Development and application of in vivo molecular traps reveals that dynein light chain occupancy differentially affects dynein-mediated processes

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The ability to rapidly and specifically regulate protein activity combined with *in vivo* functional assays and/or imaging can provide unique insight into underlying molecular processes. Here we describe the application of chemically induced dimerization of FKBP to create nearly instantaneous high-affinity bivalent ligands capable of sequestering cellular targets from their endogenous partners. We demonstrate the specificity and efficacy of these inducible, dimeric “traps” for the dynein light chains LC8 (Dylnl1) and TcTex1 (Dylnl1). Both light chains can simultaneously bind at adjacent sites of dynein intermediate chain at the base of the dynein motor complex, yet their specific function with respect to the dynein motor or other interacting proteins has been difficult to dissect. Using these traps in cultured mammalian cells, we observed that induction of dimerization of either the LC8 or TcTex1 trap rapidly disrupted early endosomal and lysosomal organization. Dimerization of either trap also disrupted Golgi organization, but at a substantially slower rate. Using either trap, the time course for disruption of each organelle was similar, suggesting a common regulatory mechanism. However, despite the essential role of dynein in cell division, neither trap had a discernable effect on mitotic progression. Taken together, these studies suggest that LC occupancy of the dynein motor complex directly affects some, but not all, dynein-mediated processes. Although the described traps offer a method for rapid inhibition of dynein function, the design principle can be extended to other molecular complexes for *in vivo* studies.

in vivo antagonist | retrograde transport | organelle kinetics | mitotic index

Cytoplasmic dynein is a microtubule-based motor protein involved in numerous essential cellular functions, including intracellular transport of membranous organelles and macromolecular complexes, mitosis, and cell migration (1). Cytoplasmic dynein consists of multiple subunits: two 532-kDa heavy chains (HCs), each containing a motor domain, and a number of accessory subunits, the intermediate, light intermediate, and light chains (ICs, LICs, and LCs, respectively). The ICs and LCs form a complex at the base of the dynein HC (residues 1–1,100) (2). The ICs have been implicated in subcellular targeting of cytoplasmic dynein through an interaction with a large accessory complex, dynactin (3–6). The LCs, in turn, form a complex with the dynein IC, but also interact with a large number of other proteins, including transcription factors, signaling molecules, RNA, and viral proteins. As such, the dynein LCs have been implicated as adaptors to link these diverse molecules to dynein for retrograde transport (1).

Our recent structural and biophysical studies aimed at understanding role of the dynein LCs, however, do not support the direct involvement of these subunits in cargo recognition and transport (7). Specifically, the structure of the LCs [LC8 (Dyln11) and TcTex1 (Dyln11)] bound to the dynein IC shows that the dynein IC occupies the same site as putative cargo (fig. 5b of ref. 8). Moreover, the LCs are homodimeric and contain two symmetric binding sites. Yet, in the structure of the IC-LC8-TcTex1 complex, both peptide binding sites on each LC are occupied by the dynein IC, occluding the peptides from putative cargo to bind to the dimeric LC. Finally, the

dynein IC, within the context of the dynein complex, is dimeric, as are most of the characterized LC-interacting proteins (e.g., nNOS, Pak1, 53BP1). This dimeric organization of the LCs and their targets gives rise to a bivalent-bivalent interaction, which is generally of higher affinity due to energy additivity compared with a tripartite interaction (e.g., target-LC-IC) (7, 9).

While it is unlikely that the LCs directly participate in bridging cargo to the dynein motor complex, recent investigations suggest the LCs may regulate specific functions of dynein such as the dynein-dynactin interaction and/or the phosphorylation of the IC. In particular, the dynein LCs can bind regions within proteins that are predicted to be intrinsically disordered (10), and recent studies in various systems have implicated such as intrinsically disordered regions frequently participate in the regulation of protein activity and/or function (11). In addition, evidence of a dynein-independent role of the LCs is beginning to emerge. LC8 has been implicated in regulating the activity of TRPS1 (12) and nNOS (13). Recent studies have also shown that LC8 participates in nuclear import of the Rabies P protein and 53BP1 (14), as well as Pak1 (15).

Because the LCs are essential for dynein function (16) and also appear to have multiple dynein-independent roles, it is difficult to address their specific cellular functions. Ultimately, the ability to target and immediately regulate the LC interaction with dynein *in vivo* through the addition of a small chemical moiety could provide significant insight into the physiological role of LCs in dynein function, especially when combined with functional readouts, including immunocytochemistry and live cell imaging. However, for a majority of macromolecular complexes, including dynein, this type of control remains elusive. In part, this is because most macromolecular interactions, including the IC-LC interaction, are spread over large interfaces and do not present sufficient cavities necessary to develop high-affinity, small-molecule antagonists. However, distributing multiple weak interactions over a large interface can lead to high apparent affinity and exquisite specificity, and appears to be a prevalent operational feature in biological systems (e.g., antigen-antibody interactions) (17). In recognition of this principle, we hypothesized that it is possible to design unique peptide-based reagents, which can then be regulated by the addition of a small molecule, to either block a macromolecular interface or strip an individual component from a complex.

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depletion of the individual components from the later elution volumes. SDS/PAGE of the fraction eluting at 10.0 mL indicated the presence of both components. We observed no shift in the absence of AP20187 (Fig. S1).

To generate a TcTex1 trap, residues 107–125 of rat dynein IC2C that specifically interact with TcTex1 were fused to the C terminus of FKBP (FKBP-IC; Fig. 2D). Native gel and SEC experiments of the admixture indicated FKBP-IC peptide bound to TcTex1 in the absence of AP20187, suggesting the interaction is of higher affinity than the IC-LC8 interaction (Fig. 2E, round 1). To reduce the affinity of the monovalent interaction, we and others have shown that the point mutation, L112 to alanine, significantly reduces the TcTex1 affinity (21). Admixtures of the mutated FKBP-IC (L112A) peptide and TcTex1 did not interact in the absence of AP20187, as judged by native PAGE and SEC (Fig. 2E, round 2). However, the addition of AP20187 to the admixture of FKBP-IC (L112A) peptide and TcTex1 produced a shift, but required a 16-fold higher concentration of AP20187 than the LC8/LC8 trap interaction (Fig. 2E, round 2). Based on this observation, we hypothesized that the sequence immediately preceding the TcTex1-binding region may interfere with the interaction, either through conformational restriction due to P109 or electrostatic repulsion due to K111. Therefore, we mutated the sequence PIK to GGS, to introduce flexible, hydrophilic residues (residues 109–111) (Fig. 2D). The modified FKBP-IC trap fully sequestered TcTex1 at ~8-fold lower concentrations of AP20187 than the FKBP-IC (L112A) peptide, or approximately twice that required for the LC8 trap (Fig. 2, round 3; Fig. S2).

Although our previous structural studies showed that LC8 and TcTex1 bind to their targets in a similar manner and to adjacent sites on the dynein intermediate chain (7), there are no known common binding partners that simultaneously bind LC8 and TcTex1 (other than the IC). In addition, there is no evidence that LC8 can bind proteins bearing a TcTex1 sequence or vice versa (e.g., LC8 binds to BimEL, Pak1, nNOS, etc.; TcTex1 binds to Doc2 α , PTHR receptor, etc.) (1). Nonetheless, based on their structural similarity, we were compelled to demonstrate that the traps were specific to their target. Using native PAGE, we showed that the LC8 trap does not bind to TcTex1, with or without AP20187 treatment, and that the TcTex1 trap does not bind to LC8 (Fig. S3). Therefore, taken together, these biochemical experiments represent a general strategy to generate an inducible, high-affinity trap to a specific target.

In Vivo Effects of Dynein LC Traps. As tests for LC function, we evaluated the effects of the traps on early endosome, lysosome, and Golgi distribution, as well as mitotic progression. First, we fused to GFP to the N terminus of both FKBP traps to identify transfected cells. Next, we transfected Cos1, Cos7, or HeLa cells with either trap or a GFP-FKBP control and labeled endosomes, lysosomes, and Golgi with EEA1, Lamp2, and GM130, respectively (Fig. 3). Expression of the traps alone resulted in a moderate increase in the fraction of cells that exhibited evidence of organelle dispersal as compared with nontransfected cells or the GFP-FKBP control. The percentage of cells expressing the GFP-LC8 or GFP-TcTex1 trap with dispersed endosomes was $14.7 \pm 1.5\%$ and $13.3 \pm 1.5\%$ compared with $11.7 \pm 3.5\%$ and $10.3 \pm 3.5\%$ for nontransfected cells and cells that expressed a GFP-FKBP control, respectively. This suggests that the LC8 and TcTex1 traps do not detectably affect the free pool of endogenous LCs. Treatment of nontransfected or GFP-FKBP expressing cells with AP20187 for 8 h did not affect the dispersion for each organelle. However, cells that expressed either trap and were treated with AP20187 exhibited dramatic reorganization of early endosomes, lysosomes, and the Golgi apparatus (Fig. 3). After 8 h of treatment with AP20187, we observed greater than 50% dispersion of the three organelle types for each trap.

To ensure the AP20187 concentration was not limiting, we followed the LC8 trap-induced dispersion of lysosomes as a function of AP20187 concentration after 8 h. The amount of lysosome dispersion increased with increasing AP20187 concentrations; how-

Fig. 3. Induction of LC traps leads to endosome, lysosome and Golgi apparatus dispersion. Cos 1 (A and C) and Cos7 (B) cells were transfected with a GFP-FKBP control, or the GFP-LC8 or GFP-TcTex1 trap (green). Cells were then treated with AP20187 for 1 h (A) or 8 h (B and C). All cells were stained with DAPI (blue). (A) Cells stained with the EEA1 marker (red) to visualize endosomes. (B) Cells stained with LAMP2 (red) to visualize lysosomes. (C) Cells stained with GM130 marker (red) to detect Golgi bodies. Cells with dispersed vesicles are marked with a *d*. Note that the endosomes and lysosomes in nontransfected cells remain compact (marked with a *c*).

ever, it reached a plateau at 100 nM, and no further increase in lysosome dispersion was seen at higher AP20187 concentrations (Fig. S4). For the TcTex1 trap, we observed efficient endosomes, lysosomes, and Golgi dispersion at AP20187 concentrations of 1 μ M or greater. Nontransfected cells did not show dispersal of these organelles at any concentration of AP20187 tested (e.g., ≤ 2 μ M).

Finally, to confirm that the traps are in fact sequestering the LCs and altering the dynein subunit composition, we performed several immunoprecipitation experiments. First, the immunoprecipitation of the LC8 trap using anti-GFP pulled down LC8 in the presence of AP20187, but not in its absence (Fig. S5). Next, anti-dynein IC (mAb 74.1; a generous gift from Kevin Pfister, University of Virginia, Charlottesville, VA) was used to immunoprecipitate the dynein motor complex from sorted cells transfected with the GFP-LC8 trap. We observe approximately a $55 \pm 10\%$ decrease of IC-precipitated LC8 in the presence of AP20187 ($n = 2$; Fig. S5). In addition, using anti-LC8 to immunoprecipitate the dynein complex from a mixture of rat brain lysate and recombinant LC8 trap we are able to pull down dynein IC in the absence, but not in the presence, of AP20187 (Fig. S5).

Because the major purpose for developing these traps was to provide temporal control, we monitored the fraction of cells with dispersed organelles as a function of time. After induction of dimerization by AP20187, effects on both early endosome and lysosome distribution were detectable at 10 min, and leveled off at ~2 h (Fig. 4 and Table S1). Intriguingly, these effects occurred at comparable rates with either the LC8 or TcTex1 trap. The most rapid effect was observed on lysosomes in cells expressing the LC8 trap. In striking contrast, Golgi dispersal using either trap occurred over a much longer time-course, requiring 8 h after induction of dimerization to reach similar levels of dispersion as for endosomes and lysosomes. Twenty-four hours after addition of AP20187, the percentage of cells with dispersed Golgi reached levels greater than 85% (Table S1). The comparable effects of LC8 and TcTex1 traps on organelle distribution support a common effect on cytoplasmic dynein.

As an alternative test to verify these observations, we monitored the distribution of Golgi in cells subjected to LC8 RNAi (Fig. S6). We observed clear Golgi dispersal in a comparable fraction of cells as determined for light-chain trapping. After 3 days of treatment, $54 \pm 2.5\%$ of transfected cells showed Golgi dispersion similar to the 8-h time point observed using either trap. The level of Golgi dispersion in cells treated with a scrambled RNAi control was $12 \pm 1.5\%$, which was similar to control experiments using the LC8 trap. We also observed that

Fig. 4. Time course of vesicle dispersion by LC sequestration. The number of cells with dispersed lysosomes (red symbols/dotted lines), endosomes (green symbols/dashed lines), or Golgi (blue symbols/dash-dot-dot lines) induced by dimerization of the LC8 trap (○) or TcTex1 trap (△) is plotted as a function of time after the induction of dimerization of AP20187. Shown as a control is the dispersion of Golgi bodies by GFP-FKBP. Each data point represents three independent measurements of 100 transfected cells for each trap or the control.

the expression level of the dynein intermediate chain remained unaffected by the RNAi treatment (Fig. S6). These observed levels of Golgi dispersion are in agreement with recently reported levels of organelle dispersion using RNAi for both light chains (22).

As a test for effects on cytoplasmic dynein functions that are distinct from vesicular transport, we monitored mitotic behavior in cells expressing either the LC8 or TcTex1 traps. We observed little effect either on mitotic index or on the fraction of mitotic cells at discrete mitotic stages with either trap (Fig. 5). We did note a small increase in defective mitotic spindle morphology in cells transfected with the LC8 trap, and cells transfected with RNAi targeting LC8 recapitulated this result, suggesting this aspect of mitotic behavior was partially under LC8 control (Fig. 5). In addition, we analyzed the time from nuclear envelope breakdown to anaphase onset in cells expressing the TcTex1 trap as one of the best current quantitative measure of mitotic dynein function. Specifically, the average times from nuclear envelope breakdown to anaphase onset for untransfected cells with or without AP20187 are 26.4 ± 4.9 min and 29.7 ± 5.8 min, respectively. The average times for cells transfected with the TcTex1 trap with or without AP20187 are 26.4 ± 6.2 min and 33.4 ± 7.1 min, respectively. A one-way ANOVA test was performed to confirm that there is no statistically significant difference between these averages ($P = 0.0085$; Fig. S7).

Finally, known methods of inhibiting cytoplasmic dynein, such as expression of dynactin polypeptides or RNAi against the dynein regulatory factors Nudel and ZW10, cause disorganization of the radial microtubule network found in cultured mammalian cells (6, 23, 24). This network did not appear disorganized in cells subjected to light-chain trapping (Fig. S8). Taken together, these findings suggest that the observed phenotypic changes in organelle distribution were a direct consequence of altered cytoplasmic dynein-mediated transport.

Fig. 5. LCs and mitotic function of cytoplasmic dynein. HeLa cells were treated with LC8 or control siRNA for 3 days or transfected with the LC8, TcTex1, or control FKBP trap for 24 h and treated with AP20187 (100 nM for LC8 trap and $1 \mu\text{M}$ for TcTex1 trap) or control solution for 8 h. Mitotic indices for all transfected cells (A), the percentage of cells in various mitotic stages (B), and the percentage of cells with various spindle defects (C) were quantified for all mitotic cells. Overexpression of dynein heavy chain, residues 1–1140, represents a control to demonstrate a dynein-specific defect during mitosis. Standard deviations for each are shown.

Discussion

We have shown that the chemically induced dimerization of low-affinity peptide ligands leads to high-affinity, bivalent traps specific for two distinct dimeric LCs. Using these reagents, we found that the LCs play a role in some, but not all, dynein-mediated processes. Because the LCs also have dynein-independent functions, we cannot rule out that defects in other pathways contribute to the phenotypes we observed. In other words, sequestering the LCs will also result in sequestering the LC from additional LC-binding proteins present in vivo. However, the binding sites for LC8 and TcTex1 on the dynein IC are adjacent, and we propose that sequestering one would affect the other and should equally affect the dynein complex (7). In addition, LC8 and TcTex1 bind to different peptide sequences and are not interchangeable, as evidenced by our biochemical data. Each trap independently produced a similar time course for the dispersion of lysosomes, endosomes, and Golgi complex, and neither showed a significant perturbation of mitotic processes. Thus, our findings support a common role for the LCs on dynein function.

The LC8 and TcTex1 traps offer a means for fine temporal control of cytoplasmic dynein in large cell populations without recourse to microinjection and in contrast to methods such as RNAi or overexpression of dominant negative polypeptides. This temporal control allowed us to observe the dramatic difference in the time course of perturbation of lysosome and endosome

localization compared with Golgi dispersal. The relatively rapid effect of the LC8 and TcTex1 traps on lysosomes and endosomes after dimerization by AP20187 suggests that the LCs are in fast equilibrium with their binding sites within cytoplasmic dynein and, therefore, are subject to sequestration. These observations also indicate that LC8 and TcTex1 are essential for minus end-directed lysosomal and endosomal transport, and that forces responsible for dispersal of lysosomes and endosomes (e.g., plus end-directed kinesins) must act within a relatively short period.

The slower dispersal of the Golgi apparatus, however, has a number of possible explanations. For example, LC8 and TcTex1 may not be associated with dynein at the Golgi. Alternatively, the LCs may be more tightly associated with the IC due to interactions unique to the Golgi, and therefore are not immediately affected by depletion of free LC8 and TcTex1 in the cytosol. It is also possible that Golgi dispersal may be rate limited by events other than plus-end transport. For example, the appearance of Golgi elements at the cell periphery in nocodazole-treated cells appears to reflect de novo formation of Golgi stacklets at ER exit sites (25). Nonetheless, the latter process occurs more quickly than the reorganization of the Golgi apparatus we report here.

In contrast to the effects on dispersal of organelles, we observed little to no effect of LC8 and TcTex1 trapping on mitosis. This observation is surprising compared with results obtained with other methods of cytoplasmic dynein inhibition (6, 26–30). This is despite the clear presence of cytoplasmic dynein at mitotic kinetochores and spindle poles, and its multiple established roles in chromosome capture (27) and attachment (30). Therefore, our findings suggest the LCs may not be functionally important in mitotic progression, or that they are not in rapid exchange with the free cytosolic pool, as was observed for endosomes and lysosomes.

Taken together, these findings raise questions concerning the role of the LCs in dynein-specific processes. As noted, the N terminus of the IC is intrinsically disordered, and such regions are frequently involved in regulating protein function. In this context, it is possible that the LCs affect the dynein–dynactin interaction through an allosteric mechanism. Dynactin, a large multisubunit complex, is an essential partner of dynein, and links vesicles to dynein and increases dynein's processivity (6, 31). The dynein–dynactin interaction is also critical in mitotic spindle checkpoint signaling. The principle dynactin binding site on the dynein IC (residues 1–106) (32) directly precedes the LC binding sites (residues 112–138). Therefore, we propose that the LC occupancy restricts and/or orients the N terminus of the IC such that the dynein–dynactin interaction is enhanced. Whether this mechanism affects other IC binding partners remains to be tested. Finally, it is possible that the LC occupancy might regulate the phosphorylation of the IC, which has been implicated in blocking the dynein–dynactin interaction (33).

In addition to the described findings pertaining to dynein function, the LC8 and TcTex1 trap design principles can be used to create inducible antagonists directed at other cellular targets. Though there are advantages in targeting homodimeric proteins, monomeric species can be targeted as well using the FKBP and FRAP peptides (Ariad Pharmaceuticals Inc.) to generate an inducible heterodimeric complex. For instance, proteins expressing multiple domains, such as an SH3 domain and a kinase, could be targeted by fusing a peptide that recognize the SH3 domain to FKBP and a kinase substrate mimic to FRAP. Individually, each fusion should have a low affinity to their respective binding partners; however, dimerization through a nontoxic rapamycin analog would create a high-affinity ligand that would interfere with its target. Likewise, peptides that bind over an extended protein surface could be broken into two halves. For instance, p27 that regulates Cdk2 and CyclinA (34) could be broken into two low-affinity peptides and fused to FKBP and FRAP (Fig. S9). In this sense, the method outlined here is similar to fragment-based drug design where the

linker between the fragments proceeds through the FKBP-AP20187 or FKBP-AP21967-FRAP complex (AP21967 is a modified rapamycin analog that does not bind endogenous targets).

Finally, it is important to note that this method differs significantly to previous applications of FKBP and FRAP to regulate protein function—for example, dimerization to initiate signaling pathways (35) or transcription (36), destabilized FKBP (37) or split ubiquitin (38) to affect protein degradation, or regulation of an inhibitory peptide (39). Specifically, the applications invariably tether FKBP and/or FRAP to the protein of interest and must express this fusion in a background containing the protein of interest or in cells where the protein of interest has been suppressed or deleted. The major advantage of the method described here is that the traps act as a competitive antagonist of the endogenous target, and thus afford the possibility of rapid inhibition of function.

Methods

Reagents. Polymerase, DNA restriction enzymes, and ligase were purchased from New England Biolabs. PC4FV1E mammalian expression vector and AP20187 were a generous gift from Ariad Pharmaceuticals, Inc. Rabbit polyclonal anti-LC8 antibody and monoclonal anti-LAMP2 were obtained from Santa Cruz Biotechnology Inc. Anti-EEA1 and anti-GM130 antibodies were from BD Biosciences. Monoclonal anti-dynein intermediate chain antibody (IC 74.1) was from Chemicon.

Molecular Biology. The LC8 and TcTex1 traps were generated by standard subcloning methods into bacterial and mammalian expression vectors (*SI Methods*). Point mutants were generated using QuikChange site-directed mutagenesis (Stratagene). Automated DNA sequencing was used to confirm each construct.

Protein Expression and Purification. The LC8 trap and TcTex1 traps (wild-type and mutants) were overexpressed at 22 °C overnight to increase solubility. Each was purified to homogeneity by either ammonium sulfate fractionation or Ni affinity chromatography followed by size exclusion chromatography (HiLoad 26/60 Superdex 75; GE Healthcare Life Sciences). The purity was monitored by SDS/PAGE. LC8 and TcTex1 were expressed independently in bacteria and purified using standard techniques as described previously (7).

Binding Assays. Analytical size-exclusion chromatography experiments were performed to determine the hydrodynamic properties of the individual proteins and their complexes with and without AP20187. Samples were run on a Superdex 75-10/300 GL column (Amersham Biosciences). The column was pre-equilibrated with 50 mM Tris (pH 8.0), 1 mM EDTA, 100 mM NaCl, and 1 mM DTT.

Immunofluorescence Microscopy. COS-7, COS-1, and HeLa cells were grown at 37 °C in DMEM with 10% FCS supplemented with penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were plated on coverslips at 70–90% confluency, transfected using Effectene (Qiagen), or LipofectAMINE²⁰⁰⁰ (Invitrogen) and OptiMEM media (Invitrogen), and examined after 24–36 h. Cells were transfected with the indicated plasmids (0.25–0.5 µg/well), and only cells expressing low to moderate levels of the traps were studied.

In the trapping experiments, various concentrations (10 nM, 100 nM, and 1–2 µM) of AP20187 were added to experimental wells 24 h post-transfection, and cells were incubated for varying periods (10 min to 24 h) of time before analysis. Cells were fixed and stained using standard protocols (*SI Methods*). Images were obtained using a Leica DMIRBE microscope equipped with a Hamamatsu ORCA 100 camera and Metamorph software (Universal Imaging Corp.). All images were visualized using either a PLAN APO 63× 1.23 NA or a C PLAN 100× 1.25 NA oil-immersion objective lens (Leica). Images were cropped and processed using Adobe Photoshop 7.0 (Adobe Systems). Unless otherwise noted, the level of dispersion was quantified by counting 100 cells per coverslip. Each experiment was performed in triplicate.

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