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Protein Kinase A, Which Regulates Intracellular Transport, Forms Complexes with Molecular Motors on Organelles

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Summary

Major signaling cascades have been shown to play a role in the regulation of intracellular organelle transport [1, 2]. Aggregation and dispersion of pigment granules in melanophores are regulated by the second messenger cAMP through the protein kinase A (PKA) signaling pathway [3, 4]; however, the exact mechanisms of this regulation is poorly understood. To study the role of signaling molecules in the regulation of pigment transport in melanophores, we have asked the question whether the components of the cAMP-signaling pathway are bound to pigment granules and whether they interact with molecular motors to regulate the granule movement throughout the cytoplasm. We found that purified pigment granules contain PKA and scaffolding proteins and that PKA associates with pigment granules in cells. Furthermore, we found that the PKA regulatory subunit forms two separate complexes, one with cytoplasmic dynein ("aggregation complex") and one with kinesin II and myosin V ("dispersion complex"), and that the removal of PKA from granules causes dissociation of dynein and disruption of dynein-dependent pigment aggregation. We conclude that cytoplasmic organelles contain protein complexes that include motor proteins and signaling molecules involved in different components of intracellular transport. We propose to call such complexes 'regulated motor units' (RMU).

Results and Discussion

In melanophores, transport of pigment granules throughout the cytoplasm is mediated by molecular motors, cytoplasmic dynein (pigment aggregation) [5], and kinesin II [6] and myosin V (pigment dispersion) [7], and is regulated by PKA (see [4] for review). Regulation by PKA usually involves localization of the PKA regulatory complex in direct proximity to its substrates (see [8] for review). To test whether PKA is associated with pigment granules, we probed the preparation of granules and granule-free supernatants from aggregated or dispersed cells with antibodies to the PKA regulatory subunit RIIα and transfected *Xenopus* melanophores treated to reduce the amount of melanin in pigment granules (as described in [9]) with RIIα-GFP fusion protein. We found that a significant fraction of intracellular RIIα is associated with pigment granule preparations in immunoblots (Figure 1A). Furthermore, we found that in transfected cells GFP localized throughout the cytoplasm in fluorescent dots also containing pigment granule marker TRP1 (data not shown), whose distribution and behavior in response to aggregation and dispersion stimuli closely resembled that of pigment granules (Figure 1B). We therefore conclude that GFP-tagged RIIα associates with pigment granules in melanophores.

Because PKA is found on pigment granules and is directly involved in the regulation of pigment transport, it is highly possible that PKA on granules directly interacts with the molecular motors involved in the transport. To test the possibility of such interaction, we isolated pigment granules, solubilized the granule proteins with 1% Triton X100, and performed immunoprecipitation of granule proteins by using a monoclonal antibody against the 74 kDa dynein intermediate chain (DIC), a polyclonal antibody against the 95 kDa chain of *Xenopus* kinesin II (Kin II), or a polyclonal antibody against the myosin V heavy chain (Myo V) (Figure 2). Dynein antibody immunoprecipitates from granules in an aggregated (Ga) or dispersed (Gd) state showed no immunoreactivity with either kinesin or myosin but contained $p150^{Glued}$, a subunit of dynactin. Immunoprecipitates from both types of granules also contained RIIα, thus indicating that dynein on pigment granules most likely forms a complex with PKA (Figure 2A). Similarly, kinesin antibody immunoprecipitate from pigment granules contained myosin V, $p150^{Glued}$, and RIIα, but no dynein (Figure 2B). Both immunoprecipitates apparently maintained their integrity in the presence of 10 mM cAMP (data not shown). Thus, dynein and myosin V/kinesin II appear to form separate complexes with RIIα on pigment granules.

In agreement with these results, immunoprecipitate with myosin V antibody also contained both RIIα and $p150^{Glued}$ but no dynein (Figure 2C, left). However, contrary to expectations, no kinesin II was found in the myosin V coimmunoprecipitate. This may be explained by the competition between the antibody and kinesin for binding to the myosin V heavy chain. To test whether the lack of kinesin II in myosin V immunoprecipitate is indeed due to the antibody-related interference, we used a recombinant fragment of myosin V, in which the N-terminal myosin motor domain was replaced by GST ("headless myosin"), precipitated with anti-GST antibody. Such precipitates contained kinesin II but no dynein (Figure 2C, right), further confirming the existence of two separate complexes in pigment granule extracts, one containing dynein, the other containing kinesin II and myosin V.

To confirm this result, we tested whether motor proteins interact with RIIα by performing a pull-down assay of proteins from pigment granule extracts with cAMP-agarose beads (Figure 2D). Incubating granule proteins with cAMP agarose beads caused nearly complete re-

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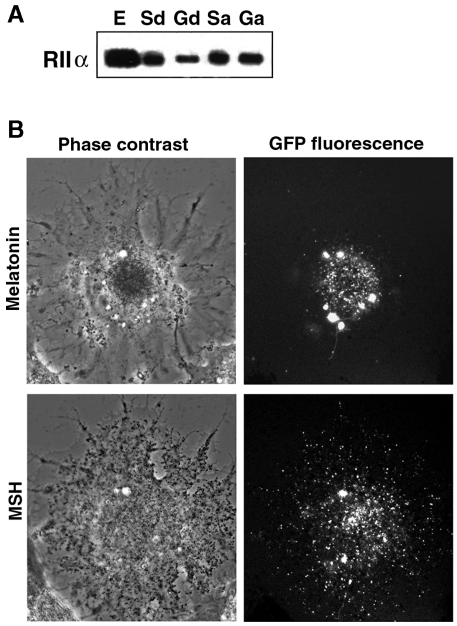

Figure 1. Protein Kinase A Is Associated with Pigment Granules In Vitro and In Vivo

(A) Immunoblotting of whole cell extract (E), granule preparations (Ga, Gd), and granule-free supernatants (Sa, Sd) from dispersed (Gd, Sd) and aggregated (Ga, Sa) cells with affinity-purified peptide antibody against human RIIα. Samples were normalized by volume to allow direct comparison of RIIα amounts. RIIα in melanophores exists in both soluble and granule bound form; although the majority of RIIα is soluble, a significant fraction of it is found on granules both in aggregated and dispersed cells.

(B) Pairs of phase contrast (left) and fluorescence (right) images of cells transfected with recombinant GFP- RIIα from cells treated with 10^{-8} M melatonin to induce pigment aggregation (top) or melanocyte-stimulating hormone (MSH) to induce dispersion (bottom). GFP- RIIα is localized as fluorescent dots whose distribution and behavior in response to hormones closely resembles that of pigment granules.

moval of RIIα from solution into the bead-containing precipitate. Such precipitate also contained p150^{Glued}, kinesin II, myosin V, and dynein, associated with the RIIα-containing beads in cAMP-sensitive manner. Thus, RIIα indeed interacts with these proteins in pigment granule extracts.

Association of RIIα with motor molecules may play a regulatory role and modulate their activity in intracellular transport. However, this interaction may also be important in the association of motor molecules with the granules because PKA normally has an independent binding site for cellular structures via A-Kinase Anchoring Proteins (AKAPs) (reviewed in [8]). We therefore tested whether there are any AKAP-like, RIIα binding proteins in the preparations of pigment granules by using a blot overlay of granule proteins with recombinant RIIα in the presence and absence of Ht31, a synthetic peptide that has been shown to prevent RIIα-AKAP interaction in vivo and in vitro and that corresponds to the consensus RIIα binding sequence of AKAPs [10]. We found that pigment granule preparation contained two bands of approximately 160 and 80 kDa, whose binding to RIIα was specifically inhibited by Ht31 but not by Ht31p, a similar peptide with two substitutions that abolish its RIIα binding activity [10] (Figure 3A). Therefore, RIIα is indeed associated with pigment granules via AKAP.

To find out the role of AKAP-RIIα interaction in the motor-mediated pigment transport, we perturbed the interaction of RIIα with AKAP by either injecting cells with synthetic peptide Ht31 or transfecting them with a truncated GFP-tagged RIIα construct containing the dimerization and AKAP binding domain (aa 1–129) [11] but lacking the C-terminal cAMP binding domain. To our surprise, both treatments had an effect on pigment transport opposite to the effect of PKA inhibition. Instead of activating, these treatments partially blocked pigment aggregation (Figures 3B and 3C). Particle-tracking analysis demonstrated that the average length of dynein-dependent MT minus-end runs reduced from 470 ± 44 nm to 272 ± 28 n, whereas the average length of kinesin II-dependent plus-end runs did not change significantly (143 ± 15 nm in control versus 108 ± 12 nm upon RIIα removal). Control treatments (injection of Ht31p peptide or transfection of the full-length RIIα subunit) had no effect on either pigment aggregation or pigment dispersion. Therefore, removing RIIα from granules primarily inhibits dynein-dependent pigment transport.

Because inhibition of PKA has been shown to result in the activation of dynein-dependent transport [12], the observed effect cannot be explained by the removal of PKA enzymatic activity from the proximity of dynein. Therefore, it is possible that the removal of RIIα from pigment granules affects the structural integrity of PKA-motor complexes. To test this possibility, we probed pigment granules purified from cells transfected with full-length and truncated GFP-RIIα constructs with antibodies against GFP, RIIα, dynein, kinesin II, myosin V, or p150^{Glued} (Figures 3D and 3E). Granules purified from cells transfected with full-length or truncated GFP-RIIα contained GFP-immunoreactive polypeptides of molecular masses 76 and 39 kDa, respectively, indicating the presence of the corresponding fusion proteins (Figure 3D). Transfection with truncated, but not full-length RIIα induced the removal of the endogenous RIIα from pigment granules (Figure 3E, top). Remarkably, this treatment also removed dynein and p150^{Glued} but not kinesin II or myosin V from the pigment granules, indicating that only the dynein-containing complex was disrupted by the removal of RIIα. This result was consistent with the observed inhibition of pigment aggregation but not pigment dispersion by PKA removal from granules.

To further test the conclusion that the effect of PKA removal from granules is related solely to the perturbation of the dynein-containing complex, we next asked the question whether the interference with p150^{Glued} interaction with the granules has the same effect on pigment transport as RIIα removal. To test this, we transfected the cells with a GFP-tagged construct containing the C-terminal half of p150^{Glued} including the site of p150^{Glued} interaction with the granule surface but not its dynein binding site. This construct was designed to replace the endogenous p150^{Glued} from granules and thus to specifically inhibit dynein interaction with pigment granules. Such transfection had the same effect on pigment transport as PKA removal, partially inhibiting dy-

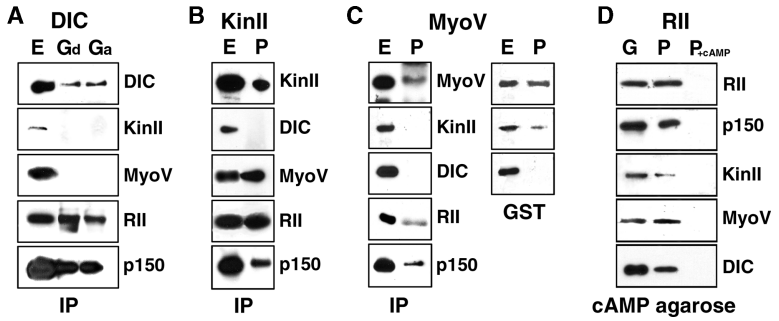

Figure 2. Motor Proteins on Pigment Granules Form Complexes with Signaling Molecules

(A) Immunoprecipitation of the pigment granule proteins from aggregated (Gd) and dispersed (Ga) cells with 74.1 antibody against dynein intermediate chain, probed with antibodies to motor proteins and signaling molecules. The left column shows immunoreactivity of each antibody with the whole cell extract (E). Dynein coimmunoprecipitates with $p150^{Glued}$ and RIIα but not with myosin V or kinesin II.

B. Immunoprecipitation of pigment granule proteins or whole-cell extract with monoclonal antibody K2.4 against the 85 kDa subunit of kinesin II. Kinesin II coimmunoprecipitates with myosin V, RIIα, $p150^{Glued}$, but not dynein.

C. Immunoprecipitation of pigment granule proteins with polyclonal antibody DIL-1 against the myosin V heavy chain (left) or a combination of GST-tagged myosin stalk-tail region and polyclonal GST antibody (right). DIL-1 immunoporecipitate contains RIIα and $p150^{Glued}$ but not dynein or kinesin II, most likely due to antibody masking of the kinesin II binding site; GST-myosin immunoprecipitate contains kinesin II and myosin V but no dynein, confirming the specificity of the kinesinII/myosinV interaction.

D. cAMP agarose-affinity chromatography of pigment granule extract; pigment granules (G) and cAMP agarose precipitate in the absence or presence of cAMP (P and P_{+cAMP}) probed with the antibodies against RIIα (RII), $p150^{Glued}$ (p150), kinesin II (KinII), myosin V (MyoV), and dynein intermediate chain (DIC). cAMP agarose beads specifically bind RIIα and also pull down $p150^{Glued}$, myosin V, kinesin II, and dynein. These interactions are completely abolished in the presence of 10 mM cAMP that specifically blocks the binding of RIIα to the agarose beads. The results of coimmunoprecipitation, GST pulldown, and cAMP agarose-affinity chromatography suggest the existence of two independent complexes of motor proteins with signaling molecules: one containing motor molecules responsible for pigment aggregation and the other—those responsible for pigment dispersion. Abbreviations are as follows: E, cell extract; P, immunoprecipitate; DIC, dynein intermediate chain; KinII, 85 kDa kinesin II chain; MyoV, Myosin V heavy chain (DIL-2 antibody); $p150^{Glued}$, 150 kDa subunit of the dynactin complex; RII, RIIα subunit of PKA.

nein-dependent pigment aggregation but not kinesin II- and myosin V-dependent pigment dispersion (Figure 3F). Thus, the removal of RIIα from granules has the same effect as the perturbation of the structural integrity of the dynein-containing complex, confirming the conclusion that PKA association with granules indeed plays a structural role.

Our results show that PKA is associated with two separate complexes on pigment granules, a dynein-containing aggregation complex and kinesin II- and myosin V-containing dispersion complex. In combination with published data about motor protein adapters on the pigment granules [13, 14], our results allow us to propose a model of the molecular arrangement of the PKA-containing motor-regulatory complexes or regulated motor units (RMU) on pigment granule surface (Figure 4). We hypothesize that the interaction of PKA with these complexes occurs through direct binding of RIIα to $p150^{Glued}$ and that this binding is sufficiently strong to induce the removal of $p150^{Glued}$ and, subsequently, dynein from the motor complexes upon the removal of RIIα, causing the inhibition of pigment aggregation. The same treatment also replaces 150^{Glued} from the kinesin/myosin-containing complex, but the motor proteins maintain their binding to the granule through an independent binding site (rab27/melanophilin, myosin V adapters [14], (see [15] for review). Thus, RIIα/150^{Glued} removal from granules does not significantly affect pigment dispersion.

An arrangement where motors involved in organelle transport are directly associated with the molecules that regulate their activity and granule interaction in response to intracellular signals, appears to be the most efficient way to switch between various kinds of transport and fine-tune their balance inside the cell. It seems likely that the RMU contain more components involved in the regulation of organelle transport, such as protein phosphatase 2A (PP2A), which has been shown to be involved in pigment transport regulation by providing an opposite signal to PKA phosphorylation [12]. What is the exact composition of RMU, and how they bind to target organelles and regulate the corresponding components of intracellular transport are exciting unanswered questions that will be in the focus of future studies.

Supplemental Data

Supplemental Data, including Experimental Procedures, are available at http://www.current-biology.com/cgi/content/full/14/20/■■■/DC1/.

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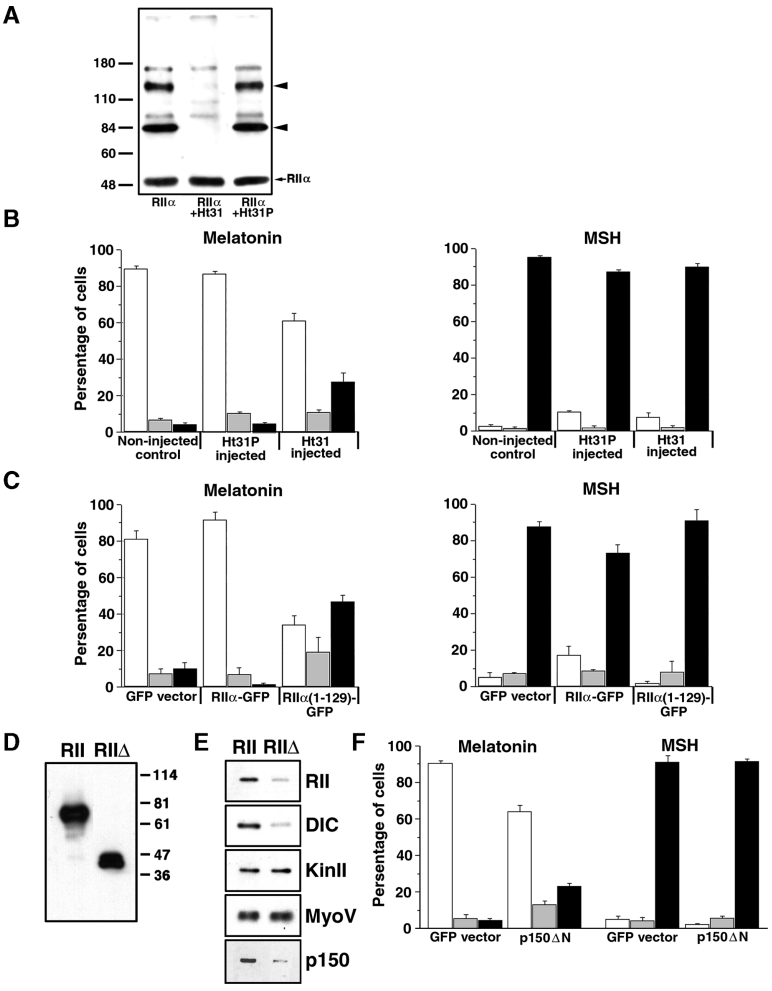

Figure 3. RIIα Interaction with Pigment Granules via AKAP Is Required for Pigment Aggregation

A. Blot overlay of pigment granule preparation with recombinant RIIα. RIIα binds to two polypeptides in the granule preparation with approximate molecular weights of 160 kDa and 80 kDa (arrowheads) as well as to itself (arrow), presumably through its dimerization domain. The binding of RIIα to the 160 and 80 kDa polypeptides, unlike other polypeptides in the preparation, is specifically inhibited by addition of Ht31, a peptide that blocks RIIα-AKAP interaction (middle), but not by the control peptide Ht31p (right).

B. Injection of the Ht31 peptide partially inhibits pigment aggregation. Data is expressed as a percentage of cells with aggregated (white bars), partially aggregated (gray bars), and dispersed (black bars) pigment. Left, pigment aggregation stimulated by melatonin. The amount of aggregated cells is similar in noninjected cells and cells injected with the control peptide Ht31p but reduced by about 35% in cells injected with Ht31, while the amount of dispersed and partially dispersed cells is low in control and Ht31p-injected cells but increases upon injection of Ht31. Right, pigment dispersion stimulated by MSH. Injections of Ht31 or Ht31p have no effect on the amount of dispersed and aggregated cells as compared to control, noninjected cells. Percentages are calculated from at least 300 cells per injection experiment.

C. Transfection of truncated (aa 1–129) but not full-length RIIα partially inhibits pigment aggregation while having no effect on pigment dispersion. Data is expressed similarly to (B). Percentages are calculated from 200 cells per treatment.

D. Pigment granule preparations from cells transfected with full-length GFP- RIIα (RII) and truncated GFP- RIIα (RIIΔ) probed with anti-GFP reveal protein bands of 76 kDa and 39 kDa, respecitvely.

E. Transfection with truncated but not full-length RIIα displaces the endogenous RIIα, p150^{Glued}, and dynein, but not kinesin or myosin V, from granules. Preparations of granules from cells transfected with full-length GFP-RIIα (RII) and truncated GFP-RIIα (RIIΔ) probed with antibodies to RIIα, dynein intermediate chain, kinesin II, myosin V, and p150^{Glued}.

F. Perturbation of dynein association with the granules has the same effect on pigment transport as RIIα removal. Data is expressed similarly to (B) and (C). In each chart, error bars represent the standard error of the mean of three independent experiments.

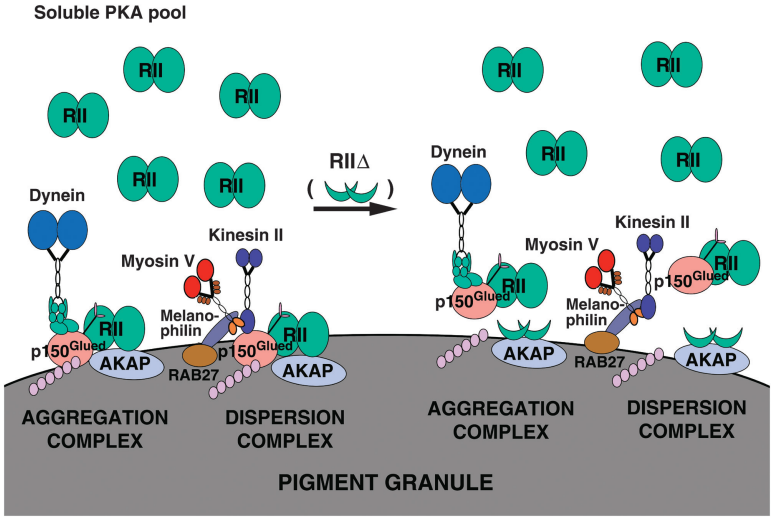

Figure 4. Hypothesis about the Arrangement of Motor and Signaling Complexes on the Surface of Pigment Granules

Dynein (blue) most likely associates with the granule through p150^{Glued} (pink), which in turn binds to the granule through the dynactin complex and also by binding RIIα -AKAP complex (green-gray). Perturbation of interaction of RIIα with AKAP inhibits interaction of p150^{Glued} with dynactin and displaces p150^{Glued} with bound dynein from the granules. Myosin V (red) and kinesin II (purple) form a complex with AKAP-bound RIIα, most likely through interaction with p150^{Glued}. Myosin has an independent binding site on the granule surface via adaptor proteins similar to rab27 (brown) and melanophilin (violet), which stabilizes the complex in the absence of RIIα-AKAP interaction.

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