(AA) inhibits the GAP activity of NF1-GRD product (16), while p120-GAP activity is only weakly affected by AA (16). When the GAP activities of two GST-NF1-GRD fusion proteins for H-ras p21 were examined in the presence of increasing concentrations of AA, AA inhibited GAP activities of both types of NF1-GRD in a concentration-dependent manner (Fig. 4B). However, the IC_{50} of AA for type I (156±34 μM, mean±SE, n=3) was significantly higher than that for type II (34±8 μM, mean±SE, n=3). Thus, these results indicate that type I and type II NF1 GRD protein possess the same ability to stimulate ras p21 GTPase activity in vitro. In addition, GAP activities of two types of NF1 proteins may be differentially regulated by lipids such as AA. It has been demonstrated that the activation of the ras oncogene product by point mutation is a rare event in gastric cancers (1). However, since ras p21 has been shown to play a crucial role in cellular proliferation and malignant transformation in a number of other cell types (17), an alteration in the ras-related signalling pathway might be involved in gastric tumorigenesis. The present results, therefore, suggest that differential expression of two types of NF1-GRD in cancer cells might affect the ras-related signal transduction through different regulation of both types of NF1-GRD by AA. Alternatively, either an insertion (type II) or a deletion (type I) of 21 amino acids may change the whole NF1 protein structure then altering its GAP activity for ras p21, but it may not affect the GAP activity of bacterially expressed NF1-GRD in vitro. Thus, if type I NF1 protein could antagonize the GAP activity of type II NF1 protein predominantly expressed in normal stomach cells, the increased level of type I NF1 protein might result in increasing the amount of ras-GTP bound form, leading to the cellular proliferation of stomach cells. To further understand the functional difference between type I and type II NF-1 gene products, transfection study using the whole cDNA of both types of the NF1 gene will be required.

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METABOLIC STUDIES IN A MOUSE MODEL OF HEPATORENAL TYROSINEMIA: ABSENCE OF PERINATAL ABNORMALITIES

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SUMMARY Radiation induced chromosomal deletions at the albino locus in the mouse, lethal when homozygous, cause abnormalities of expression of several unlinked liver specific genes. Recently, the gene encoding FAH was shown to be included in the deletions. Since in humans FAH mutations cause tyrosinemia type I, deletion homozygous mice were suspected of having tyrosinemia. Studies of plasma amino acids did not confirm this suspicion. Also, succinylacetone levels were normal in fetal and newborn livers of deletion homozygotes. The present evidence, therefore, does not support the assumption that the earlier described ultrastructural and enzyme abnormalities in deletion homozygotes are secondary effects of tyrosinemia caused by the deletion of FAH.

Hepatorenal tyrosinemia or type I hereditary tyrosinemia in humans (McKusick catalog #27670) is an autosomal recessive disorder of tyrosine metabolism that occurs in two forms. The first, the infantile form, is associated with early death due to liver failure. A chronic form is associated with nodular cirrhosis and renal tubular nephropathy leading to failure of growth . Both disorders are due to a deficiency of fumaryl acetoacetate hydrolase (1) (FAH; E.C. 3.7.1.2). Recently, the gene encoding this enzyme was cloned and mapped to a region on human chromosome 15 (2) homologous to one on mouse chromosome 7 that includes the tyrosinase locus. This led to a study of mice known to be homozygous for deletions of this region and the finding

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that the gene for FAH was included in the deletions (3). The complete absence of FAH in these mice suggested that they might represent an animal model of human hepatorenal tyrosinemia. Since, however, the deletions in the mouse are large and include multiple genetic loci (4), it is most likely that other gene products would be missing as well. A murine model for type III tyrosinemia, due to a deficiency of 4-hydroxyphenol-pyruvate dioxygenase (5), concerns a quite different disorder.

The biochemical abnormalities detected in humans affected by hepatorenal tyrosinemia type I include increases of plasma concentrations of tyrosine and methionine to several fold of normal (6). However, the diagnostic hallmarks of the condition have been elevations of succinylacetone in both blood and urine (7). This substance is a highly toxic compound and its accumulation is thought to explain the hepatorenal cellular damage associated with tyrosinemia (8). The question has been debated as to a possible prenatal onset of symptoms in human hepatorenal tyrosinemia since an increase of serum a-fetoprotein up to 1000-fold was noted and affected individuals become sick in the early post-natal period (9). However, this would present an exception to the majority of disorders of amino acid metabolism in which transfer of amino acids across the placenta and correction by the normal enzyme levels in the heterozygous mother succeed in maintaining homeostasis until birth. Since the deletion homozygous mice that lack the FAH gene seemed to offer a potential model of human hepatorenal tyrosinemia, they provided an opportunity to determine the time of perinatal onset of metabolic abnormalities in this disease, and were, therefore, subjected to the appropriate tests. Even though ultrastructural abnormalities of hepatocytes characterize these deletion homozygotes before birth (10), no metabolic abnormalities could be detected prenatally. These results suggest strongly that the previously described abnormalities of lethal albino deletion homozygotes (4) are caused by the absence of genes other than that encoding FAH.

EXPERIMENTAL PROCEDURES

Animals Mice heterozygous for the lethal albino deletion c^{3H} were bred to produce newborn albino homozygotes (c^{3H}/c^{3H}), heterozygotes (c^{3H}/c^{ch}) and homozygous normal littermates (c^{ch}/c^{ch}) distinguishable by skin and eye pigmentation (albino, light, dark). In order to obtain fetuses for prenatal studies, matings were timed by the observation of vaginal plugs, fetuses were dissected and their genotypes identified by eye pigmentation. Since deletion homozygotes (c^{3H}/c^{3H}) die within a few hours after birth, the majority of newborn mice were dissected as soon as possible. In order to maximize accumulation of possible metabolites, some newborn homozygotes were sustained with glucose injections, monitored and dissected between 3-6 1/2 hours later. The remaining newborn c^{3H}/c^{3H} homozygotes and their normal

littermates were injected after birth intraperitoneally with a combination of N^6, O^2-dibutyryladenosine 3', 5'-cyclic monophosphate (cAMP, 100 mg/kg of body weight) and dexamethasone (dex, 100 μg/kg of body weight) in 0.85% saline for possible induction of liver specific enzymes, or with 0.85% saline alone for control. Livers were removed from albino deletion homozygous and pigmented normal littermates 2 hr after injection. Blood was obtained by decapitation and collected in capillary tubes. Subsequently, plasma was pooled from identical genotypes of multiple litters. All specimens were stored at -70°C until analysis.

Determinations of Amino Acids Plasma samples (2 μl) were qualitatively screened by one dimensional thin layer chromatography for the presence of excess tyrosine and methionine (11). For quantitative determinations of amino acids, an aliquot of pooled plasma was diluted 1:5 in buffer containing 0.15 M sulfosalicylic acid, and 125 μM α-amino guanidinopropionic acid in lithium citrate buffer (Beckman 1X; pH 2.83) adjusted to pH 1.8 with solid lithium hydroxide. The sample was injected into a Beckman 119-CL amino acid analyzer.

Determinations of Liver Succinylacetone Five drops of 2N HCl and 25 μl of internal standard (1.4 mg/ml malonic acid) were added to the mouse livers which were homogenized in methanol. The sample was centrifuged for 10 min, the supernatant transferred to a conical tube and taken to dryness under a stream of air. The TMS (trimethylsilyl) derivative was prepared by the addition of 50 μl of BSTFA (N,0-bis trimethylsilyl) trifluoroacetamide/1% TMCS (trimethyl chlorosilene). The sample was heated for 15-20 min at 60°C and a 3 μl aliquot was injected onto a 60 m SPB-1 GC column. An HP 5995B mass spectrometer was used as the detector with selected-ion-monitoring at m/z 157, 169, 185, 233, 243, 287 and 302 which served to identify succinylacetone and the internal standard (m/z 233). In addition, ions 179 and 308 indicative of 4-hydroxyphenyllactic acid were monitored. The detection limit for succinylacetone under these conditions was 10 μg. Since pooled liver samples were extracted and the extract taken up in a small volume, the amount of succinylacetone detectable on a wet weight basis varied with the amount of liver extracted.

RESULTS AND DISCUSSION

Initially, plasma amino acid levels were determined for newborn mice homozygous for the c^{3H} deletion and their control littermates as described in Experimental Procedures. Visual inspection of the thin layer plate in a preliminary experiment suggested that mutant newborns had abnormally high plasma tyrosine concentrations, but no other apparent amino acid abnormalities. Plasma was then pooled separately from 19 deletion homozygous, 28 heterozygous and 24 wild type homozygous newborn mice for one set of determinations and from 15, 16, and 8 mice of the respective genotypes for a second set. As shown in Table 1, the deletion homozygous newborn mice had concentrations of methionine 1.4 times, and tyrosine 1.8 times as high as their normal littermates, both heterozygous and homozygous.

In view of the mild metabolic abnormalities detected in the newborn period, the next step addressed the question if the toxic metabolite, succinylacetone, could be identified before birth. Liver organic acid

Table 1. Plasma amino acid concentrations in newborn mice

Amino acid	micromoles/liter*			c^{3H}/c^{3H} as % of littermate averages
	c^{ch}/c^{ch}	c^{3H}/c^{ch}	c^{3H}/c^{3H}	
methionine	240.5 ± 14.5	216 ± 32	323.5 ± 14.5	142
tyrosine	172 ± 8	161.5 ± 11.5	299.5 ± 49.5	179
phenylalanine	118 ± 1	103 ± 3	131 ± 8	126
leucine	145 ± 7	111 ± 8	200.5 ± 4.5	157

*means ± std. error, 2 determinations.

determinations detected a trace of succinylacetone in only one sample which represented a pool of livers obtained from six homozygous normal 18 day old fetuses. It amounted to an approximate concentration of 26 nanograms of succinylacetone per milligram of wet weight liver. No other fetal liver samples had any detectable succinylacetone. On the other hand, 4-hydroxy phenyllactic acid was present but not related to particular genotypes (Table 2). Additional analyses of livers of eight separate sets of newborn mice representing all three genotypes also failed to show any detectable succinylacetone (Table 2).

The mutant mouse strain used in these studies is one of a group of strains carrying different overlapping deletions at and around the albino locus on chromosome 7 and characterized by neonatal lethality of deletion homozygotes. The multiple deficiencies of hepatic enzymes described before in these homozygotes (4) suggested the lack of one or more transacting regulatory factors normally encoded in the deleted genome of chromosome 7 and instrumental in the prenatal differentiation of liver specific genes mapping on other chromosomes (12).

The genes serving as targets of the effects of the deletions encode liver specific enzymes and proteins and share the ability to respond to hormones with inducible expression around the time of birth. Prenatal constitutive enzyme levels are normal in deletion homozygotes and the effects on the genes encoding the enzymes are restricted to the development of inducible gene expression. They result in the continuation of the fetal condition where constitutive expression prevails and cell type specific gene expression has not yet developed. The precise cause of the early postnatal lethality of deletion homozygotes has not been fully determined. Nevertheless, hypoglycemia resulting from the absence of normal postnatal

Table 2. Analysis of pooled liver extracts for tyrosine metabolites by GC/MS

Genotype	No. Pooled	Age	Induction Status	Succinyl-acetone†	4-OH phenyl-lactic acid
c^{3H}/c^{3H}	(6)	fetal, 18 day	-	n.d.*	+
c^{3H}/c^{3H}	(8)	fetal, 18 day	-	n.d.	-
c^{ch}/c^{ch}	(6)	fetal, 18 day	-	trace	+
c^{ch}/c^{ch} and c^{3H}/c^{ch}	(4)	fetal, 19 day	-	n.d.	-
c^{3H}/c^{3H}	(2)	fetal, 19 day	-	n.d.	-
c^{3H}/c^{3H}	(1)	fetal, 19 day	-	n.d.	-
c^{ch}/c^{ch}	(4)	fetal, 19 day	-	n.d.	+
c^{3H}/c^{3H}	(5)	newborn	-	n.d.	+
c^{ch}/c^{ch} and c^{3H}/c^{ch}	(4)	newborn	saline injected	n.d.	+
c^{ch}/c^{ch} and c^{3H}/c^{ch}	(6)	newborn	dex with cAMP	n.d.	-
c^{3H}/c^{3H}	(2)	newborn	dex with cAMP	n.d.	-
c^{3H}/c^{3H}	(4)	newborn	-	n.d.	+
c^{3H}/c^{ch}	(7)	newborn	-	n.d.	+
c^{ch}/c^{ch}	(5)	newborn	-	n.d.	+
c^{3H}/c^{3H}	(10)	newborn >4 hrs	-	n.d.	+
c^{3H}/c^{ch}	(6)	newborn >4 hrs	-	n.d.	+
c^{ch}/c^{ch}	(8)	newborn >4 hrs	-	n.d.	+
c^{ch}/c^{ch}	(2)	newborn >6½ hrs	-	n.d.	+
c^{ch}/c^{ch}	(2)	newborn >6½ hrs	-	n.d.	+

†detection limits of 21-200 ng/mg wet wt. liver.
*n.d. = not detected.

levels of gluconeogenic enzymes, is no doubt a strong factor leading to their early death (13).

Recently, two groups of investigators found the gene for murine fumarylacetoacetate hydrolase (FAH) to be included in the chromosome 7 deletions that cause the neonatal lethal phenotype (3). In fact, these authors showed that the proximal breakpoint of the c^{14CoS} deletion interrupted the transcriptional unit of FAH. Therefore the question was

examined, as reported here, whether or not deficiency of the FAH enzyme might account for the full range of phenotypic abnormalities characterizing deletion homozygotes.

Even though, as stated before, most inborn errors of mammalian metabolism are expressed after birth when soluble fetal metabolites are no longer transferred across the placenta for maternal detoxification, there exist exceptions, e.g. human fumarase deficiency where enlarged cerebral ventricles and polyhydramnios are observed prenatally (14). In the case of human hepatorenal tyrosinemia Type I, the early postnatal appearance of marked metabolic abnormalities and profound liver injury suggested the possibility that FAH deficiency might in fact be expressed prenatally. We therefore examined fetuses and newborn mice homozygous for the lethal albino deletion c^{3H} that includes the deletion of FAH in order to find out if their characteristic defects including hepatic ultrastructural abnormalities might be attributable to FAH deficiency and perinatal accumulation of the toxic substance succinylacetone. The finding of 4-hydroxy-phenyllactic acid in both wild type as well as deletion homozygous livers of fetal and newborn mice (Table 2) suggests that the pathway for tyrosine metabolism is developing in the perinatal period, comparable to observations of premature human infants. Even though mildly elevated levels of methionine and tyrosine were detected in the plasma of deletion homozygous mice, the toxic metabolite succinylacetone was not found either pre- or post-natally in the liver within the time period of study.

The early lethality of the mice homozygous for the chromosomal deletions contrasts with the human disorder of hepatorenal tyrosinemia where long survival has been noted frequently. This difference might, of course, merely reflect other differences between the intermediary metabolism of mice and humans, as observed in several other murine models in which a particular homologous mutation causes phenotypes quite different from those described in humans (15). However, in the case of the FAH deficient mice, the early lethality appears to be more easily explained by the inclusion in the chromosomal deletions, measuring more than 1.5 cM, of many genes in contrast to the single gene mutation that causes the human tyrosinemia I. Even though the normal gene product of FAH might conceivably function as a transacting regulatory factor in addition to encoding an enzyme of intermediary metabolism, it does not appear that the defects identified prenatally in fetuses homozygous for the lethal albino deletions (10) are caused by early effects of tyrosinemia.

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