Structural Basis for the Co-activation of Protein Kinase B by T-cell Leukemia-1 (TCL1) Family Proto-oncoproteins*[^S]

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Chromosomal translocations leading to overexpression of p14\_TCL1 and its homologue p13\_MTCPI are hallmarks of several human T-cell malignancies (1). p14\_TCL1/p13\_MTCPI co-activate protein kinase B (PKB, also named Akt) by binding to its pleckstrin homology (PH) domain, suggesting that p14\_TCL1/p13\_MTCPI induce T-cell leukemia by promoting anti-apoptotic signals via PKB (2, 3). Here we combined fluorescence anisotropy, NMR, and small angle x-ray-scattering measurements to determine the affinities, molecular interfaces, and low resolution structure of the complex formed between PKB-PH and p14\_TCL1/p13\_MTCPI. We show that p14\_TCL1/p13\_MTCPI target PKB-PH at a site that has not yet been observed in PH-protein interactions. Located opposite the phospholipid binding pocket and distal from known protein-protein interaction sites on PH domains, the binding of dimeric TCL1 proteins to this site would allow the cross-linking of two PKB molecules at the cellular membrane in a preactivated conformation without disrupting certain PH-ligand interactions. Thus this interaction could serve to strengthen membrane association, promote trans-phosphorylation, hinder deactivation of PKB, and involve PKB in a multi-protein complex, explaining the array of known effects of TCL1. The binding sites on both proteins present attractive drug targets against leukemia caused by TCL1 proteins.

Protein kinase B (PKB[^S]) is a 60-kDa member of the AGC superfamily of serine/threonine kinases composed of a amino-terminal PH domain and linked to a kinase domain by a 30 amino acid linker. PKB is frequently called Akt, because it is a mammalian homologue of c-Akt, a viral oncoprotein isolated from the AkT8 virus that causes T-cell leukemia in mice (4–7). Actually, the PKB family comprises three members, PKBa, PKBβ, and PKBγ (Akt1, Akt2, and Akt3), all of which display, despite some idiosyncrasies, a high level of functional redundancy (Fig. 1) (8). Protein kinase B is a central component of phosphoinositide 3'-kinase signaling pathways and has emerged as a pivotal regulator of many cellular processes including apoptosis, proliferation, differentiation, and metabolism (3, 9, 10). Deregluation of members of the PKB family has been associated with human pathologies such as cancer and diabetes (8).

PKB activation in response to growth factors and other extracellular stimuli involves membrane recruitment of PKB triggered by inositol phospholipid (PtdIns-P) binding of its amino-terminal pleckstrin homology (PH) domain. At the membrane, PKB is activated by a partially defined process involving lipid-mediated dimerization and phosphorylation of two critical residues, Thr[^S]/Thr[^S]/Thr[^S] in the kinase activation segment and Ser[^S]/Ser[^S]/Ser[^S] in the COOH-terminal hydrophobic motif, on PKBa, PKBβ, and PKBγ, respectively (6, 11–16). Residue Thr[^S]/Thr[^S]/Thr[^S] is phosphorylated by the 3-phosphoinositide-dependent kinase 1, whereas the mechanism responsible for the phosphorylation of Ser[^S]/Ser[^S]/Ser[^S] has not been resolved (6, 17).

The PH domain of PKB (PKB-PH) has been shown to be essential for mediating the targeting and co-activation of PKB by proteins of the T-cell leukemia-1 (TCL1) family (18, 19). Normally, the cellular expression of TCL1 family genes (TCL1, TCL1b, and MTCPI) is mainly restricted to the lymphoid cell lineage and to the early stages of embryogenesis (20) where they co-activate PKB, possibly to promote a growth advantage during development through PKB-stimulated cell survival (3). However, in certain T-cell malignancies, chromosomal translocations lead to changes in the expression patterns of TCL1 family genes.

Thus the TCL1 oncogene was identified because of characteristic chromosomal translocations and inversions at 14q32.1 in clonal T-cell proliferations and malignancies (21). Repositioning of T-cell receptor α/β or β-chain control sequences next to the TCL1 coding region yields deregulated T-cell-specific expression. The product of the TCL1 gene is a 14-kDa protein (p14\_TCL1) that has been shown to localize in the cytoplasm and nucleus of expressing cells (22). Crystalllographic studies indicated that p14\_TCL1 exhibits a novel β-barrel structure (23). A similar structure was found for the 13-kDa product of the MTCPI gene (p13\_MTCPI) (23–25). The MTCPI gene, located in the Xq28 chromosomal region, was the first gene to be identified in the heterogeneous group of uncommon T-cell leukemias presenting a mature phenotype (26). It is involved in the translocation t(X;14)(q28;q11), recurrently associated with this type of T-cell proliferations. In addition to structural similarity,
MTCP1 exhibits high sequence homology (40% identity, 61% similarity) with p14TCL1 and with p14TCL1b (36% identity, 63% similarity), the product of the newly identified TCL1b oncogene (27). Evidence confirming a tumorigenic role for aberrant TCL1 and MTCP1 expression has been obtained from transgenic mice (28, 29), suggesting that these proteins are the first identified members of a novel family of proto-oncoproteins.

Functionally, the association of TCL1 proteins with PKB, which is mediated by its PH domain, enhances the phosphorylation of PKB on Thr308/Thr309/Thr305 and Ser473/Ser474/Ser472, increases PKB-mediated phosphorylation of its substrates glycogen synthase kinase-3β and Bc12-antagonist cell of death, and allows PKB to enter the nucleus (18, 19). The underlying molecular mechanisms remain controversial. It was suggested that TCL1 proteins mimic PtdIns-P binding to PKB-PH (30), facilitate PKB trans-phosphorylation by the formation of heterotrimeric complexes with PKB (18), or serve as adaptor proteins to link PKB with factors containing nuclear localization

![Fig. 1. Residues involved in binding as mapped by NMR. Annotated sequence alignment of PKB family PH domains (A) and TCL1 family proteins (B) is shown. Identical positions are highlighted in blue, and homologous positions are in cyan. Residues with an above-threshold deviation from mean in NMR T2-mapping experiments are labeled according to the following: #, caused by p13MTCP1; ^, caused by p14TCL1 (A); #, caused by PKBβ-PH (B).](image-url)

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signals (19). Identification of TCL1 and PKB-PH binding surfaces is an important step in deciphering these complex mechanisms. A putative site of interaction at the TCL1 surface was previously proposed from a mutational analysis of p14TCL1 and molecular modeling (31, 32). However, the region(s) on PKB-PH that contribute to the interaction are unknown. Advances toward identifying this interaction site were provided recently by the resolution of the three-dimensional structure of the PKB-PH domain (α-and β-isoforms) and the delineation of the binding site of the PtdInsα-P (33, 34). Here, we used a combination of biochemical techniques (fluorescence anisotropy, NMR, and small angle x-ray scattering (SAXS)) to determine the structural basis of the interaction between PKB-PH and p14TCL1. Based on our experimental data, a molecular model of the complex formed between PKB-PH and p14TCL1 is proposed. Our analysis gives insights into how TCL1 family proteins promote their array of cellular effects.

MATERIALS AND METHODS

Protein Preparation—PKB-PH, p13MTCP1, and p14TCL1 were produced, purified, and, in the case of PKB-PH and p13MTCP1, 15N-labeled as described previously (24, 35, 36).

NMR Experiments—Chemical shift assignments of PKB-PH and p13MTCP1 were described previously (24, 35). All of the NMR experiments were carried out at 10 °C on a 500-MHz BRUKER AVANCE spectrometer equipped with a 5-mm z-gradient H-13C-15N triple-resonance cryoprobe. Protein samples were dissolved in 300 μl of buffer (10 mM Tris-HCl, 300 mM NaCl, pH 7.4) in Shigemi cells. In all of the experiments, the 1H carrier was centered on the water resonance and a WATERGATE (37, 38) sequence was incorporated to suppress the solvent resonance. All of the NMR spectra were acquired in the phase-sensitive mode with Digital Quadrature Detection in the F2 dimension and the hypercomplex States-TPPI (Time Proportional Phase Incrementation) method in F1 dimension (39) and processed with Gifa (version 4.22) (40) software utility.

Titrations Experiments—For each titration experiment, eleven aliquots were prepared where the concentration of 15N-labeled PKB-PH (or 15N-labeled p13MTCP1) was kept constant (60 μM) and the concentration of unlabeled p13MTCP1 (or PKB-PH) and p14TCL1 was increased from 5 to 700 μM and from 2.5 to 200 μM, respectively. [1H,15N]-HSQC (41, 42) spectra were recorded for each sample using a time domain data size of 64 τx × 2K τc complex points and 32 transients/complex τc increment. The dissociation constants (Kd) per residue were measured from the fit of the decreased intensities of all non-overlapping cross-peaks with Equation 1 (43).

\[
y = \frac{I_{max} - I_{min}}{2[P] + [L] + Kd - \sqrt{(I_{max} + I_{min} - 2[I][L])}}
\]

(Eq. 1)

where [P] and [L] are the total concentration of 15N-labeled and unlabeled proteins, respectively.

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T_2 mapping Experiments—Transverse relaxation rates (R2) were measured and analyzed following a standard protocol previously reported in detail for the backbone dynamics analysis of 15N-p13MTCP1 (33) and 15N-PKB-PH (34). In this particular case, the standard Hahn echo experiment was preferred to the classical CPMG (Carr-Purcell-Meiboom-Gill) experiment for R2 measurements because of its increased sensitivity to exchange contributions (44). R2 were measured for 15N-PKB-PH and 15N-PKB-PH “free” in solution and compared with the ones measured for the same protein in the presence of a protein from titration experiments of unlabeled partner (15N-p13MTCP1:PKB-PH (1:1), 15N-PKB-PH:13MTCP1 (1:1), and 15N-PKB-PH:PKB-PH (1:1)). In these experiments, the total (labeled plus unlabeled) protein concentration was fixed to 0.5 mM for all of the samples in order to avoid any parasitic contribution from viscosity changes.

Fluorescence Anisotropy Experiments—Proteins were labeled at pH 8.0 at 4 °C for 2 h, conditions under which labeling ratios are far from unity and that favor unique labeling of the amino terminus. The labeling ratio for the PKB-PH proteins was 16%, and that for the p13MTCP1 protein was 10%. Concentrations for the target protein were calculated for total protein, such that the concentration of fluorophore was in both cases considerably lower. Binding assays were performed with a Bruker 2000 polarization instrument (Panvera Corp., Madison, WI) at 4 °C using either Alexa488-labeled PKB-PH at a total concentration of 32 nm or Alexa488-labeled p13MTCP1 at a total concentration of 100 nm. The buffer used in the assay was 10 mM Tris-HCl, pH 7.4, and 300 mM NaCl. Anisotropy profiles were obtained in the dilution format. In this approach, data points were taken at equilibrium starting from 240 μl of a 0.1 mM solution of p14TCL1 containing 32.5 nm Alexa488-PKB-PH or 0.5 mM solution of PKB-PH containing 100 nm Alexa488-labeled p13MTCP1. For each subsequent measurement, 60-μl aliquots of solution were removed from the initial solution and replaced by 60 μl containing 32.5 nm Alexa488-PKB-PH or 100 nm Alexa488-labeled p13MTCP1 only. Anisotropy values were recorded as a function of time, and the final five or six values after stabilization were averaged. Anisotropy data were fit using a 1:1 association mass action law in a 99% confidence interval using a GraphPad Prism Fitter.

SAXS—SAXS data were recorded on beamline D24 at LURE (Orsay, France) at a wavelength of 1.49 Å using a linear 512 channel detector and sample-detector distances of 1.872 and 0.880 m. For data acquisition, the samples were kept in a quartz capillary of ~1.5 mm in diameter maintained at a temperature of 10 °C. Protein samples were dialyzed into 10 mM Tris-HCl and 300 mM NaCl at pH 7.4 and filtered through 0.22-μm pores prior to data recording. Sample concentrations were adjusted to 0.6 mg. For each detector distance, eight frames of 200 s each were averaged for both the protein samples and corresponding buffer. The individual frames did not show signs of x-ray damage and were averaged and scaled to transmitted intensity. The buffer contribution was subtracted from the protein-scattering curve. The data were merged and processed using the programs Primus and Gnom (45). Dammin and Gasbor were used for ab initio form determination (45). SAXS envelopes determined by these programs showed P2 symmetry. This symmetry constraint was imposed for subsequent ab initio shape determinations. Ten of the obtained ab initio models were averaged to obtain the most likely molecular envelope (using Damavet (45)). The χ2 values of atomic models against SAXS data were calculated with Crysol (45) using default parameters.

Molecular Modeling—Manual modeling of the PKB-PH-p14TCL1 complex was carried out using the program O (46) for visualization of atomic models and SAXS envelopes. Computational docking was carried out with the program FTDock (47). A total of 37,000 possible orientations of PH relative to p14TCL1 or p13MTCP1 were tested by FTDock for combinations of the x-ray structure of PKB-PH and NMR structure of PKB-PH with x-ray structures of p14TCL1 and p13MTCP1 and an NMR structure of p13MTCP1. This initial set of orientations was filtered to suffice the constraint that residues of the interface of both partners are <6 Å away from the other molecule. The best-scored models from FTDock were submitted to rigid body and side chain refinement by the program MultiDock (48).

An alternative approach using the HADDock (High Ambiguity Driven Docking) (49) program provided us with the means to dock the TCL1 proteins separately onto PKB-PH. The chemical shift perturbation data resulting from NMR-mapping experiments were used as ambiguous interaction restraints to drive the docking process. An ambiguous interaction restraint is defined as an ambiguous distance among all of the residues shown to be involved in the interaction. According to established criteria of the HADDock program, the “active” residues are those that have been shown to alter the HSQC spectra and also have a high solvent accessibility. The “passive” residues correspond to the residues that are surface numbers of the active residues and also have a high solvent accessibility calculated with NACCESS program. Therefore, PKB-PH residues Lys64, Glu66, and Arg67 in strand β1, and Met100, Arg101, Glu104, Met105, Asp106, and Ser109 in the COOH-terminal helix are considered to be active residues. Five neighboring PKB-PH residues, Leu62, Met63, Pro64, and Gly67, are considered to be passive residues. For the p14TCL1 protein, residues Asp1, Arg2, Trp3, Glu4, and Lys14 were considered to be active residues when the Asp12, His12, Leu24, Pro43, Pro46, Tyr79, and Asp86 are passive residues. The default HADDock parameters were used with the except that only the 300 initial complex structures were generated, the best 50 solutions in terms of intermolecular energies then were refined in water, and the 20 final structures were analyzed. All of the molecular images were produced with Assp2 or PyMOL (50).

2 M. E. M. Noble, unpublished data.
RESULTS

Affinities of the PKB-PH-p13MTCP1/p14TCL1 Interactions—
The affinity of PKBβ-PH for p14TCL1 was determined using fluorescence anisotropy. In this experiment, the PKBβ-PH protein was labeled with an amine-reactive fluorescent dye (Alexa488) under conditions that favor the selective labeling of the amino terminus. A solution of this labeled protein at a concentration of 32 nM in the presence of a high concentration of unlabeled p14TCL1 was sequentially diluted with a solution containing only the labeled protein and at a concentration of 185 at very low concentrations of p14TCL1 into 235 millianisotropy units at saturating p14TCL1.

FIG. 2. Fluorescence normalized anisotropy-derived profiles of the interactions between PKBβ-PH and p14TCL1 or p13MTCP1. Squares represent the profile obtained upon titration of Alexa488-labeled PKBβ-PH by unlabeled p14TCL1, whereas triangles correspond to the titration of Alexa488-labeled by unlabeled PKBβ-PH. Lines through the points represent the best fit to the data of a simple 1:1 binding model. We note that, in both cases, the fits were performed on the raw data prior to any manipulation and that the raw data and the fits were normalized to facilitate comparison. Buffer conditions were 4°C in 10 mM Tris-HCl, pH 7.4, and 300 mM NaCl.

The binding of PKB-PH to p13MTCP1 was also measured by fluorescence anisotropy using a p13MTCP1 sample labeled in the same manner by Alexa488 to maximize the total anisotropy change. The labeled p13MTCP1 at a concentration of 100 nM was titrated by unlabeled PKB-PH (Fig. 2, triangles). The total change in anisotropy was from a value of 110 millianisotropy units at low concentrations of PKB-PH to a value of 185 at very low concentrations of PKB-PH, indicating that the complex dissociation is slow, one should observe one set of resonance for the free protein and one set for the bound protein. During the titration, the free set will disappear and will be replaced by the bound set. Of course, for similar reasons as previously cited, only resonances belonging to the binding interface should be affected. When comparing a spectrum recorded on a 15N-PKBβ-PH/p14TCL1 (1:1) sample to a reference spectrum (15N-PKBβ-PH alone), we cannot identify any additional resonances or resonance displacements (Supplementary Material 1). This result is strongly indicative of slow-exchange conditions in the limiting case where the resonances belonging to the bound set are broadened beyond detection. Such conditions are compatible with the high molecular weight expected for the multimeric complex 15N-PKBβ-PH/p14TCL1 (56 kDa) (the dimeric protein p14TCL1 is supposed to bind two PH domains). This slow-exchange regime is also

"isoform β; PKBβ-PH) by p13MTCP1 or p14TCL1. These measurements are not redundant with fluorescence anisotropy measurements, because they are indicative of the exchange regime between the free and bound states with regard to the NMR time scale under the conditions of the NMR study. This information is mandatory for the choice of the NMR methods to be used for delineating the binding interfaces. Of course, the NMR titrations are expected to yield less accurate KD values than fluorescence anisotropy because of the relatively high concentration of the labeled target and especially because different mechanisms can participate to the line broadening of resonances belonging to residues located on the binding interface (see below), leading to an understimation of the apparent KD. Nevertheless, only a few residues are expected to belong to this category, such that only a small bias should be observed in the average result over all of the residues. Adding increasing amounts of unlabeled p13MTCP1 or p14TCL1 to a solution of 15N-labeled PKBβ-PH causes a progressive line broadening in the [1H,15N]HSQC spectrum of PKBβ-PH, indicative of an intermediate-to-slow-exchange process. Under such limiting conditions, it has been shown that a dissociation constant can be estimated from progressively disappearing resonances (51–53). With 1H and 15N assignments being available from a previous study (35), the intensity decrease can be fitted for most cross-peaks in the HSQC spectra with a simple two-state model (see "Materials and Methods"), allowing the estimation of an "apparent" dissociation constant (Kd) per residue (Fig. 3). We titrated 15N-labeled PKBβ-PH with unlabeled p13MTCP1 and p14TCL1 to obtain the binding affinity by averaging the apparent Kd per residue for all of the residues. Thus the Kd values of 410 ± 140 and 4 ± 125 µM were established for the complexes 15N-PKBβ-PH-p13MTCP1 and 15N-PKBβ-PH-p14TCL1, respectively. A similar Kd (380 ± 140 µM) was obtained for the complex PKBβ-PH-15N-p13MTCP1 when 15N-labeled p13MTCP1 was titrated with unlabeled PKBβ-PH. The Kd values obtained for both complexes, PKBβ-PH-p14TCL1 and 15N-PKBβ-PH-p13MTCP1, are in good agreement with those obtained from the fluorescence anisotropy profiles.

To distinguish between intermediate or slow-exchange regimes, highly accumulated spectra were recorded on protein samples under conditions favoring substantially high concentrations of the PKBβ-PH-15N-p13MTCP1 or 15N-PKBβ-PH-p14TCL1 complex as determined from the value of the dissociation constants (PKBβ-PH-15N-p13MTCP1 (10:1) and 15N-PKBβ-PH-p14TCL1 (1:1), respectively) and compared with reference spectra recorded on "free" species (15N-p13MTCP1 and 15N-PKBβ-PH, respectively). In the intermediate chemical exchange case in addition to extensive broadening, a displacement of the resonances should be observed from the resonance value in the free state to the resonance value in the bound state. On the other hand, if the complex dissociation is slow, one should observe one set of resonance for the free protein and one set for the bound protein. During the titration, the free set will disappear and will be replaced by the bound set. Of course, for similar reasons as previously cited, only resonances belonging to the binding interface should be affected. When comparing a spectrum recorded on a 15N-PKBβ-PH/p14TCL1 (1:1) sample to a reference spectrum (15N-PKBβ-PH alone), we cannot identify any additional resonances or resonance displacements (Supplementary Material 1). This result is strongly indicative of slow-exchange conditions in the limiting case where the resonances belonging to the bound set are broadened beyond detection. Such conditions are compatible with the high molecular weight expected for the multimeric complex 15N-PKBβ-PH-p14TCL1 (56 kDa) (the dimeric protein p14TCL1 is supposed to bind two PH domains). This slow-exchange regime is also
Fig. 3. Affinities between p13\textsuperscript{MTCP1} and PKB\textbeta-PH as measured by NMR titration experiments. A, evolution of the \([^{1}H,^{15}N]\)HSQC spectra of \(^{15}N\)-labeled sample of p13\textsuperscript{MTCP1} upon the addition of increasing amounts of unlabeled PKB\textbeta-PH. B, left panel, mean-normalized titration curve fitted from cross-peak intensity decays of some selected residues representative for the global evolution of the \([^{1}H,^{15}N]\)HSQC spectra of p13\textsuperscript{MTCP1}. Right panel, "apparent" \(K_D\) for the complex PKB\textbeta-PH-p13\textsuperscript{MTCP1} as a function of the sequence. The average values are indicated with a dashed line on the graphs, and the means ± S.D. given in the text have been calculated from all of the residue-specific \(K_D\) values over the whole sequence. C and D, affinities between p14\textsuperscript{TCL1} and PKB\textbeta-PH as measured by NMR titration experiments. C, evolution of the \([^{1}H,^{15}N]\)HSQC spectra of \(^{15}N\)-labeled sample of PKB\textbeta-PH upon the addition of increasing amounts of unlabeled p14\textsuperscript{TCL1}. D, left panel, mean-normalized titration curve fitted from cross-peak intensity decays of some selected residues representative for the global evolution of the \([^{1}H,^{15}N]\)HSQC spectra of p14\textsuperscript{TCL1}. Right panel, apparent \(K_D\) for the complex PKB\textbeta-PH-p14\textsuperscript{TCL1} as a function of the sequence. The average values are indicated with a dashed line on the graphs, and the mean ± S.D. given in the text have been calculated from all of the residue-specific \(K_D\) values over the whole sequence.
compatible with the dissociation constants measured either by NMR or by fluorescence. When comparing a spectrum recorded on a $^{15}$N-p13MTCP1 sample in the presence of PKBβ-PH (10:1) to a reference spectrum ($^{15}$N-p13MTCP1 alone), we observed an intense line broadening of most resonances. In addition, slight shifts are noticeable for the resonances of residues Val15, Arg22.
Glu^{24}, Gln^{26}, Gln^{70}, and Leu^{71} (Supplementary Material 1). These residues are likely to be in the binding site as revealed by T_{2}-mapping experiments (see below). Thus this result is in favor of intermediate exchange conditions for the complex PKBβ-PH-15N-p13MTCP1.

**Binding Interfaces within the Complexes between PKB-PH and p13MTCP1/p14TCL1**—Due to the intermediate-to-slow chemical-exchange regime, the widely used chemical shift perturbation-mapping NMR method cannot be used to map protein interfaces because it relies on fast-exchange conditions between free and bound proteins (54, 55). On the other hand, more recently two different groups have published independently an alternative NMR approach applicable under these conditions that relies on the analysis of the differential line broadening of the NMR signals. We have used this so-called NMR T_{2}-mapping method (56, 57) to determine the imprint of unlabeled p13MTCP1/p14TCL1 and PKBβ-PH, respectively, on 15N-labeled PKBβ-PH and p13MTCP1.

The theoretical principles of NMR two-site chemical exchange have been studied extensively (58–63) and the line shapes during exchange can be simulated with Equation 2 (63).

\[
I(t) = \int_0^\infty W \exp(-\omega t - \omega E t + K t + R t) dt \quad (\text{Eq. 2})
\]

Matrix R contains the transverse relaxation rates, whereas matrices K and Ω contain the chemical exchange rates and chemical shifts, respectively. Matrix W contains the probability of occurrence at each frequency, and E and 1 are the identity matrix and unity vector, respectively. Considering this equation, when the exchange between the bound and free states is slow or intermediate, two distinct situations can be considered. First, where the chemical shifts in the bound and free states are the same or very near (∆ω < 50 Hz), exchange broadening due to chemical exchange is absent or negligible and the line broadening is very sensitive to the relaxation rate of the bound state. This is the case for the resonances of residues located outside the binding surfaces. These resonances sense the same chemical environment in the free and in the bound states. Alternatively, where chemical shift differences upon binding are large (>500 Hz), the line broadening is dominated by the exchange rate in addition to the relaxation rate of the residue in the bound state. Thus NMR signals of residues that form binding contacts associated with large chemical shift perturbations have two sources of exchange broadening as opposed to one for those residues that do not undergo environmental changes upon binding. Such exchange broadening will be evident via 15N NMR T_{2} (R_{2}) relaxation measurements such as those presented here. Further exchange broadening of contact residues can also be achieved via secondary processes of conformational change at the contact site. If this conformational change is associated with large chemical shift changes, this would promote an additional relaxation pathway contributing efficiently to T_{2} relaxation. This three-site exchange mechanism can be described by modified three-site Bloch equations (64). Note that in case of fast exchange, the line broadening due to chemical exchange vanishes and a similar increase in T_{2} is expected for all of the residues, uniquely from the contribution of the relaxation rate of the bound state.

R_{2} (1/T_{2}) values were measured on the 15N-labeled protein “free” in solution and compared with the protein in the presence of a predetermined amount of unlabeled partner. The results are given as the normalized ratio (R_{2}^{ref}/R_{2}^{free} – 1) versus the protein sequence (Fig. 4) where R_{2}^{ref} and R_{2}^{free} are heteronuclear 15N transversal relaxation rates measured for the labeled protein free in solution and for a mixture of the two partners in solution (15N-p13MTCP1-PKBβ-PH (1:1), 15N-PKBβ-PH-p13MTCP1 (1:1), and 15N-PKBβ-PH-p14TCL1 (5:1)), respectively. Thus a normalized ratio of zero for a particular residue indicates no change in R_{2} between free and interacting protein, and a normalized ratio of 1.0 indicates an R_{2} value, which during interaction is double that observed in the free state. For most residues, a similar ratio is observed that corresponds to an expected nearly identical increase of R_{2} in the complex entirely due to a nearly identical increase of the correlation time for the corresponding 1H–15N vectors in the complexes. For some residues, significant deviations from the average ± S.D. are observed, resulting from significant chemical exchange contributions to R_{2} measured in the complex. These residues define finite areas on the surface of each protein, corresponding to the interaction surfaces.

The residues highlighted by NMR T_{2} mapping on PKBβ-PH upon interaction with p14TCL1 are mainly located on strands β4 (residues Val^{57} and Ala^{58}) and β5 (Lys^{64}, Thr^{65}, Arg^{67}, and Arg^{69}) of the β-sandwich, and on the COOH-terminal α-helix (Ser^{92}, Glu^{98}, Met^{100}, Arg^{101}, Ile^{103}, Glu^{104}, Met^{105}, Asn^{108}, and Ser^{109}) (Figs. 1 and 5B). The PtdIns-P binding site, formed basically by the loops VL1 and VL2 of PKBβ-PH, appears unaffected by the association with p14TCL1. No information could be obtained for the VL3 loop region because of the lack of assignments in this particular area (residues shown in black in Fig. 5B). This lack of assignment has been attributed to special dynamic behavior in this loop. Nevertheless it is unlikely that this loop interacts with TCL1 proteins because an interaction is expected to promote dynamic changes leading to additional peaks corresponding to VL3 residues in the HSQC spectrum of the bound form.

The outlined area does not show any striking features in terms of hydrophobicity or charge distribution. The imprint of p13MTCP1 on PKBβ-PH delineates principally the same region, albeit less well defined and including some residues close to the periphery or outside the area affected by p14TCL1 (residues Thr^{21}, Arg^{25}, and Tyr^{26} on β2 and residues Gly^{37} and Tyr^{38} on β3) (Figs. 1 and 4B). This can be attributed to a lower signal-to-noise level of these data, possibly due to the lower affinity of p13MTCP1 as compared with p14TCL1. Indeed, under such conditions, the “hits” due to specific interaction were barely discernable from those arising from possible nonspecific interactions.

The residues on p13MTCP1 that are highlighted upon PKBβ-PH binding are clustered on one face of the eight-stranded anti-parallel β-barrel (Figs. 1 and 5A). In addition to residues exhibiting significant deviation of the normalized ratio, a small number of resonances were no longer detectable in the experiment recorded in the presence of PKBβ-PH. These were derived from residues His^{12}, Leu^{65}, Ser^{69}, and Met^{68}. As these resonances are clearly detectable in the spectrum of the free p13MTCP1, they must be experiencing a large chemical shift change in the presence of the PH domain, thus becoming linebroadened beyond detection. Therefore, it is likely that these residues are contact points between the two proteins. The delineated area contains a hydrophobic cluster formed by Trp^{14} on β1, Leu^{67}, Met^{68}, and Leu^{71} on β3, surrounded by polar and charged residues (Gln^{20} on β3, Asn^{30} on β6, and Arg^{72} and Asp^{74} on β4). The PKBβ-PH binding site determined on p13MTCP1 by NMR mapping corresponds well to the PH binding site on p14TCL1 suggested by mutational analysis (31, 32). From the high structural and sequence homology between p13MTCP1 and p14TCL1 and the similarity of the imprint they produce on PKBβ-PH, we deduce that the molecular complexes PKBβ-PH-p13MTCP1 and PKBβ-PH-p14TCL1 are structurally comparable. In addition to the continuous area formed by these four β-strands, residues located in the α-helix seem to contrib-
ute as well by the interaction with PKBβ-PH. This small helix is located in the long flexible loop that connects the two β-membranes forming the β-barrel structure of p13MTCP1 and faces the main binding surface.

Low Resolution Structure of the Complex Formed between PKBβ-PH and p14TCL1—We used SAXS to investigate the low resolution structure of the molecular complex formed by PKBβ-PH and p14TCL1. SAXS data were collected at two different sample-detector distances (1.872 and 0.880 m). These scattering curves were merged to yield data between 241- and 14-Å resolution (Fig. 6A). Using the program Gnom (45), the pair distribution of interatomic distances \( p(r) \) was obtained from these data. Data was best fitted when using a maximum particle diameter of \( R_{\text{max}} = 105 \) Å, yielding a fit of 0.936 (classified “excellent” by Gnom) (Fig. 6B). The radius of gyration determined by the pair distribution function was \( 33.2 \pm 0.094 \) Å, very close to that obtained by Guinier analysis (32 Å). Based on these data, a molecular envelope was established by averaging the dummy atom models obtained \textit{ab initio} from 10 individual simulated annealing calculations. Through this SAXS analysis, the PKBβ-PH-p14TCL1 complex appeared as a P2-symmetric form composed by a rod-shaped form with two ellipsoid extensions (Fig. 6C). Based on the atomic structures available for PKBβ-PH (Protein Data Bank (PDB) code 1P6S) and p14TCL1 (PDB code 1JSG) and considering that in solution PKBβ-PH is monomeric and p14TCL1 is dimeric (23, 36, 65), the center of the complex was attributed to the p14TCL1 dimer and each ellipsoid extension was due to one PH domain (Fig. 6C). Indeed, the dimensions of the ellipsoid extensions and the rod-shaped central part corresponded well to those of PKBβ-PH and dimeric p14TCL1, respectively.

Without any further assumptions or modeling, these structural data revealed that, in solution, p14TCL1 binds PKBβ-PH in a 1:1 stoichiometry, forming a dimer of heterodimers where two single PKBβ-PH-p14TCL1 complexes are linked via the p14TCL1 dimer interface. In the complex, the PH domains are not in contact with each other and the PH binding site on p14TCL1 does not overlap with its dimer interface.

Molecular Modeling of the PKBβ-PH-p14TCL1 Complex—Molecular docking experiments of PKBβ-PH with TCL1 proteins, integrating NMR and SAXS data, were then attempted in order to clarify the molecular basis of their interaction. For this...
reason, we combined our experimental results with manual and computational docking and included a number of assumptions, as described below, to propose an atomic model of the complex formed between p14\textsuperscript{TCL1} and PKB-PH. However, we note that the biological and functional conclusions described in this paper were derived solely on the basis of experimental data and general structural considerations and do not depend on the detailed atomic model described below.

Structural studies indicate that the orientation of PtdIns-P\textsubscript{b}m-bound PH domains relative to the membrane is well conserved (66, 67). Imposing this PH-membrane orientation, only one possible PKB\textbeta-PH-p14\textsuperscript{TCL1} arrangement could be obtained that satisfied all of the constraints, i.e. to be contained within the limits of the SAXS envelope (both enantiomers were tested), to display surface complementarity between the ligands, and to satisfactorily involve the binding sites as determined here by NMR and previously by mutational analysis for p14\textsuperscript{TCL1} (31, 32). This PKB\textbeta-PH-p14\textsuperscript{TCL1} model was corroborated by computational docking. Under the constraint that residues mapped to the binding sites are located within 6 Å of the partner molecule, the program FTDock (47) yielded as best-scored solution a PKB-PH-p14\textsuperscript{TCL1} arrangement comparable to that determined manually (FTDock rpscore = 4.66; next best solutions were scored 3.98 and 3.80). After rigid body and side chain refinement by MultiDock (48), this model gave the best fit to SAXS data compared with the 40 next best-scored

**Fig. 5. Interaction of PKB\textbeta-PH with the TCL1 protein family.** A, footprint of PKB\textbeta-PH onto 15\textsuperscript{N}-p13\textsuperscript{MTCP1}. The solvent-accessible surfaces of residues involved in the surface contacts as revealed by the T\textsubscript{2}-mapping experiments are colored in yellow. The residues colored in red are Asp\textsuperscript{11} and Leu\textsuperscript{67} that have been also found to be involved in the PKB\textbeta-PH-p14\textsuperscript{TCL1} interaction by mutational analysis. A ribbon representation of the protein in the same orientation and with the same color code is given above. B, footprint of p13\textsuperscript{MTCP1} and p14\textsuperscript{TCL1} onto 15\textsuperscript{N}-PKB\textbeta-PH. The residues colored in yellow, blue, and green are those highlighted only by the addition of p13\textsuperscript{MTCP1} or only by the addition of p14\textsuperscript{TCL1} or by the addition of either p13\textsuperscript{MTCP1} or p14\textsuperscript{TCL1}, respectively. A ribbon representation of the protein in the same orientation and with the same color code is given above. In both panels, unaffected residues are colored in white and unassigned residues for PKB\textbeta-PH (essentially the VL\textbeta loop (34)) are colored black. The two views presented in each panel are related by an –180° rotation around the vertical axis.
models compiled from different FTDock runs ($\chi^2$ to raw data was 3.06 compared with 3.35 and 3.41 for next best solutions (Crysol (45)) (Fig. 7). The buried-accessible surface area of the modeled PKB\textsubscript{PH}-p14\textsuperscript{TCL1} complex is 1030 Å$^2$ (490 Å$^2$ on PKB-PH and 540 Å$^2$ on p14\textsuperscript{TCL1}), which is within the range observed for protein-protein associations with micromolar affinities. In the model, PKB\textsubscript{PH} docks onto p14\textsuperscript{TCL1} mainly using an interface constituted by the first $\beta$-strand of the second $\beta$-sheet and the COOH-terminal helix. On p14\textsuperscript{TCL1}, the binding site is centered around Gln77 and involves Asp16 and Trp19, which have been shown to be important for the interaction although such rearrangements cannot completely be discarded in this particular case, a low $k_{on}$ may have for origin a certain degree of aggregation of p13\textsuperscript{MTCP1}. Indeed, if the binding surface becomes temporarily masked by nonspecific aggregation, the $k_{on}$ will be significantly lower than the expected diffusion constant.

It has been shown that NMR is well suited to study such weakly interacting systems (reviewed in Ref. 55). Different NMR techniques have been developed to study these systems depending on the exchange regime. Although the chemical shift perturbation mapping is the most widely used NMR method to map protein interfaces, it cannot be used in the present case because it relies on fast-exchange conditions. On the other hand, line-broadening analysis has been shown to provide a useful alternative in the case of a slow/intermediate-exchanging system. The binding surface of p13\textsuperscript{MTCP1}, as revealed by $T_2$ mapping, is in perfect agreement with previous results obtained from single or multiple site-specific mutations (31, 32) of

### DISCUSSION

Combining fluorescence anisotropy, NMR methods, and SAXS analysis, we have determined the affinities and molecular framework of the complexes formed between TCL1 family proteins and PKB\textsubscript{PH}. Whereas the affinities measured between PKB\textsubscript{PH} and p14\textsuperscript{TCL1} using fluorescence anisotropy or NMR are compatible with the slow-exchange regime with regard to the NMR time scale, the dissociation constants measured for the PKB\textsubscript{PH}-p13\textsuperscript{MTCP1} complex are more in favor of a fast-exchange regime. Indeed, a rule of thumb is that interactions with a $K_d < 10 \mu M$ are slow exchange, whereas interactions with low affinity are immediate to fast exchange. However, exceptions can be very dramatic. Slow-exchange conditions for the interaction of a peptide with 500 $\mu M$ affinity was measured for the Hsp70 chaperones (because of a slow $k_{on}$) (52), and fast-exchange conditions were encountered for the binding of a phosphate compound to hemoglobin with an affinity of $\sim 1 \text{nM}$ (because of a multi-site binding mechanism) (68). We believe that the apparent discrepancy between the intermediate-to-slow regime and the low affinity observed for the PKB\textsubscript{PH}-p13\textsuperscript{MTCP1} complex probably arises because the $k_{on}$ is significantly slower than diffusion-limited. Usually, important conformational changes for one or more of the two partners upon binding are invoked to explain such phenomenon. Even though such rearrangements cannot completely be discarded in the present case (see below), it is unlikely that the surface recognition mechanism underlying the formation of the PKB\textsubscript{PH}-p13\textsuperscript{MTCP1} complex would require such important conformational changes. Rather, we believe that, in this particular case, a low $k_{on}$ may have for origin a certain degree of aggregation of p13\textsuperscript{MTCP1}. Indeed, if the binding surface becomes temporarily masked by nonspecific aggregation, the $k_{on}$ will be significantly lower than the expected diffusion constant.

It has been shown that NMR is well suited to study such weakly interacting systems (reviewed in Ref. 55). Different NMR techniques have been developed to study these systems depending on the exchange regime. Although the chemical shift perturbation mapping is the most widely used NMR method to map protein interfaces, it cannot be used in the present case because it relies on fast-exchange conditions. On the other hand, line-broadening analysis has been shown to provide a useful alternative in the case of a slow/intermediate-exchanging system. The binding surface of p13\textsuperscript{MTCP1}, as revealed by $T_2$ mapping, is in perfect agreement with previous results obtained from single or multiple site-specific mutations (31, 32) of
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It involves mainly residues located in the β1, β2, β5, and β6 strands of the β-barrel structure that form a continuous surface of ~788 Å², which is highly conserved among TCL1 family members. Among the p13MTCP1 residues that differ from p14TCL1, His^12^ and Arg^22^ (respectively Arg^17^ and Leu^27^ in p14TCL1) are situated in the center of the binding site and thus are likely to affect the affinity of p13MTCP1 for PKB-β-PH.

In addition, the binding site encompasses residues located in the small α-helix located in the long loop that joins the two β-sheets, forming the characteristic barrel structure of the TCL1 proteins. This helix is located on the same face of the β-barrel and is spatially close to the main binding surface as delineated from NMR experiment. This structural element was not proposed as part of the TCL1 interaction site as deduced by sequence homology analysis (32), because its sequence diverges substantially from one member to another. However, its structure is conserved within the protein family. The differences in sequence of this part of the binding surface could also contribute to the different affinities for PKB-PH measured for p13MTCP1 and p14TCL1. In addition, previous ^15^N relaxation measurements on p13MTCP1 (24) have shown that this helix as well as the entire loop connecting the two β-meanders forms the β-barrel structure experiences microsecond-to-millisecond motions. In the dimeric crystal structure of p14TCL1, contacts exist between the loops of each of the two monomers that probably restrict the flexibility of this region when compared with p13MTCP1. It is possible that these contacts restrain the helix position in the dimeric structure of p14TCL1 in a favorable orientation to bind PKB-β-PH. This favorable conformation could be reached only through the conformational changes in this highly flexible loop in the monomeric structure of p13MTCP1. Such rearrangements involve microsecond-to-millisecond motions that could explain a significant lower K_D for p13MTCP1 and thus could provide an additional explanation for the higher K_D measured for the complex p13MTCP1-PKB-β-PH.

The binding site of p14TCL1/p13MTCP1 on PKB-β-PH comprises residues located in the COOH-terminal α-helix as well as residues located in the β4 and β5 strands on one face of the β-sandwich. This surface (~830 Å²) is located opposite the PtdIns-P binding pocket and remote from the Gβγ binding site as determined for G protein receptor kinase 2-PH (66, 69), indicating that PKB-β-PH is able to engage these three interactions simultaneously (Fig. 8). The TCL1 interface on PKB-β-PH flanks the region (residues 67–77) necessary for binding to periplakin (70), a nuclear localization signal-containing plakin family protein, suggesting that these binding events are also compatible. Finally, TCL1 binding to membrane-bound PKB-PH leaves accessible PKB-PH Trp^80^, a residue potentially involved in protein-protein interactions (71). Thus TCL1 proteins could cross-link and possibly stabilize a number of PKB-PH interactions at the membrane, promoting the formation of the high molecular weight protein complexes (18) and nuclear relocation (1, 18).

Our analysis allows us to put forward a model in which dimeric p14TCL1 cross-links two PKB molecules by binding to a surface region of their PH domains, which has not yet been observed in other PH-protein interactions. It should be noted that NMR experiments can indicate contact points between two proteins, but that it is not safe to conclude that all such contacts are crucial for specificity recognition and/or any subsequent biological effect. Site-directed mutagenesis will be required to dissect the contributions of individual residues. The mapping of the contact sites offers an important starting point in such work. Nonetheless, some assumptions can be tentatively derived from the present model on how TCL1 family proteins promote their array of cellular effects.

Contrary to current models (30), the association with p14TCL1 appears compatible with the membrane anchoring of PKB-PH and even should strengthen significantly its membrane attachment by an avidity effect. Indeed, all of the PKB-β-PH-p14TCL1 orientations that satisfy the NMR and SAXS constraints share the fact that the two PtdIns-P binding sites of both PH domains point approximately in the same direction, away from the interface with dimeric p14TCL1. Together with structural data indicating that interaction with TCL1 family proteins does not affect the PtdIns-P binding site of PKB-PH (this study and Refs. 34 and 71), our analysis suggests that a p14TCL1 dimer is able to associate simultaneously with two membrane-bound PH domains (Fig. 8). The co-localization of high concentrations of p14TCL1 and activated PKB at the membrane has already led French et al. (32) to suggest that the membrane is the site of complex formation. Because constitutive membrane anchoring renders PKB oncogenic (8), we speculate that the stimulation of a prolonged membrane association...
PKB contributes to the transforming potential of p14TCL1. TCL1 proteins were suggested to mimic the conformational change induced in PKB upon PKB-PH binding to PtdIns-P, thus disrupting a postulated PKB conformation where the PH and kinase domains assemble into a catalytically inactive form (30). However, our data show that TCL1 proteins are not direct structural mimics of PtdIns-P because their respective binding sites are located on the opposite sides of PKB-PH and do not seem to be allosterically linked (this study and Refs. 34 and 71). Although the binding sites of TCL1 and PtdIns-P are non-overlapping, we cannot exclude the possibility that association with TCL1 is incompatible with the postulated “assembled” form of PKB and hence that TCL1 proteins are able to stabilize PKB in a preactivated “disassembled” conformation. Of note, a TCL1 enhancement of membrane attachment would also help to prevent PKB from dissociating from the membrane to adopt an assembled inactive conformation.

We have previously reported evidence that p14TCL1 stimulates trans-phosphorylation of PKB molecules on Thr309 in vitro (18). This is supported by our current analysis, because we show that dimeric p14TCL1 cross-links two PKB molecules via their PH domains in a way that promotes proximity between the two kinase domains. Thr309 phosphorylation enhances the catalytic activity of PKB. Therefore, it is likely that the stimulation of PKB trans-phosphorylation contributes to the transforming action of TCL1 proteins.

In agreement with cellular analysis (31), dimerization of

FIG. 8. Association of p14TCL1 with PKB-PH is nonexclusive with PKB-PH binding to membrane and Gβγ and stimulates proximity between two PKB kinase domains. Side view of the model proposed for the submembrane complex formed by PKB, p14TCL1, and Gβγ. The PtdIns-P-bound PKB-PH domains (green, PDB code 1H10 (33)) were oriented toward the membrane as suggested by crystallographic analysis (66, 67). p14TCL1 was docked onto PKB-PH as suggested by computational analysis (see “Results”). Gβγ was orientated with respect to membrane and PH domain as determined for G protein receptor kinase 2-PH-Gβγ (PDB code 1OMW (66)). PKB kinase domain was taken from Ref. 72. (PDB code 1O6L) and connected to PKB-PH by a linker modeled in an extended conformation.

FIG. 9. Conservation of the binding site within the TCL1 protein family. A, two views of p13MTCP1 showing the sequence homology of the surface residues between p13MTCP1 and p14TCL1. The same color code has been used as in Fig. 1. The two views are related by a 180° rotation around the vertical axis. B, footprint of PKB-PH onto 15N-p13MTCP1 (same color code as in Fig. 5). For each panel, a ribbon representation of the protein in the same orientation and with the same color code is given above.
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PKB proteins appear pivotal for their function. Although both p14\textsuperscript{TCL1} and p13\textsuperscript{MTCP1} co-activate PKBε and PKβ (2, 31), only p14\textsuperscript{TCL1} appears to be homodimeric in the crystal (23) as well as in solution (24). Considering that the affinity of p13\textsuperscript{MTCP1} for PKβ-β is significantly weaker than that displayed by p14\textsuperscript{TCL1}, it is likely that additional bridging molecules are necessary for the function of p13\textsuperscript{MTCP1}. When comparing the homorosilation surface of p14\textsuperscript{TCL1} with the corresponding surface on p13\textsuperscript{MTCP1}, the residue homology appears less obvious than for the surface harboring the binding site to PKβ-β (Fig. 9) on the opposite face of the barrel. This surface could likely offer potential binding sites for additional bridging molecules.

Together, the association of TCL1 proteins with PKB-β seems to stimulate an array of effects on PKB, leading to enhanced enzymatic activity as well as novel and/or extended protein–protein interactions. Targeting the herein identified PKB-TCL1 interface by molecular compounds could prove useful for therapeutic intervention against T-cell leukemias caused by the overexpression of TCL1 family proteins.

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