 GBP binds kinesin light chain and translocates during cortical rotation in *Xenopus* eggs

Carole Weaver1,2, Gist H. Farr III1,*, Weijun Pan3,*, Brian A. Rowning4,*, Jiyong Wang3, Junhao Mao5, Dianqing Wu5, Lin Li3, Carolyn A. Larabell4 and David Kimelman1,†

1Department of Biochemistry, University of Washington, Seattle, WA 98195, USA
2Molecular and Cellular Biology Program, University of Washington, Seattle, WA 98195, USA
3State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Shanghai, China
4Department of Anatomy, University of California, San Francisco, CA 94143 and Lawrence Berkeley National Laboratory, University of California, Berkeley, CA 94720, USA
5Department of Genetics and Developmental Biology, University of Connecticut, Farmington, CN 06030, USA

*These authors contributed equally to this work
†Author for correspondence (e-mail: kimelman@u.washington.edu)

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Summary

In *Xenopus*, axis development is initiated by dorsally elevated levels of cytoplasmic β-catenin, an intracellular factor regulated by GSK3 kinase activity. Upon fertilization, factors that increase β-catenin stability are translocated to the prospective dorsal side of the embryo in a microtubule-dependent process. However, neither the identity of these factors nor the mechanism of their movement is understood. Here, we show that the GSK3 inhibitory protein GBP/Frat binds kinesin light chain (KLC), a component of the microtubule motor kinesin. Upon egg activation, GBP-GFP and KLC-GFP form particles and exhibit directed translocation. KLC, through a previously uncharacterized conserved domain, binds a region of GBP that is required for GBP translocation and for GSK3 binding, and competes with GSK3 for GBP. We propose a model in which conventional kinesin transports a GBP-containing complex to the future dorsal side, where GBP dissociates and contributes to the local stabilization of β-catenin by binding and inhibiting GSK3.

Movies available online

Key words: Frat, Wnt pathway, Axis specification, Cortical rotation, Microtubules

Introduction

The formation of the dorsoanterior axis in *Xenopus* is dependent upon a series of events that occur during the first cell cycle after fertilization. Sperm entry initiates a rotation of the peripheral layer of the egg, called the cortex, relative to the inner core cytoplasm (reviewed by Harland and Gerhart, 1997; Moon and Kimelman, 1998). This event, called cortical rotation, results in a 30° displacement of the vegetal cortex toward the future dorsoanterior region (Fig. 1A). Cortical rotation coincides with the translocation of a ‘dorsalizing activity’ that also moves from the vegetal pole up toward the prospective dorsal side of the embryo. Translocation of the dorsalizing activity is both necessary and sufficient for the formation of the Spemann organizer, which regulates the formation of the embryonic axes during the gastrula stages (Yuge et al., 1990; Fujisue et al., 1993; Holowacz and Elinson, 1993; Kikkawa et al., 1996; Sakai, 1996; Kageura, 1997).

Cortical rotation and translocation of the dorsal determinants depend on the assembly of a parallel array of microtubule bundles in the periphery of the egg (Vincent and Gerhart, 1987; Elinson and Rowning, 1988; Houliston and Elinson, 1991a; Houliston and Elinson, 1991b). If microtubule polymerization is blocked during the first cell cycle, the dorsal organizer does not form and resulting embryos lack all dorsoanterior structures. The microtubule array is formed in the 4-8-μm-deep ‘shear zone’ that forms between the cortex and the dense inner core cytoplasm of the egg, and is oriented such that a population of microtubule bundles extend from the vegetal region of the egg with their plus ends directed toward the prospective dorsal region (Houliston and Elinson, 1991b). These microtubules are thought to provide tracks upon which the dorsalizing activity travels. Because a variety of studies indicate that the dorsalizing activity is distributed along a region that extends at least 30-60° from the vegetal pole, surpassing the extent of cortex displacement (Fig. 1A), it is probable that active transport beyond simple association with the cortex is used to translocate the dorsal determinants (Yuge et al., 1990; Fujisue et al., 1993; Holowacz and Elinson, 1993; Kikkawa et al., 1996; Sakai, 1996; Kageura, 1997; Rowning et al., 1997). Intriguingly, endogenous membrane-bound organelles have been observed to translocate dorsally in a microtubule-dependent manner during the first cell cycle with a velocity and saltatory behavior suggestive of transport by a plus-end-directed microtubule motor (Rowning et al., 1997). These studies and others have led to a model in which cortical rotation helps to align the microtubule array, which is then used by a kinesin-like motor...
accumulation of β-catenin, which then activates the expression of dorsal organizer genes at the onset of zygotic transcription (Schneider et al., 1996; Larabell et al., 1997; Rowning et al., 1997). Cytoplasmic transplant experiments using β-catenin-depleted embryos have shown that β-catenin is not the endogenous dorsalizing activity, but that instead this activity probably consists of proteins involved in β-catenin stabilization (Marikawa and Elinson, 1999). β-catenin is normally phosphorylated by the serine-threonine kinase glycogen synthase kinase 3 (GSK3) within a protein complex that also includes Axin and the adenomatous polyposis coli gene product (APC), and this phosphorylation targets β-catenin for degradation by the ubiquitin-proteosome pathway (reviewed by Moon and Kimelman, 1998; Biez, 1999; Polakis, 2000). Work from many laboratories has led to a model in which the localized inhibition of GSK3 in the dorsal region causes the dorsal accumulation of β-catenin (He et al., 1995; Pierce and Kimelman, 1995; Yost et al., 1996; Yost et al., 1998; Dominguez and Green, 2000; Salic et al., 2000). How GSK3 becomes locally inhibited by the dorsal determinants, however, is still an open question.

A strong candidate component of the translocating dorsalizing activity is GBP, a vertebrate-specific GSK3-binding protein (Yost et al., 1998). Depletion of endogenous GBP from the embryo with antisense oligonucleotides causes a loss of dorsal axial structures, showing that GBP is required for dorsal axis formation (Yost et al., 1998). GBP inhibits GSK3 activity by preventing its binding to Axin, thus preventing GSK3 from phosphorylating β-catenin (Farr III et al., 2000; Salic et al., 2000). When microinjected ventrally, GBP mimics the endogenous dorsal signal and induces the formation of a secondary dorsal axis (Yost et al., 1998). Overexpression of GBP also leads to GSK3 degradation in the cortical shear zone (Dominguez and Green, 2000). In addition to binding GSK3, GBP also binds directly to Dsh (Li et al., 1999; Salic et al., 2000; Lee et al., 2001; Hino et al., 2003), a positive effector of the canonical Wnt signaling pathway (Klingensmith et al., 1994; Yanagawa et al., 1995; Sokol, 1996; Rothbacher et al., 2000). Together, these two proteins potently synergize to stabilize β-catenin (Li et al., 1999; Salic et al., 2000; Hino et al., 2003).

Thus, both GBP and its binding partner Dsh have characteristics that strongly suggest that they are part of the endogenous dorsalizing activity. Furthermore, Dsh GBP has been shown to form particles in the shear zone that exhibit directed movement on microtubules, and endogenous Dsh accumulates dorsally by the end of cortical rotation (Miller et al., 1999). However, no direct molecular link has yet been established between either GBP or Dsh and the microtubule array. In this study, we demonstrate that GBP binds kinesin light chain (KLC), a component of the plus end-directed microtubule motor kinesin. Like Dsh, GBP-GFP and KLC-GFP form particles that exhibit fast, directional translocation in the shear zone during the period of cortical rotation. Our results suggest a model in which GBP acts initially as a link between the transport apparatus and the dorsalizing activity, and subsequently as an inhibitor of GSK3 in the β-catenin degradation complex.

Materials and methods
Expression constructs
FLAG epitope-tagged mouse Frat1 (Li et al., 1999), myc epitope-
tagged GBP (BP25) (Farr et al., 2000), and myc epitope-tagged wild-type (WT) (XG134) and kinase-dead (XG137) Xgsk-3 (Yost et al., 1998) have been previously described. The Glu-Glu-tagged mouse KLC1 (pGluCMV-Kinesin) was made by inserting the two-hybrid screen clone #115, containing the first 594 base pairs (bp) of 5' coding sequence, into a CMV promoter-containing expression vector (Li et al., 1999) with a Glu-Glu tag upstream of the KLC1. An HA epitope-tagged XKLC4 (XKLC4-HA) was made by amplifying full-length XKLC4 from a positive phage clone using gene-specific primers that incorporated a 3' HA epitope, cloning the PCR product first into the pCRII-TOPO vector (Invitrogen), and then subcloning XKLC4 into CS2+ (Turner and Weintraub, 1994). The EcoRI fragment of XKLC-HA containing the first 557 bp of XKLC4 was cloned into a CS2+ derivative containing three tandem HA epitope tags (CS2+3xHA, details available upon request) downstream of the HA tags to produce XKLC-N-3xHA. XKLC4-TPR was produced by amplifying the 3' two-thirds of XKLC4 beginning with bp 594 from XKLC-HA with an internal upstream primer and a downstream vector primer. This PCR product, including the HA tag from XKLC-HA, was inserted into CS2+. A derivative of CS2+ containing enhanced Green Fluorescent Protein (GFP) was used to express GFP and to construct GFP fusion proteins (GFP LT CE; kindly provided by J. Miller). A GFP-BP50 fusion (BP50) was constructed by inserting full-length GFP upstream of GFP in the GFP LT CE vector. The XKLC4-GFP fusion was produced by amplifying XKLC4 from XKLC-HA with an upstream vector primer and a downstream gene-specific primer, inserting this fragment into pCRII-TOPO, and then inserting the Xbal/ApaI fragment containing GFP and the SV40 poly A sequence from CS XLT (gift of J. Miller) downstream of the KLC sequence. The XKLC4 deletion constructs, XKLC4-D1, D2, D3, and D4, and the GBP deletion constructs ΔN-I, ΔC-I, ΔN-III and ΔC-III were made by site-directed mutagenesis using the QuikChange kit (Stratagene). ΔN-III-GFP contains additional point mutations, K139A/E140Q, that do not affect particle formation (data not shown).

Two-hybrid and Xenopus cDNA screens
A ProQuest mouse brain cDNA library (Life Technologies) was screened with full-length mouse Frat1 as bait using the ProQuest Two-Hybrid System (Life Technologies) according to the manufacturer’s instructions. One of the KLC clones obtained was cloned into the pGluCMV vector (Li et al., 1999) and used to screen a Xenopus oocyte cDNA library in λGT10 (Rebagliati et al., 1985), from which a full-length KLC clone was obtained.

Egg manipulations and confocal microscopy
Oocytes were surgically removed from Xenopus females. Stage VI oocytes were manually defolliculated and injected with 50-100 ng of synthetic RNA, as was done previously (Miller et al., 1999). After injection, oocytes were incubated at 17-18°C for 9-14 hours, then matured in Oocyte Culture Medium (OMC; 60% Leibovitz L-15 medium with 0.4% BSA, 1 mM L-glutamine, 5 μg/mL gentamycin) [modified from Kloc et al. (Kloc et al., 1996)] with 0.5-5 μM progesterone for 12-15 hours. After germinal vesicle breakdown, mature eggs were placed in 0.3X Modified Ringers (MR; 100 mM NaCl, 2 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.5) with 6% Ficoll and pricked in the top third of the animal hemisphere with a fine glass needle to induce activation and cortical rotation. Some eggs were incubated in 1 μg/mL Nile Red prior to pricking to stain yolk platelets.

To monitor the behavior of GBP-GFP particles, images were captured with a scanning laser confocal microscope (model 1024, BioRad Laboratories, Hercules, CA, USA). A 60X PlanApo oil immersion objective lens, 1.4 NA, was used to capture a field of view at a focal plane 4.8 μm from the vegetal surface of the egg. Images were collected at 1.5-3.3-second intervals and typically several 3- to 5-minute-long movies were collected for each egg during the period of cortical rotation. Image shown was collected with an electronic zoom of 2. For XKLC4-GFP particles, images were collected with a Zeiss LSM510 NLO microscope equipped with an inverted microscope. A 40X Apochromat water immersion lens, 1.2 NA, was used to capture images as for GBP-GFP at 1.5-second intervals.

To image wild-type and mutant GBP-GFP particles in fixed eggs, eggs were prepared as for live imaging and fixed 60 minutes post-activation during peak cortical rotation in room temperature fixation solution (4% paraformaldehyde, 0.1% glutaraldehyde, 0.1% Triton X-100, 100 mM KCl, 3 mM MgCl2, 5 mM HEPES, 150 mM sucrose pH 7.4). Images of the vegetal shear zone were collected using a Leica TCS SP/MP scanning confocal microscope with a 40X PlanApo oil immersion lens, 1.25 N.A. Images shown were collected with an electronic zoom of 2.

For whole-mount immunocytochemistry of eggs expressing GBP-GFP and XKLC4-HA, eggs were injected with approximately 50 ng of each RNA and prepared as for live imaging. Eggs were immersed in room temperature fixation solution 60 minutes post-activation as described for GBP-GFP alone and incubated for 1 hour at room temperature, then incubated overnight at 4°C. Blocking of nonspecific binding was performed in Super Block (Pierce) with 0.2% Triton X-100. Eggs were incubated overnight at 4°C with anti-HA antibodies (1:10,000 dilution; Covance) in Super Block/0.2% Triton X-100 followed by three washes in Super Block/0.2% Triton X-100, then incubated overnight in Alexa Fluor 568 goat anti-mouse IgG (Molecular Probes) secondary antibodies (1:600 dilution). Eggs were imaged as described for GBP-GFP.

Embryos and microinjection
Embryos were microinjected (Moon and Christian, 1989) with RNA synthesized from CS2+-derived constructs linearized with Asp718 or NotI using the Sp6 mMessage mMACHINE kit (Ambion) and purified away from unincorporated nucleotides with Microcon 100s (Millipore). Wild-type (1-1.5 ng) or mutant XKLC4-HA RNA, 0.5-1 ng WT or mutant GBP-myc RNA, and 1.5 ng WT or kinase-dead GSK3 (kdGSK3) mRNA were injected per embryo.

Immunoprecipitation and western blotting
Immunoprecipitations from CO-7 cells were performed as described (Li et al., 1999). Xenopus embryos were lysed 4-5 hours after RNA injection in 1% Triton X-100 lysis buffer (Rubinfeld et al., 1993) supplemented with Complete protease inhibitors (Roche). Embryo lysates were cleared by centrifugation in a microfuge at 14,926 g and half of the clear cytoplasmic layer retained for analysis. Antibody for immunoprecipitation (anti-HA; Covance) was used at 0.2 μl per 100 μl of lysate. Immunocomplexes were collected on Protein G beads (Amersham/Pharmacia) and washed with 1% NP-40, 50 mM Tris pH 8, 150 mM NaCl. Proteins were detected on anti-HA, anti-myc (Covance) Western blots using a goat anti-mouse IgG HRP secondary antibody (Zymed) developed with enhanced chemiluminescence (ECL). For analysis of expression of the WT GBP-GFP, ΔC-III-GFP, ΔN-III-GFP, Δ-H-II-GFP and GBP constructs in eggs, stage VI oocytes were injected with equivalent amounts of each RNA, incubated and matured in vitro as for the live confocal imaging experiments. Eggs were then processed for SDS-PAGE as in the immunoprecipitation experiments and Western blots on the total lysates were performed with an anti-GFP antibody (Covance). In Fig. 7G oocytes were injected with 92 ng of RNA each, in Fig. 7H oocytes received 88 ng of RNA each, and in both cases approximately 0.4 oocyte equivalent was loaded in each lane.

Results
GBP-GFP translocates during cortical rotation
In order to assess the behavior of GBP during the first cell cycle following fertilization, we used time-lapse confocal
microscopy to follow the localization of GBP-GFP in the vegetal region of live eggs during cortical rotation. Oocytes expressing GBP-GFP were matured in vitro and prick-activated, which induces cortical rotation and the translocation of the endogenous vesicles and the dorsalizing activity. The cortex was immobilized, allowing cortical rotation to be measured by observing the rotation of the deep inner core cytoplasm. In this situation, all components undergo the same relative displacement as in unimmobilized eggs; however, because the cortex is stationary, the core cytoplasm rotates opposite the direction the cortex would normally move (Fig. 1A) (Larabell et al., 1996; Miller et al., 1999; Rowning et al., 1997). As with previous studies, core velocity was inferred from measuring the velocity of yolk-platelet-sized dark areas in the GFP channel.

As was seen for Dsh-GFP (Miller et al., 1999), GBP-GFP localized to bright particles that were enriched in the 4-8-μm-deep subcortical shear zone relative to the deeper vegetal core cytoplasm. During rotation, a subset of the GBP-GFP particles exhibited fast, unidirectional movement opposite the rotation of the core (Fig. 1B) (see also Movie 1 at http://dev.biologists.org/supplemental/), as was demonstrated for Dsh-GFP (Miller et al., 1999). The GBP-GFP particles moved with a wide range of velocities (15-127 μm/minute) relative to the inner core. Velocities were fairly evenly distributed across this range, with a mean velocity of 47±27 μm/minute (n=31 particles from 3 eggs). These particles often appeared to stream one after the other and moved in a saltatory fashion, interspersing periods of fast transport with pauses. In all cases, we also observed a population of non-translocating particles that moved in the reverse direction at a slower rate, accompanying the rotation of the core (4-11 μm/minute). These results indicate that, like Dsh, GBP is capable of undergoing fast, directed transport away from the vegetal pole of the egg during cortical rotation.

KLC associates with GBP/Frat

In order to find new binding partners for GBP and its mammalian ortholog Frat1 (Jonkers et al., 1997; Yost et al., 1998), we performed a yeast two-hybrid screen using mouse Frat1 and a mouse brain cDNA library. From 10⁷ transformants we obtained 172 His+, β-gal+ positives of which 23 were identified as GSK3, the second most abundant type of clone. Intriguingly, the most highly represented gene among the positive clones was a member of the KLC family, mouse KLC1, obtained in 31 of the 172 positives.

To determine whether the interaction between Frat1 and KLC1 seen in yeast occurs in mammalian cells, co-immunoprecipitations were performed from COS7 cells transfected with FLAG-tagged Frat1 along with one of the KLC1 clones from the two-hybrid screen fused to a Glu-Glu epitope tag (mKLC1). Cell extracts were immunoprecipitated with anti-GFP antibody as indicated (IP), and the lysates were analyzed by Western blot as indicated (Blot). A portion of the cell lysate was reserved before immunoprecipitation and analyzed by Western blot to confirm expression of the transfected constructs (TL). Lanes 3 and 7 show the level of background binding of mFrat1-FLAG and mKLC1-GG, respectively, to the protein A/G beads used for immunoprecipitation. (B) XKLC4 co-immunoprecipitates GBP in Xenopus embryos. Embryos were injected with XKLC4-HA and GBP-myc RNAs at the 2-4-cell stage, lysed after 4 hours and immunoprecipitated (IP) with anti-HA antibody or no antibody (no Ab) as a negative control (left panel). A portion of each sample was taken prior to immunoprecipitation to show expression of injected RNAs (total lysates, right panel). Samples were analyzed by Western blot with anti-myc and anti-HA antibodies (Blot).

Fig. 2. GBP and Frat associate with kinesin light chain (KLC) in vivo. (A) KLC1 and Frat1 co-immunoprecipitate in COS-7 cells. Cells were transfected with full-length FLAG-tagged mouse Frat1 (mFrat1) and a fragment of mouse KLC1 obtained in the two-hybrid screen that consists of the N-terminal 198 residues of KLC1 fused to a Glu-Glu epitope tag (mKLC1). Cell extracts were immunoprecipitated with anti-GFP antibody as indicated (IP), and the lysates were analyzed by Western blot as indicated (Blot). A portion of the cell lysate was reserved before immunoprecipitation and analyzed by Western blot to confirm expression of the transfected constructs (TL). Lanes 3 and 7 show the level of background binding of mFrat1-FLAG and mKLC1-GG, respectively, to the protein A/G beads used for immunoprecipitation. (B) XKLC4 co-immunoprecipitates GBP in Xenopus embryos. Embryos were injected with XKLC4-HA and GBP-myc RNAs at the 2-4-cell stage, lysed after 4 hours and immunoprecipitated (IP) with anti-HA antibody or no antibody (no Ab) as a negative control (left panel). A portion of each sample was taken prior to immunoprecipitation to show expression of injected RNAs (total lysates, right panel). Samples were analyzed by Western blot with anti-myc and anti-HA antibodies (Blot).
To determine whether XKLC4 could bind GBP in *Xenopus* embryos, we performed in vivo co-immunoprecipitation experiments. *Xenopus* embryos were injected at the 2- to 4-cell stage with RNA encoding HA-tagged XKLC4 and myc-tagged GBP, and the XKLC was immunoprecipitated with an anti-HA antibody after a 4-hour incubation. As shown in Fig. 2B, GBP-myc was efficiently co-immunoprecipitated by XKLC4-HA. This interaction was maintained when immunoprecipitates were washed in RIPA buffer containing 0.1% SDS (data not shown), indicating that the binding between XKLC4 and GBP...
is robust. These results show that GBP family members from widely divergent vertebrates specifically associate with KLC.

The heptad repeat region of XKLC4 is required for GBP binding

Kinesin light chains are subunits of kinesin, a heterotetrameric, plus-end-directed microtubule motor protein. The two highly conserved domains in KLC have independent functions: the heptad repeat region binds kinesin heavy chain, the motor-containing subunit of kinesin, whereas the TPR region has recently been shown to associate with proteins involved in transporting cargo (reviewed by Kamal and Goldstein, 2002).

XKLC4 translocates during cortical rotation

Although our results demonstrated that XKLC4 could bind GBP, it was important to determine whether XKLC4 could also form particles and translocate like GBP and Dsh. As with GBP, we used time-lapse confocal microscopy to observe the localization of XKLC4-GFP in the vegetal region of immobilized, prick-activated eggs during cortical rotation. Nile

(see also the Kinesin Home Page at http://www.blocks.fhcrc.org/~kinesin/). To determine whether either of these conserved domains are important for the GBP/XKLC4 interaction, we first made constructs that consisted of the N-terminal third of XKLC4, which includes the heptad repeats (XKLC4-N) or the C-terminal two-thirds of XKLC4, which includes the TPR repeats (XKLC4-TPR), fused to an HA tag (Fig. 4A). Whereas full-length XKLC4 and XKLC4-N co-immunoprecipitated GBP-myc from *Xenopus* embryos, XKLC4-TPR failed to interact with GBP (Fig. 4B). This result is consistent with our observation that some of the KLC1 clones obtained in the two-hybrid screen, including the KLC1 clone used in the co-immunoprecipitation experiment with Frat1 (Fig. 2A), contained all of the heptad repeats but none of the TPR repeats. These results indicate that the GBP-binding domain of XKLC4 lies within the first 44 amino acids of KLC in a previously uncharacterized conserved region that is distinct from both of the known interaction domains of KLC (Fig. 3). As mouse KLC1 also contains this domain and is capable of binding mouse Frat, this interaction is likely to be conserved across vertebrate species.
Red was used to label yolk platelets to assess the direction and velocity of core rotation. Like GBP-GFP, XKLC4-GFP formed bright particles in the 4-8-μm-deep shear zone between the cortex and the inner core cytoplasm. Just prior to the initiation of cortical rotation, XKLC4-GFP particles began to exhibit random saltations (see Movie 2 at http://dev.biologists.org/supplemental/) to view time-lapse movies of XKLC4-GFP particle translocation just prior to cortical rotation, during peak cortical rotation and during late cortical rotation. (C-E) Localization of XKLC4-HA and GBP-GFP during cortical rotation. (C) Confocal image of GBP-GFP particles in the vegetal shear zone of an egg fixed during peak cortical rotation. (D) Localization of XKLC4-HA stained with anti-HA antibodies and labeled with Alexa Fluor 568 in the same confocal section. (E) Merged images of C and D. Arrowheads indicate examples of particles containing both GBP-GFP and XKLC4-HA.

The observation that both GBP-GFP and KLC-GFP form particles and translocate during cortical rotation led us to ask if they co-localized in the same particles. To test this, eggs expressing GBP-GFP and XKLC4-HA were fixed during cortical rotation, immunostained with anti-HA antibodies and imaged using confocal microscopy. We observed that GBP-GFP and XKLC4-HA co-localized in a population of particles in the vegetal shear zone (Fig. 5C-E). The co-localization of GBP-GFP and XKLC4-HA, in conjunction with their association in co-immunoprecipitations and with the fact that KLCs are components of a plus end-directed microtubule motor protein, suggests that KLC mediates the transport of GBP during cortical rotation.

**Domain III of GBP is required for binding to XKLC4**

GBP contains three domains, I, II and III, that are conserved between the *Xenopus* and mammalian proteins (Yost et al., 1998). We previously identified domain III as the site of GSK3 binding (Yost et al., 1998). No binding partners have yet been identified for domains I or II. In order to determine the binding site for KLC, we generated a series of mutant GBP constructs containing 8-15 amino acid deletions, focusing on regions within the three conserved domains (Fig. 6A). *Xenopus*
embryos expressing XKLC4-HA and either a myc-tagged mutant GBP or WT GBP-myc were lysed and immunoprecipitated with the anti-HA antibody. Deletion of domain II (Δ-II) had no effect on the ability of GBP-myc to associate with XKLC4-HA, nor did deletion of the N-terminal half of domain I (ΔN-I; Fig. 6B). Deletion of the C-terminal half of domain I (ΔC-I) slightly but consistently diminished the interaction (Fig. 6C). The most severe effects on binding were achieved by removing either the N-terminal (ΔN-III) or C-terminal (ΔC-III) half of domain III. With either of these mutations, the amount of co-precipitating GBP-myc was diminished to almost background levels (Fig. 6C; see also Fig. 6B). We conclude that domain III of GBP is required for its interaction with XKLC4, with a more minor role played by a region in the C-terminus of domain I.

The GSK3 binding site on GBP resides in the C-terminal half of domain III (Yost et al., 1998). The fact that XKLC4 also requires the C-terminal half of domain III for binding suggested that the interaction of GSK3 and XKLC4 with GBP might be mutually exclusive. To test this, XKLC4-HA and GBP-myc were coexpressed in embryos with either GSK3 or kinase-dead (kd) GSK3, which also efficiently binds GBP (data not shown). Following incubation, embryo lysates were immunoprecipitated with anti-HA antibody as a negative control (left panel). A portion of each sample was taken prior to immunoprecipitation to show expression of injected RNAs (total lysates, right panel). Samples were immunoblotted with anti-HA and anti-myc antibodies (Blot).

**Domain III of GBP is required for normal particle formation**

We predicted that a GBP mutant incapable of binding KLC should also be compromised in its ability to form particles...
GBP binds kinesin light chain

We therefore compared the localization of GFP-tagged ΔC-III, which lacks 10 amino acids in domain III (Fig. 6A), with that of GBP-GFP in prick-activated eggs. In some experiments, eggs were fixed during cortical rotation and imaged later. In obvious contrast to the punctate localization of WT GBP-GFP, ΔC-III-GFP appeared primarily as a bright, diffuse green glow throughout the shear zone, with very few particles forming (Fig. 7B). This distribution is different from what we observed for WT GBP-GFP, which also exhibited a background glow but in most cases formed hundreds of discrete particles in a single egg (Fig. 7A; see also Fig. 1B). In the live eggs, the few ΔC-III-GFP particles moved slowly in the same direction as the core, with the exception of one particle in one egg (n=14 eggs) seen to quickly dart opposite the direction of core rotation (data not shown). Analysis of the expression of WT GBP-GFP and ΔC-III-GFP by Western blot demonstrated that they were expressed at the same levels in eggs (Fig. 7G).

To further investigate the correspondence between the ability of GBP to interact with KLC and its ability to form particles, we assessed the localization of the other deletion mutant in domain III, ΔN-III-GFP, in the shear zone during cortical rotation. Like ΔC-III-GFP, ΔN-III-GFP was present as a bright, diffuse glow in the vegetal region of the egg, forming very few particles (Fig. 7E). GFP alone, as previously reported, also localized as a diffuse glow (Fig. 7C) (Miller et al., 1999). In contrast, the localization of a GBP deletion mutant that retained KLC binding in the co-immunoprecipitation assay, Δ-II, was indistinguishable from that of WT GBP-GFP (Fig. 7F). Again, analysis of the expression of the GBP-GFP constructs by Western blot demonstrated that they were expressed at equivalent levels in eggs (Fig. 7H).

We conclude that domain III of GBP is important for both in vivo association with KLC and for proper localization and translocation of GBP during cortical rotation. The residual ability of a domain III deletion mutant to form particles and translocate may be because of its ability to make contacts with other components of a larger complex of proteins, allowing some association with endogenous particles even in the absence of KLC binding. However, in general we observed a good correlation between the ability of GBP constructs to associate with KLC and their ability to form particles in the shear zone, suggesting that KLC binding is a key factor in GBP particle formation.

Discussion

In Xenopus, the formation of dorsoanterior structures depends on an early accumulation of cytoplasmic β-catenin in the future dorsal region of the embryo. This pool of stabilized β-catenin forms during the first cell cycle, when factors required for its stabilization are translocated to the prospective dorsal side of the embryo in a microtubule-dependent process. These factors are thought to act by locally inhibiting GSK3 activity. However, neither the identity of the dorsal determinants nor the mechanism of their movement is understood. Here, we show that the GSK3 inhibitory protein GBP binds KLC, and that both GBP and KLC exhibit directed translocation during the first cell cycle. This study presents the first evidence connecting a member of the early dorsal specification signaling pathway directly to a microtubule motor. Our results suggest a model in which conventional kinesin transports a GBP-containing complex to the future dorsal side, where GBP dissociates and contributes to the local stabilization of β-catenin by binding and inhibiting GSK3.
Evidence for involvement of kinesin-related proteins in the translocation of dorsal determinants

Several lines of evidence point to the involvement of an endogenous kinesin-related protein in the active transport of the dorsalizing activity to the dorsal region from the vegetal pole. First, cytoplasmic transfer experiments have demonstrated that the endogenous dorsal determinants move from the vegetal region toward the prospective dorsal side of the embryo beyond the extent of cortical displacement due to rotation, indicating that the dorsalizing activity does not just move with the cortex (Yuge et al., 1990; Fujisue et al., 1993; Holowacz and Elinson, 1993; Kikkawa et al., 1996; Sakai, 1996; Kageura, 1997). Second, hook assays have shown that approximately 90% of the subcortical microtubule bundles that make up the parallel array are aligned with their plus ends toward the future dorsal side, suggesting that active transport in this direction is performed by a plus end-directed motor (Houliston and Elinson, 1991b). Third, endogenous vesicles in the zona pellucida move dorsally from the vegetal pole with a velocity and saltatory behavior suggestive of kinesin-mediated transport (Rowning et al., 1997). Fourth, Dsh-GFP, GBP-GFP and XKLC4-GFP (Miller et al., 1999; this work) move with velocities that are in the range in which kinesin and kinesin-like proteins have been measured to move in vitro and in vivo (Howard et al., 1989; Kawaguchi and Ishiwata, 2000; Zhou et al., 2001; Yajima et al., 2002). Together, these observations support our evidence that a motor protein in the kinesin family is involved in translocating dorsal determinants along the subcortical microtubule array.

Is GBP a component of the dorsalizing activity?

GBP is known to be required for dorsal axis formation (Yost et al., 1998), but its relationship to the dorsalizing activity that translocates during the first cell cycle has been unclear. However, evidence is mounting that GBP is probably an important component of this activity. Dominguez and Green demonstrated that translocation of the dorsalizing activity leads to a decrease in GSK3 protein levels in the dorsal shear zone (Dominguez and Green, 2000), and showed that overexpressed GBP, unlike other candidate dorsalizing proteins, was able to reproduce this activity in the ventral shear zone. Our observations that GBP is capable of binding a microtubule motor and undergoing directed translocation during cortical rotation further support the role of GBP as a component of the translocating dorsalizing activity. However, work by Marikawa and Elinson suggests that the dorsalizing activity is not composed solely of GBP (Marikawa and Elinson, 1999). Before cortical rotation, the region of the shear zone where the dorsal determinants reside is called the vegetal cortical cytoplasm, or VCC. Marikawa and Elinson addressed the nature of the dorsal determinants by investigating the ability of the VCC to activate dorsal markers when transplanted into blastomeres overexpressing various centralizing molecules. Their results suggested that neither Dsh nor GBP alone is the endogenous dorsal determinant in the VCC. Salic et al. (Salic et al., 2000) subsequently demonstrated that GBP and Dsh, when expressed together, powerfully synergize to stabilize β-catenin levels (see also Li et al., 1999; Hino et al., 2003). It therefore seems probable that neither GBP nor Dsh act alone as the endogenous dorsalizing activity, but rather act in combination with each other and perhaps other β-catenin stabilizing proteins as well. It will therefore be of great importance to determine which other proteins are present in the particles formed by GBP-GFP and XKLC4-GFP. In particular, although we do not yet know whether Dsh joins these particles, the fact that Dsh binds GBP (Li et al., 1999; Salic et al., 2000) and exhibits directed translocation (Miller et al., 1999) suggests that it is likely to join KLC and GBP in a multimeric translocating complex. Future studies will investigate this issue.

A revised model for translocation of dorsal determinants

The results of this study contribute to a growing body of work that aims to describe the molecular components of the dorsalizing activity in Xenopus and the mechanism by which they travel to the dorsal region of the embryo in order to stabilize β-catenin. We propose a model in which a complex
of proteins including KLC, GBP and Dsh is assembled during oogenesis at the vegetal pole (Fig. 8A). Upon fertilization, the sperm aster introduces a minus-end microtubule organizing center into the egg, initiating microtubule assembly. Kinesin-like proteins embedded in the cortex begin to move on the microtubules, thus helping to align the forming microtubule array from the point of sperm entry (minus end) toward the future dorsal side (plus end), analogous to a comb moving through hair. As the array lines up, the GBP/KLC/Dsh particles move toward the plus end of the microtubules using a conventional kinesin motor (Fig. 8B). When the particles approach the equator of the embryo, Dsh binds to Axin and recruits GBP to the β-catenin destruction complex, causing GBP to dissociate from KLC in favor of binding to GSK3. Because GBP competes with Axin for GSK3 binding (Farr et al., 2000), this removes GSK3 from the Axin/APC/β-catenin complex, thus preventing the phosphorylation and degradation of β-catenin on the dorsal side of the embryo (Fig. 8C). Locally stabilized β-catenin then activates the expression of dorsal genes at the onset of zygotic transcription.

This model, in which the translocation of GBP and Dsh to the dorsal side accounts for the local inhibition of GSK3 activity, is simple and therefore appealing. However, the complete story will almost certainly be a more complex version of this view, and many questions remain to be answered. One issue is whether GBP is enriched in the dorsal shear zone. Potentially, GBP, like Dsh (Miller et al., 1999), could be concentrated on the dorsal side by translocation. Alternatively, GBP may be ubiquitously present but able to regulate GSK3 only when part of the translocating complex. Preliminary Western blot experiments using bisected embryos show that GBP is not restricted to the dorsal side at the 4-cell stage, although these assays may be too crude to detect local differences in protein concentration. The anti-GBP antibodies we have generated thus far are not suitable for the potentially more sensitive method of immunohistochemistry (C.W., G.H.F. and D.K., unpublished). Second, it will be important to understand exactly which endogenous dorsalizing molecules travel in kinesin particles with GBP during cortical rotation. As described above, we expect that Dsh ‘piggybacks’ on GBP during translocation. Because Dsh binds other dorsalizing molecules, such as CKIε (Peters et al., 1999; Sakanaka et al., 1999; Hino et al., 2003), and because GBP has two conserved domains of unknown function that could bind other proteins, we predict that the dorsalizing particles may be composed of a complex of proteins that as a group are particularly effective at disrupting the β-catenin destruction complex.

It will be interesting to determine the relevance of the interaction between GBP and KLC in other biological contexts. The interaction between GBP/Frat and KLC was observed in both Xenopus and mammalian cells, and the high degree of conservation within their mutual binding domains suggests that the interaction will probably occur in other vertebrate species as well. Although cortical rotation appears to be a process specific to amphibians, the translocation of signaling molecules by molecular motors is a very general phenomenon. Fertilization in zebrafish causes the formation of a parallel array of microtubules that is required for the translocation of dorsal determinants from the vegetal region of the yolk into the embryonic blastomeres, resulting in the stabilization of β-catenin (Jesuthasan and Stahle, 1997). Kinesin light chains have been shown to bind the scaffolding proteins Sunday driver/JIP3, JIP1 and JIP2 as cargo (Bowman et al., 2000; Verhey et al., 2001), which in mammalian neurons allows the kinesin-mediated transport of entire JNK signaling cassettes from the cell body to neurite tips. An interesting possibility is that components of the Wnt pathway may also be able to form multimeric signaling complexes that are transported to different subcellular locations in various cell types by virtue of the interaction between GBP and kinesin.

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