Phosphorylation-Dependent Interaction of Kinesin Light Chain 2 and the 14-3-3 Protein†

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ABSTRACT: The protein 14-3-3 is a key regulator in a cell signaling pathway mediated by protein phosphorylation. To identify the cellular targets of this protein systematically, we have employed a proteomic approach: protein components pulled down from PC 12 cells stably expressing a myc-tagged 14-3-3η isoform were analyzed by means of SDS−PAGE and mass spectrometry. This procedure allowed us to identify more than 30 proteins that include various known and unknown targets of the 14-3-3 protein. Among them are several proteins in the membrane traffic pathway, such as the heavy and light chains (KHC/KIF5B and KLC2) of conventional kinesin, a heterotetrameric mechanochemical motor involved in the ATP-dependent movement of vesicles and organelles along microtubules. Subsequent analysis showed that 14-3-3 directly binds to kinesin heterodimers through interaction with KLC2 and that this interaction is dependent on the phosphorylation of KLC2. Studies on the interaction between 14-3-3 and KLC2 variants expressed in cultured cells coupled with mass spectrometric analysis proved that Ser575 is the site of phosphorylation in KLC2 that is responsible for the in vivo interaction with the 14-3-3 protein. These data add KLC2 to the growing list of 14-3-3 targets, and suggest a role of 14-3-3 in the phosphorylation-regulated cellular transport of vesicles and organelles.

Protein phosphorylation on Ser and Thr residues has traditionally been thought of as a means to allosterically regulate enzyme activity. However, recent studies have revealed that Ser/Thr phosphorylation can also regulate the formation of signaling complexes through interactions between specific phosphorylated sequence motifs and phosphoSer/Thr-binding factors. Three classes of such binding factors have been isolated from mammalian cells. They are mitogenic proline isomerase Pin1, ubiquitin ligase Nedd4, and the highly conserved family of eukaryotic proteins collectively called the 14-3-3 protein (for a review, see ref *1*).

The 14-3-3 protein comprises a family of acidic, dimeric proteins consisting of at least seven distinct subunit isoforms (α/β, γ, δ/ζ, ϵ, η, τ, and σ; where α and δ are the phosphorylated forms of β and ζ, respectively). The crystal structures of the 14-3-3ζ and -τ isoforms have revealed that these isoforms have a similar tertiary fold consisting of a bundle of nine antiparallel α-helices, and form a conserved channel or groove in each monomer (*2, 3*). This groove has an amphipathic property, with a hydrophobic face on one side and a positively charged face on the other. Structure-based mutational analysis of 14-3-3 (*4*), as well as cocrystallization of 14-3-3ζ with phosphopeptide ligands (*5, 6*), proposed that the amphipathic groove is the ligand-binding site on the 14-3-3 molecules. The recently published crystal structure of the complex between 14-3-3ζ and serotonin *N*-acetyltransferase has supported this proposal (*7*).

The 14-3-3 family is implicated as key regulators in diverse intracellular signal transduction pathways (for a review, see ref *8*). In yeast, this protein family plays a role in checkpoint signaling for cell division, and null mutants are lethal (*9*−*11*). Recent genetic studies in *Drosophila* showed that mutations in 14-3-3 genes disrupt Ras-mediated differentiation of photoreceptor R7 cells during eye development, and decrease the capacities for olfactory learning and memory (*12*−*14*). There are currently over 70 proteins that have been identified as targets of the 14-3-3 family, such as those involved in a Ras/mitogen-activated protein kinase (MAPK)[1] signaling cascade and in an apoptosis-signaling pathway. In many cases, the capacity of a 14-3-3 protein to bind and form a complex with a target protein depends on the phosphorylation of the target, particularly at a specific Ser residue(s). On the basis of these findings, it has been proposed that the 14-3-3 family is a type of chaperone or molecular scaffold that modulates the activity, conformation,

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stability, interaction, or intracellular localization of target proteins.

In this study, we have screened 14-3-3-binding proteins in PC12 cells by means of proteomic techniques. This analysis allowed us to identify more than 30 proteins that include various known and unknown targets of the 14-3-3 protein. From among them, the heavy- and light-chain subunits (KHC/KIF5B and KLC2) of conventional kinesin (termed c-kinesin; for a review, see ref *15*) were selected for further analyses, because these subunits were found to be major constituents of the 14-3-3 immunoprecipitate. Subsequent analyses showed that 14-3-3 directly binds to c-kinesin through interaction with KLC2 and that this interaction is dependent on the phosphorylation of KLC2 at Ser575. These results suggest that 14-3-3 participates in phosphorylation-regulated, kinesin-mediated, vesicle transport processes. This finding is particularly important with respect to the recent proposal that JNK signaling may control kinesin motor activity (*16*, *17*), because 14-3-3 proteins also bind to several stress-induced MAPKKKs, such as ASK1 (*18*), MLK2 (*19*), and MEKK1, -2, and -3 (*20*), which can activate JNK and p38MAPK.

EXPERIMENTAL PROCEDURES

Materials. Anti-myc Sepharose beads were generated by covalent coupling of monoclonal myc antibody 9E10 and CNBr-activated Sepharose beads (Amersham Pharmacia Biotech). The 9E10 monoclonal antibody was purified from mouse ascites by ammonium sulfate fractionation and protein G-Sepharose chromatography. Rabbit polyclonal antibodies against 14-3-3η were provided by Immuno-Biological Laboratories. GST-14-3-3η was produced by PCR amplification and cDNA cloning into the pGEX-3X vector as described (*21*). The expressed protein was purified with glutathione-agarose beads. Phosphopeptides were synthesized and purified by C18 reverse-phase high-performance liquid chromatography as described (*21*). Oligonucleotides were purchased from Biotech International.

Plasmid Construction. The cDNA for myc-tagged 14-3-3η was generated by PCR using oligonucleotides 5′-AAG GAT CCT TAG TTC TCT CCC TCT CC-3′ and 5′-CAG GAT CCT CAG TTG CCT TCT CCG GC-3′ and bovine 14-3-3 cDNA (pAP62, ref *22*). The PCR fragment was digested with *Mlu*I and *Bam*HI, and then inserted into the cloning site of myc-tag vector pMUM1 (*23*). The myc-tagged 14-3-3η cDNA was then digested with *Hin*dIII and *Xba*I, and inserted into mammalian expression vector pcDNA3 (Invitrogen). The cDNA for myc-tagged 14-3-3η in retroviral expression vector pLNCX5 has previously been described (*24*). The cDNA for Flag-tagged full-length KLC2 (termed pWK, see Figure 3) in mammalian expression vector pCMV-Tag2C (Invitrogen) was produced by subcloning an *Eco*RI/*Sac*I fragment of mouse KLC2 cDNA (a gift from Dr. L. S. B. Goldstein, University of California) into the pUC18 vector. The KLC2 cDNA was digested with *Eco*RI and *Sal*I, and then inserted into expression vector pCMV-Tag2C. The cDNAs for truncation mutants of KLC2 (termed pMKN, pMKC1, and pMKC2; see Figure 3) were created by ligating *Bgl*II/*Xho*I, *Eco*RI/*Fsp*I, and *Eco*RI/*Apa*I fragments of pWK into the *Bam*HI/*Xho*I, *Eco*RI/*Eco*RV, and *Eco*RI/*Apa*I sites of pCMV-Tag2C, respectively. Site-directed mutagenesis was carried out as described (*24*) using the following mutagenic primers: 5′-GAG GGC CAG TCT AGC TAA CTT-3′ (for klcS575A); 5′-GGG CCG TGG CTG AGC TGG AC-3′ (for E185K); 5′-GGG CCG TGG CTG AGC TGG AC-3′ (for V180D).

PC12 Cell Culture. PC12 cells were grown on collagen-coated tissue culture plates in RPMI1640 medium supplemented with 10% heat-inactivated donor horse and 5% fetal bovine sera, as described previously (*25*). Retroviral infection was carried out as described (*24*) by adding 0.5−1 mL of a virus-containing supernatant, recovered from packaging cells (Bosc23), to 50% confluent PC12 cell cultures. Infected cells were cultured for 1 week and then subjected to selection with G418 for at least 1 month. Surviving cells were polyclonally expanded and cultured (termed PC12η cells).

Immunoprecipitation and Phosphopeptide Treatment. PC12η cells ($\sim 2 \times 10^7$ cells) were lysed in 1 mL of lysis buffer comprising 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 100 mM NaF, 10 mM EGTA, 1 mM Na_3VO_4, 1% Triton X-100, 5 μM $ZnCl_2$, 2 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 1 μg/mL leupeptin, and the lysate was centrifuged at 100000*g* for 20 min at 4 °C. The supernatant was saved and cleared by incubation with 100 μL of Sepharose beads for 60 min at 4 °C. Immunoprecipitation was then performed by incubating the supernatant (8 mg of protein) with 100 μL of anti-myc Sepharose beads for 90 min at 4 °C. The beads were washed 7 times with 1 mL of TNTG buffer comprising 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100, and once with 1 mL of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Triton X-100. Proteins bound to the immobilized 14-3-3 were dissociated from the washed beads by incubation with 1 mM synthetic phosphopeptides corresponding to cRaf-1 amino acids 250−265 for 120 min at 4 °C.

In-Gel Digestion and Mass Spectrometry. Protein bands excised from the SDS−polyacrylamide gel were placed in microtubes and cut into small pieces ($1 \times 1 \times 1$ mm). A protein-free gel piece was treated in parallel as a negative control throughout the process. The gel pieces were dehydrated with 100 μL of acetonitrile for 10 min, dried under vacuum, and then rehydrated on ice for 45 min in 10 μL of 50 mM Tris-HCl (pH 8.8) containing 25 ng/μL lysyl endopeptidase (LysC). After the excess solution had been removed, to the gel was added to 10 μL of 50 mM Tris-HCl (pH 8.8), followed by digestion at 37 °C for 16 h. The peptide solution was recovered, and the gel piece was extracted twice with 10 μL of 50% acetonitrile in 5% formic acid. The combined solution was concentrated to 5 μL in a vacuum concentrator. The peptide solution was then diluted with 400 μL of 0.1% trifluoroacetic acid and was added to 5 μL of a 0.4% (w/v) suspension of POROS R2 reversed-phase beads (Applied Biosystems). After the beads were washed twice

[1] Abbreviations: KHC, kinesin heavy chain; KIF, kinesin family member; KLC, kinesin light chain; c-kinesin, conventional kinesin; MAPK, mitogen-activated protein kinase; MAPKKKs, MAPK kinase kinases; ASK, apoptosis signal-regulating kinase; MLKs, mixed lineage Ser/Thr kinases, MEKKs, MEK kinases; PAK, p21(Cdc42/Rac1)-activated protein kinase; JNK, c-Jun N-terminal kinase; JIP, JNK-interacting protein; TPR, tetra-trico peptide repeats; GST, glutathione *S*-transferase; LysC, lysyl endopeptidase; PAGE, polyacrylamide gel electrophoresis.

[2] T. Ichimura and A. Wakamiya-Tsuruta, unpublished data.

with 400 μL of 0.1% trifluoroacetic acid, the bound peptides were recovered with 2 μL of 50% methanol in 5% acetic acid. The peptide mixture was then directly applied to a quadrupole time-of-flight mass spectrometer (Q-TOF; Micromass) equipped with a nanospray tip (Micromass). Proteins were identified using the Mascot program (Matrixscience). The database used for all sequences was the nonredundant protein sequence database maintained at the National Center for Biotechnology Information (NIH). To identify the phosphorylation site in KLC2, the excised 354−599 fragment was in-gel-digested with trypsin or LysC, and the resulting peptides were concentrated under vacuum. The peptide mixture was analyzed with the direct nano-flow LC-MS/MS system, equipped with an electrospray tip reversed-phase column and a nano-flow gradient device. The details of this system will be published elsewhere.

Far-Western Blotting. The KHC and KLC2 immunoprecipitates were subjected to 7.5% SDS−PAGE and then transferred to nitrocellulose membranes. After the membranes were blocked with 5% skim milk in TBS-T (30 mM Tris, pH 7.5, 125 mM NaCl, 0.1% Tween 20) for 60 min at room temperature, they were incubated overnight at 4 °C with 5 μg/mL GST-14-3-3η or GST in TBS-T. The bound GST proteins were detected by incubating the membranes with an anti-GST monoclonal antibody (Santa Cruz) for 90 min, followed by a horseradish perixidase-conjugated donkey anti-mouse antibody and ECL Western blotting detection system (Amersham Pharmacia Biotech). Phosphatase treatment was performed essentially according to the procedure described by Michaud et al. (*26*). Briefly, an immobilized KLC membrane (1 × 1 cm) was incubated for 60 min at room temperature with 3 units of type VII potato acid phosphatase (Sigma) in a buffer comprised of 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.0, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/mL aprotinin, and 5 μg/mL leupeptin, in a final volume of 1 mL.

Transient Expression and Binding Assay. The transfection of 293T cells was performed by the method of Chen and Okayama (*27*). The treatment and lysis of the cells were performed as described (*28*). Briefly, ~5 × 10^6 cells were lysed in 500 μL of lysis buffer, and the lysates were centrifuged at 100000*g* for 20 min. The lysates were then incubated with 20 μL of anti-Flag Sepharose beads (Sigma) for 90 min at 4 °C. The immunoprecipitates were washed 8 times with TNTG buffer and then solubilized in SDS sample buffer. Samples were analyzed by SDS−PAGE followed by Western blotting using the indicated primary antibodies.

Others. SDS−PAGE and Western blotting were performed as described (*24*). Silver staining was carried out as described previously (*29*) without glutaraldehyde.

RESULTS

Association of 14-3-3η with c-Kinesin in PC12 Cells. To isolate proteins that physically associate with the 14-3-3 protein, we used the PC12 cell line (termed PC12η cells) stably expressing a myc-tagged 14-3-3η isoform (Figure 1A). The PC12η cells were lysed, and the expressed myc-η was immunoprecipitated from the lysate with anti-myc monoclonal antibody-conjugated Sepharose beads. After the beads were washed, synthetic phosphopeptides corresponding to the Raf-binding site of 14-3-3 (LSQRQRST*p*STPNVHA, ref

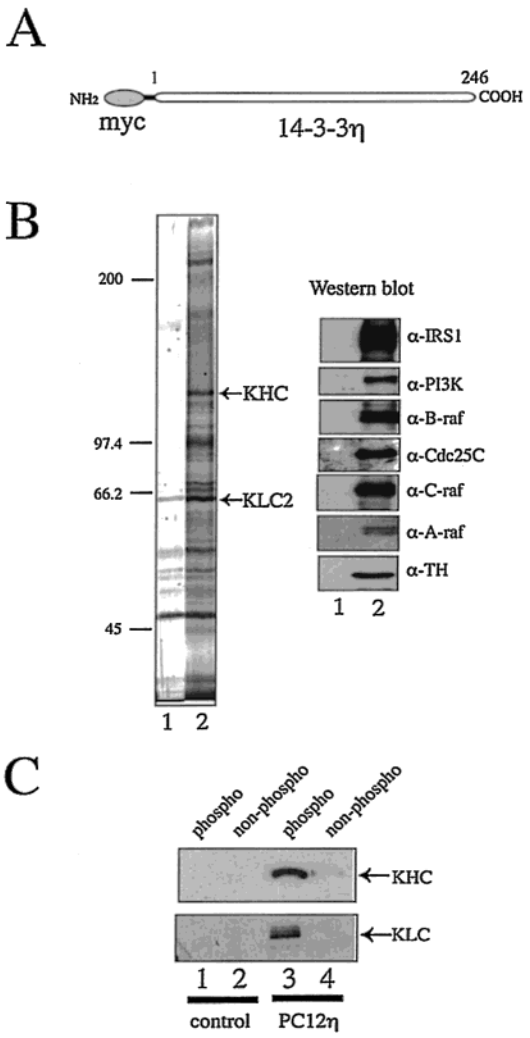

FIGURE 1: Association of 14-3-3η with c-kinesin in PC12η cells. (A) Structure of a myc-tagged 14-3-3η isoform. (B) Analysis of proteins coimmunoprecipitated with myc-14-3-3η. PC12η cells stably expressing myc-14-3-3η were lysed, and the expressed myc-η was immunoprecipitated with anti-myc monoclonal antibody-conjugated Sepharose beads. Proteins bound to the immobilized myc-14-3-3 were dissociated with phosphorylated Raf-1 peptides and then analyzed by SDS−PAGE (7.5% PAGE gel, silver staining, left panel, lane 2), or by Western blotting with the indicated antibodies (right panel, lane 2). A control experiment was performed under the same conditions with nontransfected PC12 cells (lanes 1). The weights of molecular mass markers are shown in kDa, and the positions of KHC and KLC2 are indicated by arrows. (C) Phosphopeptide-specific dissociation of c-kinesin subunits from the 14-3-3 immunocomplexes. Extracts of PC12 cells (lanes 1 and 2) or PC12η cells (lanes 3 and 4) were subjected to immunoprecipitation as in (A) except that nonphosphorylated Raf-1 peptides were used for the dissociation of bound proteins (lanes 2 and 4). Dissociated proteins were analyzed by Western blotting with anti-KHC and anti-KLC antibodies.

30) were added to the beads, and the proteins bound to the immobilized 14-3-3 were dissociated from the phosphoSer-binding groove (see also Experimental Procedures). Figure 1B (lane 2) shows SDS−PAGE photographs of the dissociated proteins. About 30 proteins with molecular masses between 35 and 300 kDa were reproducibly detected with this procedure, while only a few proteins were detected with a control lysate of cells not expressing myc-η (lane 1), suggesting that most of the proteins were co-immunoprecipitated with the expressed 14-3-3η. Western blotting with antibodies against several reported 14-3-3 targets showed that

Table 1: Identification of the Kinesin Heavy and Light Chains by NanoESI MS/MS Spectrometry

protein	accession number (GI)	molecular mass (kDa)	peptide *m/z*	charge	sequence determined	residues
kinesin heavy chain	4758648	109.5	726.4	2	SLTEYLQNVEQK	645−656
	2119280		740.9	2	EYELLSDELNQK	515−526
			764.4	2	DLAEIGIAVGNNDVK	561−575
			774.8	2	ISFLENNLEQLTK	832−844
			931.5	2	EVLQALEELAVNYDQK	490−505
kinesin light chain 2	6680578	66.6	586.6	2	LQRSEQAVAQLEEEK	120−123
			601.8	2	LSQDEIVLGTK	12−22
			607.7	2	ATMVLPREEKLSQDEIVLGTK	1−22

this protein fraction contained the IRS-1 docking protein, PI3 kinaseα, Cdc25C phosphatase, tyrosine hydroxylase, and three Raf kinase isozymes (Figure 1B, right panel).

To identify other targets of 14-3-3, the protein bands were cut out from the gel and digested with lysyl endopeptidase (LysC), and the resulting peptides were subjected to mass spectrometric (MS) analysis (see Experimental Procedures). This analysis identified 32 individual proteins including various known and unknown targets. Among the unknown targets were several protein components in membrane traffic pathways such as the heavy chain (KHC/KIF5B; GI: 2119280/4758648) and light chain (KLC2; GI:6680578) of c-kinesin (Table 1). Subsequent Western blotting revealed that these c-kinesin subunits were dissociated from the 14-3-3 immnocomplex with the phosphorylated Raf-1 peptide but not with the nonphosphorylated form (Figure 1C, lanes 3 and 4). This suggests that the interaction of c-kinesin and 14-3-3 is phosphorylation-dependent, and is probably mediated through the ligand-binding groove of the 14-3-3 protein.

14-3-3η Binds to KLC2 Directly. c-Kinesin is a tetramer composed of two KHC and two KLC subunits. To determine whether 14-3-3 binds to KHC or KLC, or both, we performed far-Western studies (*31*). The c-kinesin complex was immunoprecipitated from PC12 cells with either anti-KLC or anti-KHC antibodies, and separated by SDS−PAGE, and after transfer of the subunits to nitrocellulose membranes, the membranes were probed with GST-fused 14-3-3η. As shown in Figure 2A (top panel), GST-η bound to the membrane-immobilized KLC subunit in the c-kinesin complex that was precipitated with anti-KLC or anti-KHC antibodies. In contrast, GST-η did not bind to the KHC subunit precipitated with anti-KLC or anti-KHC antibodies (top panel). We also treated the KLC membrane with potato acid phosphatase and analyzed it for 14-3-3 binding by similar far-Western analysis. As shown in Figure 2B, this treatment abolished the binding of GST-η to KLC. These results indicate that 14-3-3η directly binds to the c-kinesin heterodimers through interaction with the phosphorylated form of its light-chain subunit (KLC2).

Interaction of Endogenous 14-3-3η and KLC2. The interaction data shown in Figures 1 and 2A,B were obtained with either myc-tagged or GST-fused 14-3-3η. To assess the interaction of endogenous 14-3-3η with KLC2, the KLC immunoprecipitate of PC12 cell extracts was analyzed by Western blotting with antibodies specific to 14-3-3η. As shown in Figure 2C, an endogenous 14-3-3η was readily detected on the analysis, confirming that the interaction occurs endogenously in the cells.

Localization of the 14-3-3-Binding Site in KLC2. KLC subunits consist of three distinct structural regions: an

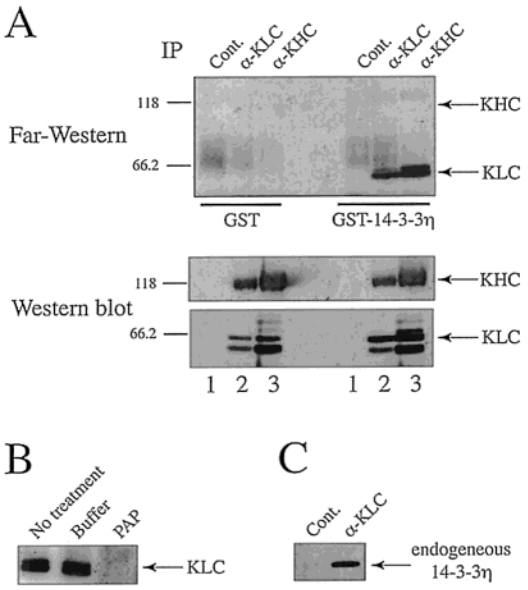

FIGURE 2: Direct binding of GST-14-3-3η to KLC. c-Kinesin heterodimers were immunoprecipitated from PC12 cells with anti-KLC (lane 2) or anti-KHC (lane 3) antibodies. The immunoprecipitates were subjected to SDS−PAGE (7.5% PAGE gel), and then transferred to nitrocellulose membranes. (A) Each membrane was overlaid with 5 μg/mL GST or GST-14-3-3η, and then the binding was detected with an anti-GST monoclonal antibody (upper panel). The membrane was then stripped and reprobed with anti-KHC and anti-KLC antibodies (lower panel). A control experiment was performed with preimmune serum (lane 1). The weights of molecular mass markers are shown in kDa, and the positions of KHC and KLC are indicated by arrows. (B) Each KLC membrane was overlaid with GST-14-3-3η as in (A) except that the membrane was treated with buffer alone (lane 2) or potato acid phosphatase (PAP, lane 3) before the overlay analysis. (C) Control and KLC immunoprecipitates obtained from PC12 cells were analyzed by Western blotting with polyclonal antibodies specific to 14-3-3η.

N-terminal coiled-coil for association with KHC, a tetratrico peptide repeat (TPR) for protein−protein interactions, and a C-terminal tail for cargo recognition and binding (see also Figure 3A). To determine the KLC2 region responsible for 14-3-3 binding, a series of deletion mutants of KLC2 were expressed in 293T cells together with myc-14-3-3η. The deletion mutants examined were three Flag-tagged KLC fragments (354−599, 1−398, and 1−562, Figure 3A) that were truncated in the amino- or the carboxyl-terminal regions of the whole molecule. After lysis and immunoprecipitaion with an anti-Flag monoclonal antibody, the immunoprecipitates were analyzed to detect 14-3-3η by Western blotting with an anti-myc monoclonal antibody. As shown in Figure 3B (right panel), 14-3-3η was detected in the immunoprecipitate of the 354−599 fragment (lane 5), which carries the C-terminal tail but lacks the coiled-coil and most TPR

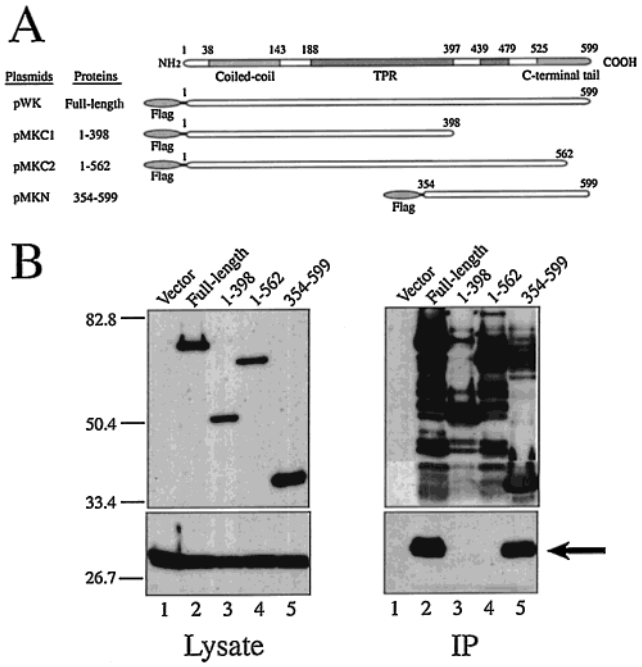

FIGURE 3: Analysis of the site of KLC2 responsible for the binding with 14-3-3. (A) Schematic illustration of the full-length and mutant forms of Flag-tagged KLC2. The relative NH_2 and COOH termini are denoted by adjacent numbers. (B) Effects of truncation mutants of KLC2 on 14-3-3 binding. 293T cells were transfected with the indicated KLC2 constructs or the vector alone and myc-14-3-3η. The lysates (10 μg of protein each) were separated by SDS−PAGE, transferred to poly(vinylidene difluoride) membranes, and then analyzed by Western blotting with an anti-Flag (upper) or anti-myc (lower) monoclonal antibody (left panel). The expressed KLC2 and its truncation mutants were immunoprecipitated from the lysates with anti-Flag Sepharose beads. The immunoprecipitates were analyzed by Western blotting with an anti-Flag (upper) or anti-myc (lower) monoclonal antibody (right panel). The weights of molecular mass markers are shown in kDa, and the arrow indicates the coprecipitated myc-14-3-3η.

regions. However, 14-3-3η was not detected in the immunoprecipitates of the 1−398 and 1−562 fragments (lanes 3 and 4), both of which have deletions of the C-terminal tail region. These data suggest that the C-terminal tail of KLC2 is important for the observed 14-3-3 binding.

To further characterize the interaction between 14-3-3 and KLC2, we prepared two structural variants of the 14-3-3η protein, 14-3-3ηV180D and -E185K, in which V and K replaced D180 and E185, respectively. It has been reported that V180D is a mutant that is 100-fold less potent in the activation of ExoS than the wild-type protein (*32*), and that E185K is a dominant-negative mutant that suppresses RAS1 signaling in photoreceptor formation (*12*). Binding experiments showed that both these variants did not bind to either the full-length KLC2 or the 354−599 fragment, indicating that the observed interaction was specific and would be functionally significant.

The C-Terminal Tail of KLC2 Contains the PhosphoSer Involved in 14-3-3 Binding. Although KLCs have been shown to exist as several phosphorylated forms in cells (*33*), there has been no information regarding the site(s) of phosphorylation. To identify the phosphoSer involved in 14-3-3 binding, we examined whether the expressed 354−599 is indeed phosphorylated in 293T cells, particularly at its C-terminal tail including the potential 14-3-3-binding site. The 354−599 fragment was transfected into 293T cells, and

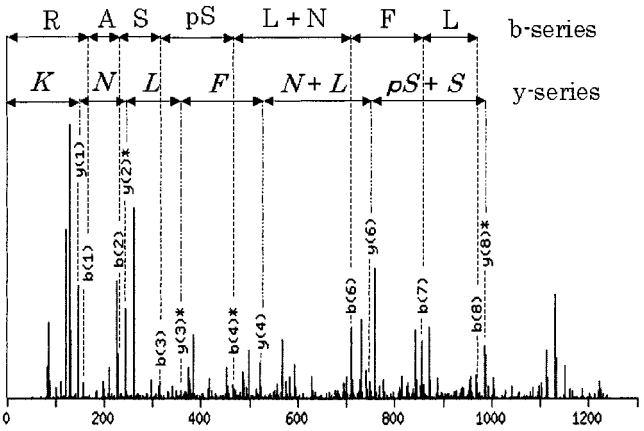

FIGURE 4: Identification of Ser575 as the in vivo phosphorylation site in KLC2. The 354−599 fragment expressed in 293 cells was immunoprecipitated with anti-Flag Sepharose beads. The immunoprecipitate was subjected to SDS−PAGE, digested with trypsin or LysC, and then analyzed by LC-MS/MS (see Experimental Procedures). The $[M+2H]^{2+}$ peptide ion with $m/z = 615.26$ was used as the parent ion for fragmentation to produce the MS/MS spectrum. The detected ions of the b- and y-ion collision series are indicated. The asterisk-denoted peaks, b(4)*, y(2)*, y(3)*, and y(8)*, represent the b(4) ion, y(2) ion, y(3) ion, and y(8) ion, respectively, which have lost their NH_3 groups (17.03 Da). The mass difference between b(3) (315.18 Da) and b(4)* (465.15 Da), 149.97 Da, is consistent with a phosphorylated Ser.

the expressed fragment was recovered by immunoprecipitation. After SDS−PAGE, the 354−599 fragment was in-gel-digested with a protease, and the resulting peptides were directly analyzed by nano-flow LC-MS/MS (see Experimental Procedures). A sequence database search with the observed MS/MS spectra identified 34 peptides that covered ~90% of the 354−599 sequence. Of them, peptide 572−580 was found to be phosphorylated at Ser575 (Figure 4). The analysis also detected nonphosphorylated peptide 572−580 (data not shown), indicating that Ser575 is not a constitutive site for phosphorylation in the cells. Nevertheless, no additional phosphopeptides were found on this analysis. These data suggested that the phosphorylation occurred in the C-terminal tail of the KLC2 molecule, particularly at Ser575, and that this site of phosphorylation mediated the interaction between KLC2 and 14-3-3.

To confirm this observation, a KLC2 mutant was prepared in which Ser575 was substituted by Ala (termed klcS575A). While this KLC2 mutant retained the ability to associate with the KHC subunit, it no longer bound to 14-3-3 (Figure 5). Thus, we concluded that phosphorylation at Ser575 is necessary and sufficient for the association of KLC2 with the 14-3-3 protein.

DISCUSSION

Kinesins are motor proteins that utilize the energy of ATP hydrolysis to move cargo along microtubules. Among the members of this superfamily, c-kinesin is the most ubiquitous motor found in a variety of cells and tissues (for reviews, see refs *15*, *34*). c-Kinesin is an elongated heterotetramer comprising two KHCs of approximately 120 kDa and two KLCs of 60−70 kDa (*35*, *36*). KHC contains the motor domain that generates the ATP-dependent force for movement along microtubules, while KLC is believed to be involved in vesicle binding and regulation of the motor

Table 2: A Conserved Sequence Motif Located in the C-Terminal Tails of Known KLCs[a]

KLC	Sequence
mouse KLC2	^{566}GGGPQEPNSRMKRASSLNFLNKSVEEPVQPGGRVFLTAAL^{599}
sea urchin KLC1-3[a]	^{593}LNKLKGRESDDDGGMKRASSMSVLPSRGNDESTPAPIQLSQRGRVGSHDNLSSRRQSGNF^{649}
mouse KLC3	^{566}DVLRRSSELLVRKLQGTEPRPSSSSMKRAASLNYLNQPNAAPLQVSRGLSASTVDLSSSS^{615}
rat KLCt	^{446}KLVSRLRGESMAGAAGMKRAMSLNMLNVDGPRAARMQLSTQHLNEASRTLSASTQDLSPR^{505}
human KLC2	^{563}KLQGGTPQEPPNPRMKRASSLNFLNKSVEEPTQPGGTGLSDSRTLSSSSMDLSRRSSLVG^{622}
human KLC3	^{295}DVLRRSSELLVRKLQGTEPRPSSSNMKRAASLNYLNQPSAAPLQVSRGLSASTMDLSSSS^{355}
consensus	MKRA(S)SLN(F)LN

[a] The indicated sequence is common to sea urchin KLC1, -2, and -3. The numbers are for sKLC1. The accession (GI) numbers are as follows: mouse KLC2 (6680578); sea urchin KLC1-3 (161526 for sKLC1, 161528 for sKLC2, 1362661 for sKLC3); mouse KLC3 (13878552); rat KLC2 (10281106); human KLC2 (13878553); and human KLC3 (13878563). [b] The conserved residues are highlighted. The consensus sequence is also indicated at the bottom.

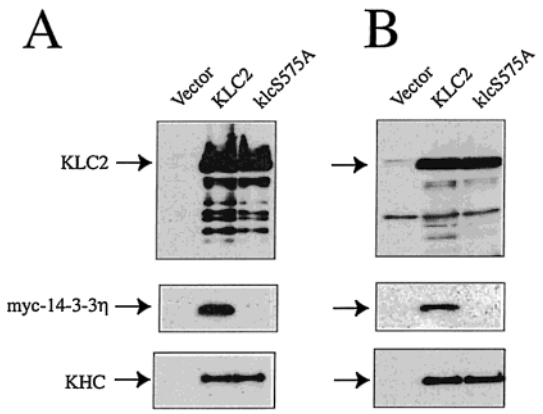

FIGURE 5: Effect of the Ser575 mutation on 14-3-3 binding. Flag-tagged KLC2 or Flag-tagged klcS575A was coexpressed with myc-14-3-3η in 293 cells (A) or PC12 cells (B), and the expressed proteins were immunoprecipitated with anti-Flag Sepharose beads. The immunoprecipitates were separated by SDS−PAGE, transferred to poly(vinylidene difluoride) membranes, and then analyzed by Western blotting with an anti-Flag or anti-myc monoclonal antibody. The immunoprecipitates were also analyzed by Western blotting with anti-KHC antibodies to assess the association of KHC with klcS575A.

activity of the kinesin protein complex. A large number of biochemical and immunocytochemical experiments have shown that c-kinesin plays roles in a variety of cellular processes during both interface and mitosis. It has also been shown that c-kinesin is required for the progress of neurogenesis, since mutations in either the KHC or the KLC gene cause defects in the number of synaptic boutons in action potential propagation and neurotransmitter release in *Drosophila* (*37*, *38*). Although the structure and function of c-kinesin are thus well characterized, little is known about the mechanisms that regulate motor activity and cargo binding. In this report, we have shown that 14-3-3 binds to c-kinesin through interaction with KLC2 and that this interaction is dependent on the phosphorylation of KLC2 in intact cells. Since 14-3-3 is a potential regulator of diverse kinase-mediated processes (*8*), we assume that this interaction is functionally significant.

The amino acid sequences of KLCs cloned from various organisms indicated that the C-terminal tails exhibit notable sequence variability, while the coiled-coil and TPF regions are highly conserved across species (*39*). To search for potential targets of 14-3-3 among the KLC family, we performed a BLAST search of the NCBI (National Center for Biotechnology Information) peptide sequence database using the C-terminal sequence of 40 amino acids in KLC2. This retrieval revealed that 7 out of 28 known KLCs shared the short unique sequence MKRA(S)*SLN(F)LN (where *S* is phosphoSer) in the C-terminal end. These include sea urchin KLC1, -2, and -3, mouse KLC3, rat KLCt, and human KLC2 and KLC3 (Table 2), suggesting that a subset of the KLC family might also be targets of the 14-3-3 protein.

It is also known that there are a number of proteins in the kinesin superfamily that are clearly distinct from c-kinesin in structural organization and function (*34*). For instance, KIF1C, involved in vesicle transport between the Golgi apparatus and the endoplasmic reticulum, is a homodimer, and contains a motor domain and cargo-binding site in each monomer (*40*). This protein was recently found to be a target of the 14-3-3 protein (*41*). In fact, KIF1C contains the consensus 14-3-3-binding sequence RxxpSxP (ref *29*) at residues 1089−1094. Likewise, the computer-assisted retrieval of the NCBI database showed that at least 5 out of 39 mouse members of this class of kinesin molecules shared the same consensus sequence for 14-3-3 binding. Thus, 14-3-3 may participate in a variety of cellular processes, which are dependent on the phosphorylation of the kinesin superfamily.

c-Kinesin binds to two distinct proteins, hsc70 and JIPs (JIP1, -2, and -3). Hsc70 is thought to be a regulator of the kinesin/vesicle interaction as it dissociates c-kinesin from the vesicle surface (*42*). JIPs are potential linkers of c-kinesin and cargo that connect c-kinesin and a transmembrane lipoprotein receptor, such as ApoER2, in the vesicle membrane (*16*). c-Kinesin binds to these molecules through the TPR motifs in its light-chain subunit (*16*, *42*). The present study showed that the light-chain subunit of c-kinesin also binds to 14-3-3, but at a site apart from the TPR motifs, that is, through its C-terminal tail. It should be noted that the interaction between c-kinesin and 14-3-3 is strictly regulated by phosphorylation at Ser575 of KLC2. Thus, it appears likely that c-kinesin can potentially associate with hsc70 and JIP molecules, and 14-3-3 through the distinct structural regions of KLC2.

Increasing evidence suggests cross-talk between the vesicle transport controlled by motor proteins and the stress-induced signaling pathway, leading to the activation of JNK and p38 MAPK. For instance, Nagata et al. (*19*) have shown that the KIF3 motor protein associates with the mixed lineage kinases MLK2 and MLK3, both of which can activate the JNK and p38MAPK cascade. It has also been shown that JIPs are scaffold proteins that bind to three kinase components of the JNK signaling pathway: JNK itself, a kinase that phosphorylates JNK such as MKK7 or MKK4, and a kinase

that phosphorylates MKK7 or MKK4 (*43*, *44*). In the previous studies (*18*−*20*), 14-3-3 was also found to bind to several stress-activated MAPKKKs, including MLK2, ASK1, and MEKK1, -2, and -3. In addition, a recent study has revealed the physiological importance of 14-3-3 in regulating stress activation (*45*), in that dominant-negative 14-3-3s increase the basal levels of JNK1 and p38 MAPK activity in cultured fibroblasts as well as murine postnatal cardiac tissue. Thus, the findings we reported here suggest a role of 14-3-3 in the stress-activation process; i.e., it could connect these signaling molecules and motor proteins in response to specific signal inputs.

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