Structural and functional dissection of the DH and PH domains of oncogenic Bcr-Abl tyrosine kinase

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The two isoforms of the Bcr-Abl tyrosine kinase, p210 and p190, are associated with different leukemias and have a dramatically different signaling network, despite similar kinase activity. To provide a molecular rationale for these observations, we study the Dbl-homology (DH) and Pleckstrin-homology (PH) domains of Bcr-Abl p210, which constitute the only structural differences to p190. Here we report high-resolution structures of the DH and PH domains and characterize conformations of the DH-PH unit in solution. Our structural and functional analyses show no evidence that the DH domain acts as a guanine nucleotide exchange factor, whereas the PH domain binds to various phosphatidylinositol-phosphates. PH-domain mutants alter subcellular localization and result in decreased interactions with p210-selective interaction partners. Hence, the PH domain, but not the DH domain, plays an important role in the formation of the differential p210 and p190 Bcr-Abl signaling networks.
The Bcr-Abl oncoprotein is expressed from the Philadelphia (Ph) chromosome, which is formed upon the t(9;22) reciprocal translocation chromosomal fusions that fuse the breakpoint cluster region (BCR) gene with the Abelson tyrosine kinase (ABL). Bcr-Abl is a constitutively active tyrosine kinase and is selectively inhibited by imatinib (Gleevec), which became a paradigm for targeted cancer therapy. Different Bcr-Abl protein isoforms are expressed, all of which contain exons 2–11 of the ABL1 gene, but, depending on the location of the translocation breakpoint in BCR, include different portions of the BCR gene. The most common Bcr-Abl isoforms are p210 and p190. p190 lacks residues 427–927 of Bcr, and thus is 501 amino acids (i.e., ~25%) shorter than p210, but otherwise contains the same domains with identical sequences. Bcr-Abl p210 is the molecular hallmark of chronic myelogenous leukemia (CML). In addition, 20–30% of adult B-cell acute lymphoblastic leukemias (B-ALL) are Bcr-Abl-positive, of which approximately 1/4 of cases express p210, whereas 3/4 express Bcr-Abl p190. The treatment of CML patients with the Bcr-Abl tyrosine kinase inhibitor (TKI) imatinib results in deep remissions and long-term survival. Although TKIs prolong survival in Bcr-Abl-positive B-ALL, acquired TKI resistance is frequent and overall survival is still dramatically low. Therefore, a deeper understanding of the Bcr-Abl oncoprotein is needed to identify additional drug targets for combinatorial therapy in B-ALL.

We have recently performed a quantitative comparative proteomics study of Bcr-Abl p210 and p190 in parallel to a second laboratory. Unexpectedly large differences in the interactome and tyrosine phosphoproteome were found, with more than a dozen proteins that preferentially interact with either p210 or p190 and ~100 differential phosphorytine (pY) sites indicating activation of distinct signaling pathways by the two Bcr-Abl isoforms. In contrast, no difference in degree and sites of p210 and p190 autophosphorylation or in vitro kinase activity of Bcr-Abl was detected that could have rationalized the observed signaling differences. These observations suggested that the functional disparities of p210 and p190 arise from regions outside the kinase domain. Hence we decided to study the differences in structure of the two isoforms. Due to the different translocation breakpoints, p210 contains a predicted Dbl-homology (DH) and PH domains that constitute the only predicted folded domains of Bcr-Abl whose structure remains undetermined. Crystallographic analysis of the N-terminus of Bcr-Abl showed that a coiled-coil domain forms antiparallel homo-tetramers and the very C-terminus of Bcr-Abl contains an F-actin binding domain that has been studied in solution by NMR.

In summary, the absence of a structural model and poorly defined biochemical specificity and regulation of the Bcr-Abl DH and PH domains, in combination with conflicting data on the role of both domains in Bcr-Abl-dependent leukemogenesis and signaling necessitates an in-depth structural and functional characterization. Here we provide high-resolution structures of the DH and PH domains that unravel unique structural features. Detailed functional characterization give no evidence for RhoGEF activity of the DH domain. In contrast, we characterize in detail DH-mediated phosphoinositide binding that affects cellular localization and the signaling network of Bcr-Abl p210.

Results

Structure of DH domain by NMR and X-ray crystallography. The DH and PH domains constitute the only predicted folded domains of Bcr-Abl whose structure remains undetermined. Therefore, we set out to structurally characterize them by crystallography. Among several constructs, the Bcr-Abl DH- domain (residues 487–702) and DH–PH-domain (residues 487–893) constructs expressed in high yields and showed good biophysical behavior (Supplementary Fig. 1). However, we were unable to obtain

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**Fig. 1** Domain organization of Bcr-Abl. The two isoforms of the fusion protein Bcr-Abl, p210, and p190, are shown with their sizes and domain arrangement. The breakpoint between Bcr and Abl is indicated with a dotted line. CC coiled-coil, DH Dbl-homology, PH Pleckstrin-homology, SH3/SH2 Src-homology 3/2, FABD F-actin binding domain.
-crystals for any of our constructs, so we turned to nuclear magnetic resonance (NMR) spectroscopy in order to get a better idea about the folding and flexibility of these proteins. The $[^{15}$N,$^1$H]-TROSY spectrum of the DH domain was clearly resolved and was indicative of a well-folded protein. We therefore embarked on the structure determination of this domain by NMR. Given the considerable size of the DH–PH tandem domain (410 residues, 47 kDa) and poor behavior of the isolated PH domain, we first concentrated on the DH domain (219 residues, 25 kDa).

The Bcr-Abl DH domain NMR structure was based on 4298 NOE upper distance limits, of which 845 were long-range (Supplementary Table 1, BMRB entry 34101). The 20 structures with the lowest target function nicely overlay with an average backbone atom root-mean-square deviation (RMSD) of 0.85 Å in the folded regions (PDB ID 5N7E, Fig. 2a). The DH domain forms a six-helix bundle arranged with a kink toward one side similar to the ‘seat-back of a chaise longue’ in line with the canonical fold of DH domains (Fig. 2b, Supplementary Table 1, Supplementary Fig. 2)\(^28\). An interesting feature that is specific to the Bcr-Abl DH domain is the extended 18-residue-long loop region between α-helices 4 and 5 covering residues 622–638, which is 10–13 residues longer than in most other DH domains. The α4–α5 loop of the Bcr-Abl DH domain is very dynamic and does not adopt a preferred conformation (Fig. 2a). Apart from this loop, only the N-terminal and C-termini of the protein are disordered.

To increase the crystallization probability of the DH–PH tandem domain, we generated monobodies, synthetic binding proteins based on the fibronectin type III domain that bind to the DH–PH domain\(^29, 30\) (Supplementary Fig. 1). Monobodies tightly bind to the target protein in a conformation-specific manner and are often effective crystallization chaperones that stabilize a low-energy state among the native conformational ensemble and create productive crystal contacts\(^31, 32\). Initial screening of monobodies generated using the DH–PH tandem domain as the target yielded only clones (termed Mb(Bcr-DH_1–4)) that bound to the DH domain with low nanomolar dissociation constants ($K_d$, Supplementary Fig. 3). Crystallization trials using these monobodies in complex with the DH domain readily gave crystals for the DH/Mb(Bcr-DH_4) complex that diffracted to 1.65 Å and enabled the structure determination (PDB ID 5N7E, Supplementary Table 2).

The crystal structure of the DH domain includes the six major α-helices in accordance with the NMR structure (Fig. 2). No electron density was observed for the extended flexible α4–α5 loop region seen in the NMR structure, so this loop was not included in the crystallographic model. Overall the comparison of the DH NMR- and crystal-structures revealed an identical overall fold with some variation in helix orientations (Supplementary Fig. 2). The monobody bound, as expected, via diversification positions in the FG loop and the βC/βD strands, to the tip of the DH domain proximal to the N-terminus (Fig. 2c, Supplementary Figs. 2, 3). To investigate the impact of monobody binding on the DH domain structure, we compared the NMR chemical shift changes upon formation of a complex between the unlabeled

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**Fig. 2** Structures of the DH and PH domains by NMR and X-ray crystallography. a Bundle of 20 DH structures as determined by NMR spectroscopy. The long and flexible α4–α5 loop region (residues 622–638) is highlighted in red. b Cartoon representation of the NMR conformer closest to the mean. The six helical elements and the α4–α5 loop region are labeled. c Crystal structure of the DH domain in complex with Mb(Bcr-DH_4) in cartoon representation. The α4–α5 loop region is missing in this structure (black arrow). The monobody interacts with the DH domain via its FG loop, which is labeled. d Crystal structure of the internally deleted PH domain (PHΔ770–829) in complex with Mb(Bcr-PH_4) in cartoon representation. The monobody interacts with the PH domain via its FG loop, which is labeled. e Cartoon representation of the PH domain (PHΔ770–829) with assignment of the secondary structure elements, the internal deletion site covering residues 770–829 and the C-terminal linker region.
monobody and the $^{15}$N-labeled DH domain. We observed large chemical shift changes throughout the N-terminal half of the DH domain, whereas the most distal region relative to the monobody binding epitope remained largely unperturbed (Supplementary Fig. 4). In summary, the solution and crystal structures of the Bcr-Abl DH domain showed the canonical six helix-fold, but with a long $\alpha_4$–$\alpha_5$ loop.

**PH domain has canonical fold with large insertion.** The Bcr-Abl PH domain exhibited limited stability at room temperature despite extensive optimization efforts. Homology modeling indicated the presence of a unique insertion of 59 amino acids (residues 770–829) between predicted strands $\beta_5$ and $\beta_6$ constituting almost a third of the entire Bcr-Abl PH domain. Since the presence of such a large, putatively flexible element can dramatically interfere with any structure determination approaches, we deleted this part of the PH domain. Still, we were not able to obtain crystals. Encouraged by our success with the DH domain, we generated additional monobodies that bound to the PH domain. Here we made use of the phage-display pool of monobodies originally enriched with the Bcr-Abl DH–PH tandem domain, but performed the secondary yeast-display screening using the PH domain as the target (Supplementary Fig. 1). We obtained three different monobodies with low nanomolar $K_d$ values (termed Mb(Bcr-PH_2–4), Supplementary Fig. 3). These monobodies bound both the full-length and the shortened PH domain (PH$\Delta$770–829) with equal efficiency (Supplementary Fig. 3). With these new constructs and tools in hand, we obtained crystals for the full-length PH domain in complex with monobody Mb(Bcr-PH_4), but we could not optimize these crystals beyond a resolution of 8 Å. In contrast, crystals of PH$\Delta$770–829 in complex with the same monobody diffracted up to 1.65 Å and allowed structure determination of this complex (Fig. 2d).

The structure of the PH domain comprises the canonical PH domain fold with a $\beta$-sandwich, in which four $\beta$-strands at the N-terminal part of the protein pack against a 3-stranded C-terminal $\beta$-sheet, and a long C-terminal $\alpha$-helix (Fig. 2e), displaying high structural similarity for example to the PH domain of Sos1 (Supplementary Fig. 2).
breakpoint with the Abl kinase and connects it to the remaining Cap region and SH3 domain in Abl\textsuperscript{20}, whereas in the full-length Bcr protein this linker would connect the PH domain to a C2 domain. Based on our biochemical data, the PH domain forms a 1:1 complex with the monobody, which binds to the β4–β5 loop and the αC helix of the PH domain via its variable FG loop (Supplementary Fig. 3).

**SAXS analysis of DH and PH domains.** To obtain a better understanding of the interplay of the DH and PH domains, we used small-angle X-ray scattering (SAXS). In other DH–PH units, the PH domain may regulate GEF activity either via intramolecular interactions with the DH domain, which could block GTPase binding or by forming an additional interface with the GTPases to facilitate nucleotide exchange\textsuperscript{28, 33}.
SAXS data for the DH, PH and DH–PH domains allowed a detailed analysis of the conformations of the constructs in solution (Supplementary Table 3, Supplementary Fig. 5). As a control, SAXS data of the isolated DH domain agreed well with the NMR and X-ray structure (Fig. 3a). Since the SAXS data of the PH domain were obtained for the full-length PH construct, fitting with the PH crystal structure with the deleted β5–β6-insertion was unsatisfactory (Fig. 3b). We therefore made a homology model for the full-length PH domain and fitted it with the SAXS data using SREFLEX34. The resulting model indicated that residues 770–829 of the Bcr-Abl PH domain protrude away from the core of the globular PH domain and add a significant volume to the overall shape of the domain (Fig. 3b). The insertion therefore does not seem to affect the PH fold nor does it interact extensively with the core PH domain. It could possibly provide an additional surface for protein–protein interactions.

The DH–PH tandem domain data were fitted using the NMR structure of the DH domain and the crystal structure of the PH domain and further analyzed using the ensemble optimization approach (EOM)35 to evaluate the inter-domain flexibility of the two domains (Fig. 3c, Supplementary Table 3, Supplementary Fig. 5). The scattering profile of the DH–PH tandem domain in solution fitted best to an ensemble of four different conformations with a flexibility of 75% (RQex = 0.75, discrepancy χ2 = 1.0, Fig. 3c), where 100% corresponds to maximum flexibility. This high flexibility between the DH and PH domains argues for a balls-on-a-string model with rather independent behavior without specific domain–domain interaction interfaces. These findings are also in agreement with results from an NMR experiment, in which only minimal chemical shift perturbations of 15N-labeled DH domain were observed upon addition of unlabeled PH domain in trans (Supplementary Fig. 4). We therefore conclude that the DH and PH domains function as independent units and have only minimal influence on each other.

The DH–PH domain has no detectable RhoGEF activity. For classical Rho GEF family members, the interaction with GTPases is mediated by two conserved regions, CR1 and CR3, which are part of the helical core of the DH domain, as well as the C-terminal α-helix 6 (Supplementary Fig. 2)36. In particular a glutamate residue in CR1 and a lysine residue in CR3 are important for catalytic activity36–38. Furthermore, residues in the ‘seat-back-region’ of the DH domains, comprising α-helix 4, α4–α5 loop and α-helix 5, determine GTPase specificity15. Notably, these residues that are important for GTPase interaction, catalysis, and selectivity are not conserved in the Bcr-Abl DH domain. Furthermore, the extended α4–α5 loop points directly into the GTPase binding interface, where it would sterically clash with binding of a Rho GTPase (Fig. 4a, Supplementary Fig. 2).

To probe our structural predictions, we first measured the interaction of the Bcr-Abl DH–PH tandem domain with the three canonical Rho GTPases RhoA, Rac1, and Cdc42. To account for a possible regulatory effect of the PH domain, all experiments were conducted with the DH domain alone, as well as the DH–PH tandem. As a positive control, we included the well-characterized RhoA- and Cdc42-selective DH–PH domain of Dbs15.

Since a number of DH–PH domains, such as Dbs, Intersectin, Tiam1, Trio, and LARG have been shown to form stable complexes with GTPases and have been co-crystallized, we first tested the interaction with recombinant RhoA, Rac1, and Cdc42 using in vitro pull-down experiments and co-migration on a size-exclusion column (Fig. 4b, c, Supplementary Fig. 6). As expected, the DH–PH domain showed very efficient pull-down of and co-elution with its cognate GTPases RhoA and Cdc42, and to a lesser extent Rac1 (Fig. 4b, c, Supplementary Fig. 6). In contrast, the Bcr-Abl DH or DH–PH domain constructs did not display detectable interactions with any of the three Rho GTPases (Fig. 4b, c, Supplementary Fig. 6). We concluded that there is no high-affinity interaction between the Bcr-Abl DH domain and the tested Rho GTPases. These observations are in line with our previous proteomics study, in which we did not detect Rho GTPases as interactors with Bcr-Abl p2108. We also conducted tandem-affinity purification (TAP) mass spectrometry experiments in the Bcr-Abl p210-expressing cell line K562 using the isolated DH–PH tandem domain to assess direct interactors of this fragment, but we found no Rho GTPase captured with the Bcr DH–PH tandem domain (Supplementary Data 1).

To exert the function of a GEF, however, a weaker or more transient interaction of the DH–PH domain with Rho GTPases may suffice. To explore this possibility, we conducted NMR titration experiments, in which we titrated RhoA, Rac1 or Cdc42 to the 15N-labelled DH domain (Fig. 4d, e, Supplementary Fig. 7). Overall, chemical shift perturbations were rather small and visible only upon addition of excess GTPase, excluding large conformational changes in the DH domain upon interaction with the respective GTPase. Additionally, line broadening at higher GTPase concentrations challenged the analysis. Nonetheless, about 10 peaks for each experiment showed concentration-dependent chemical shift perturbations throughout the titration series, which were employed for Kd determination. We obtained mean Kd values of 600 μM for the interaction with RhoA, 800 μM for Rac1 and 1900 μM for Cdc42 (Fig. 4e). We furthermore mapped the chemical shift perturbations on the DH domain structure (Supplementary Fig. 7). Interestingly, all three GTPases induced a similar pattern of chemical shift perturbation with an accumulation around the putative GTPase binding site. Collectively, these experiments indicated that the interactions with the three Rho GTPases are specific, but of much lower affinity than other GTPase-GEF interactions, which are typically in the low-micromolar range. Although the physiological significance of...
these weak interactions is not clear, the observations encouraged us to probe the catalytic GEF function of the Bcr-Abl DH–PH domain in nucleotide exchange experiments. Using fluorophore-labeled GDP, the GDP to GTP exchange was monitored in the presence of the Bcr-Abl DH or DH–PH domains, the Dbs DH–PH fragment as the positive control, and we further included a Bcr-Abl DH loop-deletion mutant into our experiments, in which we shortened the long unstructured α4–α5 loop by 8 residues (DHΔ621–628) to assess a possible steric hindrance of this long loop region (Fig. 4a, f, Supplementary Fig. 8). While the Dbs DH–PH lead to a strong acceleration of nucleotide exchange of RhoA and Cdc42 and to lesser extent for Rac1 at concentrations as low as 0.1 μM, none of the Bcr-Abl DH, DH–PH and DHΔ621–628 constructs used in these experiments showed an enhancement of the nucleotide exchange rate for any of the three GTPases, even at 10–100-fold higher concentration than in the experiment with the Dbs control (Fig. 4f–h, Supplementary Fig. 8).

Collectively, our results show that even though the Bcr-Abl DH–PH fragment interacts with very low affinity with the three

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**Fig. 5** Characterization of PIP binding to the PH domain. *a* Structure-based identification of the PIP-binding motif. The electrostatic surface representation indicates that the PIP-binding motif (black arrow) forms a positively charged patch on the PH surface. *b* The PIP-binding site is shown enlarged together with a D-myo-inositol-4,5-bisphosphate (IP$_2$) ligand fitted in the 2Fo–Fc electron-density map, contoured at 1.0 sigma. Critical residues in the binding motif are shown in sticks representation and colored by element. *c* PIP strips comparing the PIP-binding properties of PH wt, the PH R723A/K724A and R726A mutants, as well as the DH domain as a negative control. Individual signals on the strip were quantified relative to PH wild-type protein. *d* Binding of Bcr-Abl domains to surrogates of biological membranes in the liposome microarray (LiMA) assay. 0.1, 0.5, 1, and 2 μM of purified sfGFP-tagged Bcr-Abl DH, PH, DH–PH or Dbs DH–PH domains were incubated together with giant unilamellar liposomes containing the indicated concentrations of signaling lipid (in mol % of total lipids). PI3P, PI4P, PI5P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2, and PI(3,4,5)P3 are phosphoinositides (Supplementary Data 2, 3). PC phosphatidylcholine, PA phosphatidic acid, PE phosphatidylethanolamine, PI phosphatidylinositol PS phosphatidylserine, LPA lyso PA, LPC lyso PC, S1P sphingosine-1-phosphate, NBI normalized binding intensity. Not determined values are indicated with a star (*). Values are means (n = 3)
Fig. 6 Localization and signaling network of Bcr-Abl p210, p190, FABD and PH mutant proteins. a Cellular localization of Bcr-Abl p210 and p190, p210 with the PH mutations R723A/K724A or R726A, with the FABD mutant F1100E, as well as a combination of PH and FABD mutation. Cells were fixed and immunostained with an anti-HA antibody (HA-Bcr-Abl). F-actin (actin) and nuclei (DNA) were stained with rhodamine-conjugated phalloidin and Hoechst, respectively. The white scale bar in the upper left image corresponds to 10 µm. b Quantification of co-localization of Bcr-Abl with actin fibers using ImageJ. The box-and-whisker plots show the median, lower/upper quartile, and minimum/maximum of % Actin co-localization for at least six quantified cells per Bcr-Abl mutant. c Representative immunoblot analysis of anti-Bcr-Abl/Abl immunoprecipitates from BaF3 cells stably transduced with the indicated Bcr-Abl variants. Fractions of the immunoprecipitates were immunoblotted for Bcr-Abl and its interaction partner Sts1. d Quantification of the co-immunoprecipitation of Sts1 with Bcr-Abl. The quantified amount of co-immunoprecipitated Sts1 after correction for the Bcr-Abl amounts is shown. Means ± s.d. of two biological replicates analyzed in two technical replicates.
PH domain binds phosphoinositides with high affinity. To test for PH domain function, we first aimed to define the phosphatidylinositol-phosphate (PIP)-binding site, which is formed by a positively charged patch of amino acids (arginine and lysine) in the β2–β3 hairpin of PH domains. In the Bcr-Abl PH structure residues K724 and R726 in the β1–β2 hairpin, in addition to K739 and K754 in the β3–β4 hairpin may qualify for the PIP-binding interface (Fig. 5a, b, Supplementary Fig. 9). To identify the PIP-binding site of the Bcr-Abl PH domain experimentally, we soaked the PH domain crystals with a soluble PI(4,5)P2 derivative and found additional electron density at the PIP-binding interface (Fig. 5a, b, Supplementary Fig. 9). To test this hypothesis, we investigated Bcr-Abl localization by confocal immunofluorescence microscopy. We analyzed the localization of different Bcr-Abl constructs, including Bcr-Abl p210, PIP-binding-deficient mutants of p210 (R723A/K724A and R726A), as well as p190. Additionally, we combined the PH domain mutations with a mutation disrupting the binding of the F-actin binding domain (FABD; Fig. 1) in the very C-terminus of Bcr-Abl to F-actin (F1100E), which previously was shown to result in strongly reduced co-localization of Bcr-Abl p210 with cortical F-actin.

PH domain binding. In cells, PH domains are commonly involved in targeting proteins to membranes. Given the high binding affinity of the Bcr-Abl PH domain to PI(4,5)P2, this domain could contribute to Bcr-Abl subcellular localization to the plasma membrane. To test this hypothesis, we investigated Bcr-Abl localization by confocal immunofluorescence microscopy. We analyzed the localization of different Bcr-Abl constructs, including Bcr-Abl p210, PIP-binding-deficient mutants of p210 (R723A/K724A and R726A), as well as p190. Additionally, we combined the PH domain mutations with a mutation disrupting the binding of the F-actin binding domain (FABD; Fig. 1) in the very C-terminus of Bcr-Abl to F-actin (F1100E), which previously was shown to result in strongly reduced co-localization of Bcr-Abl p210 with cortical F-actin.

PH domain binding selectivity. In solution, we next investigated the PIP-binding selectivity in solution using PIP strips (Fig. 5c). We found that the Bcr-Abl PH domain bound to a wide range of mono, bis- and tris-phosphorylated PIPs, as well as phosphatidylinerine, while no lipid binding was observed with the DH domain (Fig. 5c). Mutations in the PIP-binding motif, R723A/K724A or R726A, reduced binding to all interacting PIPs by 60–80% on the PIP strips (Fig. 5a–c). To further quantify these measurements and interference preferences for specific PIPs, we employed a more physiological lipidome microarray-based assay (LiMA). In this context, we observed that the Bcr-Abl PH domain bound preferentially to bis- and tris-phosphorylated PIPs over monophosphorylated PIPs, as well as preferentially to P5-containing liposomes over other phospho- and sphingolipids (Fig. 5d, Supplementary Fig. 9, Supplementary Data 2 and 3). The estimated K_d values for binding to all bis- and tris-phosphorylated PIPs were in the range of 1–5 μM, similar to those reported for other PH domains (Supplementary Fig. 9). Again, no significant binding was observed for the DH domain alone. Interestingly, the Bcr-Abl DH–PH domain showed the same binding specificity as that of the PH domain alone, but with lower affinity. Finally, the affinity for membranes containing PIPs was strongly reduced for R726A and almost absent for R723A/K724A (Fig. 5b, d). Overall, these results indicated that the Bcr-Abl PH domain binds to several PIPs with comparable affinity, but broader specificity as compared to other PH domains.

Discussion. We have performed an extensive structural and functional characterization of the Bcr-Abl DH–PH domain. With the structures of the DH and the PH domain, the only missing domains of Bcr-Abl were finally structurally characterized and enabled us to study the molecular basis for the signaling contributions of these domains and to construct a preliminary model of the overall Bcr-Abl structure (Fig. 7).

Our structural data show that with its extended α4–α5 loop blocking the DH-GTPase interface and lack of conserved catalytic residues, the Bcr-Abl DH domain may not act as a GEF. In line with these findings, the Bcr-Abl DH domain did not interact with or directly activate the three GTPases RhoA, Rac1, and Cdc42 in our functional experiments. Importantly, in contrast to previous studies, our experimental set-up using the isolated and purified DH–PH fragment, together with purified Rho GTPases, allowed us to directly address possible GEF activity of the
DH–PH domains and to exclude possible indirect effects by other GEFs in cells, as well as downstream signaling pathways regulated by any of the other Bcr-Abl domains. We consider GEF activity on other members of the Rho family unlikely, although we cannot fully exclude it at this point. While the Rho GTPase family comprises up to 25 members, RhoA, Rac1, and Cdc42 represent the most studied members of the Rho family and have also been used in previous studies on RhoGTPase activation in Bcr-Abl expressing cells. In addition recent functional proteomics studies on six Rho GTPases (including RhoA, Rac1, and Cdc42), as well as on the Bcr-Abl interactome from others and us, did not indicate that Rho GTPase directly interact with Bcr-Abl. We also carefully checked the structural location and possible functional role of the SS09A mutation that was previously proposed to modulate leukemogenesis in a mouse model, but found no supporting evidence for these findings, as the DH domain structure or binding remains unaltered.

Diverse mechanisms for the regulation of DH-mediated GEF activity have been described, including direct intramolecular interactions with adjacent linkers of the PH domain, allosteric regulation via phospholipid binding to the PH domain, as well as post-translational modifications of the DH domain, such as phosphorylation. Our results do not support any of these regulation mechanisms in the case of the Bcr-Abl DH–PH domain. The high degree of inter-domain flexibility as observed by SAXS speaks against a regulatory impact of the PH domain on the GEF function of the DH domain. Shortening of the α4–α5 loop of the DH domain was not activating in our GEF assays, excluding a possible inhibitory role of this loop that could block the DH-GTPase interaction surface, in addition to the lack of conservation of residues that are important for GTPase interaction in other GEF proteins. Finally, three tyrosine phosphorylation sites in the DH domain, Y554, Y591, and Y644, were repeatedly mapped in the Bcr-Abl DH domain. Whereas Y544 and Y591 are conserved among other DH domains, Y644 is not, but forms part of the conserved region 3 (CR3) that builds part of the GTPase-interaction interface. The biological impact of the phosphorylation of these tyrosines may merit deeper analysis. However, in cases where the regulation upon DH domain phosphorylation has been studied, GEF activity was only mildly modulated upon phosphorylation. Collectively, based on our detailed study, there is no evidence that the Bcr-Abl DH domain acts as a RhoGEF. Hence, it may therefore be dubbed ‘pseudo-GEF’, along with six other DbI family members that lack key catalytic residues. Still, the DH domain may play a possible important role for the overall structural organization and integrity of Bcr-Abl p210. We postulate that the activation of RhoA that had been observed for Bcr-Abl p210, but not for the Bcr-Abl p190 isoform in two previous studies, may be the constitutiveness of Bcr-Abl p210, but not for the Bcr-Abl p190 isoform in two previous studies, may be the constitutiveness of the isolated PH domain determined here likely to result from a reverse affinity step. The final purification step for all constructs was a size-exclusion-chromatography step depending on the size of the constructs either on a
Superdex75 or Superdex200 16/60 column (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% glycerol, and 1 mM DTT). The samples were concentrated for the subsequent downstream application by Amicon-Ultra centrifugal filters (Millipore) and all proteins could be stored at −80 °C.

**Monobody generation and initial characterization.** The general approach for monobody generation have been previously reported. Two different monobody phage-display libraries were screened, the loop only library29 and the loop and side library. A biotinylated DH–PH domain sample in the presence of 25 μg/mL polylysine was used for sorting of phage-display libraries. Four rounds of phage-display library selection were performed at concentrations of 100, 100, 50, and 20 nM of biotinylated DH–PH domain for the first, second, third, and fourth rounds, respectively. After amplification of the cDNA encoding the resulting pBacIdapt library, the sub-library was transferred to the yeast display format, as described previously8 and selection performed at 20 nM DH–PH or 100 nM of DH domain. Clones were analyzed for target protein binding in the yeast-display format and sequenced. Binding assays in the yeast display format were performed as previously described29 with a concentration range of the biotinylated targets from 10 nM to 1 μM in Tris-buffered saline (TBS) with 0.1% BSA. Incubation of target protein, yeast cells, and a mouse anti-V5 antibody were for 30 min at RT. Streptavidin-DyLight650 and a FITC-coupled antimouse IgG was added after two washing steps and incubated for 30 min at RT. Samples were analyzed on a Gallios (Beckman Coulter) or BD Accuri C6 (BD Bioscience) flow cytometer. Plots of the mean fluorescence intensity vs. target concentration and fitting a 1:1 binding model to the data using Prism (GraphPad) was used to determine the apparent Kd values. We have established that the apparent Kd values determined using this method generally agrees with those determined from biochemical measurements of purified proteins.

**Isothermal titration calorimetry.** Isothermal titration calorimetry (ITC) measurements were performed on a MicroCal iTC200 (GE) instrument. All proteins were degassed after extensive dialysis against 25 mM HEPES (pH 7.5), 150 mM NaCl, 0.5 mM TCEP. Protein concentrations were determined by measuring UV absorbance at 280 nm. The titrations were performed in 16 steps with 0.49 μL injections for the first and 2.49 μL for the other injections. Concentrations were selected based on assumed affinities and the signal strength of the interaction. The MicroCal software was used for data analysis.

**NMR spectroscopy.** For NMR structural studies, the DH domain was expressed in minimal M9 medium supplemented with 1 g L−1 polylysine and 2 g L−1 13C-glucose (Cambridge Isotope Laboratories) or 4 g L−1 unlabelled glucose. Finally, the samples were dialyzed into an NMR compatible buffer containing 50 mM HEPES, pH 7, 50 mM NaCl, 1 mM DTT. For GTPase titration experiments, the buffer additionally contained 1 mM imidazole and 2 mM EDTA. For the DH domain, uniform, 13C, 15N-labeled DH domain (487−702) was prepared at a concentration of 850 μM. Measurements for the assignment were carried out at 303 K on Bruker Avance spectrometers equipped with cryogenic 950 MHz. Backbone assignment was achieved using TROSY-type52, 53 BEST54, 55 VSHIFT56. A consensus structure bundle of 20 conformers58. A consensus structure bundle of 20 conformers was subjected to calculations with torsion angle dynamics were started from 200 random

**Bioinformatics tools.** Sequence and secondary structure analysis relied on web-servers for BLAST (blast.ncbi.nlm.nih.gov), PSIPRED (bioinf.cs.ucl.ac.uk/psipred) and HHpred (toolkit.tuebingen.mpg.de/#tools/hhpred22−24). Homology models were created using SwissModel (swissmodel.expasy.org)64. Structural analysis and figure preparation was conducted in PyMOL (www.pymol.org) and SASPy71.

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**GTase interaction studies.** DH–PH and DH constructs were mixed with GTPases in a 1:2 molar ratio and dialyzed into either EDTA- or MgCl2-containing buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM DTT). SAXS data were collected at EMBL P12 beamline, DESY, Hamburg, Germany with protein concentration ranges of 1.6–14.4 μM. The data were collected from the first point of the Guinier region until the end of the useful range defined by SHANUM55. High- and low- concentration curves were merged to correct for concentration-dependent inter-domain interference using the program PRIMUS from the ATSAS package68. GNOM was used to obtain the r(0) and determine the corresponding Dmax and Rg values. SREFLEX was used to generate and refine high-resolution hybrid models using high-resolution structures as the starting point53. EOM was used to generate an ensemble of conformations and to assess the inter-domain flexibility of the DH–PH domain. The scattering curves from the high-resolution models were calculated using CRYSTAL59. SAXS figures were prepared using PyMOL (www.pymol.org) and SASPy71.

**X-ray crystallography.** Samples for crystallization trials were concentrated to ~10 mg mL−1. Initial screenings were set up in a 96-well plate with sitting drop crystallization using the Mosquito (TTP Labtech). Drops were composed of a mixture of protein and buffer in a 1:1 ratio (v/v) and crystals grown at 291 K. The DH/Mb(Bcr-DH_4) complex crystallized in condition F5 of the Morphotec HT screen (MD1-47, Molecular Dimensions) corresponding to 0.12 M mono-saccharides (0.2 M α-glucose; 0.2 M β-mannose; 0.2 M β-galactose; 0.2 M α-fructose; 0.2 M α-L-rhamnose; 0.2 M α-1-acetamylo-inosamine), 0.1 M buffer system pH 7.5 (sodium HEPES; MOPS (acid)) and 50% precipitant mix 1 (40% v/v PEK 500 MME; 20% v/v PEK 20000). The PH/Mb(bcr-PH_4) complex crystallized in condition 81 of the JCSG+ suite (Qagen) that contained 0.1 M potassium thiocyanate and 30% (v/v) PEK 500 producing crystals that were precipitated in the presence of 20% glycerol.

X-ray diffraction data were collected at the synchrotron SLS (Swiss Lightsource, Villigen, Switzerland), beamline 6.0.6.0 dia using a single wavelength at 100 K. The diffraction data were processed with the X-ray Detector Software (XDS, http://sds.mpimp-heidelberg.mpg.de/). Phasing was achieved by molecular replacement in Phaser using homology models curated in SwissModel64, 65. The homology models for the DH and the PH domain was based on the template with PDB ID 2ZQQ, the monobody was modeled based on template PDB ID 3TUE. Manual model building, solvent addition and refinement employed phenix.refine in the Phenix software suite66.

**Small-angle X-ray scattering.** Proteins for SAXS analysis were concentrated to 10−15 mg mL−1 and dialyzed into a SAXS compatible buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM DTT). SAXS data were collected at EMBL P12 beamline, DESY, Hamburg, Germany with protein concentration ranges of 1.6–14.4 μg mL−1. The data were collected from the first point of the Guinier region until the end of the useful range defined by SHANUM55. EOM was used to generate an ensemble of conformations and to assess the inter-domain flexibility of the DH–PH domain. The scattering curves from the high-resolution models were calculated using CRYSTAL59. SAXS figures were prepared using PyMOL (www.pymol.org) and SASPy71.

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up to 20 min, when the intrinsic exchange reached the baseline. The exchange rate constants were fitted in Prism (GraphPad) and the enhancement factor was calculated in comparison to the intrinsic exchange rate without addition of EDTA or GEF protein.

**PIP strips lipid-binding assay.** PIP strips (Echelon Biosciences) were blocked in PBS-T + 3% BSA for 1 h at room temperature before adding 20 mM of the target proteins for another 1 h at room temperature. Membranes were then washed three times in PBS-T and incubated with an anti-His antibody (Qiagen) in PBS-T + 3% BSA overnight at 4 °C. After washing again three times in PBS-T, detection was achieved with a secondary anti-mouse antibody coupled to an IRDye800 (1 h, room temperature) on the Li-Cor Odyssey imaging system (Li-Cor Biosciences) and quantification was done in the Image Studio software (Li-Cor Biosciences).

**LiMA experimental procedure.** The fabrication of liposome microarrays, the experimental procedure of protein–liposome interaction assay, the image analysis, and the calculation of normalized binding intensities (NBIs) values were described previously. Briefly, liposomes were formed in the assay buffer (10 mM HEPES, pH 7.4, 150 mM NaCl) from lipid mixtures containing various combinations of 17 signalling lipids (Supplementary Data 2). The liposomes were incubated 20 min with different concentrations of purified siGFP-tagged proteins or cell extracts incubating with the cells with plasmids with kmst in for binding to c-Abl (supplied proteins), prepared as described previously (Supplementary Fig. 8). Subsequently, the unbound material was washed and the interactions were monitored by automated microscopy. Fluorescence intensities from pixels matching liposomal membranes were extracted and used for calculation of normalized binding intensities (NBIs).

**Immunofluorescence microscopy.** HeLa cells were transfected with vectors encoding p210, mutants thereof or p190 under the control of an SV40 promoter by using PEI transfection reagent (polyethylenimine, Polysciences). After 48 h of incubation, the medium of transfected cells was removed and the cells were quickly washed with phosphate buffer saline (PBS) equilibrated at 37 °C. Coverslips were then incubated in 3% paraformaldehyde (PFA). After 15 min incubation on ice, the cells were washed three times 5 min with PBS. The cells were permeabilized for 10 min at room temperature with PBS + 0.5% Triton X-100 and blocked with a commercial 10% normal goat serum blocking solution (Invitrogen 50-1972Z) for 10 min. Incubation with the primary antibody solution (pAb mouse anti-HA.11, Covance, dilution 1:1000 in PBS or pAb anti-myc, Cell Signaling, dilution 1:8000 in PBS) was performed for 1 h and followed by three washes with PBS + 0.1% Triton X-100. Then, the secondary antibody solution (goat antimouse Alexa-488 from Thermo Scientific, R37120, dilution 1:1000 in PBS) was applied for 30 min. After application of the secondary antibody, all incubation steps were performed protected from light. After washing, the actin cytoskeleton was stained by incubating the cells with phalloidin-rhodamin for 20 min according to supplier instructions (Thermo Scientific, R415, phallolidin-rhodamin reconstituted in methanol and dilution: 5 μL methanolic stock solution into 200 μL PBS + 1% BSA). After three times 5 min washes in PBS + 0.1% Triton X-100, DNA was stained with Hoechst for 10 min (dilution 1 μg mL⁻¹ in PBS). Finally, three times 5 min washes in PBS + 0.1% Triton X-100, coverslips were mounted on a microscopic slide using Mowiol. Images were obtained on a Zeiss LSM780 confocal microscope using a Plan-Apochromat 63x/1.40 Oil DIC M27.

**Actin co-localization.** All images were acquired with the same confocal microscope settings (74 nm pixel size, 355 nm z-plane spacing, 488 nm laser excitation and 533 nm detection band for Alexa-488 immunostained constructs, 566 nm laser excitation and 634 nm detection band for phallolidin-rhodamin stained actin, with same laser powers and detector gains for all samples). Cells with similar shape and densities (NBI).

**Bcr-Abl immunoprecipitation.** A detailed description of the Bcr-Abl immunoprecipitation was described previously. Bcr-Abl isoforms and mutants were expressed in murine Ba/F3 cells (DSMZ no. ACC 380) and cell lysis and purification was performed in TAP buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5% glycerol, 0.2% (v/v) NP-40 Alternative) containing protease and phosphatase inhibitors (25 mM sodium fluoride, 1 mM vanadate, 1 mM PMSE, 10 μg mL⁻¹ TPCK, 1X Complete protease inhibitor (Roche)). Normal mouse IgG antibody (Thermo scientific, catalog no. 10400C) and the monoclonal Abl antibody (clone 24–21) were covalently coupled to NHS-activated sepharose (GE Healthcare). The lysates were pre-cleared for 1 h at 4 °C with the IgG-NHS resin and the supernatant then transferred to the Ab-NHS resin for another 3 h at 4 °C. Elution fractions containing Bcr-Abl were pooled.

**Data availability.** Coordinates have been deposited in the Protein Data Bank with the accession codes: 5N6R (DH NMR structure), 5N7E (DH/Mb(Bcr-DH_4) and 5O2C (PH/Mb(Bcr-PH_4)). NMR chemical shift assignments are deposited in BMRB entry 34101. The collected SAXS data and the generated high-resolution hybrid models have been deposited at SASDB (entry SASDC26, SASDC36, SASDC47). Other data are available from the corresponding author upon reasonable request.

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Author contributions
S.R., F.L., A.R., V.D., and F.P. conducted and analyzed experiments in Fig. 2. A.P. and D.S. conducted and analyzed experiments in Fig. 3. S.R., C.G., and A.-C.G. conducted and analyzed experiments in Figs. 5, 6. S.R. and D.H. conducted and analyzed experiments in Fig. 4. S.G. and S.P. contributed experiments to Fig. 6. B.G. contributed experiments in Fig. 5b. S.G. provided technical assistance and vital tools for all experiments. L.B. and P.G. provided important bioinformatics support for NMR structural data analysis. T.K., A.K., and S.K. designed and conducted monobody generation and analysis. S.R. and O.H. coordinated this study, designed the experiments, interpreted the data and wrote the manuscript.

Additional information
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