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Growth-Promoting Interaction of IGF-II with the Insulin Receptor during Mouse Embryonic Development

Angeliki Louvi,* Domenico Accili,† and Argiris Efstratiadis*,[1]

**Department of Genetics and Development, Columbia University, New York, New York 10032; and †Developmental Endocrinology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892*

Genetic analyses of dwarfing phenotypes resulting from targeted mutagenesis of the genes encoding the insulin-like growth factors (IGF-I and IGF-II) and their cognate type 1 IGF receptor (IGF1R) have demonstrated that this signaling system is a major determinant of mouse embryonic growth. Of the two IGF ligands, IGF-I interacts exclusively with IGF1R, whereas IGF-II recognizes an additional receptor (XR), because the growth retardation of embryos lacking both IGR1R and IGF-II (30% of normal birthweight) is more severe than that manifested in either class of single *Igf1r* or *Igf2* null mutants (45 and 60% of normal, respectively). To determine whether XR is the insulin receptor (IR), we examined embryos nullizygous for both *Igf1r* and *Insr*. While the growth of embryos lacking solely IR is affected very mildly and only at the end of gestation, concomitant absence of IGF1R results in a severe growth-deficiency phenotype (30% of normal size at birth) that is first detected at Embryonic Day 13.5 and is also characterized by transient edema, curly tail, generalized organ hypoplasia, including the muscles, developmental delays in ossification, and thin epidermis. The *Igf1r/Insr* double nullizygotes are phenotypically indistinguishable from double mutants lacking IGF1R and IGF-II and from other double and triple mutants in which all of the IGF ligand/receptor interactions have been eliminated. Therefore, these results provide genetic evidence that the growth-promoting function of IGF-II during mouse embryogenesis is mediated in part by signaling through the insulin receptor.

INTRODUCTION

The insulin-like growth factors, IGF-I and IGF-II, are mitogenic polypeptides with a structural similarity to proinsulin, which are produced by many tissues and function in an autocrine/paracrine fashion, although they may also act as classical hormones, since they circulate in the plasma in association with binding proteins (reviewed by Jones and Clemmons, 1995). The signaling of both of these ligands is mediated by the type 1 IGF receptor (IGF1R), a heterotetrameric ($\alpha_2\beta_2$) transmembrane glycoprotein that resembles the insulin receptor, and possesses extracellular ligand-binding and intracellular tyrosine kinase domains (reviewed by De Meyts *et al.*, 1994; LeRoith *et al.*, 1995; Rubin and Baserga, 1995). In mammals, the structurally unrelated cation-independent mannose 6-phosphate receptor that is involved in the trafficking of lysosomal enzymes also acts as the type 2 IGF receptor (IGF2R) serving the turnover of excess IGF-II by receptor-mediated endocytosis (reviewed by Nissley *et al.*, 1991; Kornfeld, 1992; Ludwig *et al.*, 1995).

Previously, we disrupted the mouse genes encoding the IGF ligands and receptors by targeting and studied the phenotypic consequences of the null mutations alone or in various combinations (DeChiara *et al.*, 1990; Liu *et al.*, 1993; Baker *et al.*, 1993; Ludwig *et al.*, 1996). The results of these analyses, summarized below and in Fig. 1, established the *in vivo* relationships in ligand/receptor interactions and revealed the growth-promoting developmental roles of the IGFs. Moreover, genetic evidence was obtained indicating that an additional unknown signaling receptor exists, which mediates in part IGF-II action during embryogenesis.

Because the maternal *Igf2* allele is normally silent, due to parental imprinting (reviewed by Efstratiadis, 1994), heterozygous progeny carrying a paternally derived mutated *Igf2* gene [*Igf2*(+/*p*−) mutants] are phenotypically indistin-

[1] To whom correspondence should be addressed at Department of Genetics and Development, Columbia University, 701 West 168th Street, New York, NY 10032. Fax: (212) 923-2090. E-mail: arg@cuccfa.ccc.columbia.edu.

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guishable from *Igf2* nullizygous mice (DeChiara *et al.*, 1991). The mutants of both of these classes are invariably viable dwarfs with 60% of normal birthweight (Fig. 1C). In contrast, the survival of nullizygous mutants lacking IGF-I, which also exhibit 60% of normal birthweight (Fig. 1B), depends on genetic background. The growth retardation of *Igf1r*(−/−) nullizygotes, which die immediately after birth of respiratory failure, is more severe (45% of normal birthweight; Fig. 1D). Double mutants lacking both IGF-I and IGF1R do not differ in phenotype from *Igf1r* nullizygotes, demonstrating that IGF-I interacts exclusively with IGF1R in embryos (Fig. 1G). In contrast, double mutants lacking both IGF-II and IGF1R (which are phenotypically indistinguishable from double mutants lacking both the IGF ligands) exhibit a birthweight that is 30% of normal (cf. Figs. 1F and 1H); i.e., their growth retardation is more severe than that manifested in either class of single mutants. Therefore, IGF-II interacts with IGF1R and also with an unknown receptor (XR), which is not IGF2R, as demonstrated by the following results.

Consistent with the imprinted status of the *Igf2r* gene (which is reciprocal to that of *Igf2*; Barlow *et al.*, 1991), the phenotype of heterozygotes inheriting a mutated gene maternally [*Igf2r*(+/*m*−)] is indistinguishable from that of nullizygous mutants. Mice lacking IGF2R have increased serum and tissue levels of IGF-II, exhibit overgrowth (140% of normal birthweight; Fig. 1E), and usually die perinatally (Wang *et al.*, 1994; Lau *et al.*, 1994; Ludwig *et al.*, 1996). This lethality is apparently caused by excess of IGF-II overstimulating IGF1R in the absence of IGF2R-mediated turnover, as evidenced by the fact that double mutants lacking both IGF2R and IGF-II (Fig. 1I) are fully viable (Ludwig *et al.*, 1996). Thus, when IGF-II is removed, the absence of IGF2R has no detrimental consequences. Moreover, the involvement of IGF1R overstimulation in the lethality of *Igf2r* mutants was demonstrated by the rescue of mutants lacking both IGF receptors (Fig. 1J), although each individual mutation is lethal (Ludwig *et al.*, 1996). The normal embryonic development of these *Igf1r/Igf2r* double mutants was attributed to signaling of IGF-II, being in excess, through XR, since triple mutants lacking IGF1R, IGF2R, and IGF-II are nonviable dwarfs (30% of normal size; Fig. 1K).

Considering these data and also the cross-interaction properties of the IGF and insulin systems, we asked whether XR is the insulin receptor (IR). While the affinity of insulin binding to IGF1R is 500–1000 times lower than that of the IGFs, IGF-II binds to IR quite strongly with an affinity that is only 10-fold lower than that of insulin (relative to insulin, the affinity of IGF-I for IR is at least 50- to 100-fold lower; reviewed by Nissley *et al.*, 1991; De Meyts *et al.*, 1994). Therefore, given the genetically revealed specificity of the IGF-II/XR interaction in embryos, IR was considered as a candidate for XR, although this hypothesis appeared to be inconsistent with the observation that mutants nullizygous for the *Insr* gene (encoding IR) exhibit normal embryonic development and have reportedly normal birthweights (Accili *et al.*, 1996; Joshi *et al.*, 1996). However, these mutants survive postnatally only for a few days and then die of diabetic ketoacidosis.

In an attempt to determine the identity of XR and expand our previous studies on the interactions of members of the IGF family of growth effectors during mouse embryogenesis, we generated double mutants lacking both IGF1R and IR and analyzed their phenotype.

MATERIALS AND METHODS

Breeding of Mutant Mice

The mice used in this study, which carried targeted mutations of the *Igf1, Igf2, Igf1r, Igf2r,* and *Insr* genes, have been described previously (DeChiara *et al.*, 1990; Liu *et al.*, 1993; Ludwig *et al.*, 1996; Accili *et al.*, 1996).

To generate *Igf1r*(−/−)/*Insr*(−/−) double mutants, we first crossed *Igf1r*(+/−) and *Insr*(+/−) heterozygotes and obtained F1 *Igf1r*(+/−)/*Insr*(+/−) double heterozygotes (25% of the progeny), which were then intercrossed (see Table 1A).

To generate *Igf1*(−/−)/*Igf2*(+/*p*−) or *Igf1*(−/−)/*Igf2*(−/−) double mutants, which are phenotypically identical, we first crossed *Igf1*(+/−) heterozygous males with *Igf2*(−/−) homozygous females and obtained *Igf1*(+/−)/*Igf2*(+/−) double heterozygotes (50% of the progeny), which were then intercrossed (see Table 1B).

To generate *Igf1r*(−/−)/*Igf2*(+/*p*−) double mutants, we had to rely on meiotic recombination events, since both of these genes reside on chromosome 7 at a distance of 39 c*M* apart (see Lyon *et al.*, 1996). First, we crossed *Igf2*(−/−) homozygous females with *Igf1r*(+/−) heterozygous males and obtained among the F1 progeny *Igf1r*(+/−)/*Igf2*(+/−) double heterozygous males, which were then mated to *Igf1r*(+/−) females, to produce among the F2 progeny the desired double mutants (see Table 1C).

To generate *Igf1r*(−/−)/*Igf2r*(+/*m*−)/*Igf2*(+/*p*−) triple mutants, we first crossed *Igf1r*(+/−) females with *Igf2r*(+/−) males and obtained among the F1 progeny *Igf1r*(+/−)/*Igf2r*(+/−) double heterozygous females, which were then crossed with *Igf1r*(+/−)/*Igf2r* (+/−)/*Igf2*(+/−) triple heterozygous males (see Ludwig *et al.*, 1996), to produce among the F2 progeny the desired triple null mutants (see Table 1D).

To generate *Igf1r*(−/−)/*Igf2r*(+/*m*−)/*Insr*(−/−) triple mutants, we first crossed *Igf1r*(+/−)/*Insr*(+/−) double heterozygous females with *Igf1r*(+/−)/*Igf2r*(+/−) males, and obtained triple heterozygous progeny [*Igf1r*(+/−)/*Igf2r*(+/−)/*Insr*(+/−); 1/16], which were then intercrossed (see Table 1E).

Genotyping

For genotyping by Southern or PCR analysis, DNA was prepared as described (Hogan *et al.*, 1994) from the yolk sac of e11.5–e18.5 embryos. The probes used for Southern blotting, which can detect both targeted and nontargeted alleles because of different sizes of restriction fragments, were: a 440-bp *Eco*RI–*Spe*I fragment located upstream of exon 4 of the *Igf1* gene (Liu *et al.*, 1993); a 460-bp *Bam*HI–*Hin*cII fragment located downstream of exon 3 of the *Igf1r* gene (Liu *et al.*, 1993); and a 700-bp *Bgl*II–*Hin*dIII fragment located upstream of exon 1 of the *Igf2r* gene (Ludwig *et al.*, 1996). For detection of the *Igf2* mutation by PCR amplification, we used the forward primer 5′-CTAGCTCAAAGCCTGCGTTTCTTT-3′ cor-

responding to intron sequence located 30 bp upstream from the splice junction of the first *Igf2* coding exon and a *neo* reverse primer 5′-ATCCATCTTGTTCAATGGCCGATCCC-3′. PCR cycling was for 1 min at 94°C, 1 min at 67°C, and 1 min at 72°C for 30 cycles. The intact *Insr* allele was detected using the primer pair 5′-CTTGATGTGCACCCCATGTCT-3′ (forward) and 5′-TCGGATGTTGATGATCAGGCT-3′ (reverse) corresponding to the beginning and end of exon 4, while the targeted allele was detected with the same forward *Insr* primer and a *neo* reverse primer 5′-ATATTGCTGAAGAGCTTGGCG-3′. PCR cycling was for 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C for 30 cycles.

Histological and Anatomical Analyses

For histopathology, embryos were fixed overnight in 4% paraformaldehyde, 0.1 *M* phosphate buffer, pH 7.3, washed for 24 hr at 4°C in 0.25 *M* sucrose, 0.2 *M* glycine, 0.1 *M* phosphate buffer, pH 7.3, dehydrated, and embedded in paraffin. Paraffin blocks were sectioned at 6–8 μm and stained with hematoxylin and eosin.

For skeletal analysis as described (Kaufman, 1992), embryos were eviscerated, fixed in 80% ethanol at room temperature for 1–2 days, dehydrated in 96% ethanol for 1 day, transferred to acetone for 3 days, and then stained with alizarin red S and alcian blue 8GS at 37°C for 6 hr. The tissues were cleared with 1% KOH and the skeletons were stored in glycerol.

Molecular Analyses

Northern analysis of poly(A)+ RNA was performed as described (Sambrook *et al.*, 1989), using as a hybridization probe a rat cDNA fragment corresponding to the α subunit of IGF1R, which detected an 11-kb *Igf1r* transcript (Werner *et al.*, 1989) and an additional 5.5-kb RNA species previously unreported in rodents. To provide a loading control, the same blot was stripped and hybridized to a mouse cytoplasmic β-actin probe.

For Western analysis, embryos were dissected, snap-frozen in liquid nitrogen, and stored at −70°C until use. Total extracts were prepared by dounce homogenization in 50 m*M* Hepes, pH 7.6, 150 m*M* NaCl, 1 m*M* PMSF, 1% Triton X-100, 0.5 mg/ml leupeptin, and 0.7 mg/ml pepstatin on ice. The insoluble material was pelleted and the supernatants were assayed for protein concentration (Bradford assay; Bio-Rad). Samples were analyzed by SDS–PAGE through a 5% gel. The proteins were then electrotransferred overnight at 4°C to a nitrocellulose membrane in 0.01 *M* Caps, pH 11, 10% methanol, and the blots were stained with Ponceau S to verify equal loading and protein transfer. The membrane was blocked with 5% dry nonfat milk in Tris-buffered saline, 0.05% Tween 20 for 1 hr at room temperature. After washing, the membrane was incubated at room temperature for 1 hr with 1 μg/ml of anti-IGF1Rβ polyclonal antibody (Santa Cruz Biotechnology) in blocking solution, followed by an incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Boehringer Mannheim) diluted 1:1000 in blocking solution (45 min at room temperature). After washing, the membrane was incubated for 1 min with ECL detection reagents (Enhanced Chemilluminescence; Amersham) and then exposed to X-ray film for 30 sec. A duplicate (control) blot was prepared in parallel, except that samples were analyzed by SDS–PAGE through a 15% gel and the membrane was incubated with anti-cyclophilin A polyclonal antibody (a gift from J. Luban). The intensities of the bands corresponding to the IGF1R proreceptor (135 kDa) and β-subunit (85 kDa) and the cyclophilin A polypeptide (18 kDa) were measured by a scanning densitometer (Molecular Dynamics). The derived values were corrected for small differences in the total amount of protein between loaded aliquots and mutant to wild-type ratios were calculated.

Growth Analysis

For staging of embryos, we considered noon of the day of vaginal plug appearance as e0.5. For developmental analysis of growth, embryos and their placentas were dissected and fixed in 4% paraformaldehyde. Prior to weight determination with a microbalance, the specimens were patted dry with absorbent paper. For determination of dry weights, freshly dissected embryos were weighed (wet weight determination) and then placed into tared tubes and dried by incubation at 60°C for 48 hr and 100°C for 24 hr, followed by a second weight measurement (see Babiarz *et al.*, 1988).

Regression analyses (plots of weight versus age) by application of a Gompertz equation (see Laird *et al.*, 1965) were performed by a curve-fitting computer program (SigmaPlot) using all of the weight data from each class of embryos. In the Gompertz function $W = A\ exp[-b\ exp(-kt)]$, W and A are the weight at time t and the asymptotic weight, respectively, while b and k are constants. This mathematical treatment takes into consideration that the specific rate $(1/W)\times(dW/dt)$ of the exponential embryonic growth is not constant, but declines exponentially. Specific growth rates (γ) were calculated using the equation $\gamma = -k\ ln\ (W/A)$.

RESULTS

Genetic Crosses

Previous results (Liu *et al.*, 1993; Baker *et al.*, 1993; Ludwig *et al.*, 1996) indicated that all of the *in vivo* ligand/receptor interactions of the IGF system had been accounted for as summarized in Figs. 1A–1K, except that the identity of XR remained unknown. Consistent with the interpretation of these relationships, three combinations of mutations result in identical growth-deficiency phenotypes of e18.5 embryos (and neonates), which exhibit about 30% of normal body weight (subsequently referred to as ''30% mutants,'' for simplicity). Thus, when both ligands are absent (mutants lacking IGF-I and IGF-II; Fig. 1F), the phenotype is identical with that of embryos lacking both IGF-II and IGF1R (Fig. 1H) and also with the phenotype of triple mutants lacking both IGF receptors and IGF-II (Fig. 1K). In all three cases of these 30% mutants, all of the IGF ligand/receptor interactions are eliminated. We reasoned, therefore, that if the unknown signaling receptor (XR) is the insulin receptor (IR; Fig. 1L), ablation of both IGF1R and IR should also result in the manifestation of a 30% mutant phenotype (Fig. 1N). To test the prediction of this hypothesis, we generated *Igf1r/Insr* double nullizygous embryos and compared them with known 30% mutants.

Intercrossing of *Igf1r*(+/−)/*Insr*(+/−) double heterozygotes (see Materials and Methods) generated embryos belonging to four phenotypic classes obtained in the expected 9:3:3:1 Mendelian ratio: normal embryos; mutants lacking IGF1R; mutants lacking IR; and double mutants lacking

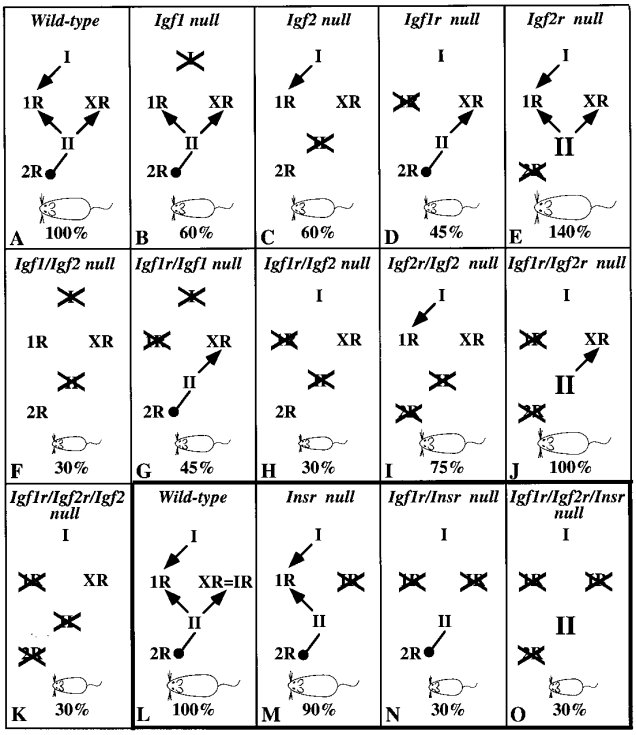


FIG. 1. Summary of mutant phenotypes. Each panel shows the interactions between IGF ligands (IGF-I = I; IGF-II = II) and receptors (IGF1R = 1R; IGF2R = 2R) in wild-type and mutant mice. The interaction of IGF-II with a previously unknown receptor (XR), which is the insulin receptor (IR), as demonstrated in this study, is also shown. Signaling interactions are represented with arrows, whereas a ''lollipop'' symbol denotes the IGF2R-mediated turnover of IGF-II. In the absence of turnover (E, J, and O), the amount of IGF-II increases (II, larger letters). The genotype of each single, double, or triple mutant is indicated on top of each panel, while the corresponding phenotype (% of normal weight of e18.5 embryos or neonates; rounded numbers) is indicated at the bottom. Ligands and/or receptors that are absent in each case are crossed-out.

both of these receptors (Table 1A). The normal embryos were genotypically wild type or heterozygous for either one or for both of the *Igf1r* and *Insr* genes. In parallel, to provide a basis for comparison, we generated known 30% mutants (lacking IGF-I and IGF-II; IGF-II and IGF1R; and IGF-II, IGF1R, and IGF2R) from appropriate matings (see Materials and Methods and Table 1B–D). As described below, our comparative analysis showed that the *Igf1r/Insr* double nullizygotes belong to the class of 30% mutants. Thus, our prediction was fulfilled demonstrating that XR is indeed IR.

To validate this conclusion, we provided additional genetic evidence based on the following considerations. Previously, the rescue of mutants lacking both IGF1R and IGF2R, which are viable and exhibit normal birthweight, was attributed to overstimulation of XR by IGF-II being in excess (Fig. 1J). This was consistent with the 30% phenotype of triple mutants lacking IGF1R, IGF2R, and IGF-II (Fig. 1K), demonstrating that, in the absence of IGF-II, XR cannot rescue the embryos from growth deficiency. In these experiments, however, the IGF-II/XR interaction was examined only from the side of the ligand. To examine the interaction from the side of the receptor and test further the hypothesis that XR is IR, we eliminated all three receptors (IGF1R, IGF2R, and IR; see Materials and Methods, Fig. 1O, and Table 1E), predicting that, if IR were absent, the excess of IGF-II could not sustain normal embryonic growth. As shown below, this prediction was also fulfilled and a 30% mutant phenotype was again observed.

Phenotype of Igf1r/Insr Double Nullizygous Embryos

Anatomical analysis. A few litters of progeny derived from intercrosses of *Igf1r/Insr* double heterozygotes were monitored at birth, but only two liveborn *Igf1r/Insr* double nullizygotes were recovered, which exhibited 30% of normal size and died immediately of respiratory failure like their *Igf1r* nullizygous littermates. As expected, *Insr* nullizygous littermates failed to thrive, although they fed normally, and survived only for 2 days. Thus, to avoid perinatal loss of mutants, we analyzed embryos between e11.5 and e18.5, considering that at the latter age the embryos are very similar to newborns.

In addition to their smaller size, the double mutant embryos lacking IGF1R and IR differed in external appearance from wild-type littermates in two features. First, at e14.5, but not earlier, the *Igf1r/Insr* double nullizygotes exhibited generalized subcutaneous edema that was most prominent on the dorsal side of the embryos and extended from the cervical region to the root of the tail and laterally to the proximal region of the limbs (Figs. 2A and 2B). In all examined cases, the accumulated fluid was clear and never hemorrhagic. From measurements of wet and dry weights, we calculated that there was an increase in water content by 21% over the wild-type value at e14.5 (Table 2). The corresponding increase in *Igf1r* nullizygotes did not exceed 7.5%, while single *Insr* mutant embryos did not exhibit edema (Table 2). In *Igf1r/Insr* double mutants, the edema persisted at e15.5 (Fig. 2C) and then declined variably in degree. Thus, the edema had disappeared in five of seven e16.5 double mutant embryos and in all three specimens examined at e17.5. However, two of nine *Igf1r/Insr* double nullizygotes recovered at e18.5 exhibited residual edema. Obvious histological abnormalities that could be associated with the development of edema were not detected and its cause remains unknown. Several spontaneous or radiation-induced mutations (see Biddle, 1990) and also some targeted mutations (see e.g., Sucov *et al.*, 1994; Kastner *et al.*, 1994; Takeuchi *et al.*, 1995) are associated with edema during embryogenesis. However, the subcutaneous edema observed in *Igf1r/Insr* double nullizygotes resembles only the edema appearing spontaneously at e14 in about 56% of embryos

TABLE 1
Breeding Data

Phenotype	Number of embryos	Observed relative frequency	Expected relative frequency	Weight (g ± SEM; e18.5)	Weight (% of normal)
(A) Intercrosses between *Igf1r*(+/−)/*Insr*(+/−) mice. Data: 764 genotyped e11.5-e18.5 embryos from 98 litters.					
Normal	438	9.2	9.0	1.32 ± 0.018 (n = 73)	
Lacking IGF1R	144	3.0	3.0	0.61 ± 0.014 (n = 23)	46.2
Lacking IR	143	3.0	3.0	1.19 ± 0.032 (n = 16)	90.2
Lacking IGF1R + IR	39	0.8	1.0	0.43 ± 0.015 (n = 9)	35.6
(B) Intercrosses between *Igf1*(+/−)/*Igf2*(+/−) mice. Data: 25 genotyped e18.5 embryos from 3 litters.					
Normal	13	4.2	3.0	1.21 ± 0.041	
Lacking IGF-I	5	1.6	1.0	0.75 ± 0.016	62.0
Lacking IGF-II	4	1.3	3.0	0.78 ± 0.047	64.5
Lacking IGF-I + IGF-II	3	1.0	1.0	0.39 ± 0.092	32.2
(C) Crosses between *Igf1r*(+/−) females and *Igf1r*(+/−)/*Igf2*(+/−) males. Data: 43 genotyped e18.5 embryos from 6 litters.					
Normal	16	7.4	7.0	1.30 ± 0.034	
Lacking IGF-II	17	7.9	8.0	0.79 ± 0.018	60.8
Lacking IGF1R	5	2.3	3.0	0.64 ± 0.029	49.2
Lacking IGF1R + IGF-II	5	2.3	2.0	0.44 ± 0.012	33.8
(D) Crosses between *Igf1r*(+/−)/*Igf2r*(+/−) females and *Igf1r*(+/−)/*Igf2r*(+/−)/*Igf2*(+/−) males. Data: 33 genotyped e18.5 embryos from 5 litters.					
Normal	3	3.5	7.0	1.41 ± 0.089	
Lacking IGF1R	3	3.5	3.0	0.58 ± 0.028	41.1
Lacking IGF2R	8	9.4	7.0	1.97 ± 0.084	139.7
Lacking IGF-II	7	8.2	8.0	0.89 ± 0.032	63.1
Lacking IGF1R + IGF2R	0	0	3.0		
Lacking IGF1R + IGF-II	1	1.2	2.0	0.46	32.6
Lacking IGF2R + IGF-II	10	11.8	8.0	0.91 ± 0.032	64.5
Lacking IGF1R + IGF2R + IGF-II	2	2.4	2.0	0.41 ± 0.049	29.0
(E) Intercrosses between *Igf1r*(+/−)/*Igf2r*(+/−)/*Insr*(+/−) mice. Data: 160 genotyped e18.5 embryos from 20 litters.					
Normal	49	9.8	9.0	1.30 ± 0.023	
Lacking IGF1R	12	2.4	3.0	0.61 ± 0.021	46.9
Lacking IGF2R	38	7.6	9.0	1.79 ± 0.029	137.7
Lacking IR	19	3.8	3.0	1.09 ± 0.019	83.8
Lacking IGF1R + IGF2R	17	3.4	3.0	1.21 ± 0.036	93.1
Lacking IGF1R + IR	3	0.6	1.0	0.40 ± 0.008	30.8
Lacking IGF2R + IR	17	3.4	3.0	1.64 ± 0.049	126.2
Lacking IGF1R + IGF2R + IR	5	1.0	1.0	0.49 ± 0.017	37.7

Note. Because the *Igf2* and *Igf2r* genes are imprinted, and the *Igf2* and *Igf1r* genes are linked on mouse chromosome 7, the expected relative frequencies of phenotypes in (B–E) differ from Mendelian ratios (see also Liu *et al.*, 1993; Ludwig *et al.*, 1996). The percentage of normal weight of mutants lacking IGF2R and IGF-II (64.5%; D) is significantly lower than previously observed (74%; Ludwig *et al.*, 1996), for unknown reasons.

of the WB/ReJ strain, which is also nonhemorrhagic and transient (it disappears by e17) and extends from the head caudally along the dorsal spine (Biddle, 1990).

A second anatomical difference from wild type evident in *Igf1r/Insr* double mutants from the earliest age examined (e12.5) onward, was a dorsal curling of the tail by more than 90° relative to the longitudinal axis (Fig. 2D). The cause of this fully penetrant trait, which permitted identification of the double mutants by visual inspection prior to genotyping, is unclear. However, this tail flexion abnormality is not associated with neural tube defects often observed in other mouse mutants with abnormal tails (see Copp *et al.*, 1990; Doolittle *et al.*, 1996) and differs in morphology from the kinky (''hairpin'') tail characterizing *Igf2r* mutants (Wang

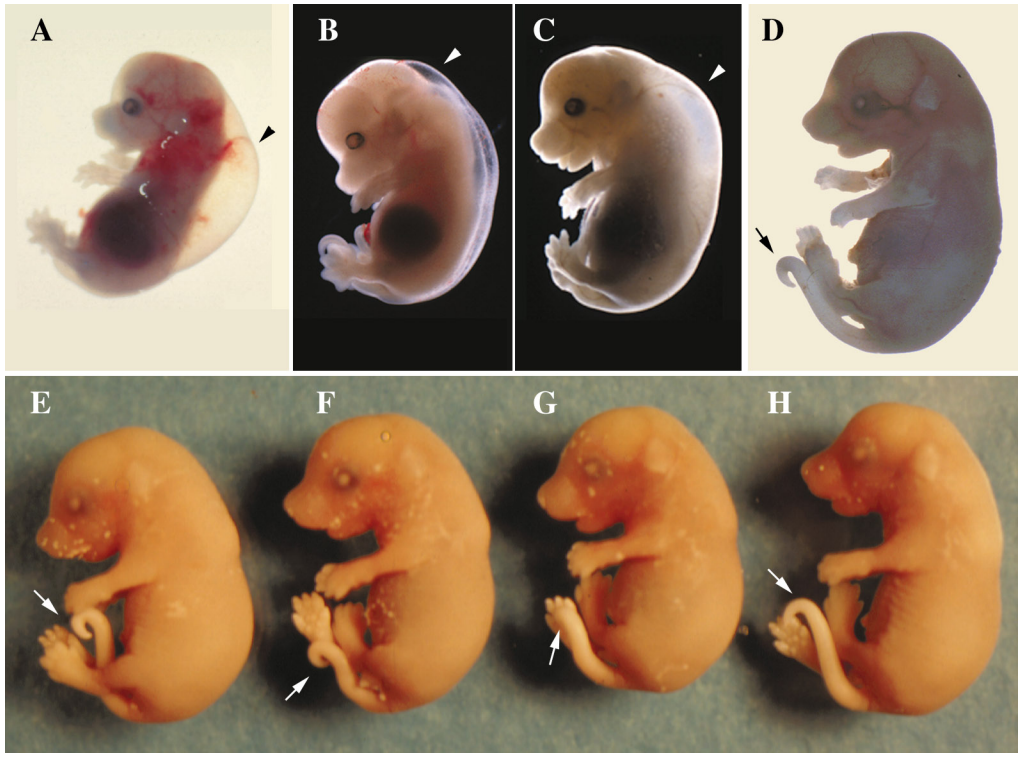


FIG. 2. *Igf1r/Insr* double nullizygous embryos at e14.5 (A and B), e15.5 (C), and e18.5 (D). The presence of subcutaneous edema, which in the case of the embryo in (A) is extreme, is indicated with an arrowhead. The embryo in (D) is compared with other 30% e18.5 mutant embryos lacking IGF-II and IGF1R (E), IGF-II, IGF1R and IGF2R (F), IGF-I and IGF-II (G), or IGF1R, IGF2R and IR (H). An arrow indicates a tail flexion abnormality.

et al., 1994; Lau *et al.*, 1994; Ludwig *et al.*, 1996). Curling of the tail was observed only in a fraction (36/138; 26%) of *Igf1r* nullizygous embryos examined between e11.5 and e18.5.

Autopsies at e18.5 revealed that, despite a generalized hypoplasia observed in most of the internal organs, the liver was disproportionately large in *Igf1r* and *Igf1r*/*Insr* nullizygotes (~11% of the body weight on average vs 6.5% in wild-type and *Insr* single mutant littermates) and appeared to compress the small thoracic cavity.

Histological analyses. While previous histopathological analyses of *Insr* nullizygous embryos (Accili *et al.*, 1996;

TABLE 2
Embryo Water Content

Phenotype	e13.5			e14.5		
	Water content[a] (±SEM)	Percentage increase over normal	Δ[b]	Water content (±SEM)	Percentage increase over normal	Δ
Normal	8.4 ± 0.05 (22)			8.0 ± 0.11 (31)		
Lacking IR	8.5 ± 0.08 (10)	1.2	−	8.1 ± 0.19 (9)	1.2	−
Lacking IGF1R	8.7 ± 0.29 (6)	3.6	−	8.6 ± 0.16 (7)	7.5	+
Lacking IGF1R + IR	8.7 ± 0.53 (2)	3.6	−	9.7 ± 0.45 (4)	21.2	+

[a] Water content per unit dry weight = (wet weight − dry weight)/dry weight. Mean values ± SEM were calculated from the number of specimens shown in parentheses. In mice, as in all mammals, total body water decreases with embryonic age (see Altman and Dittmer, 1974).

[b] The presence (+) or absence (−) of a statistically significant difference (Δ) is indicated (Student's *t* test; $P < 0.05$).

Joshi *et al.*, 1996) did not reveal significant differences from wild type, we expected that the development of bones, muscles, and skin will be affected in *Igf1r/Insr* double mutants as in *Igf1r* single mutants (Liu *et al.*, 1993), but more severely.

To ascertain the degree of bone development, we compared the skeletons of *Insr, Igf1r,* and *Igf1r/Insr* mutants and wild-type littermates at e16.5–e18.5, after staining with alcian blue and alizarin red (Figs. 3A–3L), using for simplicity a qualitative criterion and scoring for the first appearance of ossification centers, rather than determining the degree to which ossification has progressed. Despite some expected minor differences, due to developmental variability between and within litters for embryos of the same genotype, examination of several specimens of each class of embryos allowed us to reach the following conclusions.

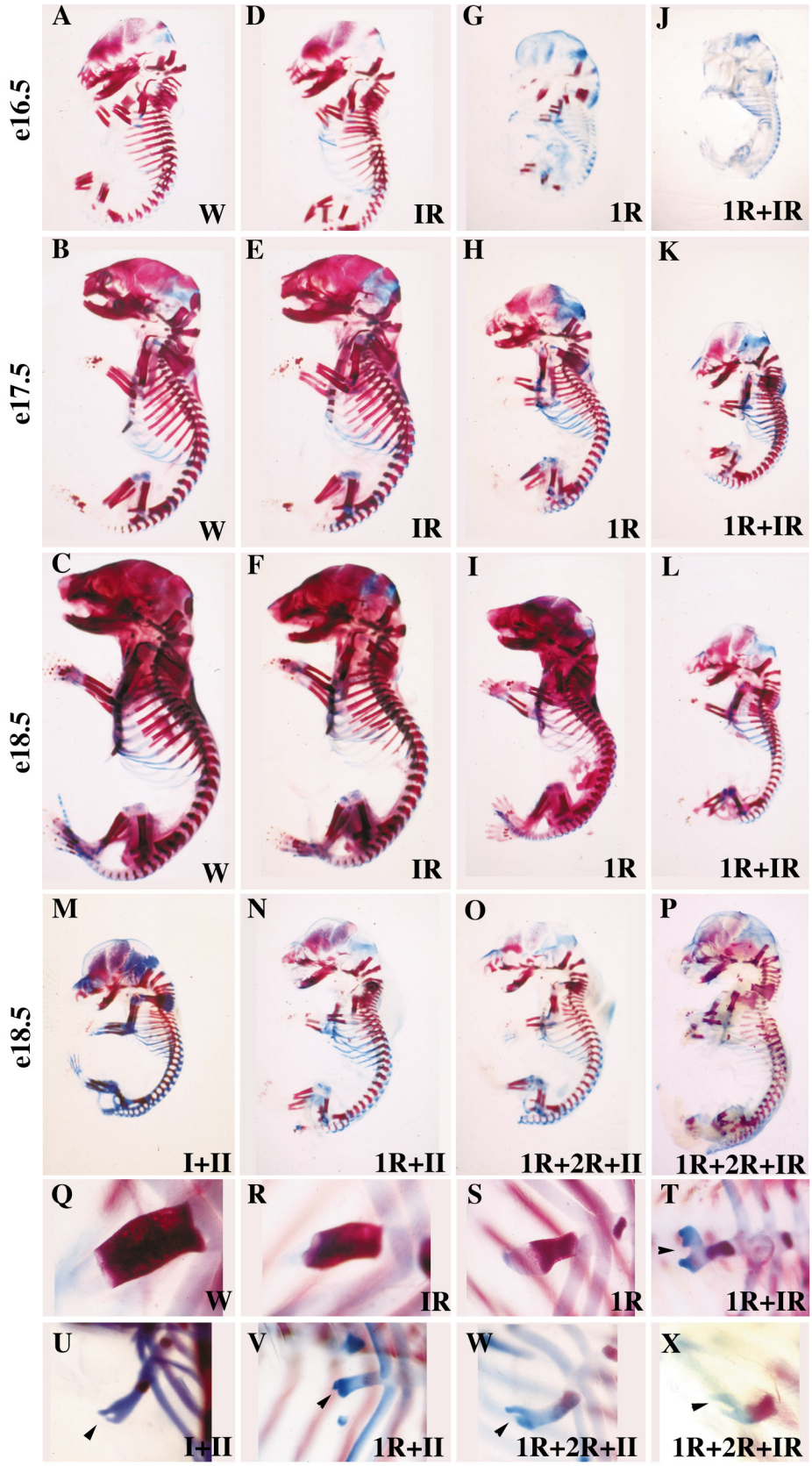
In comparison to wild type, the *Insr* mutants exhibited a developmental delay of approximately 1 embryonic day in the appearance of ossification centers in the middle digits of the forelimbs and hindlimbs, while shorter delays were detected in the bodies of cervical and caudal vertebrae and in the neural arches. Consistent with previous observations (Liu *et al.*, 1993), the ossification centers of facial and cranial bones appeared after a 2-day delay in *Igf1r* mutants in comparison with wild-type embryos, except for the interparietal bone (delay of 4 embryonic days). The time lag was 1–2 days for the bones of the trunk and extremities. In *Igf1r/Insr* double mutants, the developmental delays in the appearance of ossification centers were prolonged further. Thus, at e16.5, signs of ossification were completely absent from the double mutants, while the *Igf1r* single mutants exhibited ossification centers in several bones of the head, the body, and the extremities. Similarly, at e17.5, in contrast to the *Igf1r* mutants, the *Igf1r/Insr* double mutants were devoid of ossification centers in the zygomatic bone, the tympanic ring, the body of the atlas, the caudal vertebrae, and the distal digits of the forelimbs and hindlimbs. At e18.5, the double mutants lacked ossification centers in the bodies of the first four thoracic vertebrae and in the proximal digits of the forelimbs and hindlimbs, while the cartilage at the lower region of the xiphoid process of the sternum (xiphisternum) remained bifurcated (delay of at least 2 embryonic days; Fig. 3T).

Our histological analysis of muscle development in *Igf1r/Insr* mutants was focused on the diaphragm and the intercostal muscles, since a reduction in the number of myocytes in these respiratory muscles is one of the possible explanations for the death of *Igf1r* and *Igf1r/Igf2* mutant neonates, which exhibit generalized muscle hypoplasia and are unable to breathe (Liu *et al.*, 1993). As expected, severe hypoplasia of the diaphragm and the intercostal muscles was observed in *Igf1r/Insr* double nullizygotes in comparison with wild-type controls (cf. Figs. 4A and 4E; 4B and 4F), while the *Insr* single mutants were unaffected (not shown). The development of the diaphragm was also more severely affected in *Igf1r/Insr* double mutants than in *Igf1r* single mutants (cf. Figs. 4C and 4E), while a difference in the histological appearance of the intercostal muscles was not detected between these two groups (cf. Figs. 4D and 4F).

The skin of *Igf1r* and *Igf1r/Insr* mutant embryos is not opaque as in wild-type or *Insr* nullizygous littermates, but quite translucent. It was previously shown that this appearance of the skin in *Igf1r* mutants is due to a reduction of the epidermis in thickness relative to wild type (cf. Figs. 4O and 4P), because one of its layers (stratum spinosum) remains underdeveloped. Skin histology revealed that the epidermis of *Igf1r/Insr* double nullizygotes was even thinner than in *Igf1r* single mutants (cf. Figs. 4P and 4Q).

The *Igf1r/Insr* double mutants were then compared with triple receptor mutants (lacking IGF1R, IGF2R, and IR) and with the other 30% mutants (lacking IGF-I and IGF-II; IGF-II and IGF1R; and IGF-II, IGF1R, and IGF2R) at e18.5. These comparisons demonstrated that, within the limits of developmental variability, the phenotypic features of all classes of these embryos, in terms of body size (Table 1), dorsal tail flexion (cf. Figs. 2D and 2E–2H), delays in bone development (cf. Figs. 3L and 3M–3P; 3T and 3U–3X), respiratory muscle hypoplasia (cf. Figs. 4E and 4G, 4I, 4K, 4M; 4F and 4H, 4J, 4L, 4N), and underdevelopment of the skin (cf. Figs. 4Q and 4R–4U), were practically indistinguishable.

Molecular analyses. Altogether, the described results provided strong genetic evidence that the previously unknown IGF-II-specific receptor (XR) is IR. However, since IGF-II interacts with both IGF1R and IR during embryonic development in the mouse, the absence of severe phenotypic manifestations from *Insr* single mutants remained puzzling. Thus, we hypothesized that IGF1R can potentially compensate for the lack of IR in *Insr* mutants. To test this hypothesis, we first examined by Northern analysis whether the expression of *Igf1r* is upregulated in *Insr* mutants. Quantitation of the results from this analysis (Fig. 5A) showed that this is not the case; no difference in the level of *Igf1r* mRNAs was detected between wild-type and *Insr* mutant embryos at three ages examined (see legend to Fig. 5). To examine whether a difference could exist at the protein level, we analyzed protein extracts from whole embryos by Western blotting using a polyclonal antibody that recognizes the β-subunit of IGF1R and the IGF1R precursor (see Materials and Methods). Quantitation of reproducible results (see Fig. 5B) showed that, in comparison with the wild type, the level of IGF1R protein is increased approximately twofold in *Insr* mutants. Although the mechanism involved in this increase is unknown, the results appear to be consistent with the postulated compensatory function of IGF1R. It is notable, in this regard, that the patterns of expression of the *Igf1r* and *Insr* genes examined by *in situ* hybridization in rat embryos are very similar, except that the abundance of transcripts that are detected in virtually all tissues differs in liver and fat (high expression of *Insr* and low expression of *Igf1r;* Bondy *et al.*, 1994).



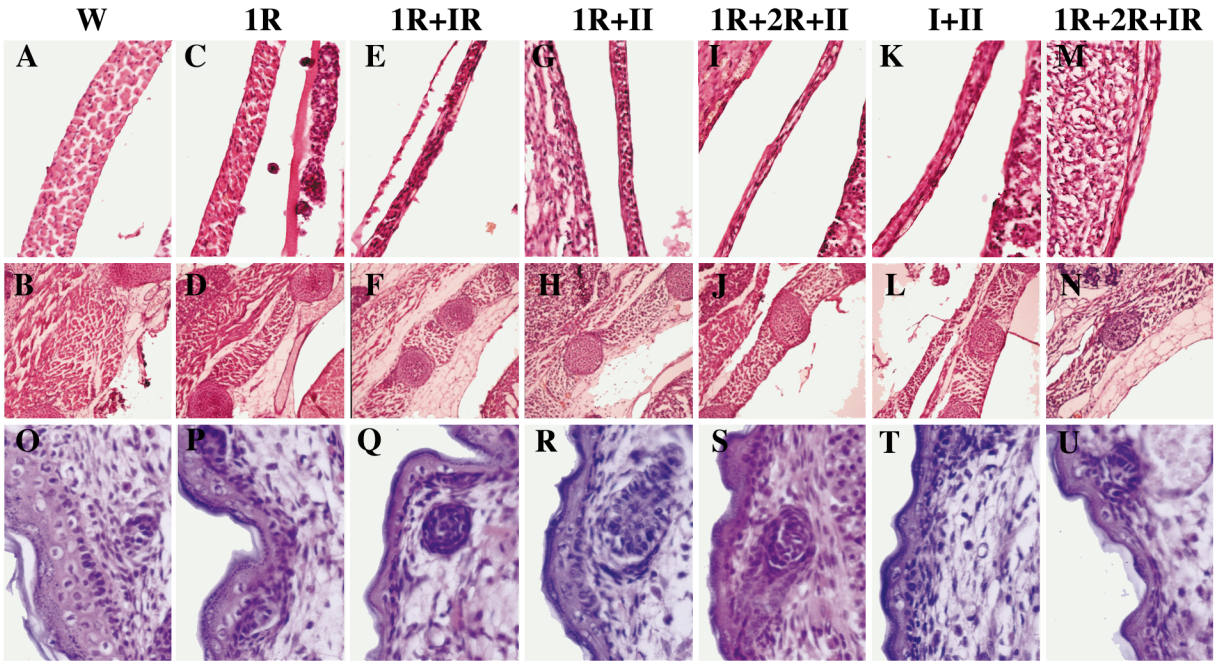


FIG. 4. Histological comparison of the development of respiratory muscles and skin in e18.5 wild-type and mutant embryos indicated on top of each column (abbreviations are as in Fig. 3). (A, C, E, G, I, K, M) diaphragm; (B, D, F, H, J, L, N) intercostal muscles; (O–U) skin sections at the level of the heart, dorsal to the spinal cord (for details, see text).

Embryonic and Placental Growth

The developmental increase of the embryo in size (growth) is effected by proliferative events increasing the total cell number. The rate of this proliferation, which determines the growth process, is reflected in the progressive increase of embryonic weight. Thus, growth curves (plots of weight versus developmental age) provide an overall growth index that can be used for comparisons of relative growth rates between wild-type and mutant embryos. However, because of developmental asynchrony of embryos with the same genotype and gestational age within and between litters, their weight values always vary within a range (see Fig. 6A). For this reason, to increase the accuracy of growth curves, we examined a large number of litters and subjected the data to statistical and regression analyses (Figs. 6A and 6B).

A comparison between wild-type embryos and mutants lacking IR showed that their growth processes were practically identical during embryogenesis, except that a statistically significant difference in average weights was detected at e18.5. Since an abrupt change in the putative compensatory role of IGF1R occurring at e18.5 appears to be unlikely, the detected growth difference of *Insr* nullizygotes from wild type can be attributed to lack of mediation of insulin action commencing late in development (see Discussion).

As expected (see Baker *et al.*, 1993), statistically significant differences in weight between wild-type embryos and mutants lacking IGF1R were detected at all times examined. A comparison between *Igf1r* single mutants and *Igf1r/Insr* nullizygotes showed that these two classes of embryos became statistically distinct in size at e13.5; double mutants were not recovered at e11.5, while the weight of a unique double nullizygote obtained at e12.5 was within the range of values observed in *Igf1r* single mutant embryos. However, in contrast to e13.5, a statistically significant difference in weights between *Igf1r* and *Igf1r/Insr* mutants was

FIG. 3. (A–L) Comparison of skeletons stained with alcian blue and alizarin red of wild-type (W) and mutant embryos lacking insulin receptor (IR), IGF1R (1R), or both of these receptors (1R + IR) at the embryonic ages indicated on the left of each row. The first appearance of ossification centers was determined from these and other specimens (for details, see text). The skeleton of an e18.5 double mutant lacking IGF1R and IR (L) is also compared with the skeletons of other 30% mutants of the same embryonic age lacking (M) IGF-I and IGF-II (I + II); (N) IGF1R and IGF-II (1R + II); (O) IGF1R, IGF2R, and IGF-II (1R + 2R + II); and (P) IGF1R, IGF2R, and IR (1R + 2R + IR). (Q–X) Magnified view of the xiphoid process of the embryos shown in (C, F, I, L) and (M–P), to demonstrate that the cartilaginous xiphisternum remains bifurcated (arrowhead) in the double and triple mutants in (T–X).

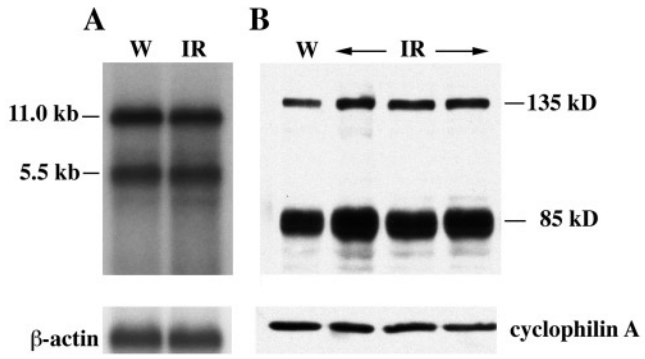


FIG. 5. (A) Northern analysis of poly(A)+ RNA from wild-type (W) and *Insr* nullizygous (IR) embryos at e18.5. The membrane was hybridized with an *Igf1r* probe and then stripped and rehybridized with a β-actin probe (see Materials and Methods). The size of detected *Igf1r* transcripts is indicated. Identical results (no difference in the level of *Igf1r* transcripts between wild-type and *Insr* null embryos) were obtained at e13.5 and e15.5 (not shown). (B) Western analysis of total protein extracts from wild-type (W) and *Insr* nullizygous (IR) embryos at e13.5 using a polyclonal antibody that detects the IGF1R receptor precursor (135 kDa) and the β-subunit (85 kDa). A duplicate (control) blot processed in parallel was analyzed with an antibody against cyclophilin A. The *Insr* nullizygous embryos were from different litters.

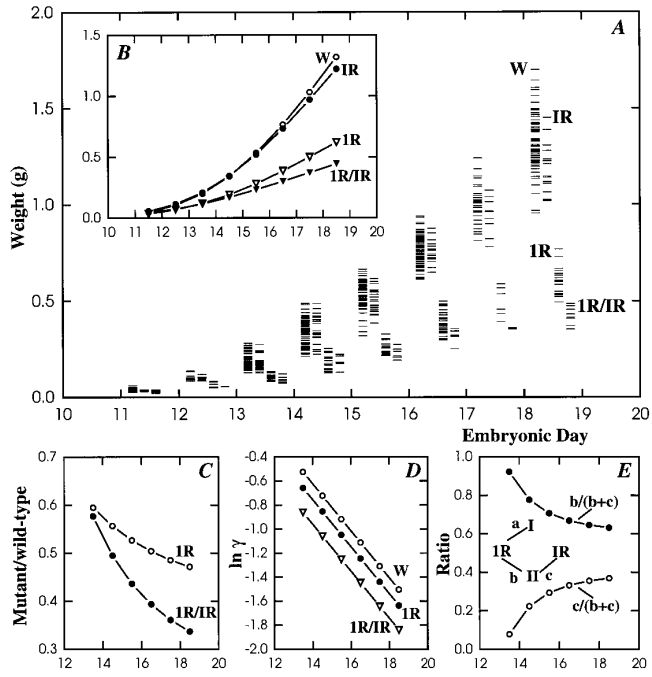


FIG. 6. Embryonic growth kinetics. (A) Plot of weights of wild-type (W), *Insr*(−/−) (IR), *Igf1r*(−/−) (1R) and *Igf1r*(−/−)/*Ir*(−/−) (1R/IR) embryos versus gestational age. The data are from 98 litters (764 genotyped embryos; Table 1A). In the plot, each symbol (dash) represents a single embryo (many dashes are superimposed). For clear display allowing comparisons, the same symbol is used for all classes of embryos. Each data group corresponding to a particular embryonic day is presented in the form of four columns centered around the cognate time point (as in histograms). Statistical analysis using Student's *t* test ($P < 0.05$) showed a significant difference in mean values between the W and IR groups only at e18.5. (B) The growth curves represent the results of regression analyses of the data in (A) using the Gompertz function (see Materials and Methods). With the exception of the e18.5 time point, the curves for wild-type embryos and mutants lacking IR are practically identical (for further details, see text). (C) The ratios of mutant to wild-type weights from e13.5 onward are shown for embryos lacking IGF1R and IGF1R + IR. These ratios were calculated from the values of the growth curves in (B). At e18.5, the weights of *Igf1r* single mutants and *Igf1r/Insr* double mutants are close to the rounded values of 45 and 30% of normal (see Fig. 1), respectively. (D) The natural logarithm of the specific growth rate (γ; see Materials and Methods) for the embryos shown is plotted versus developmental age. Calculations from this analysis reveal that throughout the period of observation the specific growth rate of *Igf1r* single mutants and *Igf1r/Insr* double mutants was 88 and 71% of that of wild-type embryos, respectively. (E) The relative fractions of embryonic weight resulting from the interactions of IGF-II with the IGF1R and IR receptors from e13.5 onward were calculated from the values in (B). The weight of *Igf1r/Insr* double mutants, in which all IGF interactions are eliminated, is the result of growth by mechanisms unrelated to the IGF system. Thus, at each time point, the weight resulting from IGF action is the remainder after subtraction of the 1R/IR weight value from the corresponding wild-type value (D and W, respectively), and consists of three components: a, weight resulting from the IGF-I/IGF1R interaction; b, weight resulting from the IGF-I/IGF1R interaction; and c, weight resulting from the IGF-II/IR interaction (i.e., W-D = a + b + c). For further details, see text.

not detected at e14.5 and e15.5, and became again evident from e16.5 onward. We attribute this deviation at e14.5 and e15.5 to retention of excess water in the double mutants (edema) affecting the weight measurements at these ages. Nevertheless, regression analysis indicated that the growth curves of *Igf1r* and *Igf1r/Insr* mutants were distinct from e13.5 onward (Figs. 6B and 6C). The growth retardation observed in these two classes of mutants was directly correlated to decreased specific growth rates in comparison with the wild-type (approximately 90 and 70% of normal in *Igf1r* and *Igf1r/Insr* mutants, respectively; Fig. 6D). As a result, the relative weights of the embryos became about 45 (*Igf1r*) and 30% (*Igf1r/Insr*) of normal at e18.5 (Fig. 6C).

What are the relative contributions to growth of the IGF-II/IGF1R and IGF-II/IR interactions? To provide a rough estimate, we calculated the respective fractions of embryonic weight resulting from these interactions, according to the following considerations. The weight D of *Igf1r/Insr* double nullizygous mutants, in which all of the IGF interactions have been eliminated (see Fig. 1), is the result of growth by mechanisms unrelated to the IGF system. Therefore, if W is the wild-type weight value, the weight resulting from IGF action at each time point is the difference W−D, which consists of three components: a, weight resulting from the IGF-I/IGF1R interaction; b, weight resulting from the IGF-II/IGF1R interaction; and c, weight resulting from the IGF-II/IR interaction, i.e., W − D = a + b + c (1) (see Figs. 1 and 6E). If II and R are the weights of *Igf2* and *Igf1r* mutants, respectively, it follows that a = II − D (2) and c = R − D (3). The value of b can then be calculated from Eq. [1]–

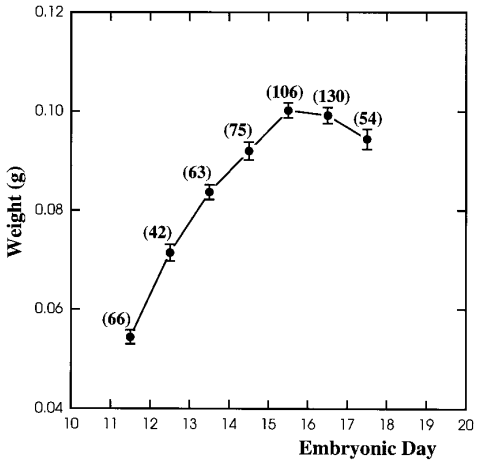


FIG. 7. Mean placental weights of a subset of the embryos in Fig. 3A are plotted versus gestational age. As expected, the placenta stops growing and actually loses weight toward the end of gestation (see McLaren, 1965). The width of the vertical bars represents 2 SEM. Statistical analysis using Student's *t* test ($P < 0.05$) did not show significant differences between wild-type controls and any of the mutants examined (lacking IR, IGF1R or both of these receptors). Thus, only average values of all four classes of embryos are shown in a single curve. The numbers of placental specimens weighed at each time point are shown in parentheses.

[3]. Although our current growth analysis (Fig. 6A) did not involve *Igf2* mutants, it was still possible to determine a and b values, because calculations from previous data (Baker *et al.*, 1993) showed that the ratios a/(a + b + c) and b/(a + b + c) are about 0.45 and 0.55, respectively, which indicated that the IGF-I/IGF1R and IGF-II/IGF1R interactions make almost equal contributions to embryonic growth. Using these ratios and applying Eqs. [1] and [3] to the data of the regression analysis (Fig. 6B), we determined the b and c components and plotted the ratios b/(b + c) and c/(b + c) (Fig. 6E). These calculations, reflecting the relative contributions of the two IGF-II interactions with the receptors, revealed that the action of IGF-II via IGF1R predominates throughout embryogenesis, but is not constant. Thus, at e13.5, the effect of the IGF-II/IR interaction on embryonic weight is small (less than 10% of the total IGF-II activity). However, the secondary, IR-mediated action of IGF-II increases with age progressively, although not linearly, becoming about 40% of the total at e18.5.

In parallel with the study of embryonic growth kinetics, we also recorded placental weights. Statistical analysis of the data demonstrated no significant differences in weights between wild-type and mutant placentas (*Insr, Igf1r,* and *Igf1r/Insr*) at any developmental age between e11.5 and e17.5 (a placental growth curve with averaged data is shown in Fig. 7). This analysis complements our previous results, which showed that placental growth is compromised only in mutants lacking IGF-II (alone or in combination with other mutations), while mutants lacking IGF-I or IGF1R have placentas of normal size (Baker *et al.*, 1993). Conversely, excess of IGF-II in mutants lacking IGF2R results in placental overgrowth (Ludwig *et al.*, 1996). This increase in placental size is also observed in *Igf1r/Igf2r* double mutants, although the birthweights of mice are normal (Ludwig *et al.*, 1996). These results, in conjunction with the data reported here, provide genetic evidence for the existence of an unknown, placenta-specific receptor (XR_p), which is distinct from IGR1R and IR and mediates the IGF-II growth-promoting role in this organ.

DISCUSSION

Functional Interplay of the IGF and Insulin Systems

We have presented conclusive genetic evidence demonstrating that the growth-promoting function of IGF-II during mouse embryogenesis is mediated via two evolutionarily and structurally related receptors, IGF1R and IR. Complementary evidence supporting this conclusion was provided by direct examination of the IGF-II/IR interaction in derivatives of cultured R^- cells, a fibroblast-like cell line originated from mutant embryos lacking IGF1R. It was shown that R^- cells overexpressing IR from a stably integrated plasmid (R^-/IR cells) are able to grow in serum-free medium (SFM) supplemented solely with insulin or IGF-II, but not with IGF-I (Morrione *et al.*, 1997). Interestingly, at any physiological concentration tested, IGF-II stimulated proliferation of R^-/IR cells at least twofold better than insulin. Moreover, when a second plasmid expressing *Igf2* was introduced into R^-/IR cells, secretion of IGF-II into the medium exerted an autocrine effect, and the cells became able to proliferate in SFM without growth factor supplementation (Morrione *et al.*, 1997).

Thus far, the generally accepted view was that under physiological conditions IR serves insulin signaling involved in metabolic effects, whereas IGF1R serves IGF signaling involved in growth. Our results indicate, however, that at least in mice the role of IR during most of the embryonic period is predominantly related to IGF-II signaling and growth control, rather than to mediation of insulin functions. Nevertheless, insulin does appear to play a minor role in growth, which cannot be compensated late in gestation, as evidenced by the results of targeting of the genes encoding IR or insulins (*Ins2* and *Ins1*). In contrast to humans and most other vertebrate species, which possess a single gene encoding insulin II, the genome of rats and mice contains an additional nonallelic gene (a functional retroposon) encoding insulin I (see Soares *et al.*, 1985). While *Ins2* or *Ins1* single nullizygotes are viable and fertile, *Ins2/Ins1* double nullizygotes mimic after birth the phenotype of neonates lacking IR and die of ketoacidosis within 48 hr (Duvillié *et al.*, 1997). At e18, these double mutants exhibit mild growth retardation (80–85% of normal weight; Duvillié *et al.*, 1997), which is comparable to the relative weight of 84–

90% of normal that we have observed for *Insr* nullizygotes at e18.5 (Table 1A and E). It is likely that, in the absence of insulin action, the reduced embryonic weights just prior to birth fall within a narrow range. Thus, despite pancreatic agenesis resulting from the elimination of insulin promoter factor 1, the birthweight of *Ipf1* nullizygous mice is reportedly either indistinguishable from wild type (Offield *et al.*, 1996) or at least 80% of normal (Jonsson *et al.*, 1994, 1995). It remains to be seen whether the birthweight of mice nullizygous for the *Pax4* gene, which lack mature insulin-producing β-cells, is indeed normal as reported (Sosa-Pineda *et al.*, 1997) or close to the normal range.

Although involved in growth control, IR is apparently unable to compensate for the growth-promoting functions of IGF1R, unless there is excess of IGF-II. Thus, when IGF1R is absent, IGF-II (presumably in normal concentrations) could continue to promote growth through IR, but this function is inadequate, and cannot prevent the severe dwarfism that results from the lack of interactions between IGF1R and its ligands. When both IGF1R and IGF2R are absent, however, the concentration of IGF-II increases significantly because of the elimination of IGF2R-mediated degradation, and overstimulation of IR by IGF-II compensates fully for the growth requirements of the embryo, which attains normal size. The genetic evidence consistent with this view was derived by generating triple mutants to demonstrate that both of the participants in the interaction, IR and excess of IGF-II, are necessary for normal growth. Thus, although double mutants lacking IGF1R and IGF2R are indistinguishable from wild-type at birth, they become nonviable dwarfs with 30% of normal size when they carry a third mutation removing either IR or IGF-II. Accordingly, excess of IGF-II in triple mutants is ineffective when IR is missing, and conversely IR is inert in the absence of IGF-II.

IGFs, Insulin, and Embryonic Growth in Humans and Mice

The significance of the IGF system not only for mouse, but also for human fetal growth was documented by the description of a 15-year-old patient carrying a homozygous partial deletion (exons 4 and 5) of the *IGF1* gene (Woods *et al.*, 1996). This mutation was manifested with severe intrauterine growth retardation (IUGR) and postnatal growth failure, including delayed bone development. Although the growth deficiency of the *IGF1* null patient was relatively more severe than that of mice lacking IGF-I, the overall phenotypic features were strikingly similar, except that the development of the reproductive system was apparently unaffected (Woods *et al.*, 1996), in contrast to the mutant mice (Baker *et al.*, 1996).

Additional information relating the IGF system to human growth was derived from studies with African pygmies. Although the exact cause of low birthweight (Bailey, 1991) and short stature of pygmies continues to remain unknown, decreased IGF1R expression and signaling were recently proposed as being involved (Hattori *et al.*, 1996). In lymphoblast cell lines derived from Efe pygmies, the steady-state level of *IGF1R* mRNA was approximately 2–13% of the control value, while the levels of the *INSR* and *GHR* (growth hormone receptor) mRNAs were normal. Moreover, although the affinity of IGF-I for IGF1R was unaffected, the number of binding sites per cell was 5.5–22% of the control value (with the exception of 1/6 lines; 65%). Interestingly, the cell lines from pygmies remained unresponsive to high concentrations of IGF-I in clonal proliferation assays (Geffner *et al.*, 1995), and residual IGF1R molecules failed to become autophosphorylated by this stimulation, although alterations in the *IGF1R* coding region were not detected by cDNA sequencing. Interestingly, however, these cell lines responded and became growth-stimulated by IGF-II (D. W. Golde, personal communication). On the basis of our observations, it can be hypothesized that this IGF-II action could be mediated by IR.

In contrast to the apparently similar actions of the IGFs in human and mouse growth, the hormonal effects of insulin exhibit a dramatic difference between the two species: absence of insulin or lack of functional IR during human embryonic development result in severe growth deficiency that is not observed in mice. IUGR of comparable severity due to lack of insulin has been reported in cases of pancreatic agenesis (Dourov and Buyl-Strouvens, 1969; Méhes *et al.*, 1976; Töpke and Menzel, 1976; Hill, 1978; Wright *et al.*, 1993; Voldsgaard *et al.*, 1994), absence of β-cells or pancreatic islets (Dodge and Laurence 1977; Jonas *et al.*, 1991; Blum *et al.*, 1993), or transient neonatal diabetes (reviewed by Fösel, 1995), a syndrome linked in familial cases to an unknown imprinted gene on chromosome 6q22-23 (Temple *et al.*, 1996). Interestingly, one case of pancreatic agenesis (Wright *et al.*, 1993) was attributed to a homozygous single-base deletion in the human *IPF1* gene causing a frame shift that resulted in the appearance of a downstream termination codon and truncation of the protein product (Stoffers *et al.*, 1997).

Insulin inaction due to IR defects occurs in leprechaunism, a genetic disorder resulting in death at infancy, which is characterized by severe IUGR, diminished muscle and subcutaneous adipose tissue, and other manifestations (see Taylor *et al.*, 1992; Koller *et al.*, 1995; Accili, 1995). Although the leprechaun syndrome is genetically heterogeneous, complete absence of IR function has been reported in three cases of homozygous mutations resulting in truncation of the protein product (Krook *et al.*, 1993; Psiachou *et al.*, 1993; Hone *et al.*, 1995; Jospe *et al.*, 1996) and in one case involving a homozygous deletion of the entire *INSR* gene (Wertheimer *et al.*, 1993). In leprechauns, insulin can reach levels as high as 400-fold over normal (see Krook *et al.*, 1993), but the overall growth is impaired, and the patients do not differ in birthweight from newborns with insulin deficiency. This indicates that, while IGF-II interacts with IR in mice and possibly in humans, insulin does not promote the growth of the whole organism via IGF1R. Therefore, as previously suggested (see e.g., Gluckman, 1995), insulin does not appear to be a major mitogen in the

context of the developing fetus, in contrast to the IGFs, and whatever growth-promoting effects it exerts can be predominantly attributed to its metabolic actions.

Growth Retardation and Developmental Timing

Body size at birth is determined by the growth rate and the duration of gestation. Thus, although the mouse embryo grows with a higher rate than the human embryo and reaches, for example, a weight of 1 g three times faster (17.5 vs 55 days; Adolph, 1970), the human newborn is much larger because of a significantly longer period of intrauterine life (280 vs 19 days). In addition to this difference in growth pattern between these two species, there is neither strict correspondence in the order of appearance of equivalent stages of various embryonic structures nor simple proportionality in the pace of development. In fact, when the ages of developmental equivalency are compared between mouse and human embryos, they cannot be correlated with the percentage of elapsed gestation time (Otis and Brent, 1954). This is reflected in the degree of maturity that the embryo has attained at the time of birth. In this regard, mice are born essentially in an ''embryonic'' state (lack of fur, low body lipid content, fused eyelids, etc.), while the human newborns are relatively more mature.

The development and maturation of the insulin system also differs between mice and humans, if birth is used as a reference time point. In the mouse embryo, insulin-producing cells in the developing pancreas appear first at e10.5–e11.5, but they are very few until e13.5 (a few hundred), increasing 20-fold in number between e13.5 and e15.5, with a further 5-fold increase occurring between e15.5 and e18.5 (Herrera *et al.*, 1991; Upchurch *et al.*, 1994). The appearance of typical islets with β-cells in the center and other endocrine cells in the periphery is a very late event beginning at e18.5 (Herrera *et al.*, 1991). Although information about the concentration of insulin in the embryonic mouse pancreas and serum is not available, data from rats indicate that a significant increase in pancreatic insulin content occurs after e14 (Rall *et al.*, 1973; equivalent to e12.5–e13 in the mouse; Rugh, 1990). In fact, more than 95% of the insulin present in the rat pancreas at the end of embryonic development accumulates between e18 and the time of birth (e22; Rishi *et al.*, 1969; Rall *et al.*, 1973; McEvoy and Madson, 1980; e16.5–e19 in the mouse). During these 4 days, the serum concentration of insulin increases about 3-fold (Félix *et al.*, 1971; Girard *et al.*, 1973; Kervan and Girard, 1974; Kervan *et al.*, 1978).

Expression of insulin in the human fetus begins at approximately 8 weeks, and the formation of well-defined islets occurs early (11–12 weeks; Goldman *et al.*, 1982; Stefan *et al.*, 1983). Until the 25th week, the pancreatic concentration of insulin is low and barely increases with age (Steinke and Driscoll, 1965; Grillo and Shima, 1966; Rastogi *et al.*, 1970; Schaeffer *et al.*, 1973; Pronina and Sapronova, 1976; Reiher *et al.*, 1983), while the insulin in serum, although detectable as early as 11 weeks of gestation (Adam *et al.*, 1969), is maintained at a low and almost constant level (Thorell, 1970; Kaplan *et al.*, 1972; Pronina and Sapronova, 1976; Economides *et al.*, 1991). After the 25th week, however, insulin increases significantly in concentration both in the pancreas and the serum (Pronina and Sapronova, 1976; Economides *et al.*, 1991), and apparently becomes involved in growth at around this time onward. It is interesting, in this regard, that ultrasound examinations in two cases of IUGR due to pancreatic agenesis (Wright *et al.*, 1993; Voldsgaard *et al.*, 1994) did not show any evidence of growth retardation at the gestational ages of 18–20 weeks.

We have shown that mouse embryos lacking IR exhibit a small degree of growth retardation detectable only during the last gestational day by statistical analysis of embryonic weights, in contrast to the severe IUGR of human fetuses due to insulin inaction. Based on the comparisons described above, a plausible explanation that we propose for this species-difference is a disparity in developmental timing between humans and mice. From the point of view of this hypothesis, elimination of insulin action cannot have significant detrimental consequences for the mouse embryo, since maturation of this hormonal system seems to occur just prior to birth. In contrast, insulin is apparently involved in human fetal development during the entire last trimester of pregnancy. Thus, in the absence of insulin action, a significant amount of time is made available for harmful effects to occur *in utero*, which result in the manifestation of a fully blown growth-deficiency phenotype in the human newborn.

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