The EMBO Journal Vol.17 No.15 pp.4304–4312, 1998

Embryonic cardiomyocyte hypoplasia and craniofacial defects in $G\alpha_q/G\alpha_{11}$-mutant mice

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Heterotrimeric G proteins of the G_q class have been implicated in signaling pathways regulating cardiac growth under physiological and pathological conditions. Knockout mice carrying inactivating mutations in both of the widely expressed $G\alpha_q$ class genes, $G\alpha_q$ and $G\alpha_{11}$, demonstrate that at least two active alleles of these genes are required for extrauterine life. Mice carrying only one intact allele [$G\alpha_q^{(-/+)};G\alpha_{11}^{(-/-)}$ or $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$] died shortly after birth. These mutants showed a high incidence of cardiac malformation. In addition, $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$ newborns suffered from craniofacial defects. Mice lacking both $G\alpha_q$ and $G\alpha_{11}$ [$G\alpha_q^{(-/-)};G\alpha_{11}^{(-/-)}$] died at embryonic day 11 due to cardiomyocyte hypoplasia. These data demonstrate overlap in $G\alpha_q$ and $G\alpha_{11}$ gene functions and indicate that the G_q class of G proteins plays a crucial role in cardiac growth and development.

Keywords: cardiac malformation/craniofacial defect/ G protein/heart defect/knockout

Introduction

During embryonic development, enlargement of the heart is primarily dependent on the increase in myocyte number. Shortly after birth, cardiac myocytes lose their proliferative capacity, and further growth of the myocardium in response to increased workloads occurs through hypertrophic enlargement of existing myocardial cells (Chien *et al*., 1993; Van Heugten *et al*., 1995; Yamazaki *et al*., 1995). Identification of the factors controlling myocardial proliferation and development is of great importance for understanding both the pathogenesis of congenital heart diseases and cardiac growth responses to physiological and pathological stimuli.

Various hormones and neurotransmitters such as angiotensin II, endothelin, norepinephrine or prostaglandin $F_{2\alpha}$ have been implicated in the development of myocardial hypertrophy (Shubeita *et al*., 1990; Knowlton *et al*., 1993; Sadoshima *et al*., 1993; Ito *et al*., 1994; Adams *et al*., 1996; Sakai *et al*., 1996; Yamazaki *et al*., 1996). Each of these agonists signals through G protein-coupled receptors which stimulate phospholipase C-β isoforms via activation of G proteins of the G_q class. Hence, the G_q/phospholipase C-β-mediated pathway has been implicated in the regulation of myocardial growth and development (Chien *et al*., 1993; La Morte *et al*., 1994; Post and Brown, 1996). Mammals express four G_q class α-subunits of which two, $G\alpha_q$ and $G\alpha_{11}$, are widely expressed and are primarily responsible for coupling receptors to phospholipase C-β (Simon *et al*., 1991; Exton, 1996). In contrast, expression of $G\alpha_{14}$ and $G\alpha_{15/16}$ is restricted to certain tissues, such as kidney and hematopoietic organs, respectively (Amatruda *et al*., 1991; Wilkie *et al*., 1991). Mice have been produced with null mutations in each α-subunit gene of the G_q class. Interestingly, only $G\alpha_q$-deficient mice revealed obvious phenotypic defects, including cerebellar ataxia and deficiencies in primary hemostasis due to a defect in platelet activation (Offermanns *et al*., 1997a,b). The observed phenotypes correlated with preferential or exclusive expression of $G\alpha_q$ in the affected cell types of the cerebellum and platelets. By contrast, heart tissue expresses both $G\alpha_q$ and $G\alpha_{11}$ (Wilkie *et al*., 1991). Since heart morphology and function in $G\alpha_q$- and $G\alpha_{11}$-deficient animals appeared to be normal we tested whether these genes have overlapping functions during heart development.

To study the role of the G_q class of G proteins in cardiac development and growth we generated mice carrying inactivating mutations of the $G\alpha_q$ gene (*Gnaq*) and the $G\alpha_{11}$ gene (*Gna11*). Genetic, morphological and pharmacological analysis of intercross offspring inheriting different combinations of these two mutations identified roles for $G\alpha_q$ and $G\alpha_{11}$ in embryonic cardiomyocyte proliferation and craniofacial development.

Results

Derivation of $G\alpha_{11}$-deficient mice

Production of $G\alpha_q$-deficient mice was as previously described (Offermanns *et al*., 1997a). To derive $G\alpha_{11}$-deficient mice, we disrupted one allele of the murine $G\alpha_{11}$ gene in mouse embryonic stem (ES) cells using the positive–negative selection method (Figure 1). The *Gna11* targeting vector was designed to replace exons 3, 4 and a portion of exon 5 with the Pgk::Neo transgene. Even in the unlikely event that alternative RNA splicing would remove the Pgk::Neo insert, the deletion should result in non-functional $G\alpha_{11}$ protein since these exons in *Gna11* encode amino acids that contribute to the guanine nucleotide binding pocket (Bourne *et al*., 1991; Mixon *et al*., 1995). Five ES cell clones were confirmed to have the predicted 5′ and 3′ restriction fragments upon hybridization with two diagnostic probes (Figure 1A and B). ES cell clones which were heterozygous for the mutation

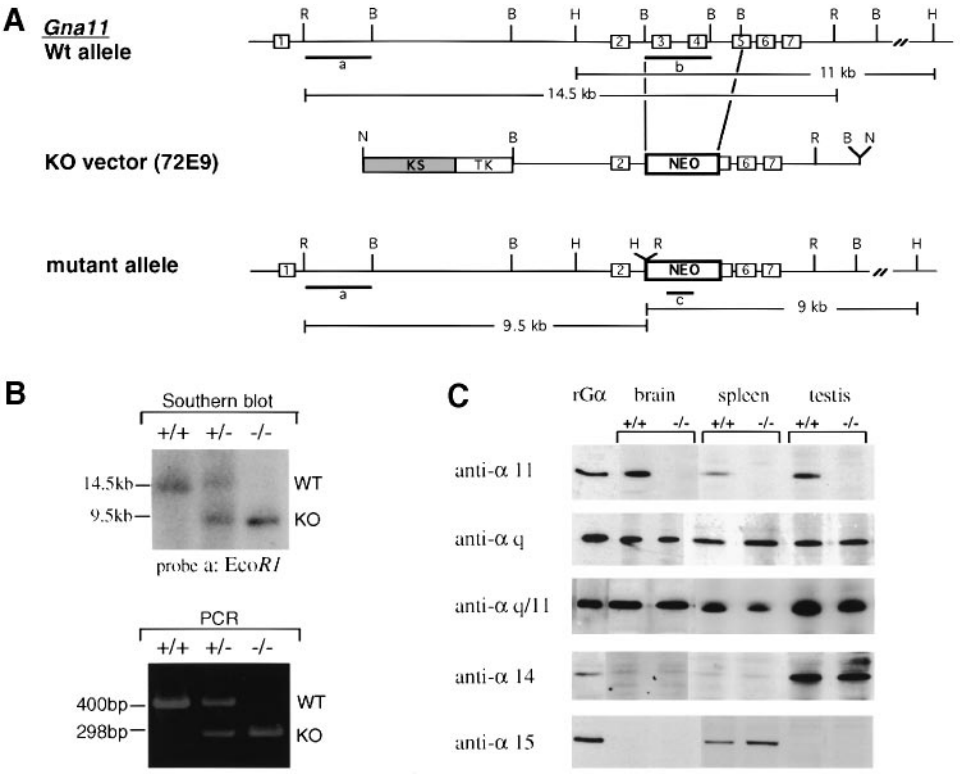

Fig. 1. Targeted disruption of the $G\alpha_{11}$ gene (*Gna11*). (**A**) *Gna11* was disrupted by homologous recombination between the wild-type (Wt) locus and the knockout (KO) vector to generate the *Gna11*-1-mutant allele. Hybridization probes (a,b and c) for Southern blots are indicated by underlaying heavy lines. The length of diagnostic restriction endonuclease fragments are indicated. B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI. NEO, PgK::Neo selectable marker gene; KS, bluescript KS+ vector sequence; TK, herpes thymidine kinase gene. (**B**) Southern blot of mouse genomic DNA from wild-type (+/+), heterozygous (+/–), and homozygous null (–/–) F2 offspring digested with *Eco*RI and hybridized with probe a. Wild-type and null alleles apppear as 14.5 and 9.5 kb bands, respectively. PCR amplification generates 400 bp and 298 bp DNA fragments from WT and KO alleles, respectively. (**C**) Western blots of membrane proteins (20 μg/lane) isolated from wild-type (+/+) and $G\alpha_{11}$-deficient (–/–) mice. This subset of tissues shows the relatively high expression of $G\alpha_q$ and $G\alpha_{11}$ in brain, $G\alpha_{14}$ in testis and $G\alpha_{15}$ in spleen. Anti-peptide polyclonal antisera are specific for the indicated G_q class α-subunits, except for anti $G\alpha_{q/11}$, which recognizes $G\alpha_q$, $G\alpha_{11}$ and $G\alpha_{14}$. The corresponding recombinant proteins (rGα) purified from bacteria are included as controls.

(*Gna11*$^{-/+}$) were injected into C57BL6 blastocysts to generate chimeric mice. Two chimeric males transmitted the *Gna11* null allele in matings to either 129/SvEv or C57BL6 female mice. Intercrosses of heterozygous mice in either background produced a normal ratio of +/+, –/+ and –/– offspring. $G\alpha_{11}$-deficient mice were viable and fertile with no apparent behavioral or morphological defects.

$G\alpha_{11}$ protein is co-expressed with functionally related proteins of the G_q class in most, if not all, tissues including the heart (Wilkie *et al*., 1991). Western blot analysis of membrane protein from a variety of tissues confirmed that *Gna11*$^{-/-}$ mice did not express $G\alpha_{11}$, and that the amount of other G_q class members expressed in these tissues was comparable with normal littermates (Figure 1C). It is possible that the normal levels of $G\alpha_q$, $G\alpha_{14}$ and/or $G\alpha_{15}$ expressed in cells could be sufficient to compensate for a deficiency of $G\alpha_{11}$ protein.

Gene dosage effects between Gnaq and Gna11

Genetic interaction between the genes encoding $G\alpha_q$ and $G\alpha_{11}$ was tested by crossing $G\alpha_q$- and $G\alpha_{11}$-mutant mice and then intercrossing their double heterozygous offspring. Double homozygous null embryos [$G\alpha_q^{(-/-)};G\alpha_{11}^{(-/-)}$] died at approximately embryonic day (e)11 (Table I). Offspring with only a single intact wild-type allele of either $G\alpha_q$ [$G\alpha_q^{(-/+)};G\alpha_{11}^{(-/-)}$] or $G\alpha_{11}$ [$G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$] died shortly after birth. By contrast, the majority of mice that inherited any combination of two or more wild-type alleles of either $G\alpha_q$ or $G\alpha_{11}$ survived to adulthood and were fertile.

$G\alpha_q^{(-/-)};G\alpha_{11}^{(-/-)}$ embryos die with heart malformations

To determine when $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/-)}$ embryos died *in utero*, we examined embryos from $G\alpha_q^{(-/+)};G\alpha_{11}^{(-/+)}$ intercrosses at different times post conception. $G\alpha_q/G\alpha_{11}$-deficient embryos were usually resorbed between e11.5 and 13.5. The gross morphology of e9.5 $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/-)}$ embryos appeared to be indistinguishable from wild-type e9.5 embryos and they represented the expected fraction of all embryos (Figure 2; Table I). In contrast, the growth of all e10.5 $G\alpha_q/G\alpha_{11}$-deficient embryos examined was clearly retarded at e10.5. Several of the recovered e10.5 $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/-)}$ embryos exhibited enlarged hearts which appeared to be filled with blood (Figure 2). Closer examination revealed that this phenomenon was due to bleeding into the space between the myocardial and the pericardial layer of the embryonic heart. The first indication of heart malformation was revealed in serial sections of $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/-)}$ embryos at e9.5 as a severe thinning of the myocardial layer of the heart, and this was even more

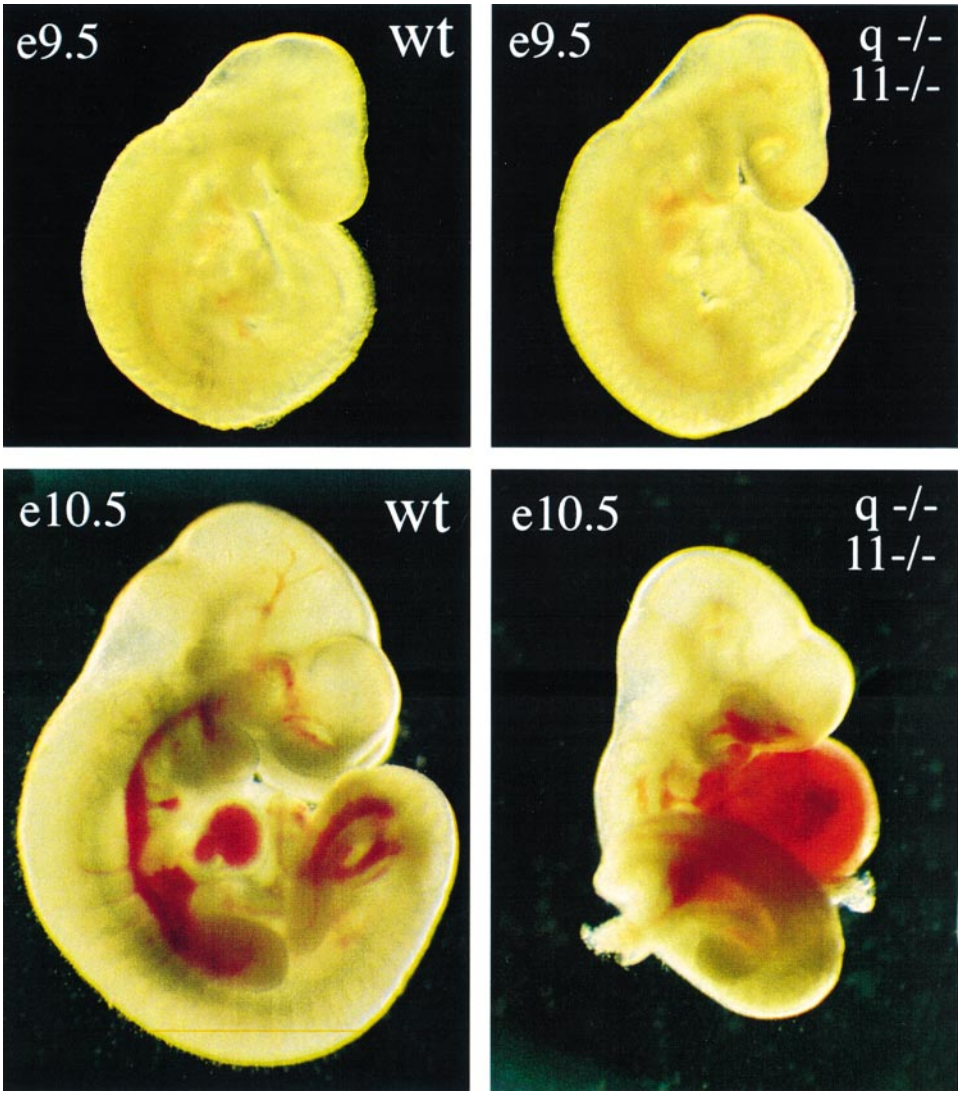

Fig. 2. Gross morphology of e9.5 and e10.5 wild-type and $G\alpha_q/G\alpha_{11}$-mutant embryos. Side view of wild-type (two left panels) and $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/-)}$ embryos (two right panels) at e9.5 (two upper panels) and e10.5 (two lower panels). $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/-)}$ embryos are clearly retarded at e10.5. Shown is a $G\alpha_q/G\alpha_{11}$-deficient embryo with a typical bleeding into the pericardial cavity.

Table I. Offspring of $G\alpha_q^{(-/+)};G\alpha_{11}^{(-/+)}$ intercrosses

Genotype		Stage						
$G\alpha_q$	$G\alpha_{11}$	e9.5	e10.5	e11.5	e13.5	P1	P28	Predicted (%)
–/–	–/–	9 (8%)	10 (6%)	5[a] (6%)	0 (0%)	0 (0%)	0 (0%)	6.25
–/–	–/+	14 (13%)	18 (11%)	10 (13%)	8 (19%)	36 (10%)	0 (0%)	12.5
–/+	–/–	13 (12%)	28 (16%)	8 (10%)	4 (9%)	41 (11%)	4 (1%)	12.5
–/–	+/+	8 (7%)	8 (5%)	3 (4%)	2 (5%)	18 (5%)	31 (4%)	6.25
+/+	–/–	6 (6%)	9 (5%)	4 (5%)	2 (5%)	23 (6%)	58 (8%)	6.25
–/+	–/+	28 (26%)	45 (26%)	26 (33%)	13 (29%)	119 (32%)	264 (38%)	25.0
–/+	+/+	13 (12%)	20 (12%)	9 (11%)	5 (12%)	51 (14%)	139 (20%)	12.5
+/+	–/+	11 (10%)	19 (11%)	7 (9%)	8 (19%)	50 (14%)	133 (19%)	12.5
+/+	+/+	7 (6%)	13 (8%)	7 (9%)	1 (2%)	30 (8%)	67 (10%)	6.25
Total		109	170	79	43	368	696	

Numbers of $G\alpha_q/G\alpha_{11}$-mutant embryos and mice genotyped at different developmental stages. e9.5–e13.5, embryonic day 9.5–13.5;P1 and P28, postnatal day 1 and 28. Percentage of all animals genotyped at each stage is given in parentheses.
[a]Resorbing.

obvious at e10.5 (Figure 3). Both the trabecular ventricular myocardium as well as the subepicardial layer appeared to be underdeveloped. Other parts of the heart were morphologically normal. A severe reduction in the thickness of the myocardium is likely to lead to cardiac failure and in some cases to myocardial rupture resulting in

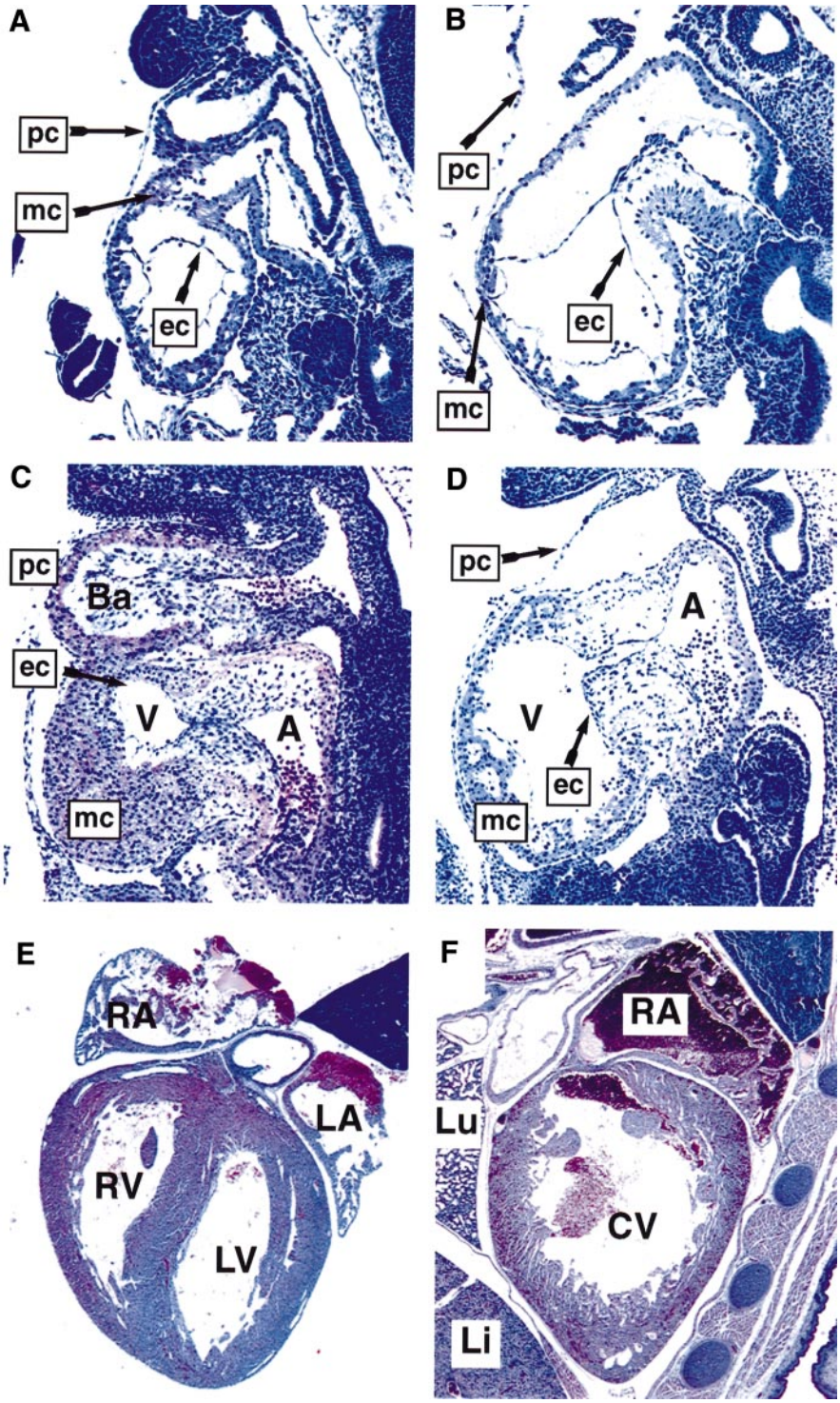

Fig. 3. Heart defects in $G\alpha_q/G\alpha_{11}$-mutant mice. Hematoxylin–eosin-stained histological sections of wild-type (**A**, **C**, **E**), $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/-)}$ (**B** and **D**) and $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$ embryos (**F**) at e9.5 (A and B), e10.5 (C and D) and e18.5 (E and F). Shown are sagittal sections of corresponding planes from wild-type and mutant mice demonstrating the myocardial hypoplasia occurring in $G\alpha_q/G\alpha_{11}$-deficient embryos (B and D) and a typical univentricular heart defect observed in a $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$-mutant (F). Ba, future aortic bulb; A, atrium; V, ventricle; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; Lu, lung; Li, liver; CV, common ventricle; pc, pericardium; mc, myocardium; ec, endocardium.

escape of blood into the pericardium. Thus, this severe heart abnormality probably accounts for the mid-gestation lethality of $G\alpha_q/G\alpha_{11}$-deficient embryos.

We examined the expression of G_q class α-subunits in embryonic mouse hearts by means of reverse transcription polymerase chain reaction (RT–PCR) with total RNA prepared from wild-type and $G\alpha_q/G\alpha_{11}$-mutant e9.5 hearts (Figure 4A). Both $G\alpha_q$ and $G\alpha_{11}$ were found to be expressed in wild-type e9.5 hearts but not in hearts of double homozygous null embryos. Of the other two known murine members of the $G\alpha_q$ class, $G\alpha_{14}$ and $G\alpha_{15}$, low levels of $G\alpha_{14}$ were found to be expressed both in wild-type and mutant hearts whereas no expression of $G\alpha_{15}$ could be detected (data not shown). Immunostaining of

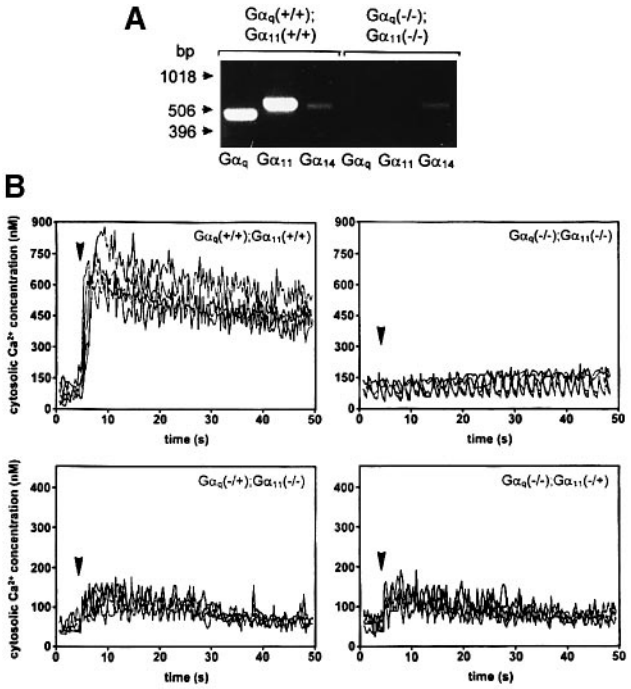

Fig. 4. Expression of $G\alpha_q$ class members in e9.5 heart and effect of angiotensin II on $[Ca^{2+}]_i$ in $G\alpha_q/G\alpha_{11}$-mutant embryonic cardiomyocytes. (**A**) RT–PCR analysis of Gα expression in embryonic heart. Total RNA was prepared from wild-type hearts and hearts from $G\alpha_q^{(-/-)}$; $G\alpha_{11}^{(-/-)}$ embryos at e9.5 and used for PCR analysis with $G\alpha_q$, $G\alpha_{11}$ and $G\alpha_{14}$ specific primers. (**B**) Effect of 1 μM angiotensin II on $[Ca^{2+}]_i$ in cardiomyocytes prepared from hearts of wild-type $[G\alpha_q^{(+/+)};G\alpha_{11}^{(+/+)}]$, $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/-)}$, $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$ and $G\alpha_q^{(-/+)};G\alpha_{11}^{(-/-)}$ embryos at e10. Shown are the results of several individual cells. Arrows indicate the time point of agonist addition.

cells derived from e9.5 hearts (see below) showed that cardiomyocytes as well as non-myocardial cells expressed $G\alpha_q/G\alpha_{11}$ consistent with the wide expression pattern of both G protein α-subunits (Strathmann and Simon, 1990; data not shown).

Several studies using neonatal cardiomyocytes have demonstrated that angiotensin II, endothelin, the α_1 adrenergic receptor agonist phenylephrine or prostaglandin $F_{2\alpha}$, all of which are agonists of G_q/G_{11}-coupled receptors, can stimulate hypertrophy (Shubeita *et al.*, 1990; Morgan and Baker, 1991; Knowlton *et al.*, 1993; Sadoshima *et al.*, 1993; Adams *et al.*, 1996). To test whether mouse embryonic cardiomyocytes at e10 expressed G_q/G_{11}-coupled receptors, we cultured embryonic cardiomyocytes from wild-type and $G\alpha_q/G\alpha_{11}$ double deficient hearts at e10 and measured the effect of various agonists on $[Ca^{2+}]_i$ (Figure 4B). In >90 % of the wild-type cardiomyocytes examined, angiotensin II induced an increase in intracellular calcium, while 20–50% of the cardiomyocytes responded to phenylephrine. In contrast, $G\alpha_q/G\alpha_{11}$ double deficient cardiomyocytes did not respond to either of these agonists. Interestingly, angiotensin II stimulated calcium responses in cardiomyocytes from embryos with only a single active $G\alpha_q$ or $G\alpha_{11}$ allele $[G\alpha_q^{(-/+)};G\alpha_{11}^{(-/-)}$ or $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}]$ were clearly reduced compared with wild-type (Figure 4B), and cells with two active alleles had intermediate responses (data not shown). These data demonstrate that embryonic mouse cardiomyocytes express G_q/G_{11}-coupled receptors and that lack of $G\alpha_q/G\alpha_{11}$ prevents elevation of $[Ca^{2+}]_i$ evoked by activation of these receptors. $G\alpha_{14}$ which was found to be expressed in $G\alpha_q/G\alpha_{11}$-deficient hearts, although at comparably low levels, was either not able to functionally compensate the loss of $G\alpha_q/G\alpha_{11}$ or is expressed in cardiac cells other than cardiomyocytes.

Craniofacial and cardiac defects in neonates with one intact $G\alpha_q$ or $G\alpha_{11}$ allele

Neonatal pups that inherited a single active gene $[G\alpha_q^{(-/+)};G\alpha_{11}^{(-/-)}$ or $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}]$ died within one to two hours of birth. Pups of all other genotypes were normal, with the exception of some $G\alpha_q^{(-/-)}$ pups (Offermanns *et al.*, 1997a). To examine pups with one intact $G\alpha_q$ or $G\alpha_{11}$ allele in greater detail, litters from additional double-intercross pregnancies were collected by Caesarian section. Pups inheriting a single active gene were runted, anoxic at birth, breathed at about half the rate of their surviving littermates, were poorly responsive to tactile stimulation and died within an hour of birth. Although no $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$ pups survived the neonatal period, four $G\alpha_q^{(-/+)};G\alpha_{11}^{(-/-)}$ (3 males, 1 female) pups out of ~100 born have survived to adulthood and at least two of the males are fertile.

$G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$ pups could be visually distinguished at birth from their $G\alpha_q^{(-/+)};G\alpha_{11}^{(-/-)}$ littermates by their foreshortened lower jaw. Staining of the skeletal system of $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$ newborns with alizarin red and alcian blue revealed malformations in the shortened mandible, vestigal tympanic ring, poorly developed otic cup and its associated bones (malleus, incus and stapes) and an abnormal hyoid bone (Figure 5). Development of these craniofacial features is dependent on immigration and proliferation of neural crest cells (Bronner-Fraser, 1995). Other neural crest-derived cell types appeared normal in mice of both mutant genotypes, including enteric neurons, choroidal melanocytes of the retina and dorsal root ganglia.

Histological analysis of newborn pups which inherited only one intact $G\alpha_q$ or $G\alpha_{11}$ allele revealed an increased incidence of cardiac malformations. $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$ pups often exhibited univentricular hearts (nine of 11 analyzed) (Figure 3F). Some hearts of $G\alpha_q^{(-/+)};G\alpha_{11}^{(-/-)}$ pups also showed malformations, a few were hypotrophic while others appeared to be hypertrophic (data not shown). All other internal organs appeared to be normal. The lungs of these pups appeared to have inflated normally. However, pups of both genotypes breathed arryhthmically and at a slower rate than wild-type pups. Serial transverse sections of brain from the cerebral cortex to the spinal cord in the upper cervical region did not reveal obvious defects. Thus, the cause of death in $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$ and $G\alpha_q^{(-/+)};G\alpha_{11}^{(-/-)}$ pups is not certain but heart malformations observed in both genotypes may be a contributing factor.

Discussion

G_q class G proteins are expressed throughout mammalian development in all cell types and tissues that have been analyzed (Wilkie *et al.*, 1991). Among the four known α-subunits of the G_q class, $G\alpha_q$ and $G\alpha_{11}$ are co-expressed in almost all cell types and are primarily responsible for coupling receptors to β-isoforms of phospholipase C. Disruption in any single α-subunit gene of the G_q class

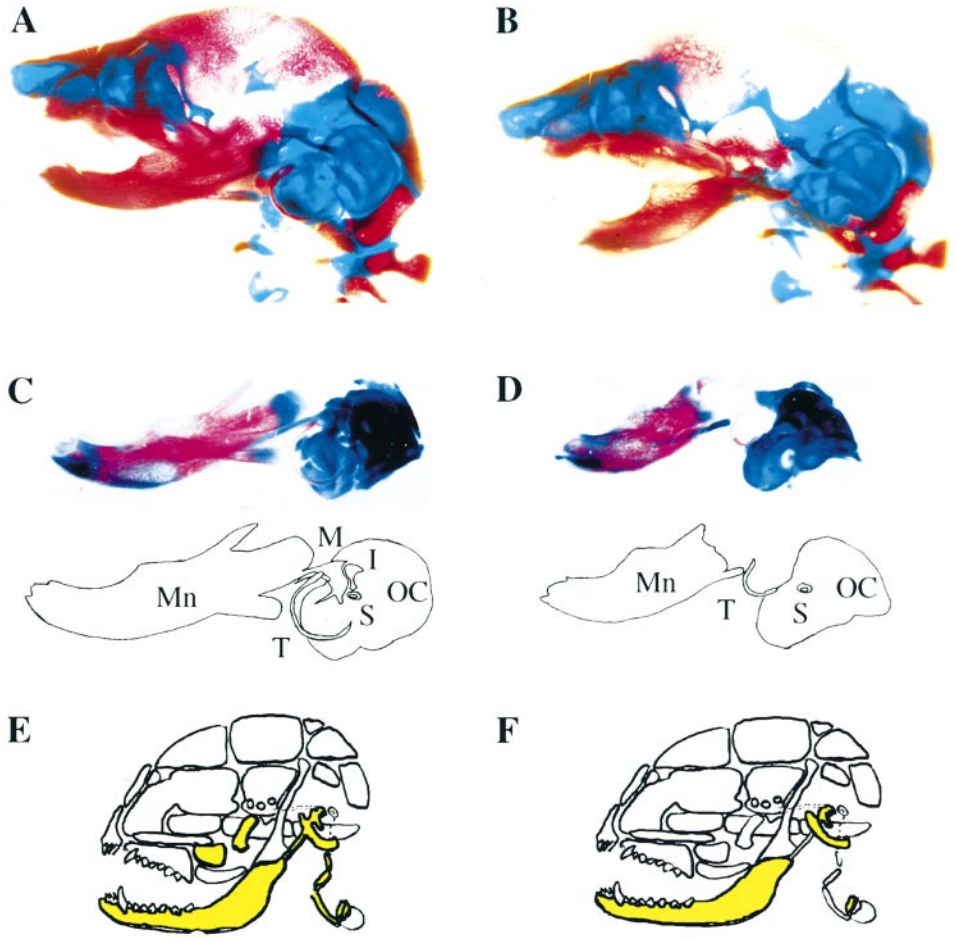

Fig. 5. Craniofacial defects in $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$ fetuses at e18.5. Skeletal morphology was analyzed following alizarin red and alician blue staining to visualize bone and cartilage, respectively. Micrographs and schematic lateral views are of the skull and dissected mandible with the middle ear bones. (**A**) and (**C**) wild-type fetus, e18.5. (**B**) and (**D**) compound heterozygous mutant fetus; $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$, e18.5. The four recognizable processes are missing in the mandibular (Mn) bone, as well as the incus (I) and malleus (M). In addition, the stapes (S), otic capsule (OC) and tympanic (T) bones are malformed. (**E** and **F**) Schematic representation of the head bones of a wild-type mouse (Matsuo *et al*., 1995) with the sites of developmental defects in endothelin (ET_A) receptor knockout mice (E) at e18.5 (Clouthier *et al*., 1998) and in $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$ mice at e18.5 (F) shown in yellow.

can be maintained in homozygous null lines, and only $G\alpha_q$-deficient mice exhibit phenotypic defects. Considerable evidence collected from biochemical, pharmacological and somatic cell genetic studies suggested that $G\alpha_q$ and $G\alpha_{11}$ have very similar, if not identical, characteristics. $G\alpha_q$ and $G\alpha_{11}$ couple to the same set of seven transmembrane receptors with the same effector specificity for phospholipase C-β isoforms (Blank *et al*., 1991; Wange *et al*., 1991; Wu *et al*., 1992; Hepler *et al*., 1993; Offermanns *et al*., 1994). This constellation of data suggested that $G\alpha_q$ and $G\alpha_{11}$ have overlapping functions; however, $G\alpha_{11}$ cannot compensate for all of $G\alpha_q$ functions. In this study, we have taken advantage of the fact that *Gna11* and *Gnaq* reside on different chromosomes (Wilkie *et al*., 1992) to analyze the phenotype resulting from homozygous null mutations in these ubiquitously expressed G_q class α-subunit genes. The first indication of a developmental defect in the complete absence of $G\alpha_q$ and $G\alpha_{11}$ proteins was thinning of the myocardium by e9 with ensuing cardiac failure and death by e11.

G_q-signaling has previously been implicated in cardiomyocyte hypertrophy in adults and hyperplasia in embryos. Cardiac hypertrophy is an adaptive response of adult hearts to hemodynamic stresses induced by hypertension, valvular heart disease or loss of cardiomyocytes due to myocardial infarction or cardiomyopathy. Adult cardiomyocyte hypertrophy has been shown to involve the induction of an embryonic gene program (Chien *et al*., 1993). Agonists of G_q-signaling pathways, including angiotensin II, endothelin, the α_1 adrenergic receptor agonist phenylephrine or prostaglandin $F_{2\alpha,}$ can stimulate hypertrophy in neonatal cardiomyocytes (Shubeita *et al*., 1990; Morgan and Baker, 1991; Knowlton *et al*., 1993; Sadoshima *et al*., 1993; Adams *et al*., 1996). It has been suggested that G_q-signaling contributes to the pathological growth of cardiomyocytes resulting in cardiac hypertrophy (Chien *et al*., 1993; La Morte *et al*., 1994; Van Heugten *et al*., 1995; Yamazaki *et al*., 1995; Post and Brown, 1996). In line with this hypothesis, transgenic expression of a constitutively activated mutant of the α_1 adrenergic receptor as well as cardiac overexpression of $G\alpha_q$ leads to cardiac hypertrophy in adult mice (Milano *et al*., 1994; D'Angelo *et al*., 1997), and transgenic overexpression of the angiotensin II (AT_{1a}) receptor in the developing atrium leads to atrial hyperplasia (Hein *et al*., 1997). Interestingly, adult cardiomyocytes are terminally differentiated post-

mitotic cells which respond to stimulatory signals by cell growth rather than proliferation. By contrast, embryonic cardiomyocytes retain the capacity to proliferate. In the double homozygous null mutants of $G\alpha_q$ and $G\alpha_{11}$ we observed a hypoplastic defect in heart development. The hypoplastic heart was apparently not able to keep up with the demands of the developing embryo, occasionally resulting in ruptured hearts, but always leading to a dramatic cessation of growth after e9.5 and death by e11 (Figures 2 and 3). This suggests that the G_q/G_{11}-signaling pathway is not only involved in cardiomyocyte hypertrophy in adult hearts but also in regulation of cell proliferation of embryonic cardiomyocytes. Thus, although adult cardiomyocytes have lost their proliferative capacity, growth regulation in both developing and adult hearts, appears to involve $G\alpha_q/G\alpha_{11}$-mediated pathways.

Activation of the G_q/G_{11}-signaling pathway in the heart results in activation of phospholipase C-β isoforms with subsequent release of intracellular Ca^{2+} and production of diacylglycerol leading to activation of various protein kinase C isozymes. To test whether mouse embryonic cardiomyocytes at e10 express receptors which are coupled to the G_q/G_{11}-mediated pathway, we cultured embryonic cardiomyocytes from wild-type and $G\alpha_q/G\alpha_{11}$-deficient hearts at e10 and measured the effect of various agonists on $[Ca^{2+}]_i$. In almost all wild-type cardiomyocytes examined, angiotensin II induced an increase in intracellular Ca^{2+}, while responses to other agonists of G_q/G_{11}-coupled receptors varied. In contrast, $G\alpha_q/G\alpha_{11}$ double-null cardiomyocytes did not respond to any of these agonists (Figure 4). These data demonstrate that embryonic mouse cardiomyocytes express G_q/G_{11}-coupled receptors and that the lack of $G\alpha_q/G\alpha_{11}$ prevents elevation of $[Ca^{2+}]_i$ induced through these receptors. The G_q/G_{11}-coupled receptors involved in the regulation of cardiac growth at mid-gestation are currently unknown. The lack of embryonic heart defects in mice deficient in angiotensinogen and endothelin-1 (Kurihara *et al*., 1994; Tanimoto *et al*., 1994) indicate that angiotensin II or endothelin-1 alone are not necessary for cardiac growth and development during embryogenesis. Interestingly, pharmacological blockade of $5\text{-}HT_{2B}$ receptors in mouse embryos resulted in abnormal organization of the subepicardial layer and the absence of the trabecular cell layer of the ventricular myocardium (Choi *et al*., 1997), and absence of both, endothelin A (ET_A) and B (ET_B) receptors resulted in midgestational cardiac failure (Yanagisawa *et al*., 1998). Additional stimuli acting through G_q/G_{11}-coupled receptors may also be involved. It is possible that there is signaling redundancy with several inputs into the $G_{q/11}$ pathway and that only deletion of both the $G\alpha_q$ and the $G\alpha_{11}$ genes results in severe phenotypic defects during early heart development due to the ablation of the G_q/G_{11}-phospholipase-C-β pathway.

Interestingly, one intact allele of the $G\alpha_q$ or $G\alpha_{11}$ gene was obviously sufficient to overcome the early developmental block in heart development resulting from the complete absence of $G\alpha_q/G\alpha_{11}$. Embryonic cardiomyocytes carrying one intact allele of the $G\alpha_q$ or $G\alpha_{11}$ gene were able to respond with elevated $[Ca^{2+}]_i$ levels to angiotensin II whereas $G\alpha_q/G\alpha_{11}$-deficient cardiomyocytes were completely unresponsive. However, at later stages, various developmental defects of the heart were observed in embryos carrying only one intact $G\alpha_q$ or $G\alpha_{11}$ allele. During mid-gestation, from e8 to e16, the embryonic heart undergoes a complex morphogenic program of differentiation, regionalization, septation and growth (Olson and Srivastava, 1996; Fishman and Chien, 1997). The early phase of organogenesis in $G\alpha_q/G\alpha_{11}$-deficient fetuses appeared normal by gross morphology at e9.5, although cardiomyocyte hypoplasia was already apparent at this stage. At later stages, at least two intact $G\alpha_q/G\alpha_{11}$ alleles were necessary for proper cardiac development. The increased incidence of obvious cardiac defects in $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$ and to a lesser degree in $G\alpha_q^{(-/+)};G\alpha_{11}^{(-/-)}$ pups reflects the requirement for threshold levels of $G\alpha_q/G\alpha_{11}$ to transduce extracellular signals which are involved in the regulation of heart development during later stages. A functional heart is required for embryonic development, and a significant fraction of preterm abortions in humans are due to congenital heart defects (Hoffman, 1995). Understanding the molecular mechanisms that underlie cardiac development may identify the cause of many congenital heart defects.

At later stages of development, G_q class signaling has also been implicated in the proliferation and/or migration of neural crest cells. Endothelin-1 and the $G_{q/11}$-coupled endothelin A (ET_A) receptor are essential for normal function of the craniofacial and cardiac neural crest. Endothelin-1 and ET_A receptor-deficient mice die shortly after birth due to respiratory failure (Kurihara *et al*., 1994, 1995; Clouthier *et al*., 1998). Severe skeletal abnormalities could be observed in their craniofacial region, including retarded mandibular bones, aberrant zygomatic and temporal bones, and absence of auditory ossicles and tympanic ring. The craniofacial defects observed in $G\alpha_q^{(-/-)};G\alpha_{11}^{(-/+)}$ mice appear to be a milder form of the endothelin-1/ET_A-receptor (–/–) phenotype. Interestingly, $G\alpha_q^{(-/+)}$; $G\alpha_{11}^{(-/-)}$ mice did not show craniofacial abnormalities suggesting that ET_A receptor-mediated neural crest development involves primarily $G\alpha_q$. It may be that a certain amount of $G\alpha_q/G\alpha_{11}$ is required for endothelin-1-dependent craniofacial development and is provided either by one intact allele of the $G\alpha_q$ gene or two intact alleles of the $G\alpha_{11}$ gene.

Our data clearly demonstrate the overlap of $G\alpha_q$ and $G\alpha_{11}$ gene functions. Interestingly, the penetrance of the $G\alpha_q/G\alpha_{11}$-mutant phenotype exhibited a strict dependence on the $G\alpha_q/G\alpha_{11}$ gene dosage. Whereas two intact $G\alpha_q/G\alpha_{11}$ alleles were necessary for extrauterine life, at least one intact $G\alpha_q$ or $G\alpha_{11}$ allele was required to bring the embryo to term. Lack of both $G\alpha_q$ and $G\alpha_{11}$, resulted in mid-gestational lethality due to myocardial hypoplasia. Given the hypoplastic heart defect in $G\alpha_q/G\alpha_{11}$ null mouse embryos and the suggested role for the $G\alpha_q/G\alpha_{11}$-mediated pathway in the development of cardiac hypertrophy, the $G\alpha_q/G\alpha_{11}$-mediated signaling pathway appears to play a pivotal role not only in pathological processes leading to cardiac hypertrophy, but also in the regulation of physiological myocardial growth during embryogenesis. It remains to be demonstrated whether the same receptors are involved in G_q/G_{11}-mediated regulation of myocardial growth under physiological and pathological conditions in developing embryos and adults.

Materials and methods

Generation of $G\alpha_{11}$-mutant mice

A genomic $G\alpha_{11}$ clone was isolated from a 129/Sv mouse genomic λ phage library (Stratagene) (Davignon *et al*., 1996). To generate a null

mutation, a fragment containing exon 3, 4 and a portion of exon 5 which together code for amino acids 108–217 of $G\alpha_{11}$ was replaced by the Pgk::Neo gene in the reverse orientation. The *Gna11* targeting construct was introduced into AB1 and JH1 ES cells (Bradley *et al*., 1992; Rosahl *et al*., 1993) by electroporation and the resulting G418- and gancyclovir-resistant clones were screened by Southern blot analysis (Ramirez-Solis *et al*., 1992). Chimeric mice were generated by injection of ES cells into C57BL6 blastocysts, and chimeras were bred with 129/SvEv and C57BL6 mice to generate hemizygous animals. Germ-line transmission was confirmed by Southern blot and PCR.

Western blotting, antibodies

Membrane proteins for Western analysis were prepared as described in Mumby *et al*. (1990). Rabbit antisera specific for $G\alpha_q$ (W082), $G\alpha_{11}$ (B825), $G\alpha_{q/11}$ (Z811) and $G\alpha_{15/16}$ (B861) have been described (Hepler *et al*., 1994). The anti-$G\alpha_{14}$ antiserum (B421) was raised against a peptide specific for mouse $G\alpha_{14}$.

Histological analysis

e9.5 or e10.5 embryos were fixed in Bouin's fixative, transferred to 70% ethanol and processed according to routine histological procedures. Paraffin-embedded specimens were sectioned sagitally. Newborn mice were fixed in buffered formalin and processed for histology. Sections were stained with hematoxylin–eosin.

Measurement of $[Ca^{2+}]_i$

Embryonic cardiomyocytes were prepared from hearts of e10 embryos. Embryonic hearts were dissected and incubated overnight at 4°C in phosphate-buffered saline containing 0.05 % trypsin and 0.4 mM EDTA. Digestion was conducted for 10 min at 37°C. Cells of individual hearts were washed and plated into small areas of glass cover slips surrounded by cloning cylinders. The majority of cells prepared in this way represented embryonic cardiomyocytes which were easily identified by their rhythmic contractions. After 2–4 days in culture, cells were washed and incubated with 2 μM fura-2 acetoxymethyl ester for 30 min at 37°C in Dulbecco's modified Eagle medium. Loaded cells were washed in 138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 5.5 mM glucose, 1 mM $CaCl_2$ and 20 mM HEPES (pH 7.4), and determinations of cytoplasmic $[Ca^{2+}]$ in single cells were performed in the same buffer using a digital imaging system (TILL Photonics, Munich, Germany). Cells were visualized with an inverted microscope. Fura-2 fluorescence was excited alternately at 340 nm and 380 nm. Maximum and minimum fluorescence were determined by addition of 10 μM ionomycin plus 10 mM $CaCl_2$ or 1 mM EGTA, respectively. Images were digitalized and analyzed using the software FUCAL 5.12 (TILL Photonics, Munich, Germany). Ratio images were generated in 0.5 s intervals.

Skeletal analysis

Bone and cartilage were prepared as described by McLeod (1980).

RT–PCR

Total RNA was prepared from e9.5 wild-type and $G\alpha_q/G\alpha_{11}$-deficient hearts and reverse transcribed with random primers and Moloney murine leukemia virus reverse transcriptase (Gibco-BRL). Oligonucleotides used in PCRs were for $G\alpha_q$ AGATCGAGCGGCACGTGCGC and GTTGTGTAGGCAGATAGGAAGG, for $G\alpha_{11}$ ACGAGGTGAAGG-AGTCGAAGC and CCATCCTGAAGATGATGTTCTCC, and for $G\alpha_{14}$ TCACTGCACTCTCTAGAGACC and GACATCTTGCTTTGGTCCT-GTG. Primer sequences were chosen so that primers hybridized to specific regions encoded by different exons in order to distinguish cDNA-dependent amplification from amplification of genomic DNA (Strathmann and Simon, 1990; Wilkie *et al*., 1991).

Acknowledgements

We thank T.Sato and members of our laboratories for comments on the manuscript, M.Yanagisawa and D.Clouthier for discussion, C.Gowan, M.Barnard, M.Staloch, V.Mancino, U.Brandt and S.Pease for technical assistance, A.Bradley and J.Herz for ES cell lines, D.Burns for histologic analysis of neural anatomy, D.Duryea for histology assistance, L.Margraf for analysis of lung morphology, P.Sternweis and J.Hepler for antisera, J.Satterfield for administrative assistance. This work was funded by NIH grant DK47890 (T.M.W.), by the Deutsche Forschungsgemeinschaft (S.O.) and additional support to T.M.W. from the March of Dimes, the Texas Advanced Research Program, the Leukemia Association of North Central Texas. T.M.W. is a recipient of the American Heart Association Established Investigator Award.

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Received April 22, 1998; revised June 5, 1998; accepted June 8, 1998