The 14-3-3 proteins play a central role in the regulation of cell growth, cycling, and apoptosis by modulating the functional activities of key signaling proteins. Through binding to a phosphoserine motif, 14-3-3 alters target proteins activities by sequestering them, relocating them, conformationally altering their functional activity, or by promoting interaction with other proteins. These functions of 14-3-3 are facilitated by, if not dependent on, its dimeric structure. We now show that the dimeric status of 14-3-3 is regulated by site-specific serine phosphorylation. We found that a sphingosine-dependent kinase phosphorylates 14-3-3 in vitro and in vivo on a serine residue (Ser58) located within the dimer interface. Furthermore, by developing an antibody that specifically recognizes 14-3-3γ phosphorylated on Ser58 and employing native-PAGE and cross-linking techniques, we found that 14-3-3 phosphorylated on Ser58 is monomeric both in vitro and in vivo. Phosphorylated 14-3-3 was detected solely as a monomer, indicating that phosphorylation of a single monomer within a dimer is sufficient to disrupt the dimeric structure. Significantly, phosphorylation-induced monomerization did not prevent 14-3-3 binding to a phosphopeptide target. We propose that this regulated monomerization of 14-3-3 controls its ability to modulate the activity of target proteins and thus may have significant implications for 14-3-3 function and the regulation of many cellular processes.

The 14-3-3 proteins belong to a highly conserved family of phosphoserine binding proteins that regulate multiple signaling pathways involved in the control of cell division, growth, and apoptosis (1–4). 14-3-3 proteins influence the function of bound phosphoserine proteins in a variety of ways: sequestering them from cellular targets, controlling their enzymatic activities, relocating them, or acting as adapter molecules in mediating the association of two distinct client proteins (2, 4). To date, 14-3-3 proteins have been found to bind to over 100 cellular “client” proteins and have been demonstrated to participate in multiple signaling pathways and biological functions (1–5). Seven isoforms of 14-3-3 have been described in humans, β, ε, γ, η, σ, τ, and ζ, which are expressed from separate genes and are relatively abundant in all tissues (with the exception of σ and τ that are predominantly expressed in epithelium and T-cells, respectively). In most cases, the regulated phosphorylation of the client protein controls their binding to 14-3-3 proteins. However, the regulation of the 14-3-3 proteins themselves by phosphorylation and the consequences are virtually unknown.

14-3-3 proteins are dimeric, being composed of two 30-kDa monomers that are each capable of binding a phosphoserine motif. Each monomer is composed of nine α helices arranged in antiparallel fashion to form an amphipathic groove that mediates phosphoserine target binding. The resolved crystal structures of 14-3-3γ and 14-3-3δ demonstrate the structural similarity between isoforms and explain the high degree of conservation across the family, with conserved residues lining the phosphoserine binding groove (6, 7). The N-terminal helices of 14-3-3 mediate dimer formation with helix 1 of monomer A interacting with helices 3 and 4 of monomer B. Hetero- as well as homodimerization of isoforms occurs (8, 9). However, it is not known whether 14-3-3 monomers exist in vivo and if so what control mechanisms may exist to regulate their formation.

It has remained a mystery exactly how 14-3-3 can regulate so many client proteins with such diverse functions. A recently proposed model provides a possible explanation (5), describing 14-3-3 as a molecular anvil that deforms a bound phosphoserine client protein, thus affecting its conformation, altering enzymatic activity, or masking/revealing phosphorylation sites or nuclear transport sequences. This deforming activity of 14-3-3 is wholly dependent on 14-3-3 protein being dimeric and binding to multiple sites in the client protein, although initially a single 14-3-3 binding motif in the client protein may act as a dominant or “gatekeeper” site for 14-3-3 interaction. In the context of this model, monomeric 14-3-3 would retain binding to a gatekeeper site but would not exhibit any “molecular anvil” activity and would therefore be unable to perform deforming functions.

Previous studies demonstrated that a sphingosine-dependent kinase (SDK)1 isolated from Balb/c 3T3 fibroblasts phosphorylated 14-3-3 proteins in vitro on Ser58 in helix 3; however, the effect of this phosphorylation was not known (10). From the crystal structure of 14-3-3γ this residue is located in the dimer interface just 5.8 Å from a conserved arginine (Arg18) in helix 1 of the opposing monomer. We have now determined the functional consequence of Ser58 phosphorylation on the structure of 14-3-3 and show that phosphorylation of this residue disrupts dimer formation. Moreover, 14-3-3 phosphorylated on Ser58 is detected in vivo in response to sphingolipid and is also found to be a monomer. These results demonstrate for the first time that the dimeric status of 14-3-3 can be regulated in vivo by site-specific phosphorylation at the dimer interface, a finding that
may have profound implications for 14-3-3 function and the regulation of many cellular processes.

EXPERIMENTAL PROCEDURES

Sphingolipids and Antibodies—Sphingolipids were purchased from Biomol. Anti-14-3-3 antibody was raised in New Zealand White rabbits using glutathione S-transferase-14-3-3 (GST-14-3-3) as the immunogen. Polyclonal anti-14-3-3 antibodies were subsequently purified from rabbit serum using a GST-14-3-3 affinity column and used for immunoblotting at a dilution of 1:2000. Anti-phospho-Ser58-14-3-3 antibody (anti-pS58) was raised by immunizing New Zealand White rabbits with the phosphopeptide CGARRSpSWRVVS peptide conjugated to keyhole limpet hemocyanin. The phospho-specific Ser58 antibodies were subsequently affinity purified from rabbit serum using the phospho- and an unphosphorylated form of the immunizing peptide, essentially as described previously (11). The purified anti-phosphoSer58 was used for immunoblotting at a concentration of 1 µg/ml. Horseradish peroxidase-conjugated anti-rabbit antibody was purchased from Pierce.

Cell Culture—Balb/c T3 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% v/v fetal calf serum. CTLL-2 cells were maintained in RPMI 1640 supplemented with 10% v/v fetal calf serum, 50 µM β-mercaptoethanol, and 100 units/ml bacteria- synthesis mouse interleukin-2.

Expression Constructs and Reconstituent Protein Production—Re-combinant 14-3-3 was produced in Escherichia coli from pGEX2T-14-3-3 (12). The recombinant 14-3-3 was cleaved from the immobilized GST-14-3-3 by resuspending 1 ml of GST-14-3-3 on glutathione resin (Sigma) in 2 ml of thrombin cleavage buffer (50 µM Tris-Cl, pH 8, 150 mM NaCl, 2.5 mM CaCl2, 2 mM diithiothreitol) and incubating overnight with 200 units of Thrombestatin (Pfizer). The cleaved protein was then purified on a Mono Q HR5/5 (Amersham Biosciences) column after dialysis into Mono Q buffer (20 mM Tris-Cl, pH 7.5, 1 mM diithiothreitol). Cleaved 14-3-3 was eluted with a 0.05-0.5 M NaCl gradient in Mono Q buffer, and the purity of the 14-3-3 was determined by SDS-PAGE and Coomassie staining. Mutations were generated in the 14-3-3 CDNA by QuikChange site-directed mutagenesis (Stratagene), and mutant protein was produced and purified as described above.

In Vitro SDK Reactions—An S100 cytosolic fraction was prepared from serum-starved, PMA-stimulated Balb/c T3 T3 fibroblasts as described previously (10). Aliquots of extract were frozen away at −70 °C and were subsequently used in SDK reactions to phosphorylate 14-3-3. SDK assays were carried out essentially as described previously (10) with the following changes: assays were carried out in 20-µl volume with 100 ng of recombinant 14-3-3. S100 extract made up half the assay volume, and SDK assay buffer was 20 mM Tris-Cl, pH 7.4, 15 mM MgCl2, 25 mM ATP, 3 mM dithiothreitol with or without 2 µM of γ-[32P]ATP. Sphingolipid was added to a final concentration of 100 µg/ml n-octyl glucoside. Reactions were incubated at 37 °C for 15 min.

Cross-linking Studies—14-3-3 phosphorylated by SDK was subjected to cross-linking with bis(sulfosuccinimidyl) suberate (BS3) (Pierce). BS3 (50 µg/ml) was added to an SDK reaction after incubation and the mixture incubated for a further 5 min at 37 °C. Cross-linking was terminated by addition of ethanolamine to a final concentration on 100 µM. Cross-linking samples were subjected to SDS-PAGE, Western transferred, and immunoblotted as indicated.

Peptide Pull-downs—14-3-3 phosphorylated by SDK was precipitated after incubation for 1 h at 4 °C with 20 µl of streptavidin–agarose (Pierce) and 2 µg of biotinylated peptide (Chiron) from 0.5 ml volume of Nonidet P-40 lysis buffer (10 mM Tris-Cl, pH 7.4, 137 mM NaCl, 10% v/v glycerol, 1% w/v Nonidet P-40) prior to lysis. The precipitated material was washed four times in Nonidet P-40 lysis buffer prior to separation by SDS-PAGE, Western transfer, and immunoblotting as indicated.

Cell Stimulation and Lysate Preparation—Balb/c T3 T3 cells were stimulated at 80% confluence in 10-cm dishes. After stimulation as indicated, cells were put on ice and washed twice with phosphate-buffered saline. Cells were released from the dishes by scraping and pelleted by centrifuging at 1000 × g for 5 min at 4 °C. The cell pellets were lysed on ice in 100 µl of Nonidet P-40 lysis buffer containing protease inhibitors (10 µM leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin) and phosphatase inhibitors (2 mM sodium orthovanadate, 1 mM sodium fluoride, and 1 mM sodium pyrophosphate, and 1 mM β-glycerophosphate). Cell debris was removed by centrifugation at 10,000 × g for 15 min at 4 °C and protein concentration determined by BCA assay (Pierce). 50 µg of lysate was subjected to native-PAGE and immunoblotted as indicated. CTCL-2 cells (2 × 106 per treatment) were stimulated as indicated and after pelleting by centrifugation at 400 × g for 5 min were washed with phosphate-buffered saline containing 1 mM β-glycerophosphate and lysed on ice in 50 µl of Nonidet P-40 lysis buffer containing protease and phosphatase inhibitors. Cell debris was removed by centrifugation at 10,000 × g for 15 min at 4 °C, and 25 µl of lysate was subjected to native-PAGE and immunoblotted as indicated.

PAGe and Immunoblotting—Native-PAGE was carried out on 12.5% PAGE gels with 5% stacking gels. Gels were prepared using the standard Laemmli recipe but excluding SDS. SDS was also excluded from the sample load buffer. Benchmark prestained protein markers (Invitrogen) were used as an indicator of protein migration. All gels were transferred to nitrocellulose by electroblotting using standard buffers, and blots were blocked in TNT (10 mM Tris-Cl, pH 8, 150 mM NaCl, and 0.05% w/v Tween 20) containing 1% (w/v) blocking reagent (Roche Applied Science). Immunoblotting was performed overnight at 4 °C with anti-phosphoSer58 antibody and for 1 h at room temperature with the anti-14-3-3 antibody. All washes were performed in TNT with 0.5% (w/v) blocking reagent added to antibody incubations. Immunoblots were developed by enhanced chemiluminescence using proprietary reagents (Amersham Biosciences).

RESULTS

The Phosphorylation of 14-3-3 on Ser58 by an SDK Activity Disrupts 14-3-3 Dimers—A SDK activity that phosphorylates Ser58 of 14-3-3 was previously identified in cytosolic extracts of PMA-stimulated Balb/c T3 fibroblasts (10). We have reproduced the in vitro SDK assay system described in the original report. As shown previously (10), phosphorylation of recombinant wild type 14-3-3 was induced by sphingosine (data not shown) and the sphingosine analogue, dimethyl sphingosine (DMS) (Fig. 1A) but not by sphingosine 1-phosphate (data not shown). Furthermore, recombinant 14-3-3 mutated at Ser58 is not phosphorylated in response to DMS confirming that Ser58 is the sole site of phosphorylation by SDK (Fig. 1A). We have now raised a polyclonal antibody to a phosphopeptide corresponding to the amino acid sequence encompassing phospho-Ser58 14-3-3: CGARRSpSWRVVS, where pS represents phosphoserine. The antibody specifically recognized the phosphorylated, but not the unphosphorylated, form of the Ser58 peptide nor a phosphopeptide corresponding to another known phosphorylation site in 14-3-3, Ser185 (Fig. 1B). Using the in
of a single monomer within a 14-3-3 dimer is sufficient to disrupt the dimeric structure of 14-3-3.

**Demonstration That 14-3-3ζ Phosphorylated on Ser^58 Is Monomeric by Cross-linking**—We employed cross-linking to examine the dimeric status of Ser^58-phosphorylated 14-3-3ζ compared with unphosphorylated 14-3-3ζ. 14-3-3ζ was phosphorylated *in vitro* in an SDK reaction and then subjected to cross-linking with BS₃. Cross-linking was terminated using ethanolamine and the cross-linked complexes separated by SDS-PAGE. Gels were Western transferrred and immunoblotted either with Ser^58-phospho-specific or anti-14-3-3 antibody (Fig. 3). As can be seen, the anti-14-3-3 antibody detected protein of molecular mass 28 kDa, corresponding to the size of denatured monomeric 14-3-3 and also a protein of 56 kDa when the SDK reaction was treated with BS₃, corresponding in size to covalently cross-linked 14-3-3 dimers (Fig. 3). In contrast, the phospho-specific antibody only detected protein of the size of denatured monomeric 14-3-3 and not covalently cross-linked dimeric 14-3-3 (Fig. 3), indicating that 14-3-3ζ phosphorylated on Ser^58 is monomeric. The absence of cross-linked phosphorylated 14-3-3ζ dimers re-affirms the previous result (Fig. 2A), showing that phosphorylation of a single monomer within a dimer is sufficient to disrupt the dimeric structure of 14-3-3ζ. Therefore, Ser^58 phosphorylation has a dominant effect with respect to dimer disruption, increasing the potency of the event.

**14-3-3ζ Phosphorylated on Ser^58 Retains the Ability to Bind Phosphoserine Peptide**—Serine 58 of 14-3-3ζ—Phosphoserine Peptide is a binding motif in the cytoplasmic domain of the granulocyte-macrophage colony-stimulating factor receptor β chain (12) was determined. Phosphorylated and unphosphorylated peptides (16) were used to pull-down 14-3-3ζ from SDK reactions carried out either in the presence or absence of DMS (Fig. 4). Immunoblotting of the pull-down material with anti-14-3-3 antibody showed that 14-3-3ζ bound to the phosphorylated but not the unphosphorylated peptide, demonstrating that interaction of 14-3-3ζ with peptide is dependent on phosphorylation of the peptide. Similarly, Ser^58-phosphorylated 14-3-3ζ also bound to phosphorylated peptide and, as expected, was detected only in SDK reactions carried out in the presence of DMS (Fig. 4). This indicates that Ser^58-phosphorylated monomeric 14-3-3ζ retains the ability to bind to a 14-3-3 binding site and that binding is dependent on phosphorylation of the site, thus demonstrating that the amphipathic binding groove in the phosphorylated 14-3-3ζ monomer is not structurally perturbed by phosphorylation.
Phosphorylation and Monomerization of 14-3-3 Can Be Induced in Vivo by Sphingolipids—Having demonstrated that phosphorylation of Ser<sup>58</sup> by SDK renders 14-3-3 monomeric <i>in vitro</i>, it is important to show that this process also occurs <i>in vivo</i>. We have examined the phosphorylation and dimerization of 14-3-3 <i>in vivo</i> in Balb/c 3T3 cells after sphingolipid treatment using the phospho-specific Ser<sup>58</sup> antibody (Fig. 5A). DMS was employed for these studies being a naturally occurring non-metabolizable analogue of sphingosine. Whole cell lysates from Balb/c 3T3 cells treated with 25 μM DMS for varying lengths of time were run on native-PAGE and immunoblotted with phospho-specific Ser<sup>58</sup> and anti-14-3-3 antibodies (Fig. 5A). The phospho-specific Ser<sup>58</sup> antibody detected a protein migrating with the seventh protein marker after 10 min of treatment with DMS corresponding to monomeric 14-3-3. This demonstrates that DMS treatment of Balb/c 3T3 fibroblasts does indeed disrupt dimeric 14-3-3 <i>in vivo</i> presumably by inducing phosphorylation of Ser<sup>58</sup>.

We extended these studies to other cell types and investigated the effect of DMS treatment on 14-3-3 phosphorylation and dimeric status in the murine lymphoid cell line, CTLL-2. Cells were stimulated with increasing doses of DMS for 30 min and whole cell lysates prepared and run on native-PAGE. After Western blotting the lysates were immunoblotted with either the phospho-specific Ser<sup>58</sup> antibody or anti-14-3-3 antibody (Fig. 5B). As can be seen, when cells were treated with 25 μM DMS, phosphorylated 14-3-3 was detected with the Ser<sup>58</sup> phospho-specific antibody migrating exclusively with the seventh protein marker (Fig. 5B). This indicates that sphingolipid-induced phosphorylation of 14-3-3 on Ser<sup>58</sup> disrupts the dimeric structure of the endogenous protein in CTLL-2 cells. Furthermore, as seen <i>in vitro</i>, no phosphorylated 14-3-3 is detected as a dimer, indicating that phosphorylation of a single monomer within a 14-3-3 dimer is sufficient to disrupt the dimer.

**DISCUSSION**

We present data here that the dimeric status of 14-3-3 protein is regulated by site-specific phosphorylation. A sphingolipid-activable cytosolic kinase activity, previously described as SDK, phosphorylates 14-3-3 <i>in vitro</i> on a serine residue (Ser<sup>58</sup>) that lies in the dimer interface of the protein (10). We have generated a phospho-specific antibody that recognizes 14-3-3<sub>58</sub> phosphorylated on Ser<sup>58</sup>, and this unique tool has allowed us to probe the effect of SDK-mediated phosphorylation of this site on 14-3-3 dimeric structure. We have employed native-PAGE and cross-linking studies to qualitatively discriminate between monomeric and dimeric forms of 14-3-3 and have found that sphingolipid induced phosphorylation of Ser<sup>58</sup> disrupts the dimeric structure of 14-3-3 both <i>in vitro</i> and <i>in vivo</i> (Figs. 2, 3, and 5). Furthermore, phosphorylation of a single monomer unit within a dimer of 14-3-3 is sufficient to disrupt the dimer, increasing the potency of the event. We show that phosphorylated monomeric 14-3-3 is competent at phosphoserine peptide binding, thereby demonstrating that phosphorylation does not have gross conformational effects on the amphipathic phosphoserine binding groove. We propose that this regulated phosphorylation and monomerization of 14-3-3 controls it’s deforming activity and may have profound implications for 14-3-3 function and the regulation of many cellular processes.

Phosphorylation of Ser<sup>58</sup> by SDK is not restricted to the 14-3-3<sub>3</sub> isoform. The original SDK report also demonstrated phosphorylation of the equivalent residues in β and η (10), and equivalent sites are found in all isoforms with the exception of σ and τ. Moreover, the amino acid sequence surrounding the serine in all phosphorylatable isoforms is highly conserved, suggesting that the ability to regulate monomerization by phosphorylation has been functionally conserved. Interestingly, not only is the sequence in the corresponding region of 14-3-3<sub>3</sub> and 14-3-3<sub>3</sub> more divergent but the homologous residue to Ser<sup>58</sup> is an alanine and therefore not susceptible to phosphorylation. It is intriguing that these more tissue-restricted isoforms are not phosphorylatable at this site and suggests that they may perform specific roles for which regulation of monomerization is not desirable.

Our findings are at odds with a recent report in which phosphorylation of Ser<sup>58</sup> in 14-3-3<sub>3</sub> by PKB/Akt was said to have no effect on dimer stability (13). However, as noted by others (8), the recombinant 14-3-3<sub>3</sub> used as kinase substrate in those studies already comprised a significant amount of monomeric 14-3-3, as determined by native-PAGE analysis. The extent of phosphorylation obtained coincided with the amount of monomer present, suggesting that 14-3-3 monomer rather than dimer may have been the substrate for Akt phosphorylation (13). In contrast, the recombinant 14-3-3<sub>3</sub> used in our <i>in vitro</i> studies is solely dimeric as determined by native-PAGE anal-
ysis, with no monomeric component detectable by immunoblotting (Fig. 2B) or Coomassie staining (Fig. 2C). Additionally, we detect monomeric 14-3-3 in vivo using the phospho-specific Ser*58 antibody (Fig. 5), indicating that the monomerization observed is physiological.

It is possible from our studies to gauge the exact stoichiometry of 14-3-3 phosphorylation in vivo as the native-PAGE system used to discriminate monomers from dimers resolves only proteins and complexes with relatively acidic isoelectric characteristics. As shown (Fig. 4), phosphorylated 14-3-3 is still able to bind to phosphoserine peptide and so would therefore be expected to interact with phosphoserine client proteins and the resulting complexes may not resolve on native-PAGE. Therefore, the amount of phosphorylated 14-3-3 detected under these conditions may underestimate the level of phosphorylation actually occurring.

The proposed functional role for Ser*58 in regulating monomerization is consistent with its location in the dimer interface (7). However, as previously noted (8), how a kinase gains access to this buried residue remains unknown. The dimeric form of the protein is inherently stable and not prone to dissociation (9), and we have found no evidence for free exchange of monomers in mixing experiments using differentially tagged forms of 14-3-3*58 (data not shown). An alternative explanation could be that 14-3-3 protein is flexible, allowing access of SDK activity to the dimer interface, but evidence suggests that this is not the case as the crystal structures of 14-3-3 unbound or bound to a protein are remarkably similar (17). It is conceivable that sphingolipid may have some direct effect on 14-3-3 that allows access of the kinase to Ser*58. Such modulation of Src kinase substrates by sphingolipids has been observed previously (18). Alternatively, in the original description of SDK activity, it was shown that the enzyme activity co-purified with 14-3-3 and could be co-immunoprecipitated with endogenous 14-3-3 (10), indicating that the kinase responsible for SDK activity is in intimate contact with 14-3-3, even in the absence of sphingolipid. Therefore, SDK may gain access to Ser*58 by conformationally altering 14-3-3 directly.

Previous studies with 14-3-3 mutants defective in dimerization indicate that although monomeric forms of 14-3-3 are capable of binding to phosphoserine target proteins, in many cases the monomer is unable to regulate client protein functions (19–22). Furthermore, 14-3-3 mutants that are defective in client protein binding owing to substitutions in the phosphoserine binding amphipathic groove exhibit dominant negative effects on several pathways due to their ability to dimerize with endogenous 14-3-3, resulting in functionally monomeric forms (23, 24). In the light of these studies we believe that the regulated monomerization of 14-3-3 demonstrated here has significant implications for the regulation of many 14-3-3 functions in cells.

To date, the most clearly defined role of 14-3-3 is to protect cells against apoptosis. A peptide with high affinity for 14-3-3, dipofein, induces apoptosis when expressed in cells, due to its ability to compete with cellular proteins for 14-3-3 binding (25). Strikingly, expression of a functionally monomeric dominant negative form of 14-3-3*58 in mouse fibroblasts enhances the cell apoptotic response to UVC irradiation, serum withdrawal, and tumor necrosis factor-α, highlighting the role of dimeric 14-3-3 in protecting cells against apoptosis (23). Therefore, regulated monomerization may play a role in inducing apoptosis by disrupting the anti-apoptotic function of dimeric 14-3-3. Consistent with this hypothesis, the sphingolipids sphingosine and DMS have both been shown to induce apoptosis in many cell types, including CTLL-2 cells (15, 26–31). Indeed, under the conditions used here to induce 14-3-3 phosphorylation in CTLL-2 cells (Fig. 5B), significant cell death was observed after 5 h of treatment with DMS (data not shown). Therefore, it is conceivable that phosphorylation and consequent monomerization of 14-3-3 contributes to apoptosis induced by these sphingolipids and may have a more general role in functions where 14-3-3 is involved.

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