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Calyculin A-induced Vimentin Phosphorylation Sequesters 14-3-3 and Displaces Other 14-3-3 Partners *in Vivo**

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14-3-3 proteins bind their targets through a specific serine/threonine-phosphorylated motif present on the target protein. This binding is a crucial step in the phosphorylation-dependent regulation of various key proteins involved in signal transduction and cell cycle control. We report that treatment of COS-7 cells with the phosphatase inhibitor calyculin A induces association of 14-3-3 with a 55-kDa protein, identified as the intermediate filament protein vimentin. Association of vimentin with 14-3-3 depends on vimentin phosphorylation and requires the phosphopeptide-binding domain of 14-3-3. The region necessary for binding to 14-3-3 is confined to the vimentin amino-terminal head domain (amino acids 1–96). Monomeric forms of 14-3-3 do not bind vimentin *in vivo* or *in vitro*, indicating that a stable complex requires the binding of a 14-3-3 dimer to two sites on a single vimentin polypeptide. The calyculin A-induced association of vimentin with 14-3-3 *in vivo* results in the displacement of most other 14-3-3 partners, including the protooncogene Raf, which nevertheless remain capable of binding 14-3-3 *in vitro*. Concomitant with 14-3-3 displacement, calyculin A treatment blocks Raf activation by EGF; however, this inhibition is completely overcome by 14-3-3 overexpression *in vivo* or by the addition of prokaryotic recombinant 14-3-3 *in vitro*. Thus, phosphovimentin, by sequestering 14-3-3 and limiting its availability to other target proteins can affect intracellular signaling processes that require 14-3-3.

The 14-3-3 protein family consists of seven highly conserved proteins of 28–31 kDa that naturally assemble as homodimers or heterodimers and bind to a wide array of intracellular proteins (1). Among the proteins known to associate with 14-3-3 *in vivo* are proteins that function in cell cycle control (*e.g.* Cdc25 (2) and Wee1 (3)), signal transduction (*e.g.* Raf (4), phosphatidylinositol 3-kinase (5), and BAD (6)), transcriptional regulation (*e.g.* FKHRL1 (7)), and cell structure (*e.g.* keratins (8)). Genetic alterations in 14-3-3 proteins have been shown to impair a variety of biologic functions, such as cell cycle timing (9) and cell cycle arrest in response to DNA damage in yeast (10), photoreceptor development in the *Drosophila* eye (11), and the Ras/Raf signaling pathway in various organisms (4, 11). The ability of 14-3-3 to bind target proteins has been shown in many instances to depend on the phosphorylation of specific sites on the target protein (1), and studies with synthetic phosphopeptides have defined two preferred 14-3-3 binding motifs as RS*X*pS*X*P or R*XXX*pS*X*P (where pS stands for phosphoserine) (12, 13). The existence of additional variations in the sequence of 14-3-3 binding sites is probable inasmuch as 14-3-3 partners such as phosphatidylinositol 3-kinase (5), tyrosine hydroxylase (1), and keratins (14) do not contain either of these motifs. Association with 14-3-3 may positively or negatively regulate the function of the partner; thus, the binding of 14-3-3 results in activation of tyrosine hydroxylase (1) but inhibition of phosphatidylinositol 3-kinase (5) and Cdc25 (15); a continuous association with 14-3-3 is necessary for physiologic regulation of Raf kinase activity (12, 16, 17). The biochemical mechanisms by which 14-3-3 proteins modify the function of the target protein may vary for each partner; for example, in the case of yeast Cdc25 (18) and FKHRL1 (7) the association with 14-3-3 (Rad24 in yeast) results in their exclusion from the nucleus. The ability of the proapoptotic protein BAD to bind and sequester the antiapoptotic factors BCL_2 and $BCLx_L$ is inhibited by the phosphorylation of BAD and subsequent binding of 14-3-3 (6). The 14-3-3 proteins have multiple functions in the regulation of c-Raf-1; the binding of 14-3-3 to Raf stabilizes the inactive state in unstimulated cells but is also required to maintain the active conformation of Raf generated after growth factor stimulation (17).

The intermediate filament (IF)[1] network provides a rigid framework that supports cell architecture during interphase (19, 20). IF proteins undergo disassembly and major reorganization during cell cycle progression (21). Inasmuch as IF proteins constitute at least 1% of total cell protein, IF disassembly creates a large pool of soluble IF proteins. The assembly/disassembly of IFs is regulated by dephosphorylation/phosphorylation of the filaments on their exposed N- and/or C-terminal domains (22). A variety of protein (Ser/Thr) kinases likely to phosphorylate IFs *in vivo* have been proposed, including protein kinase Cs, protein kinase A, CaMKII, and Cdc2 kinase. In BHK-21 cells, the IF proteins vimentin and desmin undergo a major increase in phosphorylation (to a stoichiometry of approximately 2 mol of phosphate/mol of protein) concomitant with filament disassembly that occurs as the cells move from G_2 to M (23, 24). Incubation of these cells with the protein phosphatase inhibitor calyculin A results in a rapid disassem-

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[1] The abbreviations used are: IF, intermediate filament; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; HPLC, high pressure liquid chromatography; EGF, epidermal growth factor; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ERK, extracellular signal-regulated kinase.

bly of IFs, in parallel with their hyperphosphorylation, both at sites already partially phosphorylated in interphase and at mitosis-specific sites (25). The epithelial IF protein keratin K18 associates with 14-3-3 proteins in a cell cycle- and phosphorylation-dependent manner, reaching a peak during late S-G_2/M phase (8, 14).

We now report that calyculin A treatment of COS cells induces the binding of vimentin to 14-3-3-, accompanied by the displacement of virtually all other 14-3-3 partners, including Raf-1. 14-3-3-Raf complex disassembly results in a complete inhibition of Raf kinase activity, which is, however, completely overcome by 14-3-3 overexpression *in vivo* or by the addition of recombinant 14-3-3 to the cell extract *in vitro.* These results suggest that IF phosphorylation may serve to regulate 14-3-3 availability to key proteins involved in intracellular signaling and consequently regulate their function.

MATERIALS AND METHODS

14-3-3 and Raf cDNA Constructs—GST-wt-14-3-3ζ-(1–245), GST-nt-14-3-3-(1–140), GST-ct-14-3-3-(139–245), and GST-dm-14-3-3 (E5K, L12Q/A13Q/E14R, Y82Q, K85N, E87Q) for expression in mammalian cells were in the pEBG vector. Myc-wt-14-3-3-(1–245) and Myc-c-Raf-1 were in the pMT2 vector. GST, GST-wt-14-3-3, and GST-dm-14-3-3 were expressed in *Escherichia coli* using pGEX-KG vector. The construction of these vectors is described elsewhere (17, 26, 29).

Cell Culture and Transfection—COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For transient expression of proteins, cells were transfected either with the DEAE-dextran method using 5–10 μg of DNA/10-cm dish or using LipofectAMINE (Life Technologies, Inc.) with the indicated amounts of DNA, following the manufacturer's instructions. For cell stimulation, 24–48 h after transfection cells were serum-deprived for 24 h prior to the addition of the stimulator.

Metabolic Labeling—Serum-deprived cells were washed once with medium lacking methionine/cysteine for [^{35}S]Met labeling or lacking phosphate for ^{32}P labeling and incubated for 30 min in the same media to deplete intracellular sources. Cells were labeled by incubation in the presence of 0.5 mCi/ml ^{35}S or ^{32}P for 4 or 2 h, respectively.

Cell Extraction and Protein Purification—Cells were lysed in ice-cold extraction buffer containing 50 mM Tris-CL, pH 7.5, 100 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 50 mM β-glycerophosphate, and a mixture of proteinase inhibitors (Amersham Pharmacia Biotech). Cell lysates were incubated at 4 °C for 90 min with the appropriate antibody precoupled to protein A/G-agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for immunoprecipitation or with GSH-Sepharose beads (Amersham Pharmacia Biotech) for GSH affinity purification. The beads were washed twice with the extraction buffer, twice with extraction buffer containing 0.5 M LiCl, and twice with buffer A (40 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 5 mM $MgCl_2$, and 2 mM dithiothreitol). The proteins were either eluted directly in SDS-sample buffer or served for additional experiments.

Dissociation of 14-3-3-Vimentin Complexes in Vitro—Cell extracts were incubated with 300 μM synthetic phosphopeptide corresponding to c-Raf-1 amino acids 613–627 containing a canonical 14-3-3 binding motif, LPKINRSApSEPSLHR, or with a 300 μM concentration of the same peptide in its unphosphorylated form for 30 min at 4 °C prior to protein purification.

In Vitro Binding Assays—Prokaryotic recombinant GST or GST-14-3-3 fusion variants precoupled to GSH-Sepharose beads were incubated with the various cell extracts or with the lysyl C digest of ^{32}P-labeled vimentin at 4 °C for 30–60 min in the presence or absence of phosphopeptide as indicated. The beads were washed twice with extraction buffer, twice with extraction buffer containing 0.5 M LiCl, and twice with buffer A. The associated proteins were eluted either with GSH or with SDS-sample buffer as indicated. For the phosphatase/kinase-treated vimentin binding assays, GST-14-3-3 fusions were labeled using the biotinylation kit from Amersham Pharmacia Biotech.

Raf Coupled Kinase Assay—Following anti-Myc immunoprecipitation, the Myc-Raf-containing beads were incubated in buffer A (100 μl final volume) supplemented with 100 μM ATP, 10 μCi of [γ-^{32}P]ATP, and 0.3 μg of prokaryotic recombinant GST-MEK-1 for 20 min at 30 °C and for an additional 30 min after the addition of 2 μg of prokaryotic recombinant kinase-inactive ERK-2 (K52R). Samples were separated on SDS-PAGE and transferred to PVDF membranes. The ^{32}P content in ERK-2 was quantified by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA) (data are presented as PhosphorImager units × 10^3).

RESULTS

Calyculin A Induces the Selective Association of Vimentin with 14-3-3—To survey the array of cellular polypeptides that interact with 14-3-3ζ *in vivo*, GST-14-3-3 associated proteins, extracted from [^{35}S]Met-labeled COS cells, were resolved on SDS-PAGE (Fig. 1*A*). A large number of cellular polypeptides copurified with full-length GST-14-3-3 but not with control GSH beads (Fig. 1*A*, compare *lanes 1* and *2*) or with GST-nt-14-3-3-(1–140), which lacks the carboxyl-terminal target protein-binding domain (26) (Fig. 1*A*, compare lanes 2 and 4). The amino-terminally truncated GST-ct-14-3-3-(139–245), which lacks the 14-3-3 dimerization domain (26), did not associate with endogenous 14-3-3 (Fig. 1*A*, *lane 3*) but maintained the ability to bind several other endogenous polypeptides and to bind cotransfected Myc-Raf (Fig. 1*A*, *lanes 5* and *6*). The addition of the phosphatase inhibitor calyculin A to COS cells resulted in an upshift of several of the 14-3-3-associated proteins (Fig. 1*A*, compare *lane 2* with *lane 8*) and induced the association of a 55-kDa polypeptide with full-length 14-3-3 but not with either of the 14-3-3 fragments (Fig. 1*A*, compare *lane 8* with *lanes 9* and *10*). Since following calyculin A treatment the 55-kDa polypeptide became the most abundant [^{35}S]methionine-labeled 14-3-3 partner, it was purified (Fig. 1*B*) and subjected to in-gel digestion with lysyl C. Two prominent peptides isolated by reverse phase HPLC were analyzed by automated Edman degradation, each yielding amino acid sequence identical to human vimentin (Fig. 1*C*). Confirming this identification, anti-vimentin immunoblot detected a time-dependent, calyculin A-induced association of vimentin with recombinant GST-14-3-3, *in vivo* (Fig. 1*D*). Reciprocally, calyculin A induced the appearance of both GST-14-3-3 and endogenous 14-3-3 in anti-vimentin immunoprecipitates (Fig. 1*E*). Immobilized prokaryotic recombinant GST 14-3-3 was able to bind *in vitro* vimentin from extracts of calyculin A-treated cells but not from extracts of control or EGF- or 12-*O*-tetradecanoylphorbol-13-acetate-treated cells (Fig. 1*F*). Exposure of COS cells to heat shock, okadaic acid, sodium vanadate, and extracellular hypertonicity also failed to induce vimentin/14-3-3 association (Fig. 2*C* and data not shown), pointing to a specific effect of calyculin A, probably trough inhibition of protein phosphatase-1. Calyculin A, in contrast to okadaic acid, is able to enter cells rapidly and inhibit both protein phosphatase-1 and protein phosphatase 2A while okadaic acid inhibits preferentially protein phosphatase 2A (27).

Association of Vimentin with 14-3-3 Depends on Vimentin Phosphorylation and Requires 14-3-3 Dimerization and Its Phosphopeptide-binding Domain—The ability of the phosphatase inhibitor calyculin A to induce the association of vimentin with 14-3-3 suggested that vimentin phosphorylation is involved; however, since vimentin does not contain a "perfect" 14-3-3 binding motif (19), we tested the role of vimentin phosphorylation in 14-3-3 binding. Incubation of vimentin extracted from calyculin A-treated cells with alkaline phosphatase or protein phosphatase 2A completely prevents vimentin binding to 14-3-3 *in vitro* (Fig. 2*A*, compare *lane 2* with *lanes 3* and *5*). The specificity of the inhibition was demonstrated by the ability of the alkaline phosphatase inhibitor *p*-nitrophenylphosphate (*PNPP*) to prevent the inhibition (Fig. 2*A*, *lane 4*). In support of this finding, phosphorylation of vimentin extracted from control, untreated cells with protein kinase A/AKT combination (two kinases previously shown to phosphorylate several proteins on 14-3-3 recognition sites (1, 6, 7)) induced 14-3-3 binding to vimentin (Fig. 2*A*, compare *lane 9* with *lane 10*).

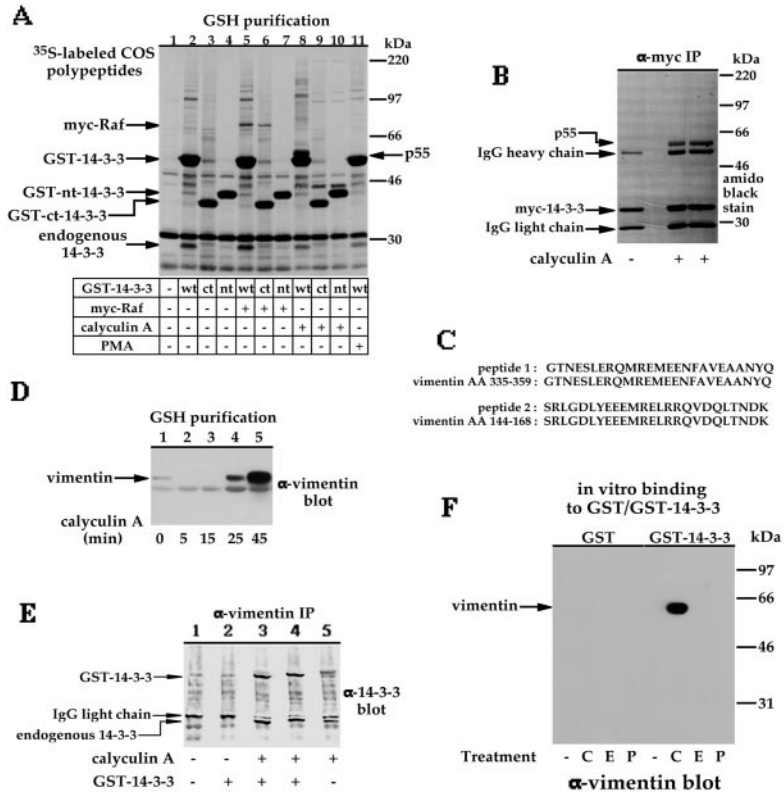

FIG. 1. **Calyculin A induces association of GST-14-3-3ζ with p55 vimentin both *in vivo* and *in vitro*.** *A*, serum-deprived COS-7 cells transiently expressing GST-14-3-3 ζ (amino acids 1–245; *wt*, *lanes 2*, *5*, *8*, and *11*), amino-terminal 14-3-3 fragment (nt-14-3-3) (amino acids 1–140; *nt*, *lanes 4*, *7*, and *10*), or carboxyl-terminal 14-3-3 fragment (ct-14-3-3) (amino acids 139–245; *ct*, *lanes 3*, *6*, and *9*) or co-expressing GST-14-3-3 and Myc epitope-tagged Raf-1 (*lanes 5–7*) were [^{35}S]Met-labeled and treated for 25 min with carrier (*lanes 1–7*), calyculin A (100 nM; *lanes 8–10*), or PMA (100 nM; *lane 11*). Following cell extraction and GSH-Sepharose affinity purification, the samples were analyzed on 12% SDS-PAGE. An autoradiogram of the ^{35}S-labeled proteins is shown. The migration of the molecular weight markers and the recombinant proteins is indicated. *B*, COS cells expressing Myc epitope-tagged 14-3-3 ζ were treated with calyculin A (300 nM) for 45 min. The Myc-14-3-3 was purified on agarose beads precoupled with anti-Myc antibodies (clone 9E10; Santa Cruz Biotechnology) and separated on SDS-PAGE. The gel was transferred to PVDF membrane and stained with Amido Black. Indicated are the migration of the molecular weight markers, the position of the IgG light and heavy chains, Myc-14-3-3, and p55. *C*, the p55 protein band from *B* was excised and digested with endoproteinase Lys-C, and the resulting peptides were separated by reverse phase HPLC. Two prominent peptides were subjected to automated Edman degradation and their amino acid sequence matched that of human vimentin. Shown are the alignments with the corresponding vimentin sequences. *AA*, amino acids. *D*, COS cells expressing GST-14-3-3 were treated with calyculin A (100 nM) for the indicated times. GST-14-3-3 proteins were purified on GSH beads and subjected to SDS-PAGE, followed by transfer to PVDF membrane. 14-3-3-associated vimentin was detected by immunoblot with anti-vimentin antibody (clone V9, Sigma). *E*, mock (*lanes 1* and *5*) or GST 14-3-3-transfected COS cells (*lanes 2–4*) were treated with vehicle (*lanes 1* and *2*) or calyculin A (100 nM; *lanes 3–5*) for 45 min. Vimentin was immunoprecipitated and resolved on SDS-PAGE. Coassociated 14-3-3 was detected by anti-14-3-3 immunoblot (pan-14-3-3; SantaCruz). Indicated are the positions of GST-14-3-3, endogenous 14-3-3, and IgG light chain. *F*, vimentin in an extract of COS cells treated for 45 min with carrier (−), calyculin A (100 nM; *C*), EGF (100 ng/ml; *E*), or PMA (100 nM; *P*) was assayed for binding to prokaryotic recombinant GST or GST-14-3-3 as described under "Materials and Methods." 14-3-3-associated vimentin was detected by anti-vimentin immunoblot.

These results demonstrate that vimentin phosphorylation mediates and is required for 14-3-3 binding. To further support our findings and to demonstrate that 14-3-3 binding to vimentin is mediated by the 14-3-3 phosphopeptide-binding region, we used a synthetic phosphopeptide corresponding to c-Raf-1 amino acids 613–627, a canonical 14-3-3 binding motif previously shown to dissociate Raf 14-3-3 complexes (12, 17), to dissociate 14-3-3/vimentin complexes *in vitro* (Fig. 2*B*). It is notable that only the phosphopeptide is able to dissociate 14-3-3-vimentin complexes, whereas the same peptide in the unphosphorylated state has no effect (Fig. 2*B*). Yaffe *et al.* (13) demonstrated that the presence of two or more phosphoserine-containing 14-3-3 binding motifs on a single synthetic phosphopeptide increases the apparent affinity for 14-3-3 by 30-fold over the same peptide containing a single phosphoserine. In view of the absence of "optimal" 14-3-3 binding motifs on vimentin and the inability of the amino-terminally truncated, dimerization-deficient GST-ct-14-3-3-(139–245) polypeptide to bind vimentin (Fig. 1*A*), we hypothesized that the coligation of two phosphoserines on a single vimentin polypeptide by a 14-3-3 dimer might be necessary to confer sufficient avidity for a stable association. Consistent with this idea, several forms of dimerization-deficient 14-3-3, although able to bind c-Raf-1 efficiently *in vivo* (Fig. 1*A* and Refs. 17 and 26) were unable to bind vimentin *in vivo* (Fig. 2*C*) or *in vitro* (Fig. 2, *A* and *D*). The requirement for a 14-3-3 dimer for vimentin binding is thus consistent with the view that phosphovimentin in calyculin A-treated cells contains two or more low affinity 14-3-3 binding sites, neither of which is sufficient by itself to bind 14-3-3, whereas their coligation by a 14-3-3 dimer enables stable association. To identify regions in vimentin required for 14-3-3 binding, a lysyl C digest of ^{32}P-vimentin, isolated from calyculin A-treated COS cells, was adsorbed to immobilized prokaryotic recombinant GST-14-3-3 or dimerization-deficient (*dm*) 14-3-3 mutant (Fig. 2*D*). A single ^{32}P-labeled peptide fragment corresponding to approximately 25% of the applied ^{32}P was retained by the wild type 14-3-3, whereas no ^{32}P-peptide was retained by the monomeric 14-3-3 (Fig. 2*D*, compare *lanes 1* and *2*); retention of the vimentin ^{32}P-peptide was inhibited by the synthetic Raf phosphopeptide (Fig. 2*D*, lane 3). The lysyl C

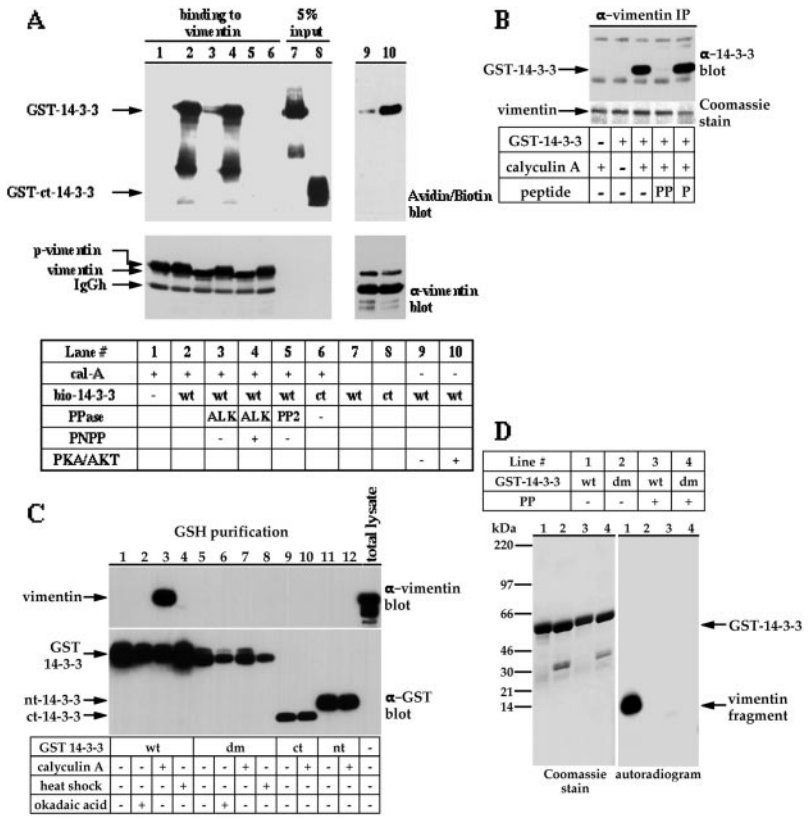

FIG. 2. **Association of vimentin with 14-3-3 depends on vimentin phosphorylation and requires 14-3-3 dimerization and its phosphopeptide-binding domain.** *A*, vimentin immunoprecipitated from COS cells treated for 45 min with carrier (*lanes 9* and *10*) or calyculin A (150 nM; lanes 1–6) was incubated for 30 min at 30 °C with buffer alone (*lanes 1*, *2*, *6*, and *9*) or with buffer containing calf intestinal alkaline phosphatase (Amersham Pharmacia Biotech) (*ALK*; 20 units, *lanes 3* and *4*), protein phosphatase 2A (*PP2*; 200 milliunits, *lane 5*), or a protein kinase A/AKT combination (20 units and 2 μg, respectively; *lane 10*) and *p*-nitrophenylphosphate (*PNPP*; 50 mM, *lane 4*). After washes, the immunoprecipitates were incubated for 30 min at 30 °C with vehicle (*lane 1*), biotinylated recombinant GST-14-3-3 (10 μg; *lanes 2–5*), or biotinylated recombinant GST-ct-14-3-3 (10 μg; *lane 6*), washed, and separated on SDS-PAGE. The bound biotinylated 14-3-3 proteins were detected by blotting with avidin-horseRadish peroxidase and ECL (*upper panel*). The *lower panel* shows equal recovery of vimentin. *Lanes 7* and *8* contain 5% input of biotinylated GST-14-3-3 and GST-ct-14-3-3, respectively. *B*, COS cells transfected with empty vector or with GST-14-3-3 were treated with calyculin A (100 nM, 30 min) or vehicle as indicated. Cell extracts were incubated in the absence or presence of 300 μM synthetic phosphopeptide corresponding to the 14-3-3 binding sequence of Raf (*PP*) or in the presence of a 300 μM concentration of the unphosphorylated peptide (*P*) at 4 °C for 1 h followed by anti-vimentin immunoprecipitation. Shown are anti-14-3-3 immunoblot (*upper panel*) and a Coomassie Blue stain (*lower panel*). *C*, COS cells transiently expressing wild type GST-14-3-3 (*wt*, *lanes 1–4*), dimerization-deficient 14-3-3 mutant GST-dm-14-3-3 (*dm*, *lanes 5–8*), GST-ct-14-3-3 (*ct*, *lanes 9* and *10*), or GST-nt-14-3-3 (*nt*, *lanes 11* and *12*) were treated with calyculin A (150 nM, 30 min), okadaic acid (3 μM, 30 min), or heat shock (42 °C, 45 min) or left untreated (−). The GST fusions were recovered on GSH-Sepharose beads and assayed for coassociated vimentin by an anti-vimentin immunoblot (*upper panel*). The recovery of GST fusions was determined by anti-GST (Upstate Biotechnology, Inc., Lake Placid, NY) immunoblot of the stripped membrane (*lower panel*). *D*, vimentin immunoprecipitated from ^{32}P-labeled COS cells treated for 60 min with calyculin A (0.3 μM) was resolved on SDS-PAGE and transferred to PVDF membrane. The ^{32}P-labeled vimentin band was excised and subjected to endoproteinase Lys-C cleavage. The digest was absorbed on immobilized wild type prokaryotic recombinant GST-14-3-3 (*wt*, *lanes 1* and *3*) or on the dimerization-deficient 14-3-3 mutant GST-dm-14-3-3 (*dm*, *lanes 2* and *4*) in the absence (*lanes 1* and *2*) or presence of 300 μM phosphopeptide (*lanes 3* and *4*). Bound polypeptides were separated on a 15% SDS-PAGE using the Tris Tricine buffer system, stained with Coomassie Blue (*left half*), and subjected to autoradiography (*right half*).

^{32}P-peptide that binds to 14-3-3 exhibits an apparent molecular mass of 14 kDa, consistent only with one predicted lysyl C fragment, corresponding to the vimentin amino-terminal region (amino acids 1–96). These results suggest that the vimentin phosphorylation sites that mediate binding to 14-3-3 are located in this amino-terminal segment.

14-3-3 Association with Vimentin Results in Its Dissociation from Raf and from Most of Its Other Target Proteins—We noticed that the progressive increase in GST-14-3-3/vimentin association *in vivo* that occurs with prolonged exposure of cells to calyculin A (300 nM, 60 min) is accompanied by the displacement of most other 14-3-3 partners (Fig. 3, *A* and *B*), when low levels of GST-14-3-3 are expressed, but not with high level GST-14-3-3 expression (Fig. 3*A*). Inasmuch as the identity of specific endogenous 14-3-3-associated polypeptide bands is not known, Myc-Raf was coexpressed with GST-14-3-3 to serve as a reporter (Fig. 3*B*). Calyculin A pretreatment abrogates completely the association of Myc-Raf with 14-3-3 (Fig. 3*B*, compare *lane 1* with *lane 4* and *lane 2* with *lane 5*); a similar inhibition is observed for the association of endogenous c-Raf-1 with GST 14-3-3 in calyculin A-treated cells (Fig. 3*C*, compare *lane 2* with *lane 4*). The loss of the 14-3-3/Raf association and 14-3-3 association with other targets caused by calyculin A *in vivo* is not due to a modification of c-Raf-1 or the other target proteins that impair their ability to bind 14-3-3, since Raf and the other targets from extracts of untreated or calyculin A-treated cells bind equally well to prokaryotic recombinant GST 14-3-3 *in vitro* (Fig. 3, *D* and *E*). Thus, a more feasible explanation for the calyculin A-induced inhibition of 14-3-3 association with Raf and other target proteins *in vivo* is that the phosphovimentin that accumulates in the calyculin A-treated cells simply outcompetes and displaces Raf and the other 14-3-3 partners.

Limitation in 14-3-3 Availability by Phosphorylated Vimentin Negatively Regulates Raf Kinase Activity—It has previously been shown that 14-3-3 serves as an indispensable cofactor for

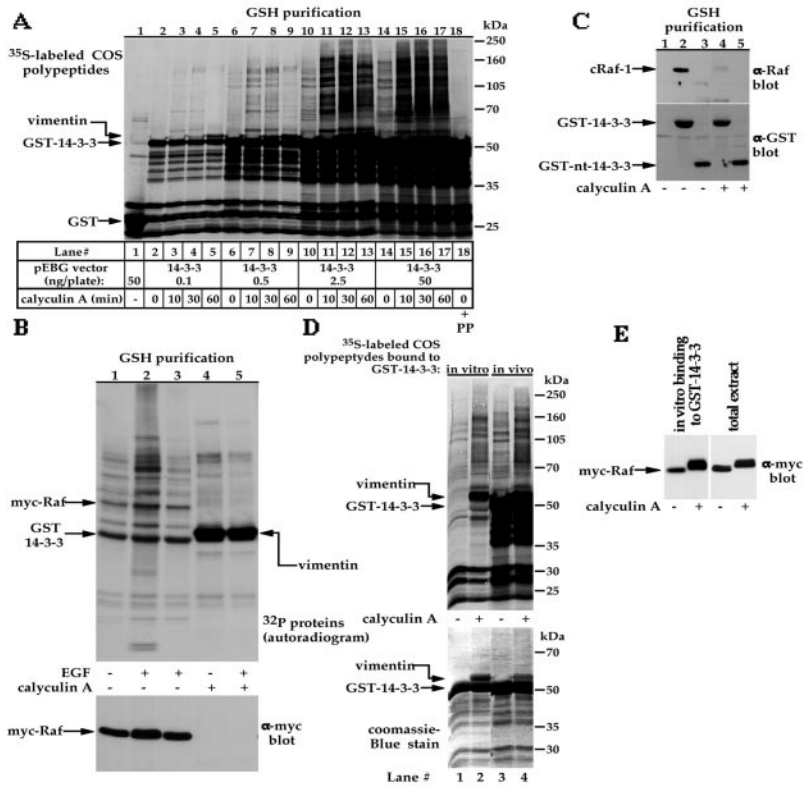

FIG. 3. **14-3-3 association with vimentin results in its dissociation from Raf and other target proteins.** *A*, COS-7 cells were transiently transfected with pEBG vector encoding GST alone (*lane 1*) or with pEBG vector encoding GST-14-3-3 fusion (*lanes 2–18*) using the indicated amounts of DNA/10-cm plate by the LipofectAMINE method (Life Technologies). 24 h post transfection, the cells were deprived of serum for 18 h and metabolically labeled as in Fig. 1*A* and treated with carrier (*lanes 2*, *6*, *10*, *14*, and *18*) or with 200 nM calyculin A for the indicated times (*lanes 3–5*, *7–9*, *11–13*, and *15–17*). Cells were extracted in the absence (*lanes 1–17*) or presence of 1 mM synthetic phosphopeptide corresponding to the 14-3-3 binding sequence of Raf (*PP*, *lane 18*), and the GSH-Sepharose-associated proteins were analyzed as in Fig. 1*A* on a 7–14% continuous gradient SDS-PAGE. An autoradiogram of the ^{35}S-labeled proteins is shown. The array of bands migrating between 35 and 50 kDa represents degradation products of GST-14-3-3 that bound to the GSH-Sepharose beads. These products are also present in GST-14-3-3 produced in bacteria (see Fig. 3*C*, *lower panel*) and are not present in mock-transfected COS cells (Fig. 3*C*, *upper panel*). Moreover, these bands were not displaced by the synthetic phosphopeptide (Fig. 3*A*, *lane 18*). *B*, serum-deprived COS cells co-expressing GST-14-3-3 and Myc-Raf-1 were ^{32}P-labeled and treated with vehicle (*lanes 1–3*) or calyculin A (300 nM; *lanes 4* and *5*) for 45 min followed by carrier (*lanes 1* and *4*) or EGF treatment (100 ng/ml; *lanes 2*, *3*, and *5*) for 15 min. GST-14-3-3 was recovered on GHS-Sepharose beads, and the samples were left untreated (*lanes 1*, *2*, *4*, and *5*) or treated with protein phosphatase 1γ for 15 min at room temperature (1 milliunit/ml; *lane 3*). 14-3-3-associated proteins were resolved on a 5–15% gradient SDS-PAGE and transferred to PVDF membrane. An autoradiogram of the ^{32}P-labeled proteins (*upper panel*) and an anti-Myc immunoblot of the same membrane (*lower panel*) are presented. The treatment with protein phosphatase-1γ (*lane 3*) is unrelated to this work. *C*, mock-expressing (*lane 1*), GST-14-3-3-expressing (*lanes 2* and *4*), or GST-nt-14-3-3-expressing (*lanes 3* and *5*) COS cells were treated with vehicle (*lanes 1–3*) or calyculin A (300 nM; *lanes 4* and *5*) for 45 min. After GSH-Sepharose purification, the association of endogenous Raf with the GST fusions was determined by anti-Raf immunoblot (*upper panel*). The recovery of the GST fusions was determined by anti-GST immunoblot (*lower panel*). *D*, COS cells were mock-transfected (*lanes 1* and *2*) or transfected with pEBG GST-14-3-3 (12.5 ng of DNA/10-cm plate; *lanes 3* and *4*). Following serum deprivation and ^{35}S-metabolic labeling, the cells were treated for 60 min with carrier (*lanes 1* and *3*) or with 200 nM calyculin A (*lanes 2* and *4*). Cell extracts were isolated on GSH-Sepharose beads (*lanes 3* and *4*) or tested for *in vitro* binding to GSH-Sepharose-immobilized prokaryotic GST-14-3-3 (*lanes 1* and *2*). The GSH-Sepharose-associated proteins were analyzed on a 7–14% continuous gradient SDS-PAGE. Shown are an autoradiogram of the ^{35}S-labeled proteins (*upper panel*) and a Coomassie Blue stain of the same gel (*lower panel*). *E*, COS cells transiently expressing Myc-Raf-1 were treated with calyculin A (300 nM, 45 min) or carrier as indicated. The Myc-Raf contained in the cell extracts was assayed for *in vitro* binding to immobilized prokaryotic recombinant GST-14-3-3. Shown are anti-Myc immunoblots of the total cell extracts (*right panel*) and of the GST-14-3-3-retained Raf (*left panel*).

Raf kinase activity (16, 17). This, together with the ability of calyculin A treatment to disrupt 14-3-3/Raf complexes *in vivo*, implied that the regulation of Raf kinase activity in calyculin A-treated cells would be impaired. Thus, we decided to use Raf kinase activity as a functional read-out assay for 14-3-3 availability in the cell. Incubation of serum-deprived COS cells with calyculin A caused a slight increase in Raf kinase activity but inhibited by 95% the EGF-induced activation of Raf (Fig. 4*A*). To confirm that this low activity was due to 14-3-3 limitation *in vivo*, we examined the ability of purified prokaryotic recombinant 14-3-3 to restore the activity of Raf extracted from calyculin A-treated cells (Fig. 4*B*). Consistent with earlier findings, the addition of recombinant 14-3-3 to Raf from unstimulated cells (Fig. 4*B*, compare *lane 2* with *lane 8*) or EGF-treated cells (Fig. 4*B*, compare *lanes 3* and *4* with *lane 9*) had no effect on Raf kinase activity. In contrast, the addition of 14-3-3 to Raf extracted from calyculin A-treated cells greatly increased Raf kinase activity (Fig. 4*B*, no EGF, compare *lane 5* with *lane 10*; with EGF, compare *lane 6* with *lanes 11* and *12*). The ability of 14-3-3 to restore Raf activity in extracts of calyculin A-treated cells was completely blocked by the Raf synthetic phosphopeptide (Fig. 4*B*, *lane 13*), demonstrating that 14-3-3 ability to reactivate Raf requires the 14-3-3 phosphopeptide binding domain. Moreover, a dimerization-deficient 14-3-3 mutant, which is unable to reactivate Raf deactivated *in vitro* after phosphopeptide-induced displacement of native 14-3-3 (17), is also unable to activate Raf extracted from calyculin A-treated cells (Fig. 4*B*, *lane 14*). To further ascertain whether the inability of EGF to activate Raf in calyculin A-treated cells is due to a limitation in the availability of endogenous 14-3-3, increasing amounts of GST-14-3-3 were cotransfected together with Myc-Raf, and the effect of calyculin A pretreatment on EGF-stimu-

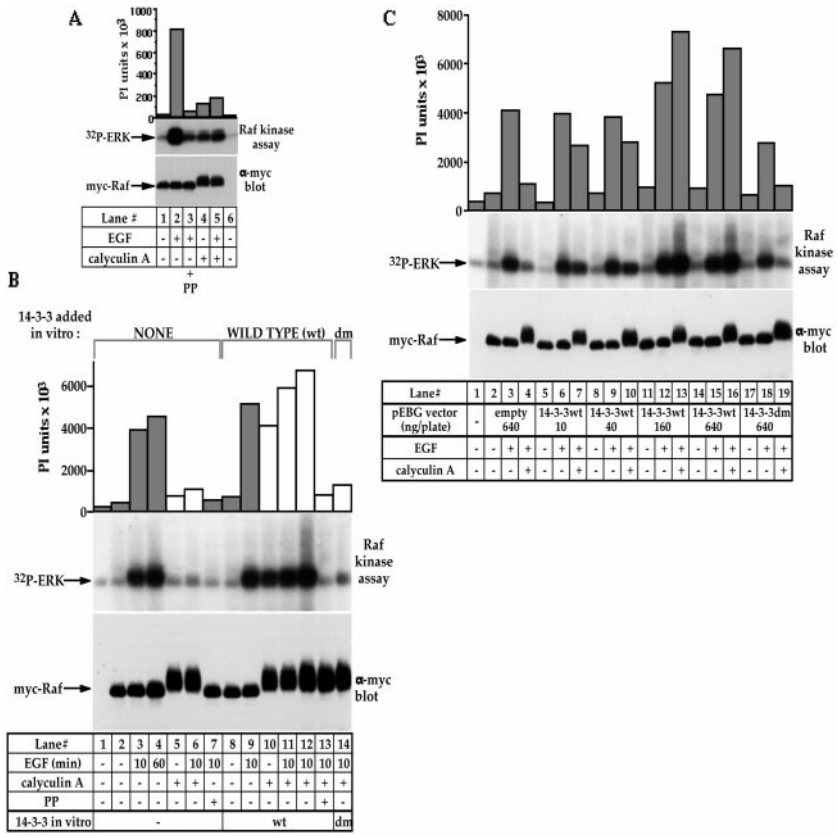

FIG. 4. **Calyculin A-induced inhibition of Raf is abrogated by the provision of excess 14-3-3, both *in vivo* and *in vitro*.** *A*, serum-deprived COS cells transiently expressing Myc-Raf were treated with vehicle (*lanes 1–3*) or calyculin A (300 nM; *lanes 4* and *5*) for 30 min, followed by carrier (*lanes 1* and *4*) or EGF treatment (100 ng/ml; *lanes 2*, *3*, and *5*) for 15 min. Myc-Raf was immunoprecipitated (clone 9E10) and assayed for Raf kinase activity in a coupled kinase assay (see "Materials and Methods"). The *upper panel* presents ^{32}P incorporation into kinase-inactive ERK (PhosphorImager units $\times 10^3$), the *middle panel* shows an autoradiogram of the ^{32}P-labeled ERK, and the *lower panel* is an anti-Myc immunoblot. *B*, serum-deprived COS cells expressing Myc-Raf were treated with vehicle (*lanes 2*, *3*, *4*, *7*, *8*, and *9*; *filled bars*) or 300 nM calyculin A (*lanes 5*, *6*, and *10–14*; *empty bars*) for 50 min, followed by 100 nM EGF treatment for the indicated times (*lanes 3*, *4*, *6*, *7*, *9*, and *11–14*). Cells were lysed in the presence of carrier (*lanes 2–6*), 50 μg of prokaryotic recombinant wild type GST-14-3-3 (*lanes 8–13*), or GST-dm-14-3-3 (*lane 14*) alone or together with 1 mM phosphopeptide (*lanes 7* and *13*). Myc-Raf immunoprecipitates were analyzed for Raf kinase activity as in *A*. *C*, COS cells were transiently transfected using the LipofectAMINE method with 2 μg/10-cm plate of DNA encoding Myc-Raf, together with the indicated amounts of pEBG vector encoding GST alone (*lanes 2–4*), GST-14-3-3 (*lanes 5–16*), or GST-dm-14-3-3 (*lanes 17–19*). 24 h post-transfection, cells were deprived of serum for 18 h and treated with vehicle (*lanes 2*, *3*, *5*, *6*, *8*, *9*, *11*, *12*, *14*, *15*, *17*, and *18*) or 300 nM calyculin A (*lanes 4*, *7*, *10*, *13*, and *19*) for 50 min, followed by carrier or EGF (100 nM) treatment for 10 min as indicated. Cells were lysed, and the Raf kinase activity in the anti-Myc immunoprecipitates was analyzed as in *A*.

lated Raf activation was assayed (Fig. 4*C*). Transfection of as little as 10 ng/plate of pEBG-GST-14-3-3 DNA was able to substantially overcome the calyculin A-induced inhibition of Raf activation, and at 160 ng/plate, transfection resulted in complete restoration of Raf activity to levels seen with EGF stimulation (Fig. 4*C*). The requirement for wild type 14-3-3 is shown by the inability of the pEBG vector (*i.e.* GST alone; Fig. 4*C*, *lanes 2–4*) as well as the pEBG-encoded dimerization-deficient mutant 14-3-3 (Fig. 4*C*, *lanes 17–19*), each added at 640 ng/plate, to increase Raf activity in calyculin A-treated cells. It is notable that the expression of recombinant 14-3-3 does not alter Raf activity in cells not exposed to calyculin A, whether unstimulated (Fig. 4*C*, compare *lane 2* with *lanes 5*, *8*, *11*, and *14*) or treated with EGF (Fig. 4*C*, compare *lane 3* with *lanes 6*, *9*, *12*, and *13*), confirming that 14-3-3 overexpression does not activate Raf *per se*. Rather, 14-3-3 overexpression specifically overcomes the inhibition caused by calyculin A pretreatment. These results demonstrate that the calyculin A-induced vimentin binding to 14-3-3 results in limitation in 14-3-3 availability to Raf and in altered regulation of its kinase activity.

DISCUSSION

14-3-3 proteins have been found to specifically associate with and participate in the regulation of many proteins including several key proteins involved in cellular signaling and cell cycle control. The present work identifies vimentin as an additional, regulated, 14-3-3 interactor. Vimentin undergoes extensive phosphorylation during cell cycle progression, and the inhibition of protein phosphatase 1 and 2A by calyculin A causes a rapid increase in vimentin phosphorylation comparable in magnitude with that occurring during mitosis, although with a somewhat different site specificity (25). The ability of calyculin A treatment to promote sufficient phosphorylation of endogenous vimentin to enable it to bind a very large fraction of cellular 14-3-3 raises two intriguing possibilities. First, it suggests that 14-3-3 may contribute to the regulation of vimentin phosphorylation/dephosphorylation and/or to the ability of vimentin to participate in filament formation. Second, the high stoichiometry of cellular vimentin phosphorylation achieved during specific phases of the cell cycle may bind sufficient cellular 14-3-3 to limit its availability, thereby modulating the function of other 14-3-3-dependent proteins.

Previous work has shown that the binding of 14-3-3 to phosphoproteins, such as Raf, can diminish their susceptibility to protein (Ser/Thr) phosphatase action (28). Thus, the association of 14-3-3 with phosphovimentin may alter vimentin dephosphorylation. As regards the impact of 14-3-3 on intermediate filament assembly, Liao *et al.* (8) demonstrated that

binding of 14-3-3 to keratin K18 could interfere with its assembly into filaments *in vitro.* 14-3-3 binding to K18 depends on keratin Ser^{33} phosphorylation; however, this phosphorylation is not sufficient by itself for conferring association (14). Since the sequence around keratin Ser^{33} does not represent a strong 14-3-3 motif, it seems that another 14-3-3 binding site(s) on K18 may collaborate with the Ser^{33} site to support 14-3-3 binding. This possibility is strengthened by our finding that vimentin, which, like K18, does not contain an optimal high affinity 14-3-3 biding site, can form a stable association only with dimeric 14-3-3 and not with various 14-3-3 monomers. The importance of 14-3-3 dimerization in 14-3-3 function has not gained much attention; the functional role usually proposed for the 14-3-3 dimer is to allow the bridging of two different proteins (29–31). For proteins that lack high affinity 14-3-3 binding sites, such as vimentin, keratin K18, and Wee1 (3), the coligation of two lower affinity sites by the 14-3-3 dimer may be necessary to enable stable target protein recognition and binding. The role of 14-3-3 dimerization extends, however, beyond the ability to bridge two different proteins and enable binding to single proteins with only low affinity sites. We recently showed that a 14-3-3 dimer is necessary to support Raf activity (17) despite the fact that Raf contains two high affinity binding sites for 14-3-3, each of which is independently sufficient to stably bind 14-3-3 (17, 32). The present report extends those findings by demonstrating that not only is the dimerization-deficient 14-3-3 unable to support the reactivation of Raf inactivated by 14-3-3 displacement *in vitro*, it is also unable to support an active configuration of Raf achieved *in vivo* in calyculin A-treated cells. Along similar lines, we recently observed that the ability of 14-3-3 to modulate the DNA binding of the *Caenorhabditis elegans* transcription factor DAF-16 requires a dimeric 14-3-3 and two independent 14-3-3 binding sites on the DAF-16 polypeptide.[2]

The finding that vimentin phosphorylation and its concomitant binding to 14-3-3 proteins results in disassembly of Raf-14-3-3 complexes and other 14-3-3-protein complexes suggests that the association of phosphovimentin with 14-3-3 may have significance in the regulation of 14-3-3 availability besides a role in vimentin dephosphorylation/disaggregation. This intriguing possibility implies that phosphorylation of vimentin and perhaps other IF polypeptides (*e.g.* keratin K18) may affect various intracellular signaling and cell cycle control pathways by modifying 14-3-3 availability, as observed with Raf in this paper. Further evaluation of the cellular distribution of the different 14-3-3 isoforms under varying physiologic conditions and during different stages of the cell cycle and the effect of IF phosphorylation on this distribution is needed to ascertain whether variation in the availability of 14-3-3 by IFs serves as a regulatory factor in cellular signaling and cell cycle control.

[2] C. M. Cahill, G. Tzivion, N. Nasrin, S. Ogg, J. Dore, G. Ruvkun, and M. Alexander-Bridges, submitted for publication.

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Calyculin A-induced Vimentin Phosphorylation Sequesters 14-3-3 and Displaces Other 14-3-3 Partners *in Vivo*

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