Activation of the JNK pathway is essential for transformation by the Met oncogene

Gerard A.Rodrigues, Morag Park[1] and Joseph Schlessinger[2]

Department of Pharmacology, New York University Medical Center, 550 First Avenue, New York, NY 10016, USA and [1]Molecular Oncology Group, McGill University, Montreal, Quebec Canada

[2]Corresponding author

The Met/Hepatocyte Growth Factor (HGF) receptor tyrosine kinase is oncogenically activated through a rearrangement that creates a hybrid gene *Tpr-Met*. The resultant chimeric p65 [Tpr–Met] protein is constitutively phosphorylated on tyrosine residues *in vivo* and associates with a number of SH2-containing signaling molecules including the p85 subunit of PI-3 kinase and the Grb2 adaptor protein, which couples receptor tyrosine kinases to the *Ras* signaling pathway. Mutation of the binding site for Grb2 impairs the ability of Tpr-Met oncoprotein to transform fibroblasts, suggesting that the activation of the *Ras*/MAP kinase signaling pathway through Grb2 may be essential for cellular transformation. To test this hypothesis dominant-negative mutants of Grb2 with deletions of the SH3 domains were introduced into Tpr-Met transformed fibroblasts. Cells overexpressing the mutants were found to be morphologically reverted and exhibited reduced growth in soft agar. Surprisingly, the Grb2 mutants blocked activation of the JNK/SAPK but not MAP kinase activity induced by the Tpr-Met oncoprotein. Additionally, cells expressing dominant-negative Grb2 mutants had reduced PI-3-kinase activity and dominant-negative mutants of Rac1 blocked both Tpr-Met-induced transformation and activation of JNK. These experiments reveal a novel link between Met and the JNK pathway, which is essential for transformation by this oncogene.
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Introduction

Growth factors and cytokines regulate a wide variety of biological activities critical for the development, maintenance and growth of multicellular organisms. Many of these events, including cell growth and differentiation, are coordinated through the interaction of these soluble factors with cell surface receptors having intrinsic tyrosine kinase activity. Accordant with their importance in cell growth, many receptor tyrosine kinases (RTKs) were initially identified as oncogenes or subsequently found to be activated in a number of transformed states (Aaronson, 1991). Constitutive activation of RTKs can occur through a variety of mechanisms that mimic ligand-induced receptor dimerization and activation (Lemmon and Schlessinger,

1994) including overexpression, mutation, deletions and gene rearrangements (Rodrigues and Park, 1994b).

Activation of RTKs is initiated upon ligand binding which promotes receptor dimerization and autophosphorylation (Lemmon and Schlessinger, 1994; Heldin, 1995). Autophosphorylation activates an RTK's intrinsic tyrosine kinase activity and creates binding sites that serve as the focus for the assembly of multiprotein signaling complexes. Components of this complex known as 'adaptor' proteins contain a Src homology (SH2) domain that recognizes phosphorylated tyrosine residues within a specific sequence context (Pawson and Schlessinger, 1993; Cohen *et al*., 1995). In this way SH2 domains serve to couple RTK to downstream effectors such as *Ras* (Schlessinger, 1993).

One such adaptor protein, Grb2, appears to be critically involved in coupling RTKs to *Ras* through its association with Sos, an exchange factor for *Ras* (Clark *et al*., 1992; Lowenstein *et al*., 1992; Buday and Downward, 1993; Egan *et al*., 1993; Gale *et al*., 1993; Li *et al*., 1993; Rozakis-Adcock *et al*., 1993; Skolnik *et al*., 1993). Grb2 is an entirely modular protein consisting of an SH2 domain flanked by two SH3 domains (Clark *et al*., 1992; Lowenstein *et al*., 1992). While the SH2 domain of Grb2 is responsible for binding phosphorylated tyrosines in a number of RTKs, the SH3 domains mediate association with proline-rich regions in Sos (Buday and Downward, 1993; Egan *et al*., 1993; Gale *et al*., 1993; Li *et al*., 1993; Rozakis-Adcock *et al*., 1993; Skolnik *et al*., 1993). Thus, Grb2 is constitutively bound to Sos and recruitment of Sos through its association with the receptor-bound Grb2 promotes the exchange of GDP for GTP on *Ras* (Chardin *et al*., 1993). As a consequence, the GTP-bound form of *Ras* becomes competent to activate downstream signaling pathways ultimately culminating in changes in gene expression. A role for Grb2 in the mitogenic action of RTKs is supported by the fact that microinjection of Grb2 with *Ras* into fibroblasts stimulates DNA synthesis (Lowenstein *et al*., 1992) while microinjection of inhibitory antibodies blocks growth factor stimulation of cell cycle progression (Matuoka *et al*., 1993).

Oncogenic activation of RTKs results in deregulated kinase activity leading to constitutive autophosphorylation thereby providing potential binding sites for immediate downstream effectors. *Ras* has been shown to be required for transformation mediated by a number of tyrosine kinases (Smith *et al*., 1986; Stacey *et al*., 1991). However, little is known about how oncogenic RTKs couple to *Ras*. This is especially relevant for those oncogenic tyrosine kinases that, because of structural alterations such as deletions or rearrangements, are no longer integral membrane proteins. Recent evidence indicates that such oncoproteins do in fact interact with many of the same adaptor proteins that their normal counterparts do, including Grb2

(Pendergast *et al*., 1993; Borrello *et al*., 1994; Crowe *et al*., 1994; Ponzetto *et al*., 1994; Puil *et al*., 1994; Fixman *et al*., 1995). The potential importance of this interaction in oncogenesis is supported by the observation that mutations that eliminate Grb2 binding impair the transforming potential of these oncogenes (Pendergast *et al*., 1993; Borrello *et al*., 1994; Crowe *et al*., 1994; Ponzetto *et al*., 1994; Puil *et al*., 1994; Fixman *et al*., 1995). Moreover, Grb2 was shown to be overexpressed in a number of human breast cancer lines and this was correlated with an increase in complex formation with Sos (Daly *et al*., 1994).

The Tpr-Met oncoprotein is the oncogenic version of the Met receptor tyrosine kinase, the receptor for Hepatocyte Growth Factor/Scatter Factor (HGF/SF). The oncoprotein has sustained a deletion of the extracellular and transmembrane domains and is thus rendered ligand-independent. In addition, due to a genomic rearrangement novel sequences at the C-terminus derived from another locus (Tpr) encode 142 novel amino acids fused in-frame with the Met kinase domain, generating a hybrid p65$^{Tpr–Met}$ oncoprotein (Park *et al*., 1986). The Tpr-Met protein retains kinase activity, which is required for its ability to transform fibroblasts and is constitutively phosphorylated on tyrosine residues *in vivo* (Rodrigues and Park, 1994a). The N-terminal Tpr sequence includes a leucine zipper region that is required for the ability of the Tpr-Met oncoprotein to transform cells by virtue of its ability to promote constitutive dimerization of Tpr-Met in the absence of ligand stimulation (Rodrigues and Park, 1993). A tyrosine residue (Y489) in the C-terminus of Met serves as a binding site for Grb2 as well as the p85 subunit of PI-3 kinase and is required for efficient transformation by the Tpr-Met oncoprotein (Ponzetto *et al*., 1994; Fixman *et al*., 1995).

To determine whether the activation of the *Ras*/MAP kinase pathway through binding of Grb2 is necessary and sufficient to transmit an oncogenic signal, dominant-negative mutants of Grb2 containing deletions of either SH3 domain were used to revert Tpr-Met transformed fibroblasts. Cell clones overexpressing these mutant Grb2 molecules were obtained and were found to be morphologically revertant. Expression of a Grb2 mutant as well as a dominant-negative Rac1 impaired the ability of the Tpr-Met oncoprotein to stimulate PI-3 kinase and the Jun kinase, providing evidence that Grb2-mediated activation of the JNK/SAPK pathway is required for Tpr-Met transformation.

Results

Grb2 SH3 deletion mutants are expressed in Met transformed fibroblasts but fail to associate with Sos

Mutation of tyrosine Y489 in the C-terminus of Met abolishes the ability of the Tpr-Met oncoprotein to associate with the Grb2 adaptor protein and impairs its ability to induce cellular transformation (Ponzetto *et al*., 1994; Fixman *et al*., 1995). However, mutation of this site also reduces association with and activation of phosphatidyl-inositol 3 kinase (PI-3 kinase) as well as association with the tyrosine phosphatase Shp-2 (Fixman *et al*., 1995). As Grb2 has been implicated in the activation of *Ras* via its
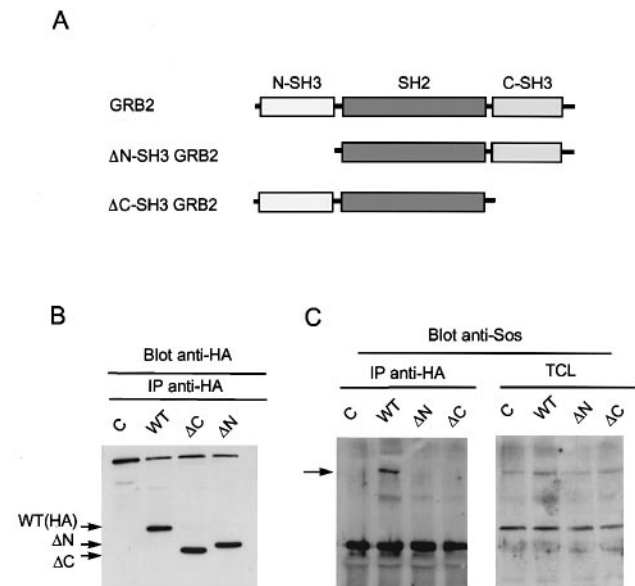

Fig. 1. Structure, expression and association of Grb2 mutants with Sos. (**A**) Schematic representation of the structure of the wild type Grb2 and the two SH3 deletion mutants. (**B**) and (**C**) Exogenously expressed Grb2 mutants were immunoprecipitated with anti-HA antibodies from lysates prepared from cell populations expressing the Tpr-Met oncoprotein following transfection with Grb2 expression vectors and selection in hygromycin. Immunoprecipitates were run on SDS–PAGE, transferred to nitrocellulose and probed with anti-HA antibodies (**B**) or anti-Sos antibodies (**C**). The Sos protein is indicated by the arrow.

association with the exchange factor Sos, we were interested to determine whether binding of the Grb2–Sos complex is required for transformation by the Tpr-Met oncoprotein. To address this question, SH3 deletion mutants of Grb2 were used to disrupt this complex in cells expressing the Tpr-Met oncoprotein.

The Grb2 protein consists of an SH2 domain flanked on both sides by SH3 domains. Mutants of Grb2 were created by deletion of either the N-terminal or C-terminal (N-SH3 or C-SH3) SH3 domains and the addition of a hemagglutinin tag (Figure 1A). Mutant Grb2 cDNAs were introduced into a Fischer Rat 3T3 cell line, Tpr-Met-1, that expresses the Tpr-Met oncoprotein and has a fully transformed phenotype (Rodrigues and Park, 1993). Following transfection of mutant cDNAs and selection in hygromycin, cell populations were examined for expression of the mutant proteins. Immunoblotting of cell lysates with anti-HA antibodies revealed the presence of proteins corresponding to the predicted sizes of the Grb2 mutants, demonstrating that these proteins could be stably expressed in these cells (Figure 1B).

As predicted, deletion of the SH3 domains was found to impair the ability of Grb2 to bind Sos. Grb2 mutants, immunoprecipitated using anti-HA antibodies, were blotted with antibodies to Sos. Despite the expression of equal amounts of mutant Grb2 in lysates as shown by blotting with anti-HA antibodies, only the wild type protein was capable of associating with Sos *in vivo* (Figure 1C).

Grb2 SH3 deletion mutants inhibit transformation of fibroblasts by the Tpr-Met oncoprotein

To test the ability of these SH3 deletion mutants to alter the transformed phenotype, Tpr-Met transformants

overexpressing these mutants were obtained. Individual cell clones were isolated, expanded and further characterized. Figure 2 shows expression of exogenously expressed Grb2 protein in representative cell lines. Total cell lysates were blotted with Grb2 antibodies directed against either the N- or C-SH3 domains. High levels of expression of both the N- and C-SH3 deletion mutants were obtained (Figure 2). However, repeated selections failed to identify clones overexpressing wild type Grb2 relative to the endogenous protein. The exogenously expressed wild type Grb2 protein is recognized by both antibodies and can be distinguished from endogenous Grb2 by its slower mobility in SDS–PAGE due to the addition of the hemagglutinin
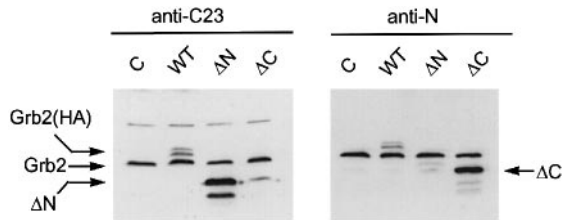
Fig. 2. Expression of Grb2 mutants in Met-transformed cells. Lysates from representative cell clones expressing the different Grb2 mutants were resolved by SDS–PAGE, transferred to nitrocellulose and immunoblotted with an antibody to the N- (anti-N) or C-terminal (C23) SH3 domains of Grb2.

(HA) tag (Figure 2). Consistent with its predicted structure, the C-SH3 deletion mutant is not detected by C23 antibodies directed against the C-terminus of Grb2 (Figure 2). Conversely, the N-SH3 deletion mutant is not detected by antibodies which recognize an epitope in the N-terminus of Grb2 (Figure 2).

To assess the effects of high levels of expression of the mutant Grb2 proteins on the ability of Tpr-Met oncoprotein to transform cells, the phenotype of cell clones expressing mutant proteins was analyzed. Clones expressing the exogenous wild type Grb2 protein retain a morphologically transformed phenotype. Cells appeared rounded and refractile with extended processes, grew in a disorganized fashion and were less adherent than their normal counterparts (Figure 3). In fact these clones appeared more transformed than the parental transformed line. This may in part explain the inability to select individual clones expressing high levels of the wild type Grb2 protein. In marked contrast, clones overexpressing the SH3 deletion mutants, especially the N- SH3 deletion mutant, appeared to have a partially revertant phenotype. The cells appeared flatter and grew in a more organized fashion. Moreover, whereas the wild type Grb2-expressing cells formed large colonies when grown in soft agar, the mutant-expressing cell lines formed fewer, smaller colonies with delayed kinetics. Again the ΔN-SH3 Grb2 mutant had a more
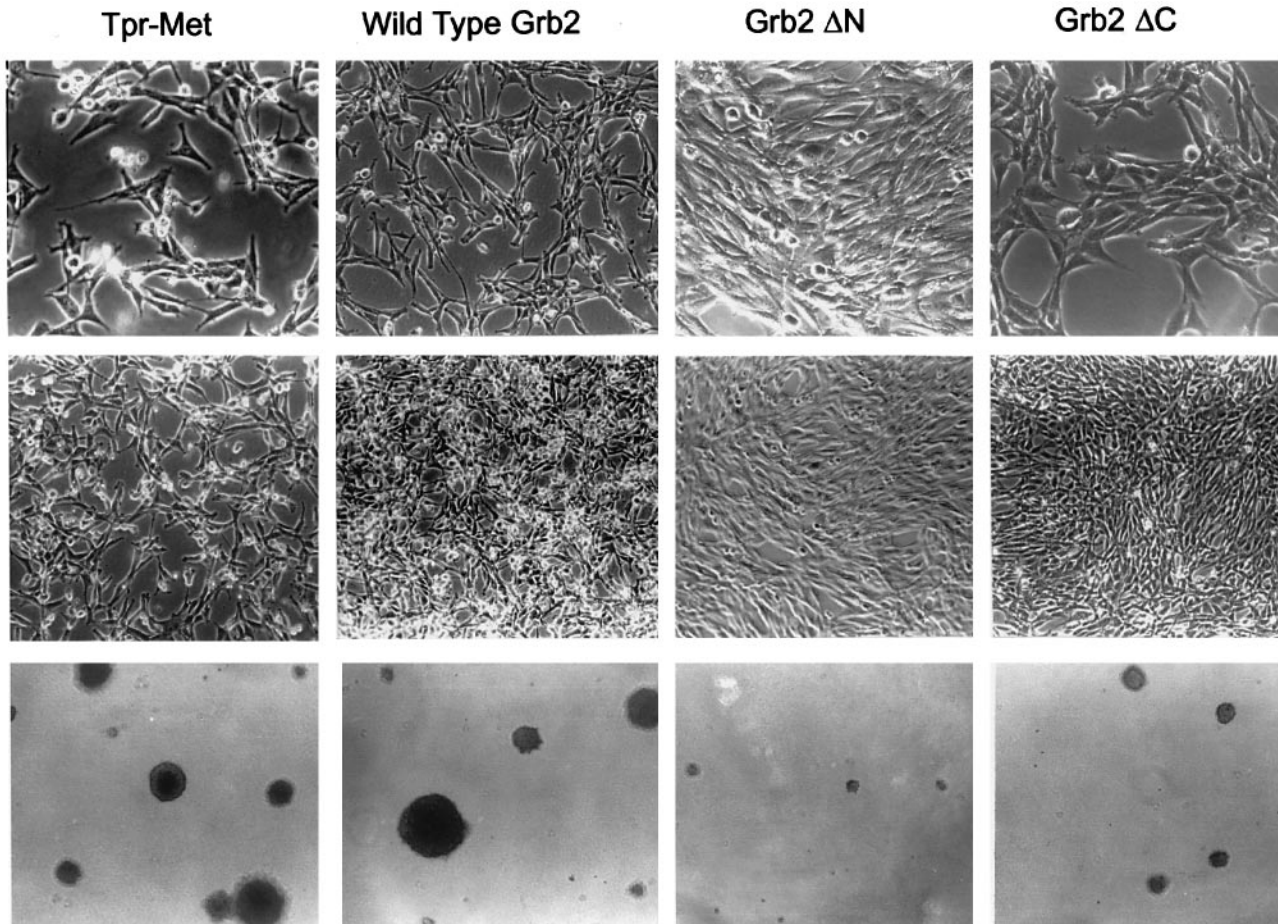

Fig. 3. Decreased transforming potential of cells coexpressing the Tpr-Met oncoprotein and Grb2 mutants. The morphology (top and middle panels) and growth in soft agar (bottom panel) of cell clones coexpressing the Tpr-Met oncoprotein are shown. The top panel is a high magnification (400×) and the middle panel is a lower magnification (100×) of representative cell clones. The bottom panel illustrates the ability of various cell clones to grow in semi-solid media. Cells were plated in soft agar as described in Materials and methods and colonies photographed 12 days later.

dramatic suppressive effect (Figure 3). To quantify differences in transforming ability of different cell lines, a focus forming assay was conducted in which cells expressing the various mutants were mixed with normal untransformed Fr3T3 cells. Focus formation was scored as a percentage relative to the number of foci obtained by mixing Fr3T3 cells with the Tpr-Met-1 cell line. In general cells expressing the dominant-negative Grb2 mutants formed fewer, smaller foci with somewhat delayed kinetics (Figure

4). Cells expressing the ΔN-SH3 Grb2 mutant were most impaired in the ability to form foci (30% of the number of foci obtained with the Tpr-Met-1 cell line) whereas the cells expressing the ΔC-SH3 Grb2 mutant had a very modest effect (70% of control) and the cells expressing wild type Grb2 had similar transforming capacity to the Tpr-Met-1 cell line (Figure 4B).

To eliminate the possibility that the observed phenotypic changes were due to loss of the Tpr-Met oncoprotein or reduced tyrosine phosphorylation of Tpr-Met in selected cell clones, anti-Met immunoprecipitates from cell clones were blotted with anti-Met and anti-phosphotyrosine antibodies. The results indicate that neither the expression level nor the amount of tyrosine phosphorylation of the Tpr-Met oncoprotein is affected by expression of the mutant Grb2 proteins (Figure 5). Moreover, phosphotyrosine immunoblots of total cell lysates revealed no significant differences in the overall level or pattern of tyrosine phosphorylated proteins in the revertant cell lines (Figure 5).
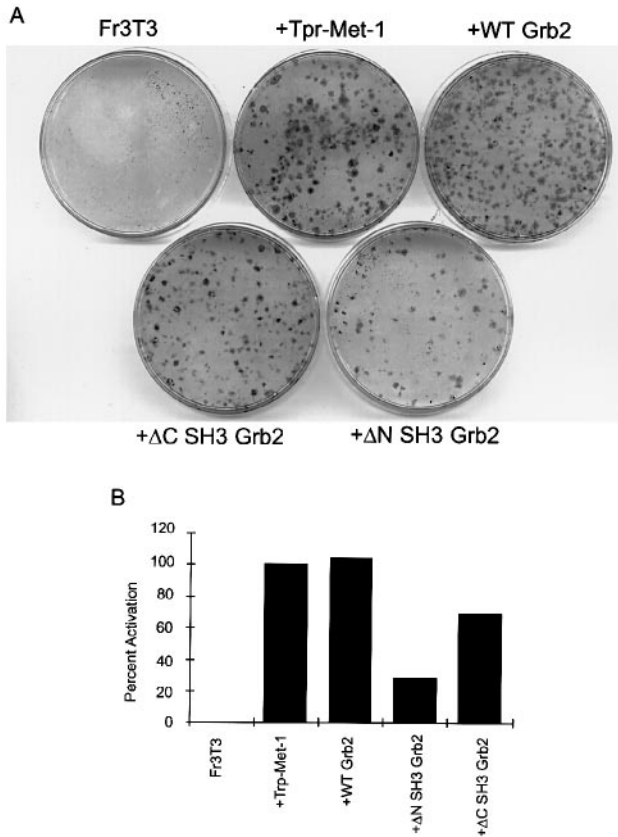
SH3 deletion mutants of Grb2 bind the Tpr-Met oncoprotein and Shc

The Grb2 SH3 deletion mutants are predicted to retain their ability to bind phosphorylated tyrosine residues but not to signal because of their inability to interact with downstream effector molecules containing proline-rich domains. To formally test this, we assessed the ability of these mutants to associate with the Tpr-Met oncoprotein and other tyrosine-phosphorylated proteins.

To determine whether there were any differences in the type or abundance of tyrosyl-phosphorylated proteins bound to deletion mutants as compared with wild type Grb2, anti-HA immunoprecipitates were probed with anti-phosphotyrosine antibodies. Figure 6 shows that a number of phosphoproteins bound to Grb2, including a 52 kDa protein which is consistent with the size of one form of Shc, a 65 kDa protein which corresponds in size to the Tpr-Met oncoprotein and a previously described unknown 110 kDa protein (Fixman *et al.*, 1995). Interestingly, this 110 kDa appears to bind more efficiently to the mutants than to wild type Grb2 when compared with the Shc protein. Additionally, a novel 210 kDa phosphoprotein appears in the immunoprecipitates of the mutants that does not appear in immunoprecipitates of the wild type Grb2 protein even upon prolonged exposure of the gel. The 110 kDa protein may correspond to the Gab1 protein
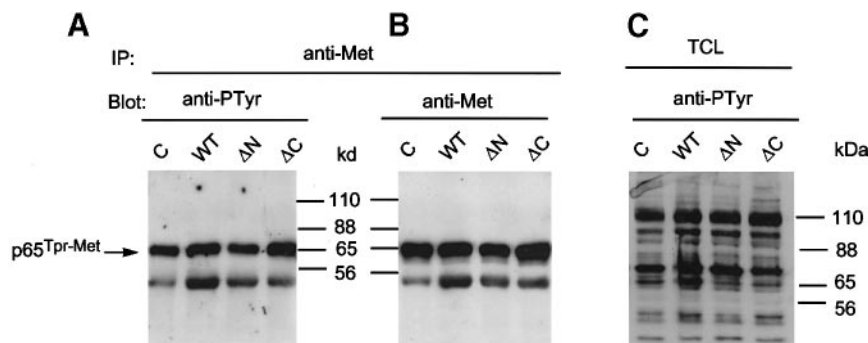

Fig. 4. Dominant-negative Grb2 mutants inhibit Tpr-Met focus formation. The effect of the dominant-negative Grb2 mutants was tested in a focus assay. Cells expressing Tpr-Met alone or with the wild type or SH3 deletion mutants of Grb2 were mixed with untransformed Fr3T3 cells, grown to confluency and foci were scored after 14 days. (**A**) Foci were visualized with Giemsa stain. (**B**) Foci were quantified and are presented as a percentage relative to the number of foci obtained with mixing Fr3T3 cells with Tpr-Met-1 cells. The number of foci used are the average of duplicate plates from three independent experiments.

Fig. 5. Expression and phosphorylation of Tpr-Met in revertant cells. Anti-Met immunoprecipitates (**A** and **B**) or cell lysates (**C**) from cells coexpressing the Tpr-Met oncoprotein and Grb2 mutants were resolved by SDS–PAGE and transferred to nitrocellulose. Membranes were immunoblotted with either anti-phosphotyrosine (A and C) or anti-Met (B) antibodies. The Tpr-Met oncoprotein is indicated by the arrow.
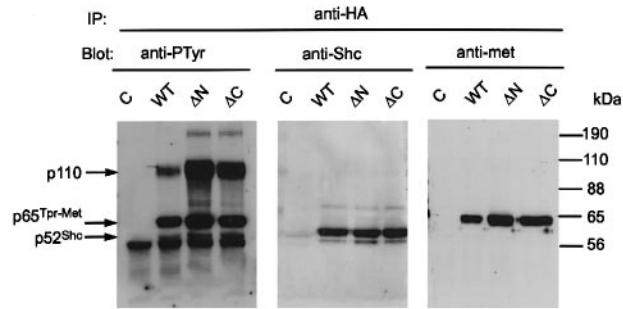
by this mutant is unrelated to inhibition of activation of $p42^{MAPK}$ or $p44^{MAPK}$.

Recently a number of experimental lines of evidence have indicated that *Ras*-induced transformation requires multiple signaling pathways which, in addition to MAP kinase, include Rac1 and Rho, members of the Rho family of GTP binding proteins (Khosravi-Far *et al.*, 1995, 1996; Michiels *et al.*, 1995; Prendergast *et al.*, 1995; Qiu *et al.*, 1995a,b). Rac1 was shown to be downstream of the Met receptor and required for HGF-induced membrane ruffling (Ridley *et al.*, 1995). Recently, Rac1 was shown to activate the JNK/SAPK signaling pathway (Coso *et al.*, 1995; Minden *et al.*, 1995). Thus we were interested in testing whether activation of the JNK/SAPK pathway was altered in Met transformed cell lines and whether this is affected by expression of dominant-negative mutants of Grb2. To test this hypothesis, a GST–N-terminal c-Jun fusion protein was used to immunoprecipitate JNK from cell lysates expressing different Grb2 mutants and was used as a substrate in an *in vitro* kinase assay to measure JNK activity. The results in Figure 8B indicate that JNK activity is enhanced dramatically in cells expressing Tpr-Met when compared with normal Fr3T3 cells. In fact the level of JNK activity in Met transformed cells is comparable with that obtained by treatment of Fr3T3 cells with UV, a potent activator of JNK. Interestingly, in the cell line expressing the ΔN-SH3 Grb2, JNK activity is significantly reduced (4-fold in Figure 8B). The levels of activity are not reduced to the low levels seen in Fr3T3 cells, consistent with the incomplete reversion of the transformed phenotype seen with this mutant.

To insure that this observed difference in JNK activity is not due to clonal variation, the levels of JNK activity were compared between three cell lines expressing different amounts of the ΔN-SH3 Grb2 mutant. The ΔN15 clone expressing the least amount of mutant protein had slightly increased levels of JNK kinase activity (Figure 8C). However, as the amount of mutant protein expressed is increased, there is a concomitant decrease in JNK kinase activity (Figure 8C). In contrast, there was a slight elevation in the amount of MAP kinase activity observed with increased expression of the ΔN-SH3 Grb2 mutant (Figure 8C). Significantly, whereas the ΔN15 clone that expressed a low level of ΔN-SH3 Grb2 has a fully transformed phenotype indistinguishable from the Tpr-Met-1 cell line, the two cell lines ΔN7 and ΔN11 that overexpress the mutant relative to endogenous Grb2 have a revertant phenotype. Thus, there is a strong correlation between the amount of ΔN-SH3 Grb2 expression, reduced JNK activity and reversion of the transformed phenotype, indicating that expression of this mutant affects activation of the JNK/SAPK pathway.

To test whether JNK activation is unique to the Tpr-Met oncoprotein or is part of the normal Met signaling response, HeLa cells were stimulated with HGF and JNK activity measured. The results in Figure 8D reveal that the normal endogenously expressed HGF receptor is capable of activating JNK upon HGF stimulation. This result suggests that the JNK/SAPK pathway is part of the normal Met signaling repertoire that is constitutively activated by the oncogenic Tpr-Met.

PI-3 kinase activity is inhibited by dominant-negative Grb2

Recently, activated PI-3 kinase has been shown to stimulate JNK (Klippel *et al.*, 1996) (S.Logan *et al.*, submitted). Moreover, Wortmannin, an inhibitor of PI-3 kinase can inhibit EGF-induced activation of JNK (S.Logan *et al.*, submitted). The fact that the Met oncogene has been shown to activate PI-3 kinase and potentially bind p85 through Y489 prompted examination of PI-3 kinase activity in cells expressing the dominant-negative Grb2 mutant. Consistent with previous results (Fixman *et al.*, 1996), potent constitutive PI-3 kinase activity was detected in anti-phosphotyrosine immunoprecipitates from Met transformed cells but not from their untransformed parental cell lines (Figure 9A). However, in lysates from cells expressing the ΔN SH3 dominant-negative mutant of Grb2 the amount of PI-3 kinase activity was significantly reduced to ~35% of that obtained with immunoprecipitates of the Tpr-Met-1 cell line (Figure 9A). The ΔC SH3 Grb2 mutant retained 65% of the activity obtained with Tpr-Met-1, consistent with its transforming capability (Figure 4). In contrast to that seen with the anti-phosphotyrosine antibodies, very little PI-3 kinase activity was detected with anti-Met antibodies (Figure 9B). However, consistent with the results obtained with anti-phosphotyrosine antibodies, the amount of PI-3 kinase activity immunoprecipitated from lysates expressing the dominant-negative Grb2 mutant was reduced to 25% of that obtained with Tpr-Met-1 (Figure 9B).

These results suggest that the dominant-negative Grb2 mutant may prevent association with and/or activation of PI-3 kinase by the Tpr-Met oncoprotein. To test whether this may occur through a competitive mechanism, the amount of Grb2-associated PI-3 kinase activity was determined in various cell lines. We were able to detect PI-3 kinase activity associated with exogenously expressed wild type Grb2 (Figure 9C). Interestingly, the ΔN-SH3 Grb2 mutant has significantly more associated PI-3 kinase activity than does the wild type Grb2 (>10 fold; Figure 9C). Again consistent with its moderate dominant-negative effect, the ΔC-SH3 Grb2 mutant had a lower fold activation relative to the wild type Grb2 (Figure 9C). These results suggest that activation of PI-3 kinase may have a role in activation of JNK by the Tpr-Met oncoprotein and that the dominant-negative Grb2 may associate with and thereby sequester PI-3 kinase.

A dominant-negative mutant of Rac1 inhibits transformation of fibroblasts by the Tpr-Met oncoprotein

The above results suggest that activation of the JNK pathway may in part mediate the transforming ability of the Met oncogene. To assess the role of the JNK pathway in transformation by Met, dominant-negative mutants of various members of the Rho family of GTP-binding proteins were tested in different biological assays. Activation of JNK has recently been shown to activate the Serum Response Element (SRE) through its phosphorylation of the transcription factor Elk (Cavigelli *et al.*, 1995; Gille *et al.*, 1995; Whitmarsh *et al.*, 1995). Therefore, we first tested the ability of these mutants to block Tpr-Met activation of the SRE driving expression of a luciferase reporter cDNA following transient cotransfection with
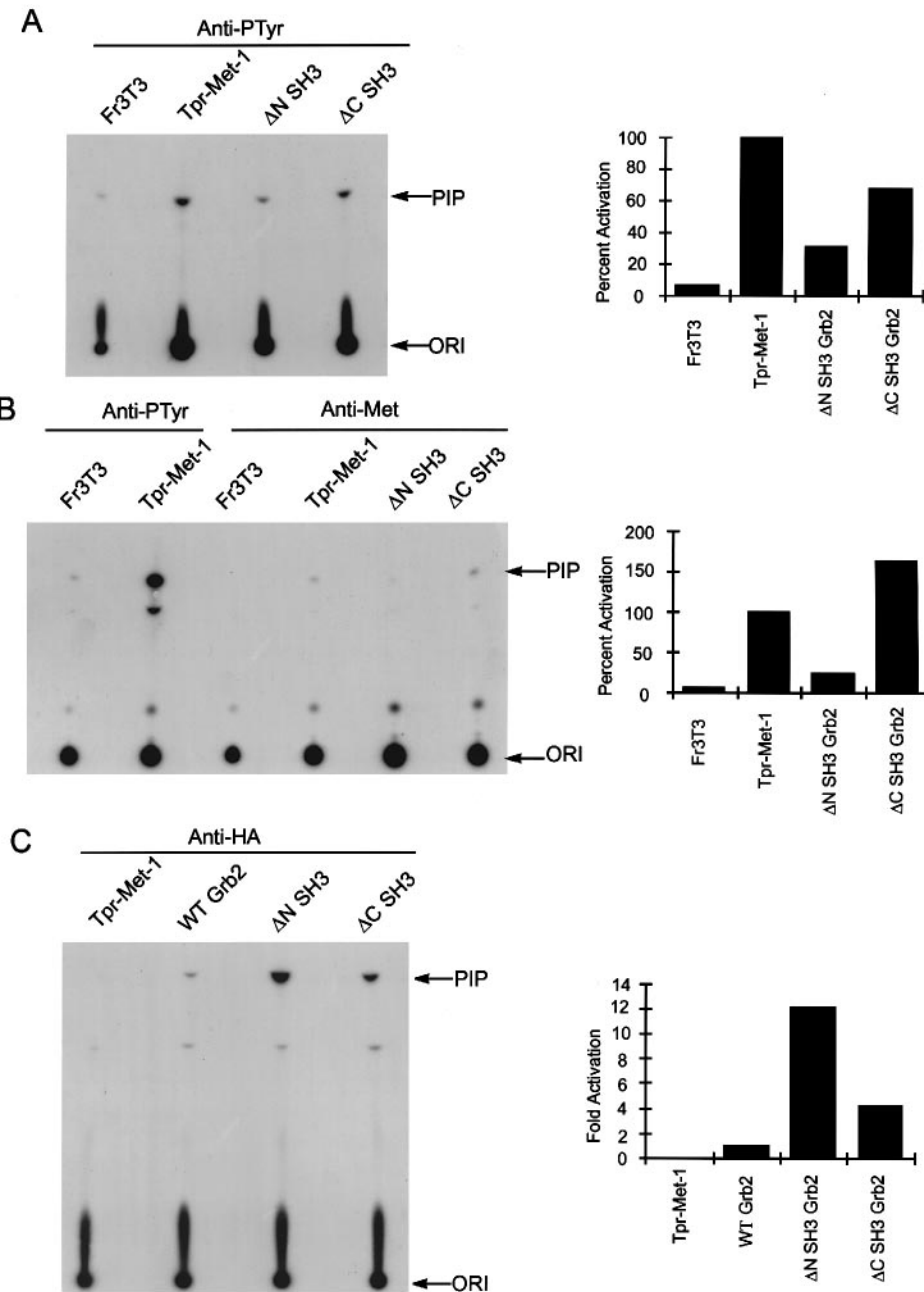
Fig. 9. PI-3 kinase activity is inhibited by expression of the ΔN SH3 Grb2 mutant. PI-3 kinase activity was measured in anti-phosphotyrosine immunoprecipitates (**A**), anti-Met immunoprecipitates (**B**) and anti-HA immunoprecipitates (**C**) from lysates of cells expressing Tpr-Met with or without the wild type or mutant Grb2. PI-3 kinase activity was measured as described in Materials and methods. The origin (ORI) of the chromatogram and the position of PI 3-phosphate (PIP) are indicated. The panels on the right are a bar graph showing the percentage activation of PI-3 kinase with the activity obtained with Tpr-Met-1 cell line being 100% (A and B) or showing the fold activation relative to cells expressing wild type Grb2 (C).

Tpr-Met into HeLa cells. The results indicate that a dominant mutant of Rac1 [Rac1(N17)] strongly inhibits Tpr-Met activation of the SRE–luciferase reporter (Figure 10A). A dominant-negative CDC42 [CDC42(N19)] was somewhat less effective but also significantly inhibited Tpr-Met activation of the SRE. In contrast, a dominant-negative Rho [Rho(N17)] had little effect on the ability of Tpr-Met to activate an SRE. This result is consistent with the fact that Rac1 and CDC42 are effective activators of JNK whereas Rho is not (Hill *et al.*, 1995).

Given its ability to potently inhibit SRE activation by the Met oncoprotein we tested the effect of dominant-

negative Rac1 on the transforming potential of this oncogene. Cell lines expressing both Tpr-Met and a Myc-tagged version of Rac1(N17) were isolated and found to be less transformed than cells expressing Tpr-Met alone. As shown in Figure 10B, Rac1(N17)-expressing cells formed colonies in soft agar that were significantly smaller than those formed by Tpr-Met-expressing cells. Tpr-Met cells expressing a dominant-negative Rho or CDC42 did not appear to be significantly impaired in their transforming potential (data not shown).

To confirm that the inhibition of transforming activity of Met correlated with alteration in JNK activity, kinase

A

B
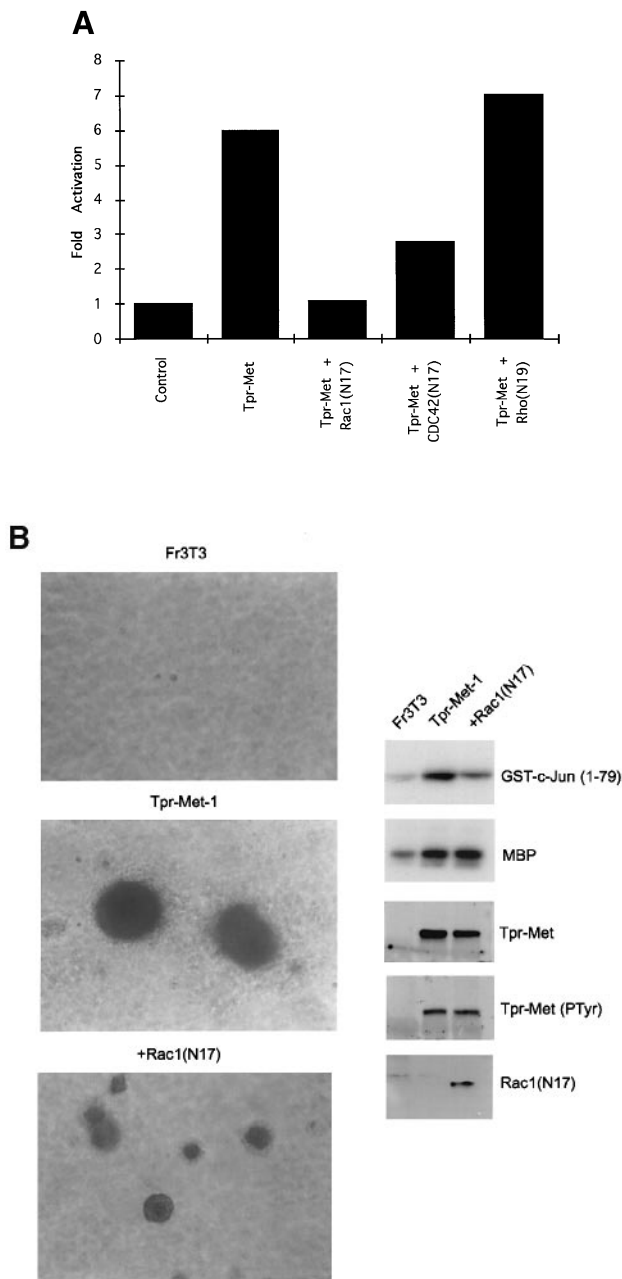
Fig. 10. Dominant-negative Rac1 inhibits transformation by the Met oncoprotein. (**A**) HeLa cells were transiently transfected with 1 μg of a SRE-luciferase reporter and 1 μg of Tpr-Met with 4 μg of the indicated dominant-negative protein or empty vector. Luciferase activity was measured 48 h post-transfection. The results are the average of two independent experiments and are expressed as fold activation over the control (cells transfected with the SRE reporter construct alone) after normalizing for β-galactosidase activity. (**B**) Expression of a Rac1(N17) inhibits growth of Tpr-Met expressing fibroblasts in soft agar. Colony growth of Fr3T3 (top panel), Tpr-Met 1 (middle panel) and cells expressing both Tpr-Met and Rac1(N17) is shown after 10 days of growth in soft agar. (**C**) JNK kinase activity is reduced in Rac1(N17)-expressing cells whereas MAP kinase activity is unaffected. Lysates from normal Fr3T3 cells, Tpr-Met-1 cells and Tpr-Met-1 cells expressing Rac1(N17) were tested in JNK and MAP kinase assays as described in the legend to Figure 7. The same lysates were immunoprecipitated with antibodies to Met and blotted with anti-phosphotyrosine and anti-Met antibodies. In addition whole cell lysates prepared from these cells were blotted with antibodies to Myc to detect expression of Rac1(N17). Western blots indicate equal expression and phosphorylation of Tpr-Met and expression of Rac1(N17).

assays were performed. The results reveal a 2-fold lower level of JNK activity in the cells expressing Rac1(N17) (Figure 10B). Control blots confirmed equal expression and phosphorylation of the Tpr-Met oncoprotein as well as expression of Rac1(N17) (Figure 10B). Thus, together with the observed effects in Grb2 revertant cell lines, cellular transformation by Tpr-Met oncogene appears to be causally related to activation of the JNK pathway.

Discussion

Based on results obtained with tyrosine mutants of Tpr-Met we hypothesized that binding of the Grb2–Sos complex is one of the principal means by which the Tpr-Met onco-protein activates *Ras* and brings about transformation. To test this proposal, dominant-negative mutants of Grb2 capable of binding Tpr-Met but not Sos were introduced into Tpr-Met transformed cells. High levels of overexpression of these mutants were able to revert the transformed phenotype induced by the Tpr-Met oncoprotein (Figures 3 and 4). Deletion of the N-SH3 domain of Grb2 had a more profound affect on the transforming ability of the Tpr-Met oncoprotein. This is in agreement with the observation that this domain appears to bind Sos with higher affinity than does the C-SH3 domain (Cussac *et al.*, 1994; Reif *et al.*, 1994; Sastry *et al.*, 1995) and therefore may play a more critical role in propagating signals.

In addition to forming complexes with growth factor receptors, Grb2 can also associate with Shc via its SH2 domain, itself an oncogene (Pelicci *et al.*, 1992). The phosphorylation state of Shc is regulated by a number of growth factors (Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992; Pronk *et al.*, 1993; Skolnik *et al.*, 1993). Furthermore, Shc phosphorylation is correlated with trans-formation by many oncogenic tyrosine kinases (McGlade *et al.*, 1992; Borrello *et al.*, 1994; Crowe *et al.*, 1994; Fixman *et al.*, 1995; Miloso *et al.*, 1995; Xie *et al.*, 1995). However, the levels of phosphorylation of Shc were unaffected by the presence of the dominant-negative Grb2 mutants (Figure 7). This is similar to what is observed when Tyr489 is mutated (Fixman *et al.*, 1995), suggesting that Shc phosphorylation is not sufficient for transform-ation by the Met oncoprotein although it may contribute to the residual transforming activity observed in these cases.

Unexpectedly, use of the dominant-negative Grb2 mutant revealed a novel connection between Met and the JNK/SAPK pathway. No detectable changes in the expression or phosphorylation state of Tpr-Met or overall phosphotyrosine levels were observed in selected clones (Figure 5). This suggested that the Grb2 SH3 mutants were blocking signaling events downstream of the Met kinase. Direct evidence that downstream events are blocked as a consequence of disruption of the Met–Grb2 complex comes from the demonstration that activation of JNK kinase is significantly reduced in cells expressing the N-SH3 Grb2 mutant in a dose-dependent manner (Figure 8). Interestingly however, MAP kinase activity was unaffected by expression of this mutant.

The fact that MAP kinase is unaffected by the expression of a dominant-negative Grb2 mutant was surprising given the established link between Grb2 and the canonical MAP kinase pathway (Schlessinger, 1993). However, this is not unprecedented, as work with Bcr-Abl demonstrated that

JNK but not MAPK is activated in cells transformed by that oncogene and that JNK activation is absolutely required for this phenotype (Raitano *et al.*, 1995). In the case of Met transformed cells both MAP kinase and JNK are activated (Figure 8). It is therefore likely that activation of both pathways is required for a fully transformed phenotype and that inhibition of either pathway would be sufficient to inhibit transformation. More curious is the observation that only one of these pathways is selectively inhibited by the ΔN-SH3 Grb2 mutant. This could be related to the fact that different aspects of the transformed phenotype are more closely associated with different signaling pathways. For example, it has been suggested that the activation of MAP kinase is an effector function of *Ras* distinct from the morphological changes observed in transformed cells (Joneson *et al.*, 1996). The inability to isolate clones that have inhibition of MAP kinase may therefore be related to its essential role in cell growth. Consistent with this notion, we failed to detect significant differences in the growth rates of Met transformed cells and those expressing the ΔN-SH3 Grb2 mutant (not shown).

Recent evidence suggests that the process of cellular transformation by *Ras* requires a number of cellular events that may involve the participation of multiple signaling pathways (Khosravi-Far *et al.*, 1995, 1996; Michiels *et al.*, 1995; Prendergast *et al.*, 1995; Qiu *et al.*, 1995a,b). *Ras* is essential for transformation by tyrosine kinase oncogenes (Smith *et al.*, 1986; Stacey *et al.*, 1991) and may therefore function as a common component of different signaling cascades. Consistent with this idea is the isolation of complementary classes of mutations within the effector region of *Ras* that selectively activate distinct signaling cascades in mammalian cells that together are required for transformation (White *et al.*, 1995; Joneson *et al.*, 1996; Khosravi-Far *et al.*, 1996). One of these signaling pathways leads to the activation of c-Jun. Transformation by oncogenic *Ras* has been shown to result in increased phosphorylation of c-Jun at the same sites phosphorylated by JNK (Smeal *et al.*, 1992; Westwick *et al.*, 1994). Moreover, dominant-negative mutants of *Ras* block activation of JNK by v-src (Minden *et al.*, 1995). Most convincingly, it was recently demonstrated that cells lacking c-Jun are unresponsive to oncogenic forms of *Ras* (Johnson *et al.*, 1996). Importantly, it has been shown that oncogenic *Ras* activates JNK and c-Jun via a pathway distinct from that of the ERKs (Smeal *et al.*, 1992; Westwick *et al.*, 1994).

Recently, JNK kinase has been shown to be part of a signaling cascade that includes members of the Rho family (Coso *et al.*, 1995; Hill *et al.*, 1995; Minden *et al.*, 1995; Olson *et al.*, 1995). Interestingly, Rho and Rac1, which were previously shown to be involved in the regulation of the actin cytoskeleton (Ridley *et al.*, 1992), have recently been implicated in transformation (Khosravi-Far *et al.*, 1995, 1996; Michiels *et al.*, 1995; Prendergast *et al.*, 1995; Qiu *et al.*, 1995a,b). Constitutively active mutants of both Rac1 and Rho potentiate *Ras* transformation and are themselves weakly transforming (Khosravi-Far *et al.*, 1995, 1996; Michiels *et al.*, 1995; Prendergast *et al.*, 1995). Conversely, dominant-negative mutants of Rac1 and Rho block transformation by *Ras* (Khosravi-Far *et al.*, 1995, 1996; Michiels *et al.*, 1995; Prendergast *et al.*,

1995; Qiu *et al.*, 1995a,b). Importantly, dominant-negative mutants of Rac1 were found to inhibit JNK activation and transformation by tyrosine kinases such as the EGF receptor and the v-src oncogene (Minden *et al.*, 1995). Furthermore, a number of exchange factors for Rho family members including Ost and Dbl activate JNK and are themselves oncogenes. Together these observations suggest that activation of both the MAP kinase and the Rac1/JNK pathways are required for efficient oncogenic transformation. Consistent with this hypothesis we observed that a dominant-negative Rac1 is capable of impairing JNK activation and cellular transformation induced by the Met oncogene (Figure 10).

Rac1 has been implicated in the process membrane ruffling (Ridley *et al.*, 1992). *Ras* transformed cells show increased membrane ruffling and microinjection of *Ras* into normal fibroblasts induces membrane ruffles (Bar-Sagi and Feramisco, 1985; Ridley *et al.*, 1992). Interestingly, HGF induces membrane ruffling, a process that is blocked by microinjection of antibodies to *Ras* and Rac1 (Ridley *et al.*, 1995). Thus, the ability of dominant-negative mutants of Grb2 to block morphological aspects of transformation through inhibition of the Rac1/JNK kinase pathway is in agreement with these observations.

How Met couples to Rac1 and JNK is still unclear. One possibility is that Sos, which, in addition to its *Ras* exchange activity, has a domain with homology to the Rho family of exchange factors, activates Rac1 (Quilliam *et al.*, 1995). However, it has not been demonstrated that Sos has any exchange activity toward Rho family members. Moreover, a membrane-targeted version of Sos that had previously been shown to constitutively activate *Ras* signaling failed to activate JNK (data not shown). Activation of Rho family members may involve coupling of *Ras* through an intermediate such as PI-3 kinase, which has been implicated in membrane ruffling (Kotani *et al.*, 1994; Wennstrom *et al.*, 1994; Hawkins *et al.*, 1995) and which has recently been shown to promote the formation of the GTP-bound form of Rac1 (Hawkins *et al.*, 1995) as well as JNK activation (Klippel *et al.*, 1996) (S.Logan *et al.*, submitted). Moreover, wortmannin, an inhibitor of PI-3 kinase, has been shown to inhibit EGF-induced activation of JNK (S.Logan *et al.*, submitted). As Tyr489 of Tpr-Met serves as the focus for the assembly of a multiple protein signaling complex which, in addition to Grb2, includes PI-3 kinase, Shc, PLCγ and Shp-2 (Ponzetto *et al.*, 1994; Fixman *et al.*, 1995), the mechanism by which the dominant-negative mutant Grb2 inhibits signaling may involve displacement of components of this complex involved in activation of JNK. PI-3 kinase activity is reduced in cells expressing dominant-negative Grb2 (Figure 9). Given the role of PI-3 kinase in activation of JNK, one possibility is that the p85 subunit PI-3 kinase is no longer bound to Met in these cells. We have been unable to detect association of p85 with the Tpr-Met oncoprotein *in vivo* and detect very little Met-precipitable PI-3 kinase activity in transformed cells, suggesting that binding of PI-3 kinase to the Tpr-Met oncoprotein may be indirect. Interestingly, we detect PI-3 kinase activity associated with exogenously expressed Grb2 mutants (Figure 9). Therefore, one possibility is that Grb2 may bind the proline-rich regions in p85, as has been previously reported (Wang *et al.*, 1995), thus sequestering PI-3 kinase

and reducing binding to Tpr-Met. It is noteworthy that there is a requirement for PI-3 kinase in EGF-induced cell transformation but not proliferation (Huang *et al*., 1996).

Recently, it has been shown that Gab1, a molecule with three tyrosines which have surrounding sequence that matches the consensus for p85 binding, binds directly to the Met receptor (Holgado-Madruga *et al*., 1996; Weidner *et al*., 1996). In addition, a number of proline-rich sequences in a favorable consensus for binding the SH3 domains of Grb2 were identified in the Met-binding domain of Gab1 (Holgado-Madruga *et al*., 1996; Weidner *et al*., 1996). An alternative model to explain our observations is that PI-3 kinase is bound to and activated by Met via an intermediate adaptor protein, potentially Gab1, rather than through Grb2. In this case the remaining SH3 domain in a SH3 deletion mutant of Grb2 that can no longer efficiently bind Sos is free to bind the proline-rich sequences in the Met-binding domain of Gab1 and thereby displace Gab1 and consequently PI-3 kinase from Met. Consistent with this model, more PI-3 kinase activity was detected in immunoprecipitates of the ΔN Grb2 mutant than in wild type (Figure 9). Moreover, more of a 110 kDa protein (the size of Gab1) was immunoprecipitated with this mutant than with wild type Grb2 (Figure 6). Another attractive feature of this model is that by analogy with IRS1, an adaptor protein such as Gab1 with numerous potential tyrosine phosphorylation sites provides an explanation for the ability of a single or pair of tyrosine residues in Met to mediate binding of multiple signaling molecules. Whatever the mechanism, the work presented here is the first to provide biochemical evidence to demonstrate a link between Met and the JNK pathway. Exact identification of molecules that couple Met to the JNK pathway should provide further insights into the mechanisms by which tyrosine kinases promote mitogenesis and cellular transformation.

Materials and methods

Cells and DNA transfection
Fischer rat 3T3 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. The isolation of the Tpr-Met-1 cell line has been described previously (Rodrigues and Park, 1993). Generation of the SH3 deletion mutants of Grb2 are described elsewhere (Gishizky *et al*., 1995). Transfections of Fischer rat 3T3 cells to generate stable cell lines was carried out using the calcium phosphate precipitation method.

Antibodies
The Met polyclonal antibody, Ab144 directed against an epitope in the C-terminus of Met has been described previously (Rodrigues *et al*., 1991). EL6 is a monoclonal antibody that recognizes an epitope in the SH2 domain of Grb2. The monoclonal antibody that recognizes an epitope in the N-terminal SH3 domain of Grb2 was purchased from Transduction Laboratories (Lexington, KY). The C23 antibody is a polyclonal antibody directed against the C-terminal SH2 domain of Grb2 and was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Myc, anti-JNK1, anti-ERK 1 (C-16) and anti-ERK2 (K23) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Sos antibodies are rabbit polyclonals generated to a GST–Sos fusion protein. Polyclonal anti-SHC antibodies were generated against a GST–SHC fusion protein.

Growth in soft agar and focus forming assays
To evaluate growth in semi-solid media ~1×10^5 cells per were seeded into 0.3% soft agar (Noble agar, Difco) that was overlaid onto 0.5% agar and grown at 37°C. Colonies were scored 10–14 days later.

To test the ability of selected cell lines to form foci, 5×10^3 cells of the line to be tested were mixed with 1×10^5 untransformed Fr3T3 cells plated into six well dishes, maintained at confluence and scored for foci 14 days later.

Immunoprecipitations and immunoblotting
Cells were lysed with Triton lysis buffer [100 mM KCl, 10 mM PIPES pH 7.0, 0.5% Nonidet P-40 (NP40), 300 mM sucrose, 3 mM MgCl$_2$]. Immunocomplexes were collected on protein A– or protein G–Sepharose, washed with lysis buffer, resuspended in Laemmli sample buffer, boiled for 5 min, subjected to SDS–PAGE and transferred to nitrocellulose. The membranes were blocked in TBST (10 mM Tris pH 7.5, 50 mM NaCl and 0.1% Triton X-100) containing 5% bovine serum albumin (BSA) for 1 h. Membranes were then incubated for 1.5 h with antibody. Membranes were washed extensively with TBST and immunoreactive proteins were detected by incubation with horseradish peroxidase-conjugated protein A for detection of polyclonal antibodies or horseradish peroxidase-conjugated anti-mouse IgG for detection of monoclonal antibodies. Proteins were visualized using enhanced chemiluminescence (ECL) and autoradiography.

JNK and MAP kinase assays
The GST–Jun protein, containing the frst 79 amino acids of c-Jun fused to GST [GST-c-Jun(1–79)] provided by Dr Michael Karin, was grown in bacteria and harvested following induction with isopropyl-β-D-thio-galactopyranoside (IPTG). Approximately 1–5 μg of protein purified on glutathione-agarose beads was used to immunoprecipitate JNKs from cell lysates. Kinase assays were carried out in 30 μl of kinase buffer (20 mM HEPES pH 7.5, 20 mM β-glycerol phosphate, 10 mM PNPP, 10 mM MgCl$_2$, 10 mM DTT 50 μM sodium vanadate) containing 20 μM cold ATP and 0.5 μl of [γ-^{32}P]ATP for 20 min at 30°C. Reactions were terminated by the addition of 2× sample buffer and proteins separated by SDS–PAGE.

Anti-ERK1 antibodies were used to immunoprecipitate MAP kinase from cells. *In vitro* kinase reactions were carried out in kinase buffer (10 mM Tris–HCl pH 7.5, 10 mM MgCl$_2$) using myelin basic protein as a substrate. Following incubation at 25°C for 15 min, reactions were terminated by the addition of sample buffer and proteins resolved by SDS–PAGE.

PI-3 kinase assays
PI-3 kinase assays were performed as previously described (Hu *et al*., 1993). Briefly, immunoprecipitates were washed three times with PBS containing 1% NP40, twice with 0.5 M LiCl/0.2 M Tris (pH 7.6), twice with TNE (10 mM Tris pH 7.6, 100 mM NaCl, 1 mM EDTA) and twice with 20 mM HEPES pH 7.5, 50 mM NaCl, 5 mM EDTA, 0.03% NP40, 30 mM tetrasodium PP$_i$, 100 μM sodium orthovanadate, 10 μg of aprotinin per ml and 1 mM phenylmethylsulfonyl fluoride. Kinase reactions were performed in 50 μl of buffer (20 mM Tris pH 7.6, 75 mM NaCl, 10 mM MgCl$_2$) with 200 μg/ml PI (Avanti), 20 μM ATP, 200 μM adenosine and 10 μCi of [γ-^{32}P]ATP for 20 min at room temperature. Reactions were terminated by the addition of 1 N HCl and the lipids extracted with a 1:1 volume of chloroform/methanol. Lipids were separated by chromotography in chloroform:methanol:4 M NH$_4$OH (9:7:2) (by vol) and visualized by autoradiography.

Luciferase assays
Forty-eight hours post-transfection, cells were washed with PBS and lysed with luciferase lysis buffer (Promega). An aliquot of cleared lysate was added to luciferase reaction buffer (25 mM gycylglycine pH 7.8, 15 mM MgSO$_4$, 1 mM ATP pH 7.0, 0.1 mg/ml BSA and 1 mM DTT). D-luciferin (1 mM) was automatically injected into the mixture and activity measured in a luminometer.

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