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Synthesis of Biotinylated Inositol Hexakisphosphate To Study DNA Double-Strand Break Repair and Affinity Capture of IP_6-Binding Proteins

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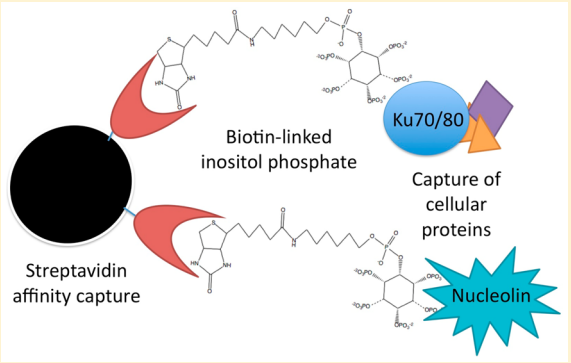
Supporting Information

ABSTRACT: Inositol hexakisphosphate (IP_6) is a soluble inositol polyphosphate, which is abundant in mammalian cells. Despite the participation of IP_6 in critical cellular functions, few IP_6-binding proteins have been characterized. We report on the synthesis, characterization, and application of biotin-labeled IP_6 (IP_6-biotin), which has biotin attached at position 2 of the *myo*-inositol ring via an aminohexyl linker. Like natural IP_6, IP_6-biotin stimulated DNA ligation by nonhomologous end joining (NHEJ) *in vitro*. The Ku protein is a required NHEJ factor that has been shown to bind IP_6. We found that IP_6-biotin could affinity capture Ku and other required NHEJ factors from human cell extracts, including the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4, and XLF. Direct binding studies with recombinant proteins show that Ku is the only NHEJ factor with affinity for IP_6-biotin. DNA-PKcs, XLF, and the XRCC4:ligase IV complex interact with Ku in cell extracts and likely interact indirectly with IP_6-biotin. IP_6-biotin was used to tether streptavidin to Ku, which inhibited NHEJ *in vitro*. These proof-of-concept experiments suggest that molecules like IP_6-biotin might be used to molecularly target biologically important proteins that bind IP_6. IP_6-biotin affinity capture experiments show that numerous proteins specifically bind IP_6-biotin, including casein kinase 2, which is known to bind IP_6, and nucleolin. Protein binding to IP_6-biotin is selective, as IP_3, IP_4, and IP_5 did not compete for binding of proteins to IP_6-biotin. Our results document IP_6-biotin as a useful tool for investigating the role of IP_6 in biological systems.



Soluble inositol polyphosphates (IPs) are the multiply phosphorylated forms of *myo*-inositol at its various positions. The role of IPs in calcium mobilization through inositol triphosphate (IP_3) and release of diacylglycerol has been accepted for many years.[1,2] Very soon after this seminal discovery, it was realized that IP_3 could be additionally phosphorylated to produce inositol tetrakisphosphate (IP_4), inositol pentakisphosphate (IP_5), and inositol hexakisphosphate (IP_6). It is now clear that eukaryotic cells harbor a large family of highly phosphorylated IPs. The phosphorylation of IPs is not limited to the inositol hydroxyl groups; numerous diphosphorylated derivatives of IP_3, IP_4, IP_5, and IP_6 and triphosphorylated IP_6 have also been described.[3]

IP_6 is one of the most abundant IPs in eukaryotes, with the intracellular concentration approaching 10−50 μM in human cells.[4−8] IP_6 metabolism in yeast and metazoans involves complex networks of IP kinases and phosphatases maintaining a high steady-state concentration of IP_6, which is in rapid flux and responsive to changes in kinase or phosphatase activity.[9−14] In human cells, the sole kinase that uses IP_5 as a substrate to produce IP_6 (inositol 1,3,4,5,6-pentakisphosphate 2-kinase, here termed the IP_5 kinase) localizes in the nucleus, and IP_6 has been implicated in important nuclear functions, including regulation of ATP-dependent chromatin remodeling, RNA editing, mRNA export, and translation.[15−20] In mice, IP_5 kinase expression is required for viability, presumably because IP_6 is essential for growth and development.[21] In zebrafish, knockdown of IP_5 kinase expression resulted in randomized placement of internal organs.[4] Further exploration of this startling result led to the discovery that the IP_5 kinase, and presumably IP_6, plays an important role in the beating of cilia, which help establish the left−right asymmetry of internal organs.[22]

We identified IP_6 as a stimulator of DNA double-strand break repair by nonhomologous end joining (NHEJ) *in vitro*.[23] Additional work demonstrated that the NHEJ factor Ku is capable of binding IP_6.[24,25] Our observations were followed by

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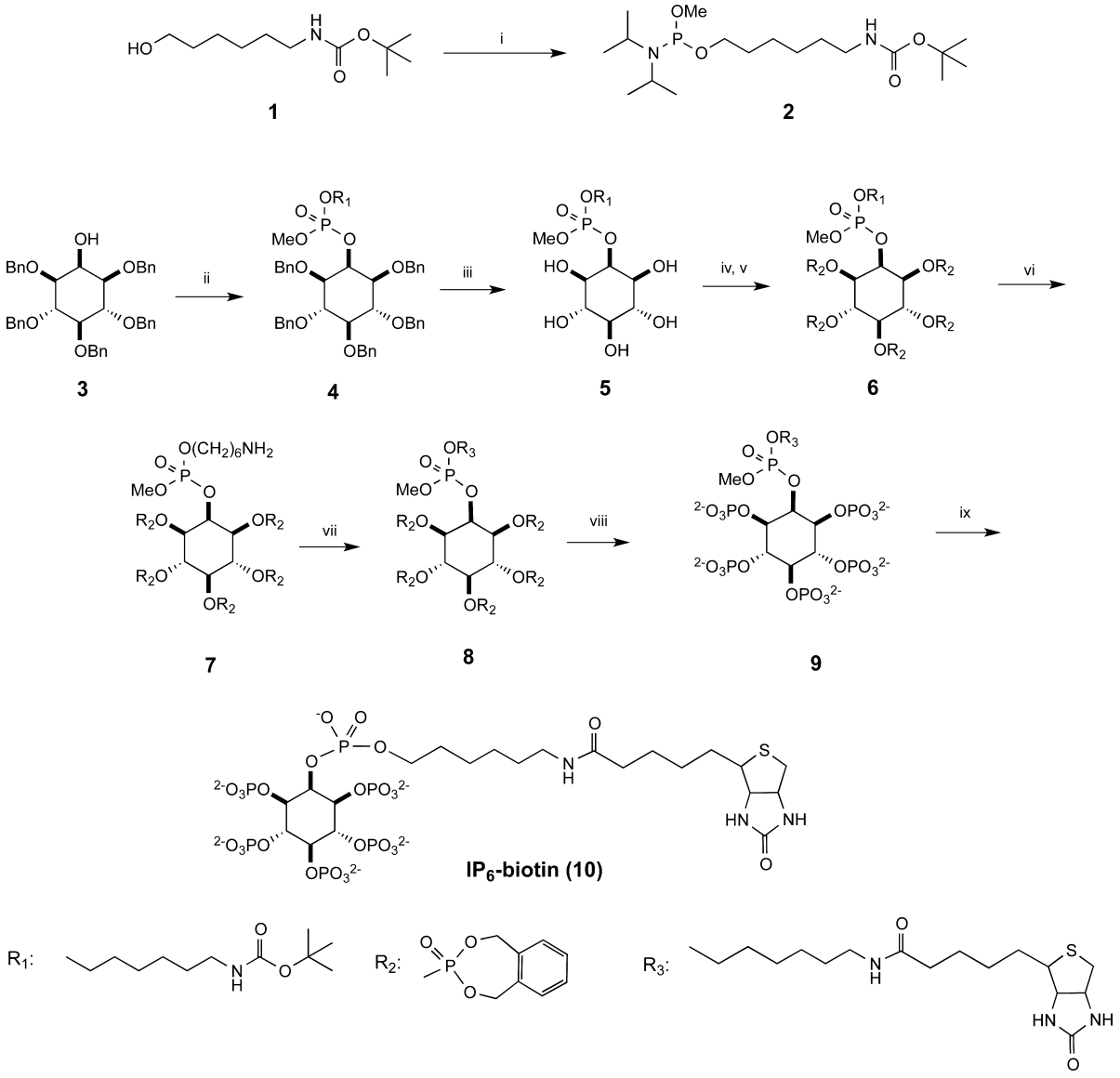
Scheme 1. Synthesis of IP_6-Biotin[a]



[a]Reagents: (i) THF, DIPEA, *P*-chloro-*N*,*N*-diisopropylmethylphosphoramidite, 71%; (ii) (a) DCM, compound **2**, tetrazole, (b) tBuOOH, 76%; (iii) EtOH, H_2, 10% Pd/CH_2, 45 psi, 59%; (iv) DCM, *O*-xylylene-*N*,*N*-diisopropylphosphoramidite, tetrazole; (v) −78 °C, mCPBA, 81%; (vi) EtOAc, 1 M HCl, 65%; (vii) DMF, DIPEA, biotin *N*-hydroxysuccinimide, 71%; (viii) H_2, 10% Pd/C, 1:1 EtOH/H_2O, 41%; (ix) 33% NH_4OH, 55 °C, 30%.

the observation that highly phosphorylated IPs can enhance the ability of Ku to move about the nucleus, presumably to locate double-strand breaks.[26] Our mutational analysis of the Ku heterodimer identified a bipartite IP_6-binding site composed of residues from both Ku70 and Ku80 subunits.[27] Despite the presence of IP_6, mutants of Ku with reduced IP_6 binding activity failed to function in NHEJ *in vitro*.[27] Most recently, we determined that Ku:IP_6 interactions play a role in regulating phosphorylation of the NHEJ proteins XRCC4 and XLF.[28] These studies demonstrate that one way in which IP_6 stimulates NHEJ *in vitro* is through direct interactions with Ku and suggest that IP_6 is a small-molecule regulator of NHEJ in human cells.

The studies of IP_6, and other highly phosphorylated IPs, were stimulated by the development of tetherable IP_6 derivatives that could be used to identify and characterize IP-binding proteins.[29−33] To further our investigation of the role of IP_6 in NHEJ, we have extended these pioneering efforts and synthesized biotin-labeled IP_6 (IP_6-biotin), a versatile IP_6 analogue that can be used as a chemical probe in solution, or combined with immobilized streptavidin resin to produce an IP_6-bioaffinity matrix. Here, we show that IP_6-biotin functions like IP_6 in NHEJ *in vitro* and that IP_6-biotin can be used to molecularly target streptavidin to Ku and inhibit NHEJ *in vitro*. Further, we found that while Ku is the only NHEJ factor that binds IP_6 with specificity, IP_6-biotin can be used to affinity capture most of the known NHEJ factors. Lastly, we show that a number of cellular proteins are affinity captured by IP_6-biotin, including casein kinase 2 (CK2), which is known to bind IP_6. We identify one of the recovered polypeptides as nucleolin and suggest that IP_6-biotin is a useful tool in the study of IP_6-responsive systems.

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EXPERIMENTAL PROCEDURES

Materials. Chemicals were purchased from Sigma-Aldrich. Anhydrous dichloromethane (DCM) was distilled from CaH_2, and anhydrous tetrahydrofuran (THF) was distilled from $LiAlH_4$. Silica gel (Merck, 60 Å, 230−240 mesh) was used for column chromatography. Thin layer chromatography was performed using silica gel on aluminum foil plates with a fluorescent indicator at 254 nm. NMR spectra were recorded with a Bruker DPX 400 spectrometer operating at 400 MHz (^{1}H) and 162 MHz (^{31}P). *N*-Boc-6-aminohexanol (**1**) was synthesized according to the published method.[34] 1,3,4,5,6-Penta-*O*-benzyl-*myo*-inositol (**3**) was synthesized according to a literature method.[35]

***O*-Xylylene *N*,*N*-Diisopropylphosphoramidite.**[36] To a solution of 1,2-benzenedimethanol[37] (1.00 g, 7.24 mmol) in 20 mL of anhydrous DCM cooled to −20 °C were added *N*,*N*-diisopropylphosphoramidous dichloride (1.46 g, 7.24 mmol) and DIPEA (2.52 mL, 14.5 mmol), and the mixture was allowed to stir at −5 °C for 3 h. The solvent was then evaporated under vacuum, and the product was purified via silica gel chromatography (hexane/Et_3N, 95:5) to give the desired product as a white solid (1.01 g, 52%): ^{1}H NMR ($CDCl_3$) δ 1.19 (m, 12H, CH_3), 3.57−3.66 (m, 2H, NCH), 7.29 (s, 4H, aromatic); ^{31}P NMR ($CDCl_3$) δ 150.0 (s).

Phosphoramidite 2 (Scheme 1). To a solution of **1** (1.0 g, 4.6 mmol) in anhydrous THF (2 mL) at −5 °C were added *P*-chloro-*N*,*N*-diisopropylmethylphosphoramidite (0.9 mL, 4.6 mmol) and *N*,*N*-diisopropylethylamine (DIPEA) (0.96 mL, 5.53 mmol). After the mixture had been stirred for 3 h at room temperature, the solvent was removed under vacuum, and the residue was purified by flash chromatography (hexane/triethylamine, 9:1) to give phosphoramidite **2** (1.25 g, 71%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.19−1.62 (m, 29H, aliphatic CH_2 and CH_3 from the isopropyl amino group), 3.11 (m, 2H, NCH), 3.39−3.43 (dd, 3H, J = 8 Hz, J = 4 Hz, POCH), 3.58−3.66 (m, 4H, aliphatic); ^{31}P NMR ($CDCl_3$) δ 147 (s).

2-*O*-[(*N*-Butoxycarbonyl-6-aminohexyl methyl)-phospho]-1,3,4,5,6-penta-*O*-benzyl-*myo*-inositol (4). To a mixture of **3** (300 mg, 0.45 mmol), tetrazole (0.127 mg, 1.8 mmol), and **2** (0.206 mg, 0.55 mmol) was added anhydrous DCM (2 mL). After the mixture had been stirred at room temperature for 4 h, *tert*-butyl hydroperoxide (5.5 M solution in decane, 0.4 mL, 2.2 mmol) was added to the reaction mixture at −5 °C, and the mixture was allowed to warm to room temperature over 30 min. The solvent was evaporated and the residue purified by flash chromatography (hexane/acetone/ethyl acetate, 3:1:1) to give **4** (330 mg, 76%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.63−1.2 (m, 17H, aliphatic CH_2 and Boc CH_3), 3.55−3.46 (m, 3H), 3.03 (m, 1H), 3.66 (d, 3H, J = 8.4 Hz, POCH), 3.95 (m, 4H), 4.47 (m, 1H), 4.58−4.61 (m, 2H, benzyl CH_2), 4.81−4.91 (m, 8H, benzyl CH_2), 5.32 (d, 1H, J = 8.4 Hz, NH), 7.31 (m, 25H, aromatic); ^{31}P NMR ($CDCl_3$) δ −0.32 (s).

2-*O*-[(*N*-*tert*-Butoxycarbonyl-6-aminohexyl methyl)-phospho]-*myo*-inositol (5). Compound **4** (330 mg, 0.36 mmol) and 10% Pd/C (70 mg) were dispersed in EtOH (20 mL), and the suspension was shaken in a Parr apparatus overnight at 45 psi of H_2. The solid was then filtered off, the solvent evaporated, and the residue purified by silica gel chromatography ($CHCl_3$/MeOH, 1:1) to give **5** (98 mg, 59%) as a white solid: ^{1}H NMR (CD_3OD) δ 1.4 (m, 17H, aliphatic CH_2 and CH_3), 3.02−3.07 (m, 2H), 3.19−3.23 (t, 1H, J = 8.8 Hz), 3.62−3.52 (m, 4H), 3.81−3.84 (d, 3H, J = 12 Hz, POCH), 4.14−4.19 (q, 2H, J = 6.4 Hz), 4.70 (m, 1H); ^{31}P NMR (CD_3OD) δ −0.37 (s).

2-*O*-[(*N*-Butoxycarbonyl-6-aminohexyl methyl)-phospho]-*myo*-inositol 1,3,4,5,6-Pentakis(xylylene phosphate) (6). To a mixture of compound **5** (100 mg, 0.21 mmol), tetrazole (258 mg, 3.70 mmol), and *O*-xylylene-*N*,*N*-diisopropylphosphoramidite (395 mg, 1.48 mmol) was added anhydrous DCM (5 mL). The solution was stirred at room temperature for 24 h, and *m*-chloroperoxybenzoic acid (*m*CPBA, 510 mg, 3.0 mmol) was added to the solution cooled to −78 °C and the solution stirred at −78 °C for 30 min and then warmed to room temperature over an additional 30 min. The solution was extracted with 10% sodium bisulfite, and the organic layer was concentrated and purified by means of silica gel chromatography (hexane/DCM/acetone/Et_3N, 1:2:2:0.2) to give compound **6** as a white solid (240 mg, 81%): ^{1}H NMR ($CDCl_3$) δ 2.94 (m, 2H, CH), 3.88 (d, 3H, J = 12 Hz, POCH), 4.19 (m, 2H), 5.7−5.1 (m, 20H, xylylene CH_2), 7.39 (m, 20H, aromatic); ^{31}P NMR ($CDCl_3$) δ −0.93 (s, 1P), −1.55 (s, 1P), −2.20 (s, 1P), −4.17 (s, 1P), −4.26 (s, 1P), −4.52 (s, 1P). Please note that the ^{31}P NMR spectrum of compound **6** contains six rather than the anticipated four signals. This is most likely due to the existence of the nonsymmetrical nonchair conformation(s) of the inositol ring.

2-*O*-[(6-Aminohexyl methyl)phospho]-*myo*-inositol 1,3,4,5,6-Pentakis(xylylene phosphate) (7). Acetyl chloride (271 mg, 3.45 mmol, 0.245 mL) was added to a cooled mixture of anhydrous MeOH (220 mg, 0.278 mL) and EtOAc (2.5 mL). An aliquot of the resulting solution (1.15 M HCl, 1.5 mL) was added to compound **6** (50 mg, 0.036 mmol) and the mixture stirred for 25 min. The solvent was evaporated under vacuum, and the residue was purified by chromatography on silica gel (DCM/MeOH, 4:1) to give compound **7** as a pale-yellow oil (31 mg, 65%): ^{1}H NMR ($CDCl_3$) δ 1.23−1.35 (m, 8H), 2.88 (m, 2H), 3.21 (m, 1H), 3.49 (m, 2H), 3.73 (m, 2H), 3.87 (d, 3H, J = 12 Hz, POCH), 4.16 (m, 2H), 4.85 (m, 1H), 4.95−5.55 (m, 20H, xylylene CH_2), 7.27−7.66 (m, 20H, aromatic); ^{31}P NMR ($CDCl_3$) δ −0.88 (s, 1P), −1.96 (s, 1P), −2.15 (s, 1P), −3.42 (s, 1P), −3.72 (s, 1P), −4.15 (s, 1P).

2-*O*-[(*N*-Biotin-6-aminohexyl methyl)phospho]-*myo*-inositol 1,3,4,5,6-Pentakis(xylylene phosphate) (8). To a solution of compound **8** (30 mg, 0.023 mM) in anhydrous DMF were added biotin *N*-hydroxysuccinimide[38] (12 mg, 0.035 mmol) and DIPEA (4.5 mg, 0.035 mmol). The solution was stirred overnight, and the solvent was removed *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 5:1) to give compound **8** as a pale yellowish oil (26 mg, 71%): ^{1}H NMR ($CDCl_3$) δ 1.63−1.31 (m, 14H), 2.16 (t, 2H, J = 6.8 Hz), 2.72 (d, 1H, J = 12.8 Hz), 2.95 (dd, 1H, J = 5.2 Hz, J = 8 Hz), 3.26 (m, 1H), 3.1 (m, 2H), 3.82 (d, 3H, J = 12 Hz, POCH), 4.13 (m, 2H), 4.25 (m, 1H), 4.44 (m, 1H), 4.65 (m, 1H), 5.77−5.03 (m, 22H), 7.42−7.25 (m, 20H); ^{31}P NMR ($CDCl_3$) δ −0.59, −0.61, −1.64, −1.75, −2.14, −2.24, −4.04, −4.12, −4.23, −4.40, −4.47.

2-*O*-[(*N*-Biotin-6-aminohexyl methyl)phospho]-*myo*-inositol 1,3,4,5,6-Pentakisphosphate (9). To a solution of compound **8** (26 mg, 0.017 mmol) in 1 mL of EtOH/H_2O solvent (1:1) was added 10% Pd/C (30 mg), and hydrogenolysis was conducted under balloon pressure of H_2. The reaction mixture was stirred overnight; the solid was filtered off and thoroughly washed with distilled water, and all solvents

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were evaporated under vacuum to give compound **9** as a yellowish gum (7 mg, 41%): ^{1}H NMR (D_2O) δ 1.63−1.31 (m, 14H), 2.16 (t, 2H, J = 6.8 Hz), 2.72 (d, 1H, J = 12.8 Hz), 2.95 (dd, 1H, J = 5.2 Hz, J = 8 Hz), 3.26 (m, 1H), 3.1 (m, 2H), 3.82 (d, 3H, J = 12 Hz, POMe), 4.18 (m, 2H), 4.33 (m, 4H, 2H aliphatic, 2H), 4.51 (m, 3H, 1H inositol, 2H); ^{31}P NMR (D_2O) δ 0.96 (1P), 0.53 (2P), −0.45 (2P), −1.22 (1P).

Biotin-IP$_6$ (10). Aqueous ammonium hydroxide (2 mL, 33%) was added to compound **9** (7.0 mg, 0.007 mmol), and the solution was heated to 55 °C overnight. The solvent was removed *in vacuo* and the residue dissolved in aqueous ammonium hydroxide (0.2 mL, 33%) and mixed with methanol (1.5 mL) in a 2 mL plastic microcentrifuge tube. The resulting solution was stored in a freezer overnight, and compound **10** precipitated out as a white solid (2 mg, 30%): ^{1}H NMR (D_2O) δ 4.76 (m, 1H, inositol), 4.55 (m, 1H), 4.36 (m, 3H), 4.06 (m, 2H), 3.95 (m, 3H), 3.26 (m, 1H), 3.1 (m, 2H, biotin and inositol), 2.95 (dd, 1H, J = 5.2 Hz, J = 8 Hz, biotin), 2.72 (d, 1H, J = 12.8 Hz, biotin), 2.16 (t, 2H, J = 6.8 Hz, biotin aliphatic), 1.63−1.31 (m, 14H, aliphatic); ^{31}P NMR (D_2O) δ 3.44 (2P), 2.28 (1P), 1.63 (2P), −1.13 (1P); HRMS (ESI) (acid form) calcd for $C_{22}H_{44}N_3O_{26}P_6S$ $(M - H)^-$ 984.0368, found 984.0361.

Extract Preparation and *in Vitro* Nonhomologous End Joining Activity Assay. The HeLa whole cell extract, prepared as previously described,[39,40] was subject to 65% ammonium sulfate precipitation and dialyzed against L buffer [20 mM Tris (pH 8.0), 10% glycerol, 0.5 mM EDTA, and 1 mM DTT] with 0.1 M KOAc at 4 °C overnight to produce AS65, or with 0.3 M KOAc for 2−4 h at 4 °C for phosphocellulose fractionation as previously described.[28] Briefly, the extract was passed over phosphocellulose equilibrated in L buffer with 0.3 M KOAc, and the column flow-through was collected, diluted to 0.1 M KOAc with L buffer, and passed over phosphocellulose equilibrated in L buffer with 0.1 M KOAc. Step elution (L buffer, 0.3 M KCl) was used to collect phosphocellulose-bound proteins, which were precipitated with 65% ammonium sulfate to concentrate the protein sample, dialyzed against L buffer with 0.1 M KOAc, and then snap-frozen on liquid nitrogen and stored at −80 °C. Extracts prepared in this manner are termed phosphocellulose C extracts (PC-C). To produce human cell extracts containing hemagglutinin (HA)-tagged nucleolin, HEK 293 cells were transiently transfected with pNtagfor,[41] which produces full-length human nucleolin with an N-terminal triple-HA tag. The extract was prepared, subjected to 65% ammonium sulfate precipitation, and dialyzed against L buffer as previously described.[40] *In vitro* end joining assays were conducted essentially as previously described.[23,27,39,40] Briefly, reactions (10 μL) were conducted in 50 mM HEPES (pH 8.0), 0.1 M KOAc, 0.5 mM $Mg(OAc)_2$, 1 mM ATP, 1 mM DTT, 0.1 mg/mL bovine serum albumin, and *Hin*dIII-linearized 5′-^{32}P-labeled pBluescript DNA (10 ng) with 20 μg of AS65 or 2.5 μg of PC-C. IP$_6$ (Calbiochem), biotin-IP$_6$, and streptavidin (Invitrogen) were added as indicated. End joining products were separated by agarose gel electrophoresis, after which the gel was dried. ^{32}P was detected with a phosphorimager (Bio-Rad PMI), and densitometry was conducted using Bio-Rad Quantity One software.

IP$_6$-Biotin Affinity Capture. Standard conditions for affinity capture were as follows. Binding reactions (250 μL) were conducted in 50 mM HEPES (pH 8.0), 0.1 M KOAc, 0.5 mM $Mg(OAc)_2$, 2 mM potassium phosphate (pH 8.0), 1 mM ATP, 1 mM DTT, 2 mM inorganic phosphate, 0.1 mg/mL bovine serum albumin, and 3% Tween 20 with 20 μL of streptavidin Dynabeads (Invitrogen), 20 μM IP$_6$-biotin, and 16 μg of purified recombinant protein, 160 μg of PC-C extract, or 500 μg of AS65 at 4 °C for 1 h with gentle end-over-end mixing. Dynabeads were collected on a DynaMag-2 magnet and washed three times with 1 mL of wash buffer [20 mM Tris (pH 8.0), 0.1 M KOAc, 2 mM potassium phosphate (pH 8.0), and 0.5 mM EDTA] with 3% Tween 20 and then once with 1 mL of wash buffer without Tween 20. After the washing had been completed, elutions were conducted by incubation with 50 μL of 12.5 mM IP$_6$ in 20 mM Tris (pH 8.0), 0.1 M KOAc, and 0.5 mM EDTA for 15 min at 37 °C with gentle agitation. Dynabeads were collected on the DynaMag. Isolated beads and the resulting supernatant were combined with 2× protein sample buffer with 10% β-mercaptoethanol and separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Ku and the XRCC4:ligase IV complex were expressed in insect cells and purified as previously described.[27,42] XRCC4 and XLF were expressed in *Escherichia coli* and purified as previously described.[28,43] Western blots were probed with anti-Ku70 rabbit polyclonal antibodies,[44] anti-Ku80 monoclonal antibodies (NeoMarkers), anti-CK2 monoclonal antibodies (AbCam), anti-his6 monoclonal antibodies, and anti-nucleolin rabbit polyclonal antibodies[41] to detect Ku70, Ku80, CK2, his6-XRCC4 and his6-ligase IV, and nucleolin, respectively. PC-C and AS65 extract affinity capture samples were detected by silver staining where indicated.

RESULTS

Synthesis of IP$_6$-Biotin. The synthesis of IP$_6$-biotin is depicted in Scheme 1. In the absence of any information related to the orientation of IP$_6$ in the IP$_6$−protein complexes, our choice of the point of attachment of the biotin tether was guided by synthetic expediency. Position 2 of inositol was chosen because it eliminated chirality of the inositol residue. Briefly, the commercially available *N*-Boc-6-aminohexanol (**1**) was phosphitylated with *N*,*N*-diisopropylmethylphosphonamidic chloride in the presence of *N*,*N*-diisopropylethylamine (DIPEA) to afford another phosphitylating agent **2**. The 2-hydroxyl group of pentabenzylinositol **3** was phosphorylated with this reagent in the presence of the tetrazole catalyst, and the intermediate phosphite (not shown) was oxidized by *tert*-butylhydroperoxide at low temperatures to form inositol derivative **4**. The benzyl protecting groups in the latter were removed by hydrogenolysis with 10% Pd/C catalyst to give pentol **5**. The exhaustive phosphorylation of compound **5** was achieved by the treatment with *O*-xylylene-*N*,*N*-diisopropylphosphoramidite, followed by oxidation with *m*CPBA to give per-phosphorylated compound **6**. The Boc group was next removed under acidic conditions to provide free amine **7**, and the liberated amino group was reacted with biotin *N*-hydroxysuccinimide under basic conditions to furnish fully protected precursor **8** of biotin-IP$_6$. The deprotection of this compound was achieved in two steps, first by hydrogenolysis to liberate the phosphomonoester groups in compound **9**, followed by the demethylation of the phosphotriester at position 2 with aqueous ammonium hydroxide to produce the final product, IP$_6$-biotin **10**.

IP$_6$-Biotin Can Replace IP$_6$ in Nonhomologous End Joining Reactions *in Vitro*. Our first step was to evaluate the ability of IP$_6$-biotin to participate in NHEJ *in vitro*. Our assay measures the conversion of monomeric DNA fragments into

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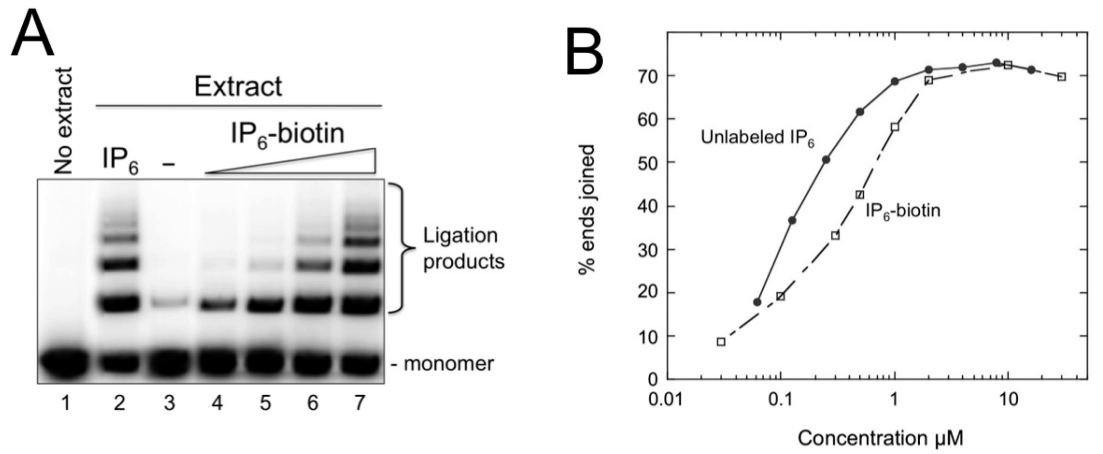

Figure 1. IP_6-biotin functions in nonhomologous end joining *in vitro*. (A) Products of nonhomologous end joining *in vitro*. Reactions were conducted using 2.5 μg of extract (PC-C) in the presence (1 μM, lane 2) or absence (lane 3) of IP_6. IP_6-biotin was used at concentrations of 0.1, 0.3, 1, and 3 μM (lanes 4−7, respectively). (B) Dependence of end joining on IP_6 and IP_6-biotin. The fraction of total counts present as ligation products (percent ends joined) is presented as a function of IP_6 or IP_6-biotin concentration: (●) unlabeled IP_6 and (□) IP_6-biotin.

oligomeric ligation products (Figure 1A), and we compared the fraction of DNA ends joined in reaction mixtures containing unlabeled IP_6 with those containing IP_6-biotin. As shown in Figure 1A, extracts alone catalyzed little end joining (12%, lane 2), the level of which was increased >5-fold by the addition of 1 μM unlabeled IP_6 (69%, lane 2). To achieve the same level of NHEJ, 2 μM IP_6-biotin was required, which indicates a 2-fold lower potency of IP_6-biotin compared to that of IP_6 (Figure 1B). These results show that the structure of IP_6-biotin is similar enough to that of unlabeled IP_6 that it can function in NHEJ *in vitro*. Additionally, these data show that the biotin moiety and its associated linker do not impede critical interactions between NHEJ factors.

IP_6-Biotin Allows Molecular Targeting of Ku To Inhibit Nonhomologous End Joining *in Vitro*. The NHEJ protein Ku specifically binds IP_6, and Ku:IP_6 interactions are important for end joining *in vitro*.[24,27,28] To determine if Ku binds IP_6-biotin, we conducted an affinity capture experiment using purified recombinant Ku. To inhibit nonspecific interactions, binding reaction mixtures contained excess inorganic phosphate (100×) and ATP (50×) relative to IP_6-biotin. Protein:IP_6-biotin complexes were captured on streptavidin Dynabeads. Unbound proteins were removed through a series of wash steps; bound proteins were eluted with 12.5 mM unlabeled IP_6, and tightly bound proteins were removed by boiling the Dynabeads in protein sample buffer. As shown in Figure 2A, purified recombinant Ku bound IP_6-biotin and was eluted with unlabeled IP_6. Retention of Ku on streptavidin Dynabeads depended upon IP_6-biotin and was markedly reduced when binding reaction mixtures contained a 20-fold excess of unlabeled IP_6.

To determine whether Ku can bind IP_6-biotin in the context of the human cell extract, we removed endogenous IPs, which might compete for binding to IP_6-biotin, through ammonium sulfate precipitation of cellular proteins followed by dialysis to remove the ammonium sulfate. Precipitation with 65% ammonium sulfate recovered most cellular proteins from HeLa whole cells extract, including all of the known NHEJ factors. We refer to this preparation as AS65. As shown in Figure 2B, Ku was affinity captured by IP_6-biotin from AS65. Unlike the purified recombinant protein (Figure 2A), Ku in HeLa cell extract bound IP_6-biotin tightly, did not exchange IP_6-biotin for unlabeled IP_6 readily, and was not eluted with unlabeled IP_6. These data may likely reflect the action of proteins that bind Ku, perhaps stabilizing Ku:IP_6-biotin interactions, reducing the level of exchange of IP_6-biotin and unlabeled IP_6 and preventing elution by unlabeled IP_6. Alternately, these data could indicate that HeLa cell Ku may be post-translationally modified and have higher affinity for IP_6-biotin than for unlabeled IP_6, although this is considered unlikely.

Ku is thought to be the first NHEJ protein to bind a DNA double-strand break and is required for recruitment and assembly of NHEJ factors at the double-strand break.[45] Mutations that disrupt interactions between Ku and other NHEJ factors impair assembly of the repair apparatus and inhibit NHEJ.[46−48] We hypothesized that IP_6-biotin might be used to molecularly target streptavidin to Ku to block interactions between Ku and other NHEJ factors and prevent NHEJ *in vitro*. As shown in Figure 2C, streptavidin had no effect on NHEJ reaction mixtures containing unlabeled IP_6 (lane 4) but inhibited end joining in reaction mixtures containing IP_6-biotin (lane 6). These data show that IP_6-biotin can be used to target Ku to inhibit NHEJ *in vitro* and suggest the exciting possibility that IP_6 analogues, synthesized using a similar methodology, might be used to target IP_6-binding proteins and inhibit specific cellular processes.

Ku Is the Only NHEJ Protein That Specifically Binds IP_6-Biotin. IP_6-biotin affinity capture experiments using human cell extracts retained Ku (Figure 2A) and most of the known NHEJ factors (Figure 3A). Recombinant Ku binds IP_6 and IP_6-biotin, while purified DNA-PKcs does not bind IP_6.[24,27] We conclude that DNA-PKcs was affinity captured from human cell lysates by IP_6-biotin through indirect interactions mediated by Ku. Purified, recombinant XRCC4 had no detectable affinity for IP_6-biotin (Figure 3B). Similar results were obtained with the XRCC4:ligase IV complex (Figure 3B), showing that neither XRCC4 nor ligase IV bound IP_6-biotin. Data presented in Figure 3 indicate that XRCC4, either alone or as part of the XRCC4:ligase IV complex, was affinity captured by IP_6-biotin through indirect interactions, likely mediated by Ku and DNA-PKcs, which is known to bind XRCC4. In similar experiments, recombinant, purified XLF was affinity captured by IP_6-biotin, but binding was unchanged by addition of IP_6, indicating that

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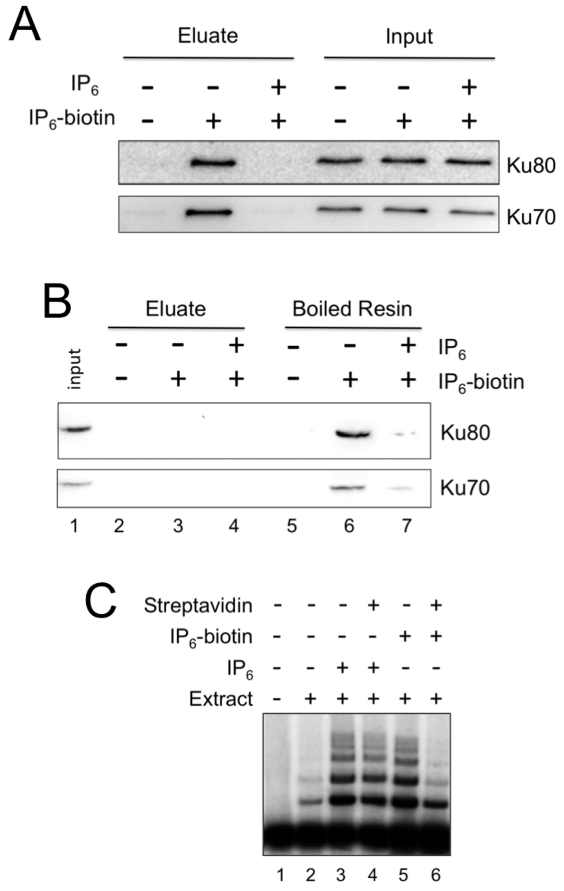

Figure 2. IP_6-biotin allows molecular targeting of Ku to inhibit NHEJ *in vitro*. (A) Purified recombinant Ku was combined with IP_6-biotin under standard conditions (20 μM IP_6-biotin, 2 mM inorganic phosphate, and 1 mM ATP) in the presence or absence of natural IP_6 (400 μM). Binding reaction mixtures were passed over streptavidin Dynabeads, and unbound protein was removed by thorough washing. In the Input lanes, 5% of input Ku shows that all reaction mixtures received equal amounts of recombinant Ku. The the Eluate lanes, bound Ku was eluted with natural IP_6 (12.5 mM). Ku70 and Ku80 were detected by Western blot analysis. (B) Crude HeLa cell extracts (AS65) were combined with IP_6-biotin under standard conditions in the presence or absence of unlabeled IP_6 (400 μM) and captured on streptavidin Dynabeads, and unbound proteins were removed by thorough washing. Bound proteins were eluted with unlabeled 12.5 mM IP_6 (Eluate), and proteins that remained associated with the resin following elution were removed by being boiled in 2× protein sample buffer (Boiled Resin). Ku70 and Ku80 were detected by Western blot analysis. (C) IP_6-biotin molecularly targets streptavidin to inhibit nonhomologous end joining. The extract (2.5 μg of PC-C) was combined with IP_6 (1 μM) or IP_6-biotin (2 μM) and streptavidin (0.15 μg) as indicated.

the binding was not specific for IP_6 (data not shown). These data identify Ku as the only NHEJ factor with specific affinity for IP_6-biotin and argue that Ku is the only NHEJ factor that binds IP_6. Further, our data suggest that IP_6 stimulates NHEJ *in vitro* by enhancing one, or more, of the functions of Ku in NHEJ.

Affinity Capture of Cellular Proteins Using IP_6-Biotin and Immobilized Streptavidin. We next sought to determine whether this technique could be used to isolate other IP_6-associated proteins from a human cell lysate. As before, we removed endogenous IPs by 65% ammonium sulfate precipitating cellular proteins from a HeLa whole cell extract,

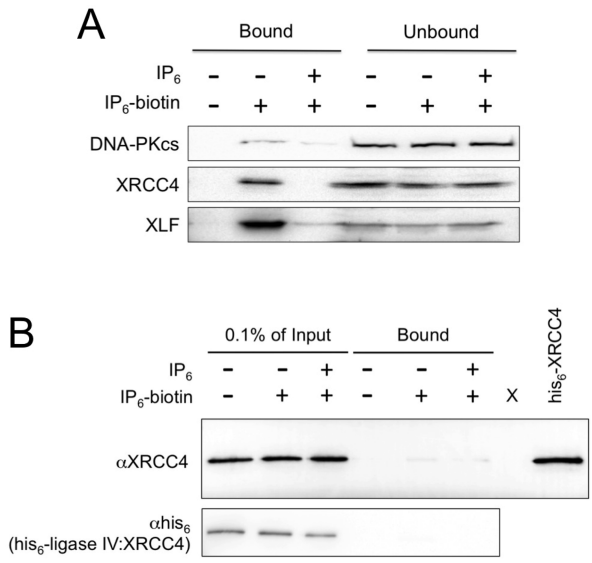

Figure 3. XRCC4 binds IP_6-biotin indirectly. IP_6-biotin affinity capture was conducted using standard conditions in the presence or absence of unlabeled IP_6 (400 μM), and unbound proteins were removed by thorough washing. Elution with unlabeled IP_6 was omitted, and all bound proteins were removed by boiling in 2× protein sample buffer. (A) Nonhomologous end joining proteins captured by IP_6-biotin from crude HeLa cell extracts. Unbound lanes contained proteins remaining in the supernatant following treatment with IP_6-biotin and streptavidin Dynabeads. (B) Affinity capture reactions conducted with purified recombinant his_6-XRCC4 or the his_6-ligase IV:XRCC4 complex, as indicated. The 0.1% of input lanes contained 0.1% of the binding reaction before treatment with IP_6-biotin and streptavidin Dynabeads.

and the reconstituted proteins were treated with IP_6-biotin-Dynabeads in the presence of a 100-fold excess of inorganic phosphate and a 50-fold excess of ATP relative to IP_6-biotin. Proteins that did not bind IP_6-biotin were removed through a series of washes; bound proteins were eluted with an excess of unlabeled IP_6, and tightly bound proteins were recovered by boiling the resin in protein sample buffer. As shown in Figure 4A, few proteins interacted with the streptavidin Dynabeads in the absence of IP_6-biotin (lane 1), and that addition of IP_6-biotin to the binding reaction mixture dramatically increased the number of proteins retained on the streptavidin resin (lane 2). Importantly, adding a 20-fold excess of unlabeled IP_6 as a specific competitor greatly reduced the number of proteins recovered by IP_6-biotin affinity capture (lane 3). This proof-of-concept experiment shows that IP_6-biotin can be used to capture proteins that directly, or indirectly, interact with highly phosphorylated IPs.

To further characterize the protein binding ability of IP_6-biotin, we examined retention of the IP_6-binding protein casein kinase 2 (CK2)[49,50] on streptavidin Dynabeads in the presence of IP_6-biotin. Western blot analysis showed that CK2 bound with high affinity to the streptavidin Dynabeads in the presence of IP_6-biotin (Figure 4B), but not when IP_6-biotin was absent, or when an excess of unlabeled IP_6 was present. Like Ku, CK2 was not eluted by incubation with excess unlabeled IP_6, which indicates that CK2 did not readily exchange IP_6-biotin for unlabeled IP_6. These data may indicate that CK2 has a higher affinity for IP_6-biotin than for unlabeled IP_6, although this is unlikely. It is more probable that these data reflect the effect of proteins that interact with IP_6-biotin-bound CK2 to stabilize CK2:IP_6-biotin interactions and reduce the rate of release of

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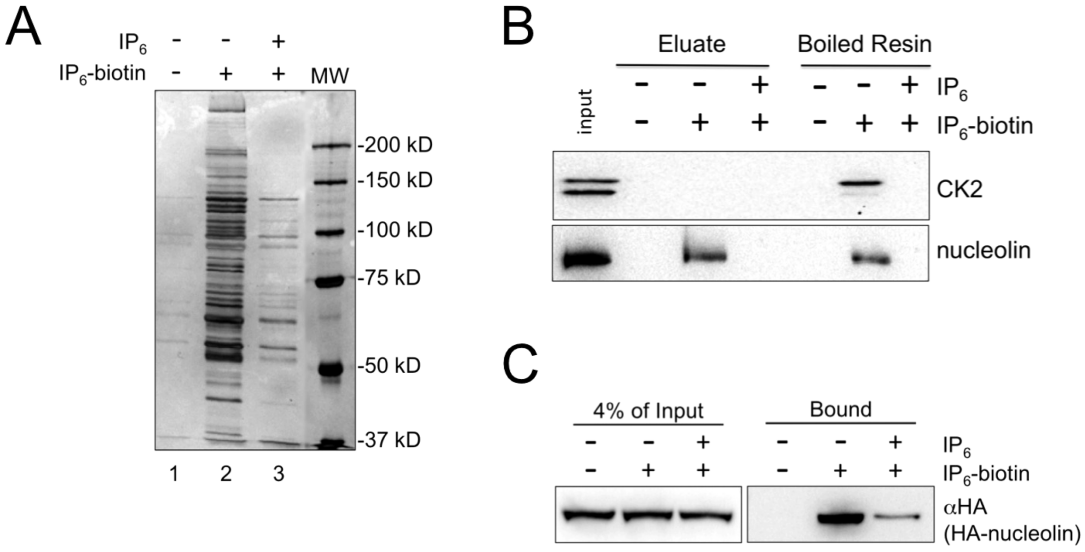

Figure 4. Affinity capture of cellular proteins using IP_6-biotin. IP_6-biotin affinity capture was conducted using standard conditions in the presence or absence of unlabeled IP_6 (400 μM), and unbound proteins were removed by thorough washing. (A) Silver stain of eluted proteins recovered from HeLa cell PC-C by IP_6-biotin affinity capture. Bound proteins were eluted with unlabeled 12.5 mM IP_6. (B) Casein kinase 2 (CK2) and nucleolin affinity captured from the crude HeLa cell extract (AS65) by IP_6-biotin. Bound proteins were eluted with unlabeled 12.5 mM IP_6 (Eluate), and proteins that remained associated with the resin following elution were removed by boiling in 2× protein sample buffer (Boiled Resin). Western blot analysis was used to detect the known IP_6-binding protein CK2 and the putative IP_6-binding protein nucleolin. (C) IP_6-biotin affinity capture of HA-tagged nucleolin. Extracts from HEK 293 cells transiently expressing HA-tagged nucleolin were subjected to IP_6-biotin affinity capture, and HA-tagged nucleolin was identified as one of the bound species. Elution with unlabeled IP_6 was omitted, and all bound proteins were removed by boiling in 2× protein sample buffer.

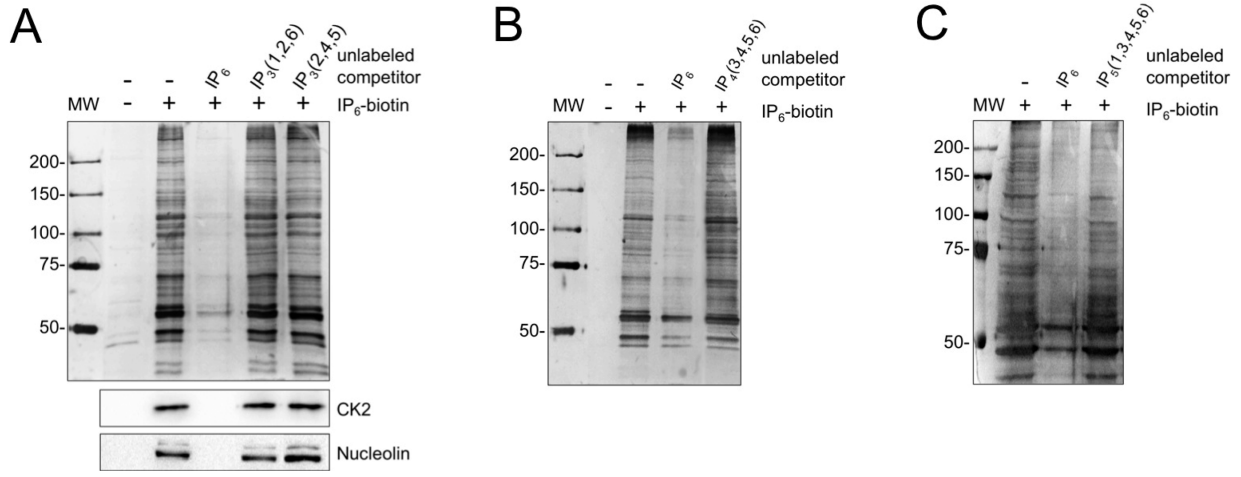

Figure 5. 1,2,6-IP_3, 2,4,5-IP_3, 3,4,5,6-IP_4, 1,3,4,5,6-IP_5, and IP_6 do not compete equally for protein binding to IP_6-biotin. IP_6-biotin affinity capture from crude HeLa cell extracts (AS65) under standard conditions in the presence or absence of the indicated unlabeled IPs (500 μM, 25-fold molar excess over IP_6-biotin). Unbound proteins were removed by thorough washing. Elution with unlabeled IP_6 was omitted, and all bound proteins were removed by boiling in 2× protein sample buffer, resolved via SDS−PAGE, and then visualized by silver staining. (A) Inclusion of IP_3 isoforms 1,2,6-IP_3 and 2,4,5-IP_3 in the binding reaction had no effect on binding of proteins to IP_6-biotin. Western blot analysis shows that binding of CK2 and nucleolin was not affected by the addition of either IP_3. (B) Inclusion of 3,4,5,6-IP_4 in the binding reaction had no obvious effect on binding of proteins to IP_6-biotin. (C) Inclusion of 1,3,4,5,6-IP_5 in the binding reaction slightly reduced the level of binding of proteins to IP_6-biotin.

IP_6-biotin, thus preventing elution by unlabeled IP_6. The input CK2 contained a lower-molecular weight band, which was captured less efficiently by IP_6-biotin (Figure 4B). We believe the faster-migrating species to be a CK2-breakdown product that lacks the ability to bind IP_6-biotin.

Nucleolin is abundant in nucleoli and distributed throughout the cell and is believed to participate in ribosome biogenesis, mRNA stability and translation, and viral infection.[51,52] While nucleolin has not previously been identified as an IP-binding protein, colocalization of nucleolin with IPK1, the primary enzyme that produces IP_6 through phosphorylation of IP_5, and phosphorylation of nucleolin by IP_7 link nucleolin with IP_6 metabolism and suggest that nucleolin may interact with IP_6.[15,53−55] Western blot analysis using anti-nucleolin antibodies identified nucleolin as one of the proteins retained by IP_6-biotin affinity capture (Figure 4B). Unlike CK2, nucleolin was both eluted by unlabeled IP_6 and remained tightly bound to the IP_6-biotin affinity resin, requiring boiling in protein sample buffer to release it from the Dynabeads. To confirm our identification of nucleolin as one of the proteins retained by

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IP_6-biotin affinity capture, we expressed HA-tagged nucleolin in HEK 293 cells, removed endogenous inositol polyphosphates by 65% ammonium sulfate precipitation, and conducted IP_6-biotin affinity capture. To simplify the data set, the 12.5 mM unlabeled IP_6 elution step was omitted and all bound proteins were recovered by boiling in protein sample buffer. We then used anti-HA antibodies to detect HA-nucleolin and found that HA-nucleolin was retained by IP_6-biotin affinity capture and that the presence of natural IP_6 reduced the extent of IP_6-biotin affinity capture of HA-nucleolin by approximately 10-fold (Figure 4C). Our data suggest that one or more forms of nucleolin can bind IP_6-biotin, either directly or indirectly through interactions with other cellular proteins.

Having determined that IP_6-biotin can be used to affinity capture bona fide IP_6-binding proteins, and possibly be used to capture and identify novel IP_6-binding proteins, we next examined the specificity of IP_6-biotin affinity capture. Although intracellular concentrations of IP_3 are lower than that of IP_6,[56] IP_3 is an important second messenger and IP_3-binding proteins are numerous. To determine whether IP_3 might compete for proteins that bind IP_6-biotin, we added a 25-fold excess of 1,2,6-IP_3 or 2,4,5-IP_3 to IP_6-biotin affinity capture binding reaction mixtures. As shown in Figure 5A, the pattern of silver-stained proteins recovered by IP_6-biotin affinity capture was not noticeably affected by the addition of 1,2,6-IP_3 or 2,4,5-IP_3, and retention of CK2 and nucleolin was unaffected. Similar results were obtained with 3,4,5,6-IP_4, and with 1,3,4,5,6-IP_5, which caused a modest reduction in the level of protein binding to IP_6-biotin (Figure 5B,C). In comparison, a 25-fold molar excess of IP_6 effectively competed for binding to IP_6-biotin (Figure 5). While we have not examined all possible isoforms of IP_3 and IP_4, our data indicate that proteins recovered by IP_6-biotin affinity capture preferentially bind IP_6.

DISCUSSION

Targeted Inhibition of Processes with Bifunctional IP_6 Analogues. We have synthesized IP_6-biotin as a tool to investigate interactions between IP_6 and IP_6-binding proteins and characterized IP_6-biotin using the Ku protein and the DNA repair mechanism NHEJ as a model system. Here, we show that IP_6-biotin stimulated NHEJ *in vitro*, with minimal loss of potency (Figure 1). These data show that biotin and the linker do not significantly impede the assembly or activity of the NHEJ apparatus. Ku binds IP_6,[24,27] and IP_6-biotin can be used to capture Ku by streptavidin affinity chromatography (Figure 2A,B). These data show that the linker separating IP_6 and biotin is long enough to permit simultaneous binding of IP_6-biotin by Ku and streptavidin. When streptavidin was added to NHEJ reaction mixtures containing IP_6-biotin, DNA ligation was inhibited (Figure 2C). We interpret these results as molecular targeting of streptavidin to Ku by IP_6-biotin and inhibition of NHEJ *in vitro*, likely through steric hindrance. Streptavidin is an ~53 kDa protein that can form multivalent tetramers, and either form of streptavidin could prevent Ku from interacting with factors that are necessary for NHEJ. On one hand, our proof-of-concept example shows how targeted delivery of streptavidin by IP_6-biotin can be used to interfere with the function of an IP_6-binding protein. On the other, our experiment suggests that tethering a functional group to an IP_6 might be used to produce highly specific targeting agents for IP_6-binding proteins. In such a bifunctional targeting molecule, the IP_6 would direct binding to IP_6-binding proteins, and a second chemical group, which replaces the biotin in our experiments, might interact with a unique aspect of the targeted protein, for example, a catalytic active site or protein-binding site.

Bifunctional IP_6 analogues, developed using the technology described here to produce IP_6-biotin, might be used to selectively inhibit cellular processes, or pathogenic processes. IP_6 is required to activate virulence factors in the Gram-positive bacteria *Vibrio cholera* and *Clostridium difficile* (*C. diff*). IP_6-biotin could be used to study the role of IP_6 in these reactions, and bifunctional IP_6 analogues might be developed as anti-toxins to prevent or treat infections resulting from exposure to these human pathogens. IP_6 has been implicated in a wide variety of highly important physiological activities, including key aspects of RNA metabolism, including RNA editing, mRNA export and translation,[5,18,20,57] neurotransmission, inhibition of protein phosphatases, and endocytic vesicle trafficking.[58−60] The technology used to produce IP_6-biotin could be adapted to develop bifunctional molecules that could be used to study or specifically control these processes.

Stimulation of NHEJ by IP_6 Occurs Primarily through Interactions with Ku. Our results indicate that although most of the factors required for NHEJ in human cells are affinity captured by IP_6-biotin, Ku is the only NHEJ factor with an intrinsic ability to specifically bind IP_6-biotin (Figures 2 and 3). It is possible that attachment of biotin to the 2-phosphate position might disrupt interactions between other NHEJ factors and IP_6, and that because of our modification of IP_6 we were unable to detect interactions with XRCC4 or the XRCC4:ligase IV complex (Figure 3B). However, if binding of IP_6 by XRCC4 or XRCC4/ligase IV was functionally important to the NHEJ reaction, and these proteins did not bind IP_6-biotin, we would expect that IP_6-biotin would be significantly less able to stimulate NHEJ *in vitro*. Because IP_6-biotin stimulates NHEJ *in vitro* with an only 2-fold loss of potency, we conclude that Ku is the primary IP_6-binding protein in the NHEJ reaction and that the effect(s) of IP_6 in NHEJ is related to one or more of the functions of Ku.

While purified recombinant human Ku could be eluted from the IP_6-biotin affinity resin with unlabeled IP_6, Ku from a HeLa cell lysate could not. These data suggest that, in the context of cell lysate, Ku produced in human cells could not exchange IP_6-biotin for unlabeled IP_6. Ku is known to interact with XLF, DNA-PKcs, and DNA ligase IV of the XRCC4:ligase IV complex as part of the NHEJ pathway for DNA double-strand break repair.[61] The higher avidity of cellular Ku for IP_6-biotin may be due to stabilization of Ku:IP_6-biotin interactions by additional cellular proteins or post-translational modification of Ku that is not reproduced by our expression system.

Affinity Capture of Proteins Binding to IP_6-Biotin. In addition to NHEJ factors, multiple other proteins were affinity captured by IP_6-biotin. To illustrate the utility of IP_6-biotin in identification of cellular IP_6-binding proteins, we tested retention of CK2, a protein known to bind IP_6. CK2 is a ubiquitous protein kinase with roles in cell growth and proliferation and suppression of apoptosis.[62] Structural and biochemical studies have shown CK2 to be a tetramer composed of two catalytic subunits (CK2α and CK2α') and two copies of the regulatory subunit CK2β. CK2 protein kinase activity is positively regulated by binding of IP_6 and negatively regulated by binding of Nopp140. IP_6 and Nopp140 compete for binding to the CK2α catalytic subunit.[63] Our experiments show that IP_6-biotin can be used to affinity capture CK2, which once bound to IP_6-biotin CK2 did not readily exchange IP_6-

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biotin for unlabeled IP_6, but when unlabeled IP_6 was included in the binding reaction, IP_6 efficiently competed for CK2 binding. The inability of CK2 to be eluted with unlabeled IP_6 might be due to binding of cellular proteins to the CK2−IP_6-biotin complex, or by binding of streptavidin blocking the IP_6-binding site and preventing access of unlabeled IP_6.[36]

Many proteins that bound IP_6-biotin were not retained on the solid phase when binding reaction mixtures contained excess unlabeled IP_6. These data suggest human cell extracts contain numerous proteins that directly or indirectly interact with IP_6. We used a candidate protein approach to determine whether a likely IP_6-binding protein was affinity captured by IP_6-biotin. Nucleolar localization of the IP_5 kinase that produces IP_6 suggests a role for IP_6 in the nucleolus.[15,53] When expressed in yeast, the human nucleolar protein nucleolin can be directly phosphorylated by the inositol pyrophosphate IP_7 (see Figure S3B[55]), which suggests a possible physical interaction with highly phosphorylated IPs such as IP_6.[54,55] While nucleolin has previously not been identified as an IP_6-binding protein, we found that nucleolin was recovered by IP_6-biotin affinity capture (Figure 4). Full-length recombinant nucleolin cannot be produced;[41] therefore, we could not assess the ability of recombinant nucleolin to bind IP_6-biotin. As an alternative, we used HA-tagged nucleolin to confirm binding of IP_6-biotin by nucleolin (Figure 4). Unlike Ku and CK2, nucleolin was eluted from the IP_6-biotin affinity resin with unlabeled IP_6, yet some nucleolin remained tightly bound to the affinity resin (Figure 4B). These data identify two populations of nucleolin, one that can exchange IP_6-biotin for unlabeled IP_6 and one that cannot. We suggest that the first represents a simple interaction in which nucleolin is directly bound to IP_6-biotin, and the second reflects complex interactions involving multiple proteins. For example, nucleolin has been shown to interact with CK2,[64] and nucleolin that could not be eluted with unlabeled IP_6 might indirectly bind IP_6-biotin through interaction with CK2.

To determine whether the proteins captured by IP_6-biotin are retained by specific interactions with IP_6, we tested the ability of less phosphorylated forms of inositol (IP_3 and IP_4) to compete for protein binding to IP_6-biotin. We were unable to detect any difference between the silver staining pattern of proteins captured in the presence or absence of a 25-fold molar excess of IP_3 or IP_4. Unlike IP_3 and IP_4, addition of a 25-fold molar excess of IP_5 reduced the level of binding of most affinity captured proteins by 50% (not shown). In contrast, addition of a 20-fold (Figure 4A) or 25-fold (Figure 5) molar excess of IP_6 dramatically reduced the amount of proteins recovered by IP_6-biotin affinity capture. All binding reaction mixtures contain inorganic phosphate and ATP as phosphate-rich nonspecific competitors, at 100- and 50-fold molar excesses, respectively. Our data (Figure 5) indicate that binding conditions prevent retention of proteins that recognize some of the less phosphorylated IPs.

In eukaryotes, IP_6 has been linked to basic cellular processes that are required for cellular viability.[15−20] In chordate development, the IP_5 kinase that produces IP_6, and presumably IP_6 itself, are required for viability in mice and play critical roles in zebrafish development.[4,21,22] While the list of processes that require IP_6 is impressive, it is likely far from complete. Deficiencies in IP_6, or in the IP_5 kinase, interfere with critical biological processes and affect multiple cellular pathways, which makes it exceedingly difficult to distinguish processes requiring direct participation of IP_6 from those indirectly affected by reduced IP_6 levels. Our data show that IP_6-biotin, and IP_6 analogues synthesized through a similar methodology, have great potential as tools for discovery and investigation of IP_6-dependent cellular processes. Proteins collected by IP_6-biotin affinity capture will likely reflect the biological systems that utilize IP_6 and may be used to identify proteins and systems not previously known to utilize inositol phosphates.

We have reported the synthesis of IP_6-biotin, a versatile IP_6 analogue that we use both as a chemical probe and as part of an affinity capture system. Our observations indicate that IP_6-biotin can be used to study the Ku:IP_6 interactions and the role of IP_6 in DNA double-strand break repair by nonhomologous end joining, and to isolate and identify proteins with affinity for IP_6. It is possible that IP_6 analogues may be developed as delivery systems for molecular targeting of biological systems that require participation of IP_6-biding proteins. We conclude that IP_6-biotin, and IP_6 analogues synthesized through a similar methodology, are powerful tools for the study of the soluble IPs and their biological functions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00642.

Spectroscopic characterization of IP_6-biotin via ^1H and ^{31}P NMR spectra and a high-resolution electrospray mass spectrum (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

IPs, soluble inositol polyphosphates; IP_3, inositol triphosphates; IP_4, inositol tetrakisphosphate; IP_5, inositol pentakisphosphate; IP_6, inositol hexakisphosphate; IP_6-biotin, biotin-labeled IP_6; CK2, casein kinase 2; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; NHEJ, nonhomologous end joining; IP_5 kinase, inositol 1,3,4,5,6-pentakisphosphate 2-kinase; DCM, dichloromethane; THF, tetrahydrofuran; $LiAlH_4$, lithium aluminum hydride; CaH_2, calcium hydride; DIPEA, *N*,*N*-diisopropylethylamine; mCPBA, *m*-chloroperoxybenzoic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *C. diff*, *C. difficile*.

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