Communication

Domain Structure of Pleiotrophin Required for Transformation*

(Received for publication, December 29, 1998, and in revised form, March 2, 1999)

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The pleiotrophin (PTN) gene (Ptn) is a potent proto-oncogene that is highly expressed in many primary human tumors and constitutively expressed in cell lines derived from these tumors. The product of the Ptn gene is a secreted 136-amino acid heparin binding cytokine with distinct lysine-rich clusters within both the N- and C-terminal domains. To seek domains of PTN functionally important in neoplastic transformation, we constructed a series of mutants and tested their transforming potential by four independent criteria. Our data establish that a domain within PTN residues 41 to 64 and either but not both the N- or C-terminal domains are required for transformation; deletion of both the N and C termini abolishes the transformation potential of PTN. Furthermore, deletion of two internal 5-amino acid residue repeats enhances the transformation potency of PTN 2-fold. Our data indicate that PTN residues 41–64 contain an essential domain for transformation and suggest the hypothesis that this domain requires an additional interaction of the highly basic clusters of the N or C terminus of PTN with a negatively charged “docking” site to enable the transforming domain itself to engage and initiate PTN signaling through its cognate receptor.

Pleiotrophin is an 18-kDa heparin-binding cytokine that was purified from bovine uterus as a weak mitogen for fibroblasts (1) and as a neurite-outgrowth promoting factor from neonatal rat brain (2). The Ptn cDNA encodes a highly basic protein of 168 amino acids with a 32-amino acid signal peptide and clusters of lysine residues of dissimilar amino acid sequence at the N and C termini (3, 4). PTN has been described as a mitogen for endothelial (6–8) and epithelial cells (7, 8) and for fibroblasts (1, 7). Expression of the Ptn gene is tightly regulated in a temporally and cell-type-specific manner during development (5). In contrast, Ptn gene expression in adults is constitutive and limited to fewer cell types than in development, such as selective populations of neurons and glia (5). The Ptn gene is a proto-oncogene (11). Cells transformed by Ptn develop into highly vasculized, aggressive tumors when implanted into the nude mouse and characteristically have significant disarray of cytoskeletal structure. Furthermore, the Ptn mRNA is highly expressed in a significant proportion of samples from different human tumors and in about one-fourth of over 40 human tumor cell lines of different origins (7, 9, 10). PTN is highly expressed in MDA-MB-231 cells, a cell line derived from a highly malignant human breast cancer that constitutively expresses high levels of the endogenous Ptn gene. A truncated mutant of PTN constitutively expressed in these cells reverted the transformed phenotype of the breast cancer cell (12), establishing the importance of endogenous PTN signaling in maintaining the malignant phenotype of these cells. To pursue the molecular basis of PTN signaling in transformation, we constructed and tested a series of mutant Ptn molecules to establish domains required for transformation of NIH 3T3 cells. We now report that an internal domain within PTN residues 41–64 is required to transform NIH 3T3 cells. We also report the interesting finding that either the N- or C-terminal lysine-rich domain is essential to enable residues 41–64 to initiate transformation and that the potency of PTN to transform is regulated by two 5-amino acid internal “repeats.”

MATERIALS AND METHODS

Expression Plasmids—The human Ptn cDNA encodes an ~18 kDa protein of 168 amino acids before cleavage of a 32-amino acid signal peptide (Fig. 1). We constructed 11 mutant human Ptn genes using PCR and “PCR mutagen” site-directed mutagenesis (CLONTECH, San Francisco, CA) (Fig. 1). The mutant genes were cloned into the KpnI/EcoRI site downstream of the SV40 early promoter in the eukaryotic expression vector pAGE103 (11), which consists of basic elements including the origin of replication, a polyadenylation splicing signal, and the neomycin-resistance gene driven by the thymidine kinase promoter. The mutant PTN proteins, PTN 1–122 (Δ123–136), PTN 1–64 (Δ65–136), and PTN 1–40 (Δ41–136) are C-terminal deletions of the PTN 136 amino acid protein. Two internal repeated amino acid sequences (GAEC) were identified at residues 41–45 and 64–68 of PTN. PTN Δ65–68 and PTN Δ42–45/Δ65–68 were constructed to ablate one or both of the two internal repeats. Ptn lys-91 → Asn/Arg-92 → Gln mutations were introduced into WT Ptn by site-directed mutagenesis. PTN 40–136 (Δ1–40), PTN 69–136 (Δ1–68), and PTN 101–136 (Δ1–100) were constructed as N-terminal deletions of human PTN. PTN 123–12 (Δ12–123–136) was created to delete both N- and C-terminal polylysine clusters. All of the Ptn constructs were confirmed by DNA sequencing (see Fig. 1).

Cells and DNA Transfections—NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% calf serum. The cells were plated at a density of 1 × 10^5 per 100-mm dish and transfected 24 h later by calcium phosphate precipitation (11). Transfectants selected with G418 at an active strength of 700 mg/ml media were changed every three days until colony appeared, and clonal cell lines were established from expansion of single colonies. Colonies with high levels of PTN expression were pooled and analyzed.

Northern Blot Analysis—Total cellular RNAs were isolated by the guanidinium thiocyanate method and analyzed as described previously (12).

Cell Growth, Focus Formation, and Soft Agar Assay—To establish rates of cell growth, 1 × 10^5 of clonally selected cells were seeded in triplicate onto 35-mm dishes, trypsinized, and counted using a hemocytometer at 24 and 48 h. For focus formation assays, 2 × 10^5 cells for each construct were plated in triplicate onto 60-mm dishes, stained with crystal violet, photographed after 16 days, and counted. Anchor-independent growth in soft agar was carried out as described previously (11). Briefly, 5 × 10^6 of cells representative of each of the stably transfected Ptn constructs were suspended in 3 ml of (0.35% w/v) agar

* This work was supported by National Institutes of Health Grants HL14147, CA66029, and CA49712. 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.
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§ The abbreviations used are: Ptn, pleiotrophin; WT, wild type; bFGF, basic fibroblast growth factor.

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Vol. 274, No. 19, Issue of May 7, pp. 12959–12962, 1999

THE JOURNAL OF BIOLOGICAL CHEMISTRY

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Vol. 274, No. 19, Issue of May 7, pp. 12959–12962, 1999

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containing Dulbecco’s modified Eagle’s medium, 10% calf serum and overlaid onto a 0.7% (w/v) agar solution in two 60-mm dishes. After 16 days, colonies of 20 cells were scored as positive using an inverted microscope equipped with a measuring grid.

Tumor Formation in Nude Mice—Tumor formation in 6-week-old female athymic nude mice (strain nu/nu; Harlan Sprague-Dawley, Indianapolis, IN) was tested by injecting subcutaneously $2 \times 10^6$ cells suspended in 200 ml of sterile phosphate-buffered saline into each flank. Animals with tumors were monitored daily starting at 10 days. After 6 weeks, selected animals were sacrificed, and tumor size was measured in two perpendicular diameters.

RESULTS

The amino acid sequences of WT PTN and mutant proteins are illustrated in Fig. 1. NIH 3T3 cells were transfected with WT or mutant Ptn expression plasmids (11), and the expression levels of each stably transfected cell line were determined by Northern blot analysis (Fig. 2). Each of the mutant and WT cDNA transcripts was readily detected. However, the levels of expression of PTN 1–40 and PTN 101–136 were low compared with others, perhaps resulting from mRNA instability. Exogenous PTN and PTN mutants expressed in the transfected cells lines were also detected in cell lysates and conditioned media by Western blot analysis (data not shown).

The growth rate of each of the stably transfected NIH 3T3 cells were then determined. The rate of growth of cells transfected with PTN 1–122, PTN 1–64, PTN $D_{65-68}$, PTN $D_{42-45/D_{65-68}}$, PTN Lys-91 $\rightarrow$ Asn/Arg-92 $\rightarrow$ Gln, PTN 41–136, and WT PTN increased nearly 1.5-fold at 24 h and 2-fold at 48 h relative to the control NIH 3T3 cells (transfected with the empty vector). Cells lines transfected with PTN 1–40, PTN 69–136, PTN 101–136, and PTN 13–122 grew at a rate similar to that of the control cells.

In focus forming assays, cell lines derived from WT PTN, PTN 1–122, PTN 1–64, PTN 41–136, PTN $D_{42-45/D_{65-68}}$, PTN $D_{42-45/D_{65-68}}$, and PTN Lys-9 $\rightarrow$ Asn/Arg-92 $\rightarrow$ Gln grew more rapidly than control NIH 3T3 cells. At confluence, they had grown to a density 2-fold higher than that of control or NIH3T3 cells stably expressing the mutant cDNAs PTN 1–40, PTN 69–136, PTN 101–136, and PTN 13–122. However,
the more rapidly growing cells grew in clusters, and cell numbers were difficult to quantitate after 3 days. The cells were highly refractile and spindled-shaped in appearance (data not shown). After 16 days, foci were readily observed in cultures of these cells (Fig. 3), whereas foci were not detected in cultures of NIH 3T3 cells expressing PTN 1–40, PTN 69–136, PTN 101–136, or PTN 13–122 or in control cells. The number of foci on each dish were counted (Table I) and compared with the control cells. The foci in cells expressing PTN 1–122, PTN Δ65–Δ68, PTN Lys-91 → Asn/Arg-92 → Gln, and PTN 41–136 were readily detected but somewhat reduced in number in comparison with WT PTN 1–136. Foci were not found in cultures expressing PTN 1–40, PTN 69–136, PTN 101–136, and PTN 13–122. Interestingly, an increase in numbers of foci was observed in cells expressing PTN 1–64 and PTN Δ42–45/Δ65–Δ68, indicating that loss of the C-terminal residues 65–136 or deletion of the two internal repeat sequences enhance the transformation potency of PTN ~2-fold in focus forming assays.

The stably transfected NIH 3T3 cells were then tested for colony formation in soft agar. After 16 days, colonies with more than 20 cells were scored as positive. NIH3T3 cells expressing WT PTN, PTN 1–122, PTN 1–64, PTN Δ65–Δ68, PTN Δ42–45/Δ65–Δ68, PTN Lys-91 → Asn/Arg-92 → Gln, and PTN 41–136 formed large colonies (Fig. 4), whereas the control cells and cells expressing PTN 1–40, PTN 69–136, PTN 101–136, or PTN 13–136 did not (Table I).

To test tumor formation in nude mice, cells were implanted in flanks of athymic nude mice. Mice were examined daily, starting 10 days after injections, and tumors were measured at 6 weeks. Of the cells tested, NIH 3T3 cells expressing PTN 1–122, PTN 1–64, PTN Δ42–45/Δ65–Δ68, and WT PTN developed readily detectable tumors within 2 weeks (Table II). NIH 3T3 cells transfected with the empty vector alone or cDNAs encoding PTN 69–136 or PTN 13–122 did not. After 6 weeks, the tumors observed in the animals injected with the NIH 3T3 cells expressing PTN or its mutants were examined. Surprisingly, significantly larger tumors were found at sites of injection of NIH 3T3 cells expressing WT PTN and PTN 1–122 when compared with PTN, PTN 1–64, and the largest tumors were observed in PTN from which the two internal repeats were deleted.

FIG. 2. The top panel shows the exogenous WT and mutant Ptn mRNA expression patterns of clonally selected, stably transfected NIH 3T3 cells. As shown in the bottom panel, the 28 and 18 S rRNAs are indicated by arrows to the right of ethidium bromide gel as loading amount controls.

FIG. 3. Focus formation of the pooled stably transfected NIH 3T3 cells with WT and mutant Ptn genes as well as pPAGE 103 vector alone as a control. A high number of foci were formed in the stably transfected NIH 3T3 cells with WT PTN (B), PTN 1–122 (C), PTN 1–64 (D), PTN Δ65–Δ68 (F), PTN Δ42–45/Δ65–Δ68 (G), PTN Lys-91 → Asn/Arg-92 → Gln (H), and PTN 41–136 (J), whereas few foci were observed in the stably transfected NIH 3T3 cells with PTN 1–40 (E), PTN 69–136 (J), PTN 101–136 (K), PTN 13–122 (L), and pAGE 103 vector alone (A).

The stably transfected NIH 3T3 cells were then tested for colony formation in soft agar. After 16 days, colonies with more than 20 cells were scored as positive. NIH3T3 cells expressing WT PTN, PTN 1–122, PTN 1–64, PTN Δ65–Δ68, PTN Δ42–45/Δ65–Δ68, PTN Lys-91 → Asn/Arg-92 → Gln, and PTN 41–136 formed large colonies (Fig. 4), whereas the control cells and cells expressing PTN 1–40, PTN 69–136, PTN 101–136, or PTN 13–136 did not (Table I).

To test tumor formation in nude mice, cells were implanted in flanks of athymic nude mice. Mice were examined daily, starting 10 days after injections, and tumors were measured at 6 weeks. Of the cells tested, NIH 3T3 cells expressing PTN 1–122, PTN 1–64, PTN Δ42–45/Δ65–Δ68, and WT PTN developed readily detectable tumors within 2 weeks (Table II). NIH 3T3 cells transfected with the empty vector alone or cDNAs encoding PTN 69–136 or PTN 13–122 did not. After 6 weeks, the tumors observed in the animals injected with the NIH 3T3 cells expressing PTN or its mutants were examined. Surprisingly, significantly larger tumors were found at sites of injection of NIH 3T3 cells expressing WT PTN and PTN 1–122 when compared with PTN, PTN 1–64, and the largest tumors were observed in PTN from which the two internal repeats were deleted.

**Table I**

| Transfection | Focus formation | Colonies formed in soft agar assay |
|--------------|----------------|-----------------------------------|
| pAGE103 vector alone | ~5 | 0 |
| WT PTN (1–136) | ~275 | ~5.7 |
| PTN 1–122 (1–132) | ~251 | ~4.3 |
| ΔHuPtn-3 (1–64) | ~331 | ~8.2 |
| PTN 1–40 ΔHuPtn-4 (1–40) | ~2 | 0 |
| PTN Δ65–68 | ~218 | ~4.9 |
| PTN Δ42–45/Δ65–Δ68 | ~489 | ~8 |
| PTN Lys-91 → Asn/Arg-92 → Gln | ~234 | ~5.2 |
| PTN 41–136 | ~192 | ~4.8 |
| PTN 69–136 | ~3 | 0 |
| PTN 101–136 | ~1 | 0 |
| PTN 13–122 | ~1 | 0 |

**DISCUSSION**

In this study, we tested different domains of PTN to determine which domains are required for transformation of NIH 3T3 cells. It was established that amino acid residues 41–64 of...
PTN are required for transformation; none of the mutant PTN proteins that lacked PTN residues 41–64 transformed NIH 3T3 cells. Although the PTN receptor has not been identified, the results suggest that residues 41–64 contain a critical domain for signaling. A surprising finding was the requirement of either but not both the N- or C-terminal lysine-rich domains together with PTN 41–64 to transform NIH 3T3 cells, indicating that these domains support a similar functional role in transformation by PTN. These two domains may function in a different way than PTN residues 41–64 because the amino acid sequence of these two domains differs significantly. However, both domains have a strong net positive charge, suggesting they may interact with the receptor or an associated second “low affinity” receptor through electrostatic forces but are unlikely to signal a specific site-mediated receptor functions. Recently, N-syndecan has been implicated as a PTN binding protein (13), however, the binding of N-syndecan to PTN is not specific to PTN because basic fibroblast growth factor (bFGF) competes for the PTN binding sites, and the glycosaminoglycan chains alone in N-syndecan bind both PTN and bFGF (13). N-syndecan functions as a low affinity receptor and appears to regulate binding of bFGF to its high affinity receptor (14, 15). It seems possible that our results are consistent with a similar model in which the N and C termini of PTN facilitate the binding of PTN residues 41–64 to sites on a high affinity receptor.

A surprising result of these experiments is the difference of NIH 3T3 cells expressing PTN 1–64 in focus and colony formation compared with tumor formation. The ability of PTN 1–64 to strongly induce focus and colony formation suggests a role of the C-terminal domain in loss of contact inhibition and anchorage independence. PTN residues 65–136 may contain a domain favoring tumor growth in vivo, such as tumor angiogenesis.

The Ptnt gene is highly expressed in breast cancers and melanomas and constitutively expressed in cell lines derived from these tumors. Ptnt gene expression is not detectable in melanocytes and normal breast cells (7, 11), suggesting that PTN signal has an important role in neoplastic growth. This view was strongly supported when introduction of a dominant negative PTN effector reversed the malignant phenotype of a human breast cancer cell line that constitutively expresses the Ptnt gene. Our findings provide a structural basis for further studies on the functions of PTN in transformation in breast cancer and other human tumors. Our findings also provide a molecular model system to dissect the functional responses in tumors constitutively expressing PTN.

We conclude that the potential of PTN to induce transformation is mediated by several functional domains. Residues 41–64 of PTN constitute an essential domain necessary for PTN-mediated transformation, whereas the C- and N-terminal lysine-rich domains function nearly equally to support residues 41–64 in PTN-dependent transformation, perhaps through a “docking” function to appropriately position PTN with its receptor. Two internal duplicates of PTN negatively regulate PTN transformation.

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