Conformational sampling of membranes by Akt controls its activation and inactivation

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The protein kinase Akt controls myriad signaling processes in cells, ranging from growth and proliferation to differentiation and metabolism. Akt is activated by a combination of binding to the lipid second messenger PI(3,4,5)P3 and its subsequent phosphorylation by phosphoinositide-dependent kinase 1 and mechanistic target of rapamycin complex 2. The relative contributions of these mechanisms to Akt activity and signaling have hitherto not been understood. Here, we show that phosphorylation and activation by membrane binding are mutually interdependent. Moreover, the converse is also true: Akt is more rapidly dephosphorylated in the absence of PI(3,4)P2, an autoinhibitory process driven by the interaction of its PH and kinase domains. We present biophysical evidence for the conformational changes in Akt that accompany its activation on membranes, show that Akt is robustly autoinhibited in the absence of PI(3,4)P2 irrespective of its phosphorylation, and map the autoinhibitory PH–kinase interface. Finally, we present a model for the activation and inactivation of Akt by an ordered series of membrane binding, phosphorylation, dissociation, and dephosphorylation events.

kinase | Akt | HDX-MS | SAXS | allostery

The protein kinase Akt/protein kinase B (PKB) plays critical roles in cell growth and survival, differentiation, and metabolism, as well as general cellular homeostasis (1). Hyperactivation of Akt is associated with cancer and tissue overgrowth disorders (2-5), while inactivation of Akt2 leads to insulin resistance in mice (6) and a severe form of inherited diabetes in humans (7). Akt is activated downstream of growth factor or hormone receptors that stimulate phosphatidylinositol-3-kinase (PI3K) activity at the plasma membrane (8). The product of PI3K, phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3], activates Akt by recruiting it to the plasma membrane, where it is phosphorylated on two sites in its kinase domain, T308 and S473, critical for its catalytic activity (9, 10). A third, constitutive, phosphorylation site at T450 in the turn motif is essential for Akt folding and stability (11).

We recently demonstrated the dependency on the signaling lipids PI(3,4,5)P3 and PI(3,4)P2 for Akt activation and activity in the cell (12). PI(3,4)P2 is produced in the cell by class II PI3K (13), or by SHIP1/2-mediated hydrolysis of PI(3,4,5)P3 (14), and, like PI(3,4,5)P3, is capable of recruiting Akt to membranes via its PH domain (12, 15). Binding to PI(3,4,5)P3 or PI(3,4)P2 results in a conformational change in Akt that relieves a sterical block to substrate binding and, together with activation loop and hydrophobic motif phosphorylation, leads to high-affinity substrate binding. Furthermore, disruption of the PH–kinase domain interaction leads to a fourfold increase in the affinity of Akt for PI(3,4,5)P3-containing membranes, indicating that the binding site is at least partially occluded in the inactive conformation. Conformational changes in Akt associated with membrane binding have previously been postulated on the basis of biochemical and cell biological studies (16–21), computational modeling (22, 23), or structures of truncated Akt in complex with inhibitors (24–26), but the exact nature of the conformational change is unknown.

We present here direct biophysical evidence for a large conformational change in Akt associated with membrane binding, and find that a mutant that disrupts the autoinhibitory interactions between the PH and kinase domains mimics these conformational changes. We show that its disruption leads to opening of the kinase and a more extended conformation. We also show, by determining the affinity of the PH–kinase domain interaction, that the inhibitory interface is relatively strong, and serves to maintain cytosolic Akt in a closed conformation 99% of the time at equilibrium. Finally, we present a detailed analysis of the conformational changes that accompany Akt activation by PI(3,4,5)P3 and demonstrate that, in addition to relieving a sterical block to substrate binding, the conformational changes govern both Akt activation and inactivation by phosphorylation and dephosphorylation, respectively. Importantly, we show that stoichiometric phosphorylation of Akt does not override PH domain-mediated autoinhibition in the absence of PI(3,4)P2. In conclusion, we show that Akt is activated by a series of conformational changes beginning with PI(3,4,5)P3 or PI(3,4)P2 binding, and followed by activation loop and hydrophobic motif phosphorylation. Conversely, reversal of those conformational changes upon dissociation from the membrane promotes Akt dephosphorylation and inactivation.

**Results**

**Akt Undergoes a Large Conformational Change upon PI(3,4)P2 Binding.** We previously described a mutation in the kinase domain of Akt1 (Akt1D323A D325A, referred to hereinafter as Akt1D4A) that

**Significance**

Akt is a paradigmatic lipid-activated kinase, which is frequently hyperactivated in human cancer. In the absence of PI(3,4,5)P3 or PI(3,4)P2, Akt is maintained in an inactive conformation by an inhibitory interaction between its membrane-binding PH domain and its kinase domain. Here, we describe the conformational changes associated with its binding to PI(3,4,5)P3, leading to disruption of the inhibitory PH–kinase interface, and its consequent activation by protein kinases. Intriguingly, we find that reversal of those conformational changes promotes its inactivation by protein phosphatases. The activation of Akt is thereby restricted to discrete membrane locations, and it is rapidly inactivated upon dissociation. We propose a model in which activation, substrate phosphorylation, and inactivation of Akt are tightly coupled to the membrane.

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confers enhanced substrate affinity, enhanced affinity for membrane-embedded PIP$_3$, and resistance to dephosphorylation (12). These observations imply a large conformational change in Akt1 that accompanies PIP$_3$ recognition. To obtain evidence for a conformational change between autoinhibited and membrane-bound Akt1, we used a combination of small-angle X-ray scattering (SAXS), hydrogen–deuterium exchange mass spectrometry (HDX-MS), and in vitro biochemistry.

All Akt1 constructs used in this study are illustrated in SI Appendix, Fig. S1. The domain architecture, phosphorylation profile (activation loop, turn motif, and hydrophobic motif), protease cleavage sites, and experimentally validated conformation of each construct are depicted. Recombinant wild-type Akt1 (Akt1$^{WT}$) isolated from baculovirus-infected insect cells is heterogeneous phosphorylated, with five major species isolated by high-resolution anion-exchange chromatography each differing by the mass of a single phosphate (SI Appendix, Fig. S2A). Tryptic digest mass spectrometry of pentakisphosphorylated Akt1 revealed up to 16 sites of phosphorylation throughout the protein, of which T308 in the activation loop and S473 in the hydrophobic motif are phosphorylated in ~10% and 0.2% of molecules, respectively (SI Appendix, Fig. S2B). To generate a chemically monodisperse sample suitable for further structural and biophysical studies, we engineered a mutant protein in which the linker is derived from the zebrafish Danio rerio, is primarily monophosphorylated when isolated from insect cells (SI Appendix, Fig. S2C and D). Human Akt1 (HsAkt1$^{WT}$), zebrafish Akt1 (DrAkt1$^{WT}$), and our chimeric Akt1$^{DrLink}$ bind P(3,4)P$_2$ equally in vitro (SI Appendix, Fig. S2E). Like HsAkt1$^{WT}$, Akt1$^{DrLink}$ is activated similarly by P(3,4)P$_2$ in vitro (SI Appendix, Fig. S2F), binds substrate with comparable affinity (SI Appendix, Fig. S2G), and is phosphorylated on both T308 and S473 in HeLa cells in response to insulin stimulation (SI Appendix, Fig. S3A). In contrast to Akt1$^{WT}$ and Akt1$^{DrLink}$, recombinant Akt1$^{DA}$ was observed to be hyperphosphorylated (SI Appendix, Fig. S3B), with ~57 to 70% of molecules phosphorylated on T308, and 26 to 28% phosphorylated on S473 (SI Appendix, Fig. S3C).

We next determined the solution structures of Akt1$^{DrLink}$ and Akt1$^{DA}$ by SAXS to characterize the conformational changes associated with disruption of the PH–kinase domain interaction. By applying samples to a size exclusion column in-line with the X-ray beam, we separated out any high molecular weight aggregates in our sample that would distort the subsequent analysis of particle parameters (SI Appendix, Fig. S4A). The raw scattering curves show a significant difference between the two proteins (Fig. 1A), readily appreciated from the calculated pair distribution functions of the two particles (Fig. 1B). Akt1$^{DA}$ did not exhibit a significant difference from Akt1$^{WT}$, while dephosphorylation of Akt1$^{WT}$ also did not significantly affect the scattering (SI Appendix, Fig. S4B–D). In contrast, Akt1$^{DA}$ exhibits a more extended conformation, with a 17% increase in its radius of gyration ($R_g$) and a 30% increase in the maximum dimension ($D_{max}$) of the particle (Fig. 1A and B and SI Appendix, Fig. S4 E–H). The Kratky plot shows that both the engineered (Akt1$^{DrLink}$) and Akt1$^{WT}$ have superimposable
bell-shaped curves, according to Porod’s law for globular macromolecules, while constitutively active Akt1 (Akt1D15), which mimics the membrane-bound conformation, exhibits an increase in random coil character (Fig. 1C). This is presumably due to the loss of interactions between the PH and kinase domains, and the consequent increase in flexibility, caused by the mutation.

We next compared the solution structure of Akt1 with the reported crystal structure of a truncated construct of Akt1 in complex with the allosteric inhibitor, inhibitor VIII (24). While the agreement between the experimental and theoretical scattering curves is not perfect (Fig. 1D), 20% of the scattering mass, including important regulatory regions (αC helix, activation loop, C-terminal tail), is missing in the crystal structure. Ab initio calculation of the molecular envelope shows that Akt1DrLink adopts a compact conformation, into which the structure of Akt1 in complex with inhibitor VIII fits reasonably well (Fig. 1D, Inset).

To evaluate the conformation of Akt1D15, we employed rigid body modeling (27) of the PH (residues 1 to 121) and kinase (residues 144 to 477) domains of Akt1 (Protein Data Bank (PDB) ID code 1UNP (28) and PDB ID code 4EKK (29), respectively) with an interdomain linker of 23 amino acids. The PH domain position was fixed and the kinase domain allowed to move according to the restraints imposed by the linker. Iterative cycles of rigid body modeling converged on a set of models in which Akt1 adopts a more compact than expected conformation, but with both the PIP3-binding surface of the PH domain and the substrate binding cleft of the kinase domain always solvent-exposed (Fig. 1E and SI Appendix, Fig. S5A).

Finally, we sought to evaluate the stability of the PH–kinase domain interface by comparing the thermal stability of Akt1DrLink with that of Akt1D15. Akt1D15 is destabilized by 8.5 °C with respect to Akt1DrLink (Fig. 1F), indicating that the PH–kinase interface considerably stabilizes the full-length protein. Dephosphorylation of both proteins (SI Appendix, Fig. S5 B and C) reduced their thermal stabilities by a further 2.5 °C to 3 °C (Fig. 1F), most likely by removing the constitutive stabilizing phosphorylation of T450 in the turn motif.

**Steady-State Autoinhibition of Akt by PH Domain Sequestration.** We previously observed that disruption of the PH–kinase domain interface not only rendered Akt1 insensitive to PIP3, but also enhanced its binding to PIP3-containing liposomes and its accumulation at the plasma membrane in response to growth factor stimulation of Akt (32). These observations imply that the PIP3-binding site is at least partially occluded in the autoinhibited conformation of Akt. Having also observed that a model substrate peptide could bind to autoinhibited Akt1, albeit at very low affinity, we concluded that inactive Akt most likely exists in an equilibrium of open and closed conformers.

To determine the position of the intramolecular equilibrium, and thereby estimate the degree of autoinhibition in the cytosol of unstimulated cells, we measured the affinity of the isolated PH domain or the isolated kinase domain for the isolated PH domain by fluorescence resonance energy transfer (FRET) (31) with that of Akt1D15. Akt1D15 is destabilized by 8.5 °C with respect to Akt1DrLink (Fig. 1F), indicating that the PH–kinase interface considerably stabilizes the full-length protein. Dephosphorylation of both proteins (SI Appendix, Fig. S5 B and C) reduced their thermal stabilities by a further 2.5 °C to 3 °C (Fig. 1F), most likely by removing the constitutive stabilizing phosphorylation of T450 in the turn motif.

PI(3,4)P2, or PIP2, binding to Akt stimulates the kinase activity and enhances its binding to PIP3 vesicles (36). This suggests that Akt activity is confined to the membrane environment, an observation corroborated by our previous findings in vivo, which showed that active Akt exhibits diffusion properties consistent with a membrane-bound species (12). However, the soluble polyphosphate inositol-1,3,4,5-tetrasphosphate (IP4) has also been recognized as an important second messenger in cells (30–32), so, to rule out the possibility that IP4 could activate Akt in the cytosol, we assayed the ability of IP4 to competitively displace the kinase domain of Akt from Ato-488-labeled PH domain. Consistent with the requirement for membrane-embedded PIP3 or PI(3,4)P2, we observed that IP4 was unable to displace the kinase domain at concentrations up to 330 μM (Fig. 2C) or activate Akt1 in an in vitro kinase assay under conditions in which Akt1 is activated by an equimolar concentration of PIP3 (Fig. 2D).

**PI(3,4)P2 Engagement Drives Conformational Changes Required for Activation.** Having established that Akt1 undergoes a large conformational change upon disruption of the PH–kinase domain interface, we sought to map the conformational changes associated with both PIP3 binding and Akt phosphorylation in more detail using HDX-MS. HDX-MS is an analytical technique that measures the exchange rate of amide hydrogens with solvent, and, as the main determinant of amide exchange is involvement in secondary structure, it can be used as a readout of protein conformational dynamics (33). It has been used as a powerful tool to examine protein conformational changes that occur upon membrane recruitment (34–36).

To evaluate the effect of both PIP3 binding and the mutation in Akt1D15, we compared HDX in Akt1DrLink and Akt1D15 both in solution and bound to PIP3-containing liposomes. We used chemically monodisperse Akt1DrLink in place of Akt1D15 for this analysis as it allowed us to examine the protein conformation of the maximally inhibited form of Akt1 (Akt1DrLink) with the hyperactivated form (Akt1D15). All HDX peptide data for Akt1DrLink and Akt1D15, in the presence and absence of PIP3-containing vesicles, can be found in SI Appendix, Figs. S7 and S8 respectively.

We observed significant changes in the rates of H/D exchange in a number of peptides corresponding to both the PH and kinase domains (SI Appendix, Fig. S9) that occurred upon membrane binding, and between the activated and inactivated forms in solution. There were no differences in the exchange rates in Akt1DrLink when exposed to liposomes lacking PIP3 (SI Appendix, Fig. S10), verifying that conformational changes were not due to a nonspecific membrane effect. To determine the conformational changes that occurred upon binding of the inhibited form of Akt1 (Akt1DrLink) to PIP3-containing membranes, we examined the differences in the rates of H/D exchange between free (unbound) and PIP3-bound Akt1 (Fig. 3A). By comparing these changes to those observed in the activated form of Akt1D15 bound to membranes, we could separate conformational changes driven by membrane interaction of the PH domain, compared with those mediated by disruption of the PH–kinase domain interface. We observed, in both proteins, that the rate of exchange is dramatically lower over the entire PH domain in the presence of PIP3 vesicles, consistent with its protection upon binding (Fig. 3A and B). Larger decreases in exchange in the PH domain of Akt1D15 compared with Akt1DrLink are most likely reflected in the competition between the kinase domain and PIP3 for the PH domain in Akt1DrLink (and the consequent enhanced affinity of Akt1D15 for PIP3) such that, under the same conditions, Akt1D15 is more tightly bound to the membrane (12) and therefore exhibits greater protection of its PH domain.

PI(3,4)P2 binding also resulted in a dramatic deprotection of residues in the kinase domain of Akt1DrLink encompassing the activation loop and helix αG in the C lobe, consistent with this surface of the C lobe being the major surface of interaction with the PH domain. Akt1D15 showed much smaller increases in exchange upon PIP3 binding, consistent with the mutation destabilizing this interface. These observations indicate that the nonphosphorylated activation loop is sequestered in the autoinhibited state of Akt, consistent with biochemical studies showing that PIP3 binding enhances phosphoinositide-dependent kinase 1 (PDK1-dependent activation loop phosphorylation (16, 17).

Curiously, increases in exchange are also seen upon membrane binding in residues 218 to 225 in strands β4 and β5 of the N lobe, but only in Akt1DrLink. Strands β4 and β5, together with helices αB and αC, were not exposed (Fig. 2E), which corresponds to a fraction of the closed, PH domain bound, conformer equal to 98.8% at equilibrium (Fig. 2B). This is further increased to 99.9% if one assumes a more compact interdomain linker (SI Appendix, Fig. S5A) rather than the maximum contour length of a 23-amino acid peptide.

Activation. The presence of PIP3, or PI(3,4)P2, suggests that Akt activity is confined to the membrane environment, an observation corroborated by our previous findings in vivo, which showed that active Akt exhibits diffusion properties consistent with a membrane-bound species (12). However, the soluble polyphosphate inositol-1,3,4,5-tetrasphosphate (IP4) has also been recognized as an important second messenger in cells (30–32), so, to rule out the possibility that IP4 could activate Akt in the cytosol, we assayed the ability of IP4 to competitively displace the kinase domain of Akt from Ato-488-labeled PH domain. Consistent with the requirement for membrane-embedded PIP3 or PI(3,4)P2, we observed that IP4 was unable to displace the kinase domain at concentrations up to 330 μM (Fig. 2C) or activate Akt1 in an in vitro kinase assay under conditions in which Akt1 is activated by an equimolar concentration of PIP3 (Fig. 2D).
αC, form the hydrophobic pocket on the N lobe of the kinase domain that accommodates the hydrophobic motif in the C-terminal tail of Akt. Q218 makes a hydrogen bond to phospho-S473 in the activation loop, and catalytic cleft (Fig. 3C, form the hydrophobic pocket on the N lobe of the kinase domain to form a PH:KD complex (black dashed line). 

Since it has been previously proposed that phosphorylation could render Akt active in the absence of PIP3 (18, 20, 38, 39), no interference from different phosphorylation states. Compared with dephosphorylated Akt1DrLink, dephosphorylated Akt1 DA exhibited depletion of the kinase C lobe and, in particular, the PH domain, consistent with exposure of the PIP3 binding site, activation loop, and catalytic cleft (Fig. 3C) and replicating the deprotection of the kinase domain exhibited by Akt1 DrLink when bound to PIP3 liposomes (Fig. 3A). This observation unambiguously rules out hyperphosphorylation of Akt1 DA as the mediator of the conformational changes.

To rule out that stoichiometric phosphorylation of Akt could drive the observed conformational changes in the absence of PIP3, we phosphorylated Akt1 DrLink and Akt1 KD with PDK1 in vitro (SI Appendix, Fig. S11A) to generate Akt1DrLink pT308 S473D and Akt1 KD. The binding curve was constructed by making serial dilutions in buffer containing 100 nM Atto488-labeled PH domain. Error bars are the SD of 50 measurements from three independent experiments. (B) Calculation of the position of equilibrium between open and closed states of Akt1 using a sphere of influence to estimate the local concentration of the two domains with respect to each other. (C) Competitive displacement of kinase domain from PH domain with IP₃ measured by fluorescence anisotropy; 100 nM Atto488-labeled PH domain was incubated with 280 μM unlabeled kinase domain to form a PH:KD complex (black dashed line). IP₃ does not result in dissociation of the PH domain (red dashed line) at concentrations up to 330 μM. Error bars are the SD of 50 independent measurements. (D) In vitro kinase assay of Akt in the presence of PIP₃-containing liposomes or soluble Ins(1,3,4,5)P₄. Error bars are the SD of triplicate measurements.

Fig. 2. Steady-state autoinhibition of Akt by PH domain sequestration. (A) Determination of the affinity of the kinase domain for the PH domain of Akt1 by fluorescence anisotropy. Akt1KD T101C was labeled in vitro with Atto488 (see Materials and Methods); 100 nM Atto488-labeled PH domain was incubated with 220 μM Akt1KD. The binding curve was constructed by making serial dilutions in buffer containing 100 nM Atto488-labeled PH domain. Error bars are the SD of 50 measurements from three independent experiments. (B) Calculation of the position of equilibrium between open and closed states of Akt1 using a sphere of influence to estimate the local concentration of the two domains with respect to each other. (C) Competitive displacement of kinase domain from PH domain with IP₃ measured by fluorescence anisotropy; 100 nM Atto488-labeled PH domain was incubated with 280 μM unlabeled kinase domain to form a PH:KD complex (black dashed line). IP₃ does not result in dissociation of the PH domain (red dashed line) at concentrations up to 330 μM. Error bars are the SD of 50 independent measurements. (D) In vitro kinase assay of Akt in the presence of PIP₃-containing liposomes or soluble Ins(1,3,4,5)P₄. Error bars are the SD of triplicate measurements.
a basic patch of residues on the N lobe comprising K158, K163, K182, and R222, all of which are found in the protected peptides.

Having established that phosphorylation does not result in major conformational changes in Akt in the absence of PIP₃, we tested the hypothesis that the kinase activity of phosphorylated Akt1 should be PIP₃-dependent. As expected, phosphorylation fails to override the activation by both P1(3,4,5)P₃ and P1(3,4)P₂ (Fig. 3F and SI Appendix, Fig. S14), consistent with the formation of the autoinhibited conformation in the absence of membrane binding, irrespective of phosphorylation.

In summary, Akt1 adopts a compact, autoinhibited conformation in the absence of PIP₃ irrespective of its phosphorylation state. In solution, the substrate binding cleft, including the activation loop and catalytic loop, is sequestered in an inactive conformation, while the PIP₃-binding pocket is at least partially occluded in the interface. Upon membrane binding, the PH domain, activation loop, and possibly also the C-terminal tail are occluded in the interface. Upon membrane binding, the PH domain, activation loop, and possibly also the C-terminal tail are occluded in the interface. Upon membrane binding, the PH domain, activation loop, and possibly also the C-terminal tail are occluded in the interface.

Membrane and ATP Binding Cooperatively Protect Akt from Dephosphorylation. We previously showed that Akt is more rapidly dephosphorylated in vivo in the presence of its PH domain (12), while other studies have demonstrated a role for ATP in stabilizing the phosphorylated, active conformation (29, 40, 41). Having observed that, in the absence of PIP₃, Akt adopts an autoinhibited conformation in which the phosphorylated activation loop is exposed (Fig. 3D), we hypothesized that ATP-dependent caging of the regulatory phosphates could only be possible in the context of membrane binding, since the PH domain occludes the docking surface on the kinase domain for the phosphorylated activation loop. To establish whether phosphorylated Akt is stabilized by ATP in the context of membrane binding, but efficiently dephosphorylated in solution, we evaluated the dephosphorylation kinetics at T308 and S473 under conditions mimicking membrane binding (Akt1DA or isolated kinase domain) or free in solution, in the presence and absence of ATP.

The active conformation of the isolated kinase domain of Akt exhibits a network of interactions between the N and C lobes of the kinase domain, stabilized by ATP, and both the phosphorylated activation loop (T308) and hydrophobic motif (S473) (41, 42). We first established that phosphorylation of these two residues leads to an almost fourfold higher affinity for ATP by comparing the constitutively active Akt1(3C) (hyperphosphorylated on T308 and S473 under conditions mimicking membrane binding (Akt1DA or isolated kinase domain)) or free in solution, in the presence and absence of ATP.

To test whether ATP-dependent caging of pT308 is context-dependent, we took advantage of 3C- cleavable Akt1(3C) (SI Appendix, Fig. S1) that contained a phosphomimetic amino acid at S473 (41, 42). To test whether whether Akt1(3C) has a PIP₃-dependent ATPase in vivo, we measured the ATPase activity of Akt1(3C) and Akt1(3C) in the presence and absence of ATP.

Having observed that, in the absence of PIP₃, Akt adopts an autoinhibited conformation in which the phosphorylated activation loop is exposed (Fig. 3D), we hypothesized that ATP-dependent caging of the regulatory phosphates could only be possible in the context of membrane binding, since the PH domain occludes the docking surface on the kinase domain for the phosphorylated activation loop. To establish whether phosphorylated Akt is stabilized by ATP in the context of membrane binding, but efficiently dephosphorylated in solution, we evaluated the dephosphorylation kinetics at T308 and S473 under conditions mimicking membrane binding (Akt1DA or isolated kinase domain) or free in solution, in the presence and absence of ATP.
dephosphorylation in the absence of ATP (Fig. 4B), it is dramatically protected from dephosphorylation in the presence of ATP compared with full-length, autoinhibited Akt1 (Fig. 4C). The protection of pT308 in the absence of ATP likely arises from a lower affinity interaction between the activation loop and the C lobe of the kinase domain even in the absence of ATP. Curiously, in the presence of ATP, both curves can be fit to a monoexponential decay with the same time constant, but, while full-length Akt1 is almost completely dephosphorylated in the course of the assay, the kinase domain is only ∼20% dephosphorylated. This suggests that a fraction of the kinase domain cannot be protected by ATP, perhaps due to substoichiometric turn motif phosphorylation.

We next compared the dephosphorylation kinetics of hyper-phosphorylated Akt1DA, which has a disrupted PH–kinase interface, with those of Akt1SC, S473D (Fig. 4D). Like the isolated kinase domain, Akt1DA exhibited identical dephosphorylation kinetics to Akt1SC S473D in the absence of nucleotide (Fig. 4E), but, in the presence of ATP, Akt1SC S473D was dephosphorylated on T308 significantly faster than Akt1DA (Fig. 4F), indicating that interaction of the PH and kinase domains renders the activation loop more accessible for dephosphorylation. While the isolated kinase domain is robustly protected by ATP (Fig. 4C), Akt1DA is completely dephosphorylated, albeit with slower kinetics than Akt1SC S473D. This is likely due to residual interaction between the kinase and PH domains of Akt1DA (Fig. 3B) that destabilize the phosphorylated activation loop even in the presence of ATP.

Finally, we investigated the stability of the phosphorylated hydrophobic motif in the context of membrane binding. To demonstrate that this is an intrinsic property of Akt, we incubated Akt1WT with liposomes containing 0 mol% or 5 mol% PIP3 in the presence or absence of ATP (Fig. 4G). We observed that Akt1 is significantly protected from hydrophobic motif dephosphorylation in the presence of PIP2-containing liposomes (Fig. 4H) and that this is further enhanced by ATP binding (Fig. 4I). It should be noted that a technical limitation of this experiment is the requirement to keep magnesium concentrations low enough to support PIP2 binding (12). Given the affinity for ATP (∼100 μM), only 50% of Akt1 will be ATP-bound under these experimental conditions (100 μM ATP, 200 μM MgCl2), and therefore the protection of the hydrophobic motif by ATP and PIP2 is significantly underestimated.

In conclusion, Akt is protected from dephosphorylation in an ATP- and PIP2-dependent manner. While PIP2-bound, Akt is protected from dephosphorylation by ATP-dependent caging of its regulatory phosphates, but, upon dissociation, Akt is rapidly dephosphorylated and inactivated.

Discussion
Protein kinases transfer phosphate from ATP to an acceptor serine, threonine, or tyrosine residue in a protein chain. While some specificity may be achieved by recognition of the primary acceptor sequence, the stereochemistry of phosphotransfer is essentially the same for the more than 500 human protein kinases (43). Therefore, in order for the cell to perform the myriad signaling reactions transduced by protein kinases, it must regulate their activity, both spatially and temporally, within the cell. Various mechanisms of protein kinase regulation have been described, including activation loop phosphorylation, steric occlusion of the catalytic cleft, requirement for accessory proteins, autoinhibition by regulatory domains, complex formation by scaffolding proteins, and spatial segregation of kinase and substrate (44–48).

The protein kinase Akt phosphorylates substrates involved in growth, survival, differentiation, and metabolism, with over 100 reported substrates (1). Despite the fact that not all substrates have been carefully validated, the evidence points to a context-dependent phosphorylation of a diverse array of downstream effectors by Akt, necessitating its tight regulation. Akt activity depends strictly on activation by PKD1 and mechanistic target of rapamycin complex 2 (mTORC2) at the plasma membrane, following growth factor stimulation. The localization of substrates in subcellular compartments distal to the plasma membrane, however, led to the proposal that Akt could disassociate from the membrane, locked in an active conformation (17, 18, 20, 38, 39). By diffusion, Akt would encounter its substrates within the cell, and its activity would be controlled by the rate at which phosphatases inactivated it (38, 39). This model, however, proposes a scenario in which Akt activity is uncoupled from its activating stimulus, as well as spatially delocalized in the cell, which would serve to diminish, rather than enhance, substrate specificity.

We recently demonstrated that the activity of Akt is strictly confined to membranes containing either PIP3 or PI(3,4,5)P3. PI(3,4,5)P3 and PI(3,4)P2 allosterically activate Akt by relieving steric occlusion of the substrate binding cleft (12). While previous studies failed to observe the direct activation of Akt by PI(3,4,5)P3 (9, 16), the reported kinase assays were performed under conditions of high magnesium (5 mM to 10 mM), which significantly attenuates Akt binding to PI(3,4,5)P3 (12) and, hence, its activation. This has likely obscured the observation of direct activation of Akt by PIP3 in previous studies.

We now show that autoinhibited Akt forms a compact structure in which the PH and kinase domains are held together by a relatively tight intramolecular interaction that sequesters the PIP2 binding site in the interface. The strength of the interaction coupled with the high local concentration of the kinase and PH domains guarantees close association of the PH–kinase domain surrounding D323 and D325 but also with the unphosphorylated activation loop conformation inaccessible to more than 99% of the time at equilibrium. Presumably, the small fraction (∼0.1 to 1.0%) of Akt in the open conformation at equilibrium is sufficient to sense and respond to PIP3, which essentially shifts the equilibrium to the open, membrane-bound conformation. The soluble polyphosphate inositol IP6 is unable to displace this inhibitory interaction or activate Akt at a concentration 100 times that reported in cells (49), reinforcing the absolute requirement for binding to PIP3 or PI(3,4)P2. The inability of IP6 to activate Akt is consistent with previous observations that the active conformation of Akt in cells is associated with a membrane-bending effect (12). This separate study has also implicated the bulk phospholipid phosphatidylserine in promoting PIP3 binding and Akt activation (50).

Mutation of two surface-exposed aspartates on the kinase domain led to PIP3-independent Akt activity. Akt hyperphosphorylation, and high-affinity substrate binding independent of membrane binding (12). Implicit from this work was a large conformational change that accompanies Akt activation, which we have now observed directly in solution. The extended conformation of Akt1DA confers a large conformational change in which the PH–kinase interface, which exerts a strong stabilizing effect over the entire Akt1WT molecule as indicated by the reduced thermal stability of Akt1DA.

HDX-MS allowed us to map experimentally the conformational changes that accompany Akt binding to membrane-localized PIP3. The surfaces of interaction identified are consistent with cross-linking mass spectrometry (19) and the relative positions of the PH and kinase domains observed in the structure of Akt1 bound to the allosteric inhibitor VIII (24). We now show, however, that the PH domain interacts not only with the C lobe of the kinase domain surrounding D323 and D325 but also with the unphosphorylated activation loop. The deprotection of the activation loop upon PIP3 binding is consistent with previously reported biochemistry that showed a dependency on PIP3 binding for PKD1 phosphorylation (16, 17). Interestingly, deprotection is also seen for the region of the N lobe that forms the hydrophobic pocket into which the hydrophobic motif of the C-terminal tail binds following its phosphorylation (37). This observation suggests that the unphosphorylated hydrophobic motif likely docks to the same pocket in the autoinhibited conformation of Akt, thereby restricting the availability of the C tail to mTORC2. The docking of the C tail to the hydrophobic pocket in the inactive state was previously proposed (23) but lacked direct evidence, while deletion of the PH domain of Akt was also observed to promote hydrophobic motif phosphorylation in the absence of mTORC2 (51). In summary, both the activation loop...
and hydrophobic motif are sequestered in the autoinhibited conformation of Akt, requiring PIP3 binding to drive their displacement and consequent accessibility for phosphorylation.

While it has been previously proposed that phosphorylation of Akt could lock it in an active conformation able to dissociate from the plasma membrane and phosphorylate substrates elsewhere in the cell (18, 20, 38), we observe that Akt adopts an autoinhibited conformation even when stoichiometrically phosphorylated. Notwithstanding the fact that this conformation blocks substrate binding, we also observed that Akt is efficiently dephosphorylated in the absence of membrane binding. Although our observations reinforce the ATP-dependent caging of pT308 and pS473 (29, 40, 39), we observe that Akt adopts an autoinhibited conformation even when stoichiometrically phosphorylated. Notwithstanding the fact that this conformation blocks substrate binding, we also observed that Akt is efficiently dephosphorylated in the absence of membrane binding.
41), we show that PIP3 binding cooperates with ATP in protecting Akt from dephosphorylation. Dissociation from PIP3 therefore not only results in PH domain-mediated autoinhibition, but also drives Akt inactivation by promoting its dephosphorylation. While not explicitly addressed, this context dependency was hinted at in a previous study in which the ATP-dependent caging of p7308 in full-length Akt1 was observed using immunoprecipitated myristoylated Akt1 incubated with lipids (40). It is therefore likely that the authors were in fact observing the ATP-dependent protection of p7308 in the context of activated, membrane-bound Akt1. Along the same lines, the paradoxical hyperphosphorylation of Akt caused by ATP-competitive Akt inhibitors (52, 53) may be a direct consequence of the inhibitor interfering with reformation of the autoinhibited conformation. Indeed, this is supported by the potentiation of membrane binding elicited by inhibitors and the requirement for a conformational change in addition to membrane localization to drive hyperphosphorylation (52). While hyperphosphorylated Akt isolated from inhibitor-treated cells was observed to be more active, increased substrate phosphorylation in vivo was not observed, consistent with the rapid dephosphorylation of Akt upon inhibitor removal. Concerns about Akt inhibitor-induced pathway activation in cancer patients are therefore likely unfounded.

In conclusion, Akt activation, substrate phosphorylation changes and phosphorylation events that accompany PIP3 pathway activation in cancer patients are therefore likely unfounded. Akt upon inhibitor removal. Concerns about Akt inhibitor-induced pathway activation in cancer patients are therefore likely unfounded.

**Materials and Methods**

**SAXS** SAXS data were collected on Akt1 proteins using an in-line size exclusion chromatography setup on BM29 at the European Synchrotron Radiation Facility (ESRF). Proteins were applied to a Superdex 200 column equilibrated in 20 mM Tris, pH 7.4, 100 mM NaCl, and 1 mM Tris carboxyethyl phosphine (TCEP), and images were acquired every second for the duration of the size exclusion run. Buffer subtraction was performed by averaging 50 frames either side of the peak. All subsequent data processing steps were performed using the ATSAS data analysis software 2.8.2. The program DAMMIF (58) was used to generate the pair distribution function $P(Q)$ for each isoform and to determine $D_{\text{max}}$ and $R_g$ from the scattering curves $I(q)$ vs. $q$ in an automatic, unbiased manner. Ab initio molecular envelopes for Akt1(Thr) were computed by 10 iterative cycles of simulated annealing starting with a dummy atom model in DAMMIF (58). The models were aligned, averaged, and filtered using DAMAVER (59). The structure of Akt1 (33) was compared with the scattering of Akt1(Thr) using CRYOSOL (60) and superimposed with the refined ab initio envelope using SUPCOMB (61). For Akt1(Thr), rigid body modeling was performed using CORAL (27), with PDB ID code 1UNP (PH domain) and PDB ID code 4EKK (chain A; kinase domain) as the starting rigid body models. Linker residues were implemented in CORAL as dummy residues. Iterative runs of CORAL were performed in which the kinase domain was allowed to move, while the PH domain was fixed.

**Differential Scanning Fluorimetry.** The thermal stabilities of Akt1 WT, Akt1(Thr), and their respective dephosphorylated species were measured by differential scanning fluorimetry (DSF). Akt1(Thr) S473D, in vitro phosphorylated on T308, was also measured. Samples contained 0.1 mg/mL of protein in 20 mM Tris pH 8.0, 100 mM NaCl, and 1 mM TCEP. Samples were measured in triplicate using a BioRad iQ5 Multicolor Real-Time PCR Detection System.

**Fluorescence Anisotropy.** The affinity of the kinase and PH domains for each other was determined by fluorescence anisotropy, using Atto488-labeled Akt1(Thr). Briefly, Akt1(Thr) harboring the mutation T101C was purified as described previously (15). The protein was incubated for 3 h at room temperature (RT) with a twofold excess of Atto488 maleimide (Atto-Tec), quenched with 0.5% (vol/vol) β-mercaptoethanol, and purified by size exclusion chromatography on Superdex 75 10/300 GL (GE Health) in 20 mM Tris, pH 7.4, 300 mM NaCl, 1 mM TCEP, and 1% (vol/vol) glycerol. Fractions containing monomeric, labeled Akt1(Thr) were collected and concentrated. The final concentration of Akt1(Thr) was determined to be 15.5 μM at 280 nm, or 7.3 μM using the extinction coefficient of the dye, indicating ~50% labeling efficiency. Concentrated Akt1(Thr) was incubated with 100 nM Atto488-Akt1(Thr) in 20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM TCEP, and 1% (vol/vol) glycerol. The binding curve was constructed by making serial dilutions of Akt1(Thr) in the same buffer containing 100 nM Atto488-Akt1(Thr). Measurements were made with a Perkin-Elmer LS50B fluorimeter ($I_0 = 502$ nm, $I_\infty = 520$ nm) at 25 °C. For each data point, 50 measurements, each with an integration time of 1 s, were averaged. Each experiment was performed three times.

**HDX-MS.** HDX-MS experiments were similar to those described in refs. 34–36. In brief, HDX experiments were conducted in 50 μL reactions with a final concentration of 400 nM for Akt1(Thr) dephos./Akt1(Thr) Thr, 400 nM for Akt1(Thr) Thr, 291 nM for Akt1(Thr) Thr, 260 nM for Akt1(Thr) Thr, 200 nM for Akt1(Thr) Thr, and 400 nM for Akt1(Thr) Thr. Eight conditions were tested: Akt1(Thr) (i) alone and (ii) with lipid vesicles (20% cholesterol, 30% phosphatidylcholine (PC), 15% phosphatidylserine (PS), 35% phosphatidylethanolamine (PE), and 5% PIP3) present at 400 μM final concentration and (iv) with lipid vesicles containing PIP3 (20% cholesterol, 30% PC, 15% PS, 35% PE, and 5% PIP3) at 400 μM final concentration and (iii) with lipid vesicles containing no PIP3 (20% cholesterol, 30% PC, 20% PS, and 35% PE) present at 400 μM final concentration; (iv) Akt1(Thr) Thr alone; (vi) Akt1(Thr) Thr alone; and (vii) Akt1(Thr) Thr alone. For conditions with vesicles, protein was allowed to incubate with lipid vesicles for 2 min before initiation of deuterium exchange. Deuterium exchange was initiated by adding 40 μL of deuterium oxide [10 mM HEPES pH 7.5, 100 mM NaCl, 98% (vol/vol) D2O]. Exchange was carried out for 4 h (10 min at 90 °C) with an additional 3-s time point on ice (2 min with ice) and terminated by the addition of 20 μL of ice-cold quench buffer (2 M guanidine HCI, 3% formic acid). Samples were immediately frozen in liquid nitrogen and stored at −80 °C.

Protein samples were rapidly thawed and injected onto an ultra-performance liquid chromatography (UPLC) system at 2 °C. Protein was run over two immobilized papain columns (porosyme, 2-3131-00; Applied Bio- systems) at 10 °C and 2 °C at 200 μL/min for 3 min, and peptides were collected onto a VanGuard precolumn trap (Waters). The trap was subsequently eluted in line with an Acquity 1.7-μm particle, 100 × 1 mm i.d. C18 UPLC column (Waters), using a gradient of 5 to 36% B (buffer A 0.1% formic acid, buffer B 100% acetonitrile) over 16 min. Mass spectrometry experiments were performed on an Impact II TQD (Bruker) acquiring over a mass range from 150 m/z to 2,200 m/z using an electrospray ionization source operated at a temperature of 200 °C and a spray voltage of 4.5 kV. Peptides were identified using data-dependent acquisition methods following tandem MS/MS experiments (0.5-s precursor scan from 150 m/z to 2,000 m/z; 12 0.25-s fragmentation scans from 150 m/z to 2,000 m/z). MS/MS datasets were analyzed using PEAKS (PEAKS), and a false discovery rate was set at 1% using a database of purified proteins and known contaminants.

**HD-Examiner Software (Sierra Analytics)** was used to automatically calculate the level of deuterium incorporation into each peptide. All peptides were manually inspected for correct charge state and presence of...
overlapping peptides. Deuteration levels were calculated using the centroid of the experimental isotope clusters. Results for these proteins are presented as relative levels of deuterium incorporation, and the only control for back-exchange was the level of deuterium present in the buffer (76.92 to 86.53%). The average error of all time points and conditions for each HDX project was 0.7% and 0.1 Da. Therefore, changes in any peptide at any time point greater than both 7% and 0.4 Da between conditions with a paired t-test value of $P < 0.05$ was considered significant and used to generate Fig. 3. All deuteration exchange data for all experiments are shown in SI Appendix, Figs. S7, S8, and S12, with deuterium incorporation graphs for selected peptides highlighted in Fig. 3 shown in SI Appendix, Figs. S9 and S13.

**Fig. 5.** Stepwise activation of Akt at membranes and inactivation in the cytosol. Autoinhibited Akt is characterized by a PH-in conformation in which its PIP$_3$-binding site is sequestered by interaction with the kinase domain and its PH domain blocks substrate binding. At equilibrium, a small fraction of Akt in which the interface has relaxed to a more open conformation is able to sample the membrane for PIP$_3$ or PIP$_2$, engagement of which leads to displacement of the PH domain from the kinase domain and concomitant exposure of the two regulatory phosphorylation sites in the activation loop (T308) and hydrophobic motif (S473). Phosphorylation of these sites by PDK1 (T308) and mTORC2 (S473) leads to conformational changes in the kinase domain, docking of the phosphorylated motifs, and the high-affinity binding of ATP.Mg$^{2+}$. As long as Akt remains membrane-bound, the phosphorylated residues are protected from dephosphorylation by the high-affinity interaction with ATP.Mg$^{2+}$. However, upon termination of the PIP$_3$ signal, dissociation from the membrane rapidly leads to inhibition of the kinase domain by the PH domain and concomitant exposure of the activation loop and hydrophobic motif for dephosphorylation. In this way, Akt activation and activity are acutely restricted to the membrane, while it is inactivated in the cytosol. States A and E are modeled on the structure of Akt in complex with inhibitor VIII (PDB ID code 3O96). Protected/deprotected residues in the PH domain are colored orange, and those on the kinase domain are blue (activation loop), magenta (catalytic loop), yellow (kinase N lobe), and cyan (C-terminal tail). The kinase domain in state D is taken from Akt in complex with a substrate peptide derived from GSK3β (green; PDB ID code 1O6K).

**In Vitro Dephosphorylation of Akt1.** Akt1 was dephosphorylated in vitro using lambda phosphatase (made in-house). Briefly, Akt1 (0.375 μM) was incubated with 100 ng to 150 ng of lambda phosphatase in a 50-μL reaction at RT. Aliquots were taken at fixed time points, mixed with SDS loading buffer, and heat-inactivated at 95 °C for 2 min. Samples were blotted onto nitrocellulose membranes and blocked with 5% BSA in 1× TBS + 0.1% TWEEN 20. Phosphorylated Akt was detected with antibodies against pT308 or pS473 (#C31E5E and #193H12, respectively; Cell Signaling Technology). Data reported in Fig. 4 B and C were obtained by Western blotting of dephosphorylation reactions containing both full-length Akt13C S473D and Akt1KD S473D. The blots were incubated simultaneously with mouse and
rabbit primary antibodies against pan-Akt and pT308, respectively, washed, and developed with IRdye-conjugated anti-mouse IgG (700 nm) and IRdye-conjugated secondary antibodies (LI-COR). Fluorescence was measured on a LI-COR Odyssey CLX infrared detector. Image data reported in Fig. 4 E, F, H, and I were obtained by developing Western blots with HRP-conjugated anti-ribosomal IgG secondary antibody and ECL. Select Western blotting detection reagent (Amersham). Chemiluminescence was measured on a Fusion FX7 Advance (Piqlab), and the phospho-Akt signal was quantified in ImageJ. For dephosphorylation of S473, 0.375 μM Akt1 was incubated with sucrose-loaded vesicles containing 0 mol % PIP3 or 5 mol % PIP3 at a total lipid concentration of 0.5 mM (25 μM PIP3). The statistical significance of the fitted values of tau or the offset (y0) was calculated with an F test.

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Lučič et al. PNAS | vol. 115 | no. 17 | E3949