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Effect of a Null Mutation of the Insulin-Like Growth Factor I Receptor Gene on Growth and Transformation of Mouse Embryo Fibroblasts

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Fibroblast cell lines, designated R^- and W cells, were generated, respectively, from mouse embryos homozygous for a targeted disruption of the *Igf1r* gene, encoding the type 1 insulin-like growth factor receptor, and from their wild-type littermates. W cells grow normally in serum-free medium supplemented with various combinations of purified growth factors, while pre- and postcrisis R^- cells cannot grow, as they are arrested before entering the S phase. R^- cells are able to grow in 10% serum, albeit more slowly than W cells, and with all phases of the cell cycle being elongated. An activated Ha-*ras* expressed from a stably transfected plasmid is unable to overcome the inability of R^- cells to grow in serum-free medium supplemented with purified growth factors but stimulates their growth in 10% serum and also induces the formation of small foci in some clones. Nevertheless, even in the presence of serum, R^- cells stably transfected with Ha-*ras*, alone or in combination with simian virus 40 large T antigen, fail to form colonies in soft agar. Reintroduction into R^- cells (or their derivatives) of a plasmid expressing the human insulin-like growth factor I receptor RNA and protein restores their ability to grow with purified growth factors or in soft agar. The signaling pathways participating in cell growth and transformation are discussed on the basis of these results.

The insulin-like growth factor I (IGF-I) receptor (IGF-IR) is expressed in a variety of cell types, both during development and in adult animals (for reviews, see references 33, 52, and 65). The activation of the IGF-IR by its ligands (IGF-I, IGF-II, and insulin at supraphysiological concentrations) plays an important role in the control of cellular proliferation of many types of cells, including fibroblasts, epithelial cells, smooth muscle cells, T lymphocytes, chondrocytes, osteoblasts, and stem cells of the bone marrow, in culture (for reviews, see references 7, 24, and 65). Interference with the function of the IGF-IR, either by antisense strategies or by using peptide analogs of IGF-I, results in inhibition of growth (8, 43, 44, 45, 47, 49). In growth-regulated cells, such as mouse 3T3 cells and human diploid fibroblasts, IGF-I alone does not stimulate cell proliferation but is a requirement for growth in serum-free medium in cooperation with other growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), or fibroblast growth factor (FGF) (14, 22, 30, 53, 57). Alterations in the IGF-IR and/or its ligands have been described in human cancer (35), and the IGF autocrine loop seems also to be involved in tumor suppression (17).

Results of gene targeting experiments in mice have shown that IGF-II is important for normal embryonic growth in vivo (15), while IGF-I has a significant growth-promoting function both during embryogenesis and postnatally (3, 32). Mice homozygous for a targeted mutation of the *Igf1r* gene die immediately after birth and exhibit a severe growth deficiency (their body weight is only 45% of normal [32]). The availability of embryos carrying this null mutation provided an opportunity to derive, by a protocol similar to that used for generation of 3T3 cells (66), cell lines of fibroblast-like cells completely lacking the IGF-IR (54). Taking advantage of the IGF-IR null background, we have used these cells to assess the significance of signaling via the IGF-IR both on cell growth and on transformation.

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MATERIALS AND METHODS

Cell cultures. Mouse embryo fibroblasts were established from 18-day embryos as previously described (54). Briefly, embryos were minced and disaggregated with trypsin for 15 min, and the single-cell suspension was seeded in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells derived from *Igf1r* gene knockout embryos were designated R^- cells, while cells derived from wild-type littermates were designated W cells. All cultures were maintained in DMEM containing 10% FBS unless otherwise noted. (*ts*A)W and (*ts*A)R^- cells are derived from W and R^- cells by stable transfection with a plasmid expressing a *ts*A mutant of simian virus 40 (SV40) T antigen. These cells have been described in detail by Sell et al. (54). p6 cells are 3T3 cells overexpressing the human IGF-IR (43).

Determination of cell growth. For all growth experiments, cells were seeded in medium containing 10% FBS and allowed to attach for 24 h, at which time the cultures were placed in serum-free medium supplemented either with different concentrations of serum or with 0.1% bovine serum albumin, 1 μM Fe_2SO_2, and the indicated growth factors. Unless otherwise noted, cells were seeded at a density of $3 \times 10^3/cm^2$. Purified growth factors (from BRL/Gibco) were used at the following concentrations: PDGF, 5 ng/ml; EGF, 20 ng/ml; IGF-I, 20 ng/ml; FGF, 20 ng/ml, transforming growth factor β (TGF-β), 20 ng/ml; IGF-II, 20 to 1,000 ng/ml; TGF-α, 20 ng/ml; and insulin, 20 to 100 μg/ml.

Cytofluorimetric analysis. Cells were fixed for flow cytom-

etry as follows. Cells were trypsinized and washed with cold phosphate-buffered saline (PBS). One million cells were then fixed by the addition of 0.5% formalin–0.25% saponin (4°C) for 1 h. Cells were then washed in cold PBS twice and treated with 75 U of RNase in PBS for 30 min. After centrifugation, the cells were stained with propidium iodide at a concentration of 50 μg/ml. A minimum of 20,000 cells were analyzed with a Coulter Epics Profile II (Coulter Electronics, Inc., Hialeah, Fla.). Cell cycle distribution of the population was analyzed with the Multi-Cycle program from Phoenix Flow Systems (San Diego, Calif.).

DNA synthesis. Tritiated thymidine was used at a concentration of 0.5 μCi/ml. Cells were placed into serum-free medium without additions for 72 h to achieve quiescence prior to the addition of growth factors. Labeled thymidine was added at the same time as growth factors, and incubations were carried out for 24 h. The cells were fixed in cold methanol and autoradiographed by standard methods. The percentage of labeled cells was determined by scoring a total of 1,000 cells.

Plasmid transfections. Both W and R^- cells were transfected by the suspension method (47). tsA58H is a plasmid containing the temperature-sensitive SV40 large T antigen *ts*A58 and the hygromycin resistance gene, which was constructed in our laboratory (47). The plasmid used to express the wild-type IGF-IR in the R^- cells was CVN Igf-I (28, 61), which contains the full-length coding sequence of the human IGF-IR cDNA and the neomycin resistance gene, both under the control of the SV40 early promoter. Selection of cells expressing the transfected IGF-IR cDNA was carried out in hygromycin, since the R^- cells were already neomycin resistant as a result of the *Igf1* gene knockout procedure. Other plasmids used were pBPV-KA and pBPV-KR, which have been described in detail by Kato et al. (27). Both express a full-length human IGF-IR with point mutations in the ATP-binding site. In KA, the amino acid was mutated from lysine to alanine; in KR, it was mutated from lysine to arginine. These plasmids were cotransfected with a hygromycin resistance plasmid (25) and selected in hygromycin (175 μg/ml). Most of the clones isolated expressed the human IGF-IR (see below).

The Ha-*ras* plasmid (a kind gift from S. Reddy) contains a genomic fragment of the human Ha-*ras* gene derived from T24 bladder carcinoma cells which has a codon 12 mutation. A *Bam*HI fragment of genomic DNA, containing the full length *ras* gene, was cloned into the *Bam*HI site of pBR322. For the generation of stable R^- cell lines carrying an activated *ras*, the *Bam*HI fragment of the *ras* gene was subcloned into the *Bam*HI site of plasmid pBSPac, which carries the gene for puromycin resistance (63). This allowed for selection of transfected cells in the presence of 20 μg of puromycin per ml.

IGF-IR cross-linking. IGF-IR cross-linking was carried out as previously described (54). Briefly, cells were seeded at a concentration of $1.25 \times 10^4/cm^2$. After 24 h, the cells were placed into serum-free medium for 48 h. [^{125}I]IGF-I was added, and the cells were incubated at 4°C for 4 h. At this time, the cell layer was washed with fresh serum-free medium. Proteins were cross-linked with disuccinyl suberate for 15 min, and the cell layer was subsequently washed with 10 mM Tris (pH 7.4). Cells were then lysed in Laemmli buffer (20% glycerol, 2% sodium dodecyl sulfate, 3% β-mercaptoethanol, 0.5% bromophenol blue). Cellular proteins were resolved on an 8% polyacrylamide gel, and cross-linked [^{125}I]IGF-I was visualized by autoradiography of the dried gel. The IGF-IR was recognized by the characteristic size of the whole receptor and its alpha subunit, as well as by competition with 100-fold nonradioactive IGF-I.

Transformation assays. For focus formation, cells were transfected with 10 μg of plasmid DNA by the calcium phosphate suspension method (47). Cells were then plated at a concentration of $4 \times 10^3/cm^2$. After 14 days in culture, cells became confluent and began to form foci. At this time, the cultures were fixed in methanol containing 0.5% crystal violet stain. Growth in soft agar was carried out as previously described (54).

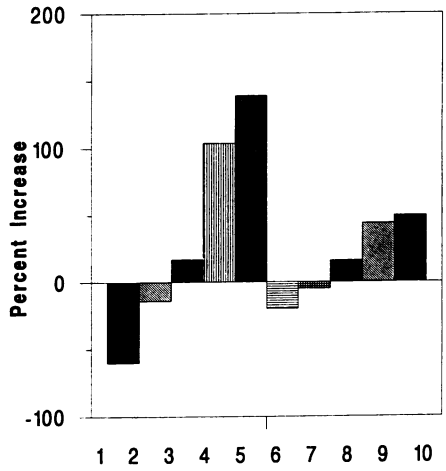

FIG. 1. Growth of precrisis W and R^- cells in different concentrations of serum. Cells were plated at a concentration of $10^4/cm^2$ in 10% serum. After 24 h (designated day 1), the growth medium was replaced with DMEM supplemented with different concentrations of FBS. Cell number was determined on day 3 and is expressed as percentage increase over the value for day 1. Bars: 1 to 5, W cells in 1, 2, 5, 7.5, and 10% serum, respectively; 6 to 10, same for R^- cells.

RESULTS

Culturing of embryonic fibroblasts. We have used homozygous *Igf1r*−/− mutant embryos and wild-type littermates (3, 32) to derive primary cultures of fibroblast-like cells that were designated R^- and W cells, respectively. These cells are partially described in a previous report (54), in which we showed that R^- cells are totally devoid of IGF-IR, whereas W cells have receptors that are normally autophosphorylated by IGF-I. Like all mouse cells established in serum-supplemented media, in the same protocol routinely used to establish 3T3 cell lines (66), both W and R^- cells underwent crisis after a certain number of passages, the R^- cells being somewhat slower to go into crisis than the W cells, presumably because of their slower growth rate (see below). Upon further passaging, some of the postcrisis clones underwent a secondary crisis and began growing at faster rates. The growth characteristics of the mutant and control cells were examined at both the pre- and postcrisis stages.

Growth of precrisis W and R^- cells in serum-supplemented medium. At the precrisis stage, the growth of both W and R^- cells depended on the concentration of serum in the culture medium. At least three clones were examined for each cell type, with essentially similar results. Neither cell type was able to grow in 1 or 2% serum (Fig. 1). In fact, some amount of cell death was observed under these conditions, which is not surprising since it occurs also with conventional 3T3 cell lines. Successful growth was observed for both cell types when the serum concentration was 5% or higher, but the growth rate of R^- cells was always slower than that of W cells (Fig. 1). Thus, in 10% serum, the growth rate of R^- cells was approximately

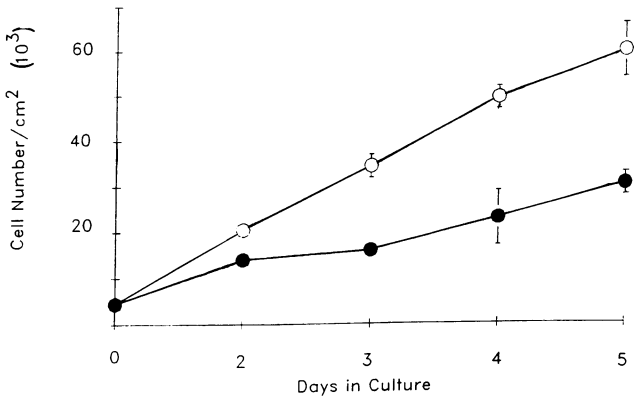

FIG. 2. Growth of precrisis W and R⁻ cells in 10% FBS. W and R⁻ cells were plated at a concentration of $2 \times 10^5/cm^2$ in DMEM supplemented with 10% FBS. Cell lines were precrisis. The number of cells (ordinate) was determined at the days after plating indicated on the abscissa. ○, W cells; ●, R⁻ cells.

45% of that of W cells, as shown in an example of growth curves for two precrisis clones (Fig. 2). Examination of several additional precrisis clones yielded similar results (not shown).

Elongation of cell cycle times in R⁻ cells. We then determined by flow cytometry analysis the cell cycle distribution of W and R⁻ cells exponentially growing in 10% serum (Table 1). At the same time, and with the same batches of cells used for flow cytometry analysis, we determined the doubling times. In this experiment, the doubling time of W cells was 43.6 h, and that of R⁻ cells was 109 h. The cell cycle distribution was substantially similar, except for a modest (1.5-fold) increase in the percentage of G_2+M cells in R⁻ cells. The cell cycle distribution was the same, whether the cells were seeded at low, medium, or high densities; furthermore, cell cycle distribution was determined in three different days of growth, with no appreciable differences (Table 1; data for day 2) despite the fact that R⁻ cells had a doubling time 2.5-fold that of W cells. We note that during these experiments, cell death was negligible (the cells were in 10% serum), and close to 100% of the cells were labeled by a 24-h exposure to [^{3}H]thymidine (not shown). Consistent with these results, we observed in an independent experiment that the percentage of cells labeled after a 1-h pulse of [^{3}H]thymidine was the same in W and R⁻ cells growing in 10% serum (not shown). From the averaged data of Table 1 and the doubling times, we have calculated the duration of the cell cycle phases in precrisis W and R⁻ cells growing in 10% serum (Table 2). The results show that as a consequence of the mutation, all phases of the cell cycle are elongated: the G_1, S, and G_2+M phases in R⁻ cells are approximately two-, three-, and fourfold, respectively, longer than in W cells. Incidentally, no appreciable difference in the size of cells between W and R⁻ could be detected by fluorescence-activated cell sorting analysis (data not shown).

TABLE 1. Cell cycle analysis of W and R⁻ cells[a]

Cells and density	% of cells in:		
	G_1	S	G_2+M
W			
Low	73	14	13
Medium	75	14	11
High	73	14	13
R⁻			
Low	65	14	21
Medium	67	15	19
High	66	19	15

[a] Precrisis cells were seeded at three densities: low ($4 \times 10^3/cm^2$), medium ($8 \times 10^3/cm^2$), and high ($1.6 \times 10^4/cm^2$). Cell growth was measured by determining cell number on a daily basis for 3 days at each cell density. All counts were done in duplicate. Flow cytometry was performed on each density every day. The percentages shown are from day 2. Average determinations (percent) ± standard deviations for each cycle throughout the experiment were as follows: for W cells, G_1, 72 ± 1.7, S, 15 ± 2.1, and G_2, 12.5 ± 2.3; for R⁻ cells, G_1, 66.5 ± 0.9, S, 14.5 ± 1.3, and G_2, 18.5 ± 2.5. The doubling times for each cell type determined during this experiment were 43.6 h for W cells and 109 h for R⁻ cells.

TABLE 2. Cell cycle parameters of W and R⁻ cells

Cells	Duration[a] (h)			
	G_1	S	G_2+M	T/c
W	33	6	5	44
R⁻	73	16	20	109

[a] Calculated directly from Table 1. T/c, length of the cell cycle.

Growth of W and R⁻ cells in serum-free medium supplemented with growth factors. To further define the role of the IGF-IR signaling pathway in the growth of embryonic mouse fibroblasts in culture, we examined the proliferative performance of R⁻ and W cells in serum-free medium supplemented with purified growth factors.

First, we used precrisis cells and tested a combination of PDGF, EGF, and IGF-I (or insulin at supraphysiological concentrations), which are the growth factors most commonly required for the growth of mouse embryonic cells. The results of a typical experiment are shown in Fig. 3. In precrisis W cells, the addition of PDGF, EGF, and IGF-I to serum-free medium stimulates growth (Fig. 3; compare bar 3 with bar 1). Insulin (at supraphysiological concentrations) is slightly less effective than IGF-1 (bar 5), while 10% serum is the most effective (sixfold stimulation; bar 7). In contrast to these results, only 10%

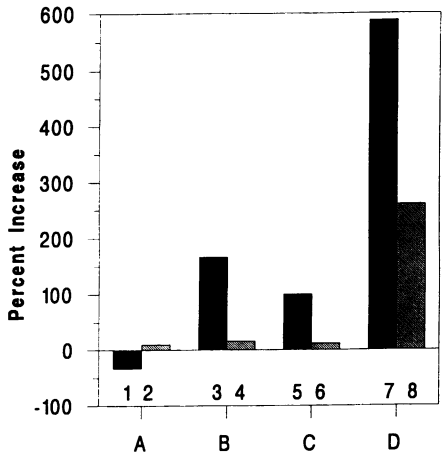

FIG. 3. Growth of precrisis W and R⁻ cells in serum-free medium supplemented with purified growth factors. Cells were seeded at a concentration of $2 \times 10^3/cm^2$ in 10% serum and then shifted to serum-free medium plus individual growth factors on day 1. Cell number (ordinate) was determined on day 3 and is expressed as percentage increase over the value for day 1. Bars: 1, 3, 5, and 7, W cells; 2, 4, 6, and 8, R⁻ cells. (A) Serum-free medium; (B) PDGF, EGF, and IGF-I; (C) PDGF, EGF, and insulin; (D) 10% serum.

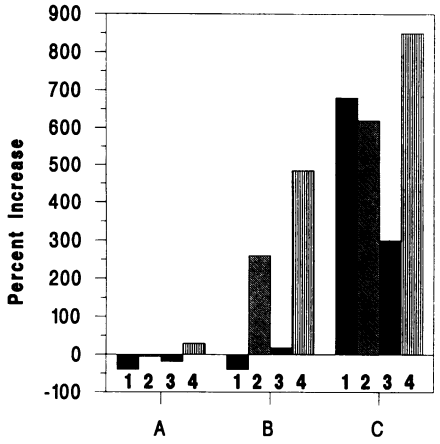

FIG. 4. Dependence of postcrisis R^- cells on serum for growth. Experiments were carried out similarly to those of Fig. 2 and 3. Number of cells (ordinate) was determined on day 3 and is expressed as percentage increase over the value for day 0 (24 h after plating). (A) Serum-free medium; (B) PDGF, EGF, and IGF-I; (C) 10% serum. Bars: 1, R^- cells postcrisis; 2, W cells postcrisis; 3, (*ts*A)R^- cells; 4, (*ts*A)W cells.

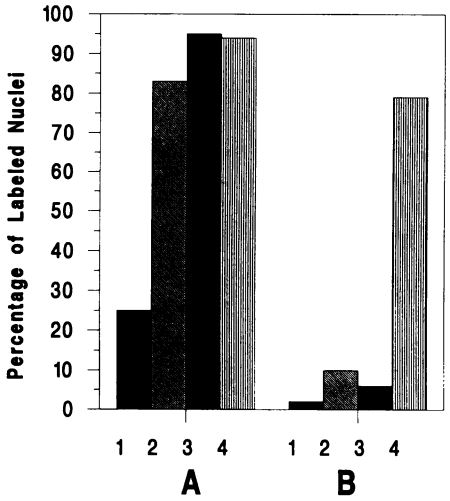

FIG. 5. Entry into S phase of postcrisis W and R^- cells. Cells were made quiescent as described in Materials and Methods and subsequently stimulated as indicated below. [^{3}H]thymidine was added at the time of stimulation; the cells were fixed after 24 h and then processed for autoradiography. (A) W cells; (B) R^- cells. The percentage of labeled cells is shown on the ordinate. Bars: 1, serum-free medium; 2, PDGF plus EGF; 3, PDGF, EGF, and IGF-I; 4, 10% serum.

serum can stimulate the growth of R^- cells (Fig. 3, bar 8). We have tested several known growth factors by themselves or in association with PDGF plus EGF. Under these conditions, the following growth factors failed to support growth of R^- cells: basic FGF, acidic FGF, IGF-II (up to 1.0 μg/ml), insulin at any concentration, hepatocyte growth factor, TGF-α and TGF-β (data not shown). With the exception of TGF-β, all of these growth factors support the growth of W cells, in association with IGF-I and/or PDGF, as in the case of 3T3 cells.

This inability of R^- cells to grow in serum-free medium supplemented with purified growth factors persists even in postcrisis cells (Fig. 4). For this experiment, we purposely selected a clone of R^- cells (postcrisis) that had undergone a secondary crisis (see above) and was growing in 10% serum at a rate even slightly higher than that of W cells. Again, in contrast to W cells, the R^- cells were unable to grow in serum-free medium supplemented with PDGF, EGF, and IGF-I. Under the same conditions, stably transfected R^- cells expressing the SV40 large T antigen (54), also failed to grow (Fig. 4).

These results indicate that the IGF-IR signaling pathway plays an important role in cell proliferation but is not an absolute prerequisite for growth. Although a set of known growth factors cannot rescue the growth phenotype of R^- cells, a presumably limiting, unidentified serum component can sustain growth, at least to a certain extent, as the results with 10% serum suggest.

Failure of R^- cells to enter into S phase. In the experiments described above, counting of cell numbers demonstrated the failure of R^- cells to grow in serum-free medium supplemented with growth factors. However, we could not discriminate from these results whether as a consequence of the mutation, the cell cycle was blocked at some point in G_1 or whether the division of R^- cells was arrested at some point after DNA synthesis. To address this question, W and R^- cells were made quiescent and then stimulated either in 10% serum or in serum-free medium supplemented with growth factors. The percentage of cells labeled by [^{3}H]thymidine was used to determine the ability of these cells to enter S phase. W cells can be stimulated to synthesize DNA by 10% serum, a combination of PDGF and EGF, or a combination of PDGF, EGF, and IGF-I, the three modalities being roughly equally effective (Fig. 5). The modest additional effect of IGF-I can be explained by the fact that both PDGF and EGF can induce the expression of IGF-I (8). In contrast, only 10% serum could stimulate R^- cells to enter S phase (Fig. 5).

Similar results were obtained with another method, i.e., a serum pulse of 1 h, which by itself is not sufficient to stimulate DNA synthesis in 3T3 cells but becomes stimulatory when followed by incubation with IGF-I or insulin at supraphysiological concentrations (68). A 1-h serum pulse had little effect on the entry into S phase of BALB/c 3T3 cells (Fig. 6A), W cells (Fig. 6B), or R^- cells (Fig. 6C). Addition of insulin or IGF-I after the serum pulse markedly stimulated 3T3 and W cells but had no effect on R^- cells. We show here the results with insulin in order to confirm that even supraphysiological concentrations of insulin have no mitogenic effect on R^- cells. These experiments, therefore, establish that with purified growth factors or equivalents, R^- cells fail to enter S phase.

Rescue of the R^- cell phenotype by transfection with the IGF-IR cDNA. To validate our conclusion, it is important to establish that the reproductive defects of R^- cells are exclusively due to the absence of the IGF-IR and not the indirect consequence of different phenotypes between W and R^- cells. For this purpose, we introduced into R^- cells an expression plasmid, encoding the full-length protein sequence of the human IGF-IR, either wild type (61) or mutated at the ATP-binding site (27). The latter plasmids, carrying point mutations that result in the replacement of a critical lysine with alanine or arginine, served as negative controls, since they express a nonfunctional IGF-IR that cannot be autophosphorylated, although it is still capable of binding the cognate ligands. The IGF-IR expression plasmids were cotransfected with a selectable marker (hygromycin resistance). Cell lines were selected and monitored for the expression of the IGF-IR by a cross-linking experiment in which radioiodinated IGF-I was cross-linked to the cell membranes. The presence of the alpha subunit of the IGF-IR was then monitored by electro-

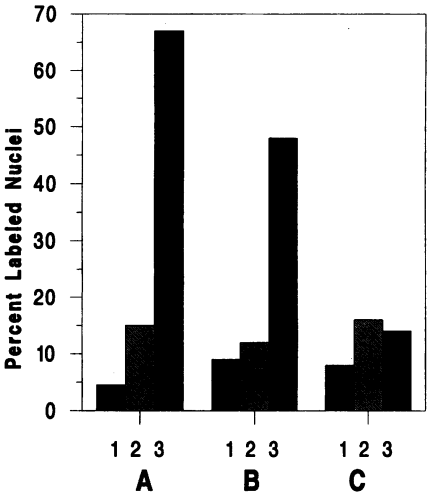

FIG. 6. Requirement for IGF-I or insulin after stimulation by serum pulses. In these experiments, quiescent cells (bar 1) were given a 1-h pulse of 10% FBS and then incubated either in serum-free medium (bar 2) or in serum-free medium supplemented with either insulin (20 μg/ml) or IGF-I. Only the data for insulin (bar 3) are given, but the same results can be obtained with IGF-I. [^{3}H]thymidine was added after the serum pulse, and the fraction of labeled cells (ordinate) was determined after 24 h. (A) BALB/c 3T3 cells; (B) W cells; (C) R^- cells.

phoretic analysis and autoradiography. Figure 7 shows an example of this analysis for some of the clones. This figure is not meant to be quantitative but is presented simply to show that several clones transfected with the wild-type and the mutant IGF-IR were expressing substantial amounts of receptor protein (lanes 4 to 9), roughly of the same order of magnitude as p6 cells (lanes 1 and 2), which are known to express large amounts of receptor (43). In contrast, no receptor was detectable by this sensitive method in R^- cells (lane 3), confirming previous results (54, 62). Several selected clones, designated R^+ cells, were expanded and tested; these included clones generated by stable transfection of a wild-type IGF-IR cDNA or of a mutant IGF-IR cDNA (alanine mutant or arginine mutant, replacing lysine at the ATP-binding site). Figure 8 shows that R^- cells expressing a wild-type human IGF-IR now grow in IGF-I almost as well as p6 cells do; however, the R^- cells expressing the mutant IGF-IRs still fail to grow under these conditions. These results confirm and extend those of Sell et al. (54), who had previously shown that a stably transfected wild-type human IGF-IR can restore to R^- cells the ability to be transformed by the SV40 T antigen. We can conclude that the growth phenotype of R^- cells depends on the absence of the IGF-IR and that only a functional receptor can restore their ability to grow in serum-free medium supplemented by purified growth factors (or to be transformed by SV40; see also below).

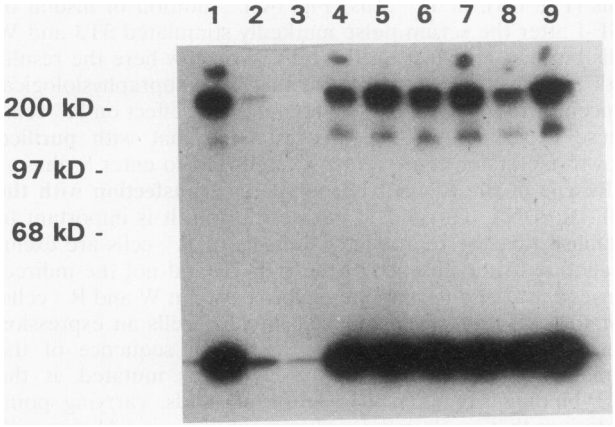

FIG. 7. Expression of the IGF-IR in R^- cells and derivative cell lines. Cross-linking of receptor to radioiodinated IGF-I was carried out as described in Materials and Methods. Lanes: 1, p6 cells; 2, p6 cells after competition with 100-fold nonradioactive IGF-I; 3, R^- cells; 4 and 5, two clones of R^- cells stably transfected with an expression plasmid coding for an IGF-IR with a single point mutation (lysine to alanine) in the ATP-binding site; 6 and 7, same as lanes 4 and 5 except that the mutation was from lysine to arginine; 8 and 9, two clones of R^- cells stably transfected with an expression plasmid coding for a wild-type human IGF-IR.

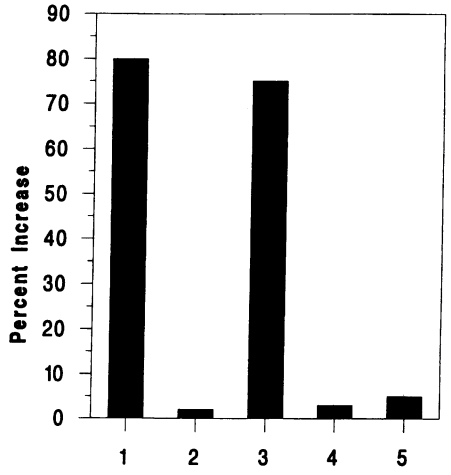

FIG. 8. Effect of IGF-I on the growth of cell lines described in Fig. 7. Growth is expressed as percentage increase over number of cells plated. The cells were grown in serum-free medium supplemented with 20 ng of IGF-I per ml. Bars: 1, p6 cells; 2, R^- cells; 3, R^- cells stably transfected with the wild-type IGF-IR plasmid; 4 and 5, R^- cells stably transfected with IGF-IR plasmids with point mutations in the ATP-binding site (lane 4, lysine to alanine; lane 5, lysine to arginine).

An activated Ha-*ras* cannot rescue the growth defect of R^- cells. The p21 product of the c-Ha-*ras* proto-oncogene (heretofore referred to simply as *ras*) is known to play a key role in growth and differentiation as a pivotal element in the signal transduction pathway of several growth factor receptors, including the PDGF, EGF, and IGF-I receptors (see Discussion). Since *ras* activation is believed to be downstream from the tyrosine kinase growth factor receptors, we thought that a plasmid expressing a p21 with a mutation in codon 12 (heretofore referred to as activated *ras*) could have the potential to bypass the growth defects of R^- cells. Cell lines were established from R^- cells stably transfected with the activated *ras*; several clones were selected and tested for expression of *ras* RNA (not shown). These clones, designated as R^-/*ras* cells, were expanded and tested for growth in PDGF, EGF, and IGF-I. As shown in Fig. 9, bar 3, R^-/*ras* cells are, like the parental cell line, incapable of growing in serum-free medium supplemented with purified growth factors. We also transfected the activated *ras* into (*ts*A)R^- cells (54), which are R^- cells expressing the SV40 T antigen. The activated *ras* had little effect on the growth of (*ts*A)R^- cells in serum-free medium

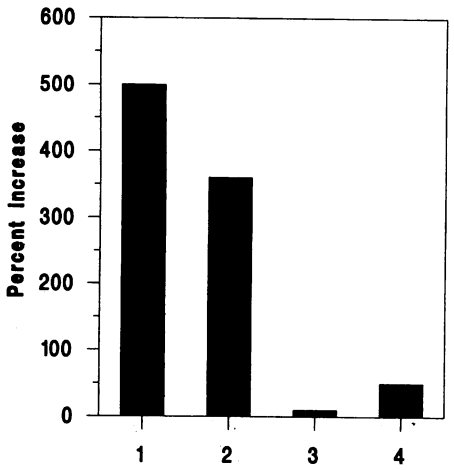

FIG. 9. Failure of activated *ras* to replace the IGF-IR for growth. R^-/*ras* cells and (*ts*A)R^- cells, expressing an activated *ras* RNA and protein, were seeded as usual and then placed in serum-free medium supplemented with PDGF, EGF, and IGF-I. The number of cells was determined 5 days later and is expressed as percentage increase over the value for serum-free medium. Bars: 1, BALB/c 3T3 cells; 2, W/*ras* cells (wild-type cells expressing an activated *ras*); 3, R^-/*ras* cells (R^- cells expressing an activated *ras*); 4, (*ts*A)R^-/*ras* cells (R^-/*ras* cells expressing the SV40 T antigen).

supplemented with growth factors (Fig. 9, bar 4). Thus, an activated *ras* cannot restore to R^- cells the ability to grow in serum-free medium supplemented with the same growth factors that sustain the growth of W cells.

We also examined whether the activated *ras* could have an effect on the growth of R^-/*ras* and (*ts*A)R^-/*ras* cells in the presence of 10% serum. Under these conditions, which can sustain the growth of R^- and (*ts*A)R^- cells by themselves, albeit at a rate slower than that of W cells, the activated *ras* did exhibit a significant effect, restoring the growth of R^-/*ras* and (*ts*A)R^-/*ras* cells close to the levels of their wild-type counterparts (not shown).

An activated *ras* can transform minimally R^- cells in the presence of serum. Since an activated *ras* has transforming potential, we tested whether this oncogene was able to transform R^- cells growing in 10% serum. For this purpose, several clones of R^-/*ras* cells were tested for focus formation. Only 4 of 10 examined clones formed foci, which were small and ranged in number from 0 to 14. We think that the ability to form foci may correlate with the levels of expression of the activated *ras*. We also tested whether the activated *ras* could affect focus formation in (*ts*A)R^- cells, which remain contact inhibited by themselves, despite the fact that they express abundantly SV40 large T antigen (54). Indeed, in the presence of 10% serum, the combination of activated *ras* and SV40 T antigen induced the formation of large foci in high numbers (more than 50 per plate; Fig. 10).

TABLE 3. Growth in soft agar of W cells expressing the SV40 T antigen and an activated *ras*

Cell type	No. of colonies[a]
Rat glioblastoma (positive control)	42, 57
W (negative control)	0, 0
W/T (T antigen)	42, 54
W/T/r (T antigen and *ras*)	128, 118
R^-/r (*ras* only)	0, 0
R^-/T (T antigen)	0, 0
R^-/T/r clone 1 (T and *ras*)	0, 0
R^-/T/r clone 4 (T and *ras*)	0, 0

[a] The cells were seeded at a concentration of 1,000 cells per well, and the number of colonies was determined 2 weeks later. Results of two determinations are presented.

Although R^- cells expressing the activated *ras* or *ras* and the SV40 T antigen can form foci in 10% serum in monolayer cultures, they still cannot grow in soft agar. Table 3 shows that W cells expressing T antigen or T antigen and *ras* form numerous foci in soft agar. No colonies were detected under the same conditions when R^- cells expressing both the T antigen and the activated *ras* (or the latter one only) were seeded.

DISCUSSION

We have used mouse embryo cells with a targeted disruption of the IGF-IR genes to investigate the role of the IGFI-R signaling pathway in cell growth and transformation. An important consideration pertaining to the interpretation of our results is that the phenotype of R^- cells was completely reversed after transfection with a plasmid expressing wild-type but not a mutant human IGF-IR cDNA (54; this report). Specifically, we can rule out a defective response to PDGF and EGF, the other two growth factors which, with IGF-I, are more commonly used for the growth of 3T3-like cells, since once R^-

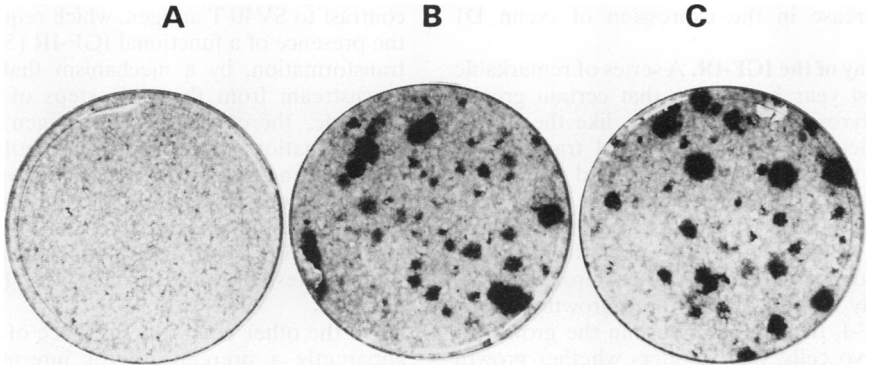

FIG. 10. Activated *ras* induces focus formation in (*ts*A)R^- cells. (*ts*A)R^- cells were transfected with the appropriate plasmid expressing an activated *ras*. Focus formation was determined as described in Materials and Methods. (A) Mock-transfected cells; (B and C) two plates of cells transfected with the *ras* expression plasmid.

cells express the human IGF-IR, they respond normally to the mitogenic action of PDGF and EGF (not shown). Thus, all of the phenotypic effects observed in the mutant cells can be attributed exclusively to IGF-IR deficiency.

Mutational effects on cell growth. The observations reported here confirm and extend previous in vivo and in vitro results (32, 54). R^- cells are unable to grow in serum-free medium supplemented with a combination of growth factors that are usually necessary and sufficient for the growth of mouse embryo cells, such as 3T3 cells and, in our case, control W cells. However, when the culture medium is supplemented with serum at a concentration of 5% or higher, R^- cells grow. We infer from these results that an as yet unidentified factor present in serum at a low concentration can sustain proliferation through a distinct signaling pathway, which either converges with the IGF-IR pathway at some step or is completely independent. However, even in 10% serum, the growth rate of R^- cells is still below that of W cells (or 3T3 cells), suggesting that the IGF-IR signaling pathway is not an absolute requirement for cell cycle progression but is necessary for optimal growth rate. Thus, in R^- cells, the cell cycle time is elongated 2.5-fold in 10% serum; i.e. the growth rate is 40% of normal (in these experiments, the cells were exponentially growing). Although the assumption of exponential growth in the whole embryo is an oversimplification (3), it seems hardly coincidental that the body weight of *Igf1r*−/− mutant mice is 45% of normal at birth (3, 32). The apparent correspondence between the in vivo and in vitro results suggests that whatever the actual cell doubling time may be in the mutant embryos, the relative elongation of the cell cycle is roughly the same as in cultured cells. Thus, the dwarfism of these mutants can be attributed to the occurrence of fewer proliferative events during a gestational period of normal length.

A significant and novel conclusion offered by our data is that the IGF-IR is needed for optimal progression through all phases of the cell cycle and not simply for the G_1-to-S transition as previously thought (12, 30, 51). The finding that in the absence of the IGF-IR, all phases of the cell cycle are lengthened raises some intriguing questions regarding the genes that the IGF-IR may induce in order to shorten the cell cycle. A relationship between the IGF-IR signaling pathway and the various cyclins and cyclin-dependent kinases (recently reviewed by Nigg [40]) is possible. It has been reported, for instance, that overexpression of cyclins D and E shortens the G_1 phase of the cell cycle (2, 41, 48). However, although the expression of *cdc2* in fibroblasts is regulated by IGF-I (58), the levels of cyclin D1 mRNA are insensitive to IGF-I and are instead regulated by PDGF (59). Curiously, though, treatment with an antisense oligodeoxynucleotide to IGF-IR RNA caused a marked increase in the expression of cyclin D1 mRNA (59).

The signaling pathway of the IGF-IR. A series of remarkable experiments in the last year has shown that certain growth factor receptors with tyrosine kinase activity, like the PDGF and EGF receptors, activate a common signal transduction pathway (for reviews, see references 13, 21, and 42) which, through Grb2, Sos, and possibly other molecules, leads to *ras* activation (18, 19, 31, 37, 50). The insulin receptor and the IGF-IR also use this pathway (34, 39), albeit through the intermediate of their docking protein, the IRS-1 protein (4, 55, 60). Since this pathway is used by the three growth factors, PDGF, EGF, and IGF-I, that together sustain the growth of wild-type mouse embryo cells, one wonders whether growth can be simply sustained by a sufficient quantitative activation of the *ras* pathway. In other words, if a sufficient number of, say, *ras* molecules is activated, a mitogenic effect is obtained, regardless of whether this effect is produced by a combination of three receptors or a single overexpressed receptor (see, for instance, reference 6). Although the evidence that *ras* activation is required for optimal cell proliferation and transformation is overwhelming (10, 11; reviewed in references 21 and 38), our present findings indicate that it is not sufficient, since a constitutively activated *ras* fails to induce growth of R^- cells in serum-free medium supplemented with individual growth factors. The isolation of recessive revertants of *ras*-transformed cells (still overexpressing *ras*) also indicates that *ras* may be required but is not sufficient for transformation (67). To account for our results, one must postulate that the IGF-IR signaling pathway at least bifurcates at some point into a second pathway, which is *ras* independent. We can only speculate on this second pathway. The facts that IGF-I induces nuclear translocation of protein kinase C (16) and that some isoforms of protein kinase C are mitogenic for cells in culture (9) indicate protein kinase C as a possibility, which can be tested. However, our preliminary experiments (not shown) indicate that neither phorbol esters nor the overexpression of protein kinase zeta (9) rescues R^- cells from their failure to grow in serum-free medium supplemented with growth factors.

Geffner et al. (23) could not detect IGF-I binding in T lymphocytes of African Pygmies, and their clonogenic response to physiological concentrations of IGF-I was vastly reduced. Higher concentrations, however, elicited a response. Interestingly, they also suggested a second pathway for the IGF-IR in Pygmy T cells, although the actual defect in the receptor or its transduction pathway was not characterized. A second pathway has also been hypothesized for the mitogenic effects of insulin (64) and serum (1, 20) and by the findings that an antibody to Grb2 (interrupting the *ras* pathway) inhibits PDGF and EGF stimulation, but not serum stimulation of DNA synthesis (36), and that *ras*-transfected mouse cells lose their dependence on EGF but still require supraphysiological concentrations of insulin for growth (29). Interestingly, when R^-/*ras* cells are grown in the presence of 10% serum, their growth is practically indistinguishable from that of W cells, suggesting that when the putative X signaling pathway is in operation, activated and overexpressed *ras* is apparently able to compensate for the ablated IGF-IR function on cell cycle progression.

Mutational effects on cell transformation. Our transformation assays, scoring for either the appearance of foci or the formation of colonies in soft agar (minimal and intermediate transformation, respectively [5, 46]), were performed by necessity in 10% serum. Under these conditions, in which the X signaling pathway is operational, an activated *ras* is able to induce the formation of a limited number of small foci in approximately half of the examined R^-/*ras* clones. Thus, in contrast to SV40 T antigen, which requires for transformation the presence of a functional IGF-IR (54), *ras* acts, for minimal transformation, by a mechanism that is independent of or downstream from the early steps of IGF-IR signaling. We conclude, therefore, that T antigen and *ras* use different transformation pathways. This is not surprising since their functions on cell growth are also nonoverlapping, as an anti-*ras* antibody does not inhibit the proliferation of SV40-transformed cells (56). Nevertheless, in (*ts*A)R^-/*ras* cells, T antigen and *ras* exhibit a cooperative effect on minimal transformation, since all tested clones formed very large foci and in large numbers.

On the other hand, the presence of a functional IGF-IR is apparently a prerequisite for intermediate transformation, since *ras* alone or in combination with T antigen is unable to induce the formation of colonies in soft agar. The presence of a functional IGF-IR seems to be also necessary for tumorige-

nicity (49a). These observations are consistent with previous data showing that NIH 3T3 cells overexpressing IGF-IR form foci in culture dishes and colonies in soft agar in the presence of IGF-I and are tumorigenic in nude mice (26).

The most intriguing aspect of our results is that they reveal a partial dissociability of mitogenesis and transformation. Although the occurrence of transformation obviously depends on proliferation, the ability of cells to grow can be separated from transformability, as evidenced by the fact that R^- cells expressing SV40 T antigen remain contact inhibited in 10% serum (54). Under the same condition, activated *ras* can induce minimal transformation but is unable to bring cells to the next stage of the transformed phenotype, growth in soft agar. Thus, as long suspected, transformation does not simply correspond to deranged growth but depends on signaling pathways, which can now be separated and identified at the molecular level.

For the moment, we can conclude that our experiments with R^- cells indicate that the signaling from the IGF-IR rests on at least two different pathways, one of which is the *ras* pathway. The identification of the second pathway will be of primary importance in our understanding of how signal transducing pathways control the proliferation and transformation of mammalian cells.

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