Phosphatidylinositol 3,4,5-trisphosphate is a phospholipid signaling molecule involved in many cellular functions including growth factor receptor signaling, cytoskeletal organization, chemotaxis, apoptosis, and protein trafficking. Phosphorylation at the 3 position of phosphatidylinositol, PtdIns(3)P, is a key event in many signaling pathways, including the mammalian target of rapamycin (mTOR) pathway. PtdIns(3)P is hydrolyzed by class II phosphoinositide 3-kinases (PI 3-kinases) to form PtdIns(3,4)P2 and PtdIns(3,4,5)P3, which serve as second messengers in various cellular processes such as cell cycle progression, proliferation, migration, and survival.

For a detailed understanding of the biological effects of phosphoinositides, very little information is available on the particular physiological roles played by these enzymes and isozymes during mammalian development. Inhibitors of PI 3-kinases such as wortmannin (17) and LY294002 (18) are very useful for probing the functions of these enzymes but as with all inhibitor studies suffer from questions of specificity as to which enzymes and isozymes are being inhibited at the dosages used in a particular study. In contrast, specific mutations in genes encoding these enzymes would provide greater specificity. We have therefore begun a systematic program of generating mice carrying deletion alleles of the PI 3-kinases. Here we report the successful targeting of the PtdIns(3,4,5)P3 kinase, Pik3ca, to create a deletion allele in the homologous state, leading to embryonic lethality at E9.5 due to a severe defect in the proliferative capacity of the embryo.

**Materials and Methods**

Cloning of Pik3ca Genomic Sequence—7.5 \( \times \) 10^6 plaques from a 129Sv mouse genomic library in the aFX II vector (Stratagene) were screened (19) with probes derived from Pik3ca cDNA. Of three positive phage clones, one (F1) was selected for further study and subcloning, and an 8-kb SauI fragment was isolated and found to contain the first three coding exons of Pik3ca (see Fig. 1). Genetic Mapping of the Pik3ca Gene—A tetranucleotide repeat (CTCT) within an intron of Pik3ca was used for chromosome mapping using the Jackson Laboratory C57Bl/6 and Mus musculus backcross DNA panel map service. Two PCR primers flanking the tetranucleotide repeat (forward, GTA GGG ATG AAG GTG GAG AGG; reverse, AGC TGA CTC AAT GGA AGT AGG G) were used for the amplification. PCR reaction was performed on 50 ng of DNA in a 20-μl volume containing 400 μM each primer, 20 μM Tris-HCl (pH 8.3), 1.5 μM MgCl2, and 0.5 unit of Taq polymerase. Reactions were denatured at 94 °C for 5 min and then subjected to 30 step cycles consisting of 94 °C for 20 s, 57 °C for 60 s, and 72 °C for 30 s. PCR products were loaded on a 3% MetaPhore agarose gel and separated at 70 V for 3 h.

Vector Construction and Pik3ca Gene Targeting in 129Sv ES Cells—The targeting construct was made in the pPNT vector (20) using an 8-kb SauI fragment from phage F1 and resulting in replacement of the first coding exon with a neomycin resistance gene transcribed in reverse orientation from a mouse PGK promoter (see Fig. 1). Electroporation into TC1 ES cells (21) and selection for homologous recombination were performed under protocols approved by the National Human Genome Research Institute's Animal Care and Use Committee under National Institutes of Health guidelines, "Using Animals in Intramural Research."
of Pik3ca—DNA was extracted from yolk sac (22) and tails (19) by published methods. Two PCR reactions were performed for genotyping. One pair of primers derived from the exon deleted in the deletion allele (P10N, forward, CTC CCC AAT GGA ATG ATA GTG, and P110N, reverse, TCT TTT CTT CAC GGT TGC CT) amplifies a 260-bp fragment only from the wild type. The second pair of primers derived from the neomycin resistance gene (Neo1, 5'-AGA GCC TAT TCG GCT ATG ACT G, and Neo2, 3'-TTC GTC CAG ATC ATC CTG ACT) amplifies a 430-bp fragment only from the disrupted Pik3ca gene. PCR amplification was performed according to the manufacturer's protocols (Life Technologies, Inc.).

RNA extracted from 9.5-day-old embryos (22) was used for reverse transcriptase (RT)-PCR per the manufacturer's protocols (Life Technologies, Inc.). Four pairs of primers were used for PCR amplification: P110N, forward, CTC CCC AAT GGA ATG ATA GTG, and reverse, TCT TTT CTT CAC GGT TGC CT, 260 bp; Neo1, 5'-AGA GCC TAT TCG GCT ATG ACT G, and Neo2, 3'-TTC GTC CAG ATC ATC CTG ACT, 431 bp; p110a, RT-1, sense primer, CTG TAT AAT GCT GGG GAG GAT and RT-3, sense primer, TCT GTC CCC TCT GAC GTG ATC, 593 bp; and RT-4, antisense primer, ATG CTC CCA GCA GGA ATT C, 396 bp. Two PCR reactions were performed for genotyping. Two PCR reactions were performed for genotyping. Two PCR reactions were performed for genotyping. Two PCR reactions were performed for genotyping.

RESULTS

Pik3ca Gene Maps to Mouse Chromosome 3—A novel tetranucleotide repeat (CCCT)n, within the fourth intron of Pik3ca was identified when this fragment was sequenced to determine the ex/intron boundaries. The repeat was found to be polymorphic between strains of C57BL/6J and M. spre-}

tus. A backcross between these two strains provided by the Jackson Laboratories Backcross DNA Panel Map Service was used to map the locus. Pik3ca maps clearly to chromosome 3 at 12.9 centimorgans between II7 and D3Mit21, with no crossovers with markers D3Br14 and #Sno. This location is syntenic with the region between D3S3682 and D3S1564 on the long arm of human chromosome 3 where human PIK3CA maps (SHGC-12912).
Genotypes of offspring from Pik3ca<sup>+/del</sup> × Pik3ca<sup>+/del</sup> crosses

| Age     | Number of viable embryos or newborn mice | Genotype | Probability |
|---------|------------------------------------------|----------|-------------|
| E8.5    | 17                                       | +/+      |             |
| E9.5    | 60                                       | +/del    |             |
| E10.5   | 16                                       | del/del  |             |
| Liveborn| 98                                       |          |             |

- Three del/del embryos were found at 10.5 days but were clearly nonviable and undergoing tissue autolysis.
- NS, not significant.

**FIG. 2. Phenotype of three Pik3ca<sup>del/del</sup> embryos.** Hemorrhage is observed at the forehead, snout, and other parts of the body. A and B represent the most typical examples of the distribution of hemorrhage. C was a less typical example in which a hemorrhagic bubble was observed on the dorsum of the embryo.

**FIG. 3. Pik3ca mRNA transcription in Pik3ca<sup>+/+</sup> and Pik3ca<sup>del/del</sup> embryos as determined by RT-PCR using total RNA isolated from E9.5 embryos.** The genotypes of the embryos were first determined by PCR using yolk sags separated from the embryos. Four pairs of primers were used for RT-PCR: pair p110F, p110R amplifies the first coding exon; primer pair neoF, neoR amplifies the neomycin resistance gene; primer pair RT-1 and RT-2 and primer pair RT-3 and RT-4 are located at the positions shown and generate fragments of 504 and 416 bp, respectively. Results demonstrate that RNA from the Pik3ca<sup>del</sup> allele is transcribed but lacks the portion of the transcript encoding the p85 binding domain. Size standard is HaeIII digest of ox174. KO, knock-out; N, normal.

Pik3ca<sup>+/+</sup>, 31 Pik3ca<sup>+/del</sup>, and 12 Pik3ca<sup>del/del</sup> as well as 9 apparent resorptions that could not be genotyped. Although there appeared to be a deficiency of Pik3ca<sup>del/del</sup> embryos, a deficiency that could be accounted for by the nine resorptions, the 17:31:12 ratio was not significantly different from the expected ratios of 15:30:15 (chi-square with Yates’ correction). At E8.5, 17 embryos showed normal Mendelian ratios (Table I). From these data, we conclude that homozygote embryos survive to E8.5, begin to die at E9.5, and do not survive to E10.5.

Most Pik3ca<sup>del/del</sup> homozygote embryos at E9.5 appeared to be viable, with obvious cardiac function, and histological analysis of E9.5 embryo sections stained with hematoxylin and eosin revealed no defect in organogenesis. They were, however, smaller than normal embryos, with fewer somites (average 22) compared with the normal embryos (average 25) from the same litter. The head region of E9.5 Pik3ca<sup>del/del</sup> homozygote embryos was smaller as compared with normal, and hemorrhage was observed at forehead, snout, and other parts of the body of the Pik3ca<sup>del/del</sup> embryos (Fig. 2). Heterozygous embryos were normal in appearance.

**Pik3Ca Transcripts—**Northern blot analysis of RNA from wild type embryos and adult mouse tissues revealed three transcripts (7.5, 5.4, and 4.4 kb) detected by a cDNA probe containing the coding region of Pik3ca cDNA (data not shown). The different transcripts result from alternative splicing in untranslated portions of the gene and are not the result of alternative splicing of coding exons (data not shown). All three transcripts appeared to be expressed at similar levels from E7 to E17 of embryonic development and in different tissues of adult mice, although there was variability in the expression levels of the different sized transcripts among various tissues. Whole mount in situ hybridization at E9.5 showed that the Pik3ca transcript was expressed uniformly throughout the whole embryo (data not shown).

RT-PCR analysis of RNA obtained from Pik3ca<sup>+/+</sup> and Pik3ca<sup>del/del</sup> embryos revealed that the deletion allele was transcribed. As shown in Fig. 3, Pik3ca transcripts were present in both Pik3ca<sup>+/+</sup> and Pik3ca<sup>del/del</sup> embryos, when assayed by RT-PCR of two segments downstream of the deletion (primer pair RT-1 and RT-2 and primer pair RT-3 and RT-4), whereas the portion of the transcript deleted by the targeted event was missing from Pik3ca<sup>del/del</sup> RNA (primer pair p110-F and p110-
R). As control, the neomycin resistance gene transcript was amplified from Pik3ca<sup>del/del</sup> embryos. 

**Proliferation and TUNEL Assay in Embryos—** The PI 3-kinases have been implicated both in signaling through growth factors (8, 27, 28) and in blocking apoptosis through signaling through the Akt Ser/Thr kinase (29, 30). The delayed embryonic development and lethality of Pik3ca<sup>del/del</sup> embryos and the inability of cells derived from these embryos to expand in culture could be due either to failure to proliferate or to excessive apoptosis or both. We tested E9.5 Pik3ca<sup>del/del</sup> embryos for their ability to proliferate using BrdUrd incorporation in vivo (24) as well as examining them for excess apoptosis by TUNEL assay (25). In wild type mice, the cells in three rapidly dividing regions of an E9.5 embryo revealed heavy BrdUrd labeling, whereas the same regions of Pik3ca<sup>del/del</sup> littermate embryos incorporated very little BrdUrd (Fig. 4). In contrast to the severe proliferative defect, no abnormality in apoptosis was seen (Fig. 5). The number of cells undergoing apoptosis was counted and averaged in six sagittal and parasagittal sections; wild type embryos contained an average of 58.8 apoptotic cells per section, whereas Pik3ca<sup>del/del</sup> contained an average of 48.0 apoptotic cells per section (p < 0.05, Student's t test). The apoptotic cells were mostly located in forebrain and hindbrain with a similar distribution in both genotypes (Fig. 5).

**Mouse Embryo Fibroblast Culture—** Cells from wild type, heterozygote, and homozygote embryos were obtained by explanting E9.5 embryos. Mouse embryonic fibroblasts grew normally from explants of wild type and Pik3ca<sup>1/del</sup> heterozygous embryos. In contrast, when explants were obtained from E9.5 Pik3ca<sup>del/del</sup> embryos, cells would adhere to tissue culture plates but could not be induced to expand in culture in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum and a variety of growth factors (bomine, phorbol 12-myristate 13-acetate, and lysophosphatidic acid), used either
man polyclonal antibody (Upstate Biotechnology Inc.) was increased in Western blot of clonal antibody in the wild type appeared to be substantially by rabbit anti-human p85 (Upstate Biotechnology Inc.) polyclonal antibody using either 40 or 25 μg of protein from cells derived from the three different genotypes N, C, and KO. The arrow indicates the absence of the fragment corresponding to p110α in the Pik3caΔdel/Δdel (lane KO) as compared with the normal Pik3ca+/− (lane N) and Pik3ca+/−/Δdel (lane C). The fragment in the lane from Pik3caΔdel/Δdel embryos is diminished in intensity, consistent with there being half-normal levels of p110α protein in heterozygous embryos carrying one targeted allele. B, anti-p85 antibody using either 40 or 25 μg of protein from cells derived from E9.5 embryos with the three different genotypes N, C, and KO. The arrow indicates the markedly increased intensity of the fragment corresponding to p85 in the Pik3caΔdel/Δdel (lane KO) as compared with the normal Pik3ca+/−/Δdel (lane N) and Pik3caΔdel/Δdel (lane C), despite loading less protein in the KO lane.

singly or in combination. These growth factors were chosen because they were reported to bypass a block in mitogenic signaling in cultured cells caused by antibodies to Pik3ca (28).

**Western Analysis of Regulatory p85/p55 in Homozygous Deletion Embryos**—The deletion mutation in Pik3ca eliminates the p85/p55 binding site, but the transcript containing the deletion could theoretically still be translated into a protein lacking the first 122 residues of the full-length p110α. Antibody directed against residues 189–390 of p110α was used for Western blot analysis of fibroblasts from wild type and Pik3caΔdel/Δdel embryos. Although this region of p110α was not deleted in the knock-out construct, no p110α protein was seen (Fig. 6A). Furthermore, by comparing two different amounts of protein (18 and 36 μg) from wild type and heterozygous embryos, the amount of p110α was clearly reduced in the heterozygotes, consistent with loss of expression of p110α resulting from the targeting event. Another difference between Pik3ca+/−/Δdel and Pik3caΔdel/Δdel embryos was also apparent when rabbit anti-human p85 polyclonal antibody (Upstate Biotechnology Inc.) was used for Western blotting of cells derived from Pik3ca+/−/Δdel and Pik3caΔdel/Δdel embryonic tissue. An 85-kDa fragment recognized by rabbit anti-human p85 (Upstate Biotechnology Inc.) polyclonal antibody in the wild type appeared to be substantially increased in Western blot of Pik3caΔdel/Δdel embryonic tissue (Fig. 6B). Other proteins detected by the antibody were either unchanged or decreased in intensity in lysate from Pik3caΔdel/Δdel embryos.

**DISCUSSION**

PtdIns 3,4,5-trisphosphate has been implicated in the response to growth factor signaling through tyrosine kinase receptors for platelet-derived growth factor, insulin, insulin-like growth factor 1, colony-stimulating factor 1, nerve growth factor, hepatocyte growth factor, and stem cell growth factor (8). Phosphoinositides phosphorylated at the 3 position of the inositol ring, including PtdIns 3,4,5-trisphosphate, are generated by the type IA PI 3-kinases, which also interact with Ras proteins in a GTP-dependent manner (31). In addition, Akt Ser/Thr protein kinases, p70 ribosomal S6 kinase and protein kinase C are considered to be downstream targets of PI 3-kinases by receptor-stimulated signaling (6). Other PI 3-kinases have also been found to participate in protein trafficking (7), cytoskeletal organization (32), and regulation of apoptosis (29, 30). Given that there are so many PI 3-kinases involved in so many cellular processes, it is important to ask to what extent each of the isozymes of PI 3-kinase is either necessary or sufficient for carrying out these many functions of PI 3-kinases. In particular, is the α isozyme of PI 3-kinase essential for normal embryonic development or is it redundant because a deficiency could be compensated for by other activities, such as the β isozyme?

We created mice homozygous for a deletion within Pik3ca by gene targeting and demonstrated loss of expression of p110α by Western analysis using an antibody directed against a region of the protein not involved in the deletion event. Pik3caΔdel/Δdel homozygotes have delayed development and undergo intrauterine death between E9.5 and E10.5 of embryonic development. The developmental delay and growth failure were due to an inability of Pik3caΔdel/Δdel embryos to proliferate in response to mitogenic stimulation. Consistent with this severe proliferation defect was our finding that all attempts to grow cells obtained from explants of E9.5 Pik3caΔdel/Δdel embryos from 129/SV inbred matings failed. Cells from Pik3caΔdel/Δdel embryos failed to expand in culture and appeared resistant to the mitogenic effects of fetal calf serum as well as defined growth factors such as bombesin, lysophosphatidic acid, and phorbol 12-myristate 13-acetate. These results were consistent with the findings of Nave et al. (33), who reported PI 3-kinase is involved in signaling through phorbol 12-myristate 13-acetate, but contrasts with the findings of Roche et al. (28), who reported that neither bombesin nor lysophosphatidic acid uses signaling pathways involving PI 3-kinase to stimulate growth.

**Knock-out Mouse Embryos**

Because of the observation that PI 3-kinases activate the AKT protein kinase and inhibit apoptosis in the nervous system (29, 30), we were particularly interested in determining whether there was increased apoptosis in the Pik3ca−/− embryos, particularly in the developing brain. In fact, embryos did not demonstrate increased numbers of apoptotic cells, either in the region of the developing brain or elsewhere. We conclude from these experiments that the PI 3-kinase encoded by Pik3ca is absolutely required for normal embryonic growth and development; the phenotype seen in Pik3caΔdel/Δdel mice results from an inability to proliferate in response to growth factor signaling rather than from unregulated apoptosis. However, we cannot eliminate the possibility that abnormalities in other phosphoinositides phosphorylated at the 3 position of inositol by the class IA PI 3-kinase we disrupted in these mice may play a role in the embryonic lethality through other unknown mechanisms.

Given the wide variety of cellular processes in which PI 3-kinases are involved, a puzzling aspect of the phenotype of Pik3caΔdel/Δdel homozygotes is that the embryos manage to survive to E9.5 of embryonic development. There may be an alternative supply of PI 3-kinase that is either lost or becomes inadequate by E9.5 if in face of a sharp increase in demand for PtdIns 3,4,5-trisphosphate that occurs normally at this stage of development. For example, a maternal contribution of p110α in the oocyte may only be sufficient to support limited early development. Alternatively, embryonic forms of PI 3-kinase with limited temporal expression might compensate early in development but not after E9.5. Finally, other PI 3-kinase catalytic subunits, such as the p110β subunit, might be sufficient to allow development to occur prior to E9.5.

The period between E9.5 and E10.5 is a time of markedly...
increased proliferation and differentiation as organogenesis and placentalization accelerate rapidly in the embryo (34). A four-chambered heart begins to beat, and angiogenesis allows the vascular system to expand in order to deliver nutrients and remove waste products. We might hypothesize that as the E9–E9.5 embryo faces markedly increased demands for proliferation, growth, and differentiation, intraterine death of Pik3ca del/del embryos occurs at this stage because the increased demand exceeds generate residual capacity of PI 3-kinase pathways to phosphatidylinositolos for proliferation. It is interesting to note that the Pik3ca del/del embryos demonstrated areas of extravasated blood, suggestive of defective angiogenesis. A role in angiogenesis for phosphatidylinositolos phosphorylated by PI 3-kinase at the 3 position of inostitol is supported by studies in which wortmannin inhibited angiogenesis in a chick embryo chorioallantoic membrane system (35).

One interesting finding in the Pik3ca del/del homozygous embryos was an apparent increase in p85/p55 as determined by Western blot analysis of embryo tissue obtained at E9.5. These data raise the intriguing possibility that one component of the deleterious effect of the Pik3ca del allele on embryonic development may be mediated through excess p85/p55 rather than simple loss of function of p110α. Overexpression of p85 can interfere with the binding of PI 3-kinases to other proteins by disrupting the binding between the Src homology 2 domains of the p85 subunits of the PI 3-kinases with their targets (36). There is little very information on the regulation of transcription and turnover of p85/p55 and an investigation into the mechanism of this elevation of p85/p55 is greatly hampered by our inability to propagate Pik3ca del/del cell lines. The increase in p85 protein seen in the Pik3ca del/del embryos points to a previously unrecognized aspect of p85 regulation that deserves further investigation.

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