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Evaluation of an LC8-Binding Peptide for the Attachment of Artificial Cargo to Dynein

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Abstract: The limited cytoplasmic mobility of nonviral gene carriers is likely to contribute to their low transfection efficiency. This limitation could be overcome by mimicking the viral strategy of recruiting the dynein motor complex for efficient transport toward the host cell nucleus. A promising approach for attaching artificial cargo to dynein is through an adaptor peptide that binds the 8 kDa light chain (LC8) found in the cargo-binding region of the dynein complex. Several viral proteins that bind LC8 have in common an LC8-binding motif defined by (K/R)-XTQT. Short peptides containing this motif have also been shown to bind recombinant LC8 in vitro. However, since the majority of intracellular LC8 exists outside of the dynein complex, it remains unclear whether peptides displaying this LC8-binding motif can access and bind to dynein-associated LC8. In this study, we employed biochemical analysis to investigate the feasibility of attaching artificial cargo to the dynein motor complex using a peptide displaying the well-characterized LC8-binding motif. We report that free intracellular LC8 bound specifically to an LC8-binding (TQT) peptide and not to a control peptide with a mutated LC8-binding motif. However, a similar binding interaction between the TQT peptide and intracellular dynein was not detected. To determine whether dynein binding of the TQT peptide was prevented by competition with free intracellular LC8 or due to the inability of the peptide to access its LC8 binding site in the dynein complex, the TQT peptide was evaluated for its ability to bind either purified LC8 or purified dynein. Our results demonstrate that, while the TQT peptide readily binds free LC8, it cannot bind to dynein-associated LC8. The results emphasize the need to identify functional dynein-binding peptides and highlight the importance of designing peptides that bind to the intact dynein motor complex.

Keywords: Dynein; gene delivery; LC8; peptide

Introduction

Nonviral gene carriers must overcome multiple biological barriers in order to successfully deliver their therapeutic cargo to the host cell nucleus. Strategies have been developed for targeting synthetic gene carriers to cell-specific receptors and for triggering their release from endosomes. However, most carriers rely on passive diffusion for transport within the cytoplasm. The cytoplasm is a crowded environment, consisting of organelles and soluble proteins enmeshed within a dense network of cytoskeletal filaments. Fluorescence recovery after photobleaching studies have shown that DNA fragments larger than 2000 bp are effectively immobile in the cytoplasm.[1] Additional studies have confirmed that translational diffusion of macromolecules in the cytoplasm is severely restricted.[2−6] For nonviral gene carriers, typically 50−200 nm in diameter, limited diffusion poses a significant

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barrier. Further improvement of nonviral gene delivery systems will benefit substantially from strategies that enable nonviral gene carriers to overcome their limited intracellular transport.

Due to the inability of macromolecules to diffuse freely within the cytoplasm, cells have developed a highly complex transport infrastructure that conveys cargo along cytoskeletal filaments. For retrograde transport toward the nucleus, dynein is the primary minus end-directed motor.[7,8] Several viruses take advantage of dynein as an intracellular shuttle for transport of viral proteins and nucleic acids toward the host nucleus.[9−13] The fact that viral particles, which are similar in size to nonviral gene carriers, recruit the dynein motor for assisted retrograde transport validates the need to develop nonviral vectors that can also actively recruit the retrograde motor machinery for efficient delivery. Despite the clear need for a retrograde transport moiety, a feasible method for the linkage of synthetic cargo to the dynein motor complex has not yet been demonstrated. We envision that nonviral vectors could be modified with short dynein-binding peptides, which would recruit the dynein motor and facilitate their microtubule-based transport toward the nucleus.

One potential target for dynein-binding peptides is the 8 kDa dynein light chain subunit (LC8), one of the three distinct classes of light chains proposed to constitute the cargo-attachment region of the dynein motor complex. Mapping of protein binding interactions with LC8 has led to the identification of two consensus motifs, (K/R)XTQT (found primarily in viral proteins that bind LC8) and GIQVD (found primarily in cellular proteins that bind LC8).[8] X-ray diffraction and NMR spectroscopy studies have revealed that these short motifs stabilize interactions with the LC8 dimer by binding inside the LC8 intermonomer groove,[14,15] while biochemical studies have confirmed that this conserved motif mediates association between LC8 and its protein partners.[16] Additionally, pepscan techniques have demonstrated the ability of short peptides containing these consensus motifs to bind recombinant LC8 in vitro.[17−19] Due to the identification of consensus amino acid motifs that bind LC8, this subunit is considered an attractive target for linking cargo to the dynein motor complex via a short peptide.

Despite the demonstrated ability of short peptides containing either the (K/R)XTQT or the GIQVD motif to bind LC8, there remains uncertainty regarding the ability of these peptides to bind LC8 when it is incorporated into the dynein complex.[20,21] Knowledge about whether LC8-binding peptides can access and bind to LC8 in dynein will guide the rational design of artificial cargo targeted to the dynein complex for assisted retrograde transport in applications such as gene delivery. Here we report that, while a peptide containing the (K/R)XTQT motif (TQT peptide) bound specifically to LC8 in cells, an intracellular interaction between these peptides and LC8 in the intact dynein motor complex was not evident. We further examined the ability of the TQT peptide to bind either purified LC8 or purified dynein. Again, the TQT peptide bound specifically to free LC8, but not to dynein-associated LC8. Our data provide novel insight into the accessibility of dynein-associated LC8

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to a peptide displaying the (K/R)XTQT motif and support the need to identify alternative peptides for attaching exogenous macromolecular cargo to the dynein complex.

Experimental Section

Biotinylation of Peptides. Peptides with C-terminal cysteines (TQT-1, PRMLHRSTQTTNC, and CP-1, PRMLHRTSGSTNC) were synthesized by Celtek Peptides (Nashville, TN), and peptides with N-terminal cysteines (TQT-2, CSSPRMLHRSTQTTN, and CP-2, CSSPRMLHRTSGSTN) were synthesized by Protein Technologies, Inc. (Tucson, AZ). Peptide disulfides were reduced using immobilized TCEP (Pierce, Rockford, IL), and then peptides were biotinylated through the cysteine residue by reaction for 2 h in PBS, pH 7.4, with biotin−PEO_2−maleimide (Pierce). Purity was assessed by HPLC, and molecular weights were verified by mass spectrometry.

Fluorescent Protein−Peptide Fusion Constructs. The plasmid encoding EGFP fused to LC8 (pEGFP−LC8) was generously provided by Dr. Yves Jacob. Fluorescent protein−peptide fusion constructs were designed such that the peptides projected from the C-terminus of the protein via an EEAAKA linker as described by Pelle et al.[22] Plasmids encoding EGFP−TQT and EGFP−control peptide fusions (pEGFP−TQT and pEGFP−CP) were constructed by inserting a cassette encoding an EEAAKA linker and the appropriate peptide into the multiple cloning site of pEGFP−C1 (Clontech, Mountain View, CA). Oligos (TQT, sense, 5′ CCG GAG AAG AAG CCG CCA AGG CCC CCA GGA TGC TGC ACA GGA GCA CCC AGA CCA CCA ACT AAG 3′; TQT, antisense, 5′ GAT CCT TAG TTG GTG GTC TGG GTG CTC CTG TGC AGC ATC CTG GGG GCC TTG GCG GCT TCT TCT 3′; CP, sense, 5′ CCG GAG AAG AAG CCG CCA AGG CCC CCA GGA TGC TGC ACA GGA CCA GCG GCA GCA CCA ACT AAG 3′; and CP, antisense, 5′′ GAT CCT TAG TTG GTG CTG CCG CTG GTC CTG TGC AGC ATC CTG GGG GCC TTG GCG GCT TCT TCT 3′′, IDT, Coralville, IA) were annealed and ligated between the *Bsp*E1 and *Bam*H1 restriction sites of the MCS. Plasmids encoding the HcRed−peptide fusions (pHcRed−TQT and pHcRed−CP) were constructed by inserting the same cassettes into the multiple cloning site of pHcRed1-C1 (Clontech). All plasmids were sequenced to verify insertion of the linker and peptide in-frame at the C-terminus. Subcloning and amplification of plasmids was performed using DH5α *Escherichia coli* cells (Invitrogen, Carlsbad, CA).

Cell Cultures and Transfection. HeLa cells were purchased from American Type Culture Collection (CCL-2) and were cultured in minimal essential medium supplemented with 10% fetal bovine serum and antibiotics. Cells were transfected with plasmids encoding fluorescent protein−peptide fusion constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For cotransfections, plasmids encoding the EGFP−LC8 and HcRed−peptide fusion constructs were mixed at a 1:1 molar ratio prior to formulation with Lipofectamine. Expression patterns were imaged in fixed cells using a Nikon TE2000-U inverted epifluorescence microscope. Colocalization of EGFP−LC8 and HcRed−TQT in intracellular aggregates was verified using a Zeiss LSM510 confocal microscope (University of Washington, Center for Nanotechnology).

Coimmunoprecipitations and Immunoblot Analysis. Lysate from HeLa cells expressing EGFP−TQT, EGFP−CP, HcRed−TQT, or HcRed−CP was collected by sonicating cells in PEM buffer (100 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9) supplemented with protease inhibitors (Roche, Indianapolis, IN). Lysate was cleared by centrifugation at 14 000 rpm for 15 min at 4 °C. To immunoprecipitate the EGFP−peptide fusions from lysate, anti-EGFP polyclonal antibodies (Clontech, Catalog 632459) were incubated with the cleared lysate for 8 h and then this mixture was added to prewashed protein A beads (Sigma Chemical Co., St. Louis, MO) and incubated for another 1.5 h. Beads were washed three times with PEM buffer containing 10% glycerol, and bound proteins were eluted by boiling the beads in 1× Laemmli sample buffer (Bio-Rad, Hercules, CA). To immunoprecipitate the dynein complex, anti-dynein intermediate chain monoclonal antibodies (IC 74.1, Chemicon, Temecula, CA, Catalog MAB1618) were added to the lysate, which was subsequently incubated with protein A beads, washed, and eluted as described above. For the EGFP−peptide co-IP samples, dynein LC8 and IC were analyzed by SDS−PAGE/immunoblot using anti-LC8 (Axxora, San Diego, CA, Catalog ALX-804−340) and anti-dynein intermediate chain (IC 74.1, Chemicon) antibodies with HRP-conjugated secondary antibodies, respectively. For the dynein IC co-IP samples, LC8 and HcRed−peptide were analyzed by SDS−PAGE/immunoblot using anti-LC8 (Axxora) and anti-HcRed (Clontech, Catalog 632452) primary antibodies with HRP-conjugated secondary antibodies, respectively.

Microtubule-Binding Assay. A microtubule-binding assay was adapted from the methods described by Kelkar et al.[23] Bovine brain tubulin (333 μg/sample, Cytoskeleton, Denver, CO) was polymerized by dissolving lyophilized tubulin in PEM-G buffer (PEM buffer with 1 mM GTP) and adding 40 μM paclitaxel at 37 °C. Microtubules were pelleted over a 60% glycerol cushion in PEM by ultracentrifugation at 55 000 rpm at 22 °C for 40 min. Microtubule pellets were resuspended in HeLa cell lysate containing 40 μM paclitaxel and were incubated for 60 min at room temperature. Microtubules with associated proteins were pelleted by ultracentrifugation. The microtubule/MAP pellet was resuspended in PEM buffer containing 40 μM paclitaxel and 10 ng of streptavidin−biotin−peptide conjugates. After 40 min

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incubation at room temperature, the microtubules and associated proteins were pelleted by ultracentrifugation and supernatant and pellet samples were resolved by 4−20% SDS−PAGE. Tubulin was detected by Coomassie blue staining, while dynein intermediate chain and streptavidin were analyzed by immunoblot using anti-dynein IC 74.1 (Chemicon) and anti-streptavidin (Abcam, Cambridge, MA, Catalog ab6676) antibodies, respectively.

Expression and Purification of Recombinant LC8. The pET-LC8 expression vector encoding LC8 fused to a His_6 tag at the C-terminus was donated to us by Dr. Ignacio Rodriguez-Crespo. Expression and purification of recombinant LC8 was conducted as previously described.[24] In summary, the pET-LC8 vector was used to transform BL21-(DE3) *E. coli* cells (Stratagene, La Jolla, CA) and a fresh colony was used to inoculate 2 mL of LB medium containing ampicillin at 100 μg/mL. This starter culture was grown at 37 °C overnight and was then used to initiate a 1 L culture in LB containing ampicillin. Once the OD_{600} of the culture reached 0.8, 1 mM IPTG was added to induce expression of recombinant LC8. After 6 h incubation with IPTG, the cells were harvested and stored as pellets at −80 °C until purification. For purification, the cell pellet was resuspended in buffer A (50 mM Hepes, pH 7.5, 100 mM NaCl, 0.1 mM PMSF, 1 mM leupeptin, 1 mM pepstatin, 1 mM antipain, 10% glycerol) plus 0.5 mg/mL lysozyme. Cells were lysed by passage through an 18-G needle and by pulse sonication on ice. After pelleting cellular debris, the supernatant was applied to a column containing Ni-NTA agarose (Qiagen, Valencia, CA) and recombinant LC8 was eluted with buffer A containing 200 mM imidazole. Purified protein eluted from the column was dialyzed against 20 mM ammonium bicarbonate $(NH_4)HCO_3$, pH 7.8, with 100 mM NaCl and was then frozen in liquid nitrogen and stored in aliquots at −80 °C. The purity of the protein was assessed by 4−20% SDS−PAGE and Coomassie blue staining.

Dynein Purification from Bovine Brain. Cytoplasmic dynein was purified from bovine brain as described by Paschal et al.[25] Briefly, white matter was collected from each of seven fresh calf brains by trimming gray matter away from the corpus callosum. White matter (54 g) was homogenized in 54 mL extraction buffer (50 mM PIPES−NaOH, 50 mM HEPES, pH 7.0, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL TAME, 1 μg/mL pepstatin A, and 1 mM DTT) by three passes through a 100 mL capacity Teflon-in-glass homogenizer (Kontes, Vineland, NJ) at 2000 rpm. Cytosolic extract was collected by centrifugation of the lysate at 24000*g* for 30 min at 2 °C, and then by high-speed centrifugation of the supernatant at 150000*g* for 60 min at 2 °C in a Beckman Ti70.1 rotor. Microtubules were polymerized by adding 20 μM paclitaxel (Sigma) to the cytosolic extract and incubating at 37 °C for 20 min. Following centrifugation at 40000*g* for 30 min at 35 °C, the microtubule pellet was washed twice by resuspension in extraction buffer supplemented with 5 μM paclitaxel followed by centrifugation at 40000*g*. Kinesin was released from the microtubules by resuspending the pellet in extraction buffer with 5 μM paclitaxel and 3 mM Mg-GTP (Sigma) and centrifuging at 40000*g*. Finally, dynein was released from the microtubules by resuspending the pellet in extraction buffer with 5 μM paclitaxel and 10 mM Mg-ATP (Sigma) and centrifuging at 150000*g* for 30 min at 25 °C. The dynein purification process was monitored by 7.5% SDS−PAGE and Coomassie blue staining. To verify copurification of LC8 with the dynein complex, samples were analyzed by 4−20% SDS−PAGE and immunoblot using an anti-LC8 primary antibody (Axxora) and an HRP-conjugated goat anti-rat IgM secondary antibody (Stressgen, San Diego, CA, Catalog SAB-210).

Pull-Down Assays and ELISA. Peptide−biotin conjugates were immobilized on streptavidin-coated agarose beads (Pierce). For pull-down of LC8 from whole cell lysate, cleared HeLa lysate was incubated with peptide-modified beads for 1 h and then beads were washed three times with Tris-KCl buffer (20 mM Tris-HCl, pH 7.6, 50 mM KCl, 5 mM $MgSO_4$, and 0.5 mM EDTA). Bound proteins were eluted by incubating beads with ImmunoPure elution buffer (Pierce) and were analyzed by immunoblot using an anti-LC8 antibody (Axxora) and an HRP-conjugated goat anti-rat IgM secondary antibody (Stressgen). For pull-down of purified proteins, 1 μg of purified LC8 or purified dynein containing 1 μg of LC8 were incubated with peptide-immobilized beads in extraction buffer for 1.5 h at room temperature. Beads were washed three times with Tris-KCl buffer, and bound proteins were eluted using ImmunoPure elution buffer (Pierce). Protein samples were neutralized by adding 1 M Tris prior to analysis. The amount of bound LC8 was measured by ELISA using a monoclonal anti-human PIN capture antibody (R&D Systems, Minneapolis, MN, Catalog MAB877), polyclonal anti-human PIN primary antibody (R&D Systems, Catalog AF877), and anti-goat IgG-HRP secondary antibody (R&D Systems, Catalog HAF109). Samples were developed by adding OPD substrate to each well (Sigma). LC8 in sample wells was quantified by comparison with an LC8 standard curve, and preferential binding to the TQT peptide was evaluated by dividing the amount of LC8 bound to the TQT-modified beads by the amount of LC8 bound to the CP-modified beads. Statistical significance was assessed using a Student's two-tailed *t* test.

Results

A Specific Binding Interaction Is Evident between the TQT Peptide and LC8 in Cell Lysate. The TQT peptide considered in this study was based on a dodecapeptide derived from the human adenovirus-associated BS69 protein, which contains an RSTQT motif. Pepscan analysis demonstrated an association between this dodecapeptide and

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Table 1. Peptide Sequences Used in the Binding Assays and Their Protein Sequence of Origin

peptide	sequence[a]	origin[b]
TQT-1	PRMLHRSTQTTNC	adenovirus-associated BS69 ^{408}PRMLHRSTQTTN^{419}
CP-1	PRMLHRTSGSTNC	modification of TQT-1 to abolish (K/R)XTQT motif
TQT-2	CSSPRMLHRSTQTTN	adenovirus-associated BS69 ^{406}SSPRMLHRSTQTTN^{419}
CP-2	CSSPRMLHRTSGSTN	modification of TQT-2 to abolish (K/R)XTQT motif

[a] Peptides were synthesized with a cysteine at either the C- or N-terminus for conjugation to biotin−maleimide and subsequent conjugation to the streptavidin protein or immobilization on streptavidin-modified surfaces. [b] Sequences were derived from the region of the adenovirus-associated BS69 protein that binds directly to LC8 via its RSTQT motif.

recombinant LC8.[18] As the glutamine residue in the (K/R)-XTQT consensus motif is believed to be critical for binding the LC8 intermonomer groove with stabilization from the flanking threonine residues,[14,15,26] a control peptide (CP) was designed to abolish the LC8-binding activity by replacing glutamine (Q) with glycine (G) and switching the order of the preceding serine (S) and threonine (T) residues. Cysteines were included at either the N- or C-terminus to enable conjugation of biotin−maleimide and subsequent immobilization of peptides on streptavidin-modified supports. Peptide sequences are provided in Table 1.

The interaction of the TQT-1 and CP-1 peptides with LC8 present in mammalian cell lysate was assessed by immobilizing biotinylated peptides on streptavidin-coated beads and incubating the beads with HeLa total cell lysate. The LC8 that bound to the peptides was detected by immunoblot (Figure 1). As expected, the TQT peptide captured LC8 from total cell lysate, thus confirming that the peptide is capable of significantly binding LC8 even in the presence of other potential binding partners in the cell. Sequence specificity of the TQT−LC8 interaction was verified by the inability of the control peptide to bind LC8, as neither the CP nor the streptavidin beads alone pulled down detectable quantities of LC8.

The TQT Peptide and Overexpressed LC8 Colocalize in Cells. The intracellular interaction between the TQT peptide and LC8 was examined by coexpressing EGFP−LC8 and HcRed−TQT or HcRed−CP fusion proteins in HeLa cells. A previous study by Petit et al. demonstrated an intracellular interaction between the retroviral Gag protein and EGFP-tagged LC8 by coexpressing these proteins in Cos6 cells.[27] By confocal microscopy, it was determined that

Figure 1. LC8 from cell lysate specifically binds to the TQT peptide. An affinity pull-down assay was designed to determine whether endogenous LC8 from cell lysate would bind to the immobilized TQT-1 peptide. Peptide−biotin conjugates were immobilized on streptavidin-coated beads and subsequently incubated with HeLa cell lysate. The specificity of this binding interaction was evaluated by immobilizing either a control peptide (CP-1) or biotin alone (no peptide, NP) on the beads. Following elution of bound protein from the beads, LC8 was detected by SDS−PAGE/immunoblot using antibodies against LC8. Cell lysate was used as a positive control for the presence of LC8 (left lane). A sequence-specific interaction between intracellular LC8 and the TQT peptide was evident (TQT-1 lane), while LC8 did not bind to the control peptide or to beads displaying no peptide (CP-1 and NP lanes).

Table 2. Sequences of Fluorescent Protein−Peptide Fusion Constructs

fusion construct[a]	amino acid sequence[b]
HcRed−TQT	<HcRed>SGEEAAKAPRMLHRSTQTTN
HcRed−CP	<HcRed>SGEEAAKAPRMLHRTSGSTN
EGFP−TQT	<EGFP> SGEEAAKAPRMLHRSTQTTN
EGFP−CP	<EGFP> SGEEAAKAPRMLHRTSGSTN
EGFP−LC8	<EGFP>CDRKAVIKNADMSEEMQQDSV ECATQALEKYNIEKDIAAHIKKEFDKK YNPTWHCIVGRNFGSYVTHETKHFIYF YLGQVAILLFKSG

[a] Fusion constructs were designed to examine the intracellular distributions and biochemical interactions of LC8 with the TQT peptide or a control peptide (CP). [b] Peptides (TQT or CP) were fused to the C-termini of HcRed1 and EGFP genes via a rigid EEAAKA linker. Coexpression of HcRed−peptide with EGFP−LC8 in cells revealed the relative intracellular distributions of the peptides and LC8. Fluorescent proteins were also used as affinity tags for coimmunoprecipitation experiments to examine biochemical interactions between the peptides, LC8, and the dynein complex.

97% of Gag colocalized with EGFP−LC8 at the microtubule organizing center (MTOC). On the basis of these findings, we hypothesized that coexpression of LC8 with the TQT peptide would result in similar patterns of colocalization, assuming efficient interaction in cells.

Fluorescent protein (HcRed or EGFP)−peptide fusion constructs were designed so that peptides extended away from the fluorescent protein by a rigid EEAAKA linker at its C-terminus (Table 2). Pelle et al. demonstrated that such a linker projected peptides away from the β-can structure of the EGFP scaffold for display in the cytoplasm.[22] EGFP−LC8 and HcRed−TQT or HcRed−CP fusion proteins were used to transfect HeLa cells either separately or as EGFP/HcRed pairs. The intracellular distribution of each of the fusion constructs expressed independently was uniform throughout the cell, as is also characteristic of EGFP or HcRed expressed alone. When EGFP−LC8 was coexpressed with HcRed−CP, the intracellular distribution of both

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proteins remained uniform and diffuse. However, when EGFP−LC8 was coexpressed with HcRed−TQT, the two proteins colocalized within the cytoplasm in a punctate pattern (Figure 2A). Some of the punctate labeling localized around the nucleus, but not at a single point, which would have been indicative of accumulation at the MTOC.

We observed a variety of distribution patterns in cells coexpressing EGFP−LC8/HcRed−TQT (Figure 2B). There was no example, however, where the intracellular distribution pattern of the TQT peptide (either in the presence or absence of overexpressed LC8) resembled that of dynein or microtubules. Instead, we hypothesize that the interaction between the TQT peptide and LC8 causes LC8 to become more hydrophobic, leading to either self-aggregation of LC8/TQT complexes or incorporation of these complexes into intracellular membranes. This theory is supported by our observation that purified LC8 precipitates from solution upon titration of the TQT peptide but not the control peptide. Considered together, the biochemical pull-down data and coexpression patterns indicate a sequence-specific binding interaction between the TQT peptide and LC8 in cells, but association of the TQT peptide with the dynein complex could not be verified.

The TQT Peptide Binds LC8 but Not Dynein in Cells. The ability of the TQT peptide to bind free LC8 in cells does not necessarily establish its ability to access and bind to LC8 in the dynein motor complex. To explore the possibility of dynein−cargo attachment through the TQT peptide, HeLa cells expressing either the EGFP−TQT or EGFP−CP fusion construct (which project the peptides from the EGFP protein via a linker at their N-terminus) (Table 2) were lysed and EGFP was immunoprecipitated using anti-EGFP antibodies. Coimmunoprecipitation of LC8 was detected in the EGFP−TQT sample, but not in the EGFP−CP sample (Figure 3A). The dynein intermediate chain did not coimmunoprecipitate with either EGFP−peptide fusion. However, when cells expressing EGFP−LC8 were subjected to immunoprecipitation using anti-EGFP antibodies, the dynein intermediate chain did coimmunoprecipitate with EGFP−LC8, suggesting that EGFP−LC8 was incorporated into the dynein complex and that the intact dynein complex could be successfully immunoprecipitated from HeLa lysate (data not shown). A complementary coimmunoprecipitation experiment was conducted in which HeLa cells expressing either HcRed−TQT or HcRed−CP were lysed and the dynein motor complex was immunoprecipitated via immobilized dynein intermediate chain antibodies. LC8 coimmunoprecipitated with dynein IC, indicating that the complex remained intact under these experimental conditions. However, neither HcRed−TQT nor HcRed−CP coimmunoprecipitated with the dynein complex (Figure 3B). These results indicate that binary interactions of TQT/LC8 and LC8/dynein can be detected by immunoprecipitation, but that the ternary complex (TQT/LC8/dynein) cannot be immunoprecipitated from cells.

Due to the close interaction between the intermediate chain and LC8 in cytoplasmic dynein,[28] it is possible that immunoprecipitation of the dynein complex using intermediate chain antibodies may interfere with the ability of the TQT peptide to access and bind to LC8 in the dynein complex. Consequently, the dynein-binding properties of the TQT peptide were further evaluated in a microtubule-binding assay, where dynein−cargo interactions can be examined in a more natural context. The microtubule-binding assay is based on the ability of paclitaxel-stabilized microtubules and their associated proteins to pellet under centrifugation. Proteins that do not bind microtubules or their associated proteins remain in the supernatant. Microtubule association through dynein can be confirmed if the cargo releases from microtubules with dynein upon addition of ATP, or if the cargo fails to bind when the microtubule-associated proteins are depleted of dynein. A dynein-dependent interaction between adenovirus and microtubules was established using these techniques.[23] For this assay, the TQT−biotin conjugate was attached to recombinant streptavidin as a model cargo. Paclitaxel-stabilized microtubules were incubated with HeLa cell lysate to obtain a microtubule pellet enriched with cytoplasmic dynein and other microtubule-associated proteins (MAPs). Streptavidin−biotin−peptide complexes were then incubated with the MT/MAP pellets and centrifuged. Supernatant and pellet samples were analyzed by immunoblot to detect dynein intermediate chain and streptavidin. Both streptavidin−biotin−TQT and the streptavidin−biotin−CP and streptavidin−biotin controls failed to pellet with the microtubules and associated dynein (Figure 4). These experiments suggest that the TQT peptide does not interact appreciably with dynein in cells.

The TQT Peptide Binds Free LC8 but Not Dynein-Associated LC8. The lack of detectable interactions between the TQT peptide and dynein in both the coimmunoprecipitation experiment and the microtubule-binding assay supports the structural prediction that the TQT binding groove in LC8 is inaccessible when LC8 is incorporated in the dynein complex. However, it is possible that the TQT peptide could physically bind to dynein but that the presence of free LC8 in the cell lysate competes effectively for peptide binding. To test whether an interaction between the TQT peptide and cytoplasmic dynein is possible, peptide−biotin conjugates were immobilized on streptavidin-functionalized agarose beads and incubated with either free recombinant LC8 or an equivalent concentration of dynein-associated LC8 isolated from calf brain. Purification of intact dynein from calf brain was monitored by SDS−PAGE, and copurification of LC8 with the heavy chain of dynein suggested that the purified dynein contained the LC8 subunit (Figure 5). The amount of LC8 in the purified dynein complex was quantified both by ELISA and by western blot analysis; these studies also confirm that LC8 in the dynein complex can be detected under these experimental conditions. Beads with immobilized TQT or control peptides were incubated with purified LC8

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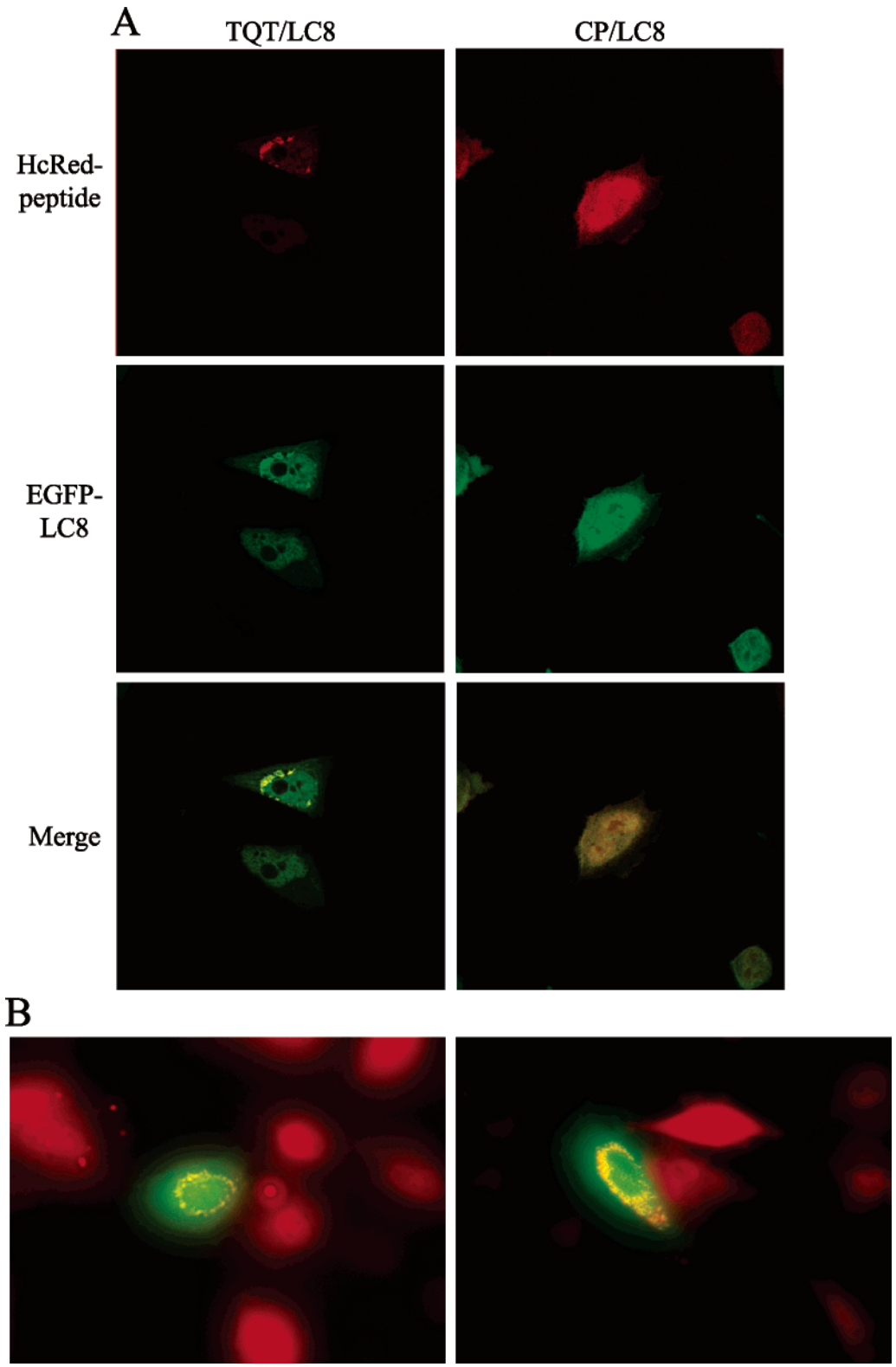

Figure 2. Coexpression of EGFP−LC8 with HcRed−TQT results in colocalization. (A) HeLa cells were cotransfected with plasmids encoding EGFP−LC8 and either HcRed−TQT or HcRed−control peptide (CP). A specific interaction between overexpressed LC8 and the TQT peptide resulted in the formation of punctate intracellular patterns. Colocalization of EGFP−LC8 and HcRed−TQT in these punctate patterns was confirmed using confocal microscopy (left panels). Both LC8 and the control peptide (CP) remained uniformly distributed throughout the cell when coexpressed (right panels). (B) Coexpression of EGFP−LC8 and HcRed−TQT resulted in a variety of intracellular distributions.

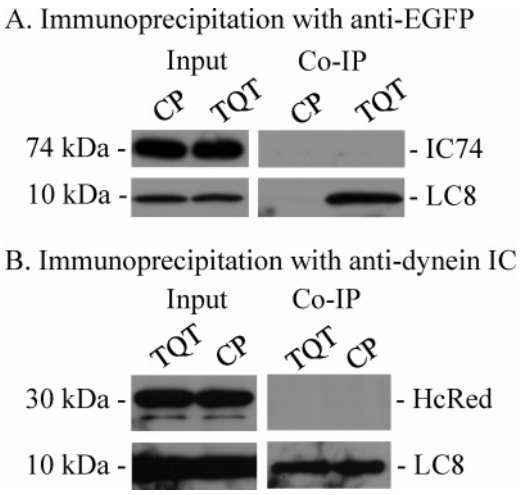

Figure 3. The TQT peptide binds to LC8 in cells but not to intact dynein. (A) HeLa cells expressing EGFP−TQT or EGFP−control peptide (CP) were lysed and EGFP was immunoprecipitated using specific antibodies. Coimmunoprecipitated LC8 and dynein intermediate chain (IC74) were detected by SDS−PAGE/immunoblot. Both dynein IC and LC8 were detected in positive control, input lysate samples (Input lanes). LC8, but not dynein IC, coimmunoprecipitated with the TQT peptide (Co-IP, TQT lane), while neither dynein IC nor LC8 coimmunoprecipitated with the control peptide (Co-IP, CP lane). (B) Cells expressing HcRed−TQT or HcRed−CP were lysed and the dynein IC was immunoprecipitated using specific antibodies. LC8 and HcRed were detected by SDS−PAGE/immunoblot. As a positive control, both HcRed and dynein LC8 were detectable in the input lysate (Input lanes). LC8 coimmunoprecipitated with the dynein IC in both samples, suggesting that the dynein complex remained intact (Co-IP lanes, bottom). However, neither HcRed−TQT nor HcRed−CP coimmunoprecipitated with the dynein complex (Co-IP lanes, top).

or dynein samples containing equivalent amounts of LC8. The amount of LC8 associated with beads was quantified using an LC8 ELISA. As an indication of the binding specificity of LC8 for the TQT peptide, the amount of LC8 bound to the TQT peptide was divided by the amount of LC8 bound to the control peptide. For recombinant LC8, there was a (5.7 ± 0.3)-fold increase for binding to the TQT peptide versus the control peptide. However, for dynein-associated LC8, there was no significant increase ((1.3 ± 0.6)-fold increase) (Figure 6). The amounts of dynein-associated LC8 bound to either the TQT or control peptide-modified beads were similar to the amount of rLC8 bound to the control peptide-modified beads, which was on the order of the background signal.

To test whether the inability of dynein-associated LC8 to bind the immobilized TQT peptide was due to steric hindrance resulting from immobilization of the peptide by its C-terminus (which is close to the LC8-binding motif), the pull-down study was repeated with the TQT peptide or CP immobilized by their N-terminus with a PEO_2 (di-polyethylene oxide) linker. This spacing allowed the LC8-

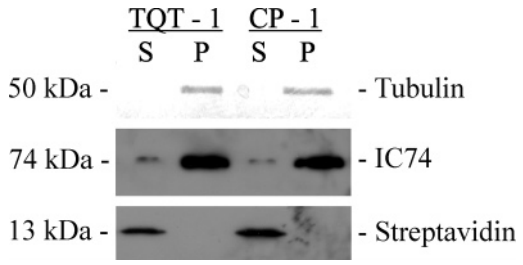

Figure 4. The TQT peptide does not copellet with dynein and microtubules in a microtubule-binding assay. Interaction between the TQT peptide and the intact dynein complex was examined in the context of a microtubule-binding assay. Purified bovine brain microtubules were incubated with HeLa cell lysate to obtain dynein-enriched microtubule/MAP (microtubule-associated protein) pellets. Streptavidin−biotin−peptide conjugates were incubated with microtubule/MAP samples and subsequently subjected to ultracentrifugation to pellet the microtubules and associated proteins. Supernatant (S) and pellet (P) samples were collected and analyzed by SDS−PAGE followed by either Coomassie blue staining to detect tubulin or immunoblotting to detect dynein (IC74) or streptavidin−biotin−peptide conjugates (Streptavidin). For both TQT-1 and CP-1 samples, streptavidin−biotin−peptide conjugates remained in the supernatant, while dynein was found in the pellet with the microtubules.

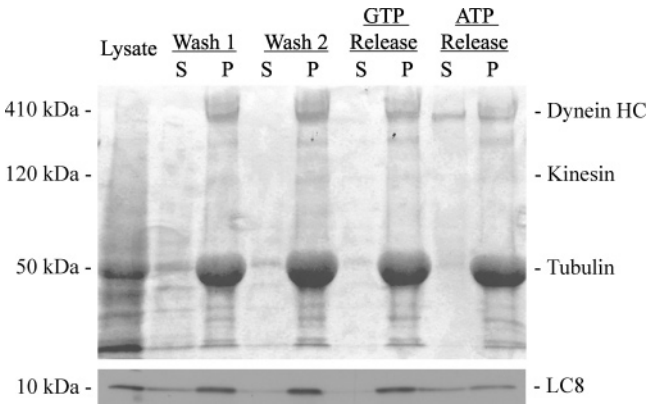

Figure 5. Intact dynein was purified from bovine brain. Cytosol from bovine brain white matter was incubated with paclitaxel to polymerize tubulin into microtubules. The microtubules were then pelleted under ultracentrifugation. Both dynein and kinesin motor proteins pelleted with the microtubules during the first and second wash steps. Incubation with GTP specifically released kinesin from the microtubule pellet. Subsequently, ATP was added to release the dynein complex. Samples of supernatant (S) and pellet (P) were collected at every step. Purification of cytoplasmic dynein from bovine brain was monitored by SDS−PAGE and immunoblot using anti-LC8 antibodies. The dynein heavy chain (HC) partially released from microtubules upon addition of ATP. Corelease of LC8 with the dynein HC suggests that the intact dynein complex was obtained.

binding motif to extend an extra seven amino acids from the surface of the agarose beads. Consistent with our previous results, only the rLC8 bound to the N-terminal-immobilized

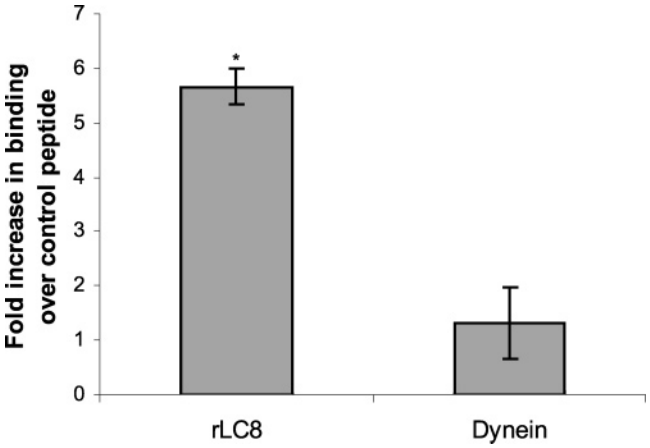

Figure 6. Free LC8, but not dynein-associated LC8, binds the immobilized TQT peptide. Equivalent concentrations of either free recombinant LC8 (rLC8) or dynein-associated LC8 were incubated with beads displaying either the TQT or control peptide (CP). Bound proteins were eluted from the beads and LC8 concentration was determined by ELISA. Preferential binding of rLC8 to the TQT-immobilized beads was evident, while dynein-associated LC8 did not bind appreciably to TQT- or CP-immobilized beads. Values presented are the mean fold increase in binding over the control peptide and standard deviation. (*) Significant statistical difference between rLC8 and dynein groups ($p = 0.014$).

TQT beads; detectable amounts of dynein-associated LC8 still did not bind.

Discussion

Strategies to improve the efficiency of nonviral gene delivery following arrival of the gene carrier at the target cell have focused on three major obstacles: (i) passage through the plasma membrane, (ii) escape from endosomes, and (iii) entry into the nucleus. However, the inability of synthetic gene carriers to efficiently traverse the cytoplasm and reach the nucleus following escape from endosomes has largely been overlooked. We hypothesize that a delivery system which exploits microtubule-based motor proteins for efficient intracellular transport will significantly improve the efficiency of nonviral transfection in many cell types, and particularly in neurons, where gene carriers must travel a long distance to reach the nucleus. Since dynein is the primary minus end-directed motor in eukaryotic cells, and since this motor is used by viruses for retrograde transport toward the host cell nucleus, our strategy is to design synthetic gene carriers that display dynein-binding peptides for enhanced intracellular transport.

In this study, a peptide that binds to LC8, a light chain subunit of dynein, was evaluated as the first potential dynein-binding peptide. The sequence of this peptide was based on a consensus LC8-binding motif, (K/R)XTQT, found in such viruses as herpes simplex virus, adenovirus, and rabies virus.[18,29] The specific 12 amino acid sequence used in these studies was derived from the adenoviral BS69 protein and was selected for evaluation because interactions between adenovirus and the dynein motor have been previously demonstrated.[23] It has been hypothesized that proteins or peptides displaying the (K/R)XTQT motif might attach to dynein through a direct interaction with its LC8 subunit.[17,18] Indeed, ample evidence supports that proteins and peptides containing this motif bind free LC8. However, it remains to be determined whether a peptide displaying the LC8-binding motif could be responsible for cargo attachment to the intact dynein motor complex. It has been estimated that only ~12% of intracellular LC8 actually associates with the dynein motor complex,[30] suggesting that LC8-binding peptides or proteins may be more likely to encounter and bind to free LC8 than dynein-associated LC8. Therefore, the ability of the TQT peptide to interact with dynein was assessed both with total cell lysate and with purified dynein. We have demonstrated that, despite the ability of the TQT peptide to bind LC8, this peptide fails to efficiently bind the dynein motor complex. This is the first experimental evidence suggesting that the LC8-binding peptide would not be suitable for attachment of cargo to dynein.

Our results support speculation, based on structural analysis, that incorporation of LC8 into the dynein complex precludes binding of a peptide to its intermonomer groove since both binding grooves are most likely occupied by the KETQT motifs of the paired dynein intermediate chains (Figure 7).[20] In fact, free intracellular LC8 has been implicated in a variety of motor-independent functions.[31−33] Therefore, it is possible that interactions between the (K/R)XTQT motif and LC8 are related to intracellular processes independent of transport. Consistent with this theory, removal of the LC8-binding motif from the rabies virus P protein resulted in minimal impairment of its retrograde transport abilities.[34,35] Additionally, our unpublished data indicate that

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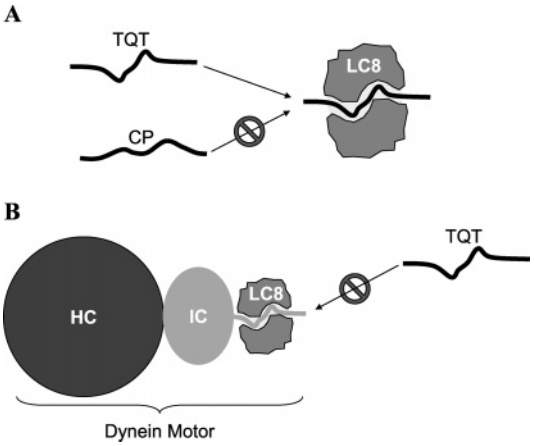

Figure 7. Model describing the possible TQT/LC8/dynein interactions. LC8 and the TQT peptide can form a binary complex in cells, but cannot form a ternary complex with dynein even in the absence of free competing LC8. (A) The data suggest that free LC8 binds specifically to the TQT peptide and not to a control peptide (CP) lacking the TQT motif. This binding interaction relies on shape complementarity and the formation of specific hydrogen bonds between the TQT motif and residues in the intermonomer groove of LC8.[15,26] (B) Our results support the inability of an exogenously introduced TQT peptide to bind to dynein-associated LC8. Instead, it is possible that LC8-binding motifs in both intermediate chains (IC) of the dynein motor complex occupy both intermonomer grooves of the LC8 dimer, preventing the attachment cargo to dynein via the TQT−LC8 interactions.

overexpression of the TQT peptides in HeLa cells does not hinder adenoviral transport or infectivity, suggesting that the (K/R)XTQT motif alone is unlikely to be responsible for adenovirus attachment to dynein. We cannot exclude the possibility, however, that certain LC8-binding sequences or display conformations might facilitate or stabilize cargo attachment to dynein through the LC8 subunit. There are limited examples in the literature where proteins bearing the (K/R)XTQT motif display retrograde transport properties.[36,37] However, since these studies did not probe for a direct interaction between the (K/R)XTQT motif and intact dynein, the mechanism of nuclear-directed transport in these systems remains an intriguing inquiry for future studies.

The fact that the peptides evaluated in this study bound robustly to free LC8 but failed to interact detectably with the intact dynein complex supports that attachment of short, (K/R)XTQT-based peptides is not a practical means for recruiting the dynein motor for gene or drug delivery systems. To support this, our unpublished data indicate that incorporation of the TQT peptide into polyplexes, when attached either to the plasmid DNA or to the cationic polymer through a spacer, confers no increase in reporter gene expression. While it remains possible that certain LC8-binding proteins can fold in such a way as to allow interaction with the intact dynein complex, we are interested in evaluating potential dynein-binding peptides rather than full-length dynein-binding proteins due to the obvious advantages of integrating short peptide sequences into multicomponent synthetic systems that may contain additional domains for cell targeting, endosomal escape, and nuclear entry.

There is significant interest in the development of artificial cargo that mimics viruses by recruiting the dynein motor for assisted transport toward the host cell nucleus. For applications in gene therapy, modification of gene carriers with dynein-binding peptides might increase their rate of transport to the nucleus by overcoming the limited diffusivity of large molecules and nanoparticles in the cytoplasm. This approach has been proposed by several groups,[38−40] although a feasible strategy for the linkage of synthetic cargo to the dynein motor complex has not yet been demonstrated. Our findings highlight the importance of designing dynein-binding peptides that not only bind to an isolated subunit of dynein but also can access the intact dynein motor complex for a functional motor−cargo interaction. Future work from our group will focus on the identification of peptides that mediate binding to the intact dynein complex by interacting with subunits such as the dynein light chain TcTex-1 or the dynein-associated dynactin complex.

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