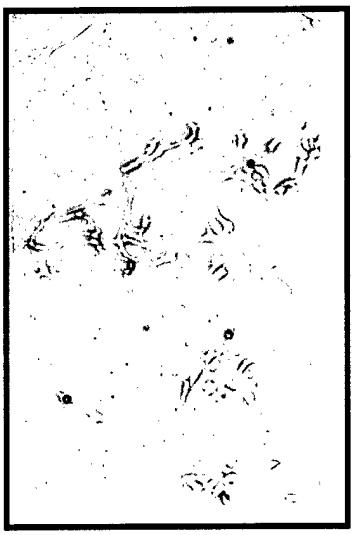


Parental MDCK Cells
Without HGF

Parental MDCK Cells
With HGF

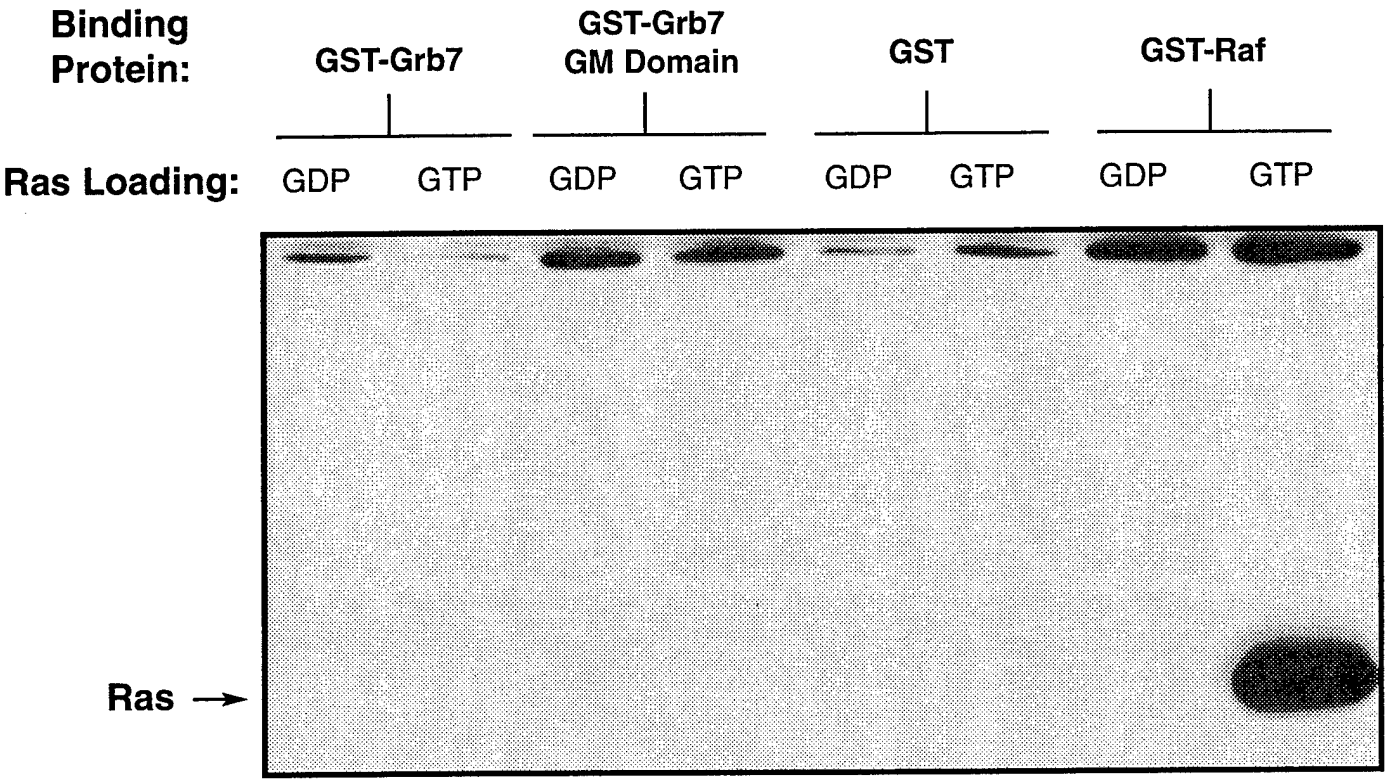


MDCK-CAAX Cells
Without HGF



MDCK-CAAX Cells
With HGF

Figure 3



Blot: Anti-Ras

Figure 4

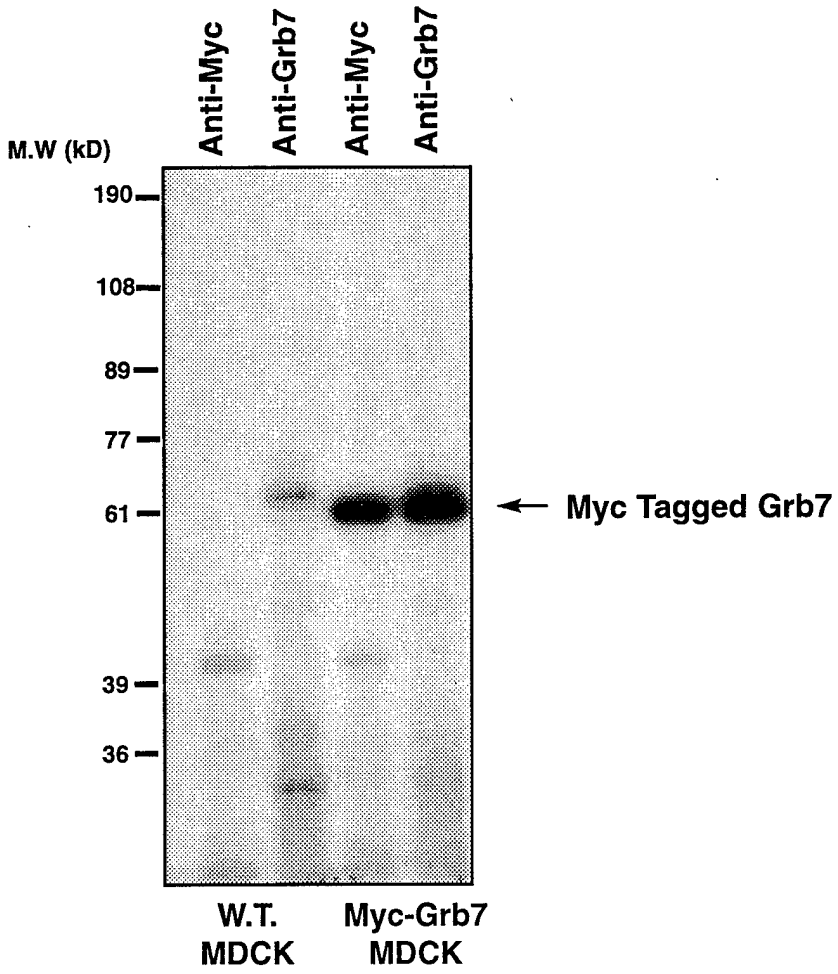
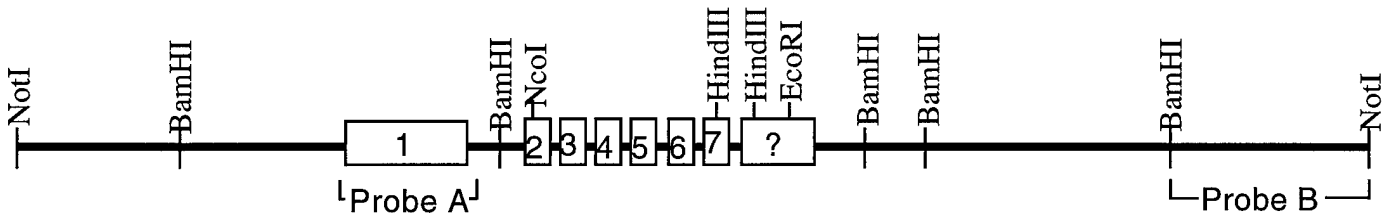
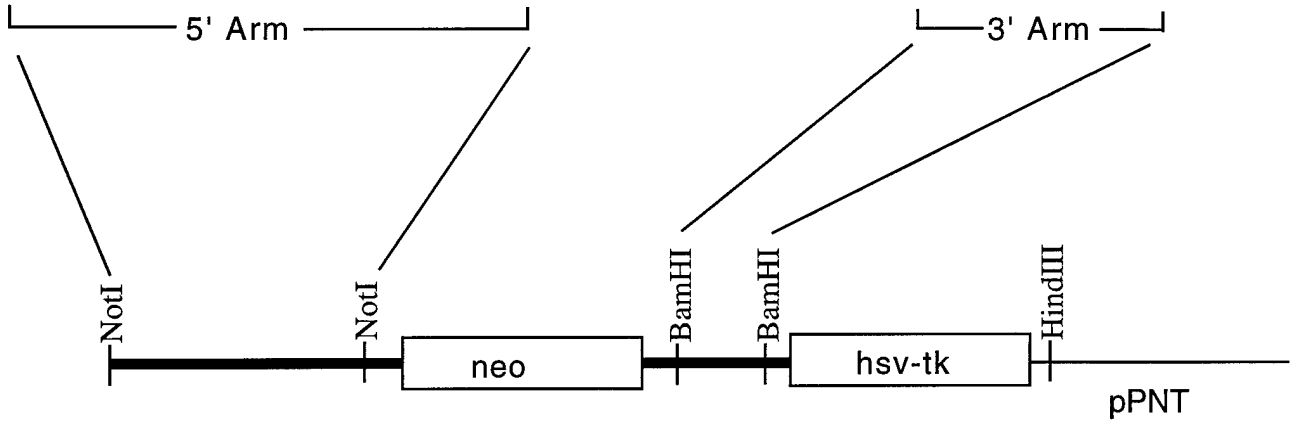

Anti-Myc
Anti-Grb7
Anti-Myc
Anti-Grb7
M.W (kD)
190
108
89
77
61
39
36
Myc Tagged Grb7
W.T.
MDCK
Myc-Grb7
MDCK

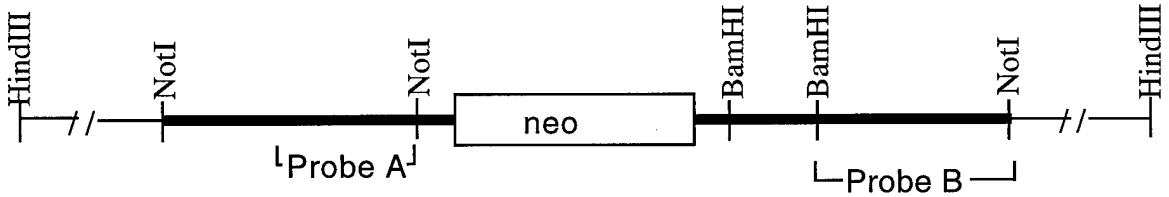
Figure 5



Wild Type Allele



Targeting Vector-pPNTGrb7



Targeted Grb7 Allele

Figure 6

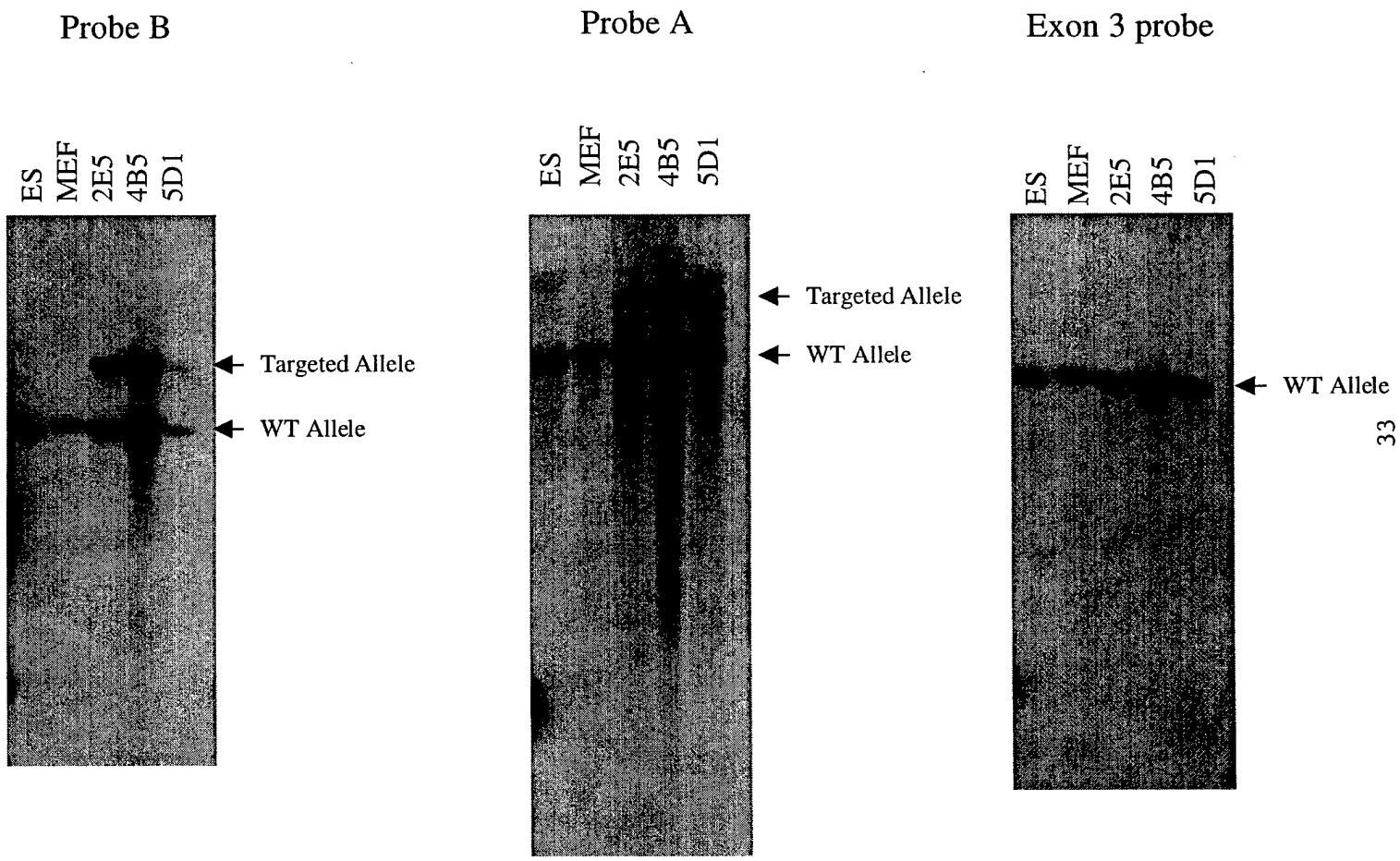
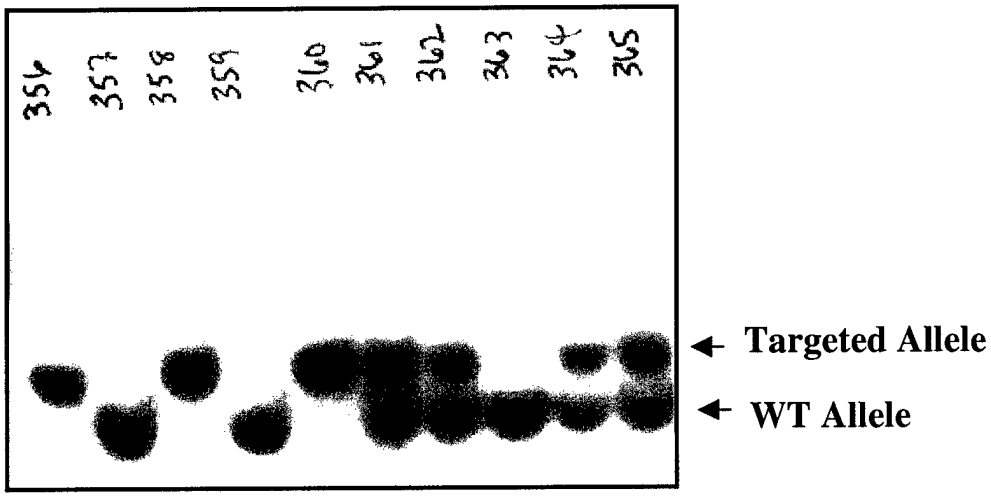

Probe B
ES
MEF
2E5
4B5
5D1
Targeted Allele
WT Allele
Probe A
ES
MEF
2E5
4B5
5D1
Targeted Allele
WT Allele
Exon 3 probe
ES
MEF
2E5
4B5
5D1
WT Allele

Figure 7

A.


356
357
358
359
360
361
362
363
364
365
Targeted Allele
WT Allele

B.

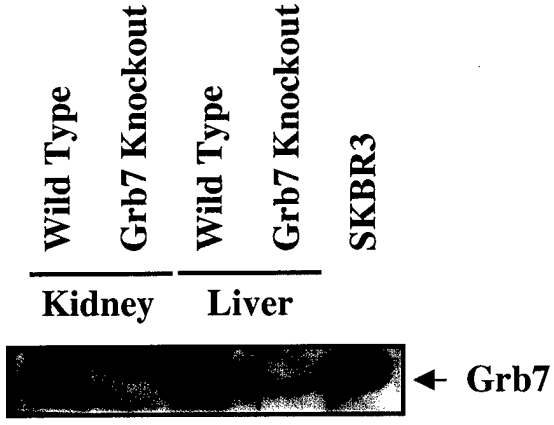

Wild Type
Grb7 Knockout
Wild Type
Grb7 Knockout
SKBR3
Kidney
Liver
Grb7

Figure 8

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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.

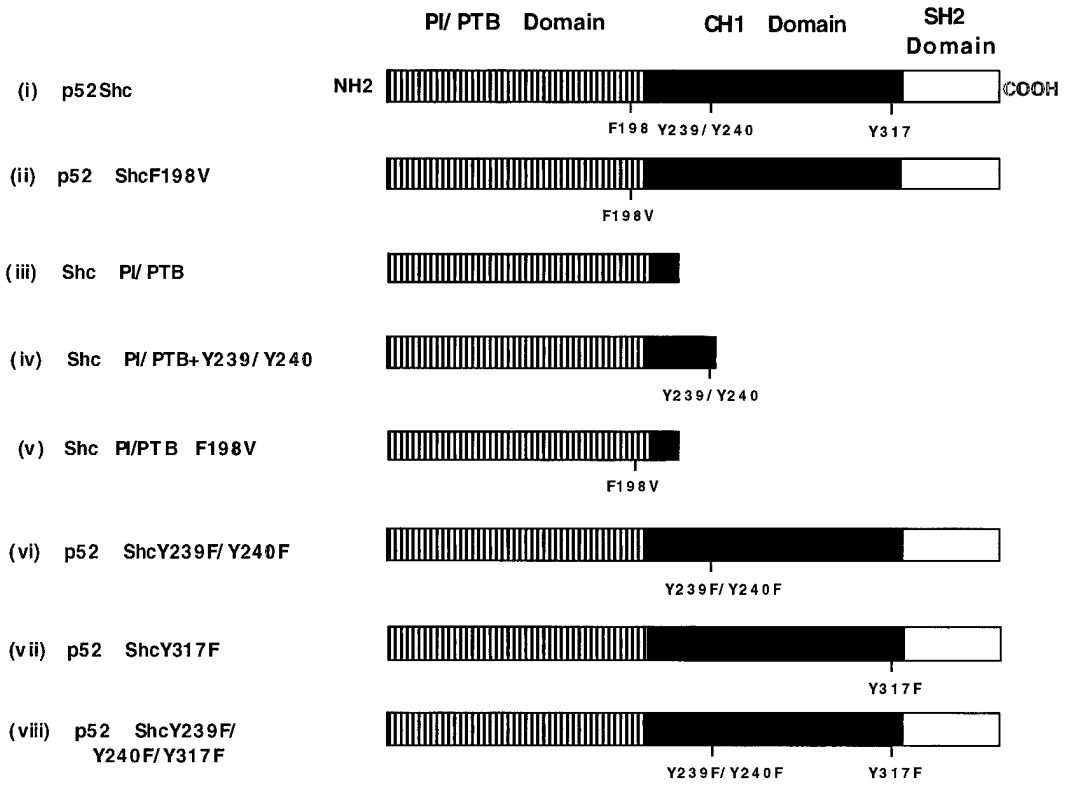

FIG. 1. **Schematic diagram depicting Shc constructs used in these studies.** Phe^{198} is an amino acid residue crucial for the binding of the PI/PTB domain to phosphopeptides which, when mutated to valine (F198V), abrogates this binding ability. Tyr^{239}/Tyr^{240} and Tyr^{317} are the major Shc tyrosine 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.

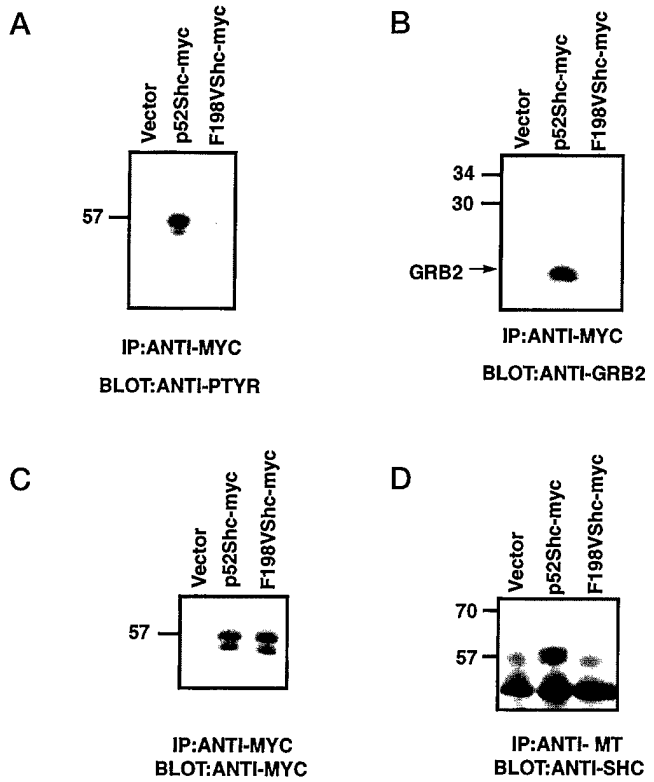

FIG. 2. **p52 Shc carrying the F198V mutation is weakly phosphorylated and does not bind Grb2 or MT.** NIH 3T3 cells stably transformed by MT and expressing either p52 Shc or p52 ShcF198V were lysed and immunoprecipitated with the anti-Myc antibody (*panels A, B,* and *C*) or anti-MT antibody (*panel 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.

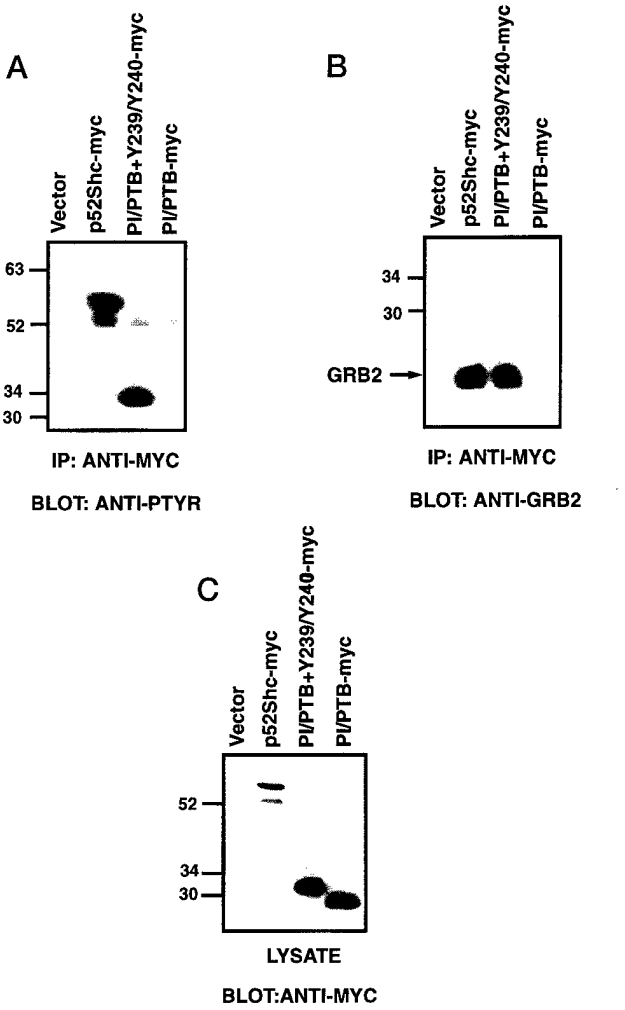

FIG. 3. **Differential binding and tyrosine phosphorylation of the Shc PI/PTB and Shc PI/PTB+Y239/Y240.** NIH 3T3 cells stably transformed by MT and expressing wild type p52 Shc, the Shc PI/PTB, or Shc PI/PTB+Y239/Y240 were lysed and immunoprecipitated with the anti-Myc antibody (*panels A* and *B*). 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}

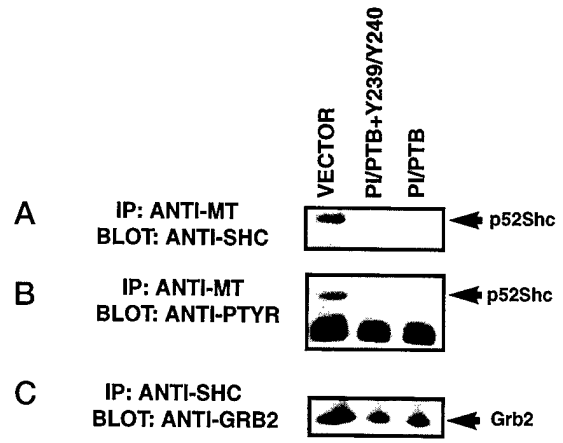

FIG. 4. **Overexpression of either Shc PI/PTB or Shc PI/PTB+Y239/Y240 in NIH 3T3 cells stably transformed by MT inhibits the binding of endogenous Shc to MT and Grb2.** NIH 3T3 cells stably transformed by MT and expressing either wild type Shc PI/PTB or Shc PI/PTB+Y239/Y240 were lysed and immunoprecipitated 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*).

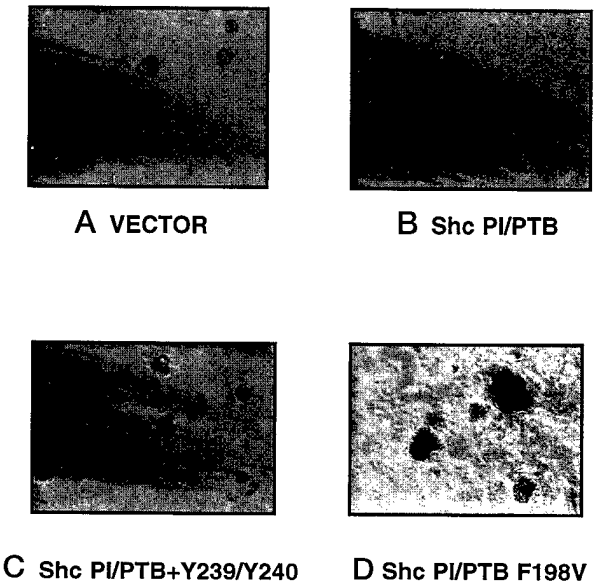

FIG. 5. **The Shc PI/PTB domain inhibits MT antigen-induced transformation.** NIH 3T3 cells stably transformed by MT expressing vector alone (*panel A*), the Shc PI/PTB (*panel B*), Shc PI/PTB+Y239/Y240 (*panel C*), or Shc PI/PTB F198V (*panel D*) were seeded in soft agar at a density of 3×10^4/well 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.

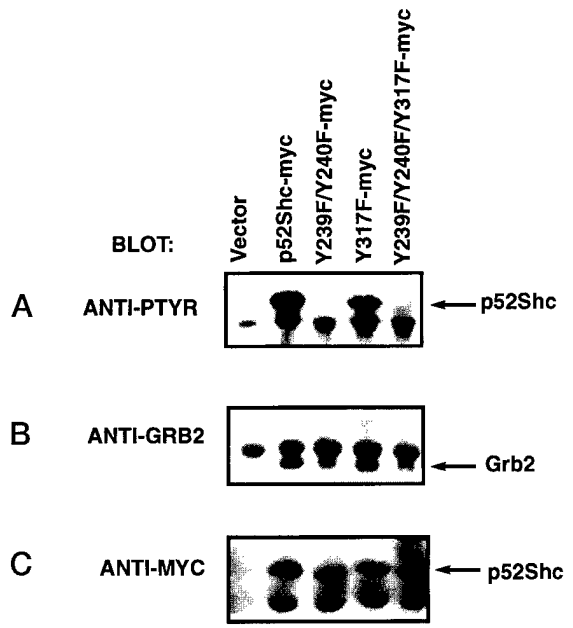

FIG. 6. **p52 Shc carrying the Y239F/Y240F mutation does not become tyrosine-phosphorylated and does not bind Grb2.** NIH 3T3 cells stably transformed by MT and expressing wild type p52 Shc, p52 ShcY239F/Y240F, p52 ShcY317F, or p52 ShcY239F/Y240F/Y317F were lysed and immunoprecipitated with the anti-Myc 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.

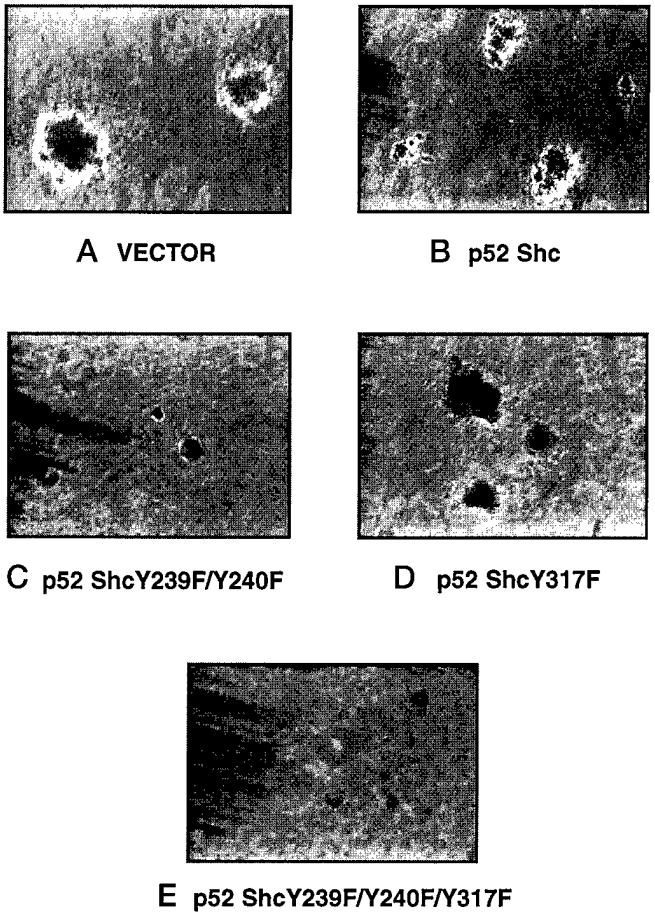

FIG. 7. **p52 ShcY239F/Y240F inhibits MT-induced transformation in soft agar.** NIH 3T3 cells stably transformed by MT expressing vector alone (*panel A*), wild type p52 Shc (*panel B*), p52 ShcY239F/Y240F (*panel C*), p52 ShcY317F (*panel D*), or p52 ShcY239F/Y240F/Y317F (*panel E*) were seeded in soft 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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