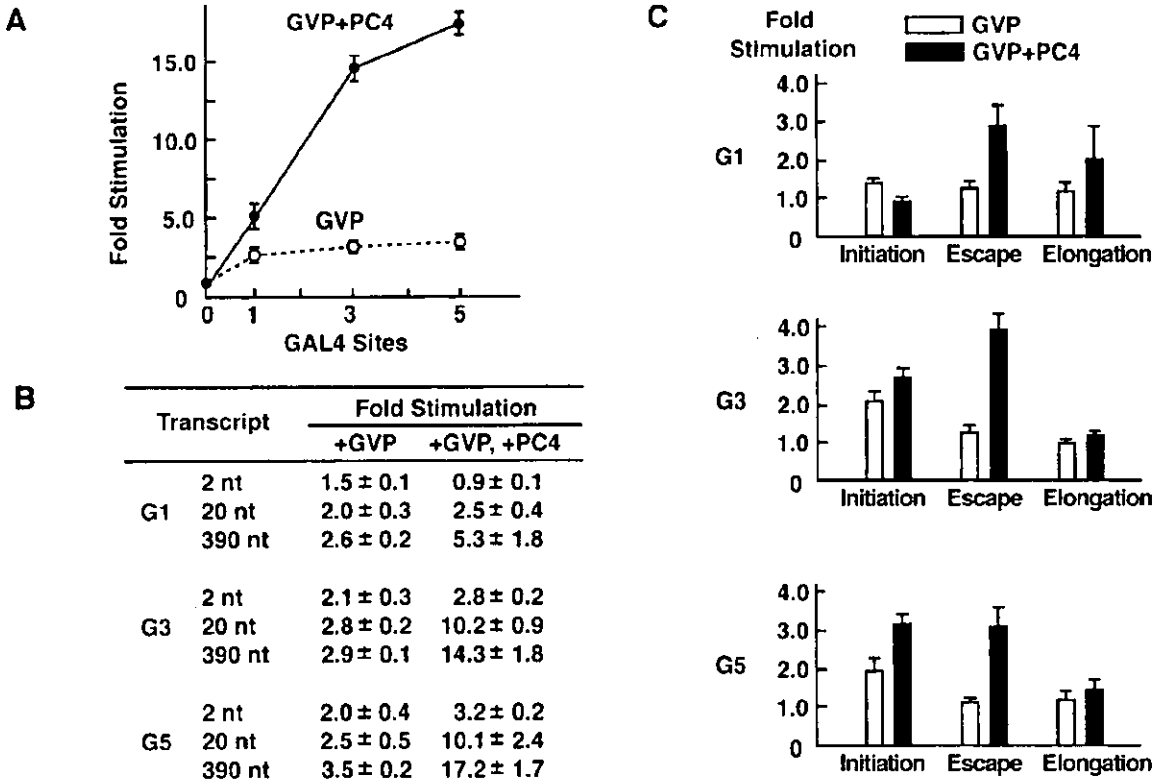

B

Transcript		Fold Stimulation	
		+GVP	+GVP, +PC4
G1	2 nt	1.5 ± 0.1	0.9 ± 0.1
	20 nt	2.0 ± 0.3	2.5 ± 0.4
	390 nt	2.6 ± 0.2	5.3 ± 1.8
G3	2 nt	2.1 ± 0.3	2.8 ± 0.2
	20 nt	2.8 ± 0.2	10.2 ± 0.9
	390 nt	2.9 ± 0.1	14.3 ± 1.8
G5	2 nt	2.0 ± 0.4	3.2 ± 0.2
	20 nt	2.5 ± 0.5	10.1 ± 2.4
	390 nt	3.5 ± 0.2	17.2 ± 1.7

FIG. 7. The stimulation of initiation, promoter escape, and elongation on the templates with different numbers of GAL4-sites. (A) The effect of coactivation by PC4 became more pronounced as the number of GAL4 sites was increased. The levels of the 390-nt transcripts shown in Fig. 6 were quantified by using Fujix BAS 2000, and the values of stimulation (*n*-fold) were calculated. The standard deviations for three independent experiments are indicated. (B) The values of activation (*n*-fold) for the 2-, 20-, and 390-nt transcripts were determined from three independent experiments and are shown as means ± standard deviations. (C) The number of GAL4 sites influences the activation of initiation, promoter escape, and elongation. The values for stimulation (*n*-fold) of initiation, promoter escape, and elongation were calculated as those presented in Fig. 1 were. On the templates with a single GAL4 site, PC4 stimulated promoter escape rather than initiation of GAL4-VP16-dependent transcription, while on the templates with three or five GAL4 sites, PC4 stimulated both initiation and promoter escape to similar extents. There were small but reproducible effects on elongation in all experiments.

tion step but little, if any, promoter escape, regardless of the number of its binding sites. Second, PC4 increases the degrees to which GAL4-VP16 stimulates initiation and promoter escape, having a more pronounced effect on promoter escape than on initiation. Third, promoter escape appears to be preferentially stimulated by GAL4-VP16 in the presence of PC4 when GAL4-VP16 is bound on a single GAL4 site. Together, these observations suggest that each GAL4-VP16 dimer bound on the promoter may stimulate a distinct step of transcription.

DISCUSSION

Although a large body of evidence indicates the functional significance of coactivators in regulating transcription in vitro and in vivo (2, 18, 20, 36, 41), far less is known about the precise mechanism(s) by which these coactivators stimulate transcription in conjunction with activators, especially in the context of naked DNA templates. In the present study, we took advantage of a well-defined reconstituted in vitro transcription system (10, 12) and demonstrated a crucial role for a coactivator, PC4, in stimulating promoter escape in activated transcription, in part through direct interaction with TFIIH.

Figure 8 depicts how PC4 enables GAL4-VP16 to achieve a high level of transcriptional activation. This model postulates at least two targets, termed targets A and B, in the basal transcription machinery, to which signals from activators are transmitted. These signals, in turn, permit target A and target B to regulate the steps leading to initiation (PIC assembly, promoter opening, and initiation) and promoter escape, respectively. Each target postulated in the model is meant to represent multiple factors rather than a single factor, and, conversely, a single factor may constitute a part of more than one target. For instance, since TFIIA and TFIID are important for facilitating both PIC assembly (7, 8, 24, 25) and promoter escape (Fig. 2), each factor must constitute parts of both target A and target B. In addition, PC4 and TFIIH (Fig. 3, 4, and 5), whose ERCC3 helicase activity is also essential for stimulating promoter escape (10), are likely to constitute the target B that regulates promoter escape. This complex network of multiple interactions may induce conformational changes, including

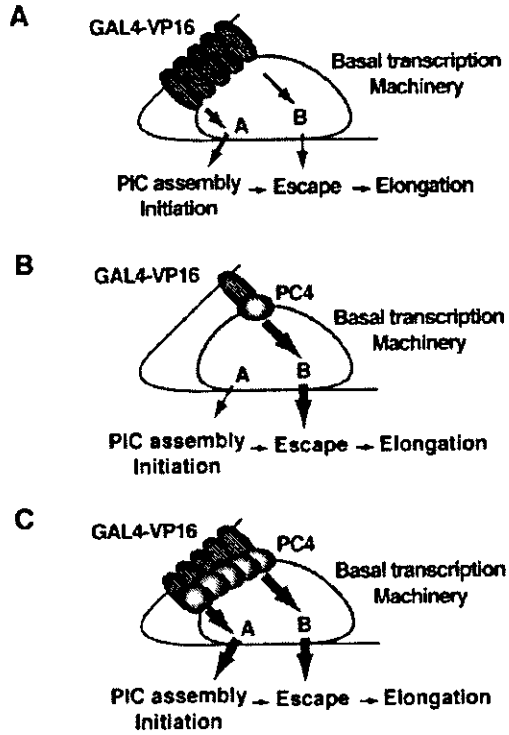

FIG. 8. A model of PC4 coactivator activity. In this model, we postulate that the basal transcription machinery contains at least two targets, termed targets A and B, through which the signals from activators are transmitted either directly or via PC4 to the individual steps of the transcription process. Target A regulates the steps leading to initiation (PIC assembly, promoter opening, and initiation) and is likely to consist of more discrete targets, whereas target B regulates promoter escape. (A) In the absence of PC4, GAL4-VP16 elicits transcriptional activation through the predominant effect on target A, regardless of the number of bound GAL4-VP16 molecules. (B) When PC4 is present, GAL4-VP16 bound at a single GAL4 site provides substantial transcriptional activation through target B. (C) When PC4 is present, multiply bound GAL4-VP16 achieves robust transcription through the synergized effects on both target A and target B, enhanced by PC4.

isomerization of the DA complex (7), that lead to stimulation of individual steps of the transcriptional process.

In the absence of PC4, GAL4-VP16 appears to function mainly through target A, and even increasing the number of GAL4-VP16 dimers bound on the template does not lead to robust transcriptional activation (Fig. 8A). In the presence of PC4, however, GAL4-VP16 can function through target B and also augment the effect through target A. Of the two postulated targets, target B seems to be preferred by the combination of GAL4-VP16 and PC4, since PC4 directs GAL4-VP16 to function predominantly through target B when the amount of GAL4-VP16 is limited, as on the G1 template (Fig. 8B). In contrast, when multiply bound GAL4-VP16 dimers are present, as on the G3 and G5 templates, PC4 permits distinct GAL4-VP16 molecules to function through both target A and target B (Fig. 8C), providing a mechanism for transcriptional synergy (6, 17, 34, 35, 46).

In this model, it is implicitly assumed that GAL4-VP16 and PC4 are capable of multiple interactions with the basal transcription machinery, interactions that obligate GAL4-VP16 and PC4 to adopt different conformations depending upon the target to which they bind. This assumption is supported not only by numerous interaction studies but also by recent structural studies showing that transcriptional activation domains, including that of VP16, are poorly structured in their free form but undergo an induced structural transition when complexed with their targets (5, 9, 30, 45, 51–53, 57, 61). In addition, the VP16 activation domain can adopt different structures whether it is bound to TBP or TFIIB (53). Moreover, this structural flexibility is also displayed by a mediator-like coactivator complex, CSRP (42, 56). Thus, given the lack of a stable three-dimensional structure within its coactivator domain (3), PC4 may form a stable structure only upon binding to activators and the basal transcription machinery. Through these interactions, PC4 could bestow activators with extra surfaces and an added conformational flexibility that permit more functionally effective links between activators and the basal transcription machinery.

Our model of PC4 action appears to contradict the widely accepted notion that PIC assembly is the primary target for activated transcription, as demonstrated by various in vivo and in vitro studies (47). In particular, using a similar in vitro transcription system, Chi et al. (7, 8) demonstrated that PIC assembly, especially DA complex assembly, is necessary and sufficient for activation, an observation supported by others (24, 25, 54, 55). Furthermore, Jacob and Luse (19) failed to detect any stimulatory effect on promoter escape by GAL4-VP16 by using HeLa nuclear extract. We believe, however, that this apparent contradiction can be reconciled for the following reasons. First, the effect on PIC assembly as inferred by the order-of-addition experiments does not necessarily dictate the actual time point at which the assembled PIC acts on steps of transcription. Thus, the effects of the assembled PIC, such as the isomerized DA complex (7), may remain far beyond the time point of their assembly. Second, we also observed the predominant effects on initiation (which may reflect PIC assembly in our assays) to overall stimulation of transcription when the amounts of factors were reduced. We suspect that, under these conditions, the stimulatory effect on promoter escape may be easily overlooked. Third, since PC4 acts as a coactivator only in its nonphosphorylated form (14, 27) and also in a highly concentration-dependent manner (13), PC4 may not have been functional as a coactivator in the transcription systems involving crude fractions (7, 8, 19, 24, 25), in which the majority of PC4 is phosphorylated (14, 27). Given these considerations, our results are not inconsistent with earlier observations that emphasized the predominant role of PIC assembly in transcriptional activation.

The exact mechanism by which PC4 assists the ERCC3 helicase of TFIIH during promoter escape remains an enigma. One attractive possibility is that PC4 stabilizes the ssDNA region exposed during promoter escape through its ssDNA-binding ability (62), thereby indirectly assisting the ERCC3 helicase. It is generally known that ssDNA-binding proteins stimulate the activities of DNA polymerases and helicases (7), and indeed, PC4 facilitates DNA replication mediated by SV40 T antigen (44). However, the possibility of this mechanism seems remote because a PC4 mutant, W89A, which has little ssDNA-binding ability (63), shows essentially the same effect

on promoter escape as wild-type PC4 does (10). Therefore, we favor alternative mechanisms by which PC4 facilitates the recruitment of TFIIH (29) or directly stabilizes the ATP-induced conformational change of TFIIH per se through protein-protein interactions, a mechanism consistent with the fact that TFIIH does not function as a classical helicase (22). Related to this idea, HBx, a coactivator-like transcriptional regulator of the hepatitis B virus (15), stimulates TFIIH helicase activities independently of its ssDNA-binding ability (48).

In conclusion, we have shown that PC4 assists GAL4-VP16 in stimulating the multiple steps of transcription and facilitates synergy by multiply bound GAL4-VP16 dimers. Future studies should address more detailed mechanistic aspects of the coactivator activity of PC4 and identify the precise factors within the basal transcription machinery that are targeted by individual GAL4-VP16 and PC4 molecules bound multiply on a single promoter. These studies may offer a paradigm for further functional analyses of diverse coactivators.

ACKNOWLEDGMENTS

We thank K. Nakagawa and Y. Miyagi for technical assistance and other members of the laboratory for materials, advice, discussion, and critical reading of the manuscript.

This study was supported by grants-in-aid from the Ministry of Education, Science, Sports and Culture, by the Maruki Memorial Prize of Saitama Medical School, and by a grant from the Sumitomo Foundation. A.F. was a Research Fellow of the Japan Society for the Promotion of Science.

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Oncogene (2002) 21, 5823–5834

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ORIGINAL PAPERS

Activation of anaplastic lymphoma kinase is responsible for hyperphosphorylation of ShcC in neuroblastoma cell lines

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Shc family of docking proteins, ShcA, ShcB and ShcC, play roles in cellular signal transduction by binding to phosphotyrosine residues of various activated receptor tyrosine kinases. Both ShcB and ShcC proteins are selectively expressed in the neural system of adult mouse tissues. In most of neuroblastoma cells, obvious tyrosine phosphorylation of ShcC was observed, whereas expression of ShcB was considerably low. Phosphoproteins associated with hyperphosphorylated ShcC were purified from neuroblastoma cell lines, and identified by mass-spectrometry. Anaplastic lymphoma kinase (ALK), which turned out to be one of these phosphoproteins, was constitutively activated and associated with the PTB domain of ShcC in three neuroblastoma cells. *In vitro* kinase assay revealed that ShcC is a potent substrate of the activated ALK kinase. The *ALK* gene locus was significantly amplified in both of these cell lines, suggesting that gene amplification leads to constitutive activation of the ALK kinase, which results in hyperphosphorylation of ShcC. Constitutive activation of ALK appeared to interfere with signals from other receptor tyrosine kinases. ALK-ShcC signal activation, possibly caused by co-amplification with the N-*myc* gene, might give additional effects on malignant tumor progression of neuroblastoma.
Oncogene (2002) **21,** 5823–5834. doi:10.1038/sj.onc.1205735

Keywords: Shc family proteins; anaplastic lymphoma kinase (ALK); constitutive phosphorylation; neuroblastoma; gene amplification

Introduction

Multiple biological events have been demonstrated to be under control of receptor tyrosine kinases (RTKs) during the human cancer development. Activated RTKs utilize adaptor molecules including Shc family and Grb2 proteins to trigger intracellular signaling pathways, such as the Ras-MAPK pathway and the phosphoinositide 3-kinase (PI3K)-Akt pathway. These adaptor molecules are, therefore, closely associated with regulation of cell proliferation, differentiation, survival and oncogenesis (Asai *et al.*, 1996; Collins *et al.*, 1999; Goga *et al.*, 1995; Gotoh *et al.*, 1996). Shc proteins are composed of an N-terminal phosphotyrosine binding (PTB) domain, a central glycine/proline-rich region (CH1) and a C-terminal Src homology 2 (SH2) domain (Pelicci *et al.*, 1992; van der Geer *et al.*, 1995). The binding affinity of the PTB domain and the SH2 domain to phosphotyrosine residues of RTKs transfers Shc proteins toward the cellular membrane upon ligand stimulation (Laminet *et al.*, 1996; Pawson *et al.*, 2001; van der Geer *et al.*, 1996). Shc proteins phosphorylated at tyrosine residues by activated RTKs subsequently recruit SH2-containing adaptor molecules such as Grb2 (Gotoh *et al.*, 1996; van der Geer *et al.*, 1996).

Shc family proteins consist of ShcA (Shc), ShcB (Sli/Sck) and ShcC (Rai/N-Shc) which are encoded by three independent genes (Nakamura *et al.*, 1996a; O'Bryan *et al.*, 1996b; Pelicci *et al.*, 1996). ShcA is expressed ubiquitously in most of the organs except the adult neural system and has three protein isoforms, $p46^{ShcA}$, $p52^{ShcA}$ and $p66^{ShcA}$. During embryogenesis, the ShcA protein plays the first critical role in the cardiovascular system at E9 (Lai and Pawson, 2000). In the neural system, the ShcA mRNA was detected in the ventricular zone of day 12.5 mouse embryo and peripheral nerves of day 19 rat embryo (Conti *et al.*, 1997; Nakamura *et al.*, 1998). In the case of ShcB, mRNA expression was detected in wide range of organs while protein expression is rather specific to brain and spinal cord with a trace amount of expression in lung, heart and stomach (Sakai *et al.*, 2000). Protein expression of ShcC ($p52^{ShcC}$ and $p67^{ShcC}$ isoforms) was restricted to the neural system in mouse (Nakamura *et al.*, 1998; O'Bryan *et al.*, 1996b; Pelicci *et al.*, 1996). Expression of ShcC is low in the embryonic brain, while remarkably elevates after

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Received 18 March 2002; revised 14 May 2002; accepted 7 June 2002

delivery (Sakai *et al.*, 2000). ShcB expression remains at the same level during pre- and postnatal development. On the other hand, ShcA expression is most significant in the prenatal period with sudden decrease after birth (Conti *et al.*, 1997). Mice lacking both ShcB and ShcC exhibit a significant loss of neurons within the superior cervical ganglia suggesting redundant roles of ShcB and ShcC in the trkA signaling (Sakai *et al.*, 2000). Functional analysis of the ShcB/ShcC on the neuronal signaling pathway indicated that these proteins in neuronal cells potentially regulate EGF or NGF signaling in a similar fashion to ShcA (Nakamura *et al.*, 1998; O'Bryan *et al.*, 1996a).

In the current study, we examined involvement of each Shc family protein in human neuroblastoma and other tumor cells of neural origin, by checking expression and tyrosine phosphorylation of these proteins. Neuroblastoma is the most common pediatric solid tumor and its origin is the sympathoadrenal lineage of the neural crest. N-*myc* gene amplification is recognized as one of the most important negative prognostic factors of neuroblastoma. Recent studies indicate that tyrosine kinase receptors, such as TRK family might be closely involved in the oncogenesis of neuroblastomas (Layfield *et al.*, 1995), but involvement of ShcB or ShcC proteins in oncogenic signals from RTKs of neural tumor cells has not been reported.

As results, expression and tyrosine phosphorylation of Shc family proteins, especially ShcC, were observed in most of neuroblastoma cell lines. Phosphoproteins associated with ShcC were immuno-affinity purified in order to identify components of ShcC signaling complex including the activated RTK(s) responsible for ShcC phosphorylation. The mass-spectrometry analysis revealed that a known RTK, anaplastic lymphoma kinase (ALK) is a major 200 kD component of ShcC associated phosphoproteins. Further genetic and biochemical analyses were performed to elucidate the molecular mechanism of ALK activation and roles of ALK-ShcC complex in the biological characteristics of neuroblastoma.

Results

Expression and tyrosine phosphorylation of Shc family proteins in neuroblastoma and other tumor cell lines of neural origin

To examine specific expression and phosphorylation of each Shc family protein, specific antibodies against CH1 domains of each Shc protein were prepared which show significant diversity among Shc family members. Specificities of anti-ShcB and anti-ShcC antibodies were previously checked using samples from ShcB/ShcC knockout mice (Sakai *et al.*, 2000). An anti-ShcA specific antiserum was raised for the current study as described in materials and methods. Twelve neuroblastoma cell lines, four other neuronal tumor cell lines, neuroepithelioma, glioblastoma and melanoma, and three non-neuronal tumor cell lines were analysed in this study (Table 1). ShcC expression was detectable

Table 1 Expression of Shc family proteins and phosphorylation of ShcC in human solid tumor cell lines

	$p52/p66^{ShcA}$	$p68^{ShcB}$	$p67^{ShcC}$	$P\text{-}p67^{ShcC}$
Neuroblastoma				
NB-39-nu	+/+	−	+	++
YT-nu	+/+	−	+	+
Nagai	+/±	−	±	++
TGW	+/+	−	+	−
KU-YS	+/+	−	−	−
GOTO	+/+	−	±	+
TNBI	±/±	−	+	+
SK-N-SH	±/±	−	+	−
SCCH-26	+/+	−	+	+
SK-N-DZ	+/+	−	+	+
NH12	ND	ND	+	+
KP-N-NS	+/±	ND	−	−
Glioblastoma				
T98G	+/+	−	+	−
Melanoma				
WM-115	ND	−	+	+
SK-MEL-1	ND	ND	−	−
Neuroepithelioma				
SK-N-MC	+/+	−	+	+
Colon cancer				
DLD-1	±	−	−	−
HCT116	±	−	−	−
Renal cancer				
VMRC-RCZ	ND	−	+	±

$P\text{-}p67^{ShcC}$: phosphorylated $p67^{ShcC}$; ND: not done; +: positive, −: negative, ±: detected at trace level

in 10 out of 12 neuroblastoma cell lines, including eight cell lines with tyrosine phosphorylation of ShcC (Table 1). The proportion of the expression level of $p52^{ShcC}$ to that of $p67^{ShcC}$ was mostly conserved in each cell line. Some of these cell lines, such as NB-39-nu and Nagai cells, showed marked tyrosine phosphorylation of ShcC, in spite of rather low expression level of ShcC (Figure 1a). Besides neuroblastoma cell lines, tyrosine phosphorylated ShcC was observed in two of three neuronal tumor cell lines which expressed ShcC. A few distinct phosphoproteins were associated with hyperphosphorylated ShcC-containing cells, with approximate molecular weights of 135 kD, 170 kD and 200 kD in NB-39-nu and Nagai cells, 110 kD and 130 kD in TNB1 cells and 80 kD, 100 kD and 120 kD in SK-N-MC cells, respectively (Figure 1a and data not shown).

Trace level of tyrosine phosphorylation of ShcC was detected in VMRC-RCZ, a human renal cancer cell line, suggesting that the neuro-specificity of ShcC might be lost in undifferentiated solid tumor cells (data not shown). No expression of ShcC was recognized in tumor cell lines of the hematopoietic lineage, such as THP-1, K562 and HELL, most of which had considerable expression of p52 ShcA (data not shown).

ShcA was also expressed in most of neuroblastoma cell lines analysed (Figure 1b and Table 1). None of the Shc family proteins were detected in the adult brain using the same antibody, showing that this antibody is not cross-reacting with ShcB or ShcC (Figure 1b). The p46 ShcA isoform could not be constantly separated from the immunoglobulin heavy chain after immuno-

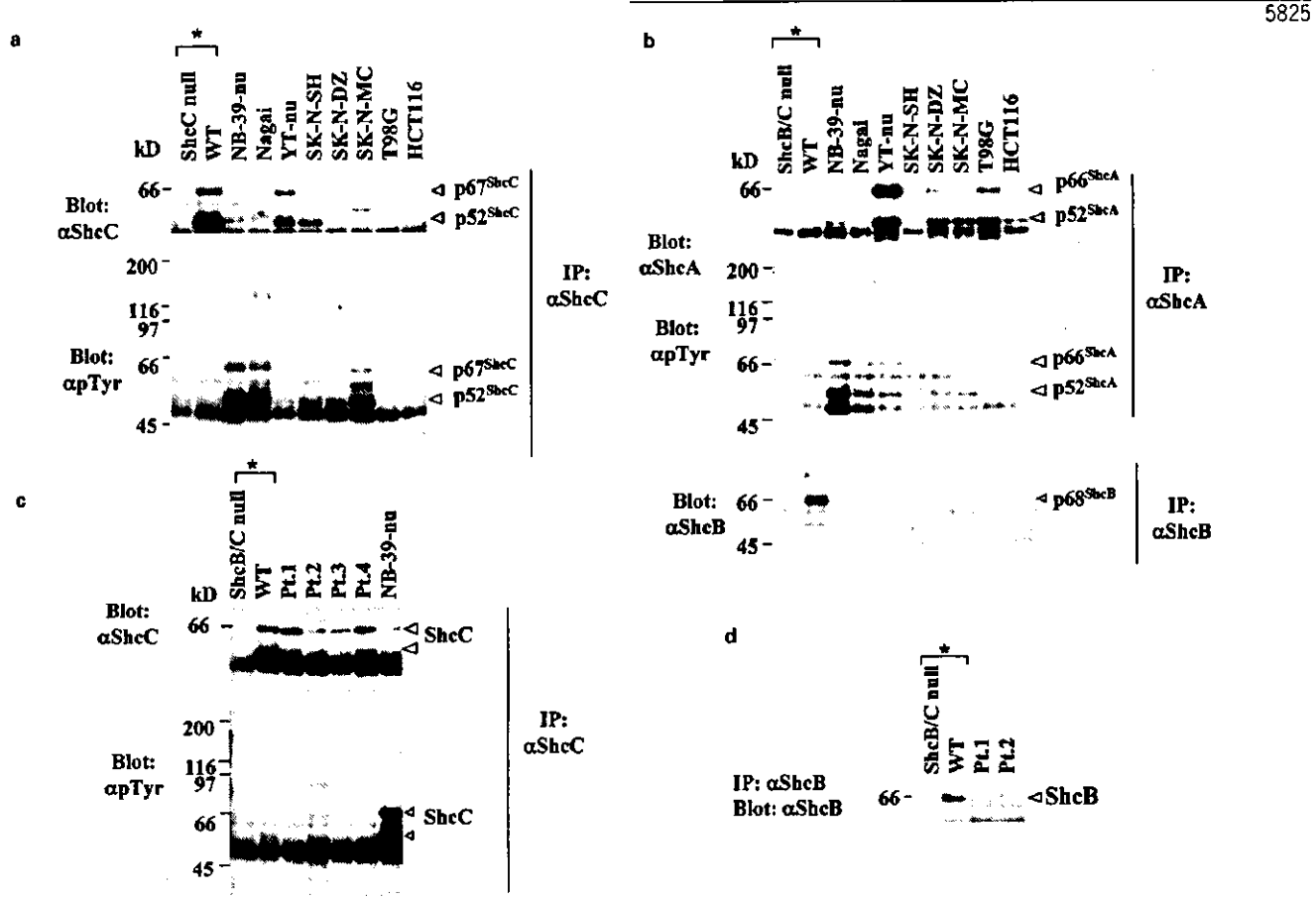

Figure 1 Expression and tyrosine phosphorylation of Shc family proteins in human neuronal tumor cell lines. (**a**) Expression (upper panel) and tyrosine phosphorylation (lower panel) of ShcC proteins. A few phosphoproteins associated with ShcC are also seen in some of cell lines (lower panel). (**b**) Expression (upper panel) and tyrosine phosphorylation (middle panel) of ShcA, and expression of ShcB (lower panel). (**c**) Expression and tyrosine phosphorylation of ShcC in neuroblastoma tissues. (**d**) Expression of ShcB in neuroblastoma tissues. Lysates were precipitated and detected by antibodies shown in the figure. Positions of molecular mass markers (kD) are shown to the left. Asterisks show samples from brain tissues of mice

precipitation, thus excluded from the study. Expression of the p66 ShcA isoform was rather restricted to neuronal tumors comparing with the p52 ShcA isoform. Tyrosine phosphorylation of $p66^{ShcA}$ was detectable in three out of six neuroblastoma cell lines in our analysis. There was obvious difference between the pattern of tyrosine phosphorylation of ShcA and ShcC among these cell lines. For example, the tyrosine phosphorylation of ShcC but not ShcA was detected in SK-N-MC and SK-N-SH and on the contrary, ShcA phosphorylation, not ShcC, was mainly detected in YT-nu. The expression level of ShcB was quite low compared with that in the brain and its phosphorylation was undetectable in any of neuronal cell lines examined (Figure 1b, lower panel).

Expression of tyrosine-phosphorylated ShcC in neuroblastoma tissues

Tissue samples of neuroblastoma were mainly derived from children in various stages and prognosis (as shown in Table 2). ShcC was significantly expressed in all of the four tumor samples, and tyrosine phosphorylation of ShcC was visible in three tumor samples (Figure 1c). Notably, tumor samples of patients of the advanced clinical stage, Pt.2 and Pt.3, showed elevated tyrosine phosphorylation of ShcC comparing with other patients. A few ShcC-associated phosphoproteins, such as 75 and 90 kD phosphoproteins were observed. In these four tumors, we could detect ShcA expression at different levels, but we failed to show tyrosine phosphorylation of ShcA (data not shown). Our mouse ShcB antibody could detect faint bands of $p68^{ShcB}$ in some of the human tissues (Figure 1d), which were not tyrosine phosphorylated.

Analysis of ShcC-binding phosphoproteins in NB-39-nu and Nagai cells

NB-39-nu and Nagai cells were brought to further analysis because they showed the most outstanding tyrosine phosphorylation of ShcC and similar consistent species of phosphotyrosine-containing proteins to each other (Figure 2a; left panel). Their molecular

Table 2 Patients characteristics of neuroblastoma tissues and cell lines

			Primary tumor		*Amplification of N-myc*
	*Age**	*Sex***	*Location*	*Clinical stage****	
Tissues					
Case					
Pt. 1	9 m	M	Adrenal gland	I	–
Pt. 2	1 y 0 m	M	Abdomen	IVa	–
Pt. 3	2 y 4 m	M	Abdomen	IVa	+
Pt. 4	6 m	M	Adrenal gland	I	–
Cell lines					
NB-39-nu	1 y 10 m	M	Adrenal gland	IV	+
Nagai	28 y	M	8th cervical nerve root	Unknown	+

*Age of onset year (y), month (m); **male (M), female (F); ***POG stage; Pt.: patient

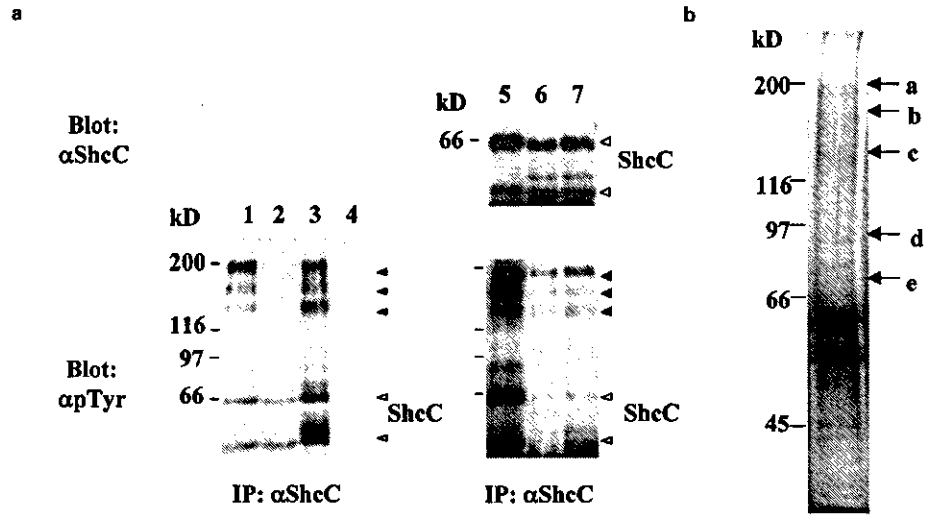

Figure 2 Detection and purification of ShcC-associated phosphoproteins. (a) Phosphotyrosine-containing proteins were precipitated with αShcC and detected by 4G10 (a, left panel) from the lysates, 1 mg of total protein for each (lane 1, 2, 3 and 4: NB-39-nu, YT-nu, Nagai, and T98G respectively). ShcC-associated phosphoprotein was also detected in the sample of nude mouse tumor derived from NB-39-nu cells (a, right panel; lane 6 and 7) (lane 5: original NB-39-nu cells). ShcC-binding phosphoproteins are marked by filled triangles. (b) ShcC-binding phosphoproteins semi-purified from NB-39-nu cells by two-step immunoaffinity purification (as described in Materials and methods). The sample indicated in the figure was obtained from 1.5×10^9 NB-39-nu cells. Aliquots were collected into one and 0.1 volume of all was subjected to silver staining to estimate the quantity. Molecular mass analyses of Lys-C fragments were performed mainly for the bands shown by arrowheads. Positions of molecular mass markers (kD) are shown to the left

weights were 70, 90, 120, 135, 170 and 200 kD. Among them, 135–200 kD phosphoproteins were also co-precipitated with the anti-ShcA antibody but not by the anti-ShcB antibody and the control serum (data not shown). Similar sets of 135–200 kD phosphotyrosine-containing proteins were shown to be associated with ShcC even in nude mouse tumors derived from these cell lines, while the phosphorylation level of ShcC was rather decreased (Figure 2a; right panel), indicating that phosphorylation of these ShcC-binding proteins is not limited to the tissue culture condition. Several antibodies against proteins which are known to be associated with Shc, such as TRK, EGFR, N-methyl-D aspartate (NMDA) receptor ε2, SHIP, ErbB-2 and Ret, failed to stain these ShcC-associated phosphoproteins, indicating that these 135–200 kD phosphoproteins are different from these molecules.

Immunoaffinity purification of ShcC-binding phosphoproteins

To identify the phosphoproteins associated with ShcC, we performed two-step immunoaffinity purification using the anti-ShcC antibody and the anti-phosphotyrosine antibody, 4G10. As a first step, the lysate of NB-39-nu cells was coupled to the anti-ShcC antibody column, eluted by the 8 M urea solution and then renatured. This denaturation-renaturation process was required to expose phosphotyrosine residues out of complexed proteins for the next affinity purification step (Sakai *et al*., 1994). The sample was then applied to anti-phosphotyrosine antibody column and phosphotyrosine-containing proteins were selectively eluted by 0.1 M phenylphosphate, an analog of phosphotyrosine. The specificity and the efficiency for each

purification step were monitored by immunoblotting using 4G10 and the silver staining. For the purpose of mass-spectrometry analysis, the cell lysate of 5×10^9 NB-39-nu cells at confluency was collected, purified through the procedure above, concentrated by lyophilization and precipitated by 10% trichloroacetate. Finally, yields of semi-purified proteins which correspond to the 135–200 kD phosphotyrosine-containing bands were estimated as 50–100 ng by silver staining of the aliquot (Figure 2b).

Identification of ShcC-binding phosphoproteins

To perform molecular mass analyses of Lys-C fragments, the sample was separated by SDS–PAGE and blotted onto a PVDF membrane, then proteins on the membrane were visualized by the Coomassie staining. We analysed nine visible bands between 70 kD and 200 kD by the MALDI-TOF MS system. After *Achrobacter* protease I (a Lys-C) digestion, p200 contained 13 peptide fragments which have exact molecular weights of fragments expected from human anaplastic lymphoma kinase (ALK) (arrow *a* in Figure 2b). Similar pattern of fragments was detected from bands of 135 and 170 kD, although amounts of the proteins were enough to be fully identified (arrows *c and b* in Figure 2b). Besides them, the bands of 70 kD and 90 kD were revealed to be FUS and EWS respectively, both of which belong to RNA-binding proteins (arrows *e* and *d* in Figure 2b). Biological significance of these two molecules is currently under investigation.

To confirm the complex formation between ShcC and ALK proteins, lysates from NB-39-nu and Nagai cells immunoprecipitated with either ShcC or ALK were analysed using anti-ALK antibodies. Multiple proteins of 135, 170 and 200 kD were detected by antibodies against C-terminal and cytoplasmic portions of ALK (Figure 3a and data not shown), indicating that the 135–200 kD ShcC-binding proteins were, as we expected, ALK and closely related molecules, such as splice variants or degradated products. In addition to that, the 200 kD allele of the ALK protein was also recognized with the anti-ALK antibody against N-terminal portion (data not shown).

In vitro pull-down assay using SH2 and PTB domains of ShcC protein fused with GST (glutathion S-transferase) demonstrated that only the PTB domain of ShcC could bring down ALK (Figure 3b; upper panel). A few unique phosphoproteins were shown to be coupled with the SH2 domain of ShcC, indicating different molecules are involved in the SH2 mediated pathway (Figure 3b; lower panel).

Constitutive tyrosine phosphorylation of ALK in NB-39-nu and Nagai cells The ALK kinase activity was examined by *in vitro* kinase assay with or without an exogenous substrate, poly-Glu/Tyr. NB-39-nu and Nagai cells possessed outstanding ALK kinase activity compared with other neural origin cells, as shown either by autophosphorylation or phosphorylation of poly-Glu/Tyr (Figure 4a). ShcC was efficiently phosphorylated by kinase activity existing in the

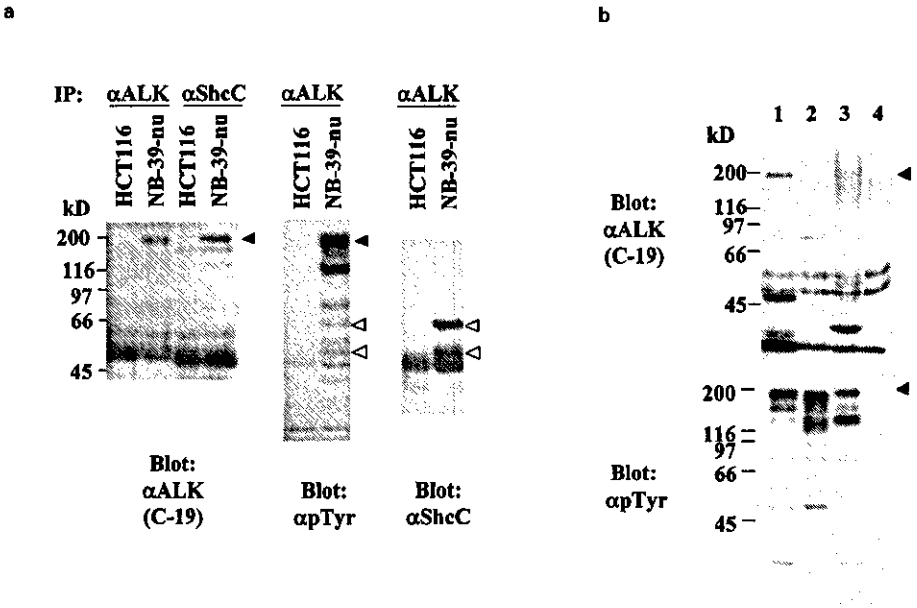

Figure 3 ShcC forms complex with ALK through its PTB domain. (a) ShcC forms a complex with the constitutively activated ALK protein in NB-39-nu cells. Lysates of HCT116 and NB-39-nu cells were used to be immunoprecipitated and detected with antibodies shown in the figure. (b) Phosphorylated ALK binds to the PTB domain of ShcC. The lysates of Nagai cells were subjected to a pull-down assay (lane 2: SH2 domain of ShcC, lane 3: PTB domain of ShcC, lane 4: GST alone) (as shown in Materials and methods). As controls, lysates were subjected to immunoprecipitation with αShcC (lane 1). ShcC and ALK proteins are marked by opened and filled triangles, respectively. Positions of molecular mass markers (kD) are shown to the left

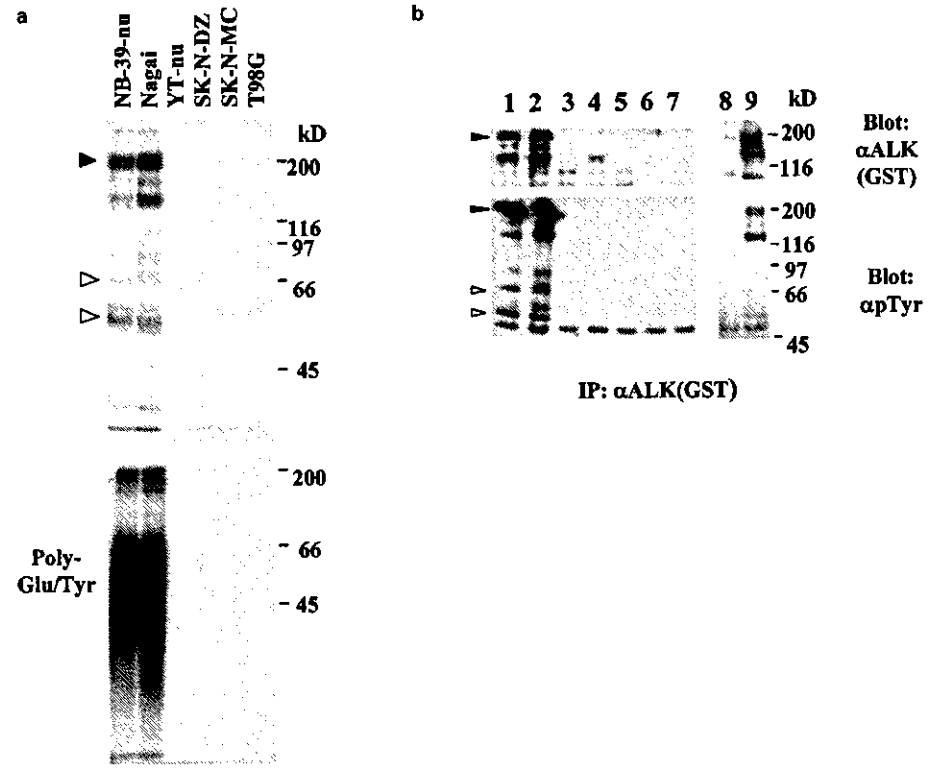

Figure 4 Significant elevation of kinase activity and expression levels of ALK detected in NB-39-nu and Nagai cells. (**a**) *In vitro* kinase assay of proteins in neuroblastoma cells immunoprecipitated with αALK(GST) (as described in Materials and methods). Kinase reaction was performed without (upper panel) or with (lower panel) poly-Glu/Tyr (4:1) as exogenous substrates. (**b**) Expression and phosphorylation of ALK in neuroblastoma cells by Western blot analysis. The sources of samples are as follows, NB-39-nu (lane 1), Nagai (lane 2), T98G (lane 3), SK-N-DZ (lane 4 and 8), YT-nu (lane 5), SK-N-MC (lane 6), HCT116 (lane 7) and NB-1 (lane 9). ShcC and ALK proteins are marked by opened and filled triangles, respectively

complex immunoprecipitated by the anti-ALK antibody, suggesting that ShcC is a potent substrate of ALK. It was also noticed that NB-39-nu and Nagai cells express much higher amount of ALK protein than others (Figure 4b), therefore marked elevation of kinase activity of ALK in these cells was at least in part due to overexpression of ALK. Slight expression of ALK protein was detected in SK-N-MC and SK-N-DZ cells, but no tyrosine phosphorylation was observed in these cells (Figure 4b, lower panel). These results indicate that the elevated kinase activity of overexpressed ALK caused hyperphosphorylation of ShcC in NB-39-nu and Nagai cells. Information from the gene chip analysis picked up another cell line, NB-1, which presents marked overexpression of ALK out of eight additional neuroblastoma cell lines. Hyperphosphorylation of ShcC and association between ALK and ShcC were also confirmed in NB-1 cells (Figure 4b, right panel, and data not shown).

We tried to use pleiotrophin (PTN), a recently identified ligand of ALK (Stoica *et al*., 2001), to investigate whether tyrosine phosphorylation of the ALK in these cells was induced by ligand-dependent or ligand-independent manner. Although various concentrations of PTN were tested in several different cell lines, we could not find any evidence that PTN functions as a ligand of ALK in neuronal cells (data not shown).

Since the tyrosine phosphorylation of ALK and ShcC was constant even after 24 h starvation in NB-39-nu and Nagai cells, we next investigated the effect of constitutive activation of ALK on the other growth factor pathways. In these cells, the EGF stimulation did not affect on the phosphorylation level of ShcC any further (Figure 5a), while prominently increased tyrosine phosphorylation of ShcC in YT-nu or T98G cells which presented no overexpression of ALK. Upon the ligand stimulation, the phosphorylated EGF receptor coprecipitated with ShcC was clearly visible at 180 kD in most of neural tumor cell lines, which appears much less in NB-39-nu and Nagai cells (Figure 5a). EGF induced slight or no activation of p44/42 MAPKs in NB-39-nu cells or Nagai cells, respectively (Figure 5b) although it could effectively activate p44/42 MAPKs in YT-nu and T98G cells. p44/42 MAPKs was also activated by the NGF stimulation in YT-nu or T98G cells, which showed no response in NB-39-nu and Nagai cells. The stimulation by NGF could not increase the levels of

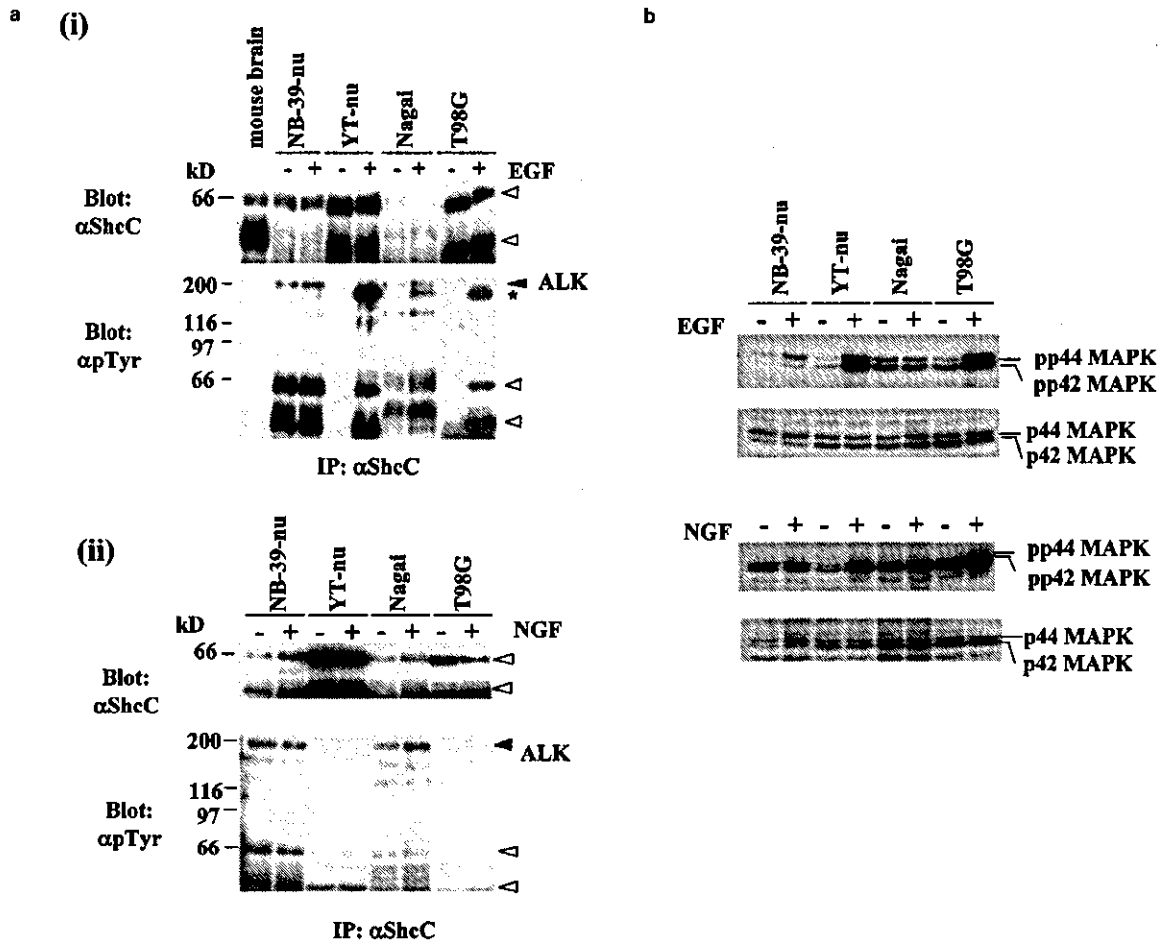

Figure 5 Phosphorylation of ShcC protein and p44/42 MAPKs are independent of stimulation by EGF or NGF in ALK-activated cells. (a) Serum-starved cells either unstimulated (−) or stimulated (+) with EGF (i) or NGF (ii) (as described in Materials and methods). ShcC, ALK and EGFR protein are marked by opened triangles, filled triangles and asterisk, respectively. Positions of molecular mass markers (kD) are shown to the left. (b) The remained lysates used in (a) were analysed to detect activation of p44/42 MAPKs. Phosphorylated MAPKs (ppMAPKs) were detected with the phospho-specific MAPK (Thr202/Tyr204) antibody (as controls, used the antibody against p44/42 which are independent of phosphorylation)

ShcC phosphorylation in any of the four cell lines indicating the TrkA-ShcC pathway might not have the main contribution to the activation of MAPKs in these tumor cell lines. These observations suggest that constitutive activation of the ALK kinase and formation of the stable ALK-ShcC complex might cause these cells to acquire unresponsiveness to the regulation by other growth factors.

Other docking molecules, IRS-1 and PLC γ are reported to be associated with ALK when it is activated as a fusion form of NPM-ALK (Iwahara *et al*., 1997; Morris *et al*., 1997). Trace levels of 200 kD phosphoproteins, possible ALK, were immunoprecipitated by IRS-1 in Nagai cells (Figure 6a) and by PLC γ in Nagai and NB-39-nu cells (Figure 6b lanes 1 and 3) suggesting formation of ALK-IRS-1 and ALK-PLC γ complexes in these cell lines. However, phosphorylation levels of IRS-1 and PLC γ were similar or even lower than in other neuronal tumor cells (Figure 6 lanes 2, 4, 6 and 8).

Overexpression and phosphorylation of ALK are associated with gene amplifications

As gene amplification could be one of the causes of overexpression of the gene products, we then checked the copy number of the *ALK* gene by Southern blot analysis using the probe for the juxtamembrane segment of the ALK cDNA. The *ALK* gene was markedly amplified in NB-39-nu and Nagai compared with other six cell lines, while the control *ShcC* gene remained unchanged (Figure 7). The N-*myc* locus (2p24) is frequently amplified in neuroblastoma and located rather close to that of *ALK* (2p23). N-*myc* gene was amplified in NB-39-nu, Nagai and NB-1 cells (Figure 7 and Ikeda *et al*., 1990). There were other cell lines, such as YT-nu, which showed amplification of N-*myc* with apparently single copy of *ALK*. Although numbers of the samples are not enough, *ALK* gene amplification might occur in some cases of N-*myc* amplification. From these results, it was shown that increased copy number of the *ALK* gene

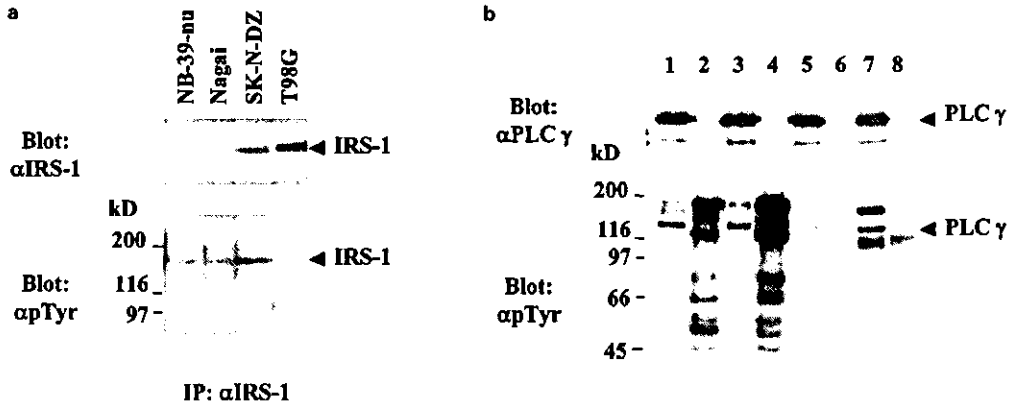

Figure 6 No significant activation of IRS-1- or PLC γ-mediated signals in NB-39-nu and Nagai cells. Lysates from neuronal tumor cells were immunoprecipitated and blotted using αIRS-1 (**a**: upper panel). The same lysates were immunoprecipitated using αPLC γ (**b**: lane 1, 3, 5 and 7) or αALK(GST) (**b**: lane 2, 4, 6 and 8), and then blotted with αPLC γ (**b**: upper panel). Each duplicated filter was detected with 4G10 (each lower panels). The sources of samples in panel **b** are as follows, NB-39-nu (lane 1 and 2), Nagai (lane 3 and 4), SK-N-DZ (lane 5 and 6) and T98G (lane 7 and 8). Positions of molecular mass markers (kD) are shown to the left

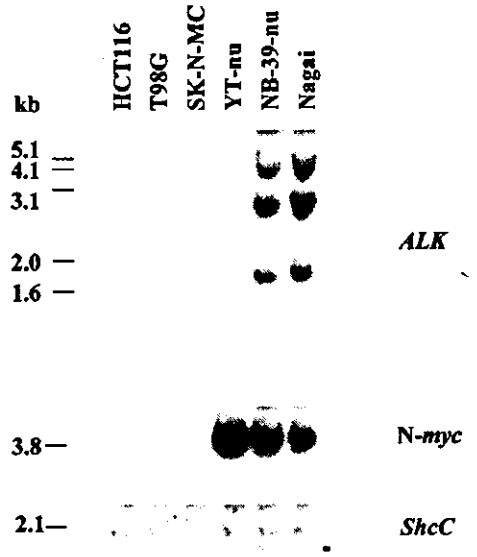

Figure 7 Marked gene amplification of the *ALK* locus in NB-39-nu and Nagai cells. To detect *ALK* gene amplification, samples of 10 μg of DNA were digested with *Hind*III. Fragments of about 1.9 kb, 2.8 kb and 4.0 kb were detected using the ^{32}P-labeled probe prepared as described in Materials and methods. The *ALK* gene was markedly amplified in the genome DNA of NB-39-nu and Nagai cells comparing with samples of other cell lines which contained equal quantity of total DNA. Similarly, amplification of the N-*myc* gene was recognized in the samples of 10 μg of DNA digested with *Eco*RI derived from YT-nu, NB-39-nu and Nagai cells. As a control for the amounts of DNA, the same filter was re-hybridized with the probe for *ShcC*

resulted in overexpression of the ALK protein in these neuroblastoma cells.

Discussion

As previously reported, the ShcA protein is constitutively phosphorylated in more than half of human tumors in association with activation of various tyrosine kinases (Pelicci *et al.*, 1995). However, no investigation about expression and phosphorylation of ShcB/ShcC in tumor cells has been published so far. We presented here expression and tyrosine phosphorylation of Shc family protein, especially ShcC, in human neuroblastoma and other neuronal tumor cells. Tyrosine phosphorylation of ShcC was observed in most of neuroblastoma cells, suggesting that ShcC might play important roles during neuronal tumorigenesis caused by perturbation of particular RTK signals in a tissue specific manner.

Extent of tyrosine phosphorylation of ShcC in neuroblastoma tissues which express ShcC, demonstrates possible correlation with the clinical stage and prognosis of neuroblastoma patients. In the case of human breast cancers, overexpression and constitutive activation of ErbB-2 protein which result in recruitment of Grb2 and Shc to the membrane are correlating with poor clinical prognosis (Dankort *et al.*, 2001). Tyrosine phosphorylation of ShcC might represent aberrant oncogenic signals initiating from activated RTKs caused by genetic alterations such as mutations and amplifications.

This is also the first report to show that the unfused form of ALK protein is constitutively activated in tumor cells by overexpression, although there are recent reports showing that expression of the ALK mRNA and protein in a wide range of human neuroblastoma cell lines and tissues (Lamant *et al.*, 2000; Passoni *et al.*, 2002). ALK is known to acquire transforming ability as a fusion protein, NPM–ALK, formed by the t(2;5) chromosomal rearrangement in non-Hodgkin's lymphoma (Bischof *et al.*, 1997; Fujimoto *et al.*, 1996; Kuefer *et al.*, 1997; Morris *et al.*, 1994). In NPM–ALK, the cytoplasmic catalytic domain of ALK is conserved, and the N-terminal NPM sequence serves oligomerization motif which leads to constitutive activation of NPM–ALK and the cellular transformation (Bischof *et al.*, 1997).

Fujimoto *et al.* (1996) indicated that cytoplasmic signals originating from the N-terminal NPM sequence is essential for the transforming activity of the fusion protein in NIH3T3 cells. The ALK protein we identified here contains both N-terminal and C-terminal portions as revealed by specific antibodies and shows an apparent molecular weight close to 200 kD, suggesting no massive deletion or rearrangement occurred in the ALK protein. We performed the RT–PCR analysis using 14 primer pairs covering the full length of human ALK cDNA (Iwahara *et al.*, 1997; Morris *et al.*, 1997), each giving an about 500-bp fragment (data not shown). The analysis revealed a small in-frame deletion of the cDNA (encoding a sequence from His-224 to Gly-318), which corresponded to a part of the extracellular domain of ALK in NB-39-nu, Nagai and YT-nu cells. This deletion was not detected by Southern blot analysis, suggesting the deletion found in the cDNAs of the three cell lines could be caused during maturation of mRNA. The fact that the deletion was also found in YT-nu cells indicates that it is not an activating mutation for the ALK kinase.

Several different mechanisms causing oncogenic activation of RTKs have been reported, such as gain of function mutations or small deletions, genomic rearrangements and gene amplification (reviewed in reference: Blume-Jensen and Hunter, 2001). Since there was no apparent activating mutation of the ALK protein in our study, it was concluded that gene amplification of ALK is principally responsible for constitutive activation of the ALK kinase in NB-39-nu and Nagai cells. As increased local concentration of receptor tyrosine kinases might lead to constitutive activation of the kinase, gene amplification can be the direct trigger of RTK activation as reported in EGFR and Neu/ErbB2 (Andrechek *et al.*, 2000), but this is the first observation of amplification of the *ALK* gene. In addition to the *ALK* gene, the N-*myc* gene amplification had been recognized in NB-39-nu, Nagai cells and also in NB-1 cells. Both *ALK* and N-*myc* are known to be at the distal short arm of chromosome 2 in the vicinity (2p23 and 2p24, respectively). The process of N-*myc* gene amplification frequently involves co-amplification of extensive regions of the DNA flanking the N-*myc* gene. The size of the N-*myc* amplicons varies among cell lines to over 1 Mb, including approximately 7 kb of the N-*myc* gene (Akiyama *et al.*, 1994; Hiemstra *et al.*, 1994). Additionally, contribution of genes co-amplified with N-*myc* to cancer progression was also proposed (George *et al.*, 1996; Wimmer *et al.*, 1999). Since no information about range of amplification or exact distance between *ALK* and N-*myc* loci was obtained, we could not conclude that a single amplicon contains both of these genes. It is possible, however, that the stage in which both *ALK* and N-*myc* are amplified and overexpressed might give additional effects on malignant phenotype of NB-39-nu, Nagai and NB-1 cells.

The phosphorylated ALK expressed in NB-39-nu and Nagai cells binds both ShcA and ShcC. It is suspected that constitutively phosphorylated ShcA and ShcC might have some redundant and overlapping roles as frequently seen in families of signaling molecules. On the contrary, they could have individual unique functions through the binding to the ALK proteins. As recently reported, ShcA and ShcC play different roles in survival and differentiation of neural stem/progenitor cells. ShcC positively affects their viability and neuronal differentiation through PI3K and MAPK pathways (Conti *et al.*, 2001). Marked elevation of ShcA mRNA was also reported in the post-injured rat hypoglossal motoneurons, whereas ShcC mRNA was down-regulated (Tanabe *et al.*, 1998).

Substrate specificity of each tyrosine kinase is known to be closely associated with its unique biological activities. ShcC is shown to be a potent substrate of the ALK kinase by *in vitro* kinase assay. Therefore, it is estimated that activation of the ALK kinase is responsible for the hyperphosphorylation of ShcC in NB-39-nu and Nagai cells. As reported in NPM–ALK, the C-terminal portion of ALK contains two potential PTB binding sites known as NPXY motifs (NPNY, aa1093–1096 and NPTY, aa1504–1507) (Iwahara *et al.*, 1997; Morris *et al.*, 1997). ShcA was shown to bind phosphorylated NPTY, whereas IRS-1 binds NPNY in NIH3T3 cells transfected with NPM–ALK (Fujimoto *et al.*, 1996). In our study, ALK did not form any stable complexes constitutively with other docking molecules, IRS-1 and PLC γ, which were phosphorylated at lower levels than in some of other neuronal tumor cells. The result indicates that the activated ALK might phosphorylate ShcC rather selectively and perturbs downstream signals, such as Ras-MAPK in neuroblastoma cells. We could not find the example of activation of the ALK protein in neuroblastoma tissues due to the limited number of samples; it is natural to think that the *ALK* gene, just like N-*myc*, is amplified in some of progressed neuroblastomas, adding more malignant phenotype to the tumors through activation of the ALK-ShcC pathway.

Materials and methods

Cell culture and tissue samples

Cell lines of Neuroblastomas; NB-39-nu, YT-nu (Ishikawa, 1977; Kuga *et al.*, 1975), SK-N-DZ (Sugimoto *et al.*, 1984) and Nagai (Ishikawa *et al.*, 1979) cells were provided by Carcinogenesis Division, National Cancer Center Research Institute. NB-39-nu and YT-nu were established from tumors that were first implanted into nude mice. TGW, GOTO, TNBI, SCCH-26, SK-N-SH, NH12 and NB-1 cells were obtained from HSRRB (Human Science Research Resource Bank). KU-YS, SK-N-SH, TNBI and KP-N-NS cells were obtained from RIKEN CELL BANK. Cell lines other than neuroblastomas; T98G cells (glioblastoma), SK-N-MC (neuroepitherioma), WM-115 (melanoma), SK-MEL-1 (melanoma), DLD-1 (colon cancer), and VMRC-RCZ cells (renal cancer) were obtained from HSRRB. HCT116 cells were kindly provided by Dr H Nojiri (Nojiri *et al.*, 1999). The

characteristics of NB-39-nu and Nagai cells are shown in Table 2. These cells were cultured in their respective medium indicated by each developer with 10% fetal calf serum (Gibco-BRL or Sigma). Cell culture was performed at 37°C and 5% CO_2.

Four frozen neuroblastoma tissues were provided by the Department of Pediatrics, University of Kitasato. Nude mouse tumors were generated by injecting 5×10^6 NB39-nu, Nagai, YT-nu or SK-N-SH cells to subcutaneous tissues of each mouse.

Preparation of anti-ShcA, ShcB and ShcC specific antibodies and anti-ALK antibody

The polyclonal antibodies against the CH1 domains of mouse ShcB (amino acid 310-477) and ShcC (amino acid 306-371) were prepared as described (Sakai *et al.*, 2000). Anti-ShcA (amino acid 271-332) antibody and anti-ALK antibody (ALK(GST)) which was against the cytoplasmic portion (amino acid 1379-1524) of human ALK were raised in rabbits and affinity-purified. An antibody against N-terminal portion of human ALK (N-19) and an antibody against C-terminal portion of human ALK (C-19) were purchased from Santa Cruz Biochemistry. An anti-phosphotyrosine antibody (4G10) was obtained from UBI. Anti-p44/42 MAPK and anti-phospho-p44/42 MAPK antibodies were purchased from BioLabs. Anti-IRS-1 and anti-PLC γ1 antibodies were purchased from Santa Cruz Biotechnology. As secondary antibodies horseradish peroxidase (HRP) conjugated anti-rabbit and anti-mouse IgG. Protein A (Amersham) and HRP-conjugated anti-goat IgG (ICN Pharmaceuticals) were used.

Cell stimulation, immunoprecipitation and immunoblotting

Cell stimulation analysis was performed with epidermal growth factor (EGF; wako) (100 ng/ml) or nerve growth factor (NGF; Wako) (100 ng/ml). Before stimulating cells were starved for 18 h and treated for 5 min with indicated concentrations. Then, cells were rinsed with ice-cold PBS and lysed in PLC-lysis buffer (Rozakis-Adcock *et al.*, 1993). Lysates containing 100 μg of total proteins per lane were applied to gels for immunoblotting, while 500-1000 μg of total proteins were mixed with 1-2 μg of each antibody for immunoprecipitation. The samples containing immune-complex were incubated at 4°C in rotating shaker with Protein A or G-Sepharose™-4B (Amersham Pharmacia) beads for 1-3 h, and then the beads were washed with PLC-lysis buffer. After boiling the lysates or immunoprecipitated materials in SDS sample buffer (2% SDS, 0.1 M Tris-HCl, pH 6, 10% glycerol, 0.01% bromophenol blue, 0.1 M dithiothreitol), each sample was resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane filters (Immobilon, Millipore). The membranes were blocked with 5% bovine serum albumin or skim-milk and proved with the appropriate diluted antibodies at room temperature. The blots were treated with horseradish peroxidase-conjugated secondary antibodies and visualized using chemical-luminescence (Renaissance, NEN™+ Life Science Products).

In vitro *pull down assays*

cDNAs encoding the SH2 (amino acid 29-189) or PTB (amino acid 377-474) domain of mouse ShcC were subcloned into the pGEX-4T2 vectors and expressed as GST fusion proteins in *E coli* after induction by isopropyl-β-D-thiogalactopyranoside (IPTG). After centrifugion of bacterial culture, the pellets were lysed in L buffer (50 mM Tris-HCl, pH 7.4, 25% sucrose, 5 mM $MgCl_2$, 0.5% Nonidet P-40) by sonication. These proteins were purified on glutathione-agarose beads (Sigma) and dialyzed with 0.1 M borate buffer (pH 8.5, 0.5 M NaCl). After coupling with CNBr-activated Sepharose 4B (Pharmacia), proteins were incubated in 50 mM Tris (pH 7.4), 150 mM NaCl, 0.05% sodium azaide. Equal amounts of each GST fusion coupling sepharose were incubated with equivalent amounts of lysates from NB-39-nu or Nagai cells at 4°C for 2 h. These mixes were washed with PLC-lysis buffer four times and re-suspended in SDS sample buffer. Then immunoblotting was performed as described.

Immunoaffinity purification of ShcC-binding phosphoproteins

Immunoaffinity purification of phosphoproteins was basically performed as described (Sakai *et al.*, 1994). Briefly, the anti-ShcC antibody-Sepharose gel and 4G10-Sepharose gel were prepared using CNBr-activated Sepharose 4B (Amersham). NB-39-nu cells lysed in PLC-lysis buffer were coupled with 1/20 volume of αShcC-Sepharose gel for 6 h at 4°C, and washed with 10 volumes of PLC-lysis buffer. Proteins eluted from the first immunoaffinity column using 8 M urea buffer (8 M urea, 1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4) were renatured by dialysis against a solution of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride, and then incubated with 0.1 volume of the 4G10-Sepharose gel and rotated slowly for 5 h at 4°C. Beads were washed with PLC-lysis buffer and 0.1% heptylglucoside, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and eluted by 0.1 M phenylphosphate buffer (0.1 M phenylphosphate, 0.1% heptylglucoside, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl). Fractions containing anti-ShcC binding phosphoproteins were collected, concentrated and precipitated with 10% trichloroacetate.

Identification of proteins by peptide mass mapping

Purified proteins separated by gel electrophoresis were blotted onto a PVDF membrane (ProBlott, Applied Biosystems) and then stained with Coomassie Stain Solution (Bio-Rad) to recognize the appropriate band to cut out for peptide mass mapping. The immobilized proteins were reduced, S-carboxymethylated, and digested *in situ* with *Achromobacter* protease I (a Lys-C) (Iwamatsu, 1992; Iwamatsu and Yoshida-Kubomura, 1996). Molecular mass analyses of Lys-C fragments were performed by Matrix-assisted Laser Desorption/Ionization time-of-flight (MALDI-TOF) mass spectrometry using a PerSeptive Biosystem Voyager-DE/RP. Identification of proteins was carried out by comparison between the molecular weights determined by MALDI-TOF/MS and theoretical peptide masses from the proteins registered in NCBInr (9.19.2001) (Ole *et al.*, 1996).

In vitro *tyrosine kinase assays*

Cell lysates containing 1 mg of proteins were mixed with 1 μg of αALK(GST) or anti-ShcC antibody and incubated for 1 h on ice. Samples were rotated with Protein G-SepharoseTM-4B (Amersham Pharmacia) for 1 h at 4°C, then the beads were washed three times in the PLC-lysis buffer and three times in kinase buffer (50 mM Tris HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$,10 mM $MnCl_2$). The kinase reaction was performed in 30 μl of kinase buffer with 5 μCi of [γ^{32}P]ATP (Amersham) at room temperature for 30 min (without

exogenous substrate) or 37°C for 10 min (with exogenous substrate). Twenty μg of poly-Glu/Tyr (4:1) (Sigma) was used as a exogenous substrate for each sample (Sakai *et al.*, 1997). Samples were boiled in the SDS sample buffer and separated by polyacrylamide gel. The gels were dried and exposed to an X-ray film and subjected to autoradiography.

DNA extraction and Southern blotting

Genomic DNAs were obtained from cultured cells as described by the procedure of Perucho *et al.* (1981). Samples of 10 μg of DNA digested with respective enzymes were electrophoresed in 0.8% agarose gel and transferred to nitrocellulose filters by the method first described by Southern (1975). The probes for detecting the *ALK* genome were prepared using the RT-PCR product (843 bp fragment) encoded the juxtamembrane segment of the ALK cDNA (3938-4781) (Morris *et al.*, 1997). The probe for the N-*myc* gene was prepared from the 2 kb *Bgl*II-*Eco*RI fragment of the genomic clone, pN-*myc*2238, kindly provided by Dr Y Taya, which was to detect 3.8 kb of the genomic N-*myc* DNA digested by *Eco*RI (Kohl *et al.*, 1986; Stanton *et al.*, 1986). As a control we used the probe for detecting *ShcC* gene, that was obtained by digesting pCMV1-T7N-Shc, human *ShcC* cDNA, kindly provided by Nakamura *et al.* (1996b). The probe contained 1.1 kb of cDNA sequence, corresponding to PTB and CH1 domains of the human ShcC protein.

Acknowledgments
We thank Dr M Ohki for comments and technical advices, and Dr H Nakadate for tissue samples of the patients. Izumi Miyake and Yuko Hakomori are the recipients of Research Resident Fellowships from the Foundation for Promotion of Cancer Research, Japan.

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Biochemical and Biophysical Research Communications 294 (2002) 635–641

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Analysis of gene expression profile in p130^{Cas}-deficient fibroblasts

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Received 10 May 2002

Abstract

p130^{Cas} (Cas) is a docking protein that becomes tyrosine phosphorylated in v-Src- or v-Crk-transformed cells and in integrin-stimulated cells. Cas −/− fibroblasts show defects in stress fiber formation, cell spreading, cell migration, and transformation by activated Src. To further characterize the role of Cas in signaling, we compared the expression profile in Cas −/− fibroblasts with that in Cas-re-expressing fibroblasts using the microarray methods. In Cas −/− fibroblasts, the expression of heme oxygenase 1 and caveolin-1 was reduced, but the expression of procollagen 1 α 1, procollagen 3 α 1, procollagen 11 α 1, elastin, periostin, TSC-36, and MARCKS was enhanced. The domains in Cas necessary for the change varied among these genes. Activated Src reduced the expression of most of these genes both in Cas −/− and in Cas +/+ fibroblasts. These results suggest the existence of signaling pathways that emanate from Cas to gene expression.

Keywords: p130^{Cas}; Microarray; Collagen; Elastin; Periostin; TSC-36; MARCKS; Caveolin; Heme oxygenase; Src

Extracellular matrix (ECM) is composed of collagens, fibronectin, vitronectin, elastin, laminins, and many other proteins. Cell-matrix interactions play a crucial role in a number of physiological and pathological processes including cell proliferation, migration, apoptosis, differentiation, metastasis, invasion, and wound healing. Focal adhesions are the site of cell-matrix attachment and integrins, cell surface receptors for ECM, are clustered to focal adhesions. Focal adhesions are composed of many other cytoskeletal and signaling proteins, such as FAK, Src, talin, paxillin, zyxin, tensin, and p130^{Cas} (Cas).

Cas was originally identified as a major tyrosine phosphorylated protein in v-Crk- or v-Src-transformed cells [1,2]. Cas contains an SH3 domain, the substrate domain that contains Crk SH2 binding motifs, the Src-binding domain, and the C-terminal domain that also binds to several proteins [2–5]. With these binding domains, Cas binds to many signaling molecules including FAK [6], PTP1B [7], PTP-PEST [8], C3G [9], CMS/CD2AP [10], CIZ [11], Nck [12], Chat [5], AND-34 [4], zyxin [13], and PI3K [14]. Cas is shown to be tyrosine phosphorylated in response to integrin stimulation [15,16] and transmits signals through several pathways, for example, through Crk-DOCK180 complex to Rac/JNK activation [17,18] and FAK/Cas/Crk complex is known to play a crucial role in cell migration [19,20].

Previously, we reported the phenotype of Cas-deficient mice [21]. Cas-deficient embryos died in utero at 11.5–12.5 days post-coitum showing marked congestion and growth retardation. Histological study of the embryo revealed disorganization of myofibrils and disruption of Z-disks in cardiocytes [21]. Furthermore, Cas-deficient fibroblasts showed impaired stress fiber formation, defects in cell migration, delayed cell spreading, and partial resistance to Src-induced transformation [21,22]. To further clarify the role of Cas in fibroblasts, we investigated the expression profile in Cas-deficient fibroblasts using microarray methods.

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0006-291X/02/$ - see front matter
PII: S0006-291X(02)00527-2

Materials and methods

Cell lines DNA transfection, and cell cloning. Primary Cas −/− fibroblasts were maintained as described earlier [21] and spontaneous cell lines were obtained. Cas-re-expressing fibroblasts were obtained by transfecting pSSRαbsr-Cas [21] using Lipofectamine (Gibco), followed by selection in 10 μg/ml blasticidin hydrochloride (Kaken Seiyaku). For the Cas mutant stable cell lines, plasmids for HA-tagged Cas mutants [23] together with pSV2bsr (Kaken Seiyaku) were transfected to Cas-deficient fibroblasts using Lipofectamine (Gibco), followed by selection in 10 μg/ml blasticidin hydrochloride (Kaken Seiyaku). Primary Cas +/+ fibroblasts, Cas −/− and a-Src cells, and Cas+/+ and a-Src cells were described earlier [21]. Briefly, activated Src (a-Src) with Myc-tag was created by replacing aa 527–536 of rat Src, which include the negative-regulatory tyrosine residues, by c-Myc epitope tag. The plasmid was introduced to Cas −/− or Cas +/+ cells using Lipofectamine (Gibco) with puromycin-resistance gene.

Microarray analysis. Total RNAs were extracted from Cas-re-expressing fibroblasts or vector control-transfected Cas −/− fibroblasts as described [24]. Poly(A) RNAs were purified using OligoTex (Roche). One μg poly(A) RNAs was sent to Incyte Genomics, and MOUSE GEM 1 microarray analysis was performed.

Western blot analysis. Proteins were extracted by lysing cells in 1% Triton buffer (10 mM Tris–HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, and 1 mM Na_3VO_4). Protein aliquots were separated by SDS–PAGE, blotted to Immobilon PVDF membranes (MILLIPORE), and probed with 1:2500 diluted anti-Cas2 antibody [2]. Positive signals were visualized using the ProtoBlot Western AP system (Promega).

Northern blot analysis. Total RNAs or poly(A) RNAs were separated on a 1% agarose gel by electrophoresis and transferred to a Hybond N nylon filter (Amersham). The cDNAs for COL1A1 and TSC-36 are gifts from Dr. Masaki Noda with the permission of Dr. Barbara E. Kream and from Dr. Kiyoshi Nose, respectively. The cDNA for Cas was described previously [2]. The other cDNAs were purchased from Incyte Genomics. For TSC-36 probe, pGEM/TSC-36 [25] was digested with *Eco*RI and the 550 bp fragment was purified using QIAquick (Qiagen). For COL1A1 probe, pBR322-rat COL1A1 was digested with *Eco*RI and the 1.4 kb fragment was purified. For COL3A1 probe, W89883 was digested with *Eco*RI and *Hin*dIII and the 450 bp fragment was purified. For COL11A1 probe, AI89400 was digested with *Eco*RI and *Pst*I and the 350 bp fragment was purified. For elastin probe, AA048878 was digested with *Eco*RI and *Hin*dIII and the 450 bp fragment was purified. For periostin probe, W82878 was digested with *Eco*RI and *Bst*XI and the 330 bp fragment was purified. For MARCKS probe, AI32268 was digested with *Eco*RI and *Pst*I and the 400 bp fragment was purified. For caveolin-1 probe, AA138963 was digested with *Eco*RI and *Bst*XI and the 360 bp fragment was purified. For heme oxygenase 1 probe, AA213167 was digested with *Eco*RI and *Hin*dIII and the 400 bp fragment was purified.

The cDNAs were labeled by a Multiprime DNA Labeling System (Amersham) and used as probes. Hybridization was carried out at 42 °C in a solution containing 50% formamide, 5× SSC, 5× Denhardt's solution, 0.5% SDS, denatured salmon sperm DNA (20 μg/ml), and a ^{32}P-labeled probe. Hybridized filters were washed twice in 2× SSC/0.1% SDS for 20 min at 55 °C, followed by exposure to BAS-III imaging plate (Fuji Film).

Results

Establishment of Cas-re-expressing fibroblasts

The spontaneous Cas-deficient fibroblast cell line was transfected with pSSRαbsr-Cas or with pSSRαbsr. The transfected cells were subjected to selection with blasticidin and the selected colonies were collected together and analyzed. Re-expression of Cas was confirmed by Western blot with anti-Cas2 antibody [2] (Fig. 1B).

Result of microarray analysis and verification by Northern blot analysis

Poly(A) RNAs were extracted from Cas-re-expressing fibroblasts or vector-control-transfected fibroblasts, and analyzed by Incyte Genomics, using MOUSE GEM 1 microarray system that includes approximately 8900 EST clones from approximately 5000 genes. Genes that showed more than 2.5-fold change in expression were presented in Table 1. All the cDNAs except for the unknown genes and for the duplicates were prepared for probes. The poly(A) RNAs used in the microarray analysis were electrophoresed, transferred to filters, and hybridized with the probes. Many of them (caveolin-1, heme oxygenase 1, procollagen type 1 α 1 (COL1A1), procollagen type 3 α 1 (COL3A1), procollagen type 11 α 1 (COL11A1), elastin, periostin, TSC-36, MARCKS, Slim1, protease nexin 1, and lumican) showed clear

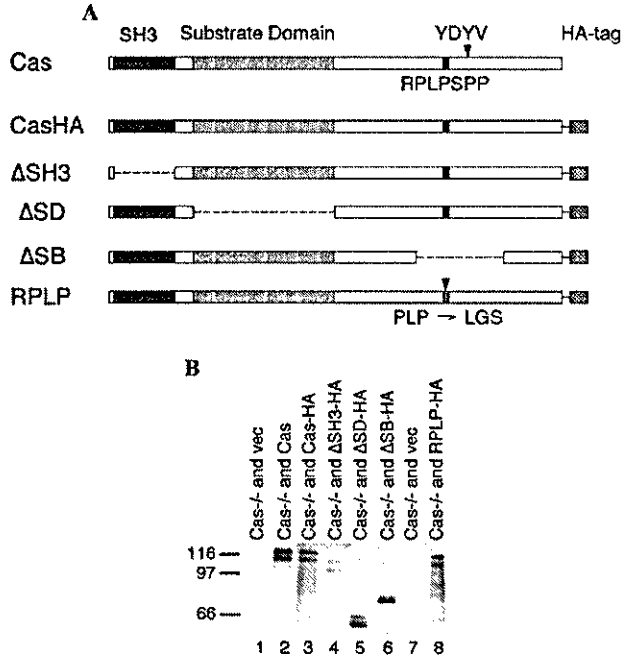

Fig. 1. (A) Schematic diagram representing the various Cas constructs used [23]. (B) Western blot for detecting the re-expression of Cas and Cas mutants. Fifty μg cell lysates extracted from Cas-deficient vector control-transfected (vec) (lane 1), Cas-re-expressing (Cas) (lane 2), CasHA-re-expressing (CasHA) (lane 3), ΔSH3-re-expressing (ΔSH3) (lane 4), ΔSD-re-expressing (ΔSD) (lane 5), ΔSB-re-expressing (ΔSB) (lane 6), vector-control-transfected (vec) (lane 7), and RPLP-re-expressing (RPLP) (lane 8) fibroblasts was separated by 7.5% SDS–PAGE, transferred to PVDF membranes, and probed with anti-Cas2 antibody [2]. The position of protein markers is shown on the left.

Table 1
Genes differentially expressed by >2.5-fold, among approximately 8900 ESTs examined

Genes down-regulated in Cas-deficient cells		
3.1	AA260520	TEL
3.1	AA260248	GRB10
2.6	AA213167	Heme oxygenase (decycling) 1
2.5	AA138693	Caveolin
Genes up-regulated in Cas-deficient cells		
Extracellular matrix		
−4.5	AA242611	TSC-36, FRP
−3.9	AA048878	Elastin
−3.7	AI894006	Procollagen, type 11, α 1 (COL11A1)
−3.4	W89883	Procollagen, type 3, α 1 (COL3A1)
−3.3	AA268082	Lumican
−3.0	AA073604	Procollagen, type 1, α 1 (COL1A1)
−3.0	W81878	Periostin (osteoblast-specific factor 2)
−2.6	AA239171	Elastin[a]
Cytoskeletal and signaling		
−3.8	AI322868	MARCKS
−3.2	AA175094	MARCKS[a]
−3.0	AA000799	Similar to synaptopodin
−2.6	AA047966	FHL/SLIM1
Metabolic		
−3.1	AI892192	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1
−2.8	AA139624	CytoC oxidase V1B
−2.6	AI322570	COQ7, CLK-1
−2.5	AA521758	MIPP65
Other known genes		
−3.3	AA051654	Metallothionein 1
−3.0	AA543573	Dactylin, hagoromo
−2.9	AA068750	Sdf-1 β
−2.7	AA218279	Spi4, PN-1 (protease-nexin1)
−2.7	AA110791	Similar to p37NB
Unknown		
−2.8	AA119804	ESTs
−2.7	W14925	ESTs
−2.6	AA266229	Public domain EST
−2.5	AA185701	ESTs

[a] There are duplicates, because several ESTs were derived from the same genes.

change of expression (Fig. 2 and data now shown). However, in the comparison between Cas −/− cells and wild-type Cas +/+ cells (Fig. 4, lanes 1 and 3), we detected no change in the expression of slim1, protease nexin 1, and lumican (data not shown). SDF1β, Dad1, COQ7, Akt, Hagoromo, cyto C oxidase, synaptopodin, p37NB, GRB10, and TEL showed only small differences in expression (data not shown). MIPP65 and metallothionein had differences of background or cross-reactive bands (data not shown). We could not detect Hmgcs1 (3-hydroxy-3-methylglutaryl-coenzyme A synthase 1) band.

Based on the above results, we picked up nine genes (caveolin-1, heme oxygenase 1, COL1A1, COL3A1, COL11A1, elastin, periostin, TSC-36, and MARCKS) in the following study. The characteristics of these nine gene products are summarized below.

Caveolin-1 is an integral membrane protein and is a major structural component of caveolae but is also found in the cytoplasm [26]. In response to integrin stimulation, caveolin-1 is known to recruit Fyn to phosphorylate Shc [27,28]. Heme oxygenase 1 is an inducible enzyme that catalyzes the conversion of heme to biliverdin, carbon monoxide and iron [29]. Heme oxygenase 1 is induced by heme products, hydrogen peroxide, ultraviolet A radiation, heavy metals, endotoxin, and oxidant stress. Collagens and elastin are extracellular matrix proteins. ECM triggers the tyrosine phosphorylation of Cas through the stimulation of integrins [15,16]. Periostin is a secreted factor and is thought to be a homolog of insect adhesion molecule fasciclin I [30]. Periostin is reported to support the attachment and spreading of MC3T3-E1 cells [31] and is expressed in the heart, playing important roles in ECM deposition following myocardial infarction and heart valve formation [32,33]. TSC-36 is a TGF-β inducible extracellular glycoprotein that has a follistatin mod-

ule [25] and is down-regulated in K-ras-transformed fibroblasts [34]. Overexpression of TSC-36 is known to cause growth inhibition of lung cancer cell lines [35] and inhibits invasion [36]. MARCKS (myristoylated alanine-rich C kinase substrate) is a substrate for PKC (protein kinase C) and is implicated in the regulation of brain development, cell migration, and adhesion as well as endo-, exo-, and phago-cytosis [37]. MARCKS is found associated both with plasma membrane and in the cytosol. Recently, MARCKS is reported to be involved in the regulation of the actin cytoskeleton and the control of lipid second messengers [37]. Overexpression of MARCKS in melanoma cells restores focal contacts [38] and decreases the cell proliferation [39,40].

The substrate domain is necessary for periostin repression by Cas

To characterize the domain necessary for this transcriptional change, we expressed a series of Cas mutants [23] (Fig. 1A) in Cas −/− fibroblasts (Fig. 1B), although we could not obtain comparable expression of ΔSH3 mutant of Cas (Fig. 1B, 3J).

Caveolin-1 and heme oxygenase 1 showed enhanced expression with the re-introduction of Cas, ΔSD, ΔSB, and RPLP but not with that of ΔSH3 (Fig. 3A and B). In contrast, COL3A1, COL11A1, and elastin showed reduced expression with the re-introduction of Cas, ΔSD, ΔSB, and RPLP but not with that of ΔSH3 (Fig. 3D–F). Periostin showed reduced expression with the re-introduction of Cas, ΔSH3, ΔSB, and RPLP but not with that of ΔSD (Fig. 3G). COL1A1 showed reduced expression with the re-expression of Cas, ΔSB, and RPLP but not with that of ΔSH3 nor ΔSB (Fig. 3C). TSC-36 and MARCKS showed reduced expression with all the mutants (Fig. 3H and I). COL3A1, COL11A1, periostin, and MARCKS showed significant down-regulation with the introduction of ΔSB or RPLP (Fig. 3C, D, G, and I).

These results indicate that the domains necessary for transcriptional change are different depending on the genes regulated, suggesting that multiple signaling pathways triggered by Cas are involved.

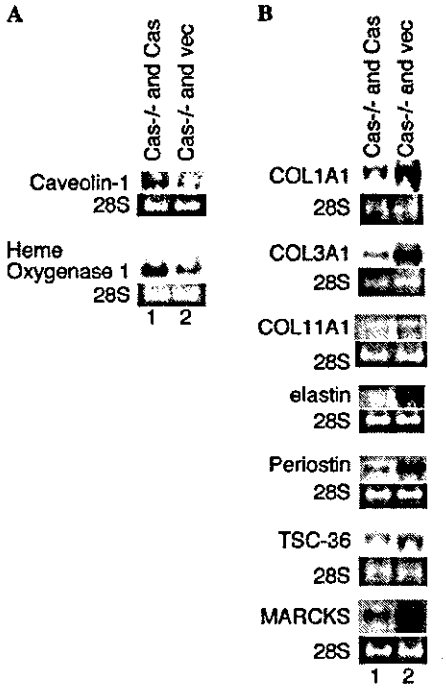

Fig. 2. Northern blots confirming altered expression of target genes. Poly(A) RNA applied to microarray analysis was used for Northern blots. Ethidium bromide-stained 28S RNA levels demonstrate similar loading in each lane. (A) Genes down-regulated in Cas −/− cells, caveolin-1 (upper), and heme oxygenase 1 (lower). (B) Genes up-regulated in Cas −/− cells, COL1A1, COL3A1, COL11A1, elastin, periostin, TSC-36, and MARCKS.

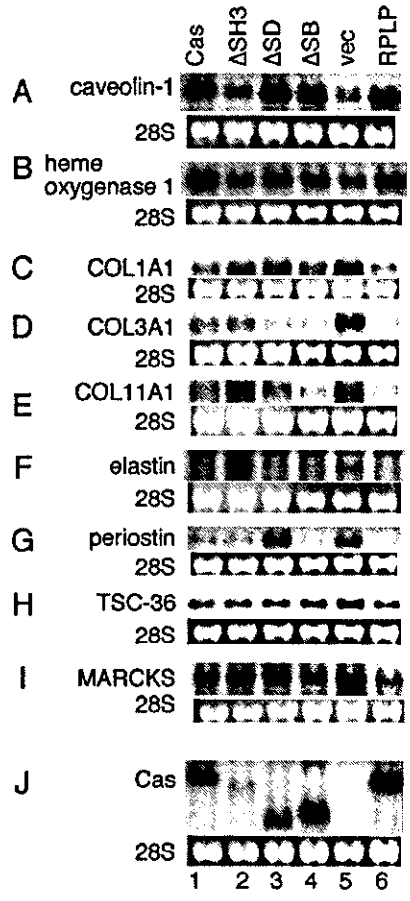

Fig. 3. Northern blots showing expression of target genes in Cas-mutant-re-expressing cells. Total RNAs harvested from Cas-HA- (lane 1), ΔSH3- (lane 2), ΔSD- (lane 3), ΔSB- (lane 4), vec- (lane 5), and RPLP (lane 6) expressing cells were used for Northern blots. Ethidium bromide-stained 28S RNA levels are shown. The probes used are for caveolin-1 (A), heme oxygenase 1 (B), COL1A1 (C), COL3A1 (D), COL11A1 (E), elastin (F), periostin (G), TSC-36 (H), MARCKS (I), and Cas (J).