Pituitary Tumor Transforming Gene (PTTG) Transforming and Transactivation Activity*

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Pituitary tumor transforming gene (PTTG) is a newly identified transforming gene, the functional mechanism of which is little understood. Computational analysis reveals a C terminus rich in Glu and Pro, a known characteristic of transcriptional activation domains. We report here that murine PTTG indeed possesses transactivation ability, which correlates highly with its transforming properties. Pro139, Ser159, Pro157-Pro158, Ser159-Pro160 (PPXP motif), and Leu120, Asp121-Phe122, Asp127-Leu124 were found to be important for transactivation. Mutation to Ala at a key Pro139 residue not only disrupted the transactivation function but also resulted in the loss of transforming ability in NIH3T3 cells. A murine PTTG cDNA that encodes a variant C-terminal tail (Gly-Lys-Gly-Val-Agr-Arg-Sn-Gly-Cys-Lys-Asp-Leu-Val-Thr) was cloned. This novel PTTG is devoid of transactivation and transforming ability; deletion of its variant C-terminal tail restores both transactivation and transforming ability. These results show a high correlation between the transforming and transactivation functions of PTTG and also indicate that the novel PTTG variant may function as an endogenous competitor to wild-type PTTG.

Oncogenes, under certain conditions, affect cellular controls of proliferation, death, migration, and adhesion leading to neoplastic transformation. Many overexpressed oncogenes such as Ras possess the ability to transform NIH3T3 cells in vitro (1). Pituitary tumor transforming gene (PTTG),1 recently isolated by our laboratory (2), encodes a novel 199-amino acid protein with no significant similarity to known proteins. Overexpression of rat PTTG in NIH3T3 fibroblasts induced cellular transformation (1). PTTG possesses a GAL4 DNA binding domain and found that PTTG indeed possesses transcriptional activation ability; mutation analysis identified several residues and regions important for this activation, and soft agar assay suggested a high correlation between transactivating and transforming abilities of PTTG. Furthermore, a novel murine PTTG transcript encoding a variant protein containing a different C-terminal tail was detected. This variant PTTG is devoid of both transactivation function and transforming ability. Deletion of the variant C-terminal tail restores both transactivation and transforming ability. These results imply that PTTG-mediated transactivation correlates with its transforming ability, and the variant C terminus may function as an endogenous competitor to wild-type PTTG.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—pcDNA3.1 and pCRII vectors were from Invitrogen, and murine PTTG was cloned in this laboratory (8) and subcloned into pcDNA3.1. pGAL4 vector, which contains the GAL4 DNA binding domain, and pGAL4-VP16 as a positive control vector were from Stratagene. pLac vector, which contains five copies of GAL4 DNA element in front of a minimal promoter, and the luciferase gene were also from Stratagene. Polyclonal antibody against a 20-amino acid peptide of rat PTTG was generated commercially (Research Genetics).

Cell Culture—Mouse fibroblast NIH3T3 cells (ATCC CCL-92) were maintained in Dulbecco’s modified Eagle’s medium low glucose medium (Life Technologies, Inc.) with 10% fetal bovine serum, and mouse MOP8 (ATCC CRL-1798) and mouse embryonal carcinoma F9 (ATCC CRL-1720) were maintained in Dulbecco’s modified Eagle’s medium high glucose medium (Life Technologies, Inc.) with 10% fetal bovine serum. All culture media were supplemented with standard antibiotics, and cells were passaged twice weekly.

Transcriptional Activation Assay—Murine PTTG cDNA was fused in-frame with pGAL4, designated pGAL4-mPTTG and used as template for all deletion and mutation analysis. pGAL4-VP16 was used as a positive control. Experimental plasmids were co-transfected with pLac and pCMV-β-Gal (as internal control). Cell lysates were prepared 48 h after transfection and assayed for luciferase activity as described (9, 10).

Rapid Amplification of 3' cDNA Ends (3 RACE) and mPTTG Variant cDNA Cloning—3' RACE was performed as suggested by Roche Molecular Biochemicals. Total RNA derived from F9 cells was used for reverse transcription, and one PTTG gene-specific primer (5'-GGTC-CAGCCGTGCCTAAAGCCAG-3') and universal primer were used in polymerase chain reaction. A probe derived from unique sequence in the alternative transcript was used to screen a F9 cDNA library, and full-length mPTTG variant cDNA was cloned and sequenced.

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Mutagenesis and Deletion—Mutagenesis and deletion of PTTG using pGAL4-mPTTG as template were performed as suggested using the ExSite polymerase chain reaction-based method (Stratagene). All mutants were sequence-verified. A total of 37 primers were used for mutation and deletion, and their sequences are available upon request.

Stable Transfection and Western Blot Analysis—NIH3T3 cells were transfected using LipofectAMINE (Life Technologies, Inc.). Expression vectors for mPTTG, mPTTG-variant, Ala139-mPTTG, Asn139-mPTTG, mPTTG-d-(171–196), and mPTTG-d-(1–100) were transfected, and G418 selection started after 48 h. Stable transfectants were confirmed by Western blot, in which cell lysates were prepared using RIPA buffer and protein concentrations were quantitated by Bio-Rad protein assay. Equal amounts of protein were separated by 12% SDS-polyacrylamide gel electrophoresis and electroblotted to polyvinylidene difluoride membrane (Millipore), and antigen detection was performed using ECL (Amersham Pharmacia Biotech).

Soft Agar Assay—The assay was performed as described previously (2). 5000 cells were seeded in triplicate for each cell line in the top layer in the presence of 0.5% agar in a 35-mm dish. 3 weeks after seeding, cell morphology was observed, and numbers of transformed colonies (>16 cells per colony) were counted. MOP8 cells were used as a positive control, and NIH3T3 cells were used as a negative control.

RESULTS

PTTG Possesses Transcriptional Activation Properties—PTTG does not exhibit significant similarity to a known protein, but computational analysis shows that the N-terminal half (1–100 aa) is rich in alkaline residues (21/100), whereas the C-terminal half is rich in acidic residues, especially glutamic acid (8/96) and proline (17/96). Because an abundance of Glu and/or Pro is characteristic of transcriptional domains for transcription factors and/or co-activators, we tested whether PTTG demonstrated transcriptional activation. Fig. 1 shows that PTTG exhibits transcriptional activation; the mutant human PTTG-M9 (replacing region 163–166 from Pro-Pro-Ser-Pro to Ala-Leu-Ala-Leu), which is devoid of transforming ability (3), also does not activate transcription. This result suggests that transforming and transcriptional activation functions of PTTG are distinct and correlate with each other.

cDNA Cloning of a Novel Murine PTTG Variant—Murine PTTG shows high homology with human and rat PTTG. Nevertheless, on Northern blot analysis, a second band (1.7-kb mPTTG alternative transcript) was detected in RNA derived from embryonic F9 cells (2). To determine the presence of this alternative PTTG transcript, 3′RACE was performed and a 1.3-kb band was amplified in addition to the band corresponding to wild-type mPTTG (Fig. 2a). Sequencing revealed that this transcript differed from wild-type mPTTG after exon 4. Using the distinct sequence obtained using this 3′RACE product as probe, an F9 cDNA library was screened and a 1.7-kb mPTTG alternative transcript was identified, which encoded a 188-aa murine PTTG cDNA variant that has been deposited in GenBank™ (accession number AF071209). Comparison of this mPTTG variant with wild-type is shown in Fig. 2b, and major differences are apparent in the C-terminal tail.

Correlation between Transactivation and Transformation Induced by PTTG—Understanding PTTG structure-function relationships in terms of its transforming ability is important for unraveling its mechanism of action as an oncogene. However, establishing stable transfectant cell lines and performing anchorage-independent soft agar assays is time-consuming and not suitable for pilot scale screening. As mutant human PTTG-M9 is devoid of both transforming and transactivation ability, we reasoned that these two functions might correlate, and therefore we tested transactivating activity to identify amino acid residues important for this function. Transactivation-related Glu and Pro, phosphorylation-related Ser and Tyr, conserved acidic and/or alkaline residues, other conserved regions, and the novel mPTTG variant C-terminal tail were mutated and/or deleted and tested for transactivating activity (Table I). Deletion of the first 100-aa mPTTG residues does not alter transactivation, indicating that this function is inherent within the C-terminal half of the mPTTG protein. Several residues were found to be important for transactivation including Pro139, Ser159, Pro134, and Glu169, and two regions including Leu120-Asp121-Phe122-Asp123-Leu124 and Pro157-Pro158-Glu159 (PPXP motif). Mutation of Pro139 to Ala or Leu resulted in complete loss of transactivation, whereas mutation of Pro139 to Asn did not disrupt transactivation, showing that the Pro139 tolerates a hydrophilic residue but not hydrophobic substitution for its activity. Mutation of Ser159 to Ala or Ser159 to Leu abrogated transactivation by 70 and 80%, respectively, whereas mutation of Ser159 to Thr only reduced transactivation by 30%. Thus, maintaining the Ser159 position hydrophilic appears critical, and this residue may be a potential phosphorylation site for regulation. Mutation of Glu169 to Gln resulted in loss of 75% transactivation activity, showing that the acidic residue is important at this position. Two regions were also found to be important for transactivation, including a highly conserved region, Leu120-Asp121-Phe122-Asp123-Leu124, which when completely deleted disrupted transactivation. The other region, a PPXP motif from aa 157 to 160 is also important for the activity. Moreover, the mPTTG cDNA variant did not exhibit transactivating ability, whereas deletion of the variant tail Gly-Lys-Gly-Val-Arg-Ser-Asn-Gly-Cys-Lys-Asp-Leu-Val-Thr (equivalent deletion of 171–196 aa in wild-type mPTTG) restored transactivation.

Although these results provided useful insights for the trans-
PTTG Transformation and Transactivation

TABLE I

Transactivation assay of mPTTG mutation and deletion mutants

| Mutant                  | Activation capability | Mutant                  | Activation capability |
|------------------------|-----------------------|------------------------|-----------------------|
|                        | %                     |                        | %                     |
| pGAL4-WT-mPTTG         | 100                   | -Ser159 → Ala         | 30 ± 2                |
| -Pro139 → Ala          | 7 ± 1                 | -Ser159 → Thr         | 60 ± 5                |
| -Pro139 → Leu          | 4 ± 1                 | -Ser159 → Leu         | 20 ± 2                |
| -Pro139 → Asn          | 100 ± 10              | -Ser177 → Ala         | 100 ± 10              |
| -Pro174 → Ala          | 30 ± 2                | -Ser178 → Ala         | 120 ± 10              |
| -Pro157 → Ala          | 100 ± 10              | -Ser181 → Ala         | 130 ± 10              |
| -Pro160 → Ala          | 45 ± 5                | -His126 → Leu         | 100 ± 10              |
| -Pro164 → Ala          | 100 ± 10              | -Arg147 → Ala         | 100 ± 10              |
| -Pro476 → Ala          | 100 ± 10              | -Lys162 → Ala         | 100 ± 10              |
| -Pro488 → Ala          | 100 ± 10              | -Lys163 → Ala         | 100 ± 10              |
| -Pro490 → Ala          | 100 ± 10              | -mPTTG variant        | 8 ± 1                 |
| -Glu126 → Gln          | 100 ± 10              | -d-(171–196)          | 85 ± 5                |
| -Glu146 → Gln          | 100 ± 10              | -d-(157–196)          | 75 ± 5                |
| -Glu146 → Gln          | 100 ± 10              | -d-(140–196)          | 50 ± 4                |
| -Glu150 → Gln          | 100 ± 10              | -d-(125–196)          | 40 ± 4                |
| -Glu169 → Gln          | 100 ± 10              | -d-(120–124)          | 7 ± 1                 |
| -Glu169 → Gln          | 50 ± 3                | -d-(1–100)           | 100 ± 10              |
| -Cys457 → Ala          | 50 ± 3                | -mut-mPTTG           | 6 ± 1                 |
| -Tyr308 → Phe          | 100 ± 10              |                        |                       |

**DISCUSSION**

PTTG is a highly conserved protein, with murine PTTG sharing 88% and 66% amino acid homology with rat PTTG and human PTTG1, respectively. Nonetheless, the C-terminal 26-amino acid tail is relatively low in homology (about 31%) and seems unlikely to be important for conserved function of the molecule. Indeed, deletion of the last 26 amino acids did not affect transcriptional activation induced by mPTTG nor did it alter the transforming ability of the protein.

These results show good correlation between transforming ability and transcriptional activation mediated by murine PTTG. Similar findings have been obtained with other oncogenes, which function as transcription factors including Jun or Myb; Jun transactivates the Myb promoter via an AP-1-like sequence, and its N terminus demonstrates the transactivation property. Interestingly, the Δ domain in human Jun, absent in the v-Jun oncogene, stabilizes the interaction of cell-specific transcriptional inhibition with activation regions (11). Expression of v-Src or oncogenic Ras disrupts the Jun-inhibitor complex, thus increasing transcriptional activity (12). The proto-oncogene Myb also contains DNA binding, transcriptional activation, and negative regulatory domains (13, 14). In both cases inactivation of relevant transcriptional activation domains also abrogates transforming abilities. Notably, deletion of PTTG N-terminal 100 aa disrupts transformation, while not affecting transactivation. It is therefore possible that the N-terminal 100 aa region interacts with DNA element(s) or other proteins whereas the C-terminal portion mainly interacts with the RNA polymerase complex for transcriptional activation. Thus without the N-terminal 100 aa, mPTTG still maintains its transcriptional ability when fused with the GAL4 DNA binding domain but is unable to interact with endogenous substrate in NIH3T3 cells to mediate its transforming effect.

The comprehensive deletion and mutagenesis analysis in the C terminal region demonstrates that it is most likely that, overall, a three-dimensional structure is important for function, and Pro139, Ser159, Pro147-Pro158-Ser159-Pro160, and Leu120-Asp121-Phe122-Asp123-Leu124 are critical contact sites for PTTG function. We have recently demonstrated that human PTTG1 C terminus is also critical for in vitro and in vivo transformation. 2 On the other hand, whether PTTG is a true transcription factor/co-activator remains to be tested via efforts to identify its endogenous binding element and/or interacting proteins.

Identification of the new murine PTTG variant is interesting. This variant possesses a different C-terminal tail and does not exhibit transforming ability. Deletion of the variant C-terminal tail restores both transcriptional activation and transforming ability, indicating an inhibitory effect of the tail on PTTG function by conformational perturbation or interaction with specific residues. Thus the intracellular balance of PTTG and this variant PTTG may compete to determine their ultimate effects on the cell. Future comparison of gene expression profiles between wild-type PTTG and variant PTTG stable transfectants should help identify downstream target genes for PTTG.

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*2 G. A. Horwitz, unpublished data."
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