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Delayed embryonic lethality in mice lacking protein phosphatase 2A catalytic subunit Cα

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ABSTRACT **Protein phosphatase 2A (PP2A) is a multimeric enzyme, containing a catalytic subunit complexed with two regulatory subunits. The catalytic subunit PP2A C is encoded by two distinct and unlinked genes, termed Cα and Cβ. The specific function of these two catalytic subunits is unknown. To address the possible redundancy between PP2A and related phosphatases as well as between Cα and Cβ, the Cα subunit gene was deleted by homologous recombination. Homozygous null mutant mice are embryonically lethal, demonstrating that the Cα subunit gene is an essential gene. As PP2A exerts a range of cellular functions including cell cycle regulation and cell fate determination, we were surprised to find that these embryos develop normally until postimplantation, around embryonic day 5.5/6.0. While no Cα protein is expressed, we find comparable expression levels of PP2A C at a time when the embryo is degenerating. Despite a 97% amino acid identity, Cβ cannot completely compensate for the absence of Cα. Degenerated embryos can be recovered even at embryonic day 13.5, indicating that although embryonic tissue is still capable of proliferating, normal differentiation is significantly impaired. While the primary germ layers ectoderm and endoderm are formed, mesoderm is not formed in degenerating embryos.**

Protein phosphorylation is probably the major regulatory mechanism employed by eukaryotic cells at the protein level (1, 2). The protein phosphatase 2A (PP2A) family of holoenzymes has been implicated in many different facets of cellular function (3, 4). Besides regulating metabolic enzymes, PP2A appears to modulate many proteins involved in signal transduction, including cell surface receptor molecules, cytosolic protein kinases, and transcription factors. The modification of proteins important for cell-cycle regulation, cell transformation, and cell fate determination affects essential processes of every cell.

Although much is known about the structure of PP2A enzymes, the regulatory features that control their activity remain to be elucidated. Many different trimeric holoenzymes of PP2A have been purified and evaluated with regard to substrate specificity and subunit composition (3, 4). All holoenzymes have in common a catalytic subunit of 36 kDa (PR36) and a 65-kDa regulatory subunit (PR65). These "core" subunits associate with a range of regulatory subunits (PR55, PR61, PR72) to form different heterotrimers.

The catalytic subunit has been cloned from a variety of species. From mammals cDNA clones encoding two different subunits, termed Cα and Cβ, were isolated. Both consist of 309 amino acids with a predicted molecular mass of 35.6 kDa. They are 97% identical, with 7 of 8 different residues located within the first 30 amino acids. The specific function of these two subunits is unknown. Analysis of the expression of the two isoforms from different porcine and rat tissues showed that the highest levels of the transcripts encoding both isoforms occur in the brain, whereas the levels in other tissues analyzed were about 1/10 as high. In all cases the Cα transcripts were about 10-fold more abundant than the Cβ transcripts (5). Interestingly, increased amounts of Cβ transcripts were reported in chemically induced rat liver tumors. Increased levels of the Cα subunit were found in several transformed cell lines (6, 7). Furthermore, solid tumors or cell lines transfected with *retII*, Ki-*ras*, or c-*raf* oncogenes have elevated catalytic subunit transcript levels (8).

To dissect the functions of the two catalytic subunits in mice, a gene targeting approach was chosen. We created a mouse strain deficient in PP2A Cα. This approach allows us to address the possible redundancy between PP2A and related phosphatases as well as between Cα and Cβ.

MATERIALS AND METHODS

Cloning. PP2A Cα genomic sequences were cloned from an isogenic λGem11 (Promega) phage genomic library prepared from AB1 [129/Sv(ev)] embryonic stem (ES) cell DNA (9). Eight phage clones were isolated by hybridization with a murine cDNA probe, and their restriction map was established. Analysis of these clones reveals that the gene organization is similar to that in humans, where the Cα locus spans approximately 30 kb and is composed of seven coding exons with the six introns intervening at identical locations (10). A 9-kb *Bam*HI fragment containing exon 1 was subcloned into the Bluescript vector (Stratagene). Sequencing from exon 1 into the promoter region revealed a striking sequence conservation between rat (10) and mouse. Since the deletion of 0.5 kb immediately upstream of the transcription start site of the rat Cα gene abolishes expression in a chloramphenicol acetyltransferase (CAT) assay (11), for a complete knockout of Cα a targeting construct (ZH13) was generated encompassing 8.1 kb of genomic sequence. An internal 0.9-kb *Stu*I/*Sma*I fragment including more than 0.5 kb of the promoter, exon 1, as well as approximately 0.3 kb of intron 1 were replaced by a neomycin-resistance gene constitutively transcribed from a phosphoglycerate kinase promoter inserted in opposite transcriptional orientation (12). In this 2-kb cassette (13), the neomycin-resistance gene (inserted in antisense orientation) is followed by the 360-bp UMS *Sal*I–*Nco*I sequence, reported to mediate transcriptional termination (14) in the sense orientation. A herpes simplex virus thymidine kinase (HSV TK) gene

Abbreviations: PP2A, protein phosphatase 2A; Cα, PP2A catalytic subunit Cα; Cβ, PP2A catalytic subunit Cβ; ES cell, embryonic stem cell; neo, neomycin-resistance gene cassette; E*n*, embryonic day *n*.

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was added at the 3′ end of the Cα sequence to allow counter selection (12). The targeting vector was then linearized with *Sac*II in the Bluescript backbone for electroporation.

ES Cell Culture and Selection. GS1 [129/Sv(ev)] ES cells were electroporated with the linearized vector as described (15). Among the 192 G418-resistant clones that were expanded, 6 had undergone specific homologous recombination as demonstrated by PCR using primers P3 (5′-ATTCG-CAGCGCATCGCCTTCTATCGCC-3′) from the 3′ end of the neo cassette and WO-1 (5′-GATATTCACTACAAAC-CAGAG-3′) corresponding to a genomic sequence 5′ from the targeting vector. These clones were shown to be heterozygous for the interrupted Cα gene as revealed by Southern blot analysis (data not shown) and used to generate chimeric animals by injecting them into blastocysts from C57BL/6 mice. Crossing of chimeras with wild-type C57BL/6 females allowed germ-line transmission for two clones.

Genotyping by PCR, Southern, and Northern Blotting. Genomic DNA was prepared as described (15). A 0.9-kb *Bam*HI/*Bgl*II fragment not present on the targeting construct was used as flanking probe for Southern blot analysis. Upon digesting genomic DNA with *Bam*HI, a 9-kb wild-type allele and a 10.1-kb mutant allele were detected. The existence of a single integration site at the targeted locus was verified by using a neo probe, giving a single 10.1-kb fragment (not shown). Extracts for PCR analysis were prepared from tail biopsies, dissected embryos, scraped paraffin sections, or blastocysts as described (15, 16).

Primers P3 and WO-6 (5′-GCTGTTGGACTAGCGCT-TAGG-3′) generated a 450-bp PCR fragment revealing the homologously recombined allele, and primers WO-6 and O-198 (5′-AGCTCCTTGGTGAACAACTTC-3′) generated a 650-bp fragment diagnostic of the wild-type PP2A Cα allele. The following primers were used for the PCR analysis of fixed tissue and scraped-off sections: Primers O-210 (5′-CAAGAT-GGATTGCACGCAGG-3′) and O-211 (5′-GCTTCAGTGA-CAACGTCGAG-3′) are internal to the neomycin gene and generate a 250-bp fragment, primers O-212 (5′-GGCATCAT-GGACGAGAAGTTG-3′) and O-213 (5′-TTCTCGCAGAG-GCTCTTGAC-3′) are internal to the Cα exon I and generate a 107-bp wild-type product.

Total RNA was extracted from brain as described (15), electrophoresed on a 0.9% formaldehyde gel with 20 μg of total RNA per lane. After separation, the RNA was stained with ethidium bromide to visualize the 28S and 18S rRNA bands for normalization, followed by a transfer to a Hybond N^+ membrane (Amersham). ^{32}P-labeled Cα exon 6 (5′-ACTTAAGAGTGTCGTCAAGT-3′) and exon 7 (5′-GACATGTGGCTCGCCTCTAC-3′) specific oligonucleotides in the antisense orientation were used as probes for hybridization.

Histology, Immunohistochemistry, and RNA *in Situ* Hybridization. Embryos within their decidua were fixed in 4% formaldehyde buffered in PBS (formalin) overnight at room temperature, dehydrated in graded alcohols, embedded in paraffin, sectioned sagitally (2- to 3-μm sections), and stained with hematoxylin and eosin. Immunohistochemical detection of PP2A C on sections used Promega rabbit antiserum V598A (directed against a C-terminal peptide shared by Cα and Cβ) and a biotinylated anti-rabbit secondary antiserum (Vector Laboratories). Detection of Cβ used rabbit antiserum 15 [directed against amino acids 22–31 of Cβ, an amino acid stretch showing a high degree of sequence diversity between Cα and Cβ (17, 18)]. Antiserum 45 (anti-Cα) (17, 18) did not work on paraffin-embedded sections. Staining was revealed by the ABC method, with the appropriate Vectastain kit (Vector Laboratories) using diaminobenzidine (and 5% $NiCl_2$) as substrate. Specificity of staining was confirmed by concurrent processing of tissue sections where the primary antibody had been omitted.

For whole-mount immunohistochemistry embryos and blastocysts [embryonic day 3.5 (E3.5)] were fixed in Dent's fixative (19), dehydrated, and permeabilized in PBS/2% milk powder/0.1% Triton X-100. Antisera V598A (anti-Cα and β) and 45 [anti-Cα; directed against the first 20 amino acids of Cα, an amino acid stretch showing a high degree of sequence diversity between Cα and Cβ (17, 18)] were used, followed by a horseradish peroxidase-conjugated anti-rabbit IgG antiserum (Dako), using diaminobenzidine as a substrate. Specificity of staining was confirmed by including controls from which the primary antibody had been omitted.

Whole mount RNA *in situ* hybridization was done following standard protocols (15, 20). Antisense RNA probes were made for brachyury (21) and goosecoid (22) transcripts as described (23), and a digoxigenin RNA labeling kit (Boehringer Mannheim) was used.

RESULTS AND DISCUSSION

Inactivation of PP2A Cα. To investigate the role of the PP2A catalytic subunit Cα during embryonic development, we generated a null mutant by using gene targeting in ES cells. A targeting vector was constructed in which the promoter, exon I, and part of intron I were replaced by a neomycin-resistance cassette (Fig. 1*a*). Among the 192 G418-resistant clones that were expanded, 6 had undergone specific homologous recombination as demonstrated by PCR and Southern blot analysis (data not shown). These clones were used to generate chimeric animals by injecting them into blastocysts from C57BL/6 mice. Crossing of chimeras with wild-type C57BL/6 females allowed germ-line transmission for two clones. Adult heterozygous mice derived from both clones were overtly normal. Cα protein levels and phosphatase activity were not reduced in heterozygous mice compared with wild-type controls (data not shown).

Embryonic Lethality of Homozygous Knockout Mice. Of more than 350 progeny derived from heterozygous crosses, none were homozygous null (−/−), demonstrating that the Cα subunit is essential for the development of viable offspring (Fig. 1*b*). We obtained identical results with mice generated from two independent ES cell clones. Analysis of brain RNA extracted from wild-type and heterozygous mice by using antisense oligonucleotide probes specific for exon 6 and exon 7 of Cα reveals that no aberrant mRNA is transcribed from the mutant allele (Fig. 1*c*). To investigate the time of the presumptive embryonic lethality, we determined the genotype of individual embryos by using PCR (Fig. 1*d*). Homozygous null blastocysts (E3.5) appeared morphologically normal and were identified at a ratio that was not significantly different from the expected Mendelian distribution (data not shown).

Histological examination revealed that all E5.5 embryos from two heterozygous crosses displayed normal morphology, whereas approximately 25% of all E6.5 and E7.0 embryos from four heterozygous crosses were degenerated and smaller (compare Fig. 2 *a* with *b* and 2 *c* with *d*) than heterozygous or wild-type embryos, which were all normal. Both normal and degenerated (−/−) embryos exhibit defined layers of cells of extra-embryonic and embryonic endoderm, as well as extra-embryonic and embryonic ectoderm, but degenerated embryos (Fig. 2 *b* and *d*) are consistently smaller, and embryonic ectoderm seems to be more degenerated than extra-embryonic ectoderm (Fig. 2*d*). Taken together, these data show that the initial development of −/− embryos is not different from that of heterozygous or wild-type embryos. Null (−/−) blastocysts display a normal morphology of both inner cell mass and trophectoderm. The embryos implant properly, and both the extra-embryonic and embryonic portion of the egg cylinder differentiate to develop the two primary germ layers, ectoderm and endoderm. It seems likely that even a proamniotic cavity can form in the absence of Cα, as some of the degenerating embryos form a cavity. At E6.5 all −/− embryos show signs

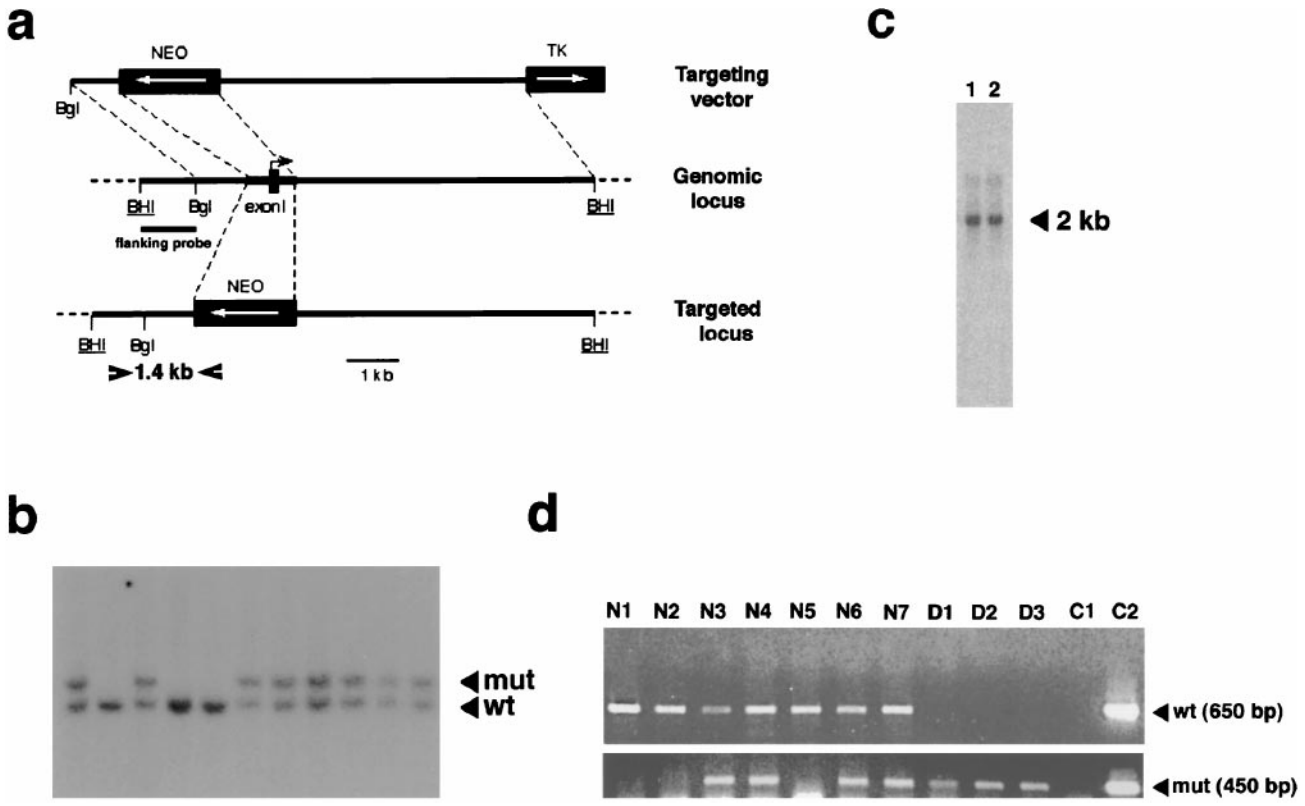

FIG. 1. PP2A Cα gene targeting and genotyping. (*a*) Homologous recombination of the Cα genomic locus resulted in the replacement of 0.5 kb of the promoter, the entire exon I, and 0.3 kb of intron I sequences by the neomycin cassette. The neomycin cassette was flanked by 1 kb and 6 kb of genomic Cα sequences, respectively. The transcriptional orientation of the Cα gene is indicated by an arrow. BHI, *Bam*HI; Bgl, *Bgl*II. The diagnostic primers WO-1 and P3 used to detect homologous recombination are indicated by arrowheads. (*b*) Southern blot analysis of viable offspring obtained from heterozygous matings. Genomic DNA was digested with *Bam*HI (BHI) and probed with a flanking *Bam*HI/*Bgl*II fragment (shown in *a*). Viable offspring were either heterozygous (+/−) or wild-type (+/+) as shown by the presence of a 9-kb wild-type and 10.1-kb mutant band, respectively. (*c*) Northern blot analysis of RNA obtained from wild-type (lane 1) and heterozygous (lane 2) brain, using a Cα-specific antisense oligonucleotide probe. (*d*) Genotyping of dissected E8.5 embryos obtained after heterozygous matings. All degenerated embryos (D1–D3) were genotypically −/− as shown by the presence of a 450-bp mutant PCR product and the absence of a 650-bp wild-type PCR product; all phenotypically normal embryos (N1–N7) were either +/+ or +/− as shown by the presence of a 650-bp wild-type PCR product. Two controls are shown for the two PCRs: For the wild-type PCR, targeting plasmid ZH13 was included as a negative control (C1) and wild-type tail lysate (C2) as a positive control. As controls for the presence of the mutant allele, wild-type (C1) and heterozygous (C2) tail lysates were included.

of degeneration, which affects all germ layers, but is most pronounced in embryonic ectoderm, leading to a block in subsequent differentiation steps (see below).

PP2A Cα Expression in Normal and Degenerated Embryos. E7.5 embryos were dissected from the decidua and analyzed by whole mount immunohistochemistry using rabbit antiserum 45, which is specific for Cα and does not cross-react with Cβ (17, 18). Cα was present in embryos with apparently normal morphology with a relatively uniform expression pattern (Fig. 3*a*). In contrast, Cα could not be detected in morphologically abnormal embryos (Fig. 3*b*), which displayed several phenotypes. Some had a proamniotic cavity and others formed an amorphous cell mass of different sizes. Genotyping revealed that the abnormal embryos were homozygous null mutants. These embryos ceased proper development shortly after implantation, demonstrating clearly that neither Cβ nor catalytic subunits of other phosphatases can compensate for the absence of Cα at this stage of development.

Partial Redundancy of the PP2A Catalytic Subunits. Despite the fact that PP2A Cα seems to be implicated in cell cycle regulation (1, 3, 24), homozygous null mutant embryos can survive until E5.5/E6.0. A first possible explanation for this finding is that prior to E6.5, Cα is not essential and its function can be assumed by other related phosphatase catalytic subunits such as Cβ. Alternatively, maternal stocks of Cα could persist until after implantation and be sufficient for the initial embryonic cell divisions. To discriminate between these possibilities, we analyzed dissected E7.5 embryos by whole mount immunohistochemistry using rabbit antiserum V598A, which is directed against an epitope common to Cα and Cβ. Previous experiments have shown that in different cell lines (10) and adult tissues (5), and also during *Xenopus* embryogenesis (25), Cα mRNA is generally far more abundant than Cβ. However, expression levels in the mouse embryo have not been described. We found that both normal and degenerated (−/−) embryos expressed PP2A C (α and β) at comparable levels (Fig. 3 *c* and *d*). This finding was confirmed by immunohistochemical analysis of sections from E6.5 decidua using antiserum V598A, which showed similar staining intensities for both normal and degenerated embryos (Fig. 4 *a* and *b*). Controls were included in which the primary antiserum had been omitted (Fig. 4*c*). In addition, serial sections were stained with antiserum 15, which is directed against a Cβ-specific epitope. Normal and degenerated embryos show comparable staining intensities (Fig. 4 *d* and *e*). The genotypes were determined by PCR analysis from embryonic tissue of serial sections. Our findings suggest that before E6.5 PP2A Cα is not essential and may be only weakly expressed in comparison to Cβ. At and beyond E6.5, PP2A Cβ may not compensate for the lack of Cα either because sufficient amounts of C protein are not provided because of the weak promoter of Cβ (10) or because Cβ cannot dephosphorylate all substrates of Cα. Comparable expression levels of C in normal and degenerating embryos at the time of onset of degeneration would argue for the latter.

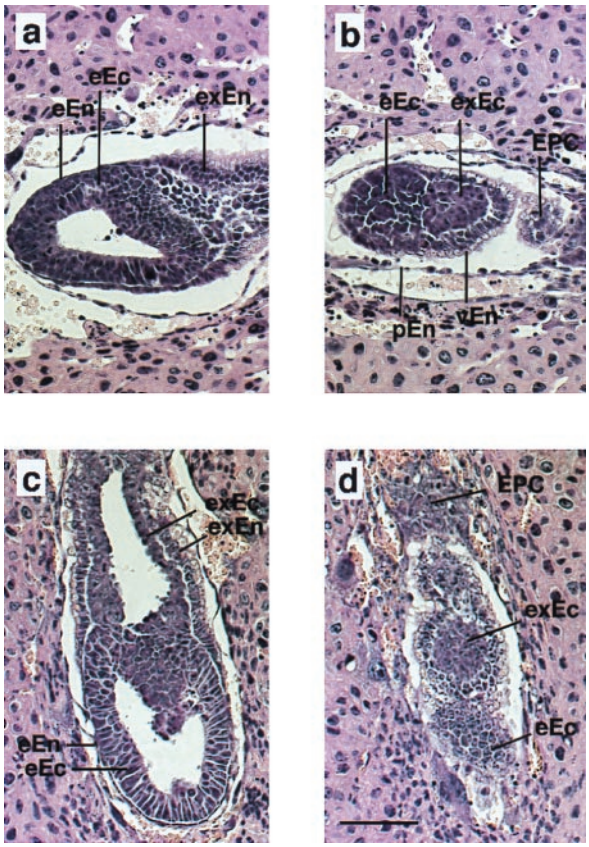

FIG. 2. Morphology of embryos obtained from heterozygous crosses. Serial sagittal sections were made from embryonic day E6.5 (*a* and *b*) and E7.0 (*c* and *d*) deciduae (29). The genotype of these embryos was determined by PCR analysis of serial sections from which embryonic tissue was scraped off. Sections were stained with hematoxylin and eosin. Normal (*a* and *c*) and degenerated (*b* and *d*) embryos exhibit defined layers of cells of extra-embryonic (exEn) and embryonic (eEn) endoderm, and extra-embryonic (exEc) and embryonic (eEc) ectoderm. Degenerated embryos (*b* and *d*) are smaller and some of them are amorphous. Embryonic ectoderm seems to be more degenerated than extra-embryonic ectoderm (*d*). Parietal endoderm (pEn), visceral (vEn) endoderm, and the ectoplacental cone (EPC) are indicated. (Scale bar = 50 μm.)

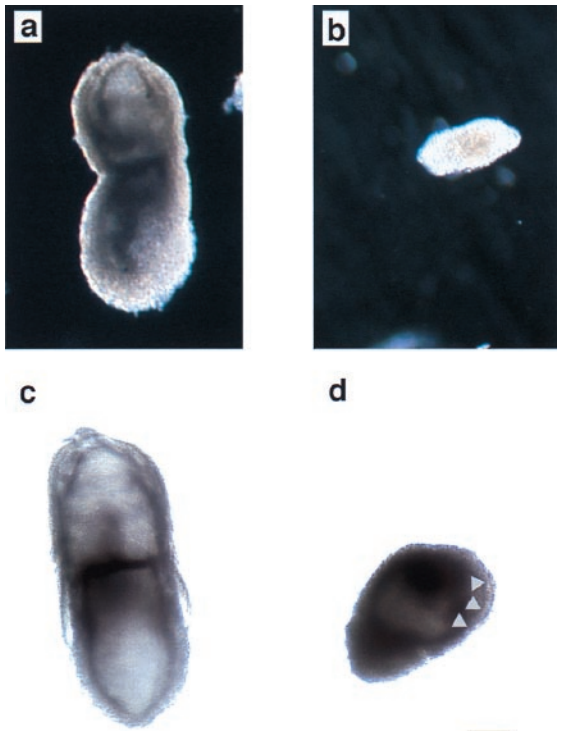

FIG. 3. Whole mount immunohistochemistry of dissected E7.5 embryos obtained from heterozygous matings. The genotype of dissected embryos was determined by PCR. (*a* and *b*) Dissected embryos were stained with antiserum 45 (17, 18), which is specific for Cα and does not cross-react with Cβ. In morphologically normal embryos (*a*) Cα is expressed almost uniformly, whereas morphologically abnormal ($-/-$) embryos (*b*) do not express Cα. (*c* and *d*) However, when an antiserum is employed that is directed against an epitope shared between Cα and Cβ, normal (*c*) and degenerated (*d*) embryos are stained equally well. Some of the degenerated embryos show a (proamniotic) cavity (*d*), the borders of which are indicated by arrowheads. (Scale bar = 50 μm.)

The second possibility, that in both wild-type and mutant embryos maternal stocks of Cα may persist, is difficult to assess because wild-type blastocysts reveal only very weak Cα staining. During normal mouse development, most maternal mRNAs are destroyed upon activation of the zygotic genome, although maternal stores of some mRNAs have been shown to persist until the blastocyst stage (26). It is unlikely that material stocks of Cα mRNA do persist, since in the 3′-untranslated region, there are five copies of the AUUUA motif, which is thought to mediate rapid mRNA turnover (27). At least in *Xenopus*, high levels of both Cα and Cβ transcripts decrease rapidly during the meiotic and first mitotic embryonic cell divisions (25).

Proliferation and Differentiation of the Degenerating Embryo. Embryos were also dissected from the decidua at later stages of embryonic development. Genotypically homozygous null embryonic cell masses could be recovered as late as E13.5 at a ratio of approximately 0.25 (data not shown). This finding suggests that the embryo or part of it is still capable of proliferating, but that normal differentiation is significantly impaired. The presence of these cell masses is surprising, as degenerating embryos are normally rapidly resorbed. Disruption of the cyclin A2 gene (28), for example, results in embryonic death around E5.5, with no residual embryonic material found at E8.5. Likewise, disruption of the β-catenin gene (16) leads to death at around E7.0 with almost complete resorption at E9.0. It can be speculated that PP2A may regulate signal transduction pathways involved in apoptosis and growth control.

As mutant embryos start to degenerate at or shortly before the primitive streak stage, when under normal conditions gastrulation is initiated (29) and cells in the embryonic ectoderm are induced to form mesoderm (E6.5), we dissected E7.0 embryos resulting from heterozygous crosses and analyzed them by RNA *in situ* hybridization. RNA probes for two mesodermal markers were employed. Brachyury transcripts (Fig. 5 *b* and *c*) (21) and goosecoid transcripts (data not shown) (22) could be detected in the primitive streak area of all phenotypically normal embryos; however, they were not found in mutant embryos. When wild-type E6.5 embryos were dissected, the pattern of Brachyury staining (Fig. 5*a*) revealed that mesoderm formation had already been initiated. The size of these wild-type embryos is comparable to that of degenerated embryos dissected at E7.0. Mutant embryos were analyzed at E7.0 and not at E6.5 as we wanted to control for a possible developmental delay of mutant embryos. As Brachyury is a very early mesodermal marker with low levels of transcript present in cells juxtaposed to the embryonic–extra-embryonic junction already the day before the primitive streak can be recognized (30), our data suggest that Cα may be required for the onset of gastrulation and that this function can neither be taken over by other phosphatases nor by PP2A containing Cβ as catalytic subunit. We therefore expect that transgenic overexpression of Cβ would not rescue the Cα knockout phenotype.

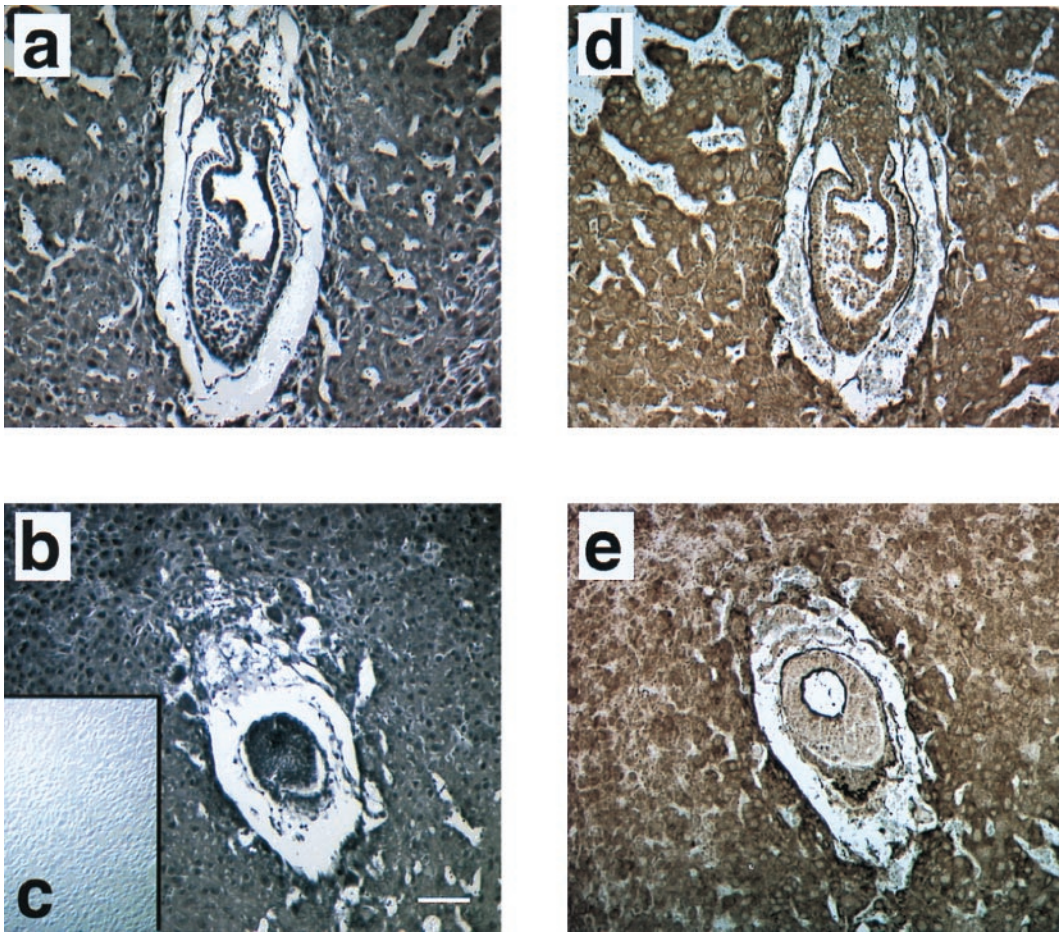

FIG. 4. PP2A C (*a–c*) and Cβ (*d* and *e*) expression of embryos obtained from heterozygous crosses. Serial sagittal sections were made from E6.5 deciduae (29). The genotype of these embryos was determined by PCR analysis of serial sections from which embryonic tissue was scraped off. (*a* and *b*) Sections were stained with antiserum V598A, which is directed against an epitope common to Cα and Cβ. The degenerated embryo (*b*) exhibits the same staining intensity for C as the normal embryo (*a*). (*Inset c*) A control is included where the primary antiserum has been omitted. (*d* and *e*) Sections were stained with antiserum 15, which has been raised with a Cβ-specific peptide. Normal (*d*) and degenerated (*e*) embryos exhibit comparable staining intensities. (Scale bar = 50 μm.)

Conclusions. Our results show that PP2A Cα is an essential gene. Despite a proposed role of PP2A in cell cycle regulation, null mutant embryos die only at around day E6.5. Presence of embryonic cell masses as late as E13.5 implies that PP2A may be involved in signal transduction pathways controlling apoptosis.

Our data suggest that Cα and Cβ exert different functions in the developing embryo. Cβ expression is sufficient to keep at least part of the embryo proliferating, but Cα expression is required for proper cell fate determination. The amino acid sequences of Cα and Cβ are 97% identical, but 7 of 8 amino acid differences are clustered in the first 30 amino acid residues, which are encoded by exon I. Therefore, it has been speculated (10) that these differences are significant and that exon I encodes sequences important for regulation and substrate specificity. PP2A Cα may be involved in early signaling

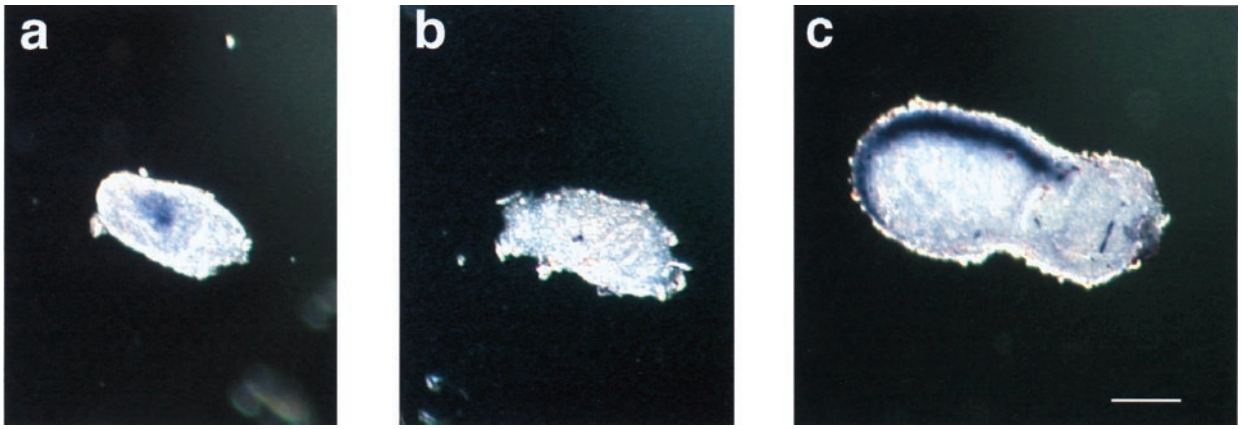

FIG. 5. RNA whole mount *in situ* hybridization of embryos analyzed for the expression of mesodermal markers (23) by using digoxigenin-labeled antisense probes for goosecoid (data not shown) and Brachyury. (*a–c*) Embryos were dissected at E6.5 (*a*) and E7.0 (*b* and *c*). Transcripts (purple staining) are present in morphologically normal embryos (*a* and *c*) but are undetectable in degenerated embryos (*b*). (Scale bar = 50 μm.)

cascades such as the wnt pathway, which controls the epithelial–mesenchymal transition during vertebrate development. Potential substrates of PP2A during embryonic development include the phosphoproteins β-catenin and glycogen synthase kinase 3β, which participate in wingless/wnt signaling (31). The expression of the β-catenin-associated cell adhesion molecule E-cadherin, for example, is dramatically reduced in −/− embryos (data not shown).

Further analysis of PP2A function will come from null mutants of the different isoforms of the regulatory subunits, which could reveal more subtle phenotypes related to PP2A function.

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