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Inositol Hexakisphosphate Is Bound in the ADAR2 Core and Required for RNA Editing

Mark R. Macbeth, Heidi L. Schubert, Andrew P. VanDemark, Arunth T. Lingam, Christopher P. Hill, Brenda L. Bass

We report the crystal structure of the catalytic domain of human ADAR2, an RNA editing enzyme, at 1.7 Ångstrom resolution. The structure reveals a zinc ion in the active site and suggests how the substrate adenosine is recognized. Unexpectedly, inositol hexakisphosphate (IP₆) is buried within the enzyme core, contributing to the protein fold. Although there are no reports that adenosine deaminases that act on RNA (ADARs) require a cofactor, we show that IP₆ is required for activity. Amino acids that coordinate IP₆ in the crystal structure are conserved in some adenosine deaminases that act on transfer RNA (tRNA) (ADATs), related enzymes that edit tRNA. Indeed, IP₆ is also essential for in vivo and in vitro deamination of adenosine 37 of tRNA by ADAT1.

One form of RNA editing is catalyzed by adenosine deaminases that act on RNA (ADARs), a family of enzymes that deaminate adenosine to form inosine in double-stranded RNA (dsRNA) (Fig. 1A) (1). ADARs are important for proper neuronal function (2–4) and also are implicated in the regulation of RNA interference (RNAi) (5–7). Inosine is recognized as guanosine by most cellular proteins and the translation machinery, and it pairs most stably with cytidine. Therefore, editing of RNA can alter a codon, create splice sites, and change its structure. The latter occurs when an AU base pair is changed to an IU mismatch and may be important for the effects of ADARs on the RNAi pathway.

ADARs from all organisms have a common domain structure consisting of one to three dsRNA binding motifs (dsRBMs) near the N terminus, followed by a conserved C-terminal catalytic domain (1, 8). Human ADAR2 (hADAR2) contains two dsRBMs, and its best characterized substrates are the pre-mRNAs of neuronal function (1, 8). The active site is indicated by an ordered zinc ion that coordinates a water molecule that presumably displaces ammonia during the deamination reaction. Coordination of the zinc ion by H394, C451, and C516, and hydrogen bonding of the water molecule by E396 (Fig. 1C), is altered function. hADAR2 also edits its own message to create a new splice site (11). Purified hADAR2 deaminates substrates in vitro (12) in the absence of any added cofactors, and deletions of N-terminal sequences, including dsRBM1, result in an active protein that accurately edits an RNA substrate (13). In addition, we found that a protein consisting of only the catalytic deaminase domain of hADAR2 (hADAR2-D, residues 299 to 701) (fig. S1A) was active in vitro, although it deaminates RNA less efficiently than full-length hADAR2 (fig. S1B).

The structure of the ADAR2 catalytic domain. To better understand the ADAR mechanism, we crystallized hADAR2-D. The structure (PDB code 1ZY7) was determined by multiple isomorphous replacement and refined at 1.7 Å resolution to an R factor of 17.4% and Rfree of 20.7% (14). The asymmetric unit includes 669 water molecules, one sulfate ion, and two hADAR2-D molecules that are essentially identical [root mean square deviation (RMSD) = 0.28Å for 358 pairs of Cα atoms].

The protein adopts a roughly spherical 40Å diameter structure (Fig. 1B) that, consistent with sizing chromatography of hADAR2-D and equilibrium ultracentrifugation of the full-length hADAR2, appears monomeric in the crystal. The active site is indicated by an ordered zinc ion that coordinates a water molecule that presumably displases ammonia during the deamination reaction. Coordination of the zinc ion by H394, C451, and C516, and hydrogen bonding of the water molecule by E396 (Fig. 1C), is

Fig. 1. (A) ADAR catalyzed hydrolytic deamination of adenosine to inosine in dsRNA. (B) Ribbon model of hADAR2-D. The active-site zinc atom is represented by a magenta sphere. The N-terminal α/β domain (residues 306 to 620) is colored cyan, with the region that shares structural similarity with CDA and TadA colored dark blue (deamination motif; residues 350 to 375, 392 to 416, 439 to 455, 514 to 525, and 542 to 551). The C-terminal helical domain (residues 621 to 700), which with contributions from the deamination motif makes the major contacts to IP₆ (ball and stick), is colored red. Ends of the disordered segment (residues 462 to 473) are indicated with asterisks. (C) Residue interactions at the active site. Shown are the zinc ion, coordinating residues (H394, C451, and C516), the nucleophilic water (blue sphere), and the proposed proton-shuttling residue, E396. The hydrogen-bond relay that connects the active site to the IP₆ is also indicated. Single-letter abbreviations for amino acid residues are defined in (42).
essentially identical to the geometry seen at the catalytic centers of cytidine deaminase (CDA) (15) and TadA (16), a member of the ADAT2 (adenosine deaminase that acts on tRNA 2) family. This similarity was predicted earlier on the basis of equivalent chemistry and sequence conservation of the four residues that coordinate zinc and water (17, 18).

Superposition of zinc, water, and coordinating residues was used as the starting point to identify residues of hADAR2-D that were structurally equivalent to those in CDA and TadA (PDB codes 1CTU and 1WWR, respectively). Inspection shows that 77 residues (RMSD = 3.05 Å on Ca atoms) form a structurally conserved “deamination motif” comprising two helices (α2 and α5), four strands (β1, β2, β5, and β8), and connecting loops (Fig. 1B, dark blue). Other hADAR2-D residues do not have structural equivalents in CDA and TadA (Fig. 2A). Further emphasizing the large evolutionary separation between these enzymes, only four of the deamination motif residues have conserved identities in all three enzymes (excluding zinc/water ligands).

**The active site of ADAR2.** The site of nucleophilic attack during the ADAR reaction (C6 of adenine) lies deep in the major groove of the dsRNA substrate. Because this site is inaccessible to an enzyme, ADARs may use a base-flipping mechanism (19, 20) like other enzymes that modify double-stranded polynucleotides (21). Consistent with this scenario, the catalytic zinc center is located in a deep pocket in the enzyme surface that is surrounded by positive electrostatic potential that likely serves as the dsRNA binding site (Fig. 2B). In contrast, TadA uses an alternative mechanism of substrate selection that probably involves recognition of the anticodon stem/loop of tRNA (16).

To model binding of substrate, we overlapped the structure of a CDA-zebularine (cytidine analog inhibitor) complex (15) onto the hADAR2 structure and built the adenosine monophosphate (AMP) portion of an ADAR substrate to maintain the same catalytic geometry. In this simple overlap based on zinc ion and coordinating residues, the zebularine ribose clashes with the hADAR2 loop containing T375 (Fig. 2C), thereby providing a plausible explanation for why ADARs do not deaminate cytidine. The steric clash is absent with AMP because of the additional distance afforded by the purine ring (Fig. 2D). The proposed AMP-binding geometry requires repositioning of the hADAR2 R455 side chain, although this could be accomplished through minor rearrangements that may occur upon dsRNA binding.

Comparison of the ADAR and CDA/TadA structures reveals an important difference in the arrangement of the two cysteine residues that coordinate zinc (fig. S5). As often found in zinc-dependent enzymes (22, 23), the two cysteines of CDA and TadA are located in a Cys-X-X-Cys motif at the N terminus of a helix. The first cysteine forms hydrogen bonds with two main-chain amide NH groups at the helix terminus; this likely contributes to catalysis by increasing the positive character of the zinc ion and nucleophilicity of the water molecule (24). In ADAR2, however, C451 and C516 are separated by a 64-residue loop, and hydrogen bonding to main-chain atoms is reduced to a single bond between C451 and the amide NH group of C516. However, a second hydrogen bond is observed between C516 and K483 (Fig. 3A and fig. S5), and thus ADARs may have evolved a compensating interaction; K483 is conserved in ADAR sequences but not seen in CDA or TadA.

**Insolit hexakisphosphate binds in the core of the catalytic domain.** One side of the deamination motif of hADAR2 contributes to a cavity, not found in CDA or TadA, that is formed mainly by C-terminal elements (Fig. 1B, red) and buries the IP6 molecule and 29 associated water molecules. The identity of IP6 was suggested by the strong, distinctive electron density (Fig. 3A and fig. S3) and the local electrostatic interactions, and was confirmed by (+) ion electrospray mass spectrometry [observed molecular weight (MW) of IP6 in complex with the protein = 660.0 Da; calculated MW = 659.9 Da] (14). IP6 is an abundant inositol polyphosphate implicated in many cellular functions, including RNA export, DNA repair, endocytosis, and chromatin remodeling (25–29). Intriguingly, the compound is reported to affect neuronal AMPA receptors (30), whose messages are edited by ADAR2 (9).

The IP6 cavity is extremely basic and lined with many arginine and lysine residues (R400, R401, and R522 and K519, K629, K662, and K690), as well as W523, W687, Y658, and Y668 (Fig. 3, A and B). Most of these residues are invariant among catalytically active ADARs, as well as in the ADAT1 family of enzymes, which deaminate A37 of certain tRNAs. (The ADAT2 family, of which TadA is a member, is distinct from ADAT1 and does not have the IP6 binding pocket or its conserved residues.) IP6 was not added during purification or crystallization but must have been acquired during expression of the human ADAR2-D protein in *Saccharomyces cerevisiae*, which like other eukaryotes has pools of IP6 (31). The presence of IP6 in the purified protein therefore indicates a tight association, consistent with the extensive array of hydrogen bonds formed with conserved side chains (Fig. 3B) and the almost completely buried environment (Fig. 3C). The structure suggests that hADAR2 will be non-functional in the absence of IP6, a view that is supported by experiments described below.
**IP₆ is required for ADAR2 activity.** In *S. cerevisiae*, the last step in the synthesis of IP₆ is the phosphorylation of IP₅ by Ipklp, and yeast harboring a deletion of IPK₁ are viable but fail to produce IP₆ (25). To test for the IP₆ requirement of hADAR₂, we expressed the protein in ipk₁Δ yeast cells and compared its activity to hADAR₂ expressed in the same strain but containing the IPK₁ gene. As substrate, we used a 27-base-pair RNA that mimics the natural arginine/glycine (R/G) editing site of the glutamate receptor B (gluR-B) pre-mRNA (Fig. 4A) and that is efficiently edited by hADAR₂ in vitro (20). IP₆ was required for hADAR₂ activity (Fig. 4B), because there was no editing of this RNA by protein expressed in the ipk₁Δ mutant. Using reverse transcription polymerase chain reaction (RT-PCR), we determined that hADAR₂ mRNA was expressed at the same levels in the wild-type and ipk₁Δ mutant strains, although the amount of hADAR₂ protein was lower by a factor of 5 to 10 in the mutant strain. Western blots were performed to determine the amount of hADAR₂ protein in each extract by comparison with a standard curve generated with known amounts of purified, histidine-tagged protein (R-D₆, an N-terminal truncation of hADAR₂) (13). This information was used to normalize amounts of hADAR₂ used for the in vitro assays, and a Western blot confirmed that amounts of hADAR₂ in wild-type and mutant editing reactions were similar (Fig. 4C).

**IP₆ is required for tRNA editing by the ADAT₁ family of deaminases.** ADATs are a class of enzymes that deaminate adenosine to generate inosine in RNA. These enzymes contain only the catalytic domain and deaminate tRNAs at adenosines 34 and 37 (A₃₄ and A₃₇) (18). On the basis of their sequences and substrates, there are three types of ADATs in eukaryotes. ADAT₁ deaminates A₃₇ of tRNA^{ala} (32), and the resulting inosine is subsequently methylated at N₁ in a reaction requiring a different enzyme and S-adenosylmethionine (33). ADAT₂ and ADAT₃ form a heterodimer that deaminates A₃₄ of various tRNAs and, consistent with the fact that this is the wobble position, unlike ADAT₁, these enzymes are essential (32, 34).

By aligning enzyme sequences, we noted that residues observed to coordinate IP₆ in the hADAR₂ crystal structure were conserved in the ADAT₁ family but not in the ADAT₂ or ADAT₃ families (Fig. 5A and fig. S6). To test the consequent prediction that ADAT₁, but not ADAT₂/₃, requires IP₆, we monitored activity of endogenous ADAT in extracts prepared from *S. cerevisiae* wild-type or ipk₁Δ strains (Fig. 6A). In contrast, there was no difference for in vitro editing of A₃₄ by the ADAT₂/ADAT₃ heterodimer in the mutant versus the wild-type extract (Fig. 6B). To confirm that the lack of activity in the ipk₁Δ strain derived from a lack of IP₆ rather than a molecule downstream in the pathway, we tested ADAT₁ activity in extracts prepared from a kcs₁Δ mutant (fig. S7). The KCS₁ gene product is downstream of IPK₁ in the inositol polyphosphate synthesis pathway and phosphorylates IP₆ to form the pyrophosphate-containing IP₇ (35). The kcs₁Δ mutation had no effect on the A₃₇ editing activity of ADAT₁, which indicates that the editing defect in the ipk₁Δ mutant is due to the IP₆ deficiency.

The existence of the ipk₁Δ mutant, and the fact that *S. cerevisiae* tRNA^{ala} is deaminated at both A₃₄ and A₃₇, provided a facile system for analyzing the in vivo requirement for IP₆. RNA was prepared from wild-type or ipk₁Δ cells, tRNA^{ala} was amplified using RT-PCR, and the RT-PCR product was sequenced (Fig. 6C). Because inosine is read as guanosine by reverse transcriptase, edited adenosines were identified as guanosine in the dideoxy sequencing reaction. Consistent with the in vitro data, we observed that A₃₄ is edited with equal efficiency in the wild-type and mutant strain, but A₃₇ is edited...
much less efficiently in the ipk1Δ strain (Fig. 6C). A37 in the wild-type strain is read as a thymidine, presumably due to N²-methylinosine at position 37. m¹I, like m¹A, may pair with reduced specificity in the reverse transcription reaction, explaining the presence of a T in the PCR product (36).

In the crystal structure of hADAR2, IP₆ binds and fills an extremely basic hole, with the center of the inositol ring more than 10 Å from the protein surface. Thus, it seems possible that ADAT2 and, by analogy, ADAT1, are unstable without IP₆. In this regard we wondered about the nature of ADAT1 expressed in the ipk1Δ mutant. Was this protein trapped in an irreversible inactive state or forming a folding intermediate that could bind IP₆ to achieve its active conformation? To explore this question, we tested whether the addition of IP₆ to extracts prepared from the ipk1Δ mutant could recover ADAT1 activity. When added to extracts prepared from the ipk1Δ strain, IP₆ recovered activity to ~50% of wild-type (Fig. 7, A and C), which suggests that the protein does not require IP₆ during its synthesis and the initial stages of folding. As expected, the addition of IP₆ to wild-type extract had no effect, because these cells are capable of synthesizing IP₆ (Fig. 7, B and C). To test for the specific requirement for IP₆ by ADAT1, we performed a similar experiment, except we substituted inositol hexakisulfate (IS₆) for IP₆. Despite its similar charge and structure, IS₆ does not recover ADAT1 activity when added to ipk1Δ extracts (Fig. 7A). This suggests that the enzyme specifically requires IP₆ for function and can discriminate between the minor differences in phosphate/sulfate chemistry (e.g., the protonation state).

So far, we have been unable to rescue the activity of hADAR2 expressed in the ipk1Δ by adding IP₆. Possibly, native S. cerevisiae ADAT1, but not the heterologous hADAR2, is associated with a host chaperone in the extract that promotes refolding in the presence of IP₆. Alternatively, this result may hint at interesting differences between the two enzymes in IP₆ accessibility. Such a difference might explain why assays of ADAT1 in ipk1Δ extracts show a small (~5%) amount of A37 deamination at the highest concentrations of extract (Fig. 6A), whereas hADAR2 expressed in this strain shows no residual activity. If the IP₆ binding site in ADAT1 was more accessible than that of hADAR2, it might bind a noncognate inositol polyphosphate, such as IP₆₂, to allow a low level of activity.

Discussion. Burial of IP₆ may reflect a novel way of using an available cellular component to define and stabilize a protein fold. This would be analogous to the use of “structural” metal ions in stabilizing the fold of metalloproteins.

To our knowledge, this represents the first example of a protein using IP₆ for this purpose. Other protein structures with bound IP₆ have been reported, such as deoxycytochrome b₅₃ and the clathrin adaptor complex AP2 (38); however, unlike ADAR2-D, in these cases the IP₆ molecule is not extensively buried and does not appear to dramatically stabilize the overall structure.

In addition to the structural requirement, IP₆ may play a subtle role in modulating catalytic efficiency by indirectly ordering the side chain of K483. Two of the IP₆ phosphate groups approach within 10 Å of the catalytic zinc ion and are indirectly coordinated to zinc by a chain of hydrogen-bonded residues that includes K519, D392, and K483 (Fig. 3A). These residues are conserved among active ADARs, and K483 may contribute to tuning the pK₆ of the nucleophilic water molecule through its interaction with C516 (Fig. 3A).

Sequence alignments indicate that ADAT1 enzymes are the evolutionary link between the other family members, ADAR2 (including TadA) and ADARs (18). ADAT1 apparently

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**Fig. 5.** (A) Schematic diagram showing the relative lengths and domain structures of hADAR2 and family members from S. cerevisiae (sc) and E. coli (ec). Proteins are anchored at the invariant zinc-coordinating histidine (H). Residues that coordinate IP₆ are red lines; double-stranded RNA binding motifs are in black. Alignments for regions surrounding the residues that coordinate IP₆ in hADAR2 are shown below, with blue numbering corresponding to hADAR2. IP₆ coordinating residues are in red, with side-chain contacts in bold. Residues N391, W523, Q669, W687, E689, and D695 are water-mediated contacts. The conserved K519, D392, and K483 are analogous to the use of a nucleophyle polymer to define and stabilize the overall structure.

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**Fig. 4.** (A) The 27-mer R/G site RNA substrate used to assay hADAR2 activity. (B) Editing of the R/G site RNA by hADAR2 expressed in wild-type or ipk1Δ yeast. The R/G site adenosine was labeled with ³²P and incubated with increasing concentrations of expressed hADAR2 in extracts. Reaction was treated with nuclease P1, the resulting ⁵ nucleotide monophosphates separated by thin-layer chromatography (TLC), and the plate exposed to a Phosphorimager screen. The amount of hADAR2 in each extract was determined by Western blotting, and extract was added to give the final ADAR2 concentrations as indicated. (C) Western blot showing the amount of hADAR2 in each reaction. Single-letter abbreviations for amino acid residues are defined in (42).
Fig. 6. (A) Editing of tRNA\(^{\text{A37}}\) in vitro by extracts of wild-type or ipk1\(\Delta\) yeast strains. tRNA\(^{\text{A37}}\), labeled with \(^{32}\text{P}\) at A37, was incubated with increasing amounts of yeast extract protein, as indicated (14). Reacted RNA was processed as in Fig. 4B, and nuclease P1 products were separated by TLC (left). The fraction of inosine in each lane was quantified, and the average of three determinations was plotted as a function of protein concentration (right; error bars, standard deviation; when error was very small, error bars are obscured by data point symbols). Solid line, editing by ADAT1 from wild-type extracts; dashed line, editing by ADAT1 from ipk1\(\Delta\) extracts. (B) As in (A) but showing editing of A34-labeled tRNA\(^{\text{A34}}\). Solid line, editing by ADAT2/3 from wild-type extracts; dashed line, editing by ADAT2/3 from ipk1\(\Delta\) extracts. (C) Editing of endogenous tRNA in vivo. tRNA was prepared from wild-type or ipk1\(\Delta\) strains, reverse transcribed, and amplified by PCR. PCR products were sequenced using dideoxy nucleotide triphosphates and a \(^{32}\text{P}\)-labeled primer that anneals to the non-template strand at the 5' end of the gene. The right panel shows an expanded view of the sequencing gel shown on the left. The dideoxy sequencing lanes are indicated at the top of each lane, and the 5' to 3' sequence to the left of the gel is read from bottom to top. Bands corresponding to A34 to G editing in the wild-type and ipk1\(\Delta\) tRNAs are labeled with daggers, and the band representing the A37 site that is not edited in the ipk1\(\Delta\) tRNA is labeled with an asterisk. Consistent with the observation of residual activity in the mutant extract in vitro (A), some editing of A37 in the mutant extract occurs.

Fig. 7. (A) Addition of IP\(_6\) but not IS\(_6\) rescued ADAT1 activity in extracts prepared from ipk1\(\Delta\) yeast. Wild-type or ipk1\(\Delta\) protein extract (0.1 \(\mu\text{g/ml}\)) was incubated with IP\(_6\) or IS\(_6\) for 15 min at 30°C before the addition of A37-labeled tRNA\(^{\text{A37}}\). IP\(_6\) and IS\(_6\) concentrations were 10-fold dilutions from 100 \(\mu\text{M}\) to \(10^{-3}\) \(\mu\text{M}\). The tRNA was processed as described in Fig. 4B. (B) Addition of IP\(_6\) had no effect on ADAT1 activity in wild-type extracts using the reaction conditions of (A). Without the addition of IP\(_6\), wild-type extracts gave 70% A to I conversion. (C) The average fraction of inosine produced in three experiments each of (A) and (B) plotted as a function of IP\(_6\) or IS\(_6\) concentration; error bars show the standard deviation (small error bars are obscured by the data point symbol). Circles, ADAT1 activity from wild-type extracts with IP\(_6\) added; squares, activity of ipk1\(\Delta\) extracts with IP\(_6\); triangles, activity of ipk1\(\Delta\) extracts with IS\(_6\).
diverged from the ADAT2/3 family by acquiring the ability to bind IP6, followed by the acquisition of one or more dsRBMs to generate an ADAR. ADAT1 may have evolved an IP6-binding function as a means of regulation. IP6 accumulates in yeast during times of stress (39) and thus could lead to increased ADAT1 activity, and consequently to an increased conversion of A37 to N1-methylinosine. Modification of position 37 is predicted to increase fidelity of protein synthesis by stabilizing the codon-anticodon interaction (40), and thus yeast may use this modification to fine-tune protein synthesis in response to environmental conditions.

Once established as a means of regulation for ADAT1, metazoa may have extended this regulatory mode for use in ADARs, which perform important roles in the nervous system and display changes in activity during development (41). For example, a feedback mechanism could act through phospholipase C in response to hormones such as angiotensin II or serotonin. Upon binding of serotonin to its 5-HT2c receptors requiring greater concentrations of serotonin. Active ADAR2, which in turn edits mRNA to attenuate the serotonin signaling pathway.

The structure of the hADAR2 catalytic domain reveals the active site architecture of a zinc-catalyzed deamination reaction and suggests how ADARs discriminate between cytidine and adenosine residues. The presence of IP6 in the protein core implied an unexpected requirement for this cofactor in ADARs, which was confirmed by assaying the RNA editing activity of enzymes lacking IP6. The finding that IP6 is required for ADAR and ADAT activity suggests many interesting links between RNA editing and diverse processes such as cell signaling and translation, thus setting the stage for future studies.

References and Notes
14. Materials and methods are available as supporting material on Science Online.
42. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Reports

Single-Molecule Torsional Pendulum

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We have built a torsional pendulum based on an individual single-walled carbon nanotube, which is used as a torsional spring and mechanical support for the moving part. The moving part can be rotated by an electric field, resulting in large but fully elastic torsional deformations of the nanotube. As a result of the extremely small restoring force associated with the torsional deformation of a single molecule, unusually large oscillations are excited by the thermal energy of the pendulum. By diffraction analysis, we are able to determine the handedness of the molecule in our device. Mechanical devices with molecular-scale components are potential building blocks for nano electromechanical systems and may also serve as sensors or actuators.

Carbon nanotubes (1, 2) are likely to be used in future nanoscale devices because of their outstanding mechanical and electrical properties. Nanoelectromechanical devices incorporating multiwalled carbon nanotubes (MWNTs) as motion-enabling elements have been demonstrated; in these devices, the MWNT serves as a torsional spring for small angular deformations (3) and torsional oscillations (4) or as a bearing for continuous rotational operation (5, 6). Here, we show that it is possible to prepare large moving objects suspended on a single molecule—a single-walled nanotube (SWNT). The cross-section of a SWNT is smaller than that of a MWNT by more than two orders of magnitude, and large deformations are possible within the elastic regime. The moving part returns to its initial position even after being turned by 180°. The ultralow torsional spring constant provided by the

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