Proto-oncogene TCL1: more than just a coactivator for Akt

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ABSTRACT **Serine threonine kinase Akt, also called PKB (protein kinase B), plays a central role in regulating intracellular survival. Deregulation of this Akt signaling pathway underlies various human neoplastic diseases. Recently, the proto-oncogene *TCL1* (T cell leukemia 1), with a previously unknown physiological function, was shown to interact with the Akt pleckstrin homology domain, enhancing Akt kinase activity; hence, it functions as an Akt kinase coactivator. In contrast to pathological conditions in which the *TCL1* gene is highly activated in various human neoplasmic diseases, the physiological expression of *TCL1* is tightly limited to early developmental cells as well as various developmental stages of immune cells. The NBRE (nerve growth factor-responsive element) of the proximal TCL1 promoter sequences can regulate the restricted physiological expression of *TCL1* in a negative feedback mechanism. Further, based on the NMR structural studies of Akt-TCL1 protein complexes, an inhibitory peptide, "*Akt-in*," consisting of the βA strand of TCL1, has been identified and has therapeutic potential. This review article summarizes and discusses recent advances in the understanding of TCL1-Akt functional interaction in order to clarify the biological action of the proto-oncogene TCL1 family and the development avenues for a suppressive drug specific for Akt, a core intracellular survival regulator.—Noguchi, M., Ropars, V., Roumestand, C., Suizu, F. Proto-oncogene TCL1: more than just a coactivator for Akt. *FASEB J.* 21, 2273–2284 (2007)**

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PROTO-ONCOGENE TCL1 MEETS AKT

THE PROTO-ONCOGENE *TCL1* (T cell leukemia 1) family protein was first identified in the translocation of human T cell prolymphocytic leukemia (T-PLL), a rare form of adulthood leukemia (1, 2). Three *TCL1* isoforms have subsequently been identified in both the human and the mouse genome: *TCL1*, *TCL1b*, and *MTCP1* (mature T cell proliferation 1) (3). In human T-PLL, both the *TCL1* and *TCL1b* gene are activated by juxtaposition onto the T cell receptor α or β loci secondary to chromosomal translocations t(14:14) (q11: q32), t(7:14) (q35: q32), or inversion (14)(q11: q32). In pathological conditions, in addition to its involvement in T-PLL, TCL1 is overexpressed in a wide variety of human diseases, including EBV-infected B cell lymphoma, ataxia-telangiectasia (A-T), seminoma, dysgerminoma, or AIDS-related lymphoma (4–6).

The proto-oncogene TCL1 family consists of the 106, 114, and 128 amino acids—*MTCP1*, *TCL1*, and *TCL1b* proteins, respectively—with a predicted molecular mass of 13 (*MTCP1*), 14 (*TCL1*), and 15 kDa (*TCL1b*). These amino acids share a relatively high sequence homology (40% identity, 61% homology between *MTCP1* and *TCL1*, 36% identity, 63% homology between *MTCP1* and *TCL1b* at the amino acid levels in humans) (**Fig. 1*A***). The NMR solution structure of human MTCP1 was elucidated first (7, 8), followed by the RX structures of human *TCL1* (9) and murine *MTCP1* (10). Subsequently, a model was proposed for both human and murine *TCL1b* based on the solved structures of the other members of the family (11). Consistent with the relatively high sequence homology among the TCL1 family proteins, these proteins share a common 3-dimensional structure that consists of an orthogonal 8-stranded β barrel with a unique topology (Fig. 1*B*). The antiparallel β strands of variable length are arranged into two very similar up-and-down, 4-stranded β-meander motifs, connected by a long, poorly structured loop that wraps around to form the barrel. Strands βA, βB, βE, and βF form four short staves on one face of the barrel while strands βC, βD, βG, and βH form the four other long staves on the opposite face. Following the criteria given by Murzin *et al.* (12), this structure is classified into the category of the "filled barrel," in which the inside of the barrel is tightly packed and hydrophobic. It is interesting that MTCP1 (and possibly TCL1b) is a monomeric protein, whereas TCL1 forms a tight dimer in the crystal and in solution. An exception to the β barrel structure of TCL1 proteins arises from the alternative splicing of MTCP1 transcripts, which results either in low expression of the 13 kDa TCL1 protein family or in moderate to high

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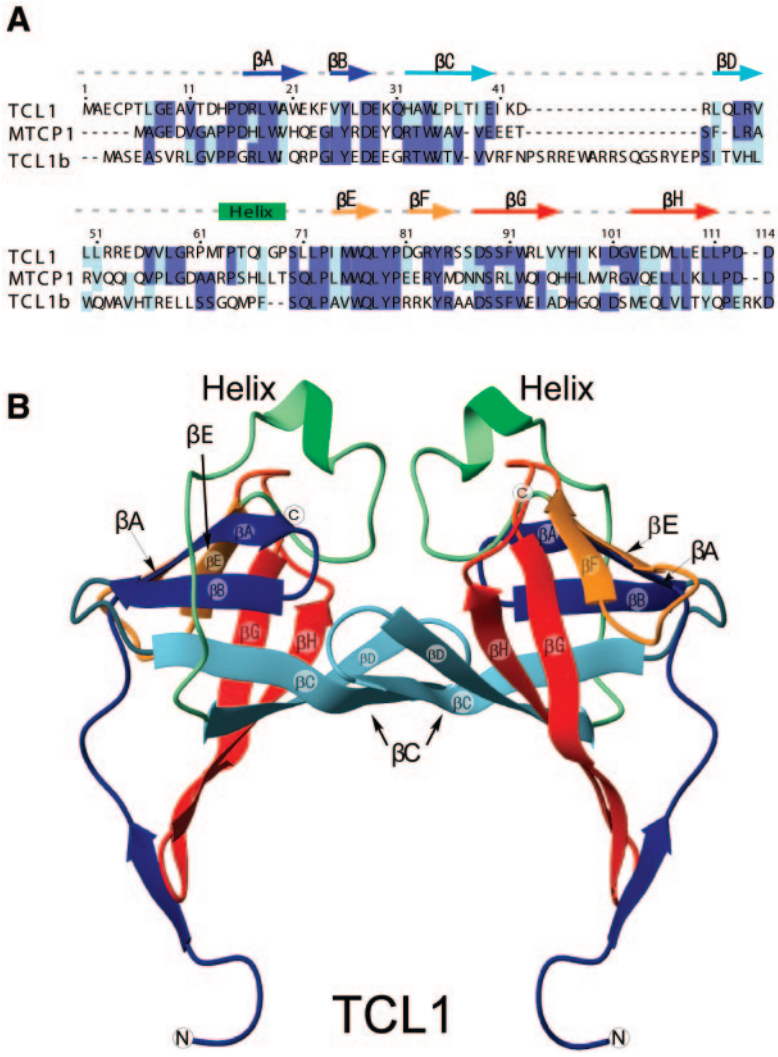

Figure 1. Structure of the TCL1 proteins. *A)* Sequence alignment of the human members of the *TCL1* proto-oncogene family. Identical residues are shaded in dark blue, homologue residues in light blue. Numbering of the sequences is one of the *TCL1* in humans. Elements of secondary structure are indicated on top of the sequence. The three TCL1 family proto-oncogenes consist of the 114, 106, and 128 amino acids—*TCL1, MTCP1,* and *TCL1b* proteins, respectively—with a predicted molecular mass of 14 (*TCL1*), 13 (*MTCP1*), and 15 kDa (*TCL1b*). *TCL1* family proteins share a relatively high sequence homology (considering only human proteins: 40% identity, 61% homology between *MTCP1* and *TCL1*, 36% identity, 63% homology between *MTCP1* and *TCL1b*) (1–3). *B)* Crystal structure (ribbon diagram) of *TCL1*. Proto-oncogene TCL1 forms a dimer in the crystal structure as well as in solution. TCL1 proteins share a common unique topology: two symmetric 4-stranded β meanders (strands βA to βD and strands βE to βH) wrap around in an 8-stranded filled β barrel analogous to the structure of MTCP1. Strands A and E (shown by arrows from the top) are involved in association with Akt PH domain. Strand C (shown by arrows at the bottom) is responsible for homodimerization of the TCL1 protein. Both Akt association and homodimerization of TCL1 are required for the complete function of TCL1 to enhance Akt kinase activity (20, 23, 47).

expression of the 8 kDa cystein-rich helical protein (13–16), with mitochondrial location and yet unknown function. The unique topology of TCL1 proteins has prompted intensive studies of their folding properties. Monitoring the unfolding reaction of MTCP1 under denaturating conditions (chemical reagents, high pressure, *etc.*) by NMR or fluorescence spectroscopy has revealed the slowest rate of unfolding ever observed for a protein (17, 18).

In yeast screening of Akt to search for an interacting Akt partner, we have demonstrated that TCL1 interacts with the pleckstrin homology (PH) domain of Akt (19). NMR studies further supported the structural basis of this interaction (20, 21). This interaction was functional, since we have demonstrated that TCL1 enhances Akt kinase activity, and therefore functions as an Akt kinase coactivator (19, 22). By means of mutational studies using "reversed" yeast two-hybrid screening, we demonstrated that both Akt interaction and dimerization of the TCL1 were required for complete function of TCL1 in enhancing Akt kinase activity (23).

Akt, also called PKB, was originally identified from the AKT8 acute transforming retrovirus, which causes mouse thymoma (24–26). The activation process of Akt has been clarified recently. After growth factors are bound to cell surface receptors, Akt is translocated to the plasma membrane secondary to bind the PH domain with phosphatidylinositol (PI)-3,4 bisphosphate (PIP_2) and PI-3,4,5-triphosphate (PIP_3). A number of growth hormones activate PI3K (phosphoinositide 3-kinase), which leads to production of PIP_2 and PIP_3 at the inner leaflet of the membrane (24, 27).

The activation process of Akt is regulated by phosphorylation at two regulatory sites, threonine 308/309/305 and serine 473/474/472 (Akt1/2/3, respectively), with phosphorylation of both required for maximum kinase activity. PDK1 (3-phosphoinositide-dependent protein kinase 1) has been identified as the primary kinase phosphorylating Akt on Thr 308. Interaction of PIP_3 with the VL1 loop of the Akt-PH domain alters the conformation, which allows PDK1 access to threonine 308/309/305 of Akt and triggers phosphorylation (28–30). Activation of Akt is also regulated by phosphorylation events within the conserved C-terminal hydrophobic motif (30, 31). Both serine 473 (Ser-473 phosphorylation and membrane anchoring are required for Thr 308 phosphorylation (32) and the complete activation of Akt.

The identity of the kinase(s)—putatively named PDK2—responsible for phosphorylation of the serine residue at 473/474/472 (Akt1/2/3, respectively) remains unclear. Several different mechanisms have been proposed including integrin-like kinase (ILK), PDK1 in combination with a fragment of another kinase (PRK-2), rictor-mTOR complex, and Akt autophosphorylation (26, 32–36). Recently, a protein phosphatase, PH domain leucine-rich repeat protein phosphatase (PHLPP), which specifically dephosphorylates the hydrophobic motif of Akt (Ser-473 in Akt1), was identified that triggers apoptosis and suppresses tumor growth (37).

THE MOLECULAR MECHANISMS OF TCL1-INDUCED AKT ACTIVATION

An essential breakthrough in understanding the function of TCL1 protein occurred when TCL1 was identified in a yeast two-hybrid search for proteins that interact with Akt. TCL1 binds to Akt and forms a hetero-oligomer within this protein complex, activating Akt (19, 22).

How does TCL1 enhance Akt kinase activity? It is well documented that dimerization can lead to activation of the surface receptor kinases (*e.g.*, VEGF-R, PDGF-R) and their intracellular responses (38–40). Similarly, intracellular nontransmembrane kinase, ASK1, has been shown to be activated by dimerization (41). It has been reported that TEL-JAK2 fusion protein causes human leukemia as a result of oligomerization and constitutive kinase activation (42). Moreover, a conditionally activated Akt fused to the hormone binding domain of the estrogen receptor was able to stimulate PHAS-1 phosphorylation (43), suggesting that Akt dimerization can promote kinase activity. The PH domain is also thought to mediate the formation of a multimeric Akt complex associated with Akt activation (44).

To dissect the molecular mechanisms of TCL1-induced Akt activation, *in vitro* kinase assays were performed using chimeric Akt molecules. This process demonstrated that oligomerization of TCL1 with Akt molecules in close physical proximity facilitated Akt transphosphorylation on the regulatory Ser-473 site (and possibly Thr-308), promoting kinase activity. PDK1 (3-phosphoinositide-dependent protein kinase 1) has been identified as the primary kinase responsible for phosphorylating Akt on Thr 308 (45). It is important that TCL1 did not trigger activation of Akt kinase, since pretreatment of PI3K inhibitor (*e.g.*, wortmannin) could compromise augmentation of Akt kinase activity. This observation indicated that some degree of Akt preactivation, presumably sensitive to wortmannin (such as association of Akt with PIP_3 and subsequent activation by PDK1), is a prerequisite for the TCL1-induced Akt activation. In this scenario, in the presence of TCL1, partially phosphorylated Akt molecules bound to TCL1 to form a hetero-oligomer in which enhancement of Akt kinase activity took place *via* a transphosphorylation reaction (**Fig. 2**) (19–21, 23, 46).

An NMR study further demonstrated that TCL1 targets the Akt-PH domain at a site that has not yet been observed in PH-protein interactions. This binding site consists of β4, β5 strands and the C-terminal helix, and is located opposite the phospholipid binding pocket (20, 21). TCL1 could bind to the Akt-PH domain *via* β4, β5, and the C-terminal α-helix of the PH domain. Dimeric TCL1 cross-linking to two Akt molecules *via* their PH domain brought the crucial phosphorylation residues Ser-473 (and/or Thr 308) close to each other and, as a consequence, enhanced their kinase activity. Akt-PH is an electrostatically polarized molecule that adopts the same fold and topology as other PH domains, which consists of a β-sandwich of seven strands capped on one top by an α-helix [Akt1 isoform (28), Akt2 isoform (21)]. The opposite face presents three variable loops (VL1, VL2, and VL3) that form the phosphoinositide binding pocket. Akt activation is initiated by the binding of PtdIns (3, 4) P_2 (PIP_2), or PtdIns (3, 4, and 5) P_3 (PIP_3) to the Akt-PH domain, recruiting the kinase to the plasma membrane. The binding surface on MTCP1 has been identified as the highly conserved face of the barrel that comprises the four short strands (βA, βB, βE, and βF). This surface is located on the opposite side of the homodimerization surface (βC strand) of TCL1. A low-resolution structure was obtained from the SAXS data for the Akt-PH/TCL1 complex (28). With the NMR information gathered on the binding surfaces for the Akt-PH/MTCP1 complex, this allowed us to build a functional molecular model (20). In this model, TCL1 cross-links two Akt molecules at the inner leaflet of the plasma membrane in a preactivated conformation without disrupting other PH-ligand interactions. The model was further supported by the observation that Akt-TCL1 complexes were preferentially present in the fraction near the plasma membrane (47). Thus, it is possible that this interaction can strengthen the membrane association, promote trans-phosphorylation, hinder the deactivation of Akt, and involve Akt in a multiprotein complex, explaining the array of known effects of TCL1. The binding affinities of the Akt-PH with TCL1 and MTCP1 have been determined by fluorescent anisotropy as well as by an NMR titration experiment. The interaction between MTCP1 and Akt was ~100-fold weaker than the TCL1-Akt interaction (K_d of 400 μM and 5 μM, respectively) (20, 21, 48). Together with the lack of dimerization interface, this suggests for *MTCP1* a distinct mechanism of Akt coactivation, or possibly a transforming mechanism separate from the augmentation of Akt activation. The molecular mechanisms of TCL1b for Akt kinase activation appear to be of interest as well, since TCL1b, unlike TCL1, does not have a structural interface for dimerization based on the predicted 3-dimensional structure. In accordance with the prediction of the TCL1b 3-dimensional struc-

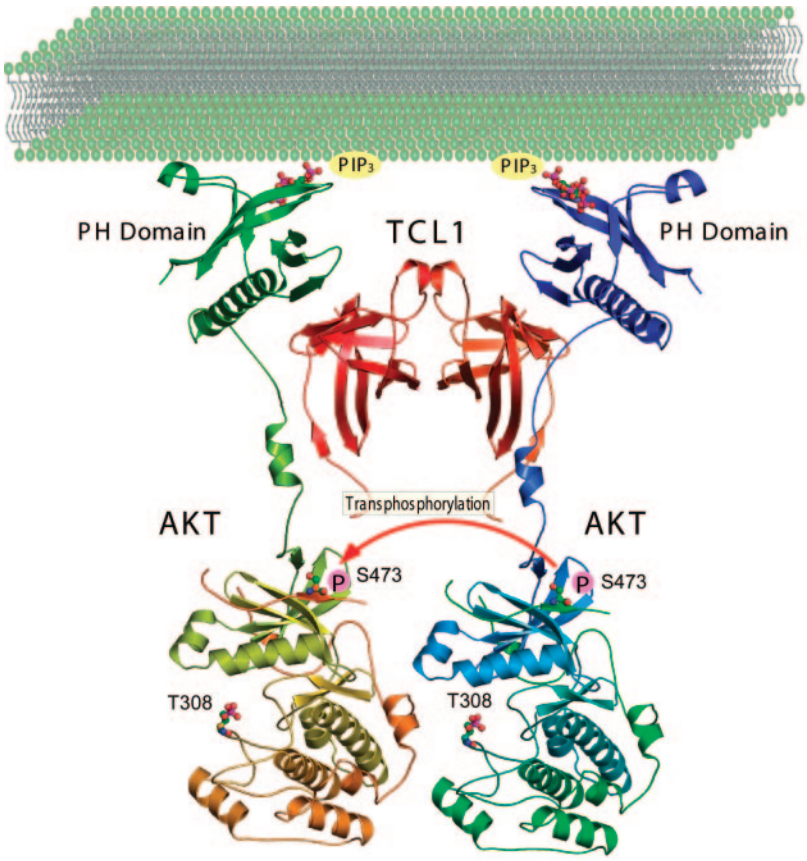

Figure 2. Model of TCL1-dependent Akt kinase activation. The proto-oncogene *TCL1* functions as a coactivator for Akt. TCL1 binds to the Akt PH domain and facilitates the formation of Akt/TCL1 hetero-oligomers at the inner plasma membrane leaflet through interaction of the PH domain of the kinase with PIP_3. The formation of the hetero-dimer brings the Akt molecules to a close physical proximity, which, as a consequence, facilitates Akt transphosphorylation on the regulatory Ser-473 (and/or Thr-308) site, promoting kinase activity and its downstream cell survival signals (19–21, 23, 46). This mechanism can explain some of the manifestations of the human T-PLL in which *TCL1* gene is up-regulated secondary to chromosomal translocations, but also promotes new insight into the molecular process of activation of Akt, the core antiapoptotic regulatory molecule.

ture, our preliminary experiments suggested that TCL1b could not form a dimer in coimmunoprecipitation assays (M. Noguchi and F. Suizu, unpublished observation).

To study the biological functions of *TCL1* and its family oncogene, transgenic mice were created for both *MTCP1* and *TCL1* in B or T cell-specific lineages. Both transgenic mice exhibited lineage-specific neoplastic diseases in the immune systems in late adulthood (49–51). Consistent with our findings, these mice exhibited a modest increase in Akt kinase activity. Rapamycin, inhibitor of mTOR, a downstream target of the PI3K-Akt pathway, suppressed tumor formation in the TCL1 transgenic mice, supporting the notion that TCL1 enhances PI3K-Akt pathways *in vivo* (52). *TCL1*-deficient female mice exhibited reduced fertility because of an impaired ability for normal cleavage and development to the morula stage. Impaired T and B cell development with decreased cellularity in the hematopoietic and lymphoid tissue (bone marrow, spleen, or thymus) is compatible with deregulation of Akt activation *in vivo* (53, 54). Incomplete suppression of Akt kinase activity observed in the *TCL1* gene target animals could be due in part to the presence of five *TCL1B* family proteins and *MTCP1* in the mouse genome. The presence of more than five isoforms of the *TCL1B* family protein in mice (3) could affect the phenotypic manifestation of TCL1 knockout mice to compensate for the function of physiological *TCL1* and its isoforms.

MOLECULAR TARGETS FOR TCL1-INDUCED AKT ACTIVATION

Akt kinase is a major downstream target of growth factor receptor tyrosine kinase that signals *via* PI3K. Accumulating evidence supports the fact that serine threonine kinase Akt plays a central role in regulating intracellular survival. The Akt substrates involved in the regulation of cell death include FKHRL; the proapoptotic Bcl-2 family member Bad; glycogen synthase kinase (GSK), which stimulates glycogen synthesis; and cyclic AMP response element binding protein (CREB) (26, 36, 55, 56).

In addition to its antiapoptotic effects, Akt plays multiple roles in regulating cellular responses in various cellular environments, including glucose metabolism, cell cycle control, angiogenesis, protein synthesis, and telomerase activity (26, 57). Akt phosphorylates the serine or threonine residue of the consensus motifs (RXRXXS/T) (58, 59). In proteomic approaches searching for a putative molecular target of Akt within the entire human genome, > 1000 molecules that bear the consensus Akt-phosphorylation motifs (RXRXXS/T) have been identified. 14–3-3 protein is an adaptor protein known to interact with serine residues within the R-S-X-S/T-X-P motifs in an Akt-dependent manner (60, 61). Since these two motifs often overlap each other, we searched the entire genome for molecular targets of Akt that bear the double consensus motifs (RXRSXS/T-X-P) of Akt phosphorylation (RXRXXS/

T), as well as 14–3-3 binding motifs (R-S-X-S/T-X-P) (**Fig. 3**). Among the putative Akt-targeting molecules identified, there were a number of kinases, phosphatases, and RNA binding proteins, including RNA splicing factors. Without full experimental verification of all the putative intrinsic factors, actual *in vivo* physiological targets of Akt in human cancer in the PI3K-Akt pathway will be a challenge. However, through these molecular targets Akt can provide cellular survival *in vivo*.

In a normal cellular environment, Akt is known to be activated through several growth factors, including PDGF and/or cytokines, *via* PI3K activation (24, 26). In contrast, the expression of TCL1 family oncogenes in physiological conditions is restricted to early developmental cells as well as the later stage immune cells such as splenic mantel zone B cells (1, 53, 62). In early developmental stages, these external signal transduction systems for Akt activation—including surface receptors, secondary messengers, and/or intracellular signaling molecules—may not be adequately developed to provide sufficient intracellular survival signals to mediate Akt activation *in vivo*. Therefore, it is plausible that, through these molecular targets, endogenous expression of TCL1, which then activates Akt, may provide intrinsic survival signals in the absence of external stimulation for Akt activation in various cellular environments. Hence, deregulation of the TCL1-Akt signaling pathway can result in human cancers *via* a diverse array of mechanisms.

DIVERGED FUNCTION OF THREE AKT AND THREE TCL1 ISOFORMS

Three isoforms of Akt have been identified: Akt1, Akt2, and Akt3 (also called PKBα, PKBβ, and PKBγ, respectively). These three isoforms bear highly conserved catalytic and regulatory domains, both of which are required for complete activation of Akt. In physiological situations, Akt1 and Akt2 seem to be ubiquitously expressed whereas the expression of Akt3 is more restricted, with predominance toward the heart, kidney, brain, testes, lung, and skeletal muscle (63–65). Therefore, it is of interest that although both Akt1 and

AKT phosphorylation Motifs	[R]-X- [R]- X- X- [ST]
14-3-3 binding Motifs	+ [R]-[S]-X- [ST]- X- [P]
AKT phosphorylation + 14-3-3 binding Motifs	[R]-X- [R]-[S]-X- [ST]- X- [P]

Genes Identified	Position	A.A.Sequencs
		RxRSx(S/T)xP
Apoptosis Related Proteins		
Forkhead box protein G1B (Forkhead-related protein FKHL1)	262 - 269:	RrRStTsP
Forkhead box protein O1A (Forkhead in rhabdomyosarcoma).	19 - 26:	RpRScTwP
Forkhead box protein O3A (Forkhead in rhabdomyosarcoma-like 1).	27 - 34:	RpRScTwP
Fork head domain transcription factor AFX1 (Forkhead box protein O4)	27 - 34:	RpRScTwP
PR domain zinc finger protein 15	57 - 64:	RwRSeSkP
Apoptotic chromatin condensation inducer in the nucleus 1	326 - 1333:	RsRSrStP
Bcl2 antagonist of cell death (BAD)	94 - 101:	RgRSrSaP
B-Raf proto-oncogene serine/threonine-protein kinase	360 - 367:	RdRSsSaP
Retinoblastoma-binding protein 6	755 - 762:	RsRSrSpP
Kinases and Phosphatases		
A-Raf proto-oncogene serine/threonine-protein kinase	209 - 216:	RiRStStP
BMP-2-inducible protein kinase	821 - 828:	RdRSgSgP
Mitogen-activated protein kinase kinase kinase 6	634 - 641:	RsRSpSsP
RAF proto-oncogene serine/threonine-protein kinase	254 - 261:	RqRStStP
BMP-2-inducible protein kinase	821 - 828:	RdRSgSgP
M-phase inducer phosphatase 2	350 - 357:	RrRSvTpP
A-Raf proto-oncogene serine/threonine-protein kinase	209 - 216:	RiRStStP
Phosphatase and actin regulator 3	14 - 21:	RgRSqSdP
Diacylglycerol kinase zeta	72 - 79:	RrRSsTvP
RNA Binding Proteins		
RNA-binding protein with serine-rich domain 1	136 - 143:	RsRSkSkP
RNA polymerase II elongation factor ELL	314 - 321:	RgRSaSpP
tRNA-splicing endonuclease subunit Sen54	200 - 207:	RkRSsSsP
Splicing factor, arginine/serine-rich 2 (Splicing factor SC35)	185 - 192:	RsRSrSpP
	202 - 209:	RsRSkSpP
FUS-interacting serine-arginine-rich protein 1	180 - 187:	RsRSkSqP
	250 - 257:	RsRSwTsP
Splicing factor, arginine/serine-rich 7 (Splicing factor 9G8).	199 - 206:	RsRSiSrP
Splicing factor 45 (45 kDa-splicing factor)	219 - 226:	RpRSpTgP
Other Enzymes		
Rho-GTPase-activating protein 9.	247 - 254:	RsRSeTnP
C-C chemokine receptor type 6	155 - 162:	RIRSrTIP
GPI mannosyltransferase 3	111 - 118:	RIRSyTyP
Ubiquitin carboxyl-terminal hydrolase 51	111 - 118:	RaRSrSqP
E3 ubiquitin-protein ligase NEDD4-like protein	443 - 450:	RpRSlSsP
Acetyl-coenzyme A synthetase,	25 - 32:	RaRSwSpP
ATP-dependent RNA helicase DHX8 (DEAH box protein 8)	225 - 232:	RsRSqSpP
Miscellaneous		
Trafficking kinesin-binding protein 2	415 - 422:	RgRSiSfP
Signaling threshold-regulating transmembrane adapter 1 precursor	68 - 75:	RgRSrShP
Thyroid receptor-interacting protein 12 (TRIP12).	233 - 240:	RsRSaSsP
Interferon-induced guanylate-binding protein 1	151 - 158:	RiRSkSsP
Immunoglobulin-like domain-containing receptor 1 precursor.	464 - 471:	RhRSySpP
Brain-specific angiogenesis inhibitor 3 precursor.	368 - 375:	RtRScTpP
Cyclin-L2	422 - 429:	RsRSdSpP
Development and differentiation-enhancing factor 2	817 - 824:	RqRSsSdP

Figure 3. Putative molecular targets of TCL1-induced Akt activation. Activated Akt phosphorylates serine/threonine residues on the intracellular targets within the consensus motifs (*RXRXXS/T*) to regulate cellular responses (58, 59). Recent proteomic analysis allowed us to identify the molecules that contain double consensus motifs of Akt phosphorylation (*RXRXXS/T*) and 14–3-3 binding motifs (*RSXS/TXP*) within the entire human genome (60, 61). Putative target molecules that contain double consensus motifs of both Akt phosphorylation and 14–3-3 binding motifs (***RXRS*XS/T**-X-***P***) are listed based on the analysis using PROSITE (http://au.expasy.org/prosite/).

Akt2 express essentially ubiquitously in normal human tissues, expression patterns in human cancer seem to be different between Akt1 and Akt2. In human cancers, Akt1, originally identified as a gene amplified in human gastric cancer, also displays increased activity in primary carcinoma of the prostate, breast, and ovary, with poor prognosis. In contrast, Akt2 is most frequently activated in a wide variety of human cancers including pancreatic, breast, and ovarian cancers, with poor prognosis. Consistent with restricted physiological expression of Akt3, activation of Akt3 in human cancers appears to be less common, the only exception being androgen-insensitive prostate cancer, progressive melanomas, or estrogen-receptor-negative breast cancer. Overexpression of wild-type Akt2, but not Akt1 or Akt3, could transform NIH 3T3 cells, inducing invasion and metastasis in human breast and ovarian cancer. To further support the distinct role of the activation of the three Akt isoforms, each Akt isoform was recently shown to play distinct biological functions. Akt1 and Akt2 have been shown to exhibit two opposing functions in cancer cell motility and invasion through the transcription factor NFAT, a vital process underlying cancer metastasis and/or progression (66–69). However, since both Akt1 and Akt2 are expressed ubiquitously in human tissues yet bear opposing functions, the *in vivo* action for transforming human cancers can be complex.

Gene-targeting mice also provided evidence that each Akt isoform has distinct biological actions *in vivo*. Akt1-deficient mice were viable but displayed impairment in organ growth, suggesting that Akt1 plays a dominant role in regulating cell survival. Mice deficient in Akt2 exhibited an impaired ability for insulin to act in skeletal muscle and/or liver. This is somewhat surprising as both Akt1 and Akt2 are ubiquitously expressed in mammalian tissues. It is important that the substitution of Akt1 and Akt3 did not compensate for the loss of Akt2. Therefore, a malfunction of glucose metabolism observed in Akt2, but not in Akt1, knockout mice could be due in part to malfunction of glucose metabolism in the liver, which could affect the overall deregulation of glucose metabolism *in vivo*. In contrast—and consistent with restricted expression of Akt3 in neurological tissues—Akt3-deficient mice exhibited a reduced brain size, affecting all major brain regions. The presence of other isoforms of Akt did not compensate for the defective phenotype of the gene-targeting animals, further supporting the distinct function of three isoforms of Akt *in vivo* (70). Since Akt1 and Akt2 are essentially expressed ubiquitously, the *in vivo* phenotype of gene-targeting animals may not be explained by the differential expression of Akt isoforms, but rather the distinct function of each Akt isoform (Akt1, Akt2, or Akt3). Given the highly conserved structure of the three Akt isoforms, it remains unclear how these divergent functions among the Akt isoforms are mediated. Since the C-terminal regions between each of these three Akt isoforms are relatively diverse (73–84%) compared with the kinase domain (90–95%), the C-terminal regions may represent functional differences among Akt1, Akt2, and Akt3. Nonetheless, the findings together could explain some of the divergent functions of Akt isoforms underlying the pathogenesis and clinical manifestations observed in human cancer patients.

In contrast to the Akt isoforms, there was only limited evidence demonstrating the divergent functions of TCL1 isoforms. The TCL1 protein family consists of three members—TCL1, MTCP1, and TCL1b—which do not contain any known functional motifs. Proteins of the TCL1 family share a relatively high degree of amino acid homology (30–50%) and a unique, symmetrical β-barrel structure based on X-ray crystallographic analysis of TCL1 (see Fig. 1). Three TCL1 isoforms could interact with the Akt PH domain and enhance Akt kinase activity. In contrast to Akt1 and Akt2, which could bind to all members of the TCL1 family, Akt3 specifically interacted with TCL1 but not with MTCP1 or TCL1b. This specific association could determine the functional specificity of Akt kinase activation, since the presence of TCL1—but not the presence of MTCP1 or TCL1b—increased Akt3 kinase activity in *in vitro* kinase assays (46). The physiological expression of TCL1 is relatively restricted to early developmental cells as well as immune cells (1, 11). Differential interaction of the three TCL1 isoforms with the three Akt isoforms may explain part of the divergent functions of the Akt isoforms. Since the three TCL1 family proteins appear to be expressed in different tissues (see below), it is possible that the three Akt family kinases and the TCL1 family play differential roles *in vivo*, underlying the pathogenesis of various human cancers. These observations also imply that each Akt isoform and/or TCL1 could be specific molecular targets for cancer therapy in various human cancers.

GENE REGULATION OF TCL1

In pathological conditions, TCL1 is overexpressed in a variety of human diseases, including EBV-infected B cell lymphoma, A-T, seminoma, dysgerminoma, and AIDS-related lymphoma (4, 5, 51, 71). In contrast to pathological conditions, the physiological expression of TCL1 is primarily restricted to early embryonic cells, $CD4^-CD8^-CD3^-$ thymocytes, $CD34^+CD19^+$ pro-B cells through IgM-negative pre-B cells, and splenic mantel zone B cells (1, 53, 62). The relatively restricted physiological expression of the TCL1 family proteins suggests that the *TCL1* gene is regulated at a transcriptional level (1, 53). Consistently, recent studies clarified that Sp1 sites around the *TCL1* transcriptional start site play a regulatory role in gene activation of the *TCL1* oncogene (72). Nucleotide sequence analysis of the 5′-*TCL1* promoter region revealed a TATA box with *cis*-regulatory elements for Nur77/NGFI-B, (NBRE, nerve growth factor-responsive element, CCAAGGTCA), NF-κB (73), FKHRL (Forkhead transcription factor) (60), and SP1 (72). It was striking that *cis*-

regulatory elements of two of the Akt substrates (FKHRL and Nur77) and related molecules (IκB for NF-κB) were present within the proximal 5′-promoter of the human *TCL1* gene.

Nur77 (NGFI-B, nerve growth factor-induced-B, TR3) was originally identified as an NGF-induced ligand-dependent transcriptional activator in PC12 cells (rat pheochromocytoma cells) (74). Two other subfamilies of NGFI-B transcription factors, Nurr1 (Nur-related factor 1) and NOR-1 (neuron-derived orphan receptor 1), have been identified in neuronal cells (74–76). Studies demonstrated that Nur77 plays a pivotal role in T cell apoptosis *in vivo* and functions on mitochondria to control cell death (77).

Akt was recently shown to interact physically with Nurr77, phosphorylate at Ser-355 and, as a consequence, down-modulate DNA binding and transcription activation (78, 79). Despite intensive study, none of the direct molecular targets of NBRE that regulate the cell death survival machinery have been identified. The molecular target of Nurr77/NGFI-B is not clear; however, one proposed target is steroid 21-hydroxylase (80), one of a group of related cytochrome P-450 enzymes that are required for steroid hormone biosynthesis.

We showed that the *TCL1* gene is a direct target of Nur77 *via* Akt-induced phosphorylation of Nur77/NGFI-B *in vivo* (81). To the best of our knowledge, TCL1-NBRE is the first direct target of Nur77 involving the regulation of intracellular cell death survival. The recognition sequences of NGFI-B orphan receptor superfamily transcription factors present within the 5′ proximal TCL1 promoter were well conserved (underlined) in humans (CC*AAGGTCA*, −396 to −388 from ATG), mice (*ACCTGGTCA*, −336 to −328), and rats (*ACGAGGTCA*, −402 to −394) (82, 83) (**Fig. 4**).

Since both TCL1 and Nur77 are expressed in early developmental cells of the immune cells, the Akt-Nur77-TCL1 regulatory loop may play a role in providing survival signals during the process of thymic selection *via* mediation of Akt activation. In this scenario, TCL1 may play a dominant role in enhancing Akt kinase activity in order to maintain cellular survival signals in early developmental cells *in vivo*.

How could this negative regulatory mechanism play a role in the pathological condition of human leukemia? It is plausible that disruption of the negative feedback regulation of the TCL1 gene underlies the molecular mechanisms of human leukemia. SupT11 cells are a T cell leukemia cell line in which the *TCL1* gene is activated by juxtaposition to the T cell receptor α or β loci by chromosomal translocations (1). Indeed, we demonstrated that PDGF stimulation did not inhibit *TCL1* gene induction in SupT 11 cells. This observation suggested that PDGF-induced Akt activation could suppress TCL1 transactivation under a physiological condition in which the *TCL1* gene was driving under an authentic *TCL1* promoter that contains NBRE, but not in a pathological condition in which the *TCL1* gene expression was primarily driven by a TCR (T cell receptor) promoter after chromosomal translocation. Loss of this negative feedback mechanism observed in SupT11 cells could contribute to constitutive activation of Akt in supT11 cells (M. Noguchi and F. Suizu, unpublished results).

The *TCL1b* gene, the third member of the *TCL1* family of oncogenes, is located adjacent to the *TCL1* oncogene on human chromosome 14q32. Both TCL1 and TCL1b are highly expressed at early developmental stages in several fetal tissues, including thymus, kidney, lung (TCL1), spleen (TCL1b), and placenta (TCL1b). Both TCL1b and TCL1 mRNAs are abundant in oocytes and two-cell embryos, but are rare in various adult tissues and in lymphoid cell lines in mice (1, 11, 50). Consistent with the differential expression pattern observed in *TCL1* and *TCL1b*, 5′-promoter sequence of *TCL1b* contains no TATA box or GC-rich sequences with YY1 and c-*myc*, which are typical for a housekeeping gene. In humans, a *TCL1b* pseudogene is also present on human chromosome 5. However, only one of each mature TCL1 family protein is expressed at a transcriptional and translational level. In the mouse

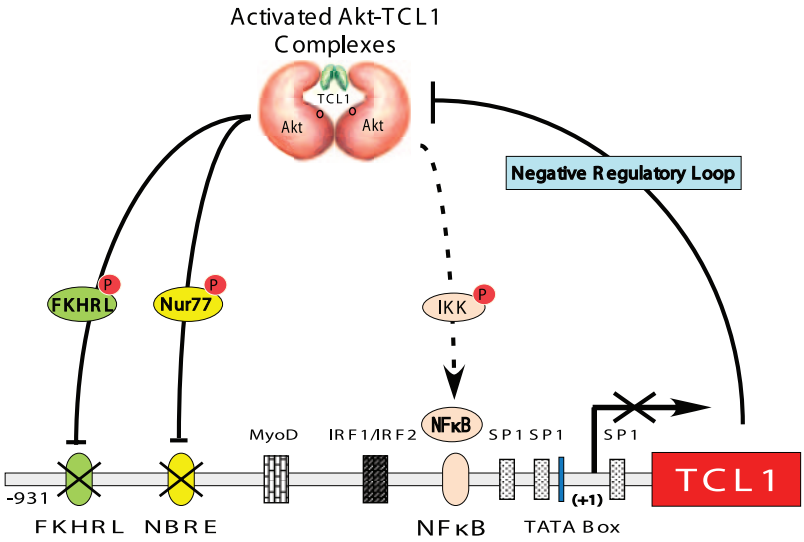

Figure 4. Negative feedback loop of Akt-Nur77-TCL1. Tightly restricted *TCL1* gene expression in early developmental cells and various developmental stages of immune cells suggests that the *TCL1* gene is regulated at a transcriptional level. The 5′-*TCL1* promoter region contains a TATA box with *cis*-regulatory elements for Nur77/NGFI-B, (NBRE, CCAAGGTCA) (82, 83), NF-κB (73), FKHRL (Forkhead transcription factor) (60, 61), and SP1 (72). Nur77/NGFI-B, an orphan receptor superfamily transcription factor implicated in T cell apoptosis, is a substrate for Akt. TCL1 induces Akt phosphorylation, prevents the interaction of Nur77 with NBRE, and in turn suppresses *TCL1* gene expression in PC12 cells *in vivo* (78, 79). TCL1-NBRE is the first direct target of Nur77 involving the regulation of intracellular cell death survival (81). It is possible that this novel regulatory mechanism may play a regulatory role in early embryogenesis and/or immunological cells *in vivo*.

genome, five *TCL1b* genes are present and coexpressed. Analogous to the structure of the *TCL1b* gene, the 5′ promoter sequence of human *MTCP1* located on the X chromosome bears no TATA box and is GC-rich, typical for a housekeeping-type promoter. In contrast to TCL1, the physiological expression of MTCP1, the third member of the TCL1 family of oncogenes, remains unclear. A recent study, however, suggested that SEB treatment induced MTCP gene expression in murine T cells *in vivo* (84). Taken together, these TCL1 isoforms are differentially expressed and, as a consequence, regulate various cellular responses. How these differentially expressed *TCL1* isoforms can regulate various cellular responses *in vivo* remains an open question.

AKT PATHWAY AS A POTENTIAL THERAPEUTIC TARGET FOR CANCER THERAPY

Kinase activities are tightly regulated in cells, and the modes of regulation are diverse and overlapping for many disease states (40). Therefore, kinases represent important therapeutic targets, and significant resources have been invested to identify therapeutically useful kinase inhibitors.

One striking discovery was that Akt is a downstream target of PI3K, a mastermind of intracellular signaling for multiple growth factors (24, 26). PI3K is a major signaling factor that catalyzes production of the second messenger PIP_3 at the cellular membrane. Since the PI3K-Akt pathway is involved in various human neoplastic diseases, Akt represents an attractive target for drug development against cancer (27, 85, 86) (**Fig. 5**). In pursuit of this aim, the binding site of TCL1 on the PH domain surface may present some interesting features. Several kinase inhibitors have been developed to target the active sites of the proteins, but the relatively conserved biochemical feature of this site often exhibited unwanted cross-inhibition among the different kinases (87, 88). Analogues of PtdIns-P head groups showed limited effect despite blocking membrane recruitment of the kinase by competing for the binding of inositol-P in the binding pocket of the PH domain (89–91). Indeed, 252 PH domains have been detected in protein sequences from an inspection of the human genome

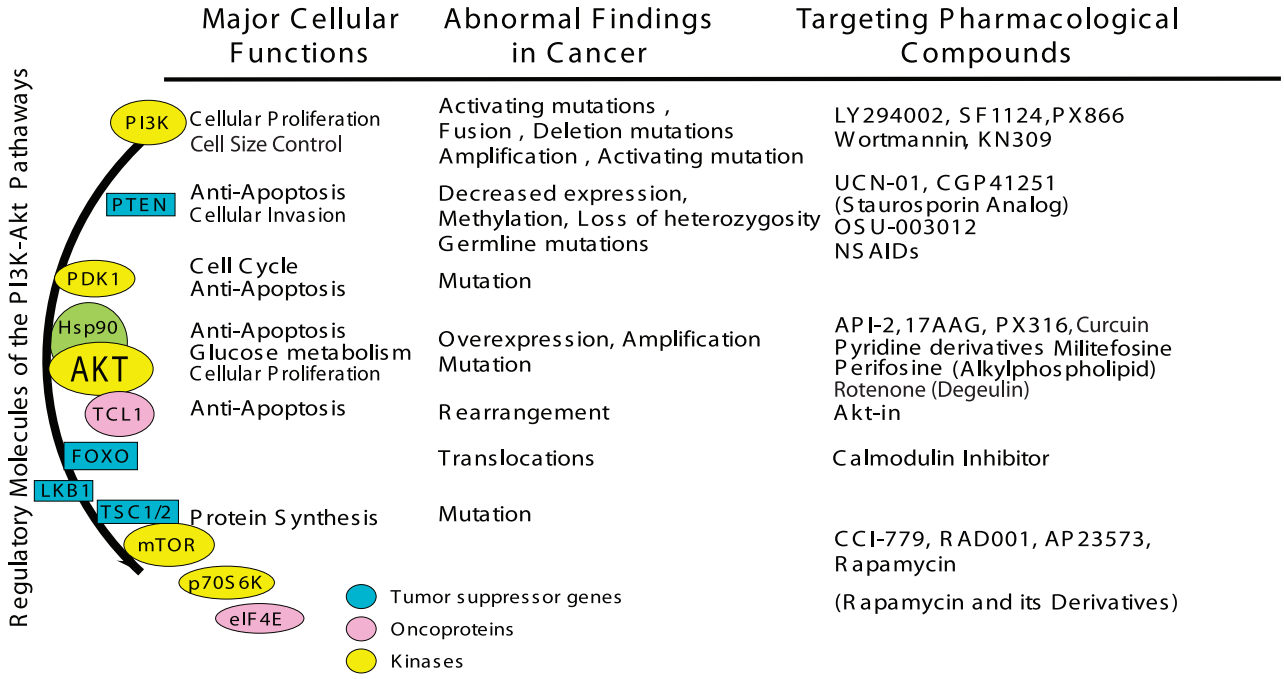

Figure 5. Targeting PI3K-Akt pathways for anti-cancer therapy. The PI3K signaling pathway is crucial to many aspects of cell growth and survival (second row from the left, major cellular function). The PI3K-Akt pathway regulates tumor suppressor genes and/or oncogenes that are structurally and/or functionally altered at the DNA level (schematic at the left). Deregulated activation of PI3K-Akt survival pathways contributes to the pathogenesis of a wide variety of human malignancies including ovarian, breast, and colon cancers (middle row, abnormal findings in cancer). Some inhibitory molecule targets for PI3K-Akt were previously developed. Shown on the right row are compounds that have been developed to control the PI3K-Akt pathway for therapeutic purposes for human cancers (right row, targeting pharmacological compounds). A synthesized phosphatidyl-inositol analog inhibited Akt by competing with phosphatidyl inositol. The plant-derived pigment curcuin reduced Akt activity, resulting in arrested cell growth in several prostate cancer cell lines. A compound synthesized from rotenone (degeulin) was a potential Akt inhibitor in malignant bronchial epithelial cells. API-2 (a nucleoside analog), also known as triciribin, is developed to suppress Akt kinase activity. However, hyperglycemia and hepato-toxicicity occurred as adverse effects in a human cancer trial. Pyridine derivatives, orally available alkylphospholipid, inhibited Akt phosphorylation, hence inducing apoptosis in cancer lines. Perifosine was also tested in a phase II trial in breast cancer. However, because of adverse effects, including severe gastrointestinal symptoms, not all the reagents listed are currently under clinical investigation (27, 33, 85, 86, 95–100).

(92), among which >100 kinases use a PH domain for their translocation to the plasma membrane and/or subsequent activation. The PH domain family appears to be a very large family of structurally homologous proteins, but shares only moderate to low sequence similarity (93, 94). The binding of PtdIns-P phospholipids is thought to be one of the common functions shared by most PH domains. In addition, PH domain can also engage specific protein-protein interactions, and the specificity of these interactions relies heavily on the specific and different nature of their surfaces. Thus, targeting a protein-protein binding surface on a kinase PH domain should yield specific inhibitors with minimum cross-reaction features.

By means of reversed yeast two-hybrid screening in combination with a random amino acid library, we have identified the βA and βE sheet of TCL1 as the Akt binding domain (23). Structural studies of Akt-TCL1 complexes further defined the physical interaction of the protein complexes at amino acid levels (20). In an attempt to develop Akt-specific inhibitors, we have generated a peptide spanning the Akt binding sequences of TCL1, named "*Akt-in*" (Akt inhibitor, NH_2-AVTDHPDRLWAWEKF-COOH), which still interacted with Akt but lacked the ability for oligomerization. Interaction of *Akt-in* with the Akt PH domain prevented phosphoinositide binding, and hence inhibited membrane translocation and activation of Akt. As revealed by NMR chemical mapping, the *Akt-in* peptide bound the Akt-PH in a surface similar to that recognized by TCL1 proteins. *Akt-in* inhibited not only cellular proliferation and antiapoptosis *in vitro*, but also *in vivo* tumor growth (48). To support the efficacy for human cancer therapy further, *in vitro* proliferations were examined using human cancer cell lines for lung and ovarian cancers. Although there were some variations in their efficacies, in principle, 12 of 12 human cancer cell lines (lung or ovarian cancers) exhibited effective inhibition of proliferation *in vitro* (supplemental data).

Since NMR studies detected slight conformational changes on the VL1 loop upon peptide binding, we first postulated that *Akt-in* prevented phosphoinositide binding, thus inhibiting membrane translocation and activation of Akt. In more recent studies, NMR titration experiments revealed only a slight decrease in the affinity of Akt-PH for PIP_4 (the head group of PIP_3) when adding saturating concentrations of peptide (C. Roumestand, personal communication). Although binding conditions in the experimental systems may have affected the interactions, the observation suggested that the mode of action of the peptide could be more complex, and remains to be unraveled.

As an additional attempt to elucidate the mechanism of cancer cell growth suppression, a DNA array was utilized to search for activated or suppressed genes using *Akt-in* to dissect the molecular nature of suppressed tumor growth. Genes induced by *Akt-in* treatment were retinal pigment epithelium-specific protein, keratin-associated protein 9.9, alpha 2, 80 syalyltransferase, interleukin 3, tectorin alpha, and interleukin 1 receptor type I. Several genes were inhibited by treatment with *Akt-in*, including TBX, SPANX family member, V-fos (osteosarcome viral oncogene homologue), Ets variant gene 5, Myos, heavy polypeptide, and growth hormone-releasing hormone receptor. It is likely that, through the activation and inactivation of these target genes, *Akt-in* could exhibit tumor cell suppression observed *in vivo* and *in vitro*. We also tried to modify the design of the inhibitory peptides that could interact with Akt-PH domains based on the corresponding amino acid sequences of the others members of the TCL1 family {[8–22 (GVPPGRLWIQRPG) in TCL1B: 5–19 (GAPPDHLWVHQEG) in MTCP1]} (1–3). However, our preliminary experiments suggested that neither TCL1B nor MTCP1 peptides showed significant affinity to the Akt-PH domain. Therefore, to obtain more efficient Akt inhibitors, further dynamic modification of the design of inhibitory molecules will be required. For this purpose, the combined use of SAR (structure/activity relationship) methodology and of chemical fragment libraries to develop chemical compounds to manipulate the suppressive function of *Akt-in* might help to design more potent Akt inhibitors with minimal off-target activities for therapeutic use.

PERSPECTIVES

More than a decade has passed since the discovery and identification of the TCL1 family proto-oncogene. In the past several years of research, the physiological function and structures of TCL1 and its isoforms have been clarified. While *TCL1* and *TCL1b* are coexpressed in human T-PLL as a result of chromosomal translocation, it remains unclear why only a prolymphocytic population of the T cell development could result in malignant transformation. In human T-PLL, both the *TCL1* and *TCL1b* genes are activated by juxtaposition onto the T cell receptor α or β loci. Therefore, it remains unclear whether TCL1b itself (independent from TCL1) bears oncogenicity. Since TCL1b lacks a dimerization domain, the nature of TCL1b-induced Akt activation is intriguing. Structural studies may answer this question. The physiological function of TCL1 and its family of proteins in the early developmental cells in the immunological compartment needs to be determined. Although it is already known that activated Akt can translocalize to the nucleus, whether or not TCL1 is indispensable for nuclear translocation of Akt needs to be clarified. It is plausible there are additional functional interacting molecules for TCL1 family proteins that have not yet been identified.

Studies are under way to solve these issues in order to understand more fully all aspects of the biological function of TCL1 and its family of oncogenes underlying various human cancer pathogenesis. FJ

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