The Heterotrimeric Protein $G_\alpha$ Is Required for the Formation of Heart Epithelium in Drosophila

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Abstract. The gene encoding the $\alpha$ subunit of the Drosophila $G_\alpha$ protein is expressed early in embryogenesis in the precursor cells of the heart tube, of the visceral muscles, and of the nervous system. This early expression coincides with the onset of the mesenchymal-epithelial transition to which are subjected the cardial cells and the precursor cells of the visceral musculature. This gene constitutes an appropriate marker to follow this transition. In addition, a detailed analysis of its expression suggests that the cardioblasts originate from two subpopulations of cells in each parasegment of the dorsal mesoderm that might depend on the wingless and hedgehog signaling pathways for both their determination and specification.

In the nervous system, the expression of $G_\alpha\alpha$ shortly precedes the beginning of axonogenesis.

In Drosophila, the larval heart (or dorsal vessel) is a simple linear tube composed of inner myoendothelial cells (cardial cells) and outer unpolarized and non-myogenic cells (pericardial cells) (Rizki, 1978; Rugendorff et al., 1994; Bodmer, 1995; Bodmer et al., 1997). It extends from the T2 thoracic segment to the A7 abdominal segment. The dorsal vessel is surrounded by a network of extracellular matrix components (Rugendorff et al., 1994), among which some are localized in specialized areas of its surface (Zaffran et al., 1995). The heart behaves as a pump to insure a continuous flow of hemolymph in the organism.

The heart precursor cells arise from a group of ventral blastoderm cells in the presumptive mesoderm anlagen which invaginates at gastrulation (Bate, 1993). Later, the mesoderm spreads and forms a monolayer of cells in close apposition to the ectoderm in the direction of the dorsal region of the embryo. Meanwhile, the cells divide three to four times during the process. A proper dorsal-lateral migration is controlled by the activity of heartless, a gene encoding a fibroblast growth factor receptor (Beiman et al., 1996; Gisselbrecht et al., 1996). Once the mesodermal cells have reached their ultimate location, they require inductive signals from the overlying ectoderm to accomplish both their determination and differentiation. The progenitors of the cardial cells derive from a particular region in the mesoderm that receives simultaneously the ectodermal signals of the Decapentaplegic (Dpp) and the Wingless (Wg) secreted proteins and that constitutes the cardio-genic region (Staehling-Hampton et al., 1994; Frasch, 1995; Lawrence et al., 1995; Wu et al., 1995; Bodmer et al., 1997).

In stage 11 embryos, the precursor cells of the cardiac lineage are metamerically organized in groups of mesenchymal cells that do not, however, fully mirror the structure of the mature heart (see for example Dunin-S. Zaffran's present address is Brookdale Center for Developmental and Molecular Biology, Mount Sinai School of Medicine, Box 1126, 1 Gustave L. Levy Place, New York, NY 10029. A. Guillèn's present address is Departamento de Bioquímica y Biología Molecular I, Facultad de Biología, Universidad Complutense, 28040 Madrid, Spain. V. Homburger's present address is Centre CNRS-INSERM de Pharmacologie et Endocrinologie, 141, Rue de la Cardonille, F-34094, Montpellier Cedex 5, France.

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been cloned and characterized. Two Gα subunits have been ascribed to any of them. In contrast, it is roles in development although no precise developmental embryogenesis in various tissues in a dynamic pattern. We are interested in the genetic control of cardiogenesis in Drosophila which is viewed as a simple model to study organogenesis. The cardiac founder cells become specified in two subpopulations from a unique mesodermal functional domain and further differentiate into cells exerting markedly different functions that are the myoendothelial cells (the cardiac cells) and the pericardial cells. A long this determination/differentiation process, the cardiac cells acquire a polarity that is the result of a typical mesenchyme-epithelium transition. To better understand these transformations at the molecular level, we have focused determination/differentiation process, the cardiac cells acquire a polarity that is the result of a typical mesenchyme-epithelium transition. To better understand these transformations at the molecular level, we have focused our attention on genes that are specifically expressed in distinct subpopulations of heart cells such as the gene encoding the α subunit of the Gα protein. Indeed, an expression of this gene early in embryogenesis in the precursors of the cardiac cells has been described (Zaffran et al., 1995) in addition to its expression in the embryonic nervous system (Guillèn et al., 1991; Wolfgang et al., 1991).

The Gα proteins, which are heterotrimeric GTPases, behave as signal transduction proteins and they are widespread proteins that have been conserved from yeast to humans. Their three subunits α, β, and γ form a complex that relay external signals inside the cell via a receptor (serpentine receptor) composed of seven transmembrane domains, activable by a variety of ligands, including hormones or neuropeptides. In the inactive complex, GDP is bound to the α subunit and activation of the receptor results in an exchange between GDP and GTP and in the dissociation of the heterotrimer in a monomer (α subunit) and a dimer (β and γ subunits). Both entities can be partners in the regulation of the activity of various enzymes and of ionic channels that in turn control the level of intracellular second messengers (see for review Clapham and Nee, 1993; Nee, 1995).

In Drosophila, genes coding for proteins that belong to the three major families of α subunits (αo, αi, and αq) have been cloned and characterized. Two Gα proteins differing by four amino acids are produced by alternative splicing of a unique gene (Quan and Forte, 1990). The unique gene for Gα produces two transcripts which encode the same protein (Provost et al., 1988). Finally, two Gα proteins have been deduced from cDNA sequence analyses and are issued from a unique gene transcribed in three different transcripts (de Sousa et al., 1989; Schmidt et al., 1989; Thambi et al., 1989; Yoon et al., 1989). Two other α subunits have been identified as homologues of vertebrate Gα (Quan et al., 1993) and Gα(3) (Talluri et al., 1995), respectively. All these proteins are expressed throughout embryogenesis in various tissues in a dynamic pattern (Wolfgang et al., 1991) and, consequently, may play major roles in development although no precise developmental function has been ascribed to any of them. In contrast, it is known that the activity of concertina which encodes the α subunit of a Gα or Gβ protein (Wilkie and Yokoyama, 1994) is necessary for the coordination of cell shape changes in the ventral furrow at gastrulation (Parks and Wieschaus, 1991). In addition, it is a likely candidate to act downstream of a putative secreted protein, Fog, whose receptor is as yet unidentified (Costa et al., 1994).

A mong the different families of Gα proteins, the Gα protein is perhaps the less well characterized in terms of the nature of its associated signal transduction pathway. On the plasma membrane, Gα proteins may regulate Ca2+ and K+ channels (van Dongen et al., 1988; Warnick et al., 1988; Leuss et al., 1991; Lédo et al., 1992), distinct types of phospholipase C (Moriarty et al., 1990), and the mitogen-activated protein kinase cascade (Van Biesen et al., 1996). In addition to interacting with heptahelical receptors such as opioid, α2H-adrenergic, D1, dopaminergic, and somatostatin receptors, the Gα protein has been described recently as a regulator of vesicular traffic (Lagriffoul et al., 1996; Asman et al., 1998).

In Drosophila, the amount of Gα protein is affected in memory mutants (Guillèn et al., 1990) and is expressed in the nervous system during development at the onset of axonal growth (Guillèn et al., 1991; Wolfgang et al., 1991). We show here that the function of Gα is required for the formation of the heart, the visceral musculature, and the nervous system. Based on the phenotype observed in mutants for the gene encoding the α subunit of the Gα protein, we have chosen brokenheart (bkh) as its name.

Materials and Methods

Generation and Analysis of Mutations

The Df(2R)47A deficiency in the bkh gene region was isolated from a cross between A O 65 (a P-element [Pw, lacZ] enhancer trap line in 47A) homozygous males which were γ-irradiated (4,000 rad) and (w; SpCy0) females. Males and females from the F1 generation which were Cy0 but not Sp, were crossed together to obtain the strain. A mong the 5,000 chromosomes which were screened, only one line affected the bkh mRN A expression: Df(2R)47A.

Three P-lines P[w, lacZ] (Török et al., 1993) l(2)k06915, l(2)k11003, and l(2)k07810, inserted on the second chromosome, had been located in the 47A region and were homozygous lethal. PCR analysis of the P-lines’ genomic DNA was used for mapping these insertions. The primers were the 31-bp terminal sequence of the P-element (5′-CGA GGG A CCAC-CTTTAGTTATTTCACTAG-3′) and bkh specific primers: bkh9′ 5′-CA GCC CAATT TGTGGTG GG-3′; bkh9′ 5′-AAC TCT CCA GCATGTA-AG CC CGCT CCT CG-3′. P07810 was localized 1.5 kb upstream of the class II cDNA ATG and the 1.5-kb PCR fragment obtained was sequenced by Genome Express. P06915 and P11003 were localized 800 bp upstream of the class I cDNA ATG. All the mutant stocks were balanced with Cy0 (w, lacZ) to identify the homozygous mutant embryos. A genomic region in the 5′ end of the bkh gene was identified with the aid of the D501563 P1 phage obtained from the Drosophila Genome Project. The 5′ end of the D501383 P1 phage was located 4.5 kb downstream of the class II cDNA ATG. A multiplication of genomic sequences from this region were performed with the pair of specific primers bkh16 and bkh16 (bkh16 5′-CTCG A C TCGA GC GGCTGC-3′). Sequences were made by Genome Express and analyzed with Gene Finder to identify transcription units.

The bkh9′ mutation was generated by P-element mobilization in the P07810 line and a cross with flies bearing (2-3) to distinguish as a source of transposase and of w; Sp/Cyo; (2-3);Sb/TM6B genotype.

P-Element–mediated Transformation

Germline transformation was performed using standard procedures as described by Rubin and Spradling (1982). The bkh cDNA (class I) was subcloned in the pU A ST vector (Brand and Perrimon, 1993) and injected at a concentration of 300 ng/μl with the (2-3) helper plasmid (100 ng/μl) (Robertson et al., 1988). Several independent transformants were obtained and mapped, and lines of interest were made homozygous with the help of a double balanced stock w; Sp/Cyo; M K R 5/TM2.

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Rescue with the GAL4 System

To rescue the Df(2R)47A phenotype at the embryonic stage, we used the yeast GAL4-directed transcription system (Brand and Perrimon, 1993). Females homozygous for a UAS-bkh class II cDNA on the X chromosome (UAS-bkh) and bearing the Df(2R)47A deficiency were crossed to homozygous males bearing the homozygous 2AB-GAL4 driver inserted on the third chromosome and the Df(2R)47A deficiency on the second chromosome. Homozygous animals were sorted out with the aid of a labeled CyO balancer and scored by observation of their phenotypes.

Immunohistochemistry and In Situ Hybridization

Digoxigenin (DIG)-labeled antisense or sense RNA probes were generated from DNA with T3 or T7 RNA polymerase (Promega) and DIG-UTP (Boehringer). They were used for whole mount in situ hybridization of fixed staged embryos prepared as described in Zaffran et al. (1997).

The DIG-labeled RNA probes were detected with the aid of a preadsorbed anti-DIG antibody coupled to alkaline phosphatase (Boehringer) and NBT/BCIP as substrate. The embryos were mounted in Geltol medium (Immunotech) for further observation. A specific probe for class I bkh cDNA was obtained by PCR amplification of genomic DNA with the oligonucleotides bkha and bkha. A probe for class II cDNA was prepared by amplification of the cDNA inserted in pBlueScript with the T7 primer and the bkhh oligonucleotide (bkhh: 5'-GCCATGTCGTAATGGCCTCAGGCGC-3'; bkha: 5'-CCGCTAGCGGTATTGCCTCAGGCGGATATT-3'; bkha, see above).

For immunohistochemistry experiments, embryos were fixed and stained with antibodies according to the protocol described by A. Burnsher (1989). β-Galactosidase in embryos was detected by using a mouse anti-β-galactosidase (Promega) antibody diluted 1,000-fold. The following primary antibodies were used: anti-Bkh-1 (1:100 dilution; Gullien et al., 1991); anti-DMEF2 (1:2,000 dilution; Bour et al., 1995); anti-E11 (1:2 dilution; Azpiazu and Frasch, 1993) and, correlatively, bkh6, anti-DMEF2 (1:2,000 dilution; Bour et al., 1995); anti-EC11 (1:2 dilution; Bodmer, 1993) and, correlatively, Tinman (tin); anti-Tin (1:800 dilution; Azpiazu and Frasch, 1993). Affinity-purified secondary antibodies (Jackson Laboratory) were used in 1:2,1:500, and 1:1,000 dilutions, respectively; Developmental Studies Hybridoma Bank; anti–beta-spectrin (1:500 dilution; Lee et al., 1993); anti-Nrt (1:2,000 dilution; Piovant and Lena, 1988) and anti–Tin (1:800 dilution; Azpiazu and Frasch, 1993).

Afinity-purified secondary antibodies (Jackson Laboratory) were either coupled to alkaline phosphatase or Biotin and used at a 1:500 dilution or conjugated to TRITC or FITC and used at a 1:100 dilution, respectively; Developmental Studies Hybridoma Bank; anti–α-spectrin (1:500 dilution; Lee et al., 1993); anti-Nrt (1:500 dilution; Piovant and Lena, 1988) and anti–Tin (1:800 dilution; Azpiazu and Frasch, 1993).

Both DIG- and NBT/BCIP labeled sequences. The location of the three enhancer trap elements P06915, P11003, and P07810 (Torok et al., 1993) is shown in 5‘ of the two cDNA’s. Numbered horizontal arrows refer to the position and sense of specific oligonucleotides described in Materials and Methods.

Results

The Dynamic Expression of bkh during Embryogenesis Suggests a Developmental Function

The organization of the gene brokenheart (bkh) encoding the α subunit of Go, as described by Yoon et al. (1989), is schematized in Fig. 1. Two classes of cDNA’s (class I and class II) have been characterized (de Sousa et al., 1989; Schmidt et al., 1989; Thambi et al., 1989; Yoon et al., 1989). They arise from an alternative splicing in a unique exon and differ only in the ATG-containing first exon. Three developmentally regulated transcripts (3.4 kb of maternal origin, 4.2 and 6 kb) were revealed (Wolfgang et al., 1991) and by using specific probes, we have shown that the class I cDNA corresponded to the 6-kb mRNA appearing after 12 h of development whereas the class II cDNA corresponded to the 4.2-kb mRNA more abundant in earlier stages (data not shown).

Both cDNA’s encoded proteins of the same size (354 amino acids) that diverged only by 7 amino acids among their 21 NH2-terminal residues and for which it is not known whether they exert different functions.

By using the class II cDNA as probe, a strong expression of bkh was observed in preblastoderm embryos due to the presence of the 3.4-kb maternal transcript (not shown). In early stage 11 embryos, the zygotic transcript could be detected for the first time in clusters of cells within 11 segments (Fig. 2, a and b). The cells appear to be cardial and visceral muscle progenitor cells since, based on bkh staining patterns in later stage 12 embryos, the cells became integrated into the conspicuous monocellular layer of cardial and visceral mesoderm cells on each side of the embryo (Fig. 2, c–e). This assumption was further supported by bkh expression in embryonic tissues which were unambiguously constituted of such cells (Fig. 2 e) and, also, in tinman (tin) loss of function mutants. In tin mutants, neither heart nor visceral muscles are formed (Azpiazu and Frasch, 1993; Bodmer, 1993) and, correlatively, bkh expression was completely abolished in the territories from which the precursor cells for these two tissues originated (Fig. 2 g). From the middle of stage 11 onwards, neuroblasts of the central nervous system (CNS) became labeled (Fig. 2 c) and bkh expression persisted in the neurons of the CNS in later stages of embryogenesis (Fig. 2, d–f). In a similar way, all the neurons of the peripheral nervous system (PNS) expressed the bkh mRNA from stage 12 onwards, slightly before the onset of axonogenesis (Fig. 2, d and f). Probes for either cDNA gave identical spatial patterns of expression although class I transcripts were quantitatively less abundant and were expressed later than the class II transcripts (not shown).

A nitrobiodes directed against a COOH-terminal peptide whose sequence was conserved in the α subunit of all Go proteins showed that the pattern of expression of the pro-
tein was superimposable to that of the mRNA during embryogenesis (Fig. 2, i and j). However, probably due to the presence of the protein of maternal origin, these antibodies were poorly efficient in detecting a significant signal in the cardial cells as early as did mRNA probes.

**bkh Is an Early Marker of the Mesenchymal-Epithelial Transition Undergone by the Cardioblasts**

In stage 11 embryos, the zygotic bkh mRNA was detected at first in the precursors of the cardioblasts in a repeated pattern of 11 clusters in the dorsal mesoderm (Fig. 3, a and b). These clusters, constituted initially of two to four cells, were located in the anterior compartment of each mesodermal parasegment in a position anterior to the domain of En ectodermal stripes (Fig. 4) and closely neighboring the Wg-expressing cells in parasegments 2–12 of the overlying ectoderm. These cardioblast precursors belonged to the functional domain of Ladybird (Lbd) and of Even-skipped (Eve) expressing cells in the mesoderm which are the precursors of a fraction of the pericardial cells and of a dorsal muscle (see for example, Lawrence et al., 1995; A-z-
Within the cluster, the expression of the bkh mRNA was initially very intense in only two of the cells and then it became detectable in the four cells with an almost uniform intensity. Due to their position relative to the parasegment boundary, we call these cells A (for anterior) cardioblasts. Soon after the onset of bkh expression in the first cluster, a second cluster of stained cells appeared posterior to the first one and not in continuity with it (Fig. 3, c and d). This group of cells called P (for posterior) cardioblasts was located in the posterior domain of the next parasegment and it never contained more than two bkh-expressing cells. Their position coincided with that of an area posterior to En stripes in the ectoderm (Fig. 4).

During germ-band retraction (late stage 11 and stage 12), the cardioblast progenitors that are mesenchymal in nature at that stage began to establish contacts between themselves, to extend filopodes (Rugendorff et al., 1994; Dunin-Borkowski et al., 1995), and to reorganize their shape to form a continuous layer on each side of the dorsal opening (Fig. 3, e–j). The origin of this process was the result of the encounter of the A and P groups of bkh-expressing cells in each parasegmental domain (Fig. 3, g and h). Later in development and due to the shortening of
The segment during germ-band retraction, the segmentally repeated six cell clusters spread along the anterior-posterior axis to finally join as a monolayer of polarized epithelial cells (Fig. 3, g–j). Still further in the process, the two rows of cardial cells, together with the pericardial cells that are attached to their basal membrane, fused at the dorsal midline to form the heart tube. Our observations led to the conclusion that a mature heart at the end of embryogenesis was constituted of 52 cardial cells per hemiembryo partitioned in six cells in every segment from T3 to A7 and in only four cells in the T2 segment. The results described above suggest the existence in each segment of two distinct populations of cells composed of four and two cardial cells that derived from mesodermal cells and that were located in domains corresponding, respectively, to the anterior and posterior compartment of each parasegment (Fig. 4). None of the cardioblasts present in parasegments 2 and 3 and only a part of those found in PS4 participated in the mature dorsal vessel, but it was, however, difficult to follow their fate since the expression of bkh vanished rapidly from these cells during development.

Similarly, by using the EC11 antibody specific for the pericardial cells (Zaffran et al., 1995), we counted 36 such cells distributed in 4 cells per segment from T2 to A7. Eve-expressing cells from the labial segment and the segment T1 did not contribute to the pool of pericardial cells and they probably gave rise to other structures that we were unable to recognize.

The cardial cells, which are also muscular cells, were determined as epithelial (Rugendorff et al., 1994; Tepass, 1997), based on the expression of several markers for polarity (see below). For example, antibodies directed against α-spectrin specifically labeled the basal-lateral membrane of the epithelium (Lee et al., 1993) (see Fig. 8 a) and the staining by anti-Neurotactin antibodies (Nrt) (Piovant and Lena, 1988) was restricted to the lateral and apical domains of the cell surface (see Fig. 8 e). In contrast, phosphotyrosyl proteins (Woods et al., 1997) and Armadillo (Müller and Wieschaus, 1996) were weakly expressed in the heart cell membranes and had no polarized localization (not shown), in good agreement with the lack of authentic Zonulae adherens in Drosophila secondary epithelia (Tepass and Knust, 1993; Tepass and Hartenstein, 1994). Finally, the basal membrane of the epithelium was visualized with the mAb E C11 (Zaffran et al., 1995). This antibody recognized an antigen probably secreted by the pericardial cells which was detected on the basal membrane of the cardial cells and around the pericardial cells (see Fig. 8 a).

In the visceral mesoderm that also undergoes a mesenchymal-epithelial transition before its differentiation into the visceral musculature, bkh was expressed in a repeated...
pattern of clusters of cells in the same 11 parasegments as the precursors of the cardial cells and anterior to them (data not shown; Azpiazu et al., 1996; Riechmann et al., 1997). These precursor cells in parasegments 4–12 were ultimately transformed in a polarized epithelial ribbon of one cell in width (Bate, 1993), whose polarity was assessed by the localization of Fasciclin III (Patel et al., 1987) to cell-cell contacts (see Fig. 8 i).

**bkh Is Required for the Formation of the Cardial and of the Visceral Mesoderm Epithelia**

The embryonic expression of bkh suggests a function of the protein G, in the formation of the cardial and visceral mesoderm epithelia as well as in axonal growth. A deficiency in the 47A region in which is localized the bkh gene was obtained by using as a starting tool the homozygous viable A Q65 line whose lac Z reporter gene was specifically expressed in the entire nervous system. Among the 5,000 chromosomes mutagenized by x-ray irradiation and screened for the loss of the phenotypic white marker, a lethal deficiency Df(2R)47A was obtained. It was shown by cytology that it had lost half of each of the A and B bands in 47 (not shown). Quantitative analyses of Western blots revealed that in animals heterozygous for that deficiency, half the amount of the BKH protein was produced as compared with its production in the initial AQ65 line (not shown). Moreover, no mRNA could be detected in homozygous mutant embryos (Fig. 2 h).

Three P-insertion lines, issued from the systematic screen of lethal P-insertions on the second chromosome (Török et al., 1993), have been located within the cytologi-

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**Figure 5.** Heart and visceral musculature phenotypes in bkh-deficient embryos. The phenotype of homozygous Df(2R)47A embryos was analyzed in the heart by using anti-E C11 (c and d) and anti-D Mef 2 (f–h) antibodies as probes. The phenotype in the visceral mesoderm was assessed with an anti-Fas III antibody (j). Figures in b, d, f, and h are enlargements of those in the left panel. (a, b, e, and i) Wild-type embryos. A frequently observed heart phenotype is interruption of the dorsal vessel (c, d, g, and h; black arrows). In f and h, the cardial cells are present in dorsal position but they are organized as clusters of cells (arrowheads) no longer forming a continuous line. The same types of defects are seen in the visceral mesoderm (j; arrowhead and black arrow). In all views, anterior is left, dorsal is up.
cal region of the bkh gene. In the P07810 insertion, the lacZ reporter gene expression was superimposable to that of the bkh mRNA (Fig. 2 k), whereas, in the P06915 and P11003 insertions, the β-galactosidase activity was restricted to the visceral mesoderm (Fig. 2 l). The different insertions were mapped by PCR in the 5' region of the class I and class II cDNAs, respectively (see Fig. 1 and Materials and Methods). The lethality in these lines was not consecutive to the insertion of the transposon but was rather due to a secondary mutation since the homozygous lethal lines were perfectly viable in trans of Df(2R)47A and of other deficiencies uncovering the same region [Df(2R)E3363, Df(2R)X1, Df(2R)Stan2, Df(2R)12].

In contrast, in the P07810 line, mobilization of the P-element has resulted in the obtention of several viable revertants and of one lethal bkh007 white revertant in trans of Df(2R)47A. Homozygous bkh007 embryos had the same heart and visceral muscle phenotype as that displayed by the homozygous Df(2R)47A-deficient animals (see below).

To analyze the molecular lesions resulting from the imprecise excision of the P07810 transposon, the 5' region of the gene in which P07810 lies has been further characterized. A P1 phage (DS01583) covering the whole length of the bkh gene contained 4.5 kb of genomic sequence upstream of the class II cDNA ATG. This fragment was cloned, fully sequenced, and analyzed for a more detailed definition of the class II transcription unit. Three canonical potential TATA boxes were located within a 510-bp sequence downstream of the P07810 insertion site. The analysis of the bkh007 allele showed that a segment of 839 bp was deleted that contained the three potential TATA boxes together with 311 bp of the 5' end of the transcription unit. As a consequence, no class II mRNA was produced in the homozygous bkh007 embryos.

Homozygous Df(2R)47A embryos (recognized by the lack of a labeled CyO balancer) harbored defects in the three tissues in which bkh was expressed with a total penetrance meaning that all homozygous Df(2R)47A embryos had defects in the heart, the visceral mesoderm, and the nervous system. When probed with anti-EC11 antibodies (Zaffran et al., 1995), the mutant embryos showed interruptions in their dorsal vessel (Fig. 5, c and d) that were also visible when DMef2 expression was investigated in all the muscle cells including the cardial cells (Fig. 5, f–h) (Bour et al., 1995). The cardial cells were unambiguously present and they had migrated properly in dorsal position, but, in some places, they were no longer arranged as a continuous layer but rather as unorganized clusters of cells (Fig. 5, f–h). Fas III expression revealed the same types of defects in the visceral mesoderm (Fig. 5 j). Similar alterations were detected in bkh007 mutants with the same total penetrance (not shown).

In the nervous system, longitudinal axons were often missing (Fig. 6 b) and important modifications were observed in the guidance and axonal growth of motoneurons (not shown). It should be recalled here that the lola gene was also deleted in the Df(2R)47A deficiency and that mutations in this gene result in missing longitudinal axons in the CNS (Fig. 5; Seeger et al., 1993; Giniger et al., 1994). Since the Df(2R)47A phenotype was slightly stronger (in terms of penetrance of the phenotype) than that provoked by a loss of function of lola, this suggests a function per se for Gα in axonal growth or guidance. This was confirmed by the analysis of the bkh007 mutation which fully complemented mutations in the lola gene but continued to display a neuronal phenotype. In homozygous bkh007 mutants, axons of the motoneurons were clearly misrouted (not shown). However, in contrast to the Df(2R)47A mutation, the longitudinal axons in the CNS were missing with a lower frequency but were often pinched (Fig. 6 d).

The heart and visceral mesoderm phenotypes could be rescued at least partially by expressing the class II cDNA under the control of 24B-GAL4 (Brand and Perrimon,
Figure 7. Expression of bkh in the whole mesoderm rescues the heart phenotype. a and b represent two different plans of focus on the same embryo, homozygous for the Df(2R)47A deficiency probed with the anti-EC11 antibody (a) and the anti–Fas II antibody (b). The class II bkh cDNA under the control of 24B-GAL4 is expressed in the whole mesoderm. The heart appears similar to that in wild-type embryos (a) while the nervous system remains disrupted (b). In both views, anterior is left and dorsal is up.

The brokenheart Gene Function Is Required for the Morphogenesis of the Heart and Visceral Musculature

In a deficiency, Df(2R)47A, which completely deleted the brokenheart gene encoding the \( \alpha \) subunit of the heterotrimeric \( G_\alpha \) protein, the morphogenesis of the heart and of the visceral mesoderm was impaired. The defects associated to the nervous system have not been studied in detail, essentially because this deficiency also uncovered the longitudinal absent gene (lola) whose requirement for axonogenesis of longitudinal fascicles is known (Seeber et al., 1993; Giniger et al., 1994). However, several arguments strongly suggest that the phenotypes associated to the heart morphogenesis and to the visceral musculature were a consequence of a loss of the function of bkh. First, in deficient animals, alterations were restricted to the tissues that expressed bkh and they appeared in synchrony with the temporal expression of bkh. Second, P-element mobilization in enhancer trap lines located within the bkh gene and imprecise excision of the P transposon led to the isolation of mutants with phenotypes identical to those observed in homozygous Df(2R)47A embryos. Finally, we were able to rescue the heart and visceral mesoderm embryonic phenotypes in homozygous bkh mutants by expressing the cDNA encoding bkh in transgenic flies. The failure to totally rescue the phenotype probably resulted from the fact that 24B-GAL4 did not drive the expression of the UAS-bkh cDNA in mesodermal cells early enough to be totally efficient (Michelson, 1994).

Although the heart was perturbed in the totality of the mutant embryos (100% penetrance), defects were observed only in some of the cells that constituted the dorsal vessel. This result does not support well the contention that bkh is an essential partner in the acquisition of polarity for the epithelial cardiac cells since, if this were the case, all the cells would be equally affected. The maternal protein which is abundant and stable until mid-embryogenesis could have partially compensated the loss of zygotic transcript. Production of germline clones lacking bkh activity could help to elucidate this point. Moreover, an-
Figure 8. The heart and visceral mesoderm epithelia as observed by confocal microscopy are not properly polarized in bkh mutants. (a, e, and i) Wild-type embryos; (b, f, and j) homozygous Df(2R)47A embryos. The images of double labeling experiments (a and b) with anti-α-spectrin (green) and anti-EC11 (red) antibodies or of labeling with anti-Nrt antibody (e and f) and anti-Fas III antibody (i and j) have been obtained with the aid of a confocal microscope. c, d, g, h, k, and l are drawings of the epithelial heart (c, d, g, and h) or of the visceral mesoderm (k and l) seen in the corresponding photographs. In these drawings, only the epithelia of interest are shown to avoid a confusing impression due to other layers that are also labeled by the antibodies. Antibody staining is in green or in red. Membranes of cells that are not labeled or of mutant cells that are poorly labeled are outlined by gray lines or gray dotted lines. (a and c) The polarized nature of the heart epithelial cells is revealed by the anti-α-spectrin antibody (green) which stains the basal-lateral but not the apical face of the cells and by the anti-EC11 antibody (red) which labels the basal membrane. The two rows of epithelial cells have not yet fused at the dorsal midline to form the heart tube. (d) In the mutant, the polarity of the cardial cells is impaired in some places as visualized by the lower level of expression of α-spectrin and its uniform localization on the surface of the cardial cells. In addition, the EC11 labeling is no longer detected on the basal face (white arrow in b). Only one row of epithelial cardioblasts is shown in the optical section below the ectodermal layer. (e and g) Nrt is mainly localized to cell-cell contacts in the cardial epithelium in a stage 16 embryo, in which the two rows of cells have joined at the midline. (f and h) Nrt is hardly detectable in some places and the cells appear round without polarity. (i and k) Fas III is localized to the lateral membrane at cell-cell contacts. At the stage shown, the visceral mesoderm is constituted of two rows of cells. (j and l) In the mutant, Fas III is expressed to a lower extent and in a nonpolarized manner (arrow). ec, ectoderm; cc, cardial cells; bm, basal membrane; am, apical membrane; vm, visceral mesoderm.
other heterotrimeric \( G \) protein is expressed in the cardial cells (Wolfgang et al., 1991) and functional redundancy might have been at work.

**The bkh Gene Function Is Required for the Acquisition of Cell Polarity in the Morphogenesis of the Heart and of the Vasculature**

The myoendothelial heart tube is considered as a secondary epithelium that forms by a mesenchymal-epithelial transition (Uegendorf et al., 1994; Tepass and Hartenstein, 1994; Zaffran et al., 1995; Tepass, 1997). The mesenchymal cardial precursor cells, after their migration from the ventral site of gastrulation in the direction of their final dorsal position, reorganize their plasma membrane to acquire their cellular polarity and establish cell junctions to build up the cardial epithelium. A signal must be received by the mesenchymal cells to create the first asymmetry on their surface (for review see Drubin and Nelson, 1996). Interactions with localized extracellular matrix components are believed to be largely responsible for this first event (Drubin and Nelson, 1996; Tepass, 1997) and Drosophila mutants, for example, in Laminin A (Yarnitzky and Volk, 1995; Tepass, 1997) or in an integrin subunit (Stark et al., 1997), present severe disruptions in their dorsal vessel. The initiating signal is probably emitted in response to inductive interactions between the mesenchymal cells and the overlying dorsal ectoderm. The newly created asymmetry could then trigger a reorganization of the cytoskeleton. Finally, the different membrane domains (apical and basal-lateral) will be established by the acquisition of different combinatorials of membrane proteins that have been specifically routed towards them; this sorting-out step relies on an absolute specificity of the vesicular traffic (for review see Rodriguez-Boulan and Powell, 1992). In the absence of bkh function, the mesenchymal cardial cells did not remodel their shape and, in the case of the most extreme phenotype, they failed to form a continuous row of cells. In addition, whenever such a layer was eventually formed, in several places some but not all cells neither acquired nor maintained a proper polarity.

Based on the timetable of bkh expression, its participation in the first step creating the asymmetry of the cardinal cells is not very likely. It might rather be required for the subsequent steps that concern cell shape change and polarization by addressing membrane proteins to their respective domains.

It has been suggested that heterotrimeric \( G \) proteins could contribute to the vesicular protein traffic by regulating early steps in the secretory pathway (for review see Nuoffer and Balch, 1994). This hypothesis stems from the observation that AIF4-\( \alpha \), an activator of heterotrimeric but not of monomeric \( G \) proteins, inhibits ER to Golgi and intra-Golgi transport as well as vesicle budding from the trans-Golgi network. In particular, \( G \) proteins have been implicated in granule exocytosis from chromaffin cells (Vitale et al., 1993; Gasman et al., 1998), insulin secretion (Lang et al., 1995), and transcytosis (Bomsel and Mostov, 1992). It has been shown that the secretion of the protease Nexin-1 by glialoma cells was under the control of \( G \)\( \alpha \)1 (Lagriffoul et al., 1996). The \( G \)\( \alpha \)1 protein was detected on the membrane of small intracellular vesicles and the secretion of Nexin-1 was stimulated by \( G \)\( \alpha \)1 overexpression and by activators of \( G \)\( \alpha \) proteins such as mastoparan. It was further suggested that the GTPase activity of the \( G \)\( \alpha \)1 protein could be stimulated in the absence of a classical serpentine receptor (Lagriffoul et al., 1996).

Thus, we are tempted to predict that the Drosophila \( G \)\( \alpha \) protein has a function in a particular type of vesicular trafficking responsible for the acquisition or maintenance of cell polarity in the cardial and visceral mesoderm cells. Preliminary observations on the subcellular localization of Bkh in embryonic cells were consistent with this prediction. In the totality of the cells examined, Bkh was located to the cytoplasm rather than associated to the cell membrane and the staining pattern revealed a typical granular appearance (data not shown).

Since the exportation and the localization to the plasma membrane of the protein markers we have used were all equally affected in bkh mutants, we conclude that bkh is involved in a general aspect of vesicular traffic rather than in the specific process of the sorting out of membrane proteins.

bkh might also be required for the reorganization of the cytoskeleton. The protein G encoded by the concertina gene participates in cell shape changes taking place at gastrulation (Parks and Wieschaus, 1991), probably via a modulation of the invaginating blastoderm cell cytoskeleton resulting from the activation of Ras (Hall, 1998).

The early role of the \( G \)\( \alpha \) protein in the formation of the heart epithelium does not exclude a function in later events leading to the formation of the heart or to the acquisition of its function. It has been shown recently that knocking-out the \( G \)\( \alpha \) gene in the mouse resulted in heart dysfunction. \( G \)\( \alpha \)-deficient mice had lost the muscarinic inhibition of isoproterenol-stimulated cardiac L-type \( \text{Ca}^{2+} \) currents (Valenzuela et al., 1997; Jiang et al., 1998). It will be interesting to investigate whether the Drosophila \( G \)\( \alpha \) protein could also be involved in such a process.

**The Lineage of the Cardial Cells**

Before the determination of cardiac precursors in the mesoderm, the overlying ectoderm is subdivided in segmentally repeated units partitioned into an anterior compartment (A-compartment) and a posterior compartment (P-compartment). A analysis of the expression of genes involved in the specification of mesodermal derivatives and other observations lend support to the idea that, after gastrulation, the mesoderm also becomes subdivided into segmentally repeated units, each of which consists of two separate domains (Drubin-Borkowski et al., 1995; Azpiazu et al., 1997; Riechmann et al., 1997). The domains that are located below the ectodermal P-compartment are subject to influences from the striped regulators eve and hh and have been termed “P-domains” or “eve-domains.” By contrast, the development of the metameric domains that are located below the A-compartment depends largely on the striped regulators wg and slp (slouchy-paired) and these domains have been termed “A-domains” or “slp-domains.”

In that scheme, three basic groups of genes are at work to pattern the mesoderm either along the dorso-ventral axis (dpp and tinman) to specify the dorsal mesoderm, or along the anterior-posterior axis (wg and slp) to subdivide
it into segmental units, or at defined positions to control tissue specification. For example, recent evidence suggests that wg, whose expression is restricted to striped domains in each of the A-compartments and which is required for a variety of inductive signaling events during embryonic development, is directly involved in heart formation, in that it is necessary for further subdividing of the dorsal mesoderm and for specifying cardiac cell fates. Elimination of the wg function shortly after gastrulation, at a time when tin becomes restricted to the dorsal mesoderm, results in the selective loss of heart progenitor cells with little effect on segmental patterning of the cuticle or other mesodermal derivatives (Baylies et al., 1995; Lawrence et al., 1995; Wu et al., 1995; Azipazu et al., 1996; Park et al., 1996). From these and other observations, a picture has emerged in which specification of precardiac and dorsal somatic muscle precursors requires intersections of the dorsal domains of dpp expression with the transverse stripes of the dorsal expression of wg (Bodmer and Frasch, 1998).

However, the results presented herein are merely consistent with some precursors of the cardiac cells originating from the P-domains and subjected to the influence of hh signaling rather than to that of wg. This hypothesis is somewhat difficult to verify because wg expression after gastrulation requires hh and vice versa (Klingensmith and Nusse, 1994), but, later in embryogenesis, the two signals become independent. Therefore, we have investigated the expression of Tin in temperature-sensitive hh

The observation that the cardiac progenitor cells can be divided into two cell subpopulations is consistent with the situation in the mature heart tube in which two genetically distinct populations of cardiac cells have been described (Bodmer et al., 1997). For example, tin (see for example Bodmer, 1993; Jagla et al., 1997) as well as β3-tubulin (Leiss et al., 1988) and several P-lacZ reporter genes from enhancer trap lines (Hartenstein et al., 1992) are expressed in only four cell pairs per segment among the six pairs present. In the same line, a D-mef2 enhancer element directed lacZ transcription in four cardiac cell pairs per segment consistent with a direct regulation by tin which is expressed in these same cells (Gajewski et al., 1997). Interestingly, these two subpopulations of cardiac cells, respectively, reside below the anterior and the posterior ectoderma parasegmental domains (Fig. 4).

These different observations could mean that the two P-cardioblasts were specified by the inductive instruction of hh rather than by that of wg. However, it is not known whether the hh pathway provides a direct late cardiogenic signal or exerts its effect via suppressing the wg function in the posterior domain. This hypothesis is unlikely in that, at that stage, reducing the function of hh in the epidermis does not lead to any visible effect on the wg signaling pathway (H eesker and D i Nardo, 1994). Indeed, wg expression in the dorsal epidermis was not expanded in a hh mutant (not shown). We predict then that hh might behave as a repressive signal for tin expression in the two P-cardioblasts. Cell heterogeneity in terms of gene expression could then be achieved along the anterior-posterior axis by an efficient cooperativity of wg and hh signals in the specification of the cardiac cells.

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References

A sburner, M. 1989. Drosophila: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

A zpiizou, N., and M. Frasch. 1993. tinman and bagpipe two homeobox genes that determine cell fates in the dorsal mesoderm of Drosophila. Genes Dev. 7:1252–1340.

A zpiizou, N., P. Lawson, J. P. Vincent, and M. Frasch. 1996. Segmentation and specification of the Drosophila mesoderm. Genes Dev. 10:3183–3194.

B ate, M. 1993. The mesoderm and its derivatives. In The Development of Drosophila melanogaster, M. Bate and A. Martinez Arias, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1013–1090.

B aylies, M.K., A. Martinez Arias, and M. Bate. 1995. Genes and development. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

B ombel, R., and M. Forte. 1990. Two forms of F as II produced by alternate splicing involving an unusual splice site. J. Cell Biol. 119:845–863.

B omsel, M., and K. Mostov. 1992. Role of heterotrimeric G proteins in interaction and intracellular signaling. J. Biol. Chem. 267:15904–15908.

B rachspuhl, A., P. M. Snow, and C. S. Goodman. 1987. Characterization and cloning of the Drosophila mkk-2 gene, a member of the mitogen-activated protein kinase family. Genes Dev. 1:1889–1899.

B rand, A., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 118:415–424.

Clapham, D., and E. Neer. 1993. New roles for G-protein a/dimers in transmembrane signaling. Nature. 365:403–406.

Costa, M. E., W. Wison, and E. Wieschaus. 1994. A putative cell signal encoded by the frizzled gene coordinates cell shape change during Drosophila gastrulation. Cell. 76:1075–1089.

de Sousa, S., L. Hoveland, S. Y. Arfittz, and J. Hurely. 1989. The Drosophila G_α-like protein encodes multiple transcripts and is expressed in the nervous system and in ovaries. J. Biol. Chem. 263:18544–18551.

Drubin, D. G., and W. J. Nelson. 1996. Origins of cell polarity. Cell. 83:335–344.

Dunin-Borkowski, E., T. D. J. Borkowski, and D. M. Borkowski. 1995. The mesoderm and its derivatives. In The Development of Drosophila melanogaster, M. Bate and A. Martinez Arias, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1013–1090.

F rasch, M. 1995. Induction of visceral and cardial mesoderm by ecdysteroid Dpp in the early Drosophila embryo. Nature. 374:464–467.

Gajewski, K., Y. Kim, Y. M. Lee, E. N. Olson, and R. A. Schulz. 1997. D-mef2 is a target for Tinman activation during Drosophila heart development. EMBO J. 16:515–522.

Garcia-Alonso, L., M. F. VanBerkum, G. Grenningloh, C. Schuster, and C. S. Goodman. 1995. Fasciclin II controls neuronal gene expression in Drosophila. Dev. Biol. 166:396–414.

Gasman, S., S. Chasserot-Golaz, P. Hubert, D. Aunis, and M. F. Bader. 1998. Identification of a potential effector pathway for the trimeric G_o protein associated with secretory granules. J. Biol. Chem. 273:16913–16920.

Ginger, F., K. John, and Y. J. 1994. Isolation and characterization of the Drosophila tinman tinman-like protein. J. Biol. Chem. 269:10501–10505.

Guillen, A., V. Jallon, J. Fehrentz, C. Pantaloni, J., Bockaert, and V. Homburger. 1990. A _α- like protein in Drosophila melanogaster and its expres-
Rubin, G.M., and A.C. Spradling. 1982. Genetic transformation of Drosophila melanogaster. Genetics. 110:461-470.

Rodriguez-Boulan, E., and S.K. Powell. 1992. Polarity of epithelial and neuronal cells. Annu. Rev. Cell. Biol. 8:395-427.

Rubin, G.M., and A.C. Spradling. 1982. Genetic transformation of Drosophila with transposable element vectors. Science. 218:348-353.

Rugendorff, A., A. Younossi-Hartenstein, and V. Hartenstein. 1994. Embryonal origin and differentiation of the Drosophila heart. Roux’s Arch. Dev. Biol. 203:266-280.

Schmidt, C., S. Garen-Fazio, Y. Chow, and E. Neer. 1989. Neuronal expression of a newly identified Drosophila melanogaster G subunit. Cell Regul. 1:125-134.

Seeger, M., G. Tear, D. Ferres-Marco, and C.S. Goodman. 1993. Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline. Neuron. 10:409-426.

Seegar, W., W. Wolfgang, N. Thambi, and M. Bate. 1994. The development of cellular junctions in Drosophila. Nature. 372:783-786.

Stark, K.A., G.H. Yee, C.E. Roote, E.L. Williams, S. Zusman, and R.O. Hynes. 1997. A novel alpha integrin subunit associates with betaPS and functions in tissue morphogenesis and movement during Drosophila development. Development. 124:4583-4594.

Talluri, S., A. Bhatt, and D. Smith. 1995. Identification of a Drosophila G-protein alpha subunit (DsGo-3) expressed in chemosensory cells and central nervous system. Proc. Natl. Acad. Sci. USA. 92:11475-11479.

Tepass, U. 1997. Epithelial differentiation in Drosophila. Bioessays. 9:673-682.

Tepass, U., and V. Hartenstein. 1994. The development of cellular junctions in the Drosophila embryo. Dev. Biol. 161:563-596.

Tepass, U., and E. Knust. 1994. dpp induces mesodermal gene expression in Drosophila. Nature. 372:783-786.

Talluri, S., A. Bhatt, and D. Smith. 1995. Identification of a Drosophila G-protein alpha subunit (DsGo-3) expressed in chemosensory cells and central nervous system. Proc. Natl. Acad. Sci. USA. 92:11475-11479.

Tepass, U. 1997. Epithelial differentiation in Drosophila. Bioessays. 8:673-682.

Tepass, U., and V. Hartenstein. 1994. The development of cellular junctions in the Drosophila embryo. Dev. Biol. 161:563-596.

Tepass, U., and E. Knust. 1994. Crumbs and Stardust act in a genetic pathway that controls the organization of epithelia in Drosophila melanogaster. Dev. Biol. 159:311-326.

Tjani, N., F. Quan, W. Wolfgang, A. Spiegel, and M. Forte. 1989. Immunological and molecular characterization of G alpha-like proteins in the Drosophila central nervous system. J. Biol. Chem. 264:18552-18560.

Török, T., G. Tick, M. Avarado, and I. Kiss. 1993. P-lacW insertional mutagenesis on the second chromosome of Drosophila melanogaster: isolation of lethal with different overgrowth phenotypes. Genetics. 110:71-80.