ShcA and Grb2 mediate polyoma middle T antigen-induced endothelial transformation and Gab1 tyrosine phosphorylation

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Middle T antigen (PymT) is the principal transforming component of polyomavirus, and rapidly induces hemangiomas in neonatal mice. PymT, a membrane-associated scaffold, recruits and activates Src family tyrosine kinases, and, once tyrosine phosphorylated, binds proteins with PTB and SH2 domains such as ShcA, phosphatidylinositol 3-kinase (PI3K) and phospholipase C γ -1 (PLC γ -1). To explore the pathways required for endothelial transformation *in vivo*, we introduced PymT mutant forms into mice. PymT variants unable to bind PI3K and PLC γ -1 directly induced hemangiomas similarly to wild type, but a mutant unable to bind ShcA was transformation compromised. Requirement for a ShcA PTB domain-binding site was suppressed by replacing this motif in PymT with YXN sequences, which bind the Grb2 SH2 domain upon phosphorylation. Surprisingly, PymT recruitment of ShcA and Grb2 correlated with PI3K activation. PymT mimics activated receptor tyrosine kinases by forming a ShcA–Grb2–Gab1 complex, thus inducing Gab1 tyrosine phosphorylation, which itself is associated with PI3K. Therefore, PymT activation of ShcA–Grb2 signaling is critical for endothelial transformation, and PymT can stimulate Grb2 signaling to both the MAP kinase and PI3K pathways.

Keywords: Gab1 phosphorylation/hemangioma/
phosphatidylinositol 3-kinase/polyomavirus middle T/ShcA

Introduction

Signaling from cell surface receptors to intracellular targets commonly involves a series of modular protein–protein interactions, typified by the binding of phosphotyrosine (pTyr)-containing motifs on activated receptor tyrosine kinases to the Src homology 2 (SH2) or pTyr-binding (PTB) domains of cytoplasmic polypeptides (Pawson and Scott, 1997). Such interactions can be usurped by viral proteins, such as polyomavirus middle

T antigen (PymT), the principal oncoprotein of the murine polyomavirus. PymT is an intrinsic plasma membrane protein that recruits cellular signaling molecules into a complex that induces transformation (Dilworth, 1995). PymT binds a Src family kinase (Courtneidge and Smith, 1983; Kornbluth *et al.*, 1987; Kypta *et al.*, 1988) phosphatidylinositol 3-kinase (PI3K) (Whitman *et al.*, 1985; Kaplan *et al.*, 1987), the ShcA docking protein (Campbell *et al.*, 1994; Dilworth *et al.*, 1994; Blaikie *et al.*, 1997), phospholipase C γ -1 (PLC γ -1) (Su *et al.*, 1995), protein phosphatase 2A (Pallas *et al.*, 1990; Walter *et al.*, 1990; Ulug *et al.*, 1992) and a 14-3-3 protein (Pallas *et al.*, 1994). Association with PymT activates Src, which consequently phosphorylates PymT on three tyrosine residues (Y250, Y315 and Y322) and thereby generates binding sites for the ShcA PTB domain (pY250) and the SH2 domains of the p85 subunit of PI3K (pY315) and PLC γ -1 (pY322). The association of these signaling proteins with the PymT–Src complex results in tyrosine phosphorylation of ShcA, and its subsequent association with downstream effectors (Dilworth *et al.*, 1994; Blaikie *et al.*, 1997), as well as in the activation of PLC γ -1 (Su *et al.*, 1995) and PI3K (Courtneidge and Heber, 1987; Kaplan *et al.*, 1987).

ShcA polypeptides have an N-terminal PTB domain that recognizes NPXpY motifs, such as the phosphorylated NPTY₂₅₀ sequence in PymT, followed by a central CH1 region and a C-terminal SH2 domain (van der Geer and Pawson, 1995; Pelicci *et al.*, 1996). The CH1 region has two conserved sites, Y₂₃₉Y₂₄₀ND and Y₃₁₇VNV, which are phosphorylated by tyrosine kinases and bind the SH2 domain of the Grb2 adaptor (Lowenstein *et al.*, 1992; Matuoka *et al.*, 1992; Rozakis-Adcock *et al.*, 1993; van der Geer *et al.*, 1996). Grb2 associates through its SH3 domains with the Ras guanine nucleotide exchange factor Sos1, and the recruitment of Grb2 to membrane-associated phosphoproteins can therefore induce activation of the Ras–mitogen-activated protein (MAP) kinase pathway (Chardin *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Simon *et al.*, 1993). Indeed, ShcA plays a physiological role in coupling activated receptors to the Ras–MAP kinase pathway (Rozakis-Adcock *et al.*, 1993; Lai and Pawson, 2000) and Grb2 is rate limiting for the induction of mammary carcinomas by PymT in transgenic mice (Cheng *et al.*, 1998). However, ShcA and Grb2 may have multiple signaling functions. ShcA has conserved binding sites for proteins other than Grb2 (Okabayashi *et al.*, 1996). Furthermore, only the N-terminal Grb2 SH3 domain appears essential for Ras activation *in vivo* (Raabe *et al.*, 1995; Cheng *et al.*, 1998), while the C-terminal SH3 domain binds polypeptides other than Sos, including Gab1 and Gab2 (Gu *et al.*, 2000; Lock *et al.*, 2000; Schaeper *et al.*, 2000; Ong *et al.*, 2001). Recent data indicate that association of ShcA with the Grb2–Gab2 complex

Fig. 1. Schematic overview of the recombinant retroviruses transducing wild-type and mutant PymT.

mediates PI3K activation by interleukin (IL)-2 and IL-3 (Gu *et al.*, 2000). The binding of Grb2 to phospho-ShcA recruits Gab2 to the IL-3 receptor complex, resulting in phosphorylation of Gab2 on YXXM motifs that bind PI3K SH2 domains (Gu *et al.*, 2000). The subsequent production of PIP₃ can generate survival signals through targets such as the Akt/PKB protein-serine/threonine kinase.

PymT rapidly induces cystic endothelial tumors or hemangiomas in newborn mice, which frequently cause lethal internal hemorrhages (Williams *et al.*, 1988; Kiefer *et al.*, 1994a). This reflects the ability of PymT selectively to transform embryonic or newborn endothelium, apparently in a single step fashion (Williams *et al.*, 1988; Kiefer *et al.*, 1994b). In contrast, transgenic mice expressing PymT in non-endothelial tissues develop tumors with relatively long latencies (Aguzzi *et al.*, 1990; Rassoulzadegan *et al.*, 1990). The exquisite sensitivity of early endothelial cells or their progenitors to PymT probably reflects an intrinsic susceptibility to signals activated by PymT.

The transforming complex assembled by PymT resembles the signaling platforms generated by activated receptor tyrosine kinases (RTKs), several of which are required for vascular development (reviewed in Gale and Yancopoulos, 1999; Carmeliet, 2000). Biochemical and genetic analysis has started to reveal potential targets of these endothelial tyrosine kinases, including ShcA and p120 Ras GTPase-activating protein (Henkemeyer *et al.*, 1995; Gale and Yancopoulos, 1999; Karkkainen and Petrova, 2000; Lai and Pawson, 2000). Interestingly, an oncogenic variant of avian PI3K induces potent endothelial malignancies *in vivo* (Chang *et al.*, 1997) and angiogenesis in a chicken embryo chorioallantoic membrane assay (Jiang *et al.*, 2000). These observations have implicated ShcA, Ras and PI3K signaling in the response of endothelial cells to growth factor stimulation.

Functional analysis of the signals activated by PymT could identify pathways important for the endothelial cells of live animals. Previous work on the contributions of signaling pathways in PymT transformation has given varying results, depending on whether PymT was introduced into cells or animals by cDNA transfection, as a transgene or by viral infection (Oostra *et al.*, 1983; Carmichael *et al.*, 1984; Markland *et al.*, 1986; Webster *et al.*, 1998). Here, we have analyzed the capacity of recombinant retroviruses transducing mutated forms of PymT to transform established mouse fibroblasts *in vitro* and to induce hemangiomas in neonatal mice. We find that PymT signals through ShcA–Grb2 pathways to induce endothelial malignancies *in vivo*, and fibroblast transformation *in vitro*, and show that PymT constitutively activates Gab1 signaling.

Results

Characterization of recombinant retroviruses transducing mutant versions of polyomavirus middle T antigen

To assess the relative contributions of ShcA, PI3K and PLCγ-1 in PymT transformation of endothelial cells, we generated PymT mutants (Figure 1) which were inserted into the retroviral vector pMSCV (Table I). We determined the focus-forming activity of these recombinant viruses in four different fibroblast cell lines (Table II). A virus transducing wild-type PymT induced intense focus formation in all cell lines. PymT with a Y250F mutation, which abolishes the ShcA-binding site, was transformation defective in NIH 3T3, Rat1A and F111/cl106 fibroblasts, but retained a reduced transforming capacity in F111/cl615 fibroblasts. Similarly, a deletion mutant lacking 10 amino acids (residues 245–254) surrounding Y250, PymT(245Δ10), was transformation deficient in all lines

except F111/cl615, where it had reduced transforming activity. Re-insertion of either YVNQ or YYND recognition motifs for the Grb2 SH2 domain into PymT(245Δ10) rescued its transforming potential, with the YYND motif being significantly more potent than YVNQ. A PymT-(245Δ10) mutant containing both motifs was similar to wild-type PymT in its transforming activity. Incorporation of one or both YXN motifs into PymT(245Δ10) indeed conferred Grb2 binding (see below, Figures 5B and 6). The insertion of Grb2-binding sites, such as those found in ShcA (YYND/YVNV) or the epidermal growth factor (EGF) receptor (YVNQ), can therefore suppress the requirement for an ShcA PTB-binding site in PymT fibroblast transformation.

Ablation of the PI3K- and PLCγ-1-binding sites (Y315,322F) was without effect in NIH 3T3 and F111/cl106 fibroblasts, but caused a strongly reduced transforming activity in Rat1A and F111/cl615 fibroblasts. A triple mutant (Y250,315,322F) was completely transformation defective in all fibroblastic lines. Thus, while ShcA binding appears most important for fibroblast transformation, all mutant PymT proteins except PymT(Y250,315,322F) retained some transforming activity depending on the cell line.

All mutant PymT proteins were stably expressed in the fibroblast cell lines and in tumor-derived endothelioma cells (see below). The mutant PymT proteins had associ-

ated *in vitro* tyrosine kinase activity capable of phosphorylating PymT itself and the associated p85 subunit of PI3K (where present), indicating that the variant PymT proteins can activate Src family kinases (Figure 2A). All mutant forms of PymT, except for the triple mutant PymT(Y250,315,322F), were phosphorylated on tyrosine in cells (Figure 2B), confirming that Y250, Y315 and Y322 are the major targets for associated Src kinases.

Loss of ShcA binding strongly attenuates PymT tumorigenicity

Susceptibility to PymT vascular tumors disappears 3–4 weeks after birth. However, 1- to 2-day-old mice inoculated intraperitoneally with 5×10^4 c.f.u. of a recombinant retrovirus transducing wild-type PymT showed signs of anemia after 5 days and lethal hemangiomas as early as 7 days after injection (Figure 3A). Cystic hemangiomas can rupture, causing severe internal hemorrhage and death, with the survival time depending on the tumor burden and location. We detected up to 50 cystic lesions of 2–10 mm diameter in the abdominal and pleural cavities of mice expressing wild-type PymT. By 10 days after injection, half of the inoculated mice had succumbed to internal hemorrhages and there were no survivors beyond 20 days (Figure 3A). No effects were observed over a 35 day period in 14 mice injected with empty MSCV retrovirus.

To test the abilities of PymT mutants to induce endothelial tumors, we inoculated newborn CD1 mice with the MSCV-PymT(Y250F), MSCV-PymT(Y315,322F) and MSCV-PymT(Y250,315,322F) viruses (Figure 3B). Mice inoculated with MSCV-PymT or MSCV-PymT-(Y315,322F) had similar tumor latencies and survival times, indicating that the PI3K- and PLCγ-1-binding sites on PymT are dispensable for transformation of endothelial cells *in vivo*. In contrast, a PymT(Y250F) virus only induced tumors after a significant delay; it took 19 days for 50% of the injected mice to develop a lethal tumor load and ~20% of the animals ($n = 8$) survived the inoculation. The triple mutant (Y250,315,322F) displayed only very weak tumorigenic potential. Over 35 days, only 3/27 inoculated mice succumbed to abdominal bleeding. Approximately half of the surviving mice carried small tumors.

Table I. Viral titers of PymT-transducing recombinant retroviruses

Recombinant virus	c.f.u. (puro)
MSCV-PymT (WT)	$3.6 \times 10^5/\text{ml}$
MSCV-PymT (Y250F)	$2.7 \times 10^5/\text{ml}$
MSCV-PymT (Y315,322F)	$2.8 \times 10^5/\text{ml}$
MSCV-PymT (Y250,315,322F)	$1.9 \times 10^5/\text{ml}$
MSCV-PymT (245Δ10)	$2.6 \times 10^5/\text{ml}$
MSCV-PymT (245Δ10-YVNQ)	$3.6 \times 10^5/\text{ml}$
MSCV-PymT (245Δ10-YYND)	$3.4 \times 10^5/\text{ml}$
MSCV-PymT (245Δ10-YVNQ/YYND)	$3.9 \times 10^5/\text{ml}$

Viral titers were determined by infection of NIH 3T3 fibroblasts with cell-free supernatants derived from stably transfected bulk cultures of GP+E86 packaging cells. Numbers of puromycin resistance transferring particles per ml of producer cell supernatant are listed.

Table II. Focus formation (%) by mutant PymT-transducing recombinant retroviruses

Recombinant virus	Fibroblast cell line			
	NIH 3T3	Rat 1A	F111/cl106	F111/cl615
MSCV (infection control)	0	0	0	0
MSCV-PymT	100	100	100	100
MSCV-PymT (Y250F)	10	<5	<5	70
MSCV-PymT (Y315,322F)	100	30	90	15
MSCV-PymT (Y250,315,322F)	0	0	0	0
MSCV-PymT (245Δ10)	<5	<5	<5	15
MSCV-PymT (245Δ10-YVNQ)	20	15	50	40
MSCV-PymT (245Δ10-YYND)	50	35	115	85
MSCV-PymT (245Δ10-YVNQ/YYND)	100	160	90	90

After infection of fibroblasts and subsequent culture in the presence of 5% calf serum for 2 weeks, cultures were screened by microscopic inspection either non-stained or after Dif-Quick staining. Successful transformation events were identified by the appearance of morphologically discernible foci. The number of foci induced by MSCV-PymT was defined as 100%.

Introduction of ShcA effector motifs into PymT lacking a PTB-binding site rescues tumorigenicity *in vivo*

To pursue the role of ShcA in endothelial cell transformation, we employed the PymT mutants carrying YYND and YVNQ motifs in place of the ShcA PTB-binding site deleted in PymT(245Δ10) (Figure 1). Intraperitoneal inoculation of newborn mice with MSCV-PymT-(245Δ10) resulted in endothelioma formation with a prolonged latency similar to MSCV-PymT(Y250F) (Figure 3C). Twenty-four days after inoculation, 50% of the infected mice had succumbed to hemorrhages and 13% ($n = 4$) survived the 50-day observation period. Re-insertion of the ShcA-derived YYND motif into PymT-(245Δ10) significantly enhanced tumorigenicity in 31 inoculated animals, with 50% lethality after 12 days. In contrast to their relative potency in focus formation, the YVNQ Grb2-binding motif in PymT(245Δ10-YVNQ) was equally if not slightly more active than YYND in restoring tumorigenicity. Fifty percent of 26 inoculated mice developed lethal tumors after 10 days. The MSCV-PymT(245Δ10-YVNQ/YVND) virus also caused the death of 50% of infected animals ($n = 34$) after 10 days (Figure 3C). These results suggest that the presence of a single Grb2-binding site within PymT fully compensates for the loss of ShcA binding in endothelial cell transformation, and argue that a key function of ShcA in PymT endothelial cell transformation is to couple PymT to Grb2.

To analyze the transforming complexes assembled by wild-type and mutant PymT proteins further (i.e. Figure 2A), we derived continuously growing endothelioma cell lines from tumors induced by the variant PymT forms. While cell lines were established from all other tumors, it was impossible to establish endothelioma cell lines from hemangiomas induced by MSCV-PymT-(Y250,315,322F).

Loss of ShcA binding is associated with a concomitant decrease in PymT-associated PI3K activity

ShcA binding appears important for PymT endothelial cell transformation, whereas loss of the PI3K- and PLCγ-1-binding sites caused only a mild reduction in PymT transforming activity (Figure 3B). This finding was curious, given the apparent importance of PI3K activity in endothelial transformation in other systems. We therefore analyzed PI3K activity associated with the different PymT mutants expressed in three fibroblast cell lines (NIH 3T3, F111/cl106 and F111/cl615) and tumor-derived endothelioma cells. In all of these cell lines, loss of either the PI3K-binding site in PymT(Y315,322F) or the ShcA-binding site in PymT(Y250F) and PymT(245Δ10) resulted in a marked decrease in PymT-associated PI3K activity (Figure 4). The reduction in associated PI3K activity was greatest for the PI3K-binding site mutant, and was slightly more pronounced in the PymT(245Δ10) mutant as compared with PymT(Y250F) (Figure 4). Insertion of the effector motifs YVNQ or YYND into PymT(245Δ10) resulted in a significant restoration of PymT-associated PI3K activity. A combination of both YXN motifs gave the strongest effect, leading to near wild-type PymT-associated PI3K in F111/cl106 fibroblasts. No detectable PI3K activity was associated with the

Fig. 2. PymT-associated *in vitro* kinase activity and *in vivo* tyrosine phosphorylation of PymT mutant proteins in retrovirally transduced fibroblast and endothelioma cell lines. **(A)** PymT-associated tyrosine kinase activity. NIH 3T3, F111/cl106 or F111/cl605 fibroblasts or endothelioma cells were starved in serum-free medium for 16–20 h and lysed. PymT proteins were immunoprecipitated and subjected to kinase assays *in vitro*. Phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. Electrophoretic mobilities of PymT proteins (white arrowheads) and the p85 subunit of PI3K (black arrowheads) are indicated. Due to the low phosphorylation levels of the triple mutant Y250,315,322F, five times the reaction products compared with the other samples were loaded. For each PymT mutant, two independently derived endothelioma cell lines are shown. **(B)** Tyrosine phosphorylation of PymT proteins in cells. PymT proteins were immunoprecipitated from lysates of NIH 3T3 fibroblasts or endothelioma cells expressing wild-type or mutant PymT. The precipitates were electrophoresed and immunoblotted with anti-pTyr or anti-PymT antibodies. The electrophoretic mobility of the mutant PymT proteins is indicated by the arrowheads.

triple mutant PymT(Y250,315,322F) (Figure 4). These results indicate that PymT can recruit PI3K either directly, or indirectly through ShcA and Grb2. The ability of YXN motifs in PymT(245Δ10) to enhance PI3K association suggests that ShcA recruits PI3K to PymT through Grb2.

Fig. 3. Tumorigenicity of mutant PymT proteins *in vivo*. (A) The indicated numbers of newborn CD1 mice were inoculated intra-peritoneally with 5×10^4 c.f.u. of recombinant retroviruses within 48 h after birth. The number of surviving animals is plotted versus the time after infection. The cause of death was established for all dead animals by necropsy. (B) In the experimental group infected with PymT(Y315,322F) (indicated by open circles), one animal died from injection-related complications within 12 h after inoculation. (C) In the group infected with PymT(245Δ10-YVNQ/YYND) (indicated by filled circles), one animal died from injection-related complications within 12 h after inoculation. *n* indicates the number of inoculated mice.

The docking protein Gab1 forms a complex with ShcA, Grb2, Sos1 and PymT in transformed cells

To investigate how ShcA–Grb2 might recruit PI3K to PymT, we analyzed the proteins associated with ShcA in fibroblasts expressing wild-type PymT or the PymT-(Y250F) mutant, which cannot bind ShcA. Blotting of ShcA immunoprecipitates from wild-type PymT-transformed cells with anti-pTyr antibodies [Figure 5A(i)] revealed both the 46 and 52 kDa ShcA phosphoproteins, and a tyrosine-phosphorylated species of ~115 kDa. In contrast, ShcA proteins were not significantly tyrosine phosphorylated in cells expressing the PymT(Y250F) mutant, and co-precipitation of the 115 kDa phosphoprotein was much reduced. Western blotting with anti-

Fig. 4. PI3K activity associated with mutant forms of PymT in fibroblasts and endothelioma cells. PymT proteins were immuno-precipitated and associated PI3K activity was determined by phosphorylation of mixed phosphatidylinositol/phosphatidylserine substrates *in vitro* in the presence of [γ - 32 P]ATP. Radioactively labeled phospholipids were extracted and separated by thin-layer chromatography on silica gel 60 and visualized by autoradiography. The origin of sample application and the chromatographic mobility of PI3-P are indicated.

Gab1 antibodies identified the 115 kDa ShcA-associated polypeptide as the docking protein Gab1 [Figure 5A(ii)]. Like Gab1, Grb2 co-precipitated with ShcA from wild-type PymT-transformed cells, but not from PymT(Y250F) cells [Figure 5A(iii)]. We conclude that ShcA, Grb2 and Gab1 associate in PymT-transformed cells in a fashion that requires the direct interaction of ShcA with PymT, probably to initiate ShcA phosphorylation and consequent recruitment of Grb2–Gab1.

To investigate whether Grb2 recruits Gab1 to PymT, lysates from NIH 3T3 cells expressing wild-type PymT, or the mutants PymT(Y250F), PymT(Y315,322F) or PymT-(245Δ10-YVNQ/YYND) were immunoprecipitated with anti-Grb2 antibodies, and blotted with anti-PymT, anti-Grb2 or anti-Gab1 antibodies (Figure 5B). The PymT-(245Δ10-YVNQ/YYND) mutant strongly co-precipitated with Grb2, and indeed this interaction was detected more readily than that of Grb2 with wild-type PymT, probably due to the latter association being indirect. Gab1 was detected in the Grb2 immunoprecipitates isolated from all cell lines, consistent with these two proteins residing in a stable complex.

The C-terminal SH3 domain of Grb2 binds a motif in the Gab1 Met-binding domain (MBD) (Lock *et al.*, 2000; Schaeper *et al.*, 2000; Ong *et al.*, 2001). We therefore tested whether the Gab1 MBD captures both Grb2 and PymT in the same complex from lysates of 293T cells

Fig. 5. (A) ShcA binding to PymT induces the association of ShcA with tyrosine-phosphorylated Gab1. Lysates from NIH 3T3 fibroblasts expressing wild-type or the Y250F mutant of PymT were immunoprecipitated with anti-ShcA antibodies. The immunocomplexes were resolved by SDS-PAGE and immunoblotted with (i) anti-pTyr, (ii) anti-Gab1, (iii) anti-Grb2 or (iv) anti-ShcA antibodies. (v) The same lysates were immunoprecipitated with anti-Gab1 antibodies, and then resolved by SDS-PAGE and immunoblotted with the same antibodies. An aliquot of 50 μ g of total cell lysates was resolved by SDS-PAGE and immunoblotted with (vi) anti-PymT or (vii) anti-Grb2. (B) A PymT mutant with two YXN motifs replacing the ShcA PTB domain-binding site associates strongly with Grb2. Lysates from NIH 3T3 fibroblasts expressing wild-type or the indicated mutant forms of PymT were immunoprecipitated with anti-Grb2 antibodies. Immunocomplexes were resolved by SDS-PAGE and immunoblotted with (i) anti-PymT, (ii) anti-Grb2 or (iii) anti-Gab1 antibodies.

transfected with wild-type or mutant forms of PymT and a small amount of v-Src, which enhances PymT phosphorylation in these cells (Figure 6). Lysates containing similar levels of PymT [Figure 6(i)] were incubated with immobilized glutathione *S*-transferase (GST)-Gab1-MBD, and bound proteins were immunoblotted with anti-PymT antibodies [Figure 6(ii)]. The GST-Gab1-MBD associated with PymT(245 Δ 10-YVNQ/YYND), containing two YXN motifs, and more weakly with PymT(245 Δ 10-YVNQ) or PymT(245 Δ 10-YYND). A GST-Grb2 SH2 domain fusion exhibited a similar pattern of binding to PymT mutants (panel iii). Equivalent amounts

Fig. 6. Coordinate association of Grb2 and Gab1 with the PymT(245 Δ 10-YYND/YYNQ) mutant. 293T cells were transfected with plasmids of vector or v-src alone or PymT and v-src (lanes 1–10 as indicated in the figure). (i) The expression of PymT and its mutants was verified by resolving equivalent amounts of the total cell lysates and immunoblotting with anti-PymT. Equivalent amounts of the lysates were incubated with (iii) Grb2(SH2)-GST or (ii–iv) Gab1(MBD)-GST bound on beads. Specifically bound proteins were eluted and resolved by SDS-PAGE, followed by immunoblotting with anti-PymT, anti-Grb2 or anti-Sos1 antibodies.

of Grb2 associated with the GST-Gab1-MBD protein in all cases, consistent with Gab1 binding the Grb2 SH3 domain (panel iv). Sos1 was also precipitated by the GST-Gab1-MBD fusion, suggesting that Sos1 and Gab1 may interact with Grb2 in the same complex (panel v). Neither Gab1 nor Sos1 was precipitated by GST alone (data not shown).

Direct binding of Grb2 to phosphorylated PymT(245 Δ 10-YVNQ/YYND) may therefore allow for the simultaneous recruitment of Sos1 and Gab1. Since Gab1 tyrosine phosphorylation creates multiple binding sites for the SH2 domain of the p85 subunit of PI3K, PymT can recruit PI3K activity indirectly through a PymT-ShcA-Grb2-Gab1-PI3K complex, potentially explaining the PI3K activity associated with the PymT(Y315,322F) mutant, which lacks an intrinsic PI3K-binding site.

Polyomavirus middle T antigen mimics growth factor-induced phosphorylation of Gab1

Through its association with Src family kinases, which induces the phosphorylation of tyrosine residues that bind the SH2 domains of ShcA, PLC γ and PI3K, PymT mimics the ability of an activated RTK to recruit cytoplasmic signaling proteins. We therefore tested whether PymT affects Gab1 tyrosine phosphorylation in a similar fashion to growth factor stimulation. In NIH 3T3 cells transformed by wild-type PymT, Gab1 was constitutively tyrosine phosphorylated (Figure 7A), even when the cells were starved of serum. Gab1 tyrosine phosphorylation was enhanced only modestly upon stimulation by platelet-derived growth factor (PDGF). In contrast, in parental cells, or in cells expressing the PymT(Y250, 315,322F) mutant, Gab1 tyrosine phosphorylation was barely detectable in serum-starved conditions and required PDGF stimulation. Gab1 is also constitutively tyrosine phosphorylated in cells transformed by the PymT(245 Δ 10-YVNQ/YYND) variant (data not shown). In contrast to

Fig. 7. PymT mimics growth factor-induced phosphorylation of Gab1. (A) Parental NIH 3T3 fibroblasts or stable transfectants expressing wild-type (wt) or the Y250,315,322F mutant of PymT were quiesced by growing in serum-free medium for 20 h, then treated with phosphate-buffered saline alone (–), or with 50 ng/ml PDGF-BB for 10 min at 37°C. The lysates were immunoprecipitated with anti-Gab1 antibodies, followed by SDS–PAGE and immunoblotting with anti-pTyr or anti-Gab1 antibodies. (B) Endothelioma cells expressing wild-type or the mutant forms of PymT indicated in the figure were cultured in complete medium and then starved in serum-free medium for 16 h before lysis. A 300 μ g aliquot of each lysate was used for immunoprecipitation with anti-Gab2 antibodies, followed by SDS–PAGE and immunoblotting with anti-pTyr antibodies. A 30 μ g aliquot of the same lysates was resolved by SDS–PAGE and blotted with anti-Gab2 antibodies.

fibroblasts, which express readily detectable levels of Gab1, Gab2 is the more abundant isoform in PymT-transformed endothelioma cell lines (Figure 7B). Both Gab1 (data not shown) and Gab2 are constitutively tyrosine phosphorylated in these cells, even when grown in serum-free medium for 16 h (Figure 7B), and Akt and Erk are also constitutively activated (data not shown). The phosphorylation of Gab2 appeared stronger in cells expressing PymT mutants that bind Grb2 directly. Taken together, these data indicate that PymT drives the constitutive tyrosine phosphorylation of Gab1 in a fashion that mimics the action of an activated growth factor receptor.

Discussion

Signaling through ShcA and Grb2 is essential for efficient transformation of endothelial cells *in vivo* by PymT

PymT shows a remarkable propensity to induce endothelial cell malignancy *in vivo*. To explore the relevant signaling pathways, we have infected mice with retroviruses expressing mutant forms of PymT that are selectively uncoupled from binding either the ShcA docking protein, or PLC γ -1 and PI3K, which regulate phosphoinositide signaling. A mutant that fails to bind

both PLC γ -1 and PI3K transforms the majority of fibroblast cell lines in culture, and induces hemangiomas with an efficiency similar to wild-type PymT in mice. In contrast, mutations that destroy the ShcA-binding site severely compromise the ability of PymT to induce hemangiomas in mice. The capacity of the PymT complex to bind and phosphorylate ShcA therefore appears important for effective endothelial cell transformation. ShcA is expressed predominantly in the developing mouse vasculature, and is required for efficient activation of the Erk1/2 MAP kinases and for embryonic cardiovascular development (Lai and Pawson, 2000). By binding ShcA, PymT may access a cytoplasmic signaling protein that normally amplifies the effects of growth factor stimulation in endothelial cells.

There are two potential binding sites for the Grb2 SH2 domain on ShcA. The YVNV motif, in particular, has been linked to Grb2 binding *in vivo* and activation of the MAP kinase pathway, while the YYND motif has also been implicated in a pathway leading to c-Myc transcription (Gotoh *et al.*, 1997; Blake *et al.*, 2000). A PymT mutant carrying the two YXN motifs in place of the NPXY₂₅₀ ShcA-binding site recognized Grb2 efficiently and induced hemangiomas with the same short latency as wild-type PymT. Indeed, either one of the YXN motifs endowed PymT with endothelial cell transforming activity. In the context of endothelial malignancy, transplantation of the YXN ShcA effector sites into PymT therefore yields the same biological activity as the ShcA-binding site. Such results indicate that PymT-associated ShcA activates biochemical pathways that stimulate endothelial cell proliferation and survival through its YXN phosphorylation sites, most probably through its ability to physically recruit Grb2.

An early proliferating endothelial or endothelial progenitor cell is likely to be the target of PymT transformation. Interestingly, vascular endothelial growth factor (VEGF)-mediated activation of the endothelial receptor tyrosine kinase Flk-1, which is most closely associated with endothelial proliferation, results in ShcA phosphorylation, and the SH2 domain of ShcB can bind to autophosphorylated Flk-1 and Flt-1 (Igarashi *et al.*, 1998; Warner *et al.*, 2000). The Flk-1 receptor and its ligand VEGF are both expressed in PymT-induced vascular tumors, suggesting that a VEGF–Flk-1 autocrine loop might contribute to hemangioma formation. However, transformed endothelial cells that are indistinguishable from tumor-derived endothelioma cells can be obtained from Flk-1-deficient embryonic stem cells (Muhlner *et al.*, 1999). Therefore, PymT appears able to bypass the requirement for Flk-1 in the proliferation and survival of early endothelium, potentially by activating signaling pathways similar to those normally stimulated by Flk-1.

ShcA induces Gab tyrosine phosphorylation in response to PymT and growth factor receptors

Biochemical analysis of PymT-transformed fibroblastic and endothelioma cell lines suggested that the association of ShcA and Grb2 with PymT might contribute to PI3K recruitment, in addition to activation of the Ras–MAP kinase pathway. This accords with previous work showing that an avian retrovirus encoding an oncogenic variant of the PI3K p110 catalytic subunit displays a remarkable

propensity to induce endothelial cell tumors (Chang *et al.*, 1997). The finding that ShcA binding is critical for the formation of PymT-induced hemangiomas, whereas the direct PI3K-binding site is dispensable, can be reconciled with the apparent sensitivity of endothelial cells to PI3K signaling, if ShcA were able to couple to PI3K as well as to the Ras–MAP kinase pathway.

Grb2 can bind through its C-terminal SH3 domain to the Gab1 and Gab2 docking proteins, which consequently become phosphorylated on sites that bind the SH2 domains of PI3K and Shp-2 (Gu *et al.*, 2000; Schaeper *et al.*, 2000; Ong *et al.*, 2001). This raised the possibility that phosphorylated ShcA in PymT-transformed cells, in addition to recruiting a Grb2–Sos1, could also associate with a Grb2–Gab1 complex, resulting in Gab1 tyrosine phosphorylation by the PymT-associated Src family tyrosine kinase. Indeed, Gab1 is constitutively tyrosine phosphorylated in PymT-transformed fibroblasts, whereas its phosphorylation in normal cells is growth factor dependent. Tyrosine-phosphorylated Gab1 co-precipitates with ShcA from cells transformed by wild-type PymT, but not from cells expressing the PymT(Y250F) mutant incapable of binding ShcA. A combination of *in vitro* binding and co-precipitation experiments supports a scheme in which tyrosine phosphorylation of YXN motifs in ShcA, induced by PymT, recruits a Grb2–Gab1 complex, which in turn leads to Gab1 tyrosine phosphorylation and the creation of binding sites for SH2 domain proteins such as PI3K. Finally, in ShcA-deficient fibroblasts, Gab1 tyrosine phosphorylation and Akt activation in response to growth factor stimulation are significantly compromised, implying a significant role for ShcA in PI3K activation (data not shown).

Expression of PymT in mammary epithelial cells also results in the rapid appearance of multifocal tumors (Guy *et al.*, 1992). Although the latency times for mammary tumorigenesis are significantly longer than those observed for hemangioma formation, it has been hypothesized that PymT expression suffices for the transformation of epithelial cells and that few, if any, additional genetic events are required. Stimulation of the Met RTK, which signals through Gab docking proteins, results in a robust proliferative response of mammary epithelial and endothelial cells (Bussolino *et al.*, 1992; Zhu *et al.*, 1994). The *in vivo* sensitivity of these tissues to PymT might therefore be due in part to the activation of Gab adaptors by PymT.

PymT apparently employs both direct and indirect mechanisms to recruit PI3K. This might augment the overall extent of PI3K activation, and potentially extend the duration of PIP₃ signaling. In this regard, it is interesting that inhibition of PI3K activity by wortmannin suppressed the ability of PymT to induce Gab1 tyrosine phosphorylation, and mutation of the direct binding site for PI3K binding (Y315) abrogated the recruitment of PI3K to the Grb2-binding mutants of PymT (data not shown). One possible explanation for this finding is that an initial burst of PIP₃ is required to attract Gab1 to the membrane, for its association (via Grb2) with phospho-ShcA. If this is indeed an important factor in PymT signaling through ShcA to PI3K, it is curious that the intrinsic binding site for PI3K (Y315) is dispensable for endothelial cell transformation. A possible explanation is provided by the observation that the MAP kinases Erk 1

and 2, as well as Akt, are constitutively activated in endothelioma cell lines, irrespective of the PymT variant, potentially reflecting deregulated activation of endogenous growth factor receptors such as Flk1 due to autocrine loops in these malignant tumor cells (Muhler *et al.*, 1999). It is therefore possible that resulting PIP₃ levels are sufficiently high to obviate the requirement for a priming event. Consistent with this notion, we have observed detectable levels of Gab1 and Gab2 tyrosine phosphorylation even in endothelioma cells expressing PymT mutants that lack the ShcA- or PI3K-binding sites, with higher levels in cells expressing Grb2-binding mutants of PymT.

A network of modular protein interactions in PymT signaling

Data from the injection of newborn mice with retroviruses encoding wild-type or mutant forms of PymT indicate that a signaling pathway involving ShcA and Grb2 is critical for efficient endothelial cell transformation. Results obtained from cultured cells indicate that ShcA, acting downstream of PymT, activates not only a Grb2–Sos1 complex to stimulate the Ras–MAP kinase pathway, as previously shown, but also recruits a Grb2–Gab1 complex resulting in the constitutive tyrosine phosphorylation of the Gab docking proteins in PymT-transformed cells. In principle, therefore, PymT-activated ShcA can up-regulate both the MAP kinase and PI3K pathways, providing the possibility of both proliferative and survival signals emanating from the phosphorylated PymT Y250 site, which could act synergistically to induce cellular transformation. Viral and bacterial proteins frequently exploit modular protein–protein interactions to re-wire cellular behavior to their own ends. In the case of signaling from the PymT Y250 site, a series of intermolecular interactions can be observed, involving PTB, SH2, SH3 and PH domains acting sequentially to stimulate specific biochemical pathways that elicit malignant transformation.

Materials and methods

DNA constructs

PymT (A3 strain) derived from J4mT (Webster *et al.*, 1998) was cloned into the retroviral vector pMSCV (Clontech). Tyrosine to phenylalanine mutations at residues 250, 315 and 322 were generated by standard PCR procedures. PymT(245Δ10) was obtained by deleting residues 245–254 (LSNPTYSVMR). PymT(245Δ10-YVNQ) was generated by replacing S~~N~~PTYSVMR with the sequence PVPEYVNQSV from the mouse EGF receptor. PymT(245Δ10-YYND) was generated by inserting the sequence IPDHQYYNDFPL from ShcA residues 282 and 283 of PymT(245Δ10). Both sequences were introduced to obtain the composite mutant, PymT(245Δ10-YVNQ/YYND). The mutations were verified by DNA sequencing.

Generation of recombinant retroviruses of PymT

Infectious retroviruses were harvested from producer cell lines GP+E86 or BOSC23 transfected with PymT plasmids and selected with puromycin (1.5 μg/ml). Viral titers were determined by infection of 1 × 10⁵ NIH 3T3 cells.

Cell lines

GP+E86 cells were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and penicillin and streptomycin, each at 100 U/ml. NIH 3T3, Rat1A, Fischer rat F111/cl106 or Fischer rat F111/cl615 fibroblasts were grown in DMEM supplemented with 5% newborn calf serum (NCS) and antibiotics. MEFs, 293T and BOSC23 cells were cultured in DMEM containing 10% FCS

and antibiotics. Transfection of 293T and BOSC23 cells was carried out using Lipofectin (Gibco-BRL). To derive endothelioma cells, mice were killed by CO₂ gassing and the tumors explanted, dissociated and cultured in DMEM containing 20% FCS. The concentration of FCS in the medium was reduced to 10% after 5 days and the cells cultured further for 2–3 weeks when endothelioma cells appeared as expanding islets consisting of a monolayer of typically spindle-shaped cells.

Focus formation assay

A total of 5×10^4 fibroblasts (NIH 3T3, Rat1A, Fischer rat F111/cl106 or Fischer rat F111/cl615) were infected with $2-4 \times 10^2$ retroviral particles of wild-type or mutants of PymT, in the presence of 5 µg/ml polybrene (Sigma). Cells were cultured in 5% calf serum and scored for the formation of foci after 2 weeks.

Tumor formation assay in newborn mice

Newborn CD1 mice were injected intraperitoneally within 48 h after birth with 5×10^4 freshly prepared retroviral particles of wild-type or mutants of PymT. Subsequently the animals were monitored for signs of anemia or the cause of death was determined by necropsy.

Antibodies and growth factors

Antibodies to PymT (Brewster *et al.*, 1997) and ShcA (Pelicci *et al.*, 1992) were described previously. Antisera against Gab1 were raised using the GST fusion protein of Gab1. Antisera for Gab2 were obtained from Dr Benjamin Neel (Beth-Israel Deaconess Medical Center, Boston, MA). Antibodies to Grb2, Sos1 and EGF receptor were from Transduction Laboratories (Lexington, KY). Anti-Flag was from Sigma (St Louis, MO). Erk1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt, anti-pS473-Akt and anti-pErk were from New England Biolabs (Beverly, MA). EGF and PDGF-BB were from Pepro Inc. (Rocky Hill, NJ).

Immunoprecipitation and in vitro binding assays

Cells were solubilized in cell lysis buffer (20 mM HEPES pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, 1 µg/ml each of leupeptin and aprotinin and 0.2 mM Na₃VO₄) and centrifuged at 10 000 g at 4°C for 15 min. The GST fusion proteins of Grb2-SH2, full-length Gab1 and Gab1-MBD were purified and used for *in vitro* binding assays as described (Holland *et al.*, 1997). The protein complexes were then washed in cell lysis buffer, eluted and analyzed by SDS-PAGE and immunoblotting with specific antibodies.

PymT-associated protein kinase and PI3K assays

Assays for protein kinase and PI3K activities associated in a complex with PymT were performed as described previously (Kaplan *et al.*, 1986; Kiefer *et al.*, 1994a).

Database accession numbers

The protein complex described in this study has been entered into the BIND database (www.bind.ca) with the accession ID numbers 1440, 6153 and 6154.

Acknowledgements

In memoriam: Werner Risau (December 18, 1953–December 13, 1998). We thank Benjamin Neel for Gab2 antibodies and helpful discussions. Postdoctoral support for S.H.O. is from the National Medical Research Council of Singapore. This work is supported by a grant from the Canadian Institutes of Health Research (CIHR), a Terry Fox Program grant from the National Cancer Institute of Canada and a Howard Hughes International Scholar award to T.P. T.P. is a Distinguished Scientist of the CIHR.

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Received July 11, 2001; revised September 5, 2001;
accepted September 7, 2001