Efficient Cellular Transformation by the Met Oncoprotein Requires a Functional Grb2 Binding Site and Correlates with Phosphorylation of the Grb2-associated Proteins, Cbl and Gab1*

(Received for publication, November 18, 1996, and in revised form, May 20, 1997)

Elizabeth D. Fixman,ab Marina Holgado-Madurga,c Linh Nguyen,ad Darren M. Kamikura,aee Tanya M. Fournier,f Albert J. Wong,ag and Morag Parkah

From the Molecular Oncology Group, Royal Victoria Hospital, Departments of aMedicine, bOncology, and cBiochemistry, McGill University, Montreal, Quebec, Canada H3A 1A1 and Departments of dMicrobiology and Immunology and ePharmacology, Kimmel Cancer Institute, Philadelphia, Pennsylvania 19107

The Tpr-Met oncoprotein consists of the catalytic kinase domain of the hepatocyte growth factor/scatter factor receptor tyrosine kinase (Met) fused downstream from sequences encoded by the tpr gene. Tpr-Met is a member of a family of tyrosine kinase oncoproteins generated following genomic rearrangement and has constitutive kinase activity. We have previously demonstrated that a single carboxyl-terminal tyrosine residue, Tyr489, is essential for efficient transformation of Fr3T3 fibroblasts by Tpr-Met and forms a multistate binding site for Grb2, phosphatidylinositol 3' kinase, phospholipase Cγ, SHP2, and an unknown protein of 110 kDa. A mutant Tpr-Met protein that selectively fails to bind Grb2 has reduced transforming activity, implicating pathways downstream of Grb2 in Tpr-Met mediated cell transformation. We show here that the 110-kDa Tpr-Met substrate corresponds to the recently identified Grb2-associated protein, Gab1. Moreover, we show that tyrosine phosphorylation of the Cbl protooncogene product as well as Gab1 required Tyr489 and correlated with the ability of Tpr-Met to associate with Grb2 and to transform cells, providing evidence that pathways downstream of Gab1 and/or Cbl may play a role in Tpr-Met-mediated cell transformation.

The receptor for hepatocyte growth factor/scatter factor (HGF/SF),1 the Met receptor tyrosine kinase (RTK), is expressed primarily in epithelial and endothelial cells in vivo and in vitro where it mediates the pleiotropic biological responses of HGF/SF (1–5). HGF/SF is a mitogen for primary hepatocytes, and stimulates scatter, invasion, and branching tubulogenesis of epithelial cells (6–9). A critical role for both HGF/SF and the Met RTK in development was demonstrated by the embryonic lethality of mice lacking genes encoding either HGF/SF or the Met RTK (10, 11). Moreover, amplification and overexpression of the Met gene is a frequent event in many human tumors (2, 12–16). Thus, activation of the Met RTK is also implicated in neoplasia. Consistent with this, the Met RTK was originally isolated as an oncogene, Tpr-Met (14). Oncogenic activation of the Met receptor occurred following translocation of sequences encoding the amino terminus of the Tpr gene located on chromosome seven (15). The product of the resulting chimeric gene, Tpr-Met, is a constitutively activated, transforming kinase (16, 17).

To define signal transduction pathways required for transformation of fibroblasts by Tpr-Met, we have shown that a single carboxyl-terminal tyrosine residue, Tyr489, is phosphorylated (18) and essential for the association of Tpr-Met with the Grb2 adaptor protein, phospholipase Cγ, and the tyrosine phosphatase, SHP2 (19, 20). This tyrosine is also required for the activation of phosphatidylinositol 3'-kinase and the tyrosine phosphorylation of and/or association with an unknown protein of 110 kDa (19). A Y489F Tpr-Met mutant transforms cells at 20% of the efficiency of the wild-type Tpr-Met oncoprotein; however, because the association of multiple signaling molecules with Tpr-Met is dependent upon Tyr489, it was unclear which of these were required for transformation. A mutant that selectively fails to associate with Grb2, yet retains the ability to associate with phospholipase Cγ, phosphatidylinositol 3'-kinase, and SHP2, transforms cells with the same efficiency as the Y489F mutant (20, 21). Thus, association of Tpr-Met with the Grb2 adaptor protein is essential for efficient transformation of fibroblasts by Tpr-Met. The identification of signaling pathways downstream of Grb2 will help define the mechanism by which Tpr-Met transforms Fr3T3 fibroblasts. In the present study we demonstrate that association of Grb2 with Tpr-Met is essential for the efficient tyrosine phosphorylation of the Cbl protooncogene product, and a previously characterized 110-kDa protein which we show corresponds to the Grb2-associated docking protein, Gab1.

MATERIALS AND METHODS

Mutagenesis—The generation of the various Tpr-Met mutant proteins has been described previously (18–20).

Cell Lines and Tissue Culture—All cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Life Technologies, Inc.) and antibiotics. Cell lines expressing either wild-type or mutant forms of Tpr-Met were generated by ecotropic retroviral infection of parental Fischer rat 3T3 (Fr3T3) fibroblasts as described previously (22).

This paper is available on line at http://www.jbc.org

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 32, Issue of August 8, pp. 20167–20172, 1997

Printed in U.S.A.

© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.

62x76 Printed in U.S.A.

1 The abbreviations used are: HGF/SF, hepatocyte growth factor/scatter factor; RTK, receptor tyrosine kinase; SH2, Src homology domain 2; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; EGF, epidermal growth factor.

2 Senior Scholar of National Cancer Institute of Canada. To whom reprint requests should be addressed: Depts. of Medicine, Oncology, and Biochemistry, McGill University, 687 Pine Ave., West, Montreal PQ, Canada H3A 1A1. Tel.: 514-842-1231 (ext. 5834); Fax: 514-843-1478.

3 This research was supported in part by operating grants from the National Cancer Institute of Canada and the Medical Research Council of Canada (to M. P.), American Cancer Society Grant CA69495, and National Institutes of Health Grant NS 34514 (to A. J. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

4 Recipient of a Fonds de la Recherche en Santé du Québec Fellowship.

5 Recipient of a Royal Victoria Hospital Research Institute Fellowship.

6 Recipient of a Steve Fonyo Research Studentship.

7 Recipient of a Royal Victoria Hospital Research Institute Studentship.

8 Recipient of a Fonds de la Recherche en Santé du Québec Fellowship.

9 This paper is available on line at http://www.jbc.org

20167
Antibodies—Antibodies which recognize Tpr-Met were generated against a carboxyl-terminal peptide of the Tpr-Met protein as described previously (22). Antibodies which recognize Gab1 were generated against bacterially expressed GST-Gab1 as described previously (23). Antibodies to Cbl were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to hemagglutinin were purchased from Berkeley Antibody Company. For anti-phosphotyrosine immunoblotting, recombinant horseradish peroxidase-conjugated antiphosphotyrosine Fab fragment, RC20H, from Transduction Laboratories, was utilized.

Cell Lysates, Immunoprecipitation, and Western Blotting—Generation of cell lysates, immunoprecipitation, and Western blotting have been described previously (19).

GST Fusion Protein Production—Bacteria expressing GST proteins fused to either full-length Grb2, SH2, SH3(N)-SH2, or SH2-SH3(C) domains of Grb2 were kindly provided by Drs. Mike Moran, Alain Charest, and Michel Tremblay. Fusion proteins were produced by iso- propyl-β-D-thiogalactopyranoside induction and purification on glutathione-agarose beads (24). Approximately 0.5–1.0 μg of protein was used in the in vitro association experiments.

Transient Transfections and in Vitro Association Assays—293 cells (8 × 10⁵) were transfected with 5 μg of expression plasmid DNA encoding either wild-type or mutant forms of the Tpr-Met proteins utilizing calcium phosphate coprecipitation. In Fig. 5B, 2 μg of an expression plasmid encoding hemagglutinin-tagged Gab1 were included. The cells were maintained for 3 days in Dulbecco’s modified Eagle’s medium and 5% fetal bovine serum. They were harvested in 0.5% Triton X-100 lysis buffer. COS-1 cells (10⁶) were transfected with 6 μg of expression plasmid DNA encoding either wild-type or mutant forms of the Tpr-Met proteins utilizing a modification of the DEAE-dextran protocol (19). The cells were maintained for 60 h in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, after which they were harvested in 0.5% Triton X-100 lysis buffer. Tpr-Met proteins from approximately 20% of each lysate were immunoprecipitated with antibody 144, the immune complexes collected on protein A-Sepharose, and washed with 0.5% Triton X-100 lysis buffer. In vitro association/kinase assays were carried out as described (19, 25). For denaturation and reimmunoprecipitation following the in vitro association/kinase assay, the immune complexes were boiled for 5 min in 900 μl of buffer composed of 0.4% SDS, 50 mM triethanolamine, 100 mM NaCl, 2 mM EDTA, and 2 μg β-mercaptoethanol. Following addition of 125 μl of Tris-Cl (pH 7.4) and 250 μl of 10% Triton X-100, the lysates were incubated with GST-Grb2 fusion proteins or anti-Tpr-Met antibody 144 for 3 h at 4°C and washed with 0.5% Triton X-100 lysis buffer. Samples were then suspended in Laemmli sample buffer, boiled for 10 min, and subjected to 9% SDS-PAGE. Proteins were visualized by autoradiography.

**RESULTS**

The carboxyl terminus of the Tpr-Met oncprotein contains three tyrosine residues, Tyr482, Tyr489, and Tyr498, two of which are followed by consensus binding sites for several SH2 binding proteins (Fig. 1) (26, 27). Consistent with Tyr489 being the only detectably phosphorylated residue in the carboxyl terminus (18), mutation of this residue to a conserved Phe residue results in a dramatic decrease in transforming activity of the Y489F mutant protein (19, 28). Utilizing carboxyl-terminal tyrosine mutant Tpr-Met proteins, Y482F, Y489F, and Y482F/Y489F, as well as a mutant that has selectively lost the ability to associate with Grb2, N491H, we have demonstrated that pathways downstream of Grb2 and Shc are required for transformation of Fr3T3 cells by Tpr-Met (20). Thus, characterization of the signaling pathways downstream of Grb2 and Shc will permit further definition of the mechanism by which Tpr-Met transforms Fr3T3 fibroblasts.

**Tyrosine Phosphorylation of a Novel 110-kDa Protein Is Dependent upon the Ability of Tpr-Met to Associate with Grb2—**

Using a sensitive in vitro association/kinase assay to detect proteins which associate with Tpr-Met, we have previously identified a highly phosphorylated protein of 110 kDa whose association with and/or phosphorylation by Tpr-Met was dependent upon the presence of Tyr489 (19) (Fig. 2A, lane 5). When compared with wild-type Tpr-Met (Fig. 2A, lane 2), a Y482F/Y489F Tpr-Met mutant failed to associate with and/or phosphorylate the 110-kDa protein (Fig. 2A, lane 6), whereas the Y482F Tpr-Met mutant retained the ability to associate with and/or phosphorylate this protein (Fig. 2A, lane 4). Interestingly, the N491H Tpr-Met mutant, which no longer binds to Grb2, was also severely impaired in its ability to associate with and/or phosphorylate the 110-kDa protein (Fig. 2A, lane 7). This suggested that the association of the 110-kDa protein with Tpr-Met was indirect, mediated by the Grb2 adaptor protein, or direct and, like Grb2, dependent upon the Asn residue two amino acids downstream of tyrosine 489.

To investigate whether the 110-kDa protein could associate directly with Grb2, the phosphorylated proteins from an in vitro association/kinase assay were denatured by boiling in buffer containing 0.4% SDS and 2 μg β-mercaptoethanol. Following addition of Tris-Cl and Triton X-100, the dissociated and denatured proteins were incubated with anti-Tpr-Met antibody 144 (22) or GST proteins encoding either full-length Grb2 or various domains of Grb2. No detectable 110-kDa protein was coimmunoprecipitated with Tpr-Met, demonstrating that the protein complexes had been efficiently dissociated (Fig. 2B, lane 6). However, following incubation of the dissociated, denatured proteins with a nondenatured GST-Grb2 fusion protein, association of the 110-kDa protein with full-length Grb2 was detected (Fig. 2B, lane 1) demonstrating the the 110-kDa protein could associate directly with Grb2. The 110-kDa protein also associated to the same extent with the SH3(SH3)(C) Grb2 fusion protein (Fig. 2B, lane 3) and to a lesser extent with the SH3(SH3)(SH3)(C) Grb2 fusion proteins (Fig. 2B, lanes 2 and 4). It did not associate with a fusion protein encoding only the SH2 domain of Grb2 (Fig. 2B, lane 5). Tpr-Met, on the other hand, associated with full-length Grb2 (Fig. 2B, lane 1) as well as the SH3(SH3)(C), SH2-SH3(C), and SH2 fusion proteins (Fig. 2B, lanes 2, 3, and 5) but not with the SH3(SH3)(SH3)(C) Grb2 fusion protein. Neither Tpr-Met nor the 110-kDa protein associated with GST (data not shown). These data demonstrate that the 110-kDa protein associated primarily with the carboxyl-terminal SH3 domain of Grb2 and suggest that the Grb2 adaptor protein acts to couple the 110-kDa protein with Tpr-Met.

**Efficient Tyrosine Phosphorylation of the Cbl Protooncogene**
Tpr-Met Transformation and Phosphorylation of Cbl and Gab1

Fig. 2. The 110-kDa protein is not associated with and/or phosphorylated by the N491H Tpr-Met mutant in vitro. A, wild-type Tpr-Met (lanes 1 and 2) or the various Tpr-Met mutant proteins (lanes 3–7) from transiently transfected COS-1 cells were immunoprecipitated and activated by phosphorylation with nonradioactive ATP. Extracts from serum-starved Fr3T3 cells (lanes 2–7) or an equivalent volume of lysis buffer (lane 1) were then added. To visualize associated proteins, the oncoprotein complexes were washed several times and then incubated in kinase buffer with [γ-32P]ATP. The complexes were resolved by 8% SDS-PAGE and visualized by autoradiography. The positions of Tpr-Met, the 110-kDa protein, and the molecular weight markers are indicated. B, following incubation with [γ-32P]ATP, Tpr-Met and associated proteins were boiled 5 min in buffer containing 0.4% SDS and 2 μL β-mercaptoethanol (see “Materials and Methods”). After adding Trit-CI and Triton X-100, the dissociated and denatured proteins were incubated with GST proteins encoding full-length Grb2 (lane 1), and the SH3(N)-SH2 (lane 2), SH2-SH3(C) (lane 3), SH3(N)-SH3(C) (lane 4), or SH2 (lane 5) domains of Grb2 bound to glutathione-Sepharose or antibody 144 that recognizes Tpr-Met (lane 6). Following several washes with lysis buffer, the proteins were resolved by 9% SDS-PAGE and visualized by autoradiography.

Product Is Dependent upon the Ability of Tpr-Met to Associate with Grb2—The Cbl protooncogene product, which is similar in size to the 110-kDa protein, is phosphorylated on tyrosine residues following stimulation of several receptor tyrosine kinases (29, 30) and in v-Src and v-Abl transformed fibroblasts (29, 31). As demonstrated by phosphorysine immunoblotting, Cbl was highly phosphorylated in the stable cell lines expressing wild-type or the highly transforming Y482F Tpr-Met mutants (Fig. 3A, lanes 2–5). However, Cbl was poorly phosphorylated in stable cell lines expressing the weakly transforming N491H, Y489F or the nontransforming Y482F/Y489F Tpr-Met mutants (Fig. 3A, lanes 6–11), although similar amounts of protein were immunoprecipitated (Fig. 3B). Thus, in a manner similar to the 110-kDa protein, the increased level of Cbl phosphorylation in stable cell lines expressing Tpr-Met was dependent upon Tyr^{489} and more specifically, the Asn residue two amino acids downstream of Tyr^{489}.

These data suggested that Cbl was binding Tpr-Met either directly, recognizing the consensus binding site, YVNV, present at position 489, or indirectly via another protein that required Tyr^{489} for association. Cbl contains several proline-rich repeats that mediate its association with SH3 domain-containing proteins, including Grb2 (32–34). Because Cbl was not highly phosphorylated in cell lines expressing Tpr-Met mutants unable to bind Grb2, the possibility that Grb2 was functioning as an adaptor coupling the Tpr-Met oncoprotein with Cbl was investigated. A GST-Grb2 fusion protein was able to bind to Cbl present in lysates prepared from both Fr3T3 fibroblasts and cell lines expressing wild-type or the mutant Tpr-Met proteins (Fig. 3C, lanes 3–10). Furthermore, while full-length Grb2 (Fig. 3D, lane 1), as well as the SH3(N)-SH2 (lane 2), SH2-SH3(C) (lane 3), SH3(N)-SH3(C) (lane 4), or SH2 (lane 5) domains of Grb2 coupled to glutathione-Sepharose. Complexes were washed, resolved by SDS-PAGE, and immunoblotted with anti-Cbl serum. Bottom, the amount of GST proteins used in the association assay were visualized by anti-GST immunoblotting.

Fig. 3. The Cbl protooncogene product is highly phosphorylated in wild-type Tpr-Met transformed cells but at low levels in cells expressing the N491H Tpr-Met mutant. Lysates from parental Fr3T3 cells (lanes 1) or stable cell lines expressing either wild-type (lanes 2 and 3) or the various Tpr-Met mutant proteins (lanes 4–11) were prepared and immunoprecipitated with an anti-Cbl serum, collected on protein A-Sepharose, and washed three times with lysis buffer. The proteins were resolved by 9% SDS-PAGE and immunoblotted with recombinant RC20H anti-phosphotyrosine antibody (A) or anti-Cbl serum (B). C, lysates from parental Fr3T3 cells (lane 3) or stable cell lines expressing wild-type Tpr-Met protein (lanes 2 and 4), the Y482F (lane 5), N491H (lanes 6 and 7), Y489F (lanes 8 and 9), or Y482F/Y489F (lane 10) Tpr-Met mutants were incubated with GST-Grb2-Sepharose (lanes 3–10) or GST-Sepharose (lane 2). Complexes were washed, resolved by SDS-PAGE, and immunoblotted with anti-Cbl serum. An anti-Cbl immunoprecipitation was also included (lane 1). D, top, lysates from parental Fr3T3 cells were incubated with GST proteins encoding full-length Grb2 (lane 1), and the SH3(N)-SH2 (lane 2), SH2-SH3(C) (lane 3), SH3(N)-SH3(C) (lane 4), or SH2 (lane 5) domains of Grb2 coupled to glutathione-Sepharose. Complexes were washed, resolved by SDS-PAGE, and immunoblotted with anti-Cbl serum. Bottom, the amount of GST proteins used in the association assay were visualized by anti-GST immunoblotting.
The novel 110-kDa protein is the Grb2-associated docking protein, Gab1—Gab1, a Grb2-binding protein of 115 kDa, was recently isolated and shown to be a multisubstrate docking protein that is tyrosine-phosphorylated following stimulation of cells with EGF and insulin (23). Because both Gab1 and the 110-kDa protein associate with Grb2 and are similar in size, the ability of Gab1 to associate with Tpr-Met was determined. In the in vitro association assay Gab1 expressed in Fr3T3 fibroblasts associated with the wild-type Tpr-Met oncoprotein (Fig. 5A, lane 1) and to a lesser extent with the highly transforming Y482F mutant Tpr-Met protein (Fig. 5A, lane 3). However, it did not associate well with the poorly transforming Y489F, N491H, or the nontransforming Y482F/Y489F mutant Tpr-Met proteins, none of which bind Grb2 (Fig. 5A, lanes 4–6).

Similarly, in 293 cells overexpressing Tpr-Met and Gab1, both the wild-type and the highly transforming Y482F mutant Tpr-Met proteins communoprecipitated with Gab1 (Fig. 5B, lanes 1 and 3), whereas the nontransforming Y482F/Y489F Tpr-Met mutant associated poorly with Gab1 (Fig. 5B, lane 5), to the same extent as the nontransforming K241A Tpr-Met mutant (Fig. 5B, lane 2). Interestingly, compared with wild-type Tpr-Met, when overexpressed, the poorly transforming Y489F and N491H mutants showed weak association with Gab1 (Fig. 5B, lanes 4 and 6). Each of the Tpr-Met proteins was expressed at approximately equal levels (Fig. 5C, lanes 1–6).

In a manner similar to the 110-kDa protein (Fig. 2B), Gab1 from serum-starved Fr3T3 cells (Fig. 5D) or Tpr-Met transformed Fr3T3 cells (Fig. 5E) associated predominantly with GST-Grb2 fusion proteins containing the carboxyl-terminal SH3 domain of Grb2 (Fig. 5, D and E, lanes 1, 3, and 4) and not detectably with a fusion protein encoding only the SH2 domain of Grb2 (Fig. 5, D and E, lanes 5). Thus, because both Gab1 and the 110-kDa protein bound preferentially to the carboxyl-terminal SH3 domain of Grb2, were impaired in their ability to interact with Tpr-Met mutants that did not bind Grb2 (Y489F and N491H, Figs. 2A and 5, A and B) and were similar in size,
we examined whether the 110-kDa protein was Gab1. In the in vitro association/kinase assay, using a lysate immunodepleted with anti-Gab1 antiserum, the 110-kDa Tpr-Met substrate was no longer detected (Fig. 5F, lane 4). However, a lysate treated similarly, but without addition of anti-Gab1 antiserum, retained the 110-kDa protein (Fig. 5F, lane 3). Thus, the 110-kDa Tpr-Met substrate corresponds to the Grb2-associated docking protein, Gab1.

**DISCUSSION**

Using a series of mutant Tpr-Met oncoproteins, we have previously demonstrated that, in the absence of direct Grb2 association and Shc phosphorylation, Tpr-Met fails to transform Fr3T3 fibroblasts (20). Thus, the identification of pathways downstream of these two adaptor molecules is crucial in defining the mechanism by which Tpr-Met transforms fibroblasts. We have shown here that tyrosine phosphorylation of the Cbl protooncogene product and the multisubstrate docking protein, Gab1, correlates with the ability of the Tpr-Met oncoprotein to associate with the Grb2 adaptor protein and to transform cells.

**Efficient Tyrosine Phosphorylation of Cbl Is Dependent upon the Ability of Tpr-Met to Associate with Grb2**—The c-Cbl protooncogene product is a 120-kDa phosphoprotein that is tyrosine-phosphorylated in cells transformed by v-Src, v-Abl, or Bcr-Abl and following activation of multiple receptor tyrosine kinases (29–31, 33, 35, 37). Consistent with tyrosine phosphorylation of Cbl playing a role in cellular transformation, Cbl was phosphorylated on tyrosine residues in cells expressing only the highly transforming wild-type or Y482F mutant Tpr-Met oncoproteins (Figs. 3A and 4A). The level of Cbl phosphorylation was reduced in cells expressing the weakly transforming Tpr-Met mutants, Y489F and N491H, which do not bind to Grb2 (Fig. 3A) (19–21). Thus, the dramatic decrease in Cbl phosphorylation in cells expressing the N491H or Y489F Tpr-Met mutants suggests that either Grb2 or the Asn residue two amino acids downstream of Tyr489 is required for subsequent phosphorylation of Cbl. Consistent with the former, Cbl contains proline-rich sequences that interact with SH3 domain-containing proteins (33, 38–40) and a GST-Grb2 fusion protein was able to bind Cbl from Fr3T3 cells and all Tpr-Met expressing cell lines irrespective of the phosphorylation status of Cbl (Fig. 3C). Moreover, an interaction between wild-type Tpr-Met and Cbl was not readily detected in Tpr-Met-transformed fibroblasts (data not shown) or in cotransfection assays where an association between Cbl and the EGF receptor was observed (Fig. 4A). Thus, while the exact mechanism by which Cbl is phosphorylated in Tpr-Met-transformed fibroblasts is unclear, the level of Cbl phosphorylation correlated with the ability of the mutant Tpr-Met proteins to transform cells.

**The Novel 110-kDa Tpr-Met Substrate Corresponds to the Grb2-associated Docking Protein, Gab1**—In addition to Cbl, the highly transforming wild-type Tpr-Met oncoprotein induced tyrosine phosphorylation of a 110-kDa protein both in vivo (19) and in vitro (Fig. 2A). Like Cbl, phosphorylation of this protein was dependent upon the ability of Tpr-Met to associate with Grb2. However, unlike Cbl, which bound primarily to GST-Grb2 fusion proteins containing the amino-terminal SH3 domain of Grb2 (Fig. 2D), the 110-kDa protein bound primarily to GST-Grb2 fusion proteins containing the carboxy-terminal SH3 domain of Grb2 (Fig. 2B). Moreover, unlike Cbl, a stable association between the 110-kDa protein and Tpr-Met was detected (Fig. 2A) and a lysate prepared from Fr3T3 fibroblasts immunodepleted of Cbl, still contained abundant levels of the 110-kDa phosphoprotein (Fig. 4D), providing further evidence that the 110-kDa protein did not correspond to Cbl. Recently a 115-kDa, Grb2 associated protein, Gab1, was identified and shown to be tyrosine-phosphorylated following stimulation of cells with EGF and insulin (23). Our data demonstrate that the highly phosphorylated 110-kDa Tpr-Met substrate in Fr3T3 fibroblasts is Gab1. Like the 110-kDa Tpr-Met substrate, Gab1 associated primarily with the carboxy-terminal SH3 domain of Grb2 (Figs. 2B and 5, D, and E) and neither Gab1 nor the 110-kDa Tpr-Met substrate associated efficiently with Tpr-Met mutant proteins that failed to bind Grb2 directly (Figs. 2A and 5, A and B). Furthermore, a lysate prepared from Fr3T3 fibroblasts and immunodepleted of Gab1 contained no 110-kDa protein (Fig. 5F), consistent with the 110-kDa protein being Gab1. While the majority of Gab1 association with Tpr-Met was dependent upon an intact Grb2 binding site at Tyr489, we also consistently observed low, but detectable binding of Gab1 to the Y489F and N491H Tpr-Met mutants that fail to bind Grb2 (Fig. 5, A and B). This may represent direct binding of Gab1 to Y482 of Tpr-Met. Interestingly, a direct interaction between Gab1 and Y1349 in the Met receptor (which corresponds to Y482 in Tpr-Met) was identified using the yeast two hybrid system (41). This is consistent with our data; however, we demonstrate here that the majority of Gab1 association with Tpr-Met is mediated by Tyr489 and Grb2 and not by direct association with Tyr482. Importantly, the near loss of Gab1 phosphorylation and reduced association of Gab1 with the Y489F and N491H Tpr-Met mutants correlated with the poor transforming activity of these mutant proteins.

**Tyrosine Phosphorylation of Both Cbl and Gab1 Correlates with Tpr-Met Transformation**—From genetic and biochemical data, Grb2 couples receptor tyrosine kinases to the Ras pathway via an interaction with SOS, a guanine nucleotide exchange factor for Ras (42–48). Activation of Ras occurs following association of Grb2 with either the activated RTK or another adaptor protein, Shc (42, 49–51). In cell lines expressing the poorly transforming Tpr-Met mutants that fail to bind Grb2, Shc is still phosphorylated and coupled to Grb2, yet these mutant oncoproteins activate Ras-dependent pathways at 50–60% the efficiency of the wild-type Tpr-Met oncoprotein (20). The poor transforming activity of these Tpr-Met mutants (20% of wild-type) was inconsistent with the observed decrease in Ras dependent signaling suggesting that other Grb2 dependent pathways, in addition to Ras, were required for efficient cell transformation by Tpr-Met. In support of this, our data demonstrate that phosphorylation of Cbl and Gab1 was dependent upon Grb2 association with Tyr489 of Tpr-Met. From sequence analysis, both Cbl and Gab1 contain proline rich sequences that interact with SH3 domain-containing proteins such as Grb2 and several tyrosine residues which, when phosphorylated, associate with multiple signaling molecules (23, 38).

Functionally, tyrosine phosphorylation of both Gab1 and Cbl is associated with an altered growth response. Constitutively phosphorylated variants of Cbl transform fibroblasts (31) and NIH3T3 cells overexpressing Gab1 have an increased growth rate in 1% serum and grow in soft agar in the presence of EGF or insulin whereas control cells do not (23). Although the function of Cbl and/or Gab1 is as yet unknown, Tpr-Met mutants impaired in their ability to couple with Grb2 and to transform fibroblasts fail to induce high levels of phosphorylation of Cbl or Gab1 consistent with one or both of these proteins functioning as important signal transducers for transformation by Tpr-Met.

**Acknowledgments**—We thank members of the Park laboratory for helpful discussions and Drs. Alain Charest, Michel Tremblay, and Mike Moran for GST fusion proteins.

**REFERENCES**

1. Bottaro, D. P., Rubin, J. S., Faletto, D. L., Chan, A.-L., Kmiecik, T. E., Vande Woude, G. F., and Aaronson, S. A. (1991) Science 251, 802–804
Tpr-Met Transformation and Phosphorylation of Cbl and Gab1