Development ePress online publication date 4 January 2006

Development 133, 459-470 doi:10.1242/dev.02219

Drosophila Cornichon acts as cargo receptor for ER export of the TGFα-like growth factor Gurken

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Drosophila Cornichon (Cni) is the founding member of a conserved protein family that also includes Erv14p, an integral component of the COPII-coated vesicles that mediate cargo export from the yeast endoplasmic reticulum (ER). During *Drosophila* oogenesis, Cni is required for transport of the TGFα growth factor Gurken (Grk) to the oocyte surface. Here, we show that Cni, but not the second *Drosophila* Cni homologue Cni-related (Cnir), binds to the extracellular domain of Grk, and propose that Cni acts as a cargo receptor recruiting Grk into COPII vesicles. Consequently, in the absence of Cni function, Grk fails to leave the oocyte ER. Proteolytic processing of Grk still occurs in *cni* mutant ovaries, demonstrating that release of the active growth factor from its transmembrane precursor occurs earlier during secretory transport than described for the other *Drosophila* TGFα homologues. Massive overexpression of Grk in a *cni* mutant background can overcome the requirement of Grk signalling for *cni* activity, confirming that *cni* is not essential for the production of the functional Grk ligand. However, the rescued egg chambers lack dorsoventral polarity. This demonstrates that the generation of temporally and spatially precisely coordinated Grk signals cannot be achieved by bulk flow secretion, but instead has to rely on fast and efficient ER export through cargo receptor-mediated recruitment of Grk into the secretory pathway.

KEY WORDS: Cargo receptor, ER export, Emp24, Axis formation, TGFα processing

INTRODUCTION

During *Drosophila* oogenesis, signals from the oocyte to the overlying follicular epithelium govern the polarization of the maturing egg and the establishment of the future embryonic body axes (Roth, 2003). These signals are mediated by the TGFα-like growth factor Gurken (Neuman-Silberberg and Schüpbach, 1993). Eggs laid by females homozygous for null alleles of *grk* are ventralized and lack anteroposterior polarity (Gonzalez-Reyes et al., 1995; Roth et al., 1995; Schüpbach, 1987). Grk consists of an extracellular growth factor domain containing one EGF repeat, a transmembrane domain and a short C-terminal cytoplasmic tail, thus resembling vertebrate TGFα (Derynck et al., 1984) and the other *Drosophila* TGFα family members Spitz (Rutledge et al., 1992) and Keren (Reich and Shilo, 2002). The mature, secreted forms of all three *Drosophila* TGFα homologues are likely to be generated by intramembrane proteases of the Rhomboid family (Lee et al., 2001; Urban et al., 2001; Urban et al., 2002). Specifically, Grk appears to be cleaved by Rhomboid-2/Brho (Ghiglione et al., 2002; Guichard et al., 2000). Processing of Spitz occurs in the Golgi apparatus, where its protease Rhomboid1 resides, and is therefore strictly dependent on the prior export of Spitz from the endoplasmic reticulum (ER), which is in turn mediated by Star (Lee et al., 2001; Tsruya et al., 2002; Urban et al., 2001). By contrast, we will show here that Grk processing occurs before export from the oocyte ER.

Generation of the Grk signals depends on the presence of Cornichon [Cni (Roth et al., 1995)] within the germline. *cni* encodes a small hydrophobic protein that is the founding member of a family of conserved eukaryotic proteins (Hwang et al., 1999; Powers and Barlowe, 1998; Roth et al., 1995). Erv14p, one of the two *S. cerevisiae* Cni homologues, was identified as an integral membrane protein of COPII-coated ER-derived vesicles (Belden and Barlowe, 1996). The COPII coat consists of several subunits that are assembled into a multimolecular coat on the surface of the ER (Barlowe et al., 1994; Bednarek et al., 1995) and serves as an external scaffold organizing the assembly of anterograde transport vesicles at the ER exit sites (Bonifacino and Glick, 2004). Direct or indirect interactions with COPII components can provide an efficient mechanism for the recruitment of cargo proteins into vesicles leaving the ER (Barlowe, 2003; Kuehn and Schekman, 1997).

Erv14p is itself recruited into such vesicles through interactions with the COPII coat (Powers and Barlowe, 2002). Loss of Erv14p results in a bud site selection defect caused by inefficient membrane transport of the bud site selection protein Axl2p (Powers and Barlowe, 1998; Roemer et al., 1996). In *erv14*Δ yeast cells, Axl2p fails to be sorted into COPII vesicles and accumulates in the ER, while other cargo molecules are secreted at normal rates. Thus, only a subset of secreted proteins depends on Erv14p for ER export (Powers and Barlowe, 1998; Powers and Barlowe, 2002). In wild-type oocytes, freshly synthesized Grk protein is efficiently and rapidly cleared from the large, continuous ER spanning the oocyte. Consistent with Cni acting as a Grk cargo receptor, reduction in Cni activity causes diffuse mislocalization of Grk protein within the ER (Herpers and Rabouille, 2004).

Here, we provide biochemical and genetic evidence for an involvement of Cni in Grk ER export and present data explaining why Cni function is essential for the spatial and temporal specificity of Grk signalling.

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Accepted 23 November 2005

MATERIALS AND METHODS

Fly stocks

The *Drosophila* strains *cni*^{AR55}, *cni*^{AA12}, *Df(2L)H60* (Roth et al., 1995), *grk*^{DC} (Schüpbach, 1987), *grk*^{2B6}, *grk*^{HF48} (Neuman-Silberberg and Schüpbach, 1993), *shi*^{TS} (*shi*^{1}) (Grigliatti, 1973) and *Df(2L)JS7* (Sekelsky et al., 1995) have all been described previously.

Immunostaining and electron microscopy

Antibody staining was performed as described (Peri et al., 1999). The antisera used were rabbit anti Grk, mouse anti Grk 1D12 (DSHB), mouse anti myc 9E10 (Dianova), rabbit anti myc A-14 (Santa Cruz), mouse anti *Drosophila* Golgi 9C1 (Abcam) and mouse anti KDEL-receptor (Abcam). For the *shi*^{TS} experiments, freshly dissected ovaries were incubated for 3 hours at either 25°C or 32°C in Schneider's *Drosophila* tissue culture medium (Sigma) before fixation and staining. Confocal imaging was performed using a Zeiss LSM 510 microscope. Ultrastructural analysis was carried out as described (Wilsch-Braeuninger et al., 1997).

Molecular techniques

cnir and dEmp24 cDNAs were amplified from an ovarian two-hybrid library (Grosshans et al., 1994). All Grk transgenes are derived from a Grk genomic rescue construct containing bases 49777- 54827 of P1 DS02110. The region encoding Grk amino acid 236-294 (transmembrane and cytoplasmic domain) was replaced using PCR generated restriction sites in domain swaps with the transmembrane or transmembrane and cytoplasmic domains of Yl (Grk-Yl TM, Grk amino acids 1-235 + Yl amino acids 1801-1825; Grk-Yl TMC, Grk amino acids 1-235 + Yl amino acids 1801-1984). In three other constructs, the cytoplasmic tail of Grk was replaced with the cytoplasmic tails of Yl (pGrk-YlCyt, Grk amino acids 1-275 + Yl amino acids 1826-1984), dEmp24 (pGrk-EmpCyt, Grk amino acids 1-275 + CG3564 amino acids 194-208) or cni (pGrk-CniCyt, Grk amino acids 1-275 + Cni amino acids 100-145).

The pcni::Cnir plasmid was cloned by replacing the *cni*-coding region with a corresponding genomic fragment of *cnir* in the published rescue construct (Roth et al., 1995). The same template was used to introduce a single myc epitope tag at the Cni C terminus. All constructs were cloned into pCasper4 (Pirrotta, 1988).

For western analysis, ovaries were ground on ice in lysis buffer (50 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 2 mM DTT, 1%Triton X-100, 1% SDS 2 mg/ml Aprotinin, 1 mg/ml Leupeptin, 0.5 mg/ml Pepstatin, 10 mM PMSF) and loaded at one to three ovaries per lane. Protein was detected using the A14 and 9E10 anti-myc antisera (Santa Cruz) or the anti-Grk monoclonal 1D12 (DSHB).

Yeast two-hybrid experiments were performed using the system of James et al. (James et al., 1996). The control pair Staufen RNA binding domain 5/CG18501 was a gift from Uwe Irion and Daniel St Johnston. Pulldown experiments were performed by bacterially expressing the first 57 amino acids of Cni and Cnir or lacZ ORF, respectively, fused to the maltose binding protein (MBP) of pMal-c2e (NEB). These fusion proteins were subsequently tested for precipitation from bacterial lysates by incubation with GST (pGEX2T, Pharmacia) or a GST-Grk fusion (GST and Grk amino acids 179-245) prebound to Glutathion-Sepharose beads (Pharmacia). The beads were spun down, washed in PBS with 300 mM NaCl and 1% Triton X-100, and MBP fusions in the pellet detected by western blots using an anti-MBP monoclonal antibody (Sigma).

RESULTS

cornichon has pleiotropic functions and colocalizes with markers of the early secretory pathway

Owing to a failure in Grk signalling during oogenesis, eggs produced by *cni* mutant females lack both anteroposterior (AP) and dorsoventral (DV) polarity (Roth et al., 1995). However, unlike mutations in *grk*, loss of *cni* function also causes defects in adult somatic tissues. Flies homozygous for the amorphic allele *cni*^{AR55} are subviable (20% of the expected number hatch), possess rough eyes (Fig. 1A-C), their postvertical, interocellar and ocellar bristles are largely missing (Fig. 1A,B), and the wings have a truncated vein 2 (Fig. 1E,F).

Transgenes expressing Cni tagged with a single C-terminal myc epitope from its endogenous promotor rescue the *cni* germline and somatic defects (Fig. 1G-I; data not shown). The tagged Cni protein is detectable in the germline from germarium stages onwards and becomes enriched within the oocyte during early and middle stages of oogenesis (Fig. 2A). Cni is also detectable in the somatic follicular epithelium (Fig. 2A), in the embryo, and in male and female somatic tissues (not shown). To address the subcellular localization of Cni, we turned to the cells of the squamous follicular epithelium, the large size and flat geometry of which minimizes colocalization artefacts. Myc-tagged Cni expressed in these cells using the CY2 driver line is found in small, discrete, dot-like structures that are only partially overlapping with the ER (Fig. 2B), labelled here using a GFP genetrap of the ER resident protein Protein Disulfide Isomerase (PDI-GFP) (Morin et al., 2001) (70% of all Cni-dots scored in three fields of view associated with PDI-GFP, *n*=291). However, whether this represents true colocalization is difficult to judge as the ER extends widely throughout the cytoplasm. Although only 8.7% of all Cni-containing structures were also stained with an antibody against the p120 Golgi protein (Fig. 2C) (*n*=346 Cni-dots scored in three fields of view), 91% were positive for staining against the KDEL-Receptor, which is involved in retrieving KDEL-tagged proteins from the Golgi to the ER (Fig. 2D) (*n*=305 Cni-dots scored in three fields of view). As biochemically determined for Erv14p (Powers and Barlowe, 1998), the subcellular localization of *Drosophila* Cni is thus consistent with cycling in and out of the ER. Although almost all Cni-containing dots were KDEL-receptor positive, the converse is not the case. Only 20.4% of the KDLR-positive dots contained Cni (*n*=641). *Drosophila* Golgi units have been shown to be biochemically heterogenous. with different enzyme and cargo content even at the same cisternal level. Presence in only a subset of retrograde vesicles may therefore reflect a prior targeting of Cni only towards specific Golgi units (Yano et al., 2005).

cornichon and *cni-related* possess partially overlapping functions

The *Drosophila* genome contains a second cni-like gene (CG17262) which we named *cni-related* (*cnir*). Cni and Cnir share 28.5% identical and 43.1% similar amino acids, but are each more closely related to specific vertebrate Cornichons, here exemplified by the human proteins. Cnirel and Cni4 form a branch distinct from other metazoan Cornichons (Fig. 3B). Overall structural properties are conserved between all family members, with a cytoplasmic N terminus and three transmembrane domains, as determined for Erv14p (Powers and Barlowe, 2002) and modelled here for Cni using the TMHMM and TMpred algorithms (Fig. 3C).

cnir is deleted by Df(2L)JS7 (Sekelsky et al., 1995). Heterozygosity for the deficiency causes synthetic lethality in an amorphic *cni* background (Table 1). In combination with the hypomorphic allele *cni*^{AA12} rare and severely malformed Df(2L)JS7 *cni*^{AR55}/*cni*^{AA12} escapers could be recovered. The synthetic lethality was completely rescued by introduction of a transgene driving expression of *cnir* under control of the *cni* promotor and 5′ and 3′ untranslated regions (pcni::Cnir), demonstrating that the initial enhancement of the *cni* phenotype to lethality was caused by the reduction of the total level of *cni*-like genes ($P<0.001$, Fisher's exact test). The construct also suppressed the loss of postvertical and ocellar bristles and the wing venation defects of amorphic *cni*^{AR55}/*cni*^{AR55} flies (Fig. 1D,F), but not the rough eye phenotype and the loss of the interocellar bristles (Fig. 1D).

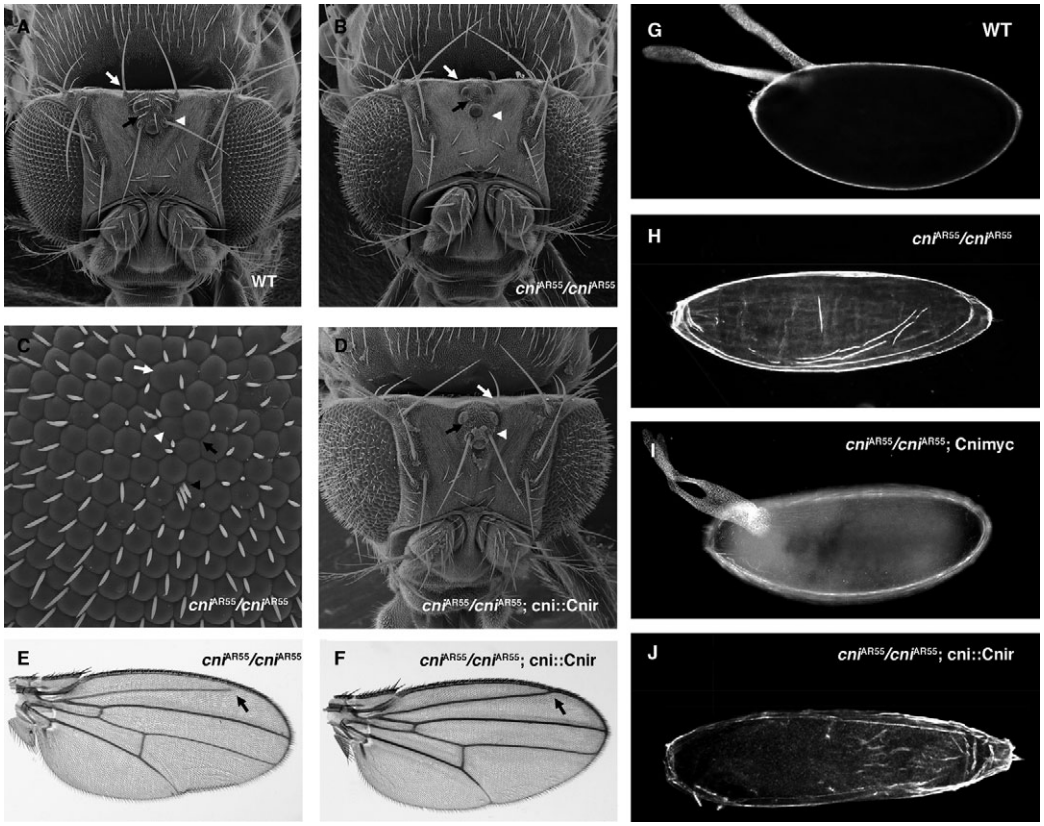

Fig. 1. *cni-related* and *cni* show partial redundancy in the soma but not the germline. (**A**) Wild-type head. Ocellar (arrowhead), interocellar (black arrow), and post-vertical bristles (white arrow) are marked. (**B**) cni^{AR55}/cni^{AR55} head. Ocellar (arrowhead), interocellar (black arrow) and post-vertical bristles (white arrow) are missing and the eyes are rough. (**C**) cni^{AR55}/cni^{AR55} rough eye phenotype with fusion of ommatidia (white arrow), specification of ommatidia with aberrant numbers of cone cells (white arrowhead), and missing (black arrow) or ectopic (black arrowhead) interommatidial bristles. (**D**) The pcni::Cnir transgene (*cnir* ORF under *cni* control) rescues ocellar (arrowhead) and post-vertical (white arrow), but not intraocellar bristles (black arrow) or the rough eye phenotype. (**E**) Homozygous cni^{AR55}/cni^{AR55} wing. Vein 2 is truncated before it reaches the margin (arrow). (**F**) One copy of the pcni::Cnir transgene suppresses the *cni* wing phenotype (arrow). (**G**) Wild-type egg with two large dorsal respiratory appendages and an anterior micropyle. (**H**) Egg from a cni^{AR55}/cni^{AR55} mother. Owing to the loss of Grk signalling, the egg is ventralized and both ends differentiate into anterior structures. (**I**) Egg from a cni^{AR55}/cni^{AR55} female carrying a pCnimyc transgene. Establishment of the anteroposterior axis is completely rescued and that of the dorsoventral axis largely rescued. (**J**) Egg from a cni^{AR55}/cni^{AR55}; pcni::Cnir female. Expression of *cnir* under control of *cni* promotor and UTR sequences fails to rescue Grk signalling.

In contrast to rescue constructs expressing native or myc-epitope tagged *cni* from either the *cni* or *bicoid* promotors (Fig. 1I and data not shown), analogous constructs containing *cnir* were unable to compensate for the loss of *cni* during oogenesis (Fig. 1J; data not shown). Cnir is therefore able to substitute for Cni in some contexts, whereas other processes, most notably Grk signalling by the oocyte, specifically require Cni function.

Grk protein is mislocalized to the interior of *cni* and grk^{DC} oocytes

We re-examined the Grk protein distribution in *cni* ovaries by light and immunoelectron microscopy, comparing the results with wild-type and a *grk* mutation (grk^{DC}) that produces a non-secreted Grk protein (Queenan et al., 1999). In wild-type stage 10 ovaries, Grk is concentrated between the oocyte nucleus and the adjacent

Table 1. Hemizygosity for a *cnir* deficiency causes specific synthetic lethality in *cni* mutant flies

(A) *Df(2L)JS7 b cni^{AR55} pr cn/SM6a* × *b cni^{AA12} pr cn bw/CyO*				
Genotype scored	N (mut)	N (total)	*P* (mut)	*P* (expected)
Df(2L)JS7 b cni^{AR55} pr cn/b cni^{AA12} pr cn bw	2	246	0.01	0.33
(B) *Df(2L)JS7 b cni^{AR55} pr cn/SM6a* × *b cni^{AR55} pr cn bw/CyO*				
Df(2L)JS7 b cni^{AR55} pr cn/b cni^{AR55} pr cn bw	0	193	0	0.33
(C) *Df(2L)JS7 b cni^{AR55} pr cn/SM6a* × *b cni^{AR55} pr cn bw/CyO; pcni::Cnir/pcni::Cnir*				
Df(2L)JS7 b cni^{AR55} pr cn/b cni^{AR55}pr cn bw; pcni::Cnir/+	117	386	0.30	0.33

N (mut), observed number of hatching flies mutant for *cni* and hemizygous for the tested deficiency; N (total), total number of hatching flies; *P* (mut), observed frequency of flies mutant for *cni* and hemizygous for the tested deficiency; *P* (expected), expected frequency of flies mutant for *cni* and hemizygous for the tested deficiency under the assumption that there is no synthetic lethality (A and B) or that the rescue construct is fully functional (C).

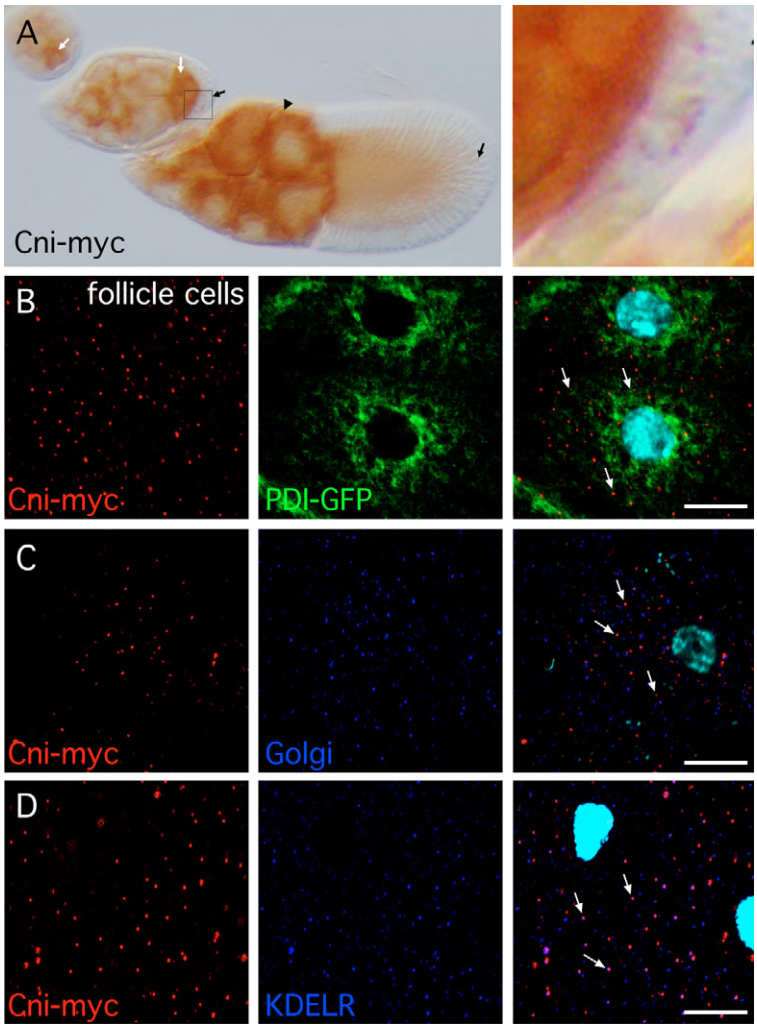

Fig. 2. Cni protein localization. (**A**) Myc-tagged Cni (Cni-myc) expressed from the endogenous promotor accumulates in the oocyte during early and middle oogenesis (white arrows). At stage 10, strong expression in the nurse cells indicates maternal loading (arrowhead). The protein is also detectable in the somatic follicular epithelium (black arrows). Right panel, magnification of boxed area. (**B**) C-terminally myc-tagged Cni protein (Cni-myc, red) expressed in the squamous follicle epithelium forms discrete punctate structures, that are often associated (arrows) but not necessarily tightly colocalized with the large reticulate ER of these cells labelled with PDI-GFP (green). (**C**) Cni-myc (red) does in most cases (arrows) not colocalize with the punctate and disperse Golgi apparatus of the same cells, marked by the p120 Golgi protein (blue). (**D**) Cni-myc containing structures (red) are largely co-stained (arrows) with an antibody against the KDEL receptor (blue). This protein cycles between ER and Golgi. The total pool of KDEL-receptor positive structures exceeds that co-staining with Cni-myc. (B-D) DNA (DAPI) is shown in light blue. Scale bars: 10 μm.

oolemma (Fig. 4A) (Neuman-Silberberg and Schüpbach, 1996). Immunoelectron microscopy shows that during vitellogenesis the oolemma and the apical sides of the follicle cells have enlarged surfaces covered by microvillous processes to facilitate rapid yolk uptake by the oocyte (King, 1970) (Fig. 4D). Grk protein could at that stage be detected at the oocyte surface, especially on the microvilli. Grk was also found at the microvilli covering the apical surface of the follicular epithelium and within follicle cells (Fig. 4D), confirming Grk release from the oocyte (Peri et al., 1999). grk^{DC} is representative of a distinct class of amorphic *grk* alleles with amino acid substitutions affecting a conserved alanine residue within the transmembrane domain (Queenan et al., 1999). These mutations apparently block proteolytic release of the extracellular growth factor domain (see below, Fig. 6C), which has been shown to be necessary for Grk activity (Ghiglione et al., 2002). Grk distribution in grk^{DC} oocytes is indistinguishable from wild type before the onset of vitellogenesis (stage 10A), when the oocyte increases its endocytosis activity. Grk protein can then also be found in posterior and ventral parts in homo- or heterozygous grk^{DC} oocytes, from where it is completely absent in wild-type ovaries (Fig. 4B). At the ultrastructural level internalized Grk protein associates with the membranous cortex of yolk granules in heterozygous grk^{DC} ovaries (Fig. 4E). Yolk granules are endosomal derivatives that grow by fusion with endocytic vesicles internalizing yolk proteins (DiMario and Mahowald, 1987). Both the timing of the Grk^{DC} mislocalization and the association of the mutant protein with the cortex of the yolk granules therefore suggest that the Grk^{DC} protein is transiently inserted into the plasma membrane, and then reinternalized during yolk uptake.

By comparison, Grk is more smoothly distributed within *cni* mutant oocytes and seems to diffuse away from a source near the nucleus (Fig. 4C). At the ultrastructural level, the comparison with grk^{DC} and wild-type ovaries shows that Grk protein in *cni* oocytes is neither clustered at the cortex of yolk granules nor enriched at the plasma membrane adjacent to the oocyte nucleus (Fig. 4F). In contrast to earlier suggestions (Queenan et al., 1999), the mutant Grk^{DC} protein in wild-type ovaries therefore appears to reside in a different endomembrane compartment than wild-type Grk protein in *cni* mutant egg chambers.

Grk transport to the plasma membrane requires *cni* function

To confirm a requirement for Cni during Grk transport to the plasma membrane, we attempted to trap the apparently membrane-tethered Grk^{DC} protein at the oocyte surface in the presence or absence of Cni by inactivating a temperature-sensitive allele of the *Drosophila* dynamin homologue *shibire* (shi^{TS}) (Chen et al., 1991; Grigliatti, 1973; van der Bliek, 1991).

Incubation of dissected shi^{TS} ovaries heterozygous for grk^{DC} at a restrictive temperature (32°C) resulted in the expansion of Grk protein staining around the anterior margin of these oocytes (Fig.

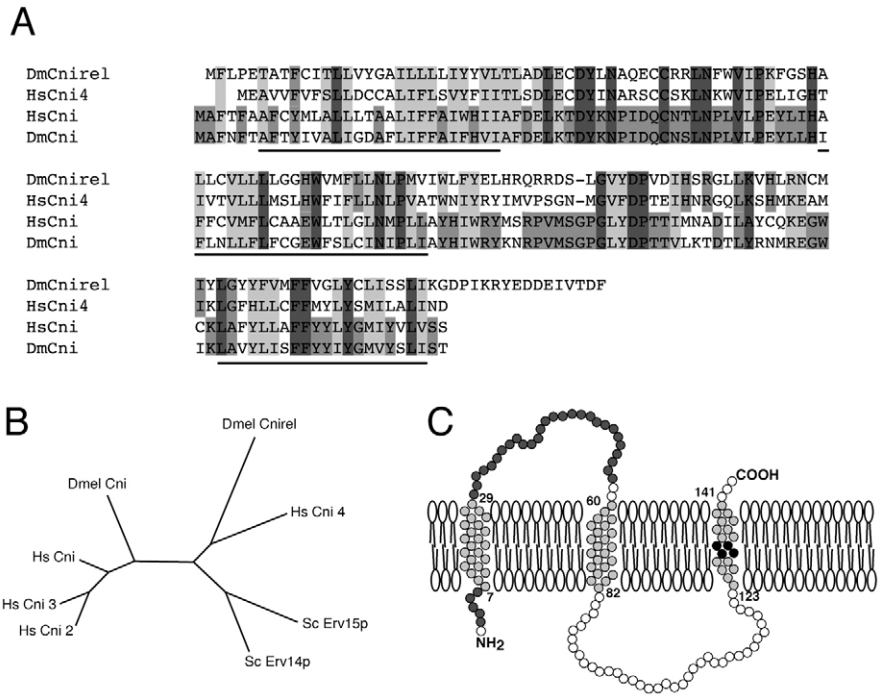

Fig. 3. Cornichon belongs to a conserved protein family. (**A**) *Drosophila* Cni and Cni-related, and their human homologues HsCni (gb| AAD20960.1) and HsCni4 (gb| NP_054903.1) share 21.5% identical (dark grey) and 43.9% similar (light grey) amino acids. Conservation is much higher between pairs of homologues: 66.7% identitical/85.4% similar in the case of Cni and HsCni, and 51.2% identitical/81.8% similar for Cnirel and HsCni4 (residues identical between homologues in medium grey). Conserved residues are distributed along the entire length of the proteins, predicted transmembrane domains are underlined. (**B**) Rootless phylogenetic tree for the fly, human and yeast Cni family members. Cnirel and human Cni4 form a distinct branch. Tree constructed by neighbour-joining, bootstrap support>98% for all nodes. (**C**) Model of the membrane topology predicted for Cni using TMHMM (www.cbs.dtu.dk/services/TMHMM) and TMpred (www.ch.embnet.org), with the N terminus facing the cytoplasm. The three transmembrane domains are labelled in light grey, while the Grk-binding domain (dark grey) maps to the N terminus, including the first lumenal loop. Black residues denote the conserved diaromatic motive.

5D). Such an expansion was not observable in control ovaries that contained only wild-type *grk* (Fig. 5E) or were heterozygous for shi^{TS} (not shown). Incubation at the permissive temperature never resulted in Grk accumulation at the plasma membrane, irrespective of the presence of the grk^{DC} allele (Fig. 5A,B). These observations support our model that grk^{DC} encodes a cleavage-resistant protein that localizes to the plasma membrane prior to its reinternalization.

By contrast, Grk^{DC} protein could not be trapped at the plasma membrane of oocytes from shi^{TS}/shi^{TS}; grk^{DC} Df(2L)H60/cni^{AR55} females. Both at permissive and restrictive incubation temperatures for shi^{TS}, all Grk protein remained diffusely distributed throughout the oocyte (Fig. 5C,F), in a pattern indistinguishable from the distribution of wild-type Grk in homozygous *cni* ovaries. Thus, Cni is required for the transport of mutant Grk^{DC} protein and, by extension, wild-type Grk to the oocyte plasma membrane.

Cni acts after the Grk proteolytic processing step

We generated a fully functional (not shown) C-terminally tagged version of Grk by replacing the intracellular domain, which is dispensable for function (Queenan et al., 1999), with five myc epitopes (Grk5myc). When Grk5myc was expressed from the endogenous promotor, only a short fragment corresponding in size to the expected C-terminal cleavage remnant (23 kDa) could be detected in western blots by antisera directed against the C-terminal epitope tags (Fig. 6A). Thus, at near endogenous expression levels Grk protein is quantitatively present in the processed form. As the same band was observed in extracts from flies mutant for *cni* (Fig. 6A), Grk processing does not require Cni function.

In ovary lysates from flies overexpressing Grk5myc in the germline under control of the maternal α-Tubulin Gal4 or nanos driver lines both the cleavage remnant and a longer protein species (ca. 70 kDa) could readily be detected (Fig. 6B and data not shown). The 70 kDa band corresponds to an uncleaved precursor form, as it can be detected both by a monoclonal antibody directed against the extracellular growth factor domain (Fig. 6B, right panel) as well as by antibodies against the intracellular, C-terminal epitope tag (Fig. 6B, left panel). Formation of the C-terminal Grk cleavage remnant is independent of *cni* also under overexpression conditions (Fig. 6B, left panel). However, in the absence of Cni, another species accumulates (48 kDa), which is recognized by the extracellular but not the C-terminal antisera, and therefore appears to be the N-terminal cleavage product corresponding to the mature growth factor. Supporting this interpretation, a band of this size has previously been identified as the active secreted Grk ligand in cell culture experiments (Ghiglione et al., 2002).

Together, these experiments suggest that *cni* is not required for proteolytic processing of Grk, and that in wild type, the generation, secretion and eventual degradation of the mature growth factor is a rapid process with low steady state levels of the protein. Although the cleavage process is saturable when Grk is overexpressed in wild

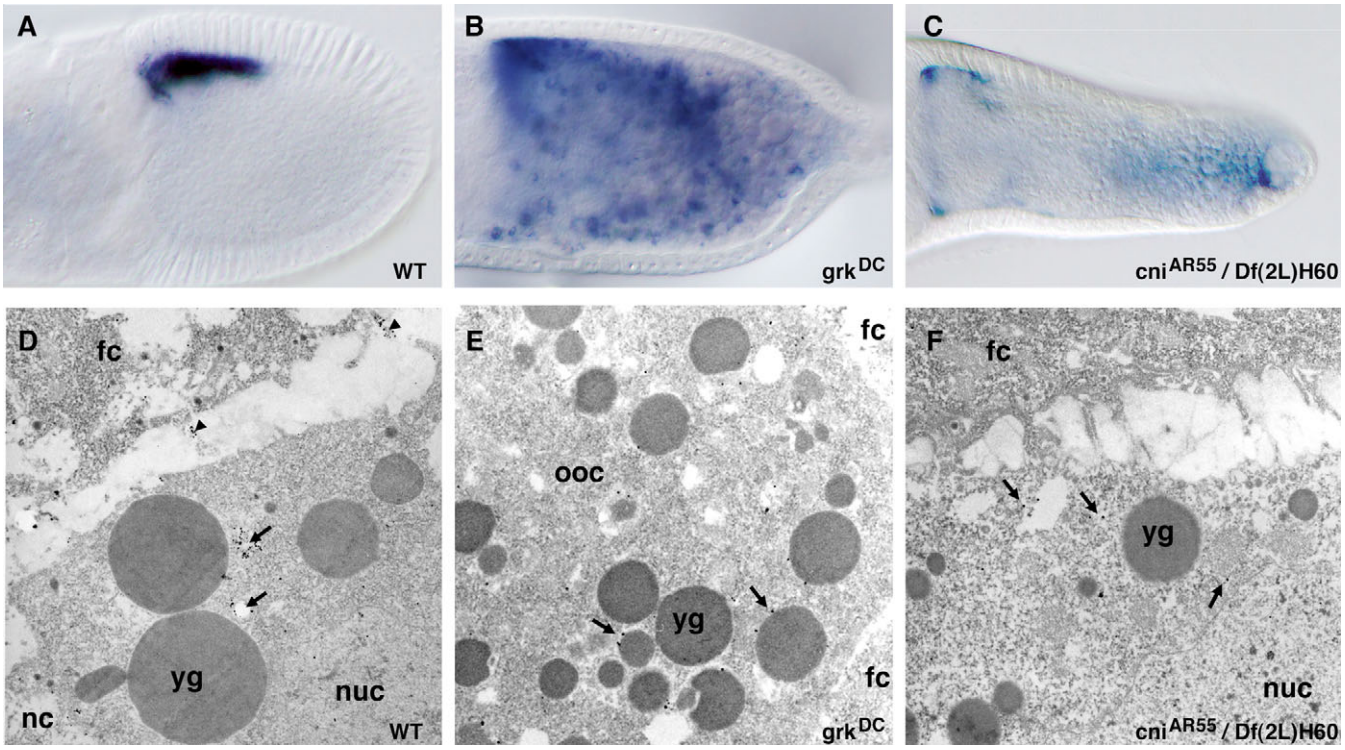

Fig. 4. Grk protein distribution in wild-type, *grk*^{DC} and *cni* mutant egg chambers. (**A**) Stage 10 wild-type egg chamber. Grk protein is concentrated at the anterodorsal corner of the oocyte in the vicinity of the nucleus. Some Grk can be seen in the follicle cells receiving the Grk signal. (**B**) Stage 10 *grk*^{DC}/*grk*^{DC} egg chamber. Grk protein is mislocalized to the interior of the oocyte. The staining appears patchy and granular, with high protein concentration near the nucleus. Grk is not detectable in the follicular epithelium. (**C**) Stage 10 *cni*^{AR55}/Df(2L)H60 egg chamber. Grk protein is distributed in a gradient with a high point near the posteriorly mislocalized oocyte nucleus and in a cortical ring at the anterior end of the oocyte adjacent to the nurse cells. There is no detectable Grk uptake into the follicular epithelium. (**D**) Immunoelectron microscopy of the anterodorsal corner of a stage 10 wild-type egg chamber. Grk immunogold staining (small black dots) can be detected in the oocyte (ooc) near the nucleus (nuc, arrows) and at the plasma membrane facing both follicle cells (fc) and nurse cells (nc). Grk is also present at the microvillous processes of the follicle cell surface (arrowheads) and within the follicle cells. Grk is absent from the cortex of yolk granules (yg). (**E**) Immunoelectron microscopy of the ventral posterior part of a heterozygous stage 10 *grk*^{DC}/*CyO* oocyte. Most Grk staining is associated with the cortex of growing yolk granules (arrows). (**F**) Immunoelectron micrograph of a stage 10 *cni*^{AR55}/Df(2L)H60 egg chamber. Grk staining is found scattered throughout the oocyte (arrows) but does not accumulate at yolk granules or the oolemma.

type, causing accumulation of the uncleaved precursor, the N-terminal cleavage product is detected only after overexpression in a *cni* mutant background.

Cni is required for ER export of Grk

We next asked where processing of Grk occurs along the secretory pathway. We generated a C-terminally tagged version of Grk that resembles Grk5myc, but in addition carries the point mutation that induces the A to V amino acid exchange found in *grk*^{DC} (GrkDC5myc). In contrast to the functional cleavable Grk5myc protein, GrkDC5myc expressed from the endogenous promotor accumulates in the ovary as a high molecular weight form (70 kDa, Fig. 6C) corresponding in size to the precursor band found in the overexpression situation (Fig. 6B). This supports the notion that the *grk*^{DC} point mutation prevents proteolytic cleavage of the Grk precursor. In lysates from *cni* heterozygous control ovaries, the GrkDC5myc signal is smeared out into several high molecular weight bands, which is indicative of glycosylation of the protein in the Golgi (Fig. 6C). Strikingly, this smearing of the GrkDC5myc band does not occur in the absence of Cni. We therefore checked the glycosylation state of the N-terminal Grk5myc cleavage product accumulating in *cni* mutant ovaries (Fig. 6B). The molecular weight of this fragment could be decreased (Fig. 6D) both by treatment with Endoglycosidase H (EndoH) and Protein N-glycosidase F (PNGaseF). Although PNGaseF is capable of removing all N-linked sugar modifications, EndoH sensitivity is characteristic for the high mannose type modifications added already in the ER, which will subsequently be trimmed and replaced by the final sugar modifications upon entry into the Golgi (Kornfeld and Kornfeld, 1985). Thus, in the absence of Cni, proteolytic cleavage of the Grk precursor occurs normally, but the extracellular/lumenal fragment that in wild type forms the mature, secreted growth factor accumulates in the ER and retains its pre-Golgi glycosylation signature.

Proteolytic processing of Grk therefore differs from that of Spitz which has to be exported from the ER to be processed by Rhomboid1 within the Golgi (Lee et al., 2001; Urban et al., 2001; Urban et al., 2002). By contrast, Grk is processed at the ER level, and requires Cni function for efficient export of the mature ligand. These in vivo observations are consistent with tissue culture data showing that the presumptive Grk protease Rhomboid2 (Guichard et al., 2000) is able to process Grk retained within the ER (Ghiglione et al., 2002).

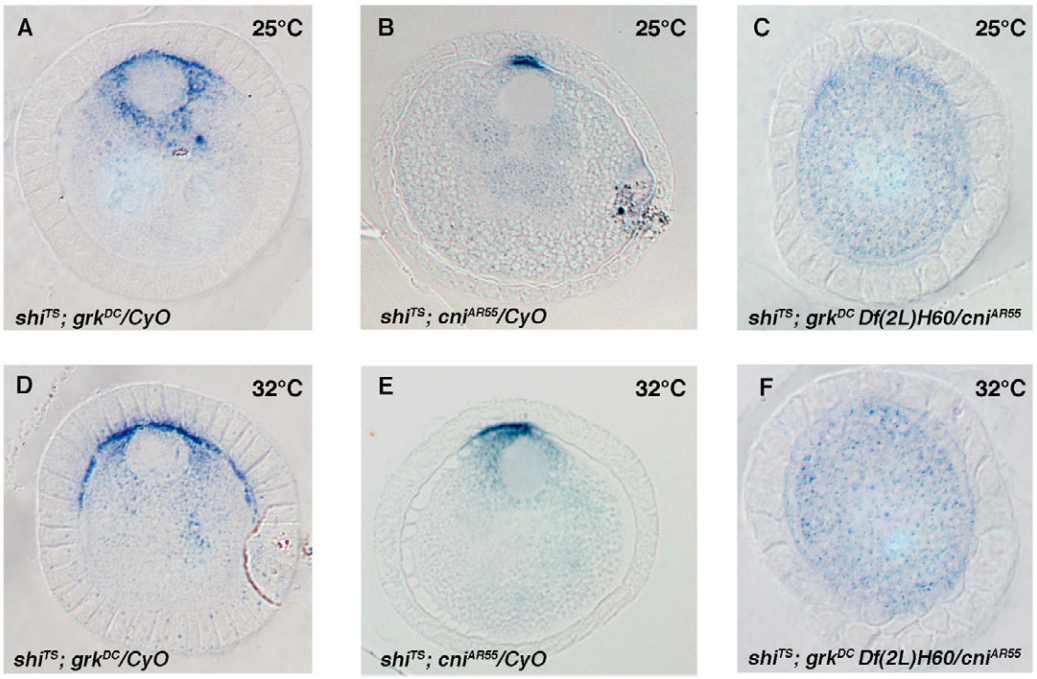

Fig. 5. Trapping of Grk^{DC} protein at the plasma membrane through inactivation of the *Drosophila* Dynamin homologue Shibire requires Cni function. (**A**) *shi*^{TS}; *grk*^{DC} / *CyO* egg chamber incubated at the permissive temperature (25°C). Grk staining at the membrane is limited to the vicinity of the nucleus. Ovaries preincubated in Schneider's medium exhibit higher than usual background, irrespective of their genotype. (**B**) *shi*^{TS}; *cni*^{AR55}/*CyO* egg chamber incubated at 25°C. Grk is distributed in the wild-type pattern. (**C**) *shi*^{TS}; *cni*^{AR55}/*grk*^{DC} *Df(2L)H60* egg chamber incubated at 25°C. Grk is diffusely distributed throughout the oocyte. (**D**) Incubation at 32°C blocks endocytosis in *shi*^{TS}; *grk*^{DC} /*CyO* oocytes. Membrane tethered Grk^{DC} protein accumulates at the oolemma. (**E**) Blocking endocytosis in *shi*^{TS}; *cni*^{AR55}/*CyO* ovaries has no effect on the wild-type Grk protein not anchored to the oocyte plasma membrane. (**F**) *shi*^{TS}; *cni*^{AR55}/*grk*^{DC} *Df(2L)H60* egg chamber incubated at the restrictive temperature. In the absence of *cni*, Grk^{DC} protein cannot be trapped at the plasma membrane but remains mislocalized to the interior of the oocyte.

Grk/Yolkless domain swaps can rescue the oogenesis phenotype of *grk* but not *cni*

Secretion of Grk depends both on the presence of Cni protein and the Grk transmembrane domain, and it has therefore been suggested that an interaction within the membrane might be responsible for the role of Cni in Grk secretion (Queenan et al., 1999). Unlike Grk, the *Drosophila* vitellogenin receptor Yolkless (Yl) is efficiently transported to the plasma membrane of *cni* mutant oocytes as seen from the formation of yolk vesicles in these cells (Fig. 4D) (DiMario and Mahowald, 1987). We therefore generated transgenic flies expressing domain swap constructs from the endogenous *grk* promotor, in which either the Grk intracellular domain, the transmembrane domain, or both, were replaced with the corresponding fragments of Yl. Although Yl itself is not proteolytically released, it possesses, like Grk, an alanine residue at the position mutated in the cleavage resistant allele *grk*^{DC} (Ala245Val). All three constructs were able to rescue the oogenesis phenotype of *grk* mutant flies (Fig. 7D; data not shown). As Grk activity depends on prior proteolytic release of the extracellular growth factor (Ghiglione et al., 2002), these domain swaps do not interfere with the processing of Grk despite the reported substrate selectivity of Rhomboid type proteases (Urban and Freeman, 2003). By contrast, signalling by all three fusion proteins still depends on Cni function, as neither construct was able to suppress the *cni* oogenesis phenotype (Fig. 7E; data not shown). The region crucial for the role of Cni in Grk secretion shown above must therefore lie within the extracellular domain of Grk.

Cni interacts with the membrane proximal part of the Grk extracellular domain

We tested for potential corresponding protein interactions using a yeast two-hybrid system selecting for adenine and histidine autotrophy (James et al., 1996) with bait constructs containing different portions of the Grk extracellular domain extending to the beginning of the transmembrane domain (amino acids 74-245, 179-245, 215-245). Introduction of a prey construct containing the Cni ORF into this background conferred the ability to grow under stringent selection conditions, indicating an interaction between the two proteins (Table 2). Thus, the membrane proximal 31 residues of the Grk extracellular domain are sufficient to mediate

Table 2. Two-hybrid interactions

	Bait			
Prey	Grk amino acids 74-245	Grk amino acids 179-245	Grk amino acids 215-245	CG 18501
Cni amino acids 2-144	+++*	+++	+++	–
cni N-terminal amino acids 2-57	n.d.	+++	+++	n.d.
Cnir amino acids 2-157	n.d.	–	–	–
Staufen dsRNA BD 5	–*	–	–	+++

*Assayed with His Ade double selection to suppress Grk autoactivation. n.d. not done.
Grk binds to Cni but not to Cnir in a two-hybrid assay. The regions responsible for binding can be narrowed down to the 31 membrane proximal residues of the Grk extracellular domain and the first 57 amino acids of the Cni N terminus, containing the lumenal loop of the protein (Powers and Barlowe, 2002).

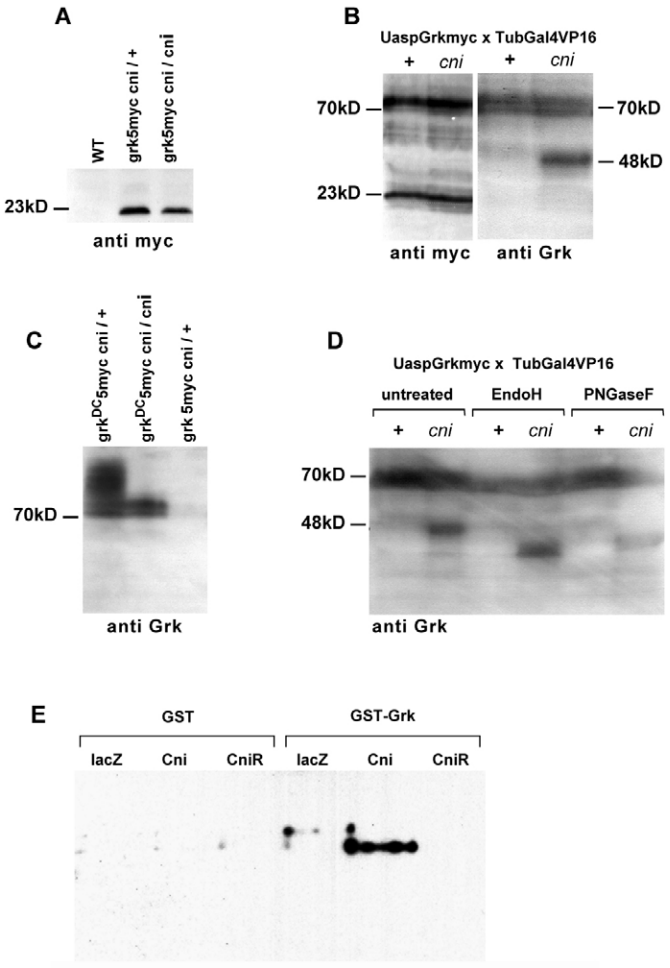

Fig. 6. Western blots with epitope tagged Grk protein and GST pull-downs. (**A**) Grk tagged C-terminally with five myc tags (Grk5myc) under endogenous control. Only the C-terminal cleavage residue but not a full-length precursor can be detected using the 9E10 anti-myc monoclonal. No differences are visible between homozygous *cni*^{AR55} mutant ovaries and heterozygous sibling controls. (**B**) Grk5myc overexpressed in wild-type (+) and homozygous *cni*^{AR55} (*cni*) ovaries using the TubGal4VP16 driver. The 9E10 anti-myc monoclonal reveals two bands. Although the lower band corresponds in size to the expected C-terminal cleavage residue, the upper band, corresponding to an uncleaved precursor, is larger than predicted from the protein sequence, indicative of glycosylation. In addition to the precursor band, the 1D12 anti-Grk monoclonal reveals a shorter band corresponding in size to the released N-terminal growth factor fragment in the mutant but not the wild-type lane. (**C**) A protein species corresponding in size to the uncleaved Grk precursor accumulates in lysates expressing a Grk5myc construct carrying the *grk*^{DC} point mutation from the endogenous promotor (GrkDC5myc). This does not occur in ovaries expressing the functional Grk5myc version. Smearing of the GrkDC5myc band into several high molecular weight species indicative of Golgi glycosylation only occurs in the presence of Cni. (**D**) The N-terminal growth factor fragment accumulating in *cni* mutant ovaries upon Grk overexpression is sensitive to EndoH and PNGaseF, indicating ER-type high Mannose glycosylation. (**E**) Grk and Cni interact directly. MBP fusion proteins with *lacZ* or the N-terminal 57 amino acids of Cni or Cnir were pulled down using GST or GST-Grk (GST fused to Grk amino acids 179-245) prebound to beads. Probing the pellets with anti-MBP antibody reveals a specific interaction between the juxtamembrane domain of Grk and the Cni N terminus.

binding to Cni in a yeast two-hybrid assay. Interaction was also observed with a prey construct encoding only the first 57 amino acids of Cni. By contrast, neither of the Grk bait constructs nor the CG18501 control were able to bind to a prey construct containing Cnir. These two hybrid data were confirmed by pull-down experiments, where a GST fusion protein containing amino-acids 197 to 245 of Grk could specifically co-purify a MBP fusion construct containing the N-terminal 57 amino acids of Cni, but not one with the corresponding Cnir domain or a *lacZ* control (Fig. 6E). This difference between Cni and Cnir in their ability to bind Grk may underlie the strict requirement for Cni during Grk secretion, even though the two *Drosophila* Cni-like proteins exhibit redundancy in other contexts.

Fusion of Grk with ER exit signals or massive overexpression can partially alleviate the dependence on Cni

Yeast Emp24p is a cargo receptor cycling between the ER and the intermediate compartment (Schimmoller et al., 1995). Exit of Emp24p and associated cargo from the ER is in part mediated by binding of Emp24p to components of the COPII coat through diaromatic amino acid pairs in the C-terminal cytoplasmic tail. We replaced the intracellular domain of Grk with the short cytoplasmic tail of one of the *D. melanogaster* Emp24 homologues (CG3564 amino acids 194-208). A transgene expressing this fusion protein from the endogenous promotor (pGrk-EmpCyt) fully rescued the loss of *grk*, indicating that the transgene produced normal amounts of active Grk ligand (not shown). Interestingly, it also restored some Grk signalling activity in the absence of *cni* (Fig. 7F). Eggs laid by homozygous *cni* females containing one copy of pGrk-EmpCyt had normal anteroposterior polarity. Some eggs also possessed recognizable dorsal appendage material, indicating low to intermediate levels of Grk signalling in these egg chambers (Fig. 7F). Thus, fusing Grk to a domain known to mediate selective recruitment into COPII vesicles partially alleviates its dependence on Cni.

Similar results were achieved using an analogous transgene replacing the Grk intracellular domains with a Cni fragment consisting of the C terminus after the second predicted transmembrane domain (Cni amino acids 100-145, pGrk-CniCyt). The transgene fully rescued the loss of *grk* (data not shown) and restored some signalling activity in the absence of *cni*. Eggs laid by *cni* mutant females carrying one copy of this transgene had normal anteroposterior polarity and showed slight and variable rescue of the dorsoventral axis (Fig. 7G). The C-terminal domains of Cni-like and Emp24-like proteins may therefore be functionally equivalent.

If Cni were only functioning as a cargo receptor for ER export of Grk, massive overexpression of Grk should result in bulk flow ER to Golgi transport and might thus overcome the requirement for *cni*. To test this assumption, we analyzed the egg phenotypes produced by those *grk* overexpression lines that were used for the western blots described above. Expression of *grk* with the help of the maternal α-Tubulin Gal4 driver leads to a strong increase in the amount of Grk protein in stage 9 egg chambers when compared with endogenous Grk levels (Fig. 8A,C,E). When overexpressed in a wild-type background, the bulk of *grk* mRNA is still transported to the vicinity of the nucleus (data not shown). Grk protein remains asymmetrically distributed, although the region with high Grk protein levels within the oocyte is more expanded when compared with wild type (Fig. 8A,C). This might be due to the saturation of the mechanisms normally responsible for retention of the protein near its site of

translation and the subsequent secretion through a few local ER exit sites (Herpers et al., 2004). The resulting egg chambers maintain DV polarity although they are severely dorsalized. The operculum, the dorsal-most chorion structure that is specified in follicle cells receiving maximal Grk levels, is expanded while the dorsal appendages normally specified at more lateral positions experiencing slightly lower Grk signalling are shifted to the ventral side of the egg (Fig. 8B,C).

Overexpression of Grk in a *cni* mutant background results in uniform high levels of Grk protein within the oocyte (Fig. 8E). Interestingly, the resulting eggs possess variable amounts of dorsal appendage material (Fig. 8F,G), indicating restoration Grk signalling, albeit to lower levels than in the presence of Cni. However, the eggs lack DV and frequently even AP polarity, as can be seen by the patchy induction of dorsal appendage material around the entire egg circumference (Fig. 8F,G) and the presence of a posterior micropyle (Fig. 8G), respectively.

These observations show that the requirement for Cni can be overcome by Grk overexpression. Thus, *cni* function is not essential for the formation of an active ligand per se, but the Cni-mediated increase in the efficiency of Grk secretion is a prerequisite for the precise temporal and spatial control of Grk signalling.

DISCUSSION

Recruitment within the ER is essential for the efficient ER exit of many different proteins. Cargo proteins may be concentrated into outgoing COPII-coated vesicles at the ER exit sites either by binding directly to vesicle or coat components, or through indirect

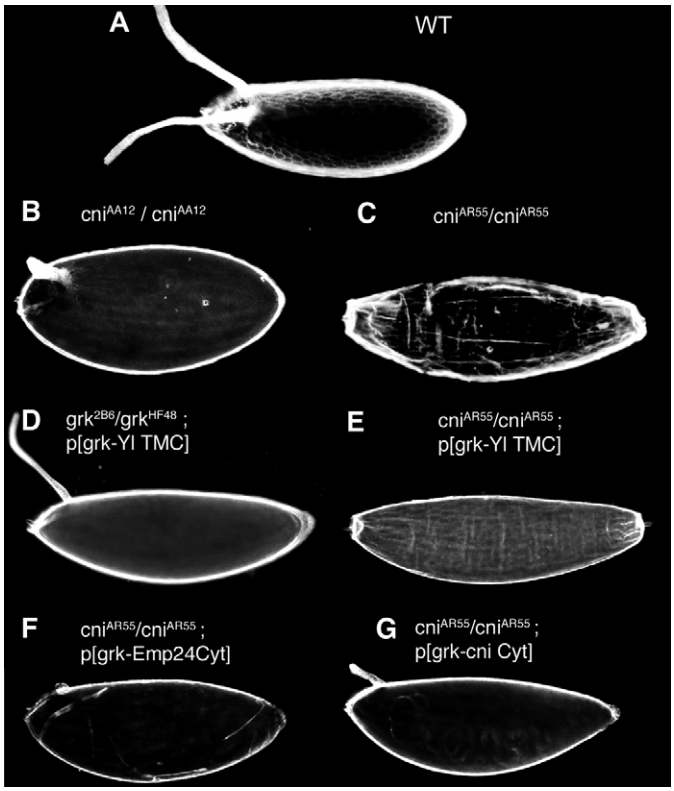

Fig. 7. Fusions of Grk to the cytoplasmic tails of Yl, dEmp24 or Cni. (**A**) Wild-type egg. The anterior pole bears a micropyle, and the dorsal side carries two respiratory appendages. (**B**) Egg from a *cni*^{AA12}/*cni*^{AR55} female. The egg is ventralized (reduction of dorsal appendages), but the anterior and posterior structures differentiate correctly. (**C**) Egg from a *cni*^{AR55}/*cni*^{AR55} female. The egg is completely ventralized and both ends differentiate into anterior structures (micropyle), indicating complete loss of Grk function. (**D**) Egg from a *grk*^{HF48}/*grk*^{2B6} female carrying a transgene replacing the Grk transmembrane and cytoplasmic domains with the corresponding domains of Yl (grk-Yl TMC). The transgene is able to rescue the *grk* oogenesis phenotype. (**E**) The grk-Yl TMC transgene shows no function in an amorphic *cni* background. (**F**) Egg from a *cni*^{AR55}/*cni*^{AR55} female expressing the Grk extracellular and transmembrane domains fused to the dEmp24 cytoplasmic domain (grk-Emp24 Cyt). The egg is partially ventralized, but the anteroposterior axis is correctly specified. (**G**) Egg from a *cni*^{AR55}/*cni*^{AR55} female expressing the Grk extracellular and transmembrane domains fused to the presumptive cytoplasmic domain of Cni (grk-Cni Cyt). The anteroposterior axis is correctly polarized and the dorsal appendages are partially rescued.

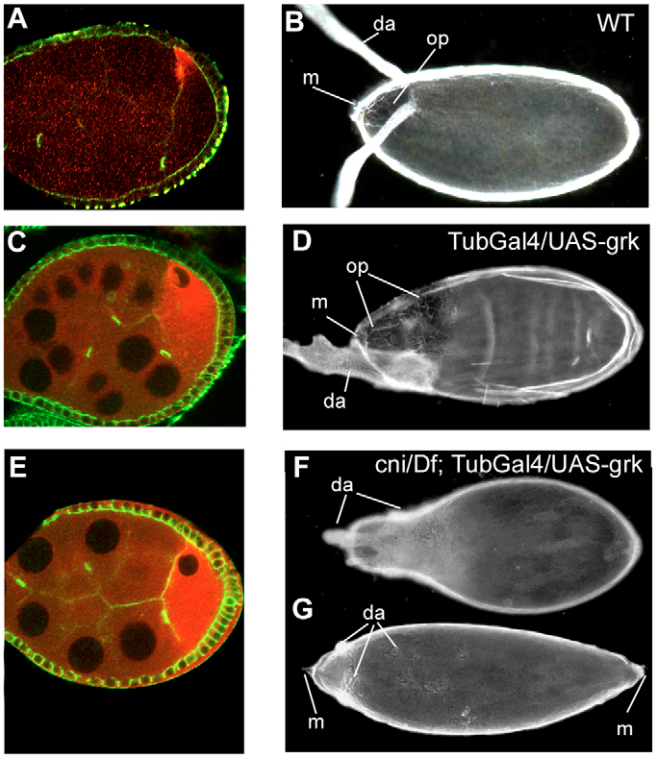

Fig. 8. Gal4-mediated Grk overexpression in the germline can overcome the secretion block in *cni* ovaries. (**A**) Wild-type egg chamber. Endogenous Grk (red) is tightly localized to the dorsal anterior corner of the oocyte. Cells are outlined with Phalloidin (green). (**B**) Wild-type egg. The dorsalmost chorion structure is the operculum (op). Dorsal appendages (da) are derived from more lateral positions. (**C**,**D**) Grk overexpression in a wild-type background using the maternal tubulin:Gal4-VP16 driver. (C) Despite a massive increase in the amount of Grk protein, there is still a Grk gradient within the oocyte with highest levels close to the oocyte nucleus. (D) The eggs maintain DV polarity although they have an expanded operculum (op) and the dorsal appendages (da) are shifted to the ventral side. (E-G) Overexpression of Grk in a *cni*^{AR55}/*Df(2R)H60* background using the maternal tubulin:Gal4-VP16 driver line. (**E**) High amounts of Grk protein are evenly distributed within the oocyte. (**F**) The eggs lack DV polarity, but the presence of dorsal appendage material (da) and the suppression of a posterior micropyle indicate low levels of Grk signalling. (**G**) Despite formation of dorsal appendage material (da), 30% of the eggs lack anteroposterior polarity as can be seen from the presence of a posterior micropyle (m). This indicates a lack of temporal control of Grk signalling.

recruitment using additional adaptors (Barlowe, 2003; Bonifacino and Glick, 2004; Kuehn and Schekman, 1997). Cni partially colocalizes with the ER resident protein marker PDI-GFP, associates with the KDEL-receptor (a protein involved in retrieval of escaped ER proteins) but is largely excluded from the Golgi. This suggests that Cni, like its yeast homologue Erv14p (Powers and Barlowe, 1998; Powers and Barlowe, 2002), has a pre-Golgi localization.

Using the cleavage-resistant Grk^{DC} protein, we have demonstrated that loss of *cni* blocks Grk transport to the oocyte plasma membrane, and in the absence of Cni overexpressed Grk protein accumulates within the oocyte and retains an ER-type glycosylation. Together with the accumulation of Grk within the ER of hypomorphic *cni* mutant oocytes (Herpers and Rabouille, 2004), these data show that the requirement for Cni in Grk export lies at the level of ER export. Our two hybrid data and Grk domain swap experiments suggest that Cni binds Grk through an interaction between its lumenal hydrophilic loop and the membrane-proximal extracellular spacer between the transmembrane and EGF domains of Grk. This interaction is consistent with the membrane topology proposed by Powers and Barlowe (Powers and Barlowe, 2002) for Erv14p, and suggests that Cni is the cargo receptor for Grk ER export. The inability of Cnir to bind to Grk in the same assay also correlates with its failure to suppress the *cni* oogenesis phenotype.

Processing of Spitz by Rhomboid1 depends on prior export of the transmembrane precursor from the ER (Lee et al., 2001; Tsruya et al., 2002; Urban et al., 2001). However, Spitz, as well as Grk, can be processed by Rhomboid2 and Rhomboid3 while still within the ER (Ghiglione et al., 2002; Urban et al., 2002). In the absence of Cni, Grk is effectively processed in the oocyte although it cannot leave the ER. Thus, during oogenesis Grk processing must occur within the ER, suggesting that the presumptive Grk protease Rhomboid2 (Ghiglione et al., 2002; Guichard et al., 2000) either resides in, or cycles through, the oocyte ER.

Processed Spitz generated before export of the precursor to the Golgi is specifically retained in the ER (Schlesinger et al., 2004). By contrast, our data suggest that Cni serves to specifically ensure efficient export of Grk after cleavage in the oocyte ER. We propose that Grk interacts with Cni prior to its proteolytic processing, and that the proteins remain associated at least until the mature growth factor is recruited into an outgoing vesicle (Fig. 9A). By contrast, soluble Grk protein lacking a membrane anchor would diffuse away into the lumen after synthesis, precluding recruitment by Cni at the ER membrane, explaining why truncated Grk protein lacking a transmembrane domain is not secreted from the oocyte and corresponding *grk* alleles are nonfunctional (Queenan et al., 1999). The same fate would await Grk released from its membrane anchor through proteolytic processing in a *cni* mutant oocyte (Fig. 9B).

Constructs expressing Grk fused to the cytoplasmic parts of either Cni or dEmp24p are partly able to restore Grk signalling in the absence of Cni, but not to wild-type levels. We suggest that the heterologous cytoplasmic tails, which contain the respective domains shown in yeast to mediate the COPII interactions (Schimmoller et al., 1995; Powers and Barlowe, 2002), are rapidly recruiting unprocessed Grk fusion proteins towards prospective vesicle budding sites. Because the ER exit motives are separated from the growth factor part during the processing step, most of the processed protein will in the absence of Cni still escape into the ER lumen, explaining the low rescue efficiency of the fusion proteins. However, proteolytic cleavage would preferentially occur in the vicinity of the outgoing vesicles, locally increasing the concentration of the soluble mature growth factor (Fig. 9C). This appears to be sufficient to ensure inclusion of some processed Grk into the outgoing vesicles in the absence of Cni, but cannot reconstitute wild-type rates of Grk signalling.

Conversely, the hypomorphic mutation cni^{AA12} truncates Cni after the first two putative membrane-spanning domains (Roth et al., 1995). It therefore deletes the second, cytoplasmic loop shown to mediate COPII interaction in Erv14p (Powers and Barlowe, 2002), but still possesses the first, lumenal loop binding to Grk. The truncated Cni^{AA12} protein may therefore remain able to keep processed Grk at the ER membrane. This would limit diffusion of processed Grk to the two dimensions of the ER membrane, rather than the three dimensions of the lumen, thereby enriching it to some degree in vesicles leaving the oocyte ER (Fig. 9D). However, to achieve the full rate of Grk secretion further cargo concentration into outgoing vesicles through interaction of the Grk-Cni complexes with the COPII coat would be required. Consistently, cni^{AA12} is clearly a hypomorphic allele with readily detectable remaining Grk signalling activity (Fig. 7B), but in mutant oocytes, Grk protein is diffusely mislocalized within the large, continuous ER and can no longer be found concentrated at ER exit sites (Herpers and Rabouille, 2004).

Grk is translated from a localized mRNA and becomes translocated into a giant ER spanning the entire oocyte and containing around 1000 active exit sites. Nevertheless, in the presence of its cargo receptor Cni, Grk is exclusively secreted through a few of these sites and their associated Golgi stacks at the dorsal anterior corner where the *grk* mRNA is found (Herpers and Rabouille, 2004), giving rise to a spatially tightly confined signal to

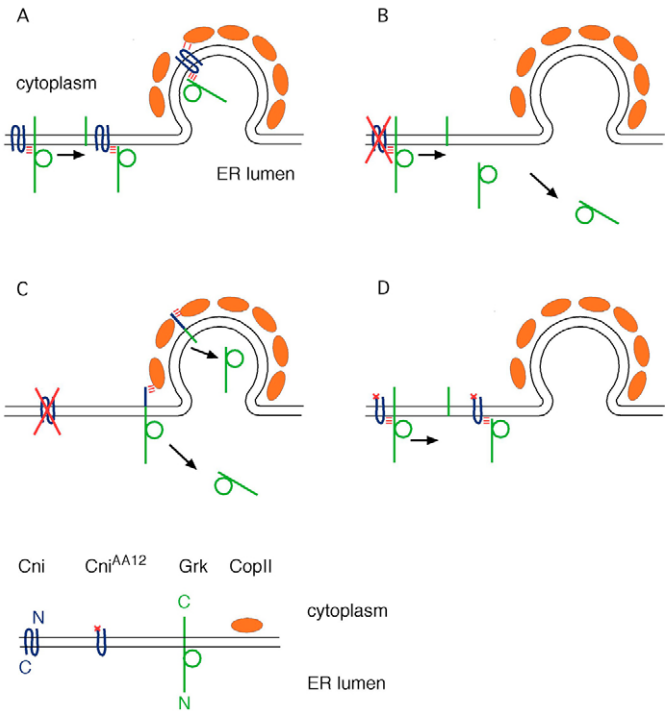

Fig. 9. Schematic representation of Cni function. (**A**) Wild-type situation. Grk protein binds to Cni after processing in the ER and the released growth factor is recruited into COPII coated vesicles by Cni. (**B**) In the absence of Cni, processing still occurs but the released growth factor is lost into the ER lumen. (**C**) Loss of Cni can be partially overcome by fusion of Grk with a COPII targeting motive from Cni or dEmp24. Processing will preferentially occur near ER exit sites, locally increasing the concentration of the soluble growth factor. (**D**) The truncated Cni^{AA12} protein can keep processed Grk at the membrane, but fails to efficiently recruit it into outgoing vesicles.

the neighbouring follicle cells. Concentration-driven bulk flow export from the ER can support secretion if cargo proteins are synthesized at sufficient rates to allow their accumulation within the ER (Martinez-Menarguez et al., 1999). Correspondingly, we have shown that massive overexpression of Grk in the oocyte can in principle restore signalling in the absence of Cni function, most likely via bulk flow ER export. However, in comparison to the wild-type situation the spatial and temporal precision of the Grk signals is lost, with severe consequences for the subsequent steps of pattern formation.

Cni is also required independently from Grk in somatic tissues, where it appears to act redundantly with Cnir. The reduced viability and life span of flies lacking *cni* function and the synthetic lethality when the gene dose of *cnir* is reduced in a *cni* mutant background indicate a more general cellular function of the Cni proteins. Erv14p, the *cni* homologue from *S. cerevisiae*, is involved in recruiting the golgin Rud3p to the cis-Golgi stacks (Gillingham et al., 2004). Besides its function in Axl2p recruitment, Erv14p therefore may play a more general role in establishing cis-Golgi identity. It will be interesting to find out whether Cni proteins in general might have a more fundamental cell biological function, e.g. in establishing Golgi polarity, that may so far have been masked by redundancy and more easily detected phenotypes caused by their roles as cargo receptors.

We thank Jürgen Berger for the *Drosophila* SEM images, Birgitta Lattemann, Inge Zimmermann and Heinz Schwarz for their help with TEM; Oliver Karst for technical assistance; Andrea Klaes for making transgenic lines; and Jörg Grosshans and Uwe Irion for materials. We are grateful to Anne-Marie Queenan and Trudi Schüpbach for sharing results prior to publication, and we thank Francesca Peri, Katia Litiere, Antoine Guichet and Slawek Bartoszewski for suggestions. The project was supported by SFBs 446, 243 and 572 of the Deutsche Forschungsgemeinschaft and the Graduierten Kolleg Genetik zellulärer Systeme (DFG) at the University of Cologne.

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