Increased Basal cAMP-dependent Protein Kinase Activity Inhibits the Formation of Mesoderm-derived Structures in the Developing Mouse Embryo*

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A targeted disruption of the RIIα isoform of protein kinase A (PKA) was created by using homologous recombination in embryonic stem cells. Unlike the other regulatory and catalytic subunits of PKA, RIIα is the only isoform that is essential for early embryonic development. RIIα homozygous mutant embryos fail to develop a functional heart tube at E8.5 and are resorbed at approximately E10.5. Mutant embryos show significant growth retardation and developmental delay compared with wild type littermates from E7.5 to E10.5. The anterior-posterior axis of RIIα mutants is well developed, with a prominent head structure but a reduced trunk. PKA activity measurements reveal an increased basal PKA activity in these embryos. Brachyury mRNA expression in the primitive streak of RIIα mutants is significantly reduced, consistent with later deficits in axial, paraxial, and lateral plate mesodermal derivatives. This defect in the production and migration of mesoderm can be completely rescued by crossing RIIα mutants to mice carrying a targeted disruption in the Ca c catalytic subunit, demonstrating that unregulated PKA activity rather than a specific loss of RIIα is responsible for the phenotype. Primary embryonic fibroblasts from RIIα mutant embryos display an abnormal cytoskeleton and an altered ability to migrate in cell culture. Our results demonstrate that unregulated PKA activity negatively affects growth factor-mediated mesoderm formation during early mouse development.

The cAMP-dependent protein kinase (PKA) is a multisubstrate serine/threonine protein kinase responsible for modulating a vast number of cell physiological processes ranging from the maintenance of basal transcription of specific genes (1, 2) to rapid reorganization of the cytoskeleton (3, 4). The mechanism by which PKA modulates cellular physiology is by covalent modification of polypeptide substrates via reversible enzymatic transfer of a phosphate moiety to a consensus phosphorylation motif in the target protein (5). The number and range of protein substrates phosphorylated by PKA is as varied as the cell physiological processes which it is known to regulate. These substrates occur in all cellular compartments, including the nucleus, cytoplasm, and plasma membrane (6).

The PKA holoenzyme is a heterotrimer composed of two N-terminally dimerized regulatory (R) subunits combined with two catalytic (C) subunits (5). PKA is unique in that the regulatory domain of the enzyme is encoded by separate genes located on different chromosomes. PKA is also unique in that the mechanism of activation involves simple subunit dissociation upon binding of cAMP to the regulatory subunits, thereby liberating catalytically active C subunits that diffuse throughout the cell, phosphorylating protein substrates containing appropriate consensus phosphorylation motifs.

Four regulatory (RIα, RIβ, RIIα, and RIIβ) and two catalytic (Ca and Cβ) isoform genes have been described in the mouse (7). Messenger RNA and protein for the α isoforms of PKA (RIα, RIIα, and Ca) are found ubiquitously in the mouse and are expressed during early embryogenesis, whereas the β isoforms (RIβ, RIIβ, and Cβ) have more tissue-specific patterns of expression. RIβ expression is restricted to neurons, whereas RIIβ expression is highest in brain and brown and white adipose tissues (8, 9). Expression of Cβ is highest in brain with lower levels in all tissues (10). The pattern of expression of R and C isoforms has been examined at day 14 of embryogenesis in the mouse (11). However, a more detailed study of the expression and function of PKA isoforms during early stages of mammalian development is lacking.

The cAMP/PKA signaling system is likely to play an important role in early development, and several lines of experimentation have suggested important pathways that are modified by PKA activity. A potential requirement for PKA activity in the regulation of zygotic gene activation in the preimplantation mouse embryo has been documented (12, 13). Studies of Drosophila imaginal disks demonstrate that imaginal disk cells lacking PKA activity behave as if they have received an excessive sonic hedgehog signal, resulting in dramatic pattern re-specifications (14–18). These observations led to the hypothesis that PKA acts to antagonize sonic hedgehog signaling and thus repress hedgehog-responsive genes such as decapentaplegic, patched, and wingless. By using constitutively active and dominant negative forms of PKA, two independent studies have shown that PKA acts as a common negative regulator of sonic hedgehog signaling in the zebrafish embryo (19–21). Apart from the role of PKA as a general negative regulator of sonic

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The abbreviations used are: PKA, cAMP-dependent protein kinase; ES, embryonic stem; HPLC, high pressure liquid chromatography; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; oligos, oligonucleotides; DAPI, 4,6-diamino-2-phenylindole; FAK, focal adhesion kinase.
hedgehog signaling, research to date has not uncovered any other specific roles for PKA in vertebrate development. One recent report (22) has implicated PKA in even earlier stages of vertebrate embryogenesis. In this study it was demonstrated that dissociated zebrafish blastula cells required PKA activity for activin induction of the early mesoderm marker genes goosecoid and no tail. However, only mild effects were observed in vivo by injection of a dominant negative regulatory subunit of PKA on gooseoid and no tail expression. Furthermore, when Joore et al. (22) injected 2–4 cell stage zebrafish embryos with a constitutively active form of the catalytic subunit of PKA, it had no effect. This is in direct contrast to injections of a similar construct into 2–4 cell stage zebrafish embryos made by Hamschmidt and McMahon (20), who observed that blastula stage zebrafish embryos were exquisitely sensitive to a constitutively active form of the catalytic subunit of PKA, resulting in failure of a large fraction of these embryos. Recent evidence in our laboratory has confirmed the sensitivity of vertebrate embryos to a constitutively active form of PKA catalytic subunit. In this study we present evidence that increased basal PKA activity resulting from targeted disruption of the RIIα isoform of PKA affects signaling in the primitive streak, causing profound deficits in the production of all mesodermal derivatives including the heart.

EXPERIMENTAL PROCEDURES

Mapping the RIIα Gene—The mouse RIIα genomic sequences were isolated from a Charon 4A λ phage library containing partial HindIII/Aval fragments of BALB/c mouse genomic DNA. The promoter sites for goosecoid and 1.5-kb of 3'UTR of RIIα were isolated that contains a 5'UTR of the gene was then subcloned into a pUC18 vector. The vector was linearized at the HindIII site and stored at −80°C. Subsequently, male chimeras were generated from the pUC18 polylinker. This replacement type gene were determined by primer extension and S1 nuclease mapping.

RIIαRec3 Targeting Vector—In order to make a targeted disruption in the RIIα gene, a 5.0-kb genomic HindIII fragment was isolated from a 129svJ mouse λ phage library using plaque hybridization screening with a 514-bp XhoI-EcoRI [32P]cDNA probe from RIIα. A genomic fragment was isolated that contains a 5' 3.2-kb intron, the 170-bp exon 3, and 1.5-kb of 3' sequence including exons 4 and 5. This genomic fragment was then subcloned into a pUC18 vector. The vector was linearized at the XhoI site in exon 3 corresponding to amino acid 77 in the RIIα coding sequence. A neomycin resistance cassette containing the SV40 promoter, the neomycin phosphotransferase cDNA, and poly(A) sequence was inserted 5' to the genomic sequence using EcoRI and BamHI sites in the pUC18 polylinker. This replacement type targeting vector was designated RIIαRec3 and was linearized at the BamHI site before electroporation into ES cells.

Generation of ES Cells and Generation of Germ Line Chimeras—The generation of germ line-competent ES cells in our laboratory has been described (23). REK2 or REK3 ES cells were electroporated with 25 μg of BamHI-linearized RIIαRec3 per 109 cells using a ProgenitorTM II PG200 electroporator (Hofer Scientific Instruments) and subsequently grown under double selection in G418 (280 μg/ml) and ganciclovir (2 μM). DNA from ES cell clones was analyzed by Southern blot using a 1-kb Sau3AI/HindIII RIIα genomic probe located 3' to the targeting construct. Positively targeted clones were expanded and injected into C57BL/6 blastocysts that were subsequently implanted into BL6/CBAF1 pseudopregnant females. The resulting male chimeras were mated with C57BL6 females, and agouti offspring were genotyped by Southern blot analysis. Both REK2- and REK3-derived male chimeras passed the mutation through the germ line resulting in agouty agouti offspring. Heterozygous agouti mice were then interbred and thus maintained on the mixed (129svJ × C57BL6) background for all subsequent analyses.

Generation of RIIα−/−; Cα−/− Animals for Interbreeding—Targeting of the Ca gene and creation of Ca null mutant mice are the subject of a separate study (24). In order to generate RIIα−/−; Cα−/− mice, RIIα heterozygous female mice were crossed to Cα heterozygous male mice, which are both on the 129svJ/C57BL6 mixed background. Genotyping of offspring for RIIα was carried out using the Southern blot strategy described above. Genotyping of offspring for Ca was carried out using a Southern blot strategy in which a 315-bp HindIII/EcoRI probe located outside the Ca targeting construct was hybridized to BamHI-digested genomic tail DNA. The targeted Ca allele yields a 1.4-kb band, whereas the wild type allele yields a 4.1-kb band.

PCR Analysis of Yolk Sac DNA—Individual yolk sacs from the embryos in M2 medium (Sigma) and transferred to 1× SET buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 1% SDS) followed by overnight digestion with 300 μg/ml proteinase K in a 45°C water bath. Yolk sac DNA was then extracted twice with phenol/chloroform and precipitated. DNA was resuspended in a total volume of 25 and 1 μl of the resuspended yolk sac DNA was used for PCR analysis with TaqDNA polymerase using the following oligos at 1 μM final concentration: (A) RIIα 5’ exon 3, 5’-GAGGAGGCGAACGAGAT-3’; (B) RIIα 3’ exon 3, 5’-CTTCTAACCAGTGAGGG-3’; (C) Neo 5’-oligo, 5’-TCGCATATGTGAA-CAAG-3’; (D) Neo 3’-oligo, 5’-AGACGAAGGAGGCTG-3’. Reaction conditions for RIIα exon 3 oligos were 94°C for 1 min, 45°C for 1 min, and 72°C for 1.5 min for 35 cycles. Reaction conditions for the neomycin oligos were 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min for 35 cycles. Reaction conditions were optimized using the PCR OptimizerTM Kit (Invitrogen). In order to genotype embryos from RIIα−/−; Cα−/− × RIIα+/−/Ca−/− interscroses, yolk sac DNA was isolated as described above, and the following two sets of Ca-specific primers were used to genotype for Ca: (A) Ca 5’ exon 6, 5’-CTGACCTTTGAGATTCTGAC-3’; (B) Ca 3’ exon 7, 5’-GTCACCAGAGGCTCAATGA-3’; (C) Neo 5’-oligo, 5’-ACACTGACCTCGCATCAG-3’; (D) Neo 3’-oligo, 5’-AGACGAAGGAGGCTG-3’. Reaction conditions for the Ca exon 6 and 7 oligos were 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s for 30 cycles. Reaction conditions for the Neo and Ca intron 7 oligos were 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min for 30 cycles.

Western Analysis and Kinase Assays—For Western analysis only, E8.5 embryos were isolated in M2 media, rinsed through several changes of ice-cold PBS, and placed directly into 20 μl of 1× Laemmli sample buffer and boiled for 5 min. Lysates were loaded directly onto 10% SDS-PAGE gels and subsequently transferred to nitrocellulose membranes. Blots were blocked overnight, probed with an affinity-purified polyclonal antibody to RIIα, and visualized using the Amersham Biosciences ECL®TM system. Kinase assays were performed as described previously (8). Briefly, E8.5 embryos were isolated in M2 media, rinsed through several changes of ice-cold PBS, and subsequently homogenized on ice in 150 μl of lysis buffer (20 mM Tris, pH 7.5, 250 mM sucrose, 0.1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 1 mM dithiothreitol, 1 μg/ml leupeptin, 3 μg/ml aprotinin, 40 μg/ml soybean trypsin inhibitor, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride) in a Dounce homogenizer followed by sonication using a Branson Sonifier 250 (5 times). Homogenates were cleared by centrifugation, cooled and stored at −80°C. Homogenates were thawed on wet ice and kinase assays performed in triplicate in the presence and absence of 5 μM cAMP with Leu-Arg-Ala-Ser-Leu-Gly (Kemptide) as the substrate. Assays were also performed in the presence of 40 μM/ml protein kinase inhibitor peptide (5–24) to demonstrate that the kinase activity was PKA-specific (25). Bradford assays (Bio-Rad) were performed to determine protein concentration, and kinase activity was normalized to milligrams of protein.

HPLC Analysis—HPLC analysis of RIIα wild type and knockout E8.5 primary embryonic fibroblasts was performed as described (26). Briefly, fibroblasts were grown on gelatin-coated 60-mm dishes in 10% FBS/DMEM and incubated in a 5% CO2 incubator at 37°C for 5 daily changes of ice-cold PBS, and subsequently lysed in homogenization buffer and stored at −80°C until the day of the assay. The sample was diluted with homogenization buffer to a final concentration of 1–2 mg/ml, loaded onto a DEAE/HPLC column, and eluted using a linear salt gradient from 0 to 250 mM. Immediately following the HPLC separation, fractions were collected and assayed for kinase activity in the presence and absence of 5 μM cAMP with Kem tide as a substrate (8).

Histology—Embryos were isolated in M2 media and fixed in Methacarnoys fixative (3:1.5:0.5 methanol/chloroform/glacial acetic acid) at room temperature. After fixation, embryos were processed through methanol (3 times for 15 min), methyl benzoate (1 times for 30 min), and xylene (2 times for 15 min) and embedded in paraffin. Paraffin blocks were sectioned at 8 μm on a Reichert-Jung microtome, and histological sections were stained with hematoxylin and eosin. Sections were subsequently dehydrated through graded ethanol into xylene and coverslipped with Permount®. Embryo sections were photographed on a Nikon Microphot-FXA microscope.

Whole Mount in Situ Analysis—Whole mount in situ analysis was performed as described (27). The following cRNA probes were generated as runoff transcripts using T3, T7, or SP6 RNA polymerase to generate sense and antisense probes: (A) Brachyury (gift from Virginia Papaio-
annou) linearized with BamHI (antisense) or HindIII (sense) and (B) Pax3 (gift from Peter Gruss) linearized with HindIII (antisense) or PstI (sense). Whole mounts were photographed on a Nikon SMZ-U Dissecting Scope.

Generation of Primary Embryonic Fibroblasts—The derivation of primary embryonic fibroblasts has been described (28). Briefly, E8.5 mouse embryos were dissected out from the decidua, and all decidual tissues were removed from the yolk sacs, including Reichardt’s membrane. Yolk sacs were removed from each individual embryo and kept for PCR analysis, and the amniotic membrane and allantochorion were removed and discarded. Ria mutant E8.5 embryos were easily identified by their morphology. Mutant or wild type embryos were pooled and placed in the tip of a 1-ml syringe in PBS and then triturated several times with a 20-gauge needle into a 96-well plate containing 10% FBS/DMEM and subsequently incubated in a 5% CO₂ incubator at 37 °C. Primary embryonic fibroblasts were expanded from 96- to 24-well to 60-mm dishes over several weeks. The genotype of Ria mutant primary embryonic fibroblasts was confirmed by yolk sac PCR and Western analysis.

Cell Cycle Analysis Using Primary Embryonic Fibroblasts—Analysis of cell cycle using DAPI staining has been described (29, 30). Briefly, subconfluent Ria or wild type primary embryonic fibroblasts growing in 10% FBS/DMEM in 5% CO₂ at 37 °C were gently trypsinized and subsequently washed twice through PBS and resuspended in 200 μl of buffer containing 4,6-diamino-2-phenylindole (10 μg/ml DAPI, 0.1% Triton X-100 in PBS, pH 7.5, 150 mM NaCl). The cells were triturated with a 26-gauge needle and analyzed using a Coulter ELITE cytometer with ultraviolet excitation and DAPI emission collected at >450 nm. DNA content and cell cycle were analyzed using the software program Multicycle.

In Vitro Migration Assay Using Primary Embryonic Fibroblasts—Wild type and Ria mutant primary embryonic fibroblasts were seeded onto extracellular matrix coverslips in 12-well tissue culture dishes in 10% FBS/DMEM and grown to confluency in a 5% CO₂ incubator at 37 °C. By using a p200 pipette tip, a wound was incised across the central area of the coverslip, and a line perpendicular to the incision was made on the bottom of the 12-well plate to mark the location for image collection. Images were collected at 4-h intervals using a Leica inverted scope interfaced with a Kodak 290 Digital camera and the Kodak Capture 1D 290 software.

Confocal Imaging of Fibroblasts—For actin cytoskeletal visualization, fibroblasts grown in 10% FBS/DMEM at 37 °C in a 5% CO₂ incubator were seeded onto 1% gelatin-coated coverslips in 12-well plates. At the time of staining, cells were fixed with 4% paraformaldehyde in PBS for 20 min, washed, delipidated with PBS containing 1% Triton X-100 for 5 min, washed, blocked with 1% bovine serum albumin in PBS for 20 min, and subsequently stained for 20 min with Texas Red phalloidin (Amersham Biosciences). After staining, cells were washed again and coverslipped in phosphate-buffered glycerol containing 0.1% Triton X-100 for 5 min, washed, delipidated with PBS containing DAPI, and then mounted on slides for analysis.

RESULTS

Structure of the Mouse Ria Gene—Mapping and sequencing of a λ clones generated a map of the Ria locus showing that the gene contains 11 exons and spans about 18 kb in the mouse genome. The position of intronic sequences within the coding region corresponds to the genomic structure of the closely related mouse Rfβ gene (31). A previous mapping of the human RIA gene showed only 10 exons (32), but a recent report demonstrates 11 exons (33) indicating conservation between the human and mouse Ria genes. The promoter site for the mouse Ria gene was mapped by primer extension and S1 mapping and revealed multiple start sites within a GC-rich 5'-flanking region with no recognizable TATA or CCAAT box homologies. This promoter and first non-coding exon corresponds to promoter 1a as characterized for the human RIA gene. Other promoter regions are used in humans (32) but all of these first exons are non-coding and splice to the first coding exon, exon 2, to give identical protein products. Fig. 1A shows a diagram of the mouse Ria gene and the overlapping λ clones that were analyzed, and Fig. 1B contains the sequence of the exons and exon/intron boundaries as well as the sequence of the 1a promoter region.

Targeting the Ria Gene in Mice Results in Increased Basal PKA Activity—In order to address the role of the Ria isofrom of PKA in mouse development, we generated a targeted disruption of the gene. The targeting vector, RiaREK3, contained a neomycin resistance cassette inserted into exon 3 of a 5-κb 129svJ genomic fragment of the Ria gene (Fig. 2A) and a thymidine kinase cassette flanking the Ria genomic coding sequence. This targeting vector was electroporated into REK2 and REK3 ES cell lines (34), and Southern blot analysis identified clones from both REK2 and REK3 ES cells that had homologously recombined the replacement vector sequence into the Ria gene (Fig. 2B). The targeting efficiency observed for the construct over several electroporations was ~1 in 30. Targeted REK2 and REK3 ES cell lines were injected into C57BL/6 blastocysts, and the resulting male chimeric offspring were bred to C57BL/6 females. Chimeras generated from the two independently derived targeted ES cell lines transmitted the mutation through the germ line. The analysis of Ria mutant embryos in this study are derived from mice generated from REK2- and REK3-targeted ES cell clones on the mixed 129svJ × C57BL/6 background.

Mice carrying a heterozygous mutation in Ria were viable, fertile, and morphologically indistinguishable from wild type littermates; however, interbreeding of Ria heterozygotes to generate Ria homozygotes resulted only in wild type and heterozygous offspring in a 1:2 ratio. To determine the point at which Ria homozygous embryos were no longer viable, embryos from heterozygous intercross matings were analyzed at successive stages of development. Only resorption sites were found upon initial inspection of embryos at E11.5; however, Ria mutant embryos were found in Mendelian ratios from E7.5 up to E10.5 (Table I). PCR genotyping of yolk sac DNA using 5' and 3'- oligonucleotides to Ria exon 3 confirmed that the mutant embryos do indeed carry the disruption in exon 3 (Fig. 2C). To confirm the absence of Ria protein in homozygous mutant embryos, mutant and wild type or heterozygote embryos were homogenized directly in sample buffer and run on an SDS-PAGE gel followed by probing with an affinity-purified polyclonal antibody to Ria (Fig. 2D). Western blot analysis confirms that Ria protein is indeed absent in the phenotypically mutant embryos.

To determine whether loss of the Ria regulatory subunit results in increased or decreased PKA activity, we performed kinase assays on pooled E8.5 mutant and wild type or heterozygote embryos (Fig. 3E). Although the total PKA activity is decreased by ~40%, basal PKA activity is increased substantially in Ria mutant embryos. The reduction in total PKA activity in the absence of the Ria subunit is consistent with previous research in cell culture that has demonstrated that the PKA catalytic subunit is unstable when not complexed to regulatory subunits (35), suggesting that there is an increase in free C subunit in Ria mutant embryos that is also more rapidly
degraded. Approximately 52% of the kinase activity in RIo
mutant embryos is still cAMP-regulated, and Western blots
confirm the presence of RIIo in RIo mutant embryos (Fig. 3E,
insert). Interestingly, up-regulation of RIIo is not observed in
RIo mutants, which is consistent with observations made in
cell culture where PKA catalytic subunit was overexpressed.
In fact, a decrease in RIIα levels is seen in the RIα knockout embryos that is also observed in primary embryonic fibroblasts isolated from these embryos (Fig. 7B, inset).

**Phenotype of RIα Mutant Embryos**—One of the most striking features of RIα mutant embryos when first observed was severe growth retardation combined with developmental delay. Although not morphologically evident in mid- and late-streak stage embryos, the phenotype became clearly apparent from the head-fold stage forward (Fig. 3B). Examination of over 30 RIα mutant embryos at E9.5 revealed a very consistent mutant phenotype. The anterior-posterior axis of RIα mutant embryos is clearly apparent, with a prominent head structure followed by a greatly reduced trunk structure compared with wild type E9.5 littermates (Fig. 3, B and D). The most striking feature of the RIα mutant embryo when compared with an equivalently sized E8.0 wild type embryo is the absence of a definitive heart tube (Fig. 3, B and D). The cardiogenic plate is present (Fig. 3B), but rather than being displaced more caudally by the formation of

| Table 1 |
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| **Distribution of genotypes in embryos and adult mice from RIα heterozygous matings** |
| Age | +/+ | +/- | --/-- |
| 7.5 dpc | 11 (33%) | 19 (58%) | 3 (9%) |
| 8.5 dpc | 7 (21%) | 20 (59%) | 7 (21%) |
| 9.5 dpc | 8 (27%) | 13 (43%) | 9 (30%) |
| 10.5 dpc | 5 (15%) | 19 (58%) | 9 (27%) |
| Postnatal | 66 (33%) | 124 (66%) | 0 (0%) |

* Histology on E10.5 mutants revealed extensive necrosis of tissues. dpc, days post-coitum.
the heart tube, it is tightly juxtaposed to the proencephalon. The absence of a heart tube also results in a more radical angle of entry of the foregut (Fig. 3D).

At E8.5, RIA mutant embryos can be easily recognized by their small size, flattened morphology (Fig. 3B), and ruffled yolk sac membrane (not shown). Mutant embryos at this stage resemble presomite head-fold stage wild type embryos and are situated appropriately within the yolk sac and amniotic membranes. At E7.5 the developmental delay is less apparent, although E7.5 RIA mutant embryos are smaller than wild type littermates and have the characteristic flattened appearance and ruffling of the yolk sac membrane.

Abnormal Cardiac Morphogenesis in RIA Mutant Embryos—As mentioned in the previous section, although the cardiogenic plate is present in E8.5 mutant embryos, no heart tube is formed. Morphological and histological examination of the cardiogenic plate reveals the presence of bilateral cardiocytes located within the putative intraembryonic coelomic cavities that have failed to fuse and thus form the pericardium (Fig. 4, C and D). Examination of one E11.5 litter containing three RIA mutant embryos revealed the presence of bilaterally located cardiocytes within enlarged coelomic cavities that contracted rhythmically at room temperature in M2 media at 36
beats/min for several hours (not shown). Histological examination of these embryos revealed the presence of healthy bilateral aggregations of cardiocytes, whereas the embryos themselves were completely necrosed. These observations support the conclusion that at least some mesodermal cells arising from the posterior epiblast of Rlα mutant embryos have ingresed through the primitive streak and migrated in the appropriate lateral and anterior direction together with the definitive endoderm to arrive at the heart field (36). Interestingly, the Rlα mutants never initiate the turning process that would normally occur at the 7–8 somite transition (37). The absence of turning is also evident in the BMP 2 and GATA 4 knockouts that display aberrant cardiac morphogenesis (38, 39). In the case of BMP 2, a failure in the closure of the proamniotic canal results in the formation of a single heart tube outside the amniotic membrane (39). In the GATA 4 knockout, a failure of lateral to ventral folding results in the formation of fully developed bilateral heart tubes located dorsal to the aberrant foregut (38).

In the Rlα mutants, we hypothesize that the failure of the bilateral cardiocytes to fuse ventrally arises from a deficiency in the total number of mesodermal cells fated to become cardiocytes.

Axial and Paraxial Mesoderm Is Formed and Expresses Appropriate Markers but Is Greatly Reduced in Size—As mentioned previously, the anterior-posterior axis of Rlα mutant embryos is well defined, including axial and paraxial mesodermal derivatives. A morphological node is apparent in E7.5 mutant embryos (Fig. 3A), and the notochord is clearly present in rostral sections of E9.5 mutant embryos (not shown). Although paraxial mesoderm is formed and is segmented in Rlα mutants, the somites are very small and irregularly shaped (Fig. 4, E and F). The absence of fusion of somites along the ventral midline suggests that notochord material must be present along the rostro-caudal axis of Rlα mutants (Fig. 4F), as the complete absence of notochord normally results in ventral fusion of somites (40–42). Although the somites are small and irregularly shaped, they do express the dermomyotomal marker Pax 3, suggesting that dorsolateral differentiation of the somites is progressing normally (Fig. 4E).

Reduced Primitive Streak and Abnormal Migration of Mesoderm in Rlα Mutants—Due to the deficiencies observed in mesodermal derivatives, we chose to look at earlier stages of mesoderm formation in Rlα mutant embryos. Histological examination of E6.5 Rlα mutant embryos compared with E6.5 wild type littermates revealed aberrant migration of mesodermal cells away from the primitive streak. Although wild type late-stage streak embryos always exhibit very tightly ordered columns of mesodermal cells that extend smoothly in a lateral and anterior direction, the mutants display highly disorganized movement away from the primitive streak (Fig. 5, A and B). Quantitation of mesodermal cells that have exited the streak in the embryonic portion of late-streak stage Rlα mutant embryos indicates that mutants have approximately one-third the number of mesodermal cells that have exited the streak and moved laterally and anteriorly (Fig. 5C). Reduction in primitive streak mesoderm is also clearly visible 2 days later in the E8.5 Rlα mutant embryo, where a significant reduction in brachyury mRNA is observed (Fig. 5D). An accumulation of presumptive mesoderm beneath the primitive streak that has failed to migrate out laterally and anteriorly is observed in the E9.5 embryo (not shown).

Loss of Rlα Alters Cell Morphology, Migration, and Localization of C Subunit in Primary Embryonic Fibroblasts—To address the hypothesis that increased PKA activity inhibits growth factor signaling involved in the proliferation and migration of mesoderm from the primitive streak stage forward, we isolated mesodermal primary embryonic fibroblasts from E8.5 Rlα mutant and wild type embryos. Fibroblasts isolated from these embryos were extremely unusual with respect to overall morphology. Rather than showing the typical spindled morphology of wild type fibroblasts, Rlα mutant fibroblasts are flattened and show a box-shaped or rounded morphology. Examination of the actin cytoskeleton revealed that the Rlα mutant fibroblasts have multiple actin organizing centers that extend in all directions and an extensive subcortical actin cytoskeleton, whereas wild type fibroblasts display sparse parallel actin fibers running from the leading to the lagging edge of the cell (Fig. 6, A and B). To assess localization of C subunit, confocal imaging was used on wild type and Rlα knockout E8.5 primary embryonic fibroblasts. C subunit is uniformly distributed throughout the cytoplasm of the wild type fibroblasts (Fig. 6C); however, in Rlα knockout fibroblasts, C subunit was preferentially localized to the perinuclear space (Fig. 6D). Examination of Rlα localization revealed a uniform cytoplasmic distribution in wild type fibroblasts (Fig. 6E), similar to that observed for Cα, whereas no staining was observed in Rlα knockout fibroblasts (Fig. 6F). Cell cycle analysis of wild type and Rlα mutant fibroblasts showed no difference in the percentage of cells in G1/G0, S, and G2/M (Fig. 7A). cAMP activation curves revealed high basal PKA activity in Rlα mutant fibroblasts consistent with the unregulated PKA activity observed in the Rlα knockout embryos (Fig. 7B). A small decrease in total PKA activity was observed compared with wild type fibroblasts combined with a decrease in RIIα levels in Rlα knockout fibroblasts (Fig. 7B, inset). HPLC analysis of wild type E8.5 primary embryonic fibroblasts reveals significant quantities of type II holoenzyme along with type I holoenzyme (Fig. 7C). Quantitation of the type II and type I holoenzyme peaks indicates a type II to type I ratio of ~3.5 to 1 in the wild type cells and as expected no type I kinase in the mutants (Fig. 7C).

Migration assays using an in vitro wound model demonstrated that Rlα mutant fibroblasts completed wound healing by ~18 h, whereas wild type fibroblasts were significantly slower at completing this process (Fig. 7D). This difference in migration and “wound healing” was similar when fibronectin-coated dishes were used in place of uncoated plastic. The significantly faster closure of a scrape “wound” in cell culture by Rlα mutant fibroblasts is superficially at odds with the observed deficiency in mesoderm formation and migration out of the primitive streak in Rlα knockout embryos. However, it is likely that the complex environment at the primitive streak in vivo, involving FGF-dependent receptor tyrosine kinase signaling, E cadherin down-regulation, epithelial to mesenchymal transition, and subsequent integrin-dependent migration of nascent mesoderm over the endoderm is not adequately modeled by this in vitro assay.

The Deficits in Mesodermal Derivatives Can Be Rescued by Crossing Rlα Mutants to Cα Mutants—Based upon the hypothesis that the defects observed in the mesoderm of Rlα mutants is a result of increased basal PKA activity, we attempted to rescue the Rlα mutant phenotype by crossing Rlα mutants to Cα mutant mice that carry a null mutation in the Cα gene (24). As Cα homozygous mutant mice are infertile and Rlα homozygous mutant mice are embryonic lethals, we generated Rlα/Cα double heterozygotes (Rlα+/Cα+/-) by crossing Rlα heterozygous females to Cα heterozygous males. The resulting Rlα+/-/Cα+/- double heterozygous animals were viable and were interbred to generate Rlα knockout animals on the Cα heterozygous and Cα homozygous background. The first 32 offspring from a total of four litters yielded Rlα+/-/Cα+/- and Rlα+/-/Cα-/- offspring at the expected Mendelian ratios of 1:8
and 1:16. RIA+/−/Ca+/− embryos were easily identified by their morphology and display an intermediate phenotype clearly indicative of partial rescue of the RIA mutation (Fig. 8C). One of the most dramatic features of RIA+/−/Ca+/− is the presence of a definitive heart tube located within a fluid-filled pericardial cavity (Fig. 8C). Morphological and histological examination of these embryos reveals a significant increase in all axial, paraxial, and lateral plate mesodermal derivatives, including increased head mesenchyme, increased somite size and number, and increased trunk length. Unlike the RIA mutants, RIA+/−/Ca+/− embryos also seem to have initiated the turning process (Fig. 8C). RIA+/−/Ca−/− embryos showed an even greater degree of rescue compared with the RIA mutant phenotype (Fig. 8, B and D). RIA+/−/Ca−/− shows dramatic increases in trunk length, somite number and size, branchial arch and limb bud development, and head mesenchyme. Unlike RIA mutants, RIA+/−/Ca−/− also completes the turning process and has a heart tube located within the pericardial cavity (Fig. 8, B and D). Kinase activity measurements from the various genotypes revealed a predictable decrease in total PKA activity but also a graded decrease in basal PKA activity as Ca alleles are deleted on the RIA knockout background (Fig. 8E). Genotyping of newborn pups from several RIA+/−/Ca+/− interbreedings has failed to yield viable RIA knockout animals, suggesting that the lack of RIA protein on the Ca knockout background is still lethal. Examination of RIA+/−/Ca−/− embryos at midgestation (E10.5) revealed an expanded fluid-filled pericardial cavity, suggesting cardiovascular failure in the absence of RIA at a later stage in development.

DISCUSSION

We have utilized targeted disruption of PKA subunit genes to determine the developmental and physiological roles of individual isoforms of the PKA family of genes in the mouse. Disruption of the RIIα subunit that is expressed ubiquitously at all stages of development did not lead to any developmental deficits, and the animals were healthy and fertile (43). However, in this report we show that disruption of the other ubiquitous R subunit, RIIα, results in a dramatic developmental phenotype. This phenotype is characterized by mesodermal insufficiency, and the early manifestations are caused by inappropriately regulated C subunit activity because we can partially rescue the RIIα mutant embryos by breeding them onto a Ca knockout genotype that reduces the total level of C subunit in the mutants.

The observation that receptor-tyrosine kinases are critical for mesoderm formation combined with the substantial literature on PKA-dependent inhibition of receptor tyrosine kinase

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signaling led to the hypothesis that increased PKA activity in the Rlα mutants might be antagonizing growth factor-dependent signaling in the primitive streak. This possibility is consistent with the greatly reduced brachyury whole mount in situ staining in the primitive streak of Rlα mutants and the deficiencies in anterior mesoderm-derived structures. The accumulation of nascent mesoderm at the base of the primitive streak also suggests deficits in integrin-dependent cell migration over the endoderm which also depends upon growth factor receptor-tyrosine kinases and extracellular matrix signals (44). Targeted disruption in mice of several key proteins involved in integrin-mediated signaling and migration, including fibronectin and focal adhesion kinase (FAK), result in the failure of embryos at gastrulation (45–47). FAK-deficient and fibronectin-deficient embryos bear a striking resemblance to each other and to Rlα mutant embryos, including growth retardation, developmental delay, failure of cardiac morphogenesis, and anterior mesoderm deficits. The similarities in the phenotypes of FAK, fibronectin, and Rlα mutant embryos combined with the literature describing PKA-dependent regulation of the actin cytoskeleton support the hypothesis that growth factor/ integrin signaling may be affected (3, 4). Activation of PKA causes dephosphorylation of paxillin via decreasing a tyrosine phosphatase activity (48). More recent evidence demonstrates that PKA can phosphorylate and activate Shp2, a tyrosine phosphatase that localizes to focal adhesion complexes and dephosphorylates both paxillin and FAK (49, 50). Related studies reveal that the p21-activated kinases are also critical for the integration of growth factor and integrin-extracellular matrix signals with the actin cytoskeleton (51, 52). Significantly, PKA activity also negatively regulates p21-activated kinase and thus interferes with its ability to mediate anchorage-dependent growth factor responses (53). Isolation of primary embryonic fibroblasts from E8.5 Rlα mutant embryos reveals a profound disruption of the actin-based cytoskeleton. These fibroblasts bear a striking resemblance to FAK-deficient and Shp2-deficient primary embryonic fibroblasts, including a dramatic increase in focal adhesions and condensed F-actin aggregation at the cell periphery (47, 54). Expression of a dominant negative Shp2 tyrosine phosphatase prevents paxillin and FAK dephosphorylation and results again in increased focal adhesions and impaired migration (49), suggesting that successive cycles of phosphorylation/dephosphorylation are required for directed
cell migration and that PKA may also be involved in this process.

We hypothesize that the defects observed in mesodermal derivatives result from antagonism of growth factor-mediated production and migration of mesoderm out of the primitive streak. The fact that we can incrementally rescue all the mesodermal derivatives of the primitive streak by reducing the total amount of C subunit supports the hypothesis that excessive catalytic subunit activity is antagonizing growth factor signaling in the primitive streak. These observations support a model in which PKA plays a role as a general negative regulator of growth factor signaling in the primitive streak, not unlike its role as a general negative regulator of sonic hedgehog signaling in vertebrates (19, 20). Whether the cAMP/PKA pathway is normally involved in regulating receptor tyrosine kinase signaling in the primitive streak remains to be demonstrated.

Is there a specific role for RI\(\alpha\) in mouse embryo development? C\(\alpha\) knockout animals are developmentally normal, and it is only postnatally that they manifest growth retardation and a failure to thrive (24). The observation that RI\(\alpha\) knockout embryos on the C\(\alpha\) knockout background suffer cardiovascular failure at midgestation suggests a specific and novel role for RI\(\alpha\) in cardiac development. It is unlikely that the midgestation cardiovascular failure of RI\(\alpha\) knockouts on the C\(\alpha\) knockout background is due to an overall decrease in C subunit activity, as C\(\alpha\) knockouts show a significant decrease in C subunit activity in the heart and survive to adulthood. Furthermore, mice with only one remaining C subunit allele (C\(\alpha\) or C\(\beta\)) also survive to birth and have normal cardiac development (55). There is evidence for specific localization of RI\(\alpha\) containing holoenzyme by interaction between scaffolding proteins and the N terminus of RI\(\alpha\). For example, RI\(\alpha\) localizes to the neuromuscular junction in skeletal muscle and the fibrous sheath in mammalian spermatozoa (56, 57).

A unique role for the RI\(\alpha\) regulatory subunit has been demonstrated recently in the human disease known as Carney complex (33, 58). One of the disease loci in humans has been mapped to the RI\(\alpha\) gene located on human chromosome 17q24 and consists of heterozygous mutations in the RI\(\alpha\) coding sequence or splice junctions that prevent RI\(\alpha\) protein expression and also appear to lead to mRNA degradation. Patients suffering from loss of heterozygosity due to somatic mutations in the remaining RI\(\alpha\) allele present with a multiple neoplasia syndrome characterized by spotty pigmentation of the skin, endocrine tumors, melanotic schwannomas, and cardiac myxomas. The mechanism by which the loss of heterozygosity leads to these clinical manifestations is unclear. One possible mechanism is increased basal PKA activity due to loss of regulation; however, direct analysis of adrenal and ovarian tumors did not show an increase in basal but instead revealed an increase in total PKA activity (58). The RI\(\alpha\) heterozygous mice should serve as an animal model and provide an opportunity to explore the underlying mechanisms leading to the pathology of Carney complex.

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FIG. 8. RI\(\alpha\) mutants can be rescued on the C\(\alpha\) mutant background. Morphology of an E9.5 RI\(\alpha^{+/+}\)C\(\alpha^{+/+}\) wild type littermate (A), E9.5 RI\(\alpha^{-/-}\)C\(\alpha^{+/+}\) mutant (B), E9.5 RI\(\alpha^{-/-}\)C\(\alpha^{-/-}\) mutant (C), and an E9.5 RI\(\alpha^{-/-}\)C\(\alpha^{-/-}\) mutant (D). Scale bars = 500 µm. E, measurement of basal and total PKA activity from all four genotypes. Animals were staged and embryos collected from two litters (14 total embryos) to obtain rescued embryos with the genotypes indicated. Embryos were homogenized, sonicated, and stored at \(-80^\circ C\) until the day of the assay.
