CK1 activates minus-end–directed transport of membrane organelles along microtubules

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ABSTRACT **Microtubule (MT)-based organelle transport is driven by MT motor proteins that move cargoes toward MT minus-ends clustered in the cell center (dyneins) or plus-ends extended to the periphery (kinesins). Cells are able to rapidly switch the direction of transport in response to external cues, but the signaling events that control switching remain poorly understood. Here, we examined the signaling mechanism responsible for the rapid activation of dynein-dependent MT minus-end–directed pigment granule movement in *Xenopus* melanophores (pigment aggregation). We found that, along with the previously identified protein phosphatase 2A (PP2A), pigment aggregation signaling also involved casein kinase 1ε (CK1ε), that both enzymes were bound to pigment granules, and that their activities were increased during pigment aggregation. Furthermore we found that CK1ε functioned downstream of PP2A in the pigment aggregation signaling pathway. Finally, we discovered that stimulation of pigment aggregation increased phosphorylation of dynein intermediate chain (DIC) and that this increase was partially suppressed by CK1ε inhibition. We propose that signal transduction during pigment aggregation involves successive activation of PP2A and CK1ε and CK1ε-dependent phosphorylation of DIC, which stimulates dynein motor activity and increases minus-end–directed runs of pigment granules.**

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INTRODUCTION

Intracellular transport is a key mechanism responsible for spatial organization of the cytoplasm and the delivery of organelles and particles to their destinations inside the cell (Lane and Allan, 1998; Allan and Schroer, 1999; Guzik and Goldstein, 2004). The driving force for intracellular transport is provided by molecular motors bound to the surface of cargo organelles (Allan and Schroer, 1999; Vale, 2003). Molecular motors recognize the polarity of cytoskeletal transport tracks, microtubules (MTs), or actin filaments, and move specifically to their plus- or minus-ends (Allan and Schroer, 1999; Vale, 2003). MT-dependent motors include kinesins, which generally support transport to the MT plus-ends (Goldstein, 2001; Hirokawa *et al.*, 2009), and dyneins, which are exclusively minus-end–directed (King, 2000; Vallee *et al.*, 2004). Kinesins and dyneins are usually bound to the surface of the same cargo organelles and therefore MT-based movement is bidirectional and involves stochastic transitions between three states, uninterrupted displacements to the minus- or plus-ends, respectively, and pauses (Gross, 2004; Welte, 2004). The direction of the net movement is controlled by intracellular signals, which change the lengths of MT runs in one or both directions (Gross, 2004; Welte, 2004). Although the changes of run length reflect regulation of motor proteins, the signaling mechanisms responsible for modulation of dynein and kinesin activities remain largely unknown.

An excellent model system for studies of regulation of MT motors by signaling events is *Xenopus* melanophores (Nascimento *et al.*, 2003; Aspengren *et al.*, 2009). The major function of these cells is fast and synchronous redistribution of thousands of

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Abbreviations used: CK1, casein kinase 1; DIC, dynein intermediate chain; GFP, green fluorescent protein; MSH, melanocyte-stimulating hormone; MT, microtubule; PKA, protein kinase A; PP2A, protein phosphatase 2A; SEM, standard error of the mean.

membrane-bounded pigment granules, which accumulate in the cell center (pigment aggregation) or uniformly distribute throughout the cytoplasm (pigment dispersion) (Nascimento *et al.*, 2003; Aspengren *et al.*, 2009). During aggregation and dispersion, pigment granules are moved by MT motors dynein and kinesin-2 along cytoplasmic MTs organized into a polarized radial array (minus-ends at the cell center, plus-ends at the periphery) (Tuma *et al.*, 1998). Pigment aggregation involves a sudden dramatic increase in the length of dynein-dependent MT minus-end runs and a simultaneous decrease in the length of plus-end runs generated by kinesin-2 (Gross *et al.*, 2002; Zaliapin *et al.*, 2005). These changes cause rapid movement of pigment granules to the cell center. Pigment dispersion involves opposite changes in the lengths of MT plus- and minus-end MT runs, which result in the redistribution of pigment granules to the cell periphery (Gross *et al.*, 2002; Zaliapin *et al.*, 2005).

Pigment aggregation and dispersion are regulated by a signaling pathway that involves changes of the cytoplasmic levels of the second messenger cAMP and the activity of protein kinase A (PKA) (Nascimento *et al.*, 2003; Kashina and Rodionov, 2005; Aspengren *et al.*, 2009). An increase in cAMP levels stimulates PKA activity and causes pigment dispersion, whereas a decrease in cAMP levels inhibits PKA activity and induces aggregation (Nascimento *et al.*, 2003; Kashina and Rodionov, 2005; Aspengren *et al.*, 2009). Pigment aggregation also requires the activity of protein phosphatase 2A (PP2A) (Reilein *et al.*, 1998; Kashina and Rodionov, 2005). PKA and PP2A control pigment aggregation and dispersion by modifying the length of MT plus- and minus-end runs of individual pigment granules (Rodionov *et al.*, 2003; Kashina and Rodionov, 2005). Therefore PKA and PP2A directly or indirectly regulate kinesin-2 and dynein activities that generate pigment granule movement along MTs. The signaling mechanisms that link PKA and PP2A activities to the regulation of MT motors, however, remain unknown.

In this study, we examined the signaling pathway responsible for the activation of minus-end–directed MT movement of pigment granules during pigment aggregation. We hypothesized that, along with the activity of PP2A, this pathway involved casein kinase 1 (CK1), which has been shown to work together with PP2A in several signaling cascades (Porter and Sale, 2000; Vielhaber and Virshup, 2001; Virshup *et al.*, 2007; MacDonald *et al.*, 2009). We found that melanophores expressed several CK1 isoforms, but only CK1ε was bound to pigment granules. In full agreement with our hypothesis, we found that CK1 inhibitors prevented pigment aggregation in cells with dispersed pigment granules and induced pigment dispersion in melanophores with aggregated pigment granules. The activity of pigment granule-associated CK1ε increased upon stimulation of pigment aggregation, and this increase was partially blocked by inhibition of PP2A activity. Stimulation of pigment granule aggregation caused an increase in the phosphorylation of dynein intermediate chain (DIC), and inhibition of CK1ε activity partially prevented this increase. On the basis of these results, we propose a model for a signal transduction pathway for pigment granule aggregation in which a drop in cAMP levels and down-regulation of PKA activity increases the activities of PP2A and CK1ε, followed by CK1ε-dependent phosphorylation of DIC that stimulates dynein motor activity and results in minus-end–directed MT movement of pigment granules.

RESULTS

Several CK1 isoforms are expressed in melanophores, but only CK1ε is associated with pigment granules

To examine the possibility that CK1 is involved in pigment granule transport in melanophores, we asked whether melanophores expressed CK1. We used immunoblotting to probe whole-cell extracts and preparations of purified pigment granules with antibodies raised against major CK1 isoforms. We found that antibodies specific for CK1α, CK1δ, and CK1ε isoforms recognized relevant protein bands in whole-cell extracts (Figure 1A). Remarkably, only the CK1ε-specific antibody, but not antibodies to the other two isoforms, reacted with preparations of purified pigment granules (Figure 1A). This result indicated that several CK1 isoforms were expressed in melanophores but only the CK1ε was bound to purified pigment granules. This localization is expected for a signaling enzyme that regulates activities of granule-bound MT motors (Kashina *et al.*, 2004; Kashina and Rodionov, 2005).

To confirm the binding of CK1ε to pigment granules, we tested whether CK1ε was associated with pigment granules in cells using a

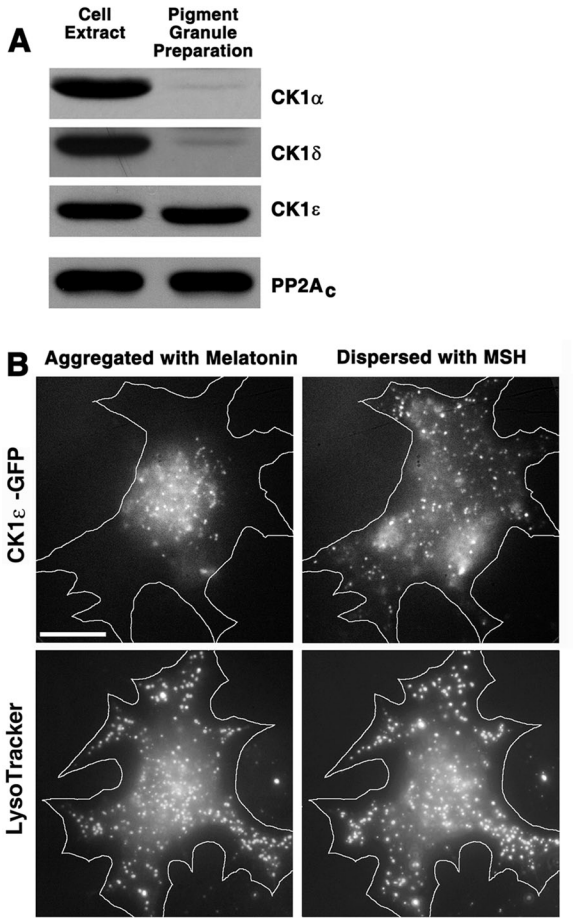

FIGURE 1: CK1ε and PP2A are bound to pigment granules. (A) Immunoblots of cell extracts (left lanes) or preparations of pigment granules (right lanes) probed for CK1 isoforms (top) or the catalytic subunit of PP2A (bottom). PP2A and CK1ε, but not other CK1 isoforms, are enriched in preparations of purified pigment granules. (B) Fluorescence images of melanophores expressing GFP-CK1ε (top row) or stained with LysoTracker (bottom row) sequentially treated with melatonin (left column) and MSH (right column) to induce aggregation and dispersion of pigment granules. GFP-CK1ε is localized to fluorescent dots that accumulate in the cell center or redisperse throughout the cytoplasm after treatment with melatonin and MSH, respectively, as would be expected from pigment granules, whereas the distribution of lysosomes does not change significantly in response to hormones. Scale bar, 20 μm.

CK1ε–GFP fusion. We found that CK1ε–GFP was localized in fluorescent dots that accumulated in the cell center or distributed homogeneously throughout the cytoplasm in response to treatment with the hormones that induced pigment aggregation (melatonin) or dispersion (melanocyte-stimulating hormone [MSH]), and therefore behaved similarly to pigment granules (Figure 1B). Control experiments showed that melatonin and MSH did not induce significant changes in the distribution of other membrane organelles, such as lysosomes (Figure 1B). We conclude that the CK1 isoform CK1ε is bound to pigment granules in melanophores and therefore is a good candidate for regulating MT-based pigment granule transport.

CK1 activity is required for the induction and maintenance of pigment granule aggregation

To directly test whether CK1 activity is involved in the regulation of pigment granule transport, we examined melatonin-induced aggregation and MSH-induced dispersion of pigment granules in melanophores treated with two chemically distinct CK1 inhibitors. D4476 is a broad-specificity CK1 inhibitor (Rena *et al.*, 2004), and IC261 is a selective inhibitor of CK1ε and CK1δ (Mashhoon *et al.*, 2000). Because we found that IC261 partially depolymerized cytoplasmic MTs in melanophores, which could interfere with MT-based transport, we pretreated cells with the MT-stabilizing drug paclitaxel (Taxol) at 0.1 μM. Control experiments showed that 0.1 μM Taxol prevented MT depolymerization induced by IC261, but did not significantly affect the density or distribution of cytoplasmic MTs (unpublished data).

We found that D4476 and IC261 both markedly inhibited melatonin-induced pigment aggregation as evidenced from the increase in the number of cells that completely or partially failed to aggregate pigment granules (Figure 2, A and B). In agreement with our previously published data (Lomakin *et al.*, 2009), treatment of cells with Taxol alone partially inhibited pigment granule aggregation (Figure 2B). IC261 further enhanced this inhibition in a dose-dependent manner (Figure 2B). At the highest tested concentration of IC261 (100 μM), more than 80% of melanophores completely failed to aggregate pigment granules in response to melatonin treatment. In contrast to aggregation, neither D4476 nor IC261 significantly affected MSH-induced dispersion of pigment granules (Figure 2C, and unpublished data). Remarkably, IC261 treatment of the melatonin-stimulated melanophores with aggregated pigment granules induced rapid granule dispersion (Figure 2D). We conclude that CK1 activity is essential for both the induction and maintenance of pigment granule aggregation.

Pigment aggregation stimuli increase the activity of pigment granule-bound CK1ε

The results of our experiments showed that CK1ε was bound to pigment granules and that CK1 activity was required for granule aggregation. To determine whether the pigment granule-bound CK1ε was constitutively active or was stimulated by granule aggregation signals, we measured the enzymatic activity of CK1 in the preparations of pigment granules isolated from melanophores treated with melatonin or MSH, to aggregate or disperse pigment granules, respectively. We found that CK1 activity was approximately twofold higher in preparations of pigment granules isolated from melatonin-treated cells, as compared with MSH-treated cells (Figure 3A). We conclude that CK1ε bound to pigment granules is activated during pigment aggregation.

Activation of granule-bound CK1ε requires PP2A

Our data identified CK1ε as the pigment granule-bound CK1 isoform and showed that its activity increased during granule aggregation. Therefore our results strongly suggested that pigment granule aggregation signals stimulated the activity of CK1ε. CK1ε activation is known to involve the dephosphorylation of amino acid residues in the C-terminal autophosphorylation inhibitory domain of the CK1ε molecule (Gietzen and Virshup, 1999; Knippschild *et al.*, 2005; Virshup *et al.*, 2007). CK1ε dephosphorylation can be catalyzed by PP2A, which has been shown to activate CK1ε in vitro (Gietzen and Virshup, 1999). Furthermore it has been demonstrated that PP2A activity is essential for aggregation of pigment granules in melanophores (Reilein *et al.*, 1998). We therefore hypothesized that activation of pigment granule-associated CK1ε required PP2A activity.

To test this hypothesis, we first examined whether PP2A colocalized with CK1ε on pigment granules, as would be expected for signaling enzymes involved in the same signal transduction pathway. We also investigated whether activity of granule-bound PP2A was elevated in cells stimulated to aggregate pigment granules. We performed Western blotting of granule preparations using an antibody against the catalytic subunit of PP2A, and measured PP2A activity in preparations of purified pigment granules isolated from cells

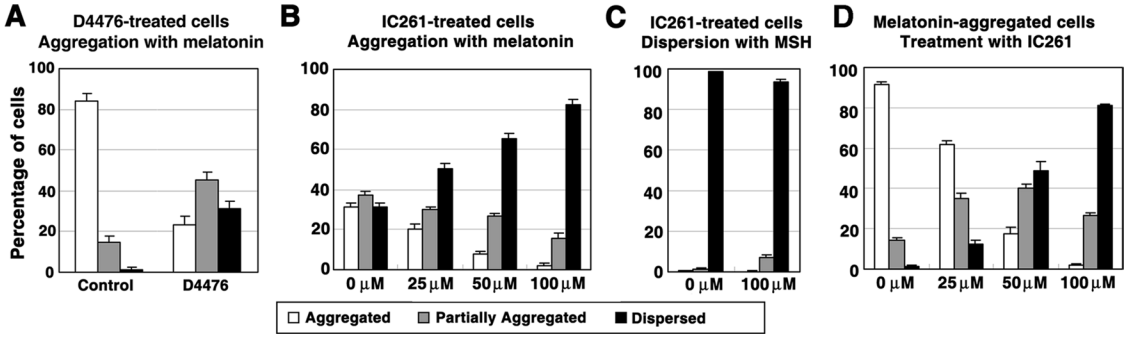

FIGURE 2: CK1 activity is required for pigment granule aggregation and maintenance of the aggregated state. Quantification of responses of pigment granules to pigment aggregating or dispersing hormones melatonin (A and B) or MSH (C) in cells pretreated with various concentrations of a broad-specificity CK1 inhibitor D4476, or the CK1δ and CK1ε specific inhibitor IC261. (D) Responses of pigment granules to IC261 in cells with pigment aggregated with melatonin. Data are expressed as the percentage of cells with aggregated (white bars), partially aggregated (gray bars), or dispersed (black bars) granules. In panel B, the large fraction of cells with dispersed pigment granules among IC261-untreated melanophores (0 μM) is explained by reduced rate of pigment aggregation in the presence of Taxol, which was used in this experiment to prevent MT depolymerization induced by IC261. D4476 and IC261 treatments inhibit melatonin-induced aggregation, but not MSH-induced dispersion of pigment granules; IC261 causes pigment dispersion in melatonin-treated cells with aggregated granules.

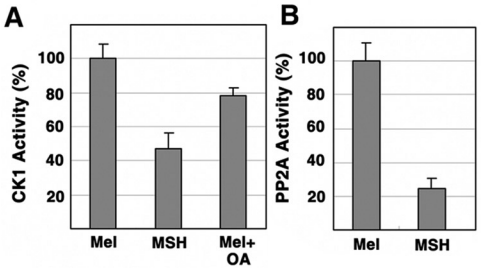

FIGURE 3: Enzymatic activities of the pigment granule-bound CK1 and PP2A are increased upon pigment aggregation. Activities of CK1 (A) or PP2A (B) measured in preparations of pigment granules isolated from melanophores stimulated with melatonin (Mel) or MSH, to aggregate or disperse pigment granules, respectively. The bar labeled Mel+OA (panel A) shows the data for melatonin-stimulated cells pretreated with the PP2A inhibitor okadaic acid. Average values determined for melatonin-treated cells are set at 100%. PP2A and CK1 activities are significantly higher in preparations of pigment granules obtained from melatonin- than MSH-stimulated cells, which shows that the activities of both enzymes increase during granule aggregation; okadaic acid treatment reduces the activity of CK1, indicating that CK1 activity is stimulated by PP2A.

stimulated to aggregate or disperse pigment. We found PP2A present in the pigment granule preparations (Figure 1A, bottom). We also found that PP2A activity was approximately four times higher in pigment granule preparations from cells with aggregated versus dispersed granules (Figure 3B). Therefore highly active PP2A copurified with pigment granules.

We next tested whether CK1ε functions downstream of PP2A in pigment granule aggregation. We examined whether suppression of granule aggregation in cells with inhibited PP2A activity could be rescued by overexpression of a constitutively active CK1ε mutant (Swiatek *et al.*, 2004). Such a CK1ε mutant was generated by replacing Ser and Thr amino acid residues with Ala in the inhibitory autophosphorylation sites (Swiatek *et al.*, 2004). We compared the inhibitory effect of the highly specific PP2A inhibitor okadaic acid on pigment granule aggregation in cells expressing this mutant versus cells expressing wild-type CK1ε at similar levels (Figure 4B), which were significantly lower than the level of expression of endogenous CK1ε (the ratio of expression levels of wild-type to endogenous CK1ε was 0.14 ± 0.03; mean ± standard error of the mean [SEM]). To identify transfected cells, wild-type and mutant CK1ε were expressed as green fluorescent protein (GFP) fusions. We found that okadaic acid dramatically inhibited pigment granule aggregation in control nontransfected cells or cells expressing GFP, as well as in wild-type CK1ε–expressing cells (Figure 4A). This result was consistent with published data that showed that PP2A activity was required for pigment granule aggregation (Reilein *et al.*, 1998). Remarkably, in cells expressing the constitutively active CK1ε mutant the fraction of melanophores with aggregated pigment granules was markedly increased. This effect was statistically significant (the p value for nontransfected and the constitutively active CK1ε-expressing cells was 0.00061 compared with 0.503 and 0.497 for cells expressing GFP or wild-type CK1ε). Thus, expression of the constitutively active CK1ε mutant partially rescued the inhibition of pigment aggregation induced by okadaic acid. This finding indicates that CK1ε acts downstream of PP2A in the granule aggregation signaling pathway.

Finally, we directly examined whether pigment granule-bound CK1ε was regulated by PP2A, by comparing CK1 activities in preparations of pigment granules isolated from control and okadaic acid–treated cells. We found that the activity of CK1 was significantly reduced in the granule preparations isolated from cells treated with okadaic acid (Figure 3B, Mel+OA and Mel). Therefore suppression of PP2A activity markedly inhibited the activity of CK1ε bound to pigment granules. Taken together, our data indicate that pigment granule aggregation signals increase PP2A activity and that PP2A stimulates the activity of CK1ε during granule aggregation.

CK1 regulates the length of MT minus-end runs of pigment granules

Our results indicated that CK1 inhibitors dramatically suppressed pigment granule aggregation. This aggregation involves granule runs toward the MT minus-ends focused at the cell center (Rodionov *et al.*, 2003; Kashina and Rodionov, 2005). Thus CK1 inhibitors likely suppressed pigment aggregation by reducing either the length or the velocity of minus-end runs of pigment granules. To test how the inhibition of CK1 activity affected MT minus-end runs, we tracked individual granules in cells treated with the CK1ε inhibitor IC261 at 25 μM. At this concentration, IC261 reduced the rate of pigment granule aggregation but did not block it completely (Figure 2B). This partial inhibition allowed us to measure the velocity and length of bidirectional granule movements. Comparison of granule movement parameters between the IC261-treated and control untreated cells showed that partial inhibition of CK1 activity reduced the average length of minus-end runs of pigment granules to 69% of the control length, without changing the average velocity of movement

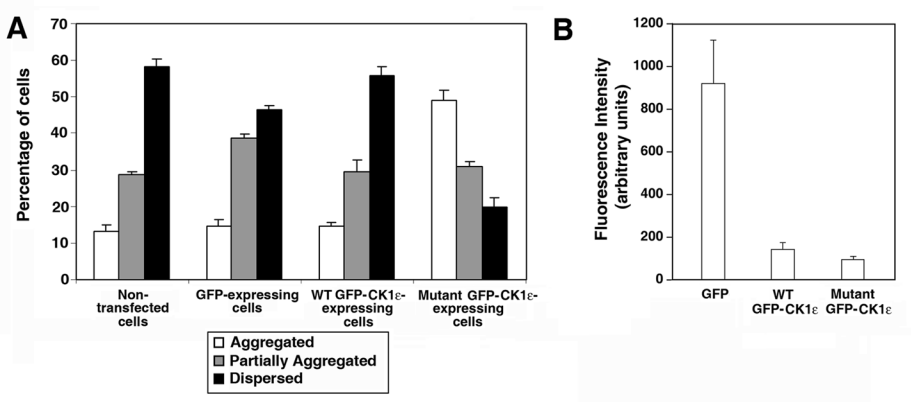

FIGURE 4: Overexpression of a constitutively active CK1ε mutant partially rescues the inhibition of pigment granule aggregation by the PP2A inhibitor okadaic acid. (A) Quantification of response to melatonin of nontransfected melanophores, or melanophores expressing GFP, wild-type GFP-CK1ε, or the mutant GFP-CK1ε with constitutively high enzymatic activity; prior to stimulation with melatonin cells were treated with PP2A inhibitor okadaic acid (OA). Data are expressed as the percentage of cells with aggregated (white bars), partially aggregated (gray bars), or dispersed (black bars) pigment. OA treatment significantly inhibits aggregation of pigment granules in nontransfected melanophores and in cells expressing GFP or wild-type GFP-CK1ε. Expression of the constitutively active CK1ε mutant partially rescues OA-induced inhibition of pigment granule aggregation as evidenced from the increased fraction of cells with aggregated granules. (B) Comparison of the levels of expression of GFP and CK1ε constructs; wild-type and the constitutively active mutant CK1ε are expressed at approximately the same levels, whereas GFP is expressed at significantly higher levels compared with CK1ε constructs.

Parameter	Control	IC261 (25 μM)
Velocity of minus-end runs (nm/s)	348.8 ± 7.4	327.7 ± 6.8
Length of minus-end runs (nm)	134.4 ± 9.1	92.5 ± 5.4
Number of examined minus-end runs	1306	1606
Velocity of plus-end runs (nm/s)	296.1 ± 9.0	312.1 ± 8.4
Length of plus-end runs (nm)	42.4 ± 1.8	34.7±1.1
Number of examined plus-end runs	1037	1341
Duration of pauses (s)	0.64 ± 0.2	0.65 ± 0.2
Number of examined pauses	428	499
Number of examined trajectories	76	85

TABLE 1: Parameters of bidirectional movement of single melanosomes along MTs during pigment aggregation in melanophores treated with CK1 inhibitor IC261. Numbers are average ± SEM.

(Table 1). The length of plus-end runs was reduced to some extent as well (Table 1), which is consistent with the observation that transport in either direction requires the activity of opposite-polarity motor proteins (Ally *et al.*, 2009). Thus the inhibition of CK1 activity suppressed pigment aggregation by shortening the length of minus-end granule runs. We conclude that CK1 activity is required to increase the length of minus-end runs of pigment granules during their aggregation.

CK1 phosphorylates DIC in vivo and in vitro

It is known that MT minus-end runs of pigment granules are generated by cytoplasmic dynein and that dynein activity increases during granule aggregation (Nilsson and Wallin, 1997; Gross *et al.*, 2002; Rodionov *et al.*, 2003; Zaliapin *et al.*, 2005). Our data showed that the increase in the length of minus-end runs required the activity of CK1. Therefore we assumed that CK1 was involved in the stimulation of dynein activity. Although dynein regulation remains a mystery, the existing experimental data suggest that the regulation mechanism involves reversible phosphorylation of dynein subunits (Reilein *et al.*, 2001). We therefore performed experiments to determine whether the phosphorylation levels of dynein subunits changed after the stimulation of pigment aggregation and whether inhibition of CK1 activity prevented these changes.

To detect changes in the levels of phosphorylation of dynein subunits, we compared the patterns of protein phosphorylation in dynein immunoprecipitates prepared from cells stimulated to aggregate or disperse pigment granules. We incubated melanophores overexpressing GFP-DIC or control nontransfected melanophores with ^{32}Pi, stimulated cells with melatonin or MSH to induce granule aggregation or dispersion, and immunoprecipitated dynein from total extracts of stimulated cells using an anti-GFP antibody. Autoradiography indicated that ^{32}P incorporated predominantly into the GFP-DIC (Figure 5A, middle) identified on the basis of electrophoretic mobility and immunoblotting with the anti-DIC antibody 74.1 (unpublished data). This incorporation was specific because it was not observed in immunoprecipitates prepared from control nontransfected cells (Supplemental Figure 1). Incorporation of ^{32}P into the endogenous DIC could not be detected because the immunoblotting data indicated that its amounts in the GFP immunoprecipitates were small. Remarkably, ^{32}P incorporation into GFP-DIC was significantly higher in the melatonin- than MSH-stimulated cells (Figure 5A). Furthermore the levels of ^{32}P incorporation into GFP-DIC were also reduced in dynein immunoprecipitates obtained from melatonin-stimulated cells pretreated with the CK1ε inhibitor IC261 (Figure 5A). Quantification of the ^{32}P signal showed that the levels of ^{32}P incorporation into GFP-DIC in cells treated with MSH and IC261 were 61.0 ± 13.0% and 80.8 ± 8.5% (mean ± SEM; n = 3) of the levels in melatonin-treated cells. Pretreatment of cells with the broad-specificity CK1 inhibitor D4476 also reduced ^{32}P incorporation into GFP-DIC (Supplemental Figure 2). Thus our results indicate that granule aggregation signals increase the phosphorylation of DIC and that this increase is diminished upon

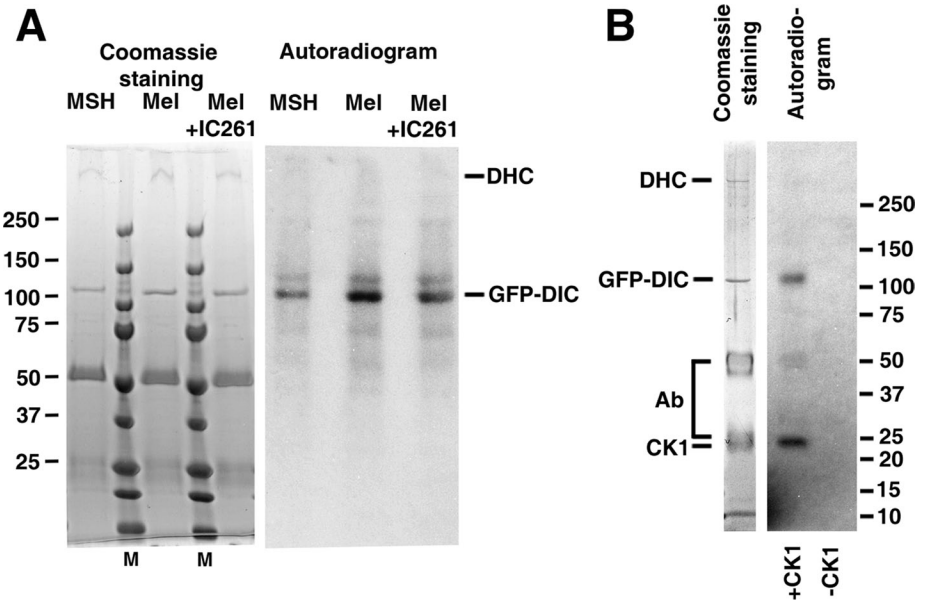

FIGURE 5: CK1 phosphorylates DIC in vivo and in vitro. (A) Immunoprecipitation of dynein with GFP antibody from extracts of GFP-DIC–expressing melanophores metabolically labeled with ^{32}P. Left panel, Coomassie-stained gel; right panel, autoradiograph; MSH, Mel, and Mel+IC261 on top of each panel indicate extracts of cells treated with MSH (to induce pigment dispersion), melatonin (to induce aggregation), or IC261 and melatonin (to inhibit CK1 activity prior to induction of aggregation), respectively; M, lanes with molecular weight markers. In immunoprecipitates, ^{32}P incorporates predominantly into GFP-DIC; this incorporation is increased in cells stimulated to aggregate pigment granules, and this increase is diminished by inhibition of CK1 activity. (B) In vitro phosphorylation by CK1 of dynein immunoprecipitated with an anti-GFP antibody from the extracts of GFP-DIC–expressing cells; left, Coomassie-stained gel of immunoprecipitate; right, autoradiograph of immunoprecipitate incubated with γ-^{32}P[ATP] in the presence (left) or absence (right) of recombinant CK1; DHC, GFP-DIC, antibody, and CK1 indicate positions of dynein heavy chain, GFP-DIC fusion protein, antibody heavy and light chains, and recombinant CK1, respectively. CK1 predominantly phosphorylates the protein with electrophoretic mobility similar to GFP-DIC.

inhibition of CK1ε activity, suggesting that CK1ε directly or indirectly regulates DIC phosphorylation.

To examine whether CK1 could directly phosphorylate DIC, we immunoprecipitated dynein from extracts of GFP-DIC–expressing melanophores stimulated to disperse pigment granules and incubated the immunoprecipitates with ^{32}P[ATP] in the presence or absence of recombinant CK1. We found that ^{32}P incorporated predominantly into the GFP-DIC and that this incorporation did not occur in the absence of CK1 (Figure 5B). Thus DIC was the major substrate of CK1 in dynein immunoprecipitates. We conclude that DIC might be directly phosphorylated by CK1 during pigment granule aggregation.

DISCUSSION

In this study, we identified CK1 as a key regulator of MT minus-end–directed MT pigment granule transport in *Xenopus* melanophores. This conclusion is based on several lines of experimental evidence. First, the CK1 isoform CK1ε was bound to pigment granules and therefore was properly localized to regulate dynein. Second, the activity of pigment granule-bound CK1 was significantly increased in cells stimulated to aggregate granules, as would be expected for a signaling enzyme regulating minus-end–directed granule transport. Finally, the aggregation of pigment granules was markedly suppressed by treatment of melanophores with CK inhibitors, which confirmed that CK1 activity was essential for minus-end–directed MT transport. Taken together, these results show that CK1 regulates minus-end–directed MT transport of pigment granules in melanophores.

Our data are consistent with the results of studies in yeast and mammalian cells, which link CK1 to transport of membrane organelles. In yeast cells, knockout of CK1 blocks the movement of membrane vesicles containing uracil permease from the plasma membrane to the vacuole (Marchal *et al.*, 2002). In mammalian cells, CK1 isoforms interact with endomembrane compartments such as the endoplasmic reticulum, Golgi apparatus, and transport vesicles (Gross and Anderson, 1998; Behrend *et al.*, 2000; Faundez and Kelly, 2000; Milne *et al.*, 2001; Yu and Roth, 2002), and are involved in synaptic vesicle exocytosis (Kreutz *et al.*, 1997; Dubois *et al.*, 2001). Therefore experimental evidence indicates that CK1 participates in the transport of membrane organelles. The mechanism by which CK1 participates in membrane organelle transport was not elucidated in these studies, however, to our knowledge. Our work for the first time reveals a specific function of CK1 in regulating dynein-based transport of membrane organelles.

Furthermore we found that PP2A was bound to pigment granules along with CK1ε, and that this phosphatase functioned upstream of CK1 in the pigment aggregation signaling pathway. We also found that the increase of CK1 activity observed during granule aggregation was suppressed by okadaic acid, a highly specific PP2A inhibitor. Our data are consistent with the results of in vivo studies that suggest that the PP2A-induced stimulation of CK1 activity is involved in other major signaling pathways, including canonical Wnt signaling (Swiatek *et al.*, 2004), chromosome cohesion (Ishiguro *et al.*, 2010), and flagellar motility (Wirschell *et al.*, 2007). Our data are also consistent with in vitro experiments that show that PP2A activates CK1 isoforms, such as the granule-bound CK1ε, by the direct dephosphorylation of inhibitory autophosphorylation sites (DeMaggio *et al.*, 1992; Fish *et al.*, 1995; Graves and Roach, 1995; Zhai *et al.*, 1995; Swiatek *et al.*, 2004). Therefore in melanophores activation of CK1 during aggregation of pigment granules likely involves a dephosphorylation step catalyzed by PP2A.

Signal transduction during granule aggregation that induces increase of CK1 activity implicates the activation PP2A. This conclusion is based on our data that show that signaling during granule aggregation enhances activity of PP2A bound to pigment granules. The major mechanism of PP2A regulation implicates phosphorylation of amino acid residues in the conserved C-terminal part of the PP2A catalytic subunit, which inhibits its enzymatic activity (Goldberg, 1999; Lechward *et al.*, 2001; Sontag, 2001). Reactivation occurs through the unique ability of PP2A to dephosphorylate itself. (Goldberg, 1999; Lechward *et al.*, 2001; Sontag, 2001). The autodephosphorylation reaction occurs at a steady rate, and therefore intracellular signals regulate PP2A activity indirectly through control over activities of protein kinases responsible for PP2A inactivation (Goldberg, 1999; Lechward *et al.*, 2001; Sontag, 2001). Thus, in melanophores aggregation signals likely activate PP2A by suppressing activities of inhibitory protein kinase(s). An attractive possibility is that PP2A is regulated by a direct phosphorylation by PKA and that PP2A activation results from the reduction of PKA activity caused by a drop in cAMP level during granule aggregation.

Our data show that the activation of minus-end movement of pigment granules driven by cytoplasmic dynein correlates with the increase in phosphorylation of the granule-bound DIC, and that CK1 inhibition prevents this phosphorylation. Our data also demonstrate that recombinant CK1 phosphorylates DIC in vitro. It is therefore possible that CK1 stimulates dynein motor activity via the direct phosphorylation of DIC. In support of this possibility, studies in *Chlamydomonas* indicate that CK1 regulates flagellar motility through the phosphorylation of a 138 kDa intermediate chain of axonemal dynein (Yang and Sale, 2000; Gokhale *et al.*, 2009). Partial suppression of DIC phosphorylation in the presence of high concentrations of a selective CK1 inhibitor observed in our experiments indicates, however, that dynein regulation is likely complex and might involve other protein kinases that cooperate with CK1 in phosphorylating DIC, leading to dynein activation. Nonetheless, our data show that CK1 is essential for dynein activation and suggest that the role of CK1 in this process is in the phosphorylation of DIC.

On the basis of our results, we propose a model for the activation of minus-end–directed transport of pigment granules along MTs in melanophores (Figure 6). We suggest that stimulation involves sequential changes in the activities of PKA, PP2A, and CK1ε bound to pigment granules. First, a drop in cytoplasmic cAMP levels caused by melatonin treatment reduces the activity of PKA,

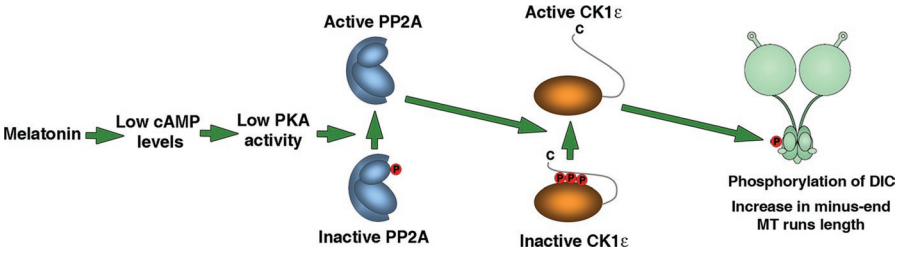

FIGURE 6: Model for the activation of MT minus-end–directed transport of pigment granules during pigment aggregation in melanophores. Melatonin decreases intracellular levels of cAMP, which in turn reduces the activity of PKA. PKA inactivation relieves inhibition of PP2A, which then dephosphorylates and activates CK1ε. CK1ε phosphorylates DIC, and this phosphorylation stimulates dynein activity and increases the length of MT minus-end–directed runs of pigment granules, leading to their aggregation in the cell center.

which allows PP2A to dephosphorylate and activate itself. Second, PP2A dephoshorylates amino acid residues in the inhibitory autophosphorylation sites of CK1ε, which increases CK1ε kinase activity. Finally, CK1ε induces phosphorylation of DIC, which is required to stimulate dynein activity. This dynein activation leads to an increase in the length of minus-end runs, which results in efficient pigment granule aggregation. Testing predictions of our model is an exciting new direction for future research.

MATERIALS AND METHODS

Cell culture

Xenopus melanophores (Kashina *et al.*, 2004) were cultured in *Xenopus* tissue culture medium (70% L15 medium supplemented with antibiotics, 20% fetal bovine serum, and insulin at 5 μg/ml). To induce pigment aggregation or dispersion, cells were placed in serum-free medium 1 h before hormone addition. Aggregation or dispersion was induced by treatment with 10^{-8} M melatonin or MSH, respectively. Cells with a reduced amount of melanin in their pigment granules were obtained as described (Rogers and Gelfand, 1998).

To quantify aggregation or dispersion responses, melanophores were treated with melatonin or MSH for 20 min and fixed with formaldehyde. The number of cells with aggregated, partially aggregated, or dispersed pigment granules was determined by counting cells in each category by phase-contrast microscopy, as described previously (Kashina *et al.*, 2004).

Treatment of melanophores with CK1 inhibitors

Cells were incubated with the CK1 inhibitor IC261 (EMD Chemicals, Gibbstown, NJ) at concentrations of 25–100 μM for 10 min. During the IC261 treatment, cytoplasmic MTs were stabilized with paclitaxel (Taxol; Sigma-Aldrich, St. Louis, MO) (0.1 μM) to prevent IC261-induced MT depolymerization. The CK1 inhibitor D4476 (150 μM) was applied to cells for 2 h in a mixture with Fugene 6 (Roche Applied Science, Indianapolis, IN) (Rena *et al.*, 2004) to promote solubility.

Immunoblotting

Immunoblotting was performed as described previously (Kashina *et al.*, 2004). Primary antibodies were mouse monoclonal antibodies specific for CK1ε (BD Biosciences, San Jose, CA), CK1α (Cell Signaling Technology, Danvers, MA), the PP2A catalytic subunit (Millipore, Billerica, MA), DIC (74.1; Covance, Princeton, NJ), or a goat polyclonal affinity-purified antibody raised against CK1δ (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were detected with SuperSignal West Femto maximum-sensitivity substrate (Thermo Scientific, Rockford, IL).

CK1ε DNA constructs and cell transfection

The constitutively active CK1ε mutant MM2 (Cegielska *et al.*, 1998) was a gift from David Virshup (Cancer and Stem Cell Biology Program, Duke–NUS Graduate Medical School, Singapore). To exclude the possibility of MM2 regulation by PKA, an additional mutation (S377A) was introduced into the potential inhibitory PKA phosphorylation site (Giamas *et al.*, 2007) using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Wild-type and constitutively active CK1ε were subcloned into the enhanced green fluorescent prottein pEGFP-C1 vector (Clontech). Transfection of melanophores with GFP-tagged wild-type or constitutively active CK1ε was performed using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The expression levels of the wild-type and mutant CK1ε and GFP were compared by measuring the GFP fluorescence within cell outlines. Expression levels of endogenous CK1ε and wild-type GFP-CK1ε were compared using quantitative immunoblotting. Protein bands corresponding to CK1ε and GFP-CK1ε were revealed by immunostaining with a mouse monoclonal CK1ε antibody (BD Biosciences, San Jose, CA) and IRDye800-conjugated affinity-purified anti-mouse immunoglobulin G (Rockland Immunochemicals, Gilbertsville, PA). The intensity of the infrared signal was quantified with the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). The results of quantification were normalized for the fraction of cells expressing GFP-CK1ε, which was determined by counting the number of GFP-CK1ε expressing cells using a fluorescence microscope.

Fluorescence staining of lysosomes

For fluorescence staining of lysosomes, melanophores were incubated with LysoTracker Yellow (0.5 μM; Invitrogen) for 30 min at room temperature. LysoTracker solution was replaced with fresh tissue culture medium, and cells were observed using a fluorescence microscope.

Image acquisition and analysis

Fluorescence images of cells were acquired using a Nikon Eclipse TE300 inverted microscope equipped with a Plan ×100 1.25 NA objective lens using an Andor iXon EM-CCD camera (Andor Technology, Windsor, CT) driven by Metamorph image acquisition and analysis software (Molecular Dynamics, Downington, PA).

The movement of individual pigment granules was recorded and analyzed as described previously (Zaliapin *et al.*, 2005). The data were collected 10 min after the stimulation of cells with melatonin or MSH.

Isolation of pigment granules and measurement of PP2A and CK1 activities in pigment granule preparations

Pigment granules were purified as described previously (Kashina *et al.*, 2004). Preparations of pigment granules isolated from cells with aggregated or dispersed granules were normalized by melanin content (Ozeki *et al.*, 1996). PP2A activity was measured by estimating the release of P_i from the PP2A substrate peptide using the Ser/Thr phosphatase assay system (Promega, Madison, WI). CK1 activity was determined using the Casein Kinase I Activity Assay Kit (Sigma, St. Louis, MO) by measuring the phosphorylation with γ-^{32}P[ATP] of the CK1 substrate peptide derived from the CK1 phosphorylation site at Ser10 of rabbit muscle glycogen synthase. The phosphorylated substrate was separated from the radioactive reagent by absorption on P81 cellulose phosphate paper squares. After extensive washing with 0.5% phosphoric acid, ethanol, and acetone, the radioactivity absorbed on the paper was counted using a scintillation counter.

Phosphorylation of dynein in vivo and in vitro

To determine the levels of phosphorylation of dynein subunits in cells stimulated to aggregate or disperse pigment granules or treated with a CK1 inhibitor, melanophores were transfected with mouse DIC2 DNA subcloned into the pEGFP-C1 vector. After overnight culturing in phosphate-free L15 medium (United States Biological, Swampscott, MA), cells were incubated for 1 h in the presence of ^{32}Pi (22 μCi/cm^2 of cell monolayer; PerkinElmer, Shelton, CT), stimulated with melatonin or MSH for aggregation or dispersion of pigment granules, or treated with IC261 (100 μM) before melatonin stimulation to inhibit CK1 activity, and lysed with a Triton X-100–containing buffer as described previously (Kashina *et al.*, 2004). Dynein was immunoprecipitated from cell lysates with an anti-GFP antibody (Abcam, Cambridge, MA) immobilized on Protein

A Agarose (Millipore, Billerica, MA). Dynein immunoprecipitates were examined using SDS–PAGE and autoradiography. To quantify the levels of phosphorylation of GFP-DIC, autoradiograms were scanned and integrated gray scale densities of GFP-DIC bands were measured using Metamorph software.

For the measurement of the phosphorylation of DIC by CK1 in vitro, dynein was immunoprecipitated with the anti-GFP antibody from extracts of melanophores expressing GFP-DIC treated with MSH to disperse pigment granules. Immunoprecipitates were incubated with recombinant CK1 (100 U/μl; Sigma) in the presence of γ-^{32}P[ATP] (PerkinElmer) and examined using SDS–PAGE and autoradiography.

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