The Kinesin-like Motor Protein KIF1C Occurs in Intact Cells as a Dimer and Associates with Proteins of the 14-3-3 Family*

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Proteins of the kinesin superfamily are regulated in their motor activity as well as in their ability to bind to their cargo by carboxyl-terminal associating proteins and phosphorylation. KIF1C, a recently identified member of the KIF1/Unc104 family, was shown to be involved in the retrograde vesicle transport from the Golgi-apparatus to the endoplasmic reticulum. In a yeast two-hybrid screen using the carboxyl-terminal 350 amino acids of KIF1C as a bait, we identified as binding proteins 14-3-3 β, γ, ε, and ζ. In addition, a clone encoding the carboxyl-terminal 290 amino acids of KIF1C was found, indicating a potential for KIF1C to dimerize. Subsequent transient overexpression experiments showed that KIF1C can dimerize efficiently. However, in untransfected cells, only a small portion of KIF1C was detected as a dimer. The association of 14-3-3 proteins with KIF1C could be confirmed in transient expression systems and in untransfected cells and was dependent on the phosphorylation of serine 1092 located in a consensus binding sequence for 14-3-3 ligands. Serine 1092 was a substrate for the protein kinase casein kinase II in vitro, and inhibition of casein kinase II in cells diminished the association of KIF1C with 14-3-3γ. Our data thus suggest that KIF1C can form dimers and is associated with proteins of the 14-3-3 family.

Kinesin-like proteins (KLPs), also known as kinesin family proteins (KIFs), form a superfamily of microtubule-based mechanochemical motors. All KLPs share a conserved motor domain of ~340 amino acids, and similarities between this domain have been used to group the known members of the kinesin superfamily into a number of subfamilies. Currently, 7 subfamilies and several outgroups comprise more than 250 KLPs from different organisms. The members of each subfamily share a common domain organization, exhibit sequence homology outside of the motor domain, and have similar motility properties and cellular functions (for recent reviews see

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¶The abbreviations used are: KLP, kinesin-like protein; CKII, casein kinase II; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; DSS, disuccinimidyl suberate; GST, glutathione S-transferase; KIF, kinesin family protein; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid.

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Furthermore, we have shown that in contrast to the related proteins KIF1A and KIF1B, the carboxy terminus of KIF1C mediates the formation of KIF1C dimers.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—The carboxy terminus of KIF1C (amino acids 755–1103) was cloned into the LexA fusion protein vector pBTM116 (kindly provided by S. Hollenberg and J. Cooper) and transformed into Saccharomyces cerevisiae strain L40 (MATa trpl leu2 his3 lys2::leu HIS3 ura3::leu-lacZ) generating the L40 lexA-KIF1C-C17 strain. A human skeletal muscle cDNA library fused to the GAL4 activation domain in the pGAD10 vector and a human brain cDNA library cloned into the similar pACT2 vector (CLONTECH) were transformed into the L40 lexA-KIF1C-CT strain, and 5 × 10⁶ transformants were screened for interaction as described by Hollenberg et al. (22). Yeast plasmid DNA was isolated from His+ β-galactosidase colonies, rescued in Escherichia coli HB101, transformed into L40 lexA-KIF1C-C17, and assayed for β-galactosidase activity and growth on drop-out medium with complete supplement lacking Trp, Leu, Lys, and His (Bio 101). The specificity of the interaction between the carboxy terminus of KIF1C and potential candidates was proven by transforming the candidate plasmid also into the L40 LexA and the L40 LexA-laminin strain.

Antiserum—The KIF1C rabbit polyclonal antiserum was raised against a glutathione S-transferase (GST) fusion protein of KIF1C (amino acids 362–725) and has been described earlier (21). The antiserum against GST was produced by immunizing rabbits with bacterially expressed GST protein. The antiserum against the different 14-3-3 isoforms were obtained from Santa Cruz Biotechnology.

Construction of Expression Plasmids—For transient expression in 293 cells, all cDNAs were cloned by standard procedures into the cytomegalovirus immediate early promoter-based expression plasmid pRK5 (23). The GST-KIF1C construct contains the GST of the pGEX1 vector with the restriction endonucleases HincII and BamHI and cloned in frame to a sequence coding for the hemagglutinin peptide followed by the open reading frame of the KIF1C-cDNA. The amino-terminus deletion mutant of KIF1C, KIF1C-NT430, contained the amino acids 570–1103, and the carboxy-terminal deletion mutants KIF1C-ΔCT622, KIF1C-ΔCT802, and KIF1C-ΔCT1042 contained the amino acids 1–622, 1–802, and 1–1042, respectively. For generation of the KIF1C-SA mutant, the codon of serine 1092 was mutated to a codon for alanine using the method of Kunkel et al. (24).

Cell Lines and Cell Culture—NiH3T3, RD, and C6C12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) and 2 mM glutamine. 293 cells were maintained in F-12/Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) and 2 mM glutamine. The inhibitor of protein kinase CKII CT622, KIF1C-CT802, and KIF1C-CT1042 were cloned into the similar pACT2 vector (CLONTECH). Approximately 5 × 10⁶ transformants were screened for expression of the reporter genes HIS3 and LacZ, and five different types of clones were found to interact specifically with KIF1C. Four types of clones represented members of the 14-3-3 family of proteins (see below), and one type encoded a peptide consisting of the carboxy-terminal amino acids 814–1103 of KIF1C. This result was rather unexpected since the KLPS of the KIF1C/UNC104 subfamily have been described as monomers (4, 8).

To investigate the dimerization of KIF1C in intact cells, we first applied coimmunoprecipitation analysis. We constructed a fusion protein between GST and KIF1C that differs in size from wild-type KIF1C by 26 kDa and thus allows a separation of GST-KIF1C and KIF1C by SDS-PAGE. Coexpression of KIF1C and GST-KIF1C in 293 cells resulted in the formation of a dimer between these two proteins. As shown in Fig. 1, immunoprecipitation using the αGST antibody, which did not react with KIF1C, resulted in a coprecipitation of KIF1C with GST-KIF1C. Since both proteins were detected by Western blot analysis using the αKIF1C antibody, the amount of coprecipitated KIF1C almost equaled the amount of the immunoprecipitated GST-KIF1C. These data demonstrate that under the conditions of transient overexpression, the full-length KIF1C proteins do form dimers with GST-KIF1C.

Since a dimerization of endogenous KIF1C cannot be proven by coimmunoprecipitation, we tested two cross-linking reagents for their ability to cross-link KIF1C. We applied DFDNB, which differ in their reactivity against amino acids and their spacing distance, DSS is an amine-selective cross-linking reagent, which can span approximately 1.13 nm. The aryl halide DFDNB spans only over 0.3 nm but in addition to primary amines also reacts with thiol and phenol groups as they occur in cysteine and tyrosine, respectively. Since our coimmunoprecipitation experiments clearly showed a...
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Dimerization between KLPs occurs by the formation of a coiled-coil structure that is formed by the carboxyl-terminal part of KIF1C coded for the proteins β, γ, ε, and η of the 14-3-3 family. In our studies we concentrated on the 14-3-3γ isoform. To confirm the interaction of KIF1C with this 14-3-3 family protein, in vitro association studies were performed. The cDNA of 14-3-3γ was cloned into the pGEX1 vector, and the GST-14-3-3γ fusion protein was bacterially expressed and purified. We immobilized 0.5 μg of GST-14-3-3γ or as a control 0.5 μg of GST protein on glutathione-Sepharose 4B beads. The lysates from 293 cells transiently overexpressing KIF1C and 14-3-3γ were added and incubated with 20 μl of GST-14-3-3γ fusion protein or GST. The reactions were stopped by increasing the Tris concentration to 50 mM.

Association of 14-3-3γ with the Carboxy-terminal of KIF1C—The additional clones that were found interacting with the carboxy-terminal part of KIF1C coded for the proteins β, γ, ε, and η of the 14-3-3 family. In our studies we concentrated on the 14-3-3γ isoform. To confirm the interaction of KIF1C with this 14-3-3 family protein, in vitro association studies were performed. The cDNA of 14-3-3γ was cloned into the pGEX1 vector, and the GST-14-3-3γ fusion protein was bacterially expressed and purified. We immobilized 0.5 μg of GST-14-3-3γ or as a control 0.5 μg of GST protein on glutathione-Sepharose and incubated it with 200 μl of lysate from 293 cells overexpressing KIF1C. As shown in Fig. 4A, KIF1C specifically associated with the GST-14-3-3γ fusion protein but not with GST.

To prove the association of KIF1C and 14-3-3γ in intact cells, we transiently overexpressed KIF1C and 14-3-3γ in 293 cells.
and immunoprecipitated either KIF1C or 14-3-3γ using the αKIF1C or α14-3-3γ antibodies, respectively. For specificity control of the antibodies, protein was also precipitated from lysates expressing only 14-3-3γ or KIF1C, respectively. After separation of the immunocomplex by SDS-PAGE and Western blot analysis, we could detect KIF1C in 14-3-3γ or GST immunoprecipitates and also 14-3-3γ in KIF1C immunoprecipitates (Fig. 4B). Interestingly, KIF1C was also coprecipitated from lysates of cells overexpressing only KIF1C but not 14-3-3γ. In these cells, the amount of endogenous 14-3-3γ was sufficient to associate with overexpressed KIF1C. Since less 14-3-3γ and consequently less KIF1C was precipitated from these cell lysates than from the lysate of cells overexpressing KIF1C and 14-3-3γ, the coprecipitation of KIF1C did not result from unspecific binding of KIF1C to the antibody but from an association of the overexpressed KIF1C with endogenous 14-3-3γ.

These data suggested a high affinity of 14-3-3γ for KIF1C, and we therefore investigated the association between the two proteins in untransfected fibroblasts and skeletal muscle cells. After lysis of human 293 or mouse NIH3T3 fibroblasts, we

immunoprecipitated 14-3-3γ, separated the proteins by SDS-PAGE, and detected KIF1C and 14-3-3γ in the precipitates by Western blot analysis (Fig. 4C). This association was also detected in the skeletal muscle C2C12 and rhabdomyosarcoma RD cell line (data not shown). To investigate whether the 14-3-3β, -ε, and -ζ isoforms also interact with KIF1C in intact cells, we constructed cDNAs that were expressed with a carboxyl-terminal Myc tag. Co-overexpression of KIF1C and these 14-3-3 proteins revealed that all isoforms associated with similar affinities with KIF1C (data not shown). These data thus clearly demonstrate an association between the motor protein KIF1C and the 14-3-3γ protein, and it is likely that the β, ε, and ζ isoforms act similarly.

Localization of the 14-3-3γ Binding Site in KIF1C—By using the amino acids 755–1103 as a bait in the yeast two-hybrid screen, we limited the 14-3-3γ interaction site of KIF1C to the carboxyl-terminal part of the protein. To locate the binding site more accurately and to exclude additional binding sites in KIF1C we coexpressed deletion mutants of KIF1C with 14-3-3γ in 293 cells. The deletion mutants of KIF1C were either the amino-terminal truncated protein, KIF1C-ΔNT570, or the different carboxyl-terminal truncated proteins KIF1C-ΔCT629, KIF1C-ΔCT692, and KIF1C-ΔCT1042. After lysis and immunoprecipitation with the α14-3-3γ and αKIF1C antibodies, we analyzed the immunocomplexes for coprecipitated KIF1C and 14-3-3γ, respectively. In KIF1C immunoprecipitates 14-3-3γ was only detected by Western blot analysis in cells that were expressing either the wild-type or the amino-terminal truncated KIF1C protein. Consequently, if 14-3-3γ was immunoprecipitated from these cell lysates, only wild-type KIF1C and KIF1C-ΔNT570 coprecipitated (Fig. 5A). From these data we confined the binding site of 14-3-3γ to KIF1C to the last 60 amino acids of the protein.

Recently, the binding of 14-3-3 proteins to their ligands has been described to be dependent on a phosphorylated serine residue in the amino acid context of RRXpS/SpXP (where X indicates any amino acid; pS indicates phosphoserine (28)). This sequence motif occurs also in the very carboxyl terminus of KIF1C and is located around the serine residue Ser1092 (RRQBSAP). To test if this serine indeed is involved in 14-3-3 binding, we mutated Ser1092 to alanine so that the association of KIF1C with 14 [h]phényl]3-3-proteins should be abolished. Immunoprecipitation of 14-3-3γ from lysates of 293 cells transiently expressing the KIF1C-SA mutant did not result in a coprecipitation of the KIF1C-SA (Fig. 5B) although the protein was expressed (data not shown). This indicates that Ser1092 indeed is mediating the association between KIF1C and 14-3-3γ.

Phosphorylation of KIF1C by CKII Is Required for the Association with 14-3-3γ—Since the binding of 14-3-3 proteins normally depends on a phosphorylation of the serine residue, we tried to identify the responsible kinase. Analysis of the amino acid sequence of KIF1C identified Ser1092 as a potential substrate sequence for the protein kinase CKII. CKII can be inhibited by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), which was shown to be specific for CKII and does not inhibit other serine/threonine kinases like protein kinase A (29, 30). If CKII indeed is involved in the phosphorylation of Ser1092 an incubation of cells with DRB should reduce its phosphorylation and thus diminish the association of KIF1C with 14-3-3γ. To test this hypothesis, 293 cells were either left untreated or incubated with 30 μM DRB for 18 h, lysed, and the 14-3-3γ protein immunoprecipitated. The immunocomplexes were separated by SDS-PAGE and analyzed for the presence of KIF1C. As shown in Fig. 6, the amount of KIF1C coprecipitating with 14-3-3γ was strongly reduced in cells treated with the CKII
inhibitor DRB. The reduction was dependent on the incubation time of the cells in the presence of DRB. In cells incubated for more than 16 h a significant reduction of KIF1C bound to 14-3-3 was detected (data not shown), indicating that phosphorylation of Ser 1092 is rather stable. These data show an involvement of CKII in the regulation of the association of KIF1C with 14-3-3 and provide additional evidence for the specificity of the association of 14-3-3 with KIF1C.

To investigate if KIF1C is indeed a direct substrate of CKII or if the kinase CKII is regulating another kinase, which in turn then phosphorylates KIF1C, we performed an in vitro kinase assay with CKII. KIF1C and KIF1C-SA were expressed in 293 cells and purified by immunoprecipitation. To remove any phosphorylation that had occurred in 293 cells, the immunoprecipitates were incubated with alkaline phosphatase before starting the in vitro kinase assay. Then the immunoprecipitates were incubated with CKII in the presence of 25 μCi γ-[32P]ATP for different times as described under “Experimental Procedures.” To ensure that the observed phosphorylation represents only CKII activity and does not result from a kinase coprecipitated with KIF1C, 1 aliquot was incubated under the same conditions without adding the CKII enzyme. In Fig. 7A the phosphorylation of KIF1C by CKII is shown. It indicates that KIF1C is readily phosphorylated by purified CKII, whereas KIF1C-SA is phosphorylated to a lower extent. The densitometric analysis of the amount of incorporated phosphate into KIF1C and KIF1C-SA is shown in Fig. 7B. According to these data, in addition to Ser 1092 CKII phosphorylates KIF1C at one more amino acid residue.

**DISCUSSION**

Since the purification of kinesin in 1985 (31) more than 250 different KLPs have been cloned from different organisms. They all contain a motor domain responsible for the binding of microtubules and the hydrolyzation of ATP for the generation of force, allowing these proteins to move along the microtubules. Although some progress has been made in the understanding of the mechanochemical basis of this movement, the regulation of motor activity and vesicle binding remains to be elucidated. In this report we describe the association of KIF1C with 14-3-3 proteins and also provide evidence for a dimerization of KIF1C in intact cells.

**Dimerization of KIF1C—** Whereas all characterized KLPs do form either homo- or heterodimers, the members of the KIF1/Unc104 subfamily are believed to exist as monomers only. Recombinant KIF1A and KIF1B were purified as monomers, by applying a sucrose gradient centrifugation, gel chromatography, and rotary-shadowing electron microscopy (4, 8). Under these conditions other KLPs like kinesin or KIF3 were found as dimeric complexes. The proteins of the KIF1/Unc104 subfamily were thus believed to represent the first class of monomeric microtubule-based motor proteins that must obtain processivity by a different mechanism than kinesin.

By using the carboxyl terminus of KIF1C as a bait in the yeast two-hybrid system, we have found that KIF1C can dimerize. We confirmed this result in fibroblasts with overexpressed
as well as endogenous KIF1C in coimmunoprecipitation and chemical cross-linking experiments. Analysis of the sequence of KIF1C revealed a probability to form coiled-coil structures only for short stretches that was similar to that of KIF1B (Fig. 3), which has been predicted not to dimerize (4). However, O’Shea et al. (32) described a dimerization of the GCN4 protein mediated by coiled-coils formed by stretches of only 28–35 amino acids. Thus, it remains possible that KIF1C dimerizes like the other KLPs by formation of coiled-coils, but compared with kinesin the dimerization should be rather weak.

Association of KIF1C with 14-3-3 Proteins—In this study we identified the β, γ, ε, and ζ members of the 14-3-3 protein family as binding partners of KIF1C. In a transient expression system the four isoforms coimmunoprecipitated with similar affinities with KIF1C. The isoform investigated in more detail, 14-3-3ζ, is known to have the highest affinity for KIF1C (19) and is strongly expressed in the brain and therefore should be represented in the library used (33).

The binding sequence of 14-3-3ζ in KIF1C was identified as the motif including the phosphorylated serine residue 1092. Mutation of this residue abolished the association of overexpressed KIF1C-SA with 14-3-3ζ in 293 cells. This indicates that the sequence RRQRS(p)AP, which is very similar to the peptide sequences reported by Yaffe et al. (28), efficiently binds 14-3-3 proteins. Ser1092 is located in a consensus sequence for phosphorylation by the protein kinase CKII and was indeed a substrate of CKII in vitro. In addition to Ser1092, at least one more serine/threonine residue of KIF1C was phosphorylated by CKII under these conditions. The inhibition of CKII in intact cells by the specific inhibitor DRB resulted in a diminished association of KIF1C with 14-3-3ζ. Although we cannot exclude that the effect of CKII is only indirect, the in vitro phosphorylation data provide strong evidence for a direct interaction between KIF1C and CKII. Like KIF1C and 14-3-3 proteins, the protein kinase CKII is a ubiquitous serine/threonine kinase with established roles in cell proliferation and signal transduction. Although the majority of CKII has been found in the nucleus, CKII activity was also detected at the Golgi apparatus and the endoplasmic reticulum (34, 35). Identified substrates of CKII include transcription factors and enzymes involved in transcription and translation, receptor tyrosine kinases like the insulin receptor, and cytoskeletal and structural proteins like β-tubulin or clathrin (for review see Ref. 36). Recently, Karki et al. (26) identified cytoplasmic dynein in a complex with CKII and showed that CKII was also able to phosphorylate the dynein intermediate chain in vitro. Although the function of this CKII-derived phosphorylation of dynein is not known yet, serine/threonine phosphorylation was described to regulate the cargo association of KLPs and dynein superfamily motor proteins. For example, kinesin showed a reduced association with synaptic vesicles when phosphorylated by protein kinase A. However, the mechanism and the protein interactions affected by this phosphorylation remain to be elucidated (37).

The 14-3-3 protein family consists of highly conserved members and contains several isoforms with overlapping functions. Like KIF1C, the β, γ, and ζ isoforms have been found at the Golgi apparatus (38, 39). Since the structure of the 14-3-3 dimer allows simultaneous binding to two proteins (40, 41), it was suggested that 14-3-3 proteins provide a scaffold on which other proteins interact. We identified KIF1C in intact cells as a dimer that associates with the also dimeric 14-3-3 proteins. It is therefore tempting to imagine a KIF1C-14-3-3 heterodimer in analogy to the KHC-KLC complex. Unfortunately, it was not possible to chemically cross-link 14-3-3 proteins with KIF1C, and thus this hypothesis remains only speculative. In contrast to the kinesin complex, which is formed constitutively, the generation of a KIF1C-14-3-3 complex is dependent on the serine phosphorylation of KIF1C. In mammals, five different KLC isoforms are known so far that have homologous amino-terminal sequences for binding to KHC but vary in their carboxyl-terminal sequences that are supposed to be involved in vesicle binding (16). Thus, the attachment of KHC to different vesicles and organelles is probably regulated by the corresponding KLC. Four different isoforms of the 14-3-3 family proteins, which like the KLC also differ in their properties, have been shown to interact with KIF1C. It remains to be elucidated whether they differentially regulate the activity of KIF1C. The γ and ζ isoforms, which in contrast to the β and ε isoform bind phospholipids (42), could act as cofactors for the association of KIF1C with the membranes of the Golgi apparatus or the transported vesicles. In earlier studies 14-3-3 proteins have been found at the membrane of synaptic vesicles (43) where they stimulate calcium-dependent exocytosis (44, 45). In addition, the yeast homologues of 14-3-3, BMH1, and BMH2 are also involved in vesicular trafficking as well as in the Ras signaling pathways (46).

Recent data support the idea of a cross-talk between vesicle transport controlled by motor proteins and the stress-induced signaling pathway leading to an activation of the c-Jun amino-terminal kinase and the p38 kinases. Nagata et al. (19) de-
scribed a complex of the mixed lineage kinases MLK2 and MLK3 with the motor protein KIF3X and 14-3-3 proteins. The ε and ζ isoform of the 14-3-3 proteins were described to interact with the MEK1-3 kinases upstream of the c-Jun amino-terminal kinase and ERK1/2 kinases (38). Since MEKK2 is localized at the Golgi apparatus (38), future experiments should explore a connection between the regulation of KIF1C involved in the retrograde Golgi-endoplasmic reticulum membrane transport and the cellular response to extracellular stimuli by mitogen-activated kinase/extracellular signal-regulated kinase kinases.

In conclusion, we have demonstrated a dual role for the carboxy-terminal 350 amino acids in KIF1C as follows: a motif around Ser1092 interacts in a regulated manner with members of the 14-3-3 family, and most likely a stretch of 50 amino acids around position 850 forms coiled-coil structures and is likely to mediate dimerization with another KIF1C protein. Future experiments will clarify the physiological role of these interactions.

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