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Insulin-Like Growth Factor I (IGF-I) Receptor Overexpression Abolishes the IGF Requirement for Differentiation and Induces a Ligand-Dependent Transformed Phenotype in C2 Inducible Myoblasts*

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ABSTRACT

Insulin-like growth factors (IGFs) stimulate both proliferation and differentiation of myogenic cell lines, and these actions are mostly mediated through the type I IGF receptor (type I IGF-R). To further investigate the role of this receptor in phenotypic characteristics of C2 murine myoblasts, we overexpressed the human type I IGF-R in the inducible clone of C2 cells, which requires IGFs in the differentiation medium to undergo terminal differentiation.

Inducible myoblasts were transfected with either the eukaryotic expression vector pNTK or pNTK containing the human type I IGF-R complementary DNA, and we isolated two clones named Ind-Neo and Ind-R, respectively. Binding and autophosphorylation experiments indicate that Ind-R cells express about 10 times as much type I IGF-R compared with Ind-Neo control cells and that the transfected type I IGF-R is functional in Ind-R cells.

We show that overexpression of the human type I IGF-R makes inducible myoblasts able to differentiate spontaneously, as assessed by expression of the myogenic transcription factors MyoD and myogenin, detection of the muscle-specific protein troponin T, and myotube formation. Moreover, when exposed to IGF-I, Ind-R cells lose contact inhibition, grow in the presence of a low level of growth factors and form colonies in soft agar, which is characteristic of a ligand-dependent transformed phenotype.

It emerges from this study that 1) the type I IGF-R is strongly involved in the phenotypic differences between inducible and permissive cells with respect to the differentiation program; and 2) overexpression causes this receptor to act as a ligand-dependent transforming protein in muscle cells. We suggest that type I IGF-R abundance and level of activation may determine the efficiency of the autocrine mode of action of IGFs and discriminate their biological functions. (*Endocrinology* **138:** 5210–5219, 1997)

INSULIN-LIKE growth factors (IGFs), their receptors (IGF-Rs), and their binding proteins constitute a paracrine/autocrine growth factor system involved in the regulation of various cellular processes, including cell proliferation (1), cell differentiation (2, 3), neoplastic transformation (4), and inhibition of cell apoptosis (5). IGFs bind to the type I IGF-R, the IGF-II/mannose-6-phosphate receptor, and IGF-binding proteins, but their effects are mostly mediated via the type I IGF-R (6–8). The type I IGF-R belongs to the class 2 receptor tyrosine kinase family, which includes the insulin receptor and an orphan receptor termed insulin receptor-related receptor (9, 10). These receptors are heterotetrameric proteins with two ligand-binding α-subunits and two β-subunits. The β-subunit includes a tyrosine kinase domain responsible for autophosphorylation and initiation of the signaling pathways through the receptor (9, 11). Several type I IGF-R variants, which differ in electrophoretic mobility (12), binding properties (13), or immunoreactivity (14), have been reported in various tissues, arising from splice variants (15), post-translational modifications (16), or hybrid formation with the insulin receptor (17). It has been well established that muscle cells express all the components of the IGF system (18–20) and that IGFs stimulate both proliferation and differentiation of muscle cells (21). Therefore, muscle cell lines provide a suitable model to study and discriminate the different IGF effects and their mechanism of action. They also prove to be of particular interest in the study of mutually exclusive cellular programs, such as proliferation and differentiation. During skeletal muscle differentiation, proliferating undifferentiated myoblasts cease dividing and fuse to form multinucleated myotubes. This process is accompanied by the coordinate expression of several muscle-specific genes under the control of the members of the myogenic transcription factor family (Myf 5, MyoD, myogenin, and MRF4) (22). It has been demonstrated that IGFs can induce the expression of myogenin and MyoD genes (23, 24); however, the particular mechanism by which type I IGF-R activation modulates the expression of myogenic genes is not precisely known. To further investigate the role of the type I IGF-R in myoblast differentiation, we took advantage of the inducible variant of the C2 murine muscle cell line (25), which exhibits interesting

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features with respect to the IGF system. Unlike the parental cells, referred to as permissive, which are autonomous for differentiation, inducible myoblasts require exogenous IGFs to differentiate (26). Two differences at the molecular level have been implicated in this phenotype: 1) inducible myoblasts produce 200 and 10 times less IGF-I and IGF-II than permissive myoblasts, respectively (27); and 2) although expression of MyoD is constitutive in permissive cells, this gene is only induced during the differentiation program in inducible myoblasts (28). Interestingly, we have shown that inducible and permissive cells also differ with respect to their IGF-Rs. Indeed, we observed an atypical $\alpha_2\beta_2$ IGF-R in quiescent inducible, but not permissive myoblasts. This receptor has higher mol wt α- and β-subunits than the classical type I IGF-R and exhibits higher affinity for IGF-II than for IGF-I (29, 30). In this study we overexpressed the classical human type I IGF-R in inducible myoblasts and examined the effects of this overexpression on differentiation and proliferation. We found that type I IGF-R overexpression abrogates the exogenous IGF requirement for differentiation and induces a ligand-dependent transformed phenotype in Ind-R cells, but not in control cells.

Materials and Methods

Cell lines and cell culture conditions

The C2 cell line was isolated from satellite cells of adult mice by Yaffe *et al*. (25). The subclone of C2–7 myoblasts, named Permissive (Per), was derived from C2 myoblasts, and inducible (Ind) myoblasts were isolated from permissive C2–7 myoblasts by Pinset *et al*. (26).

Proliferating myoblasts were routinely maintained in a growth medium consisting of DMEM-Ham's F-12 (1:1) supplemented with 20% FCS (Life Technologies, Cergy Pontoise, France) and incubated at 37 C under 5% CO_2.

To achieve differentiation, myoblasts grown for 3 days in proliferation medium supplemented with dexamethasone (10^{-6} M) were switched to low serum differentiation medium consisting of DMEM supplemented with 0.5% FCS with or without IGF-I (10^{-8} M; gift from Dr. H. H. Peter, Ciba Geigy, Basel, Switzerland). The initial cell density was 640 cells/cm^2.

Monoclonal anti-type I IGF-R antibody αIR3 was obtained from Calbiochem and used in culture medium at 1 μg/ml.

Transfection of C2 cells with human type I IGF-R-expressing vector

The pNTK eukaryotic expression vector containing or lacking the 4.4-kilobase (kb) human type I IGF-R-coding sequence was used. Transcription of the receptor complementary DNA (cDNA) is under the control of the thymidine kinase promotor, and the vector contains the neomycin phosphotransferase gene that confers resistance to the antibiotic G418. Inducible cells were transfected using Lipofectamine reagent (Life Technologies) as described by the supplier. Stably transfected cells were selected in the presence of 1 mg/ml G418 (Geneticin, Life Technologies). Individual colonies were isolated after 5 days, passaged into stable cell lines, and cloned by limiting dilution. Two clones, named Ind-R (expressing human type I IGF-R) and Ind-Neo (control cells, transfected with pNTK), were used for the following studies.

Binding studies

For type I IGF-R binding assays, membrane samples (150 μg protein, determined by the Bio-Rad protein assay, Hercules, CA), prepared as previously described (29), were incubated overnight at 4 C with [^{125}I]IGF-I (50 pM) and an appropriate dilution of unlabeled IGF-I in a final volume of 0.3 ml buffer A (20 mM Tris-Cl, pH 7.5, and 10 mM $MgCl_2$) containing 0.2% BSA (Sigma Chemical Co., St. Louis, MO). Nonspecific binding was determined by adding an excess of IGF-I (5×10^{-8} M). Membrane-bound and free hormone were separated by adding 3 ml ice-cold buffer A and centrifuging at 5000 $\times$ *g* for 30 min at 4 C. The supernatant was removed, and the radioactivity of the pellets was measured in a γ-counter (Kontron, St. Quentin en Yvelines, France).

Autophosphorylation of type I IGF-R

Microsomal membranes were solubilized, and IGF-R were partially purified using a wheat germ agglutinin (WGA)-Sepharose column, as previously described (31). WGA-purified receptors (5–30 μg protein) were preincubated for 30 min at 4 C in 50 μl 50 mM HEPES (pH 7.8)-2.5 mM $MnCl_2$ buffer in the presence or absence of IGF-I (10^{-9} M). The kinase reaction was initiated by the addition of 1 μM [γ-^{32}P]ATP and 50 μM sodium orthovanadate and, after 10-min incubation at 20 C, was stopped by the addition of 200 μl chilled stop mix [5 mM HEPES (pH 7.8), 2% Triton X-100, 150 mM NaCl, 0.4 mM sodium orthovanadate, 4 mM EDTA, 4 mM EGTA, and 0.4 mM phenylmethylsulfonylfluoride containing 40 μg each of leupeptin, aprotinin, and soybean trypsin inhibitor/ml]. The mixture was incubated overnight at 4 C in the presence of polyclonal antibody P2, specific for the tyrosine kinase domain of the receptor (32). Immune complexes were recovered by centrifugation after incubation for 1 h at 4 C with protein A-Sepharose beads and washed as previously described (31). Autophosphorylated receptors were eluted by the addition of denaturing Laemmli buffer containing 1 M 2β-mercaptoethanol and 0.2 M dithiothreitol, boiled for 3 min, and subjected to electrophoresis (6.5% SDS-PAGE). Then, the gel was exposed to autoradiography film, and the radiolabeled band corresponding to the β-subunit of the receptor was cut out for Cerenkov counting.

Immunofluorescence assays

Cells were fixed for 5 min in PBS containing 3.7% formaldehyde (wt/vol), followed by a 30-sec extraction in glacial acetone. Expression of troponin T was assayed using a 1-h incubation of cells with 1:100 dilution of a mouse monoclonal antibody against troponin T (Sigma, T-6277) followed by a 30-min incubation with fluorescein-conjugated antimouse antibodies (Cappel, Organon Technica, Fresnes, France). Cells were stained with Hoechst B2883 dye and mounted in Airvol 205 [15% Airvol 205 (Air Products, Utrecht, The Netherlands), 33% glycerol, and 0.1% NaN_3 in PBS, pH 7]. Stained cells were observed on a microscope (Axiophot, Carl Zeiss, Thornwood, NY) using a planapochromat $\times$40 objective. Fluorescent images were recorded onto TriXpan 400 film (Eastman Kodak, Rochester, NY).

RNA extraction and Northern blot analysis

Total RNA was prepared using the guanidinium thiocyanate method, as previously described (33). For Northern analysis, total RNA (20 μg) was separated on a 2 M formaldehyde-containing 1% agarose gel, transferred, and bound to nylon membranes (Hybond-N, Amersham, Arlington Heights, IL) as recommended by the supplier. Filters were prehybridized for at least 2 h in a buffer containing 50% formamide, 5 $\times$ SSPE (1X SSPE = 0.15 M NaCl, 0.01 $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA), 0.1% SDS, 100 μg/ml denatured DNA salmon sperm, and 5 $\times$ Denhardt's solution and hybridized in the same medium containing the appropriated random-primed ^{32}P-labeled probe (10^6 cpm/ml) at 42 C for 6 h. Filters were washed at room temperature in 2 $\times$ SSC (standard saline citrate) buffer-0.1% SDS for 5 min and twice at 65 C for 20 min each time in 0.2 $\times$ SSC-0.1% SDS. Filters were then exposed to X-Omat Kodak x-ray films at -70 C.

RT-PCR amplification of MyoD

First strand cDNA synthesis of RNA was carried out at 37 C for 1 h in a final volume of 50 μl containing 1 μg total RNA, 2 μg random primers, 5 U RNasin (Promega, Madison, WI), 1 mM of each deoxy-NTP, and 200 U reverse transcriptase Moloney murine leukemia virus ribonuclease H$^-$ (Promega).

PCR amplification of a 144-bp fragment of MyoD was performed using 1 $\times$ standard PCR reaction buffer [50 mM KCl, 10 mM Tris-Cl (pH 9 at 25 C), 0.1% Triton X-100, 2 mM $MgCl_2$], 200 μM deoxy-NTPs, 2.5 U *Taq* polymerase (Promega), and 20 pmol of each primer in a 50-μl reaction. The oligonucleotide primers used were MyoD identical and

MyoD complementary, previously described by Montarras *et al.* (34). Amplification of 5 μl of the template first strand cDNA was carried out in ultrathin-walled tubes in a model PTC-150 DNA thermocycler (MJ Research, Watertown, MA) with the following program: 1 min at 93 C and 30 cycles of 1 min at 93 C, 1 min at 50 C, and 1 min at 72 C. Amplified cDNA was analyzed after electrophoretic migration in 2% agarose gels by alkaline Southern blotting onto positively charged nylon membrane (Hybond-N$^+$, Amersham) and hybridization with a random primed ^{32}P-labeled MyoD probe (35).

Soft agar assay

To test the ability of cells to form colonies in soft agar, a double layer culture technique was used. Per 60-mm dish, 1.8×10^4 cells were seeded in 3 ml proliferation medium containing 0.2% agar (Life Technologies) with or without IGF-I (10^{-8} M) and placed over a bottom agar consisting of 4 ml proliferation medium containing 0.5% agar. Cells were fed once a week with 200 μl proliferation medium with or without IGF-I (10^{-8} M). Two weeks after seeding, colonies were scored.

When used to inhibit colony formation, αIR3 was included in top agar medium at a final concentration of 1 μg/ml.

Results

Type I IGF-R expression

C2 inducible cells were used to generate two stably transfected cell lines, Ind-Receptor and Ind-Neomycin, by transfection with pNTK eukaryotic expression vector containing (Ind-Receptor) or lacking (Ind-Neomycin) the 4.4-kb coding region of the human type I IGF-R, respectively. The expression of human type I IGF-R was assessed by Northern blot analysis and [^{125}I]IGF-I binding. The fact that the human type I IGF-R level in the polyclonal population of transfected cells was low and decreased after few cell culture passages led us to clone several cell lines by limiting dilution. Among the clones expressing human type I IGF-R, a clone named Ind-R was expanded in culture for further analysis, and a control clone was randomly chosen among Ind-Neomycin clones and named Ind-Neo.

The overexpression of the human type I IGF-R in Ind-R cells was characterized by Northern blot analysis (Fig. 1A), [^{125}I]IGF-I binding studies (Fig. 1B) and autophosphorylation experiments (Fig. 1C).

Northern blots showed that Ind-R cells express a major transcript of about 4.8 kb and a minor transcript of about 3.1 kb, both of which correspond to the transfected human type I IGF-R messenger RNA (mRNA; Fig. 1A). In addition, Ind-R cells and Ind-Neo cells express a transcript of about 7 kb and a minor transcript of about 11 kb (which appears after longer exposure). These two latter transcripts correspond to the endogenous type I IGF-R mRNA, in agreement with the multiple type I IGF-R transcripts previously reported in mouse cells (36). Densitometric analysis of autoradiographs showed that mRNAs expressed from the pNTK expression vector are 20 times more abundant than mRNAs transcribed from endogenous gene.

Binding displacement curves of [^{125}I]IGF-I (Fig. 1B) and

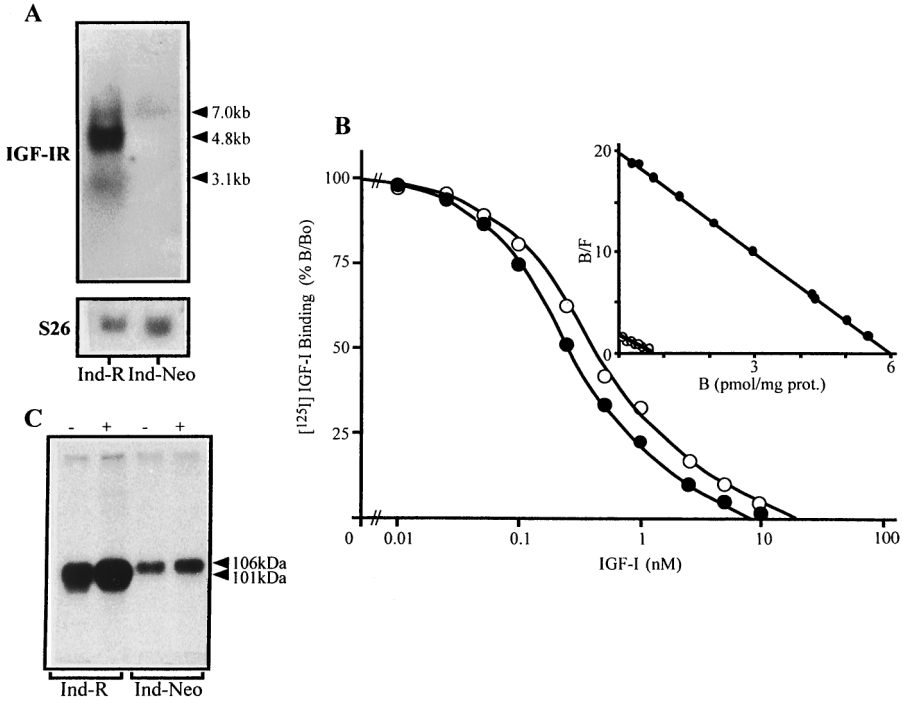

FIG. 1. Overexpression of human type I IGF-R in Ind-R cells. A, Northern blot analysis of human type I IGF-R expression. Total RNA (20 μg/lane) of Ind-R and Ind-Neo cells were subjected to Northern blot analysis using a human type I IGF-R (position 1–900), random primed, ^{32}P-labeled probe (11), as described in *Materials and Methods*. The ribosomal S26 protein transcript was probed onto the same blot to control the amount of RNA loaded on the gel [the hamster ribosomal S26 protein cDNA probe was obtained from Dr. S. Vincent (53)]. B, Displacement curve of [^{125}I]IGF-I (50 pM) by IGF-I to microsomes from Ind-Neo (○) and Ind-R (●) cells. B, Specific binding; nonspecific binding represented 7% of total binding. *Inset*, Scatchard analysis of the data concerning [^{125}I]IGF-I binding displacement by IGF-I to microsomes from Ind-Neo and Ind-R cells. Dissociation constants were 3×10^{-10} and 5×10^{-10} M for Ind-R and Ind-Neo, respectively. B/F, Bound to free ratio. C, Autophosphorylation response of WGA-purified type I IGF-R from Ind-Neo and Ind-R cells. Samples were autophosphorylated in the absence (−) or presence (+) of IGF-I (10^{-9} M), immunoprecipitated with antihuman type I IGF-R antipeptide antibody P2, and subjected to 6.5% SDS-PAGE under reducing conditions as described in *Materials and Methods*.

Scatchard analysis of data (Fig. 1B, *inset*) indicated that the maximal binding capacity is about 9 times as much in Ind-R cells (6 pmol/mg protein) as that in Ind-Neo cells (0.7 pmol/mg protein) and that the transfected human type I IGF-R exhibits an affinity for IGF-I of 3×10^{-10} M. This value is in the same range as the affinity for the type I IGF-R in Ind-Neo cells (Fig. 1B) as well as in Per and Ind cells (29). The affinity for IGF-II is 10^{-9} M (not shown).

Autophosphorylation experiments (Fig. 1C) showed that the β-subunits of the type I IGF-R are labeled in Ind-R and Ind-Neo cells. In both cell lines we observed a 106-kDa band corresponding to the atypical type I IGF-R previously described in Ind cells (30). Ind-R cells also exhibited a major band at 101 kDa, which represents the β-subunit of the transfected receptor. The two bands cannot be distinguished in Fig. 1C, but they are visible in experiments in which less protein is loaded (data not shown). The 101-kDa major band in Ind-R cells is labeled 10-fold more strongly than the 106-kDa band in Ind-Neo cells. This agrees with the 9-fold higher IGF-I binding level present in Ind-R cells. In the absence of IGF-I stimulation, the labeled β-subunit moieties show that the endogenous type I IGF-R as well as the transfected human type I IGF-R display a basal autophosphorylation activity. IGF-I (10^{-9} M) induces a 2-fold increase in the phosphorylation of the β-subunit moieties in both Ind-R and Ind-Neo receptor preparations. This indicates that the transfected human type I IGF-R and the endogenous type I IGF-R respond similarly to this ligand.

Taken together, these data demonstrate that Ind-R myoblasts express about 10 times as much type I IGF-R as control Ind-Neo myoblasts and that the transfected human type I IGF-R is fully functional.

Differentiation of Ind-R cells occurs without any IGF-I addition

Because C2 inducible cells exhibit an atypical $\alpha_2\beta_2$ IGF-R and require addition of IGFs to undergo terminal differentiation, we hypothesized that overexpression of the classical human type I IGF-R in these cells could abrogate their IGF requirement for differentiation. Therefore, we examined the kinetics of differentiation of Ind-R cells in the absence of added IGF-I, which is usually termed spontaneous differentiation. Ind-R and Ind-Neo myoblasts were grown for 3 days in proliferation medium (20% FCS), then switched to differentiation medium (0.5% FCS) and assayed for the appearance of myotubes at 24-h intervals for 4 days. Per cells, which differentiate spontaneously, were used as a positive control. We confirmed the validity of Ind-Neo cells as a control by also testing untransfected Ind cells in each experiment. Throughout this study, similar results were obtained with Ind-Neo and Ind cells.

To be certain that our results were not due to differences in cell number at the time of induction of differentiation, cell counts were performed on day 3 in proliferation medium. No difference in cell density in Ind-R and Ind-Neo cell cultures was observed.

Activation of muscle-specific genes is controlled by the MyoD family of transcription factors. Among these transcription factors, two were of interest in this study: MyoD (which is not expressed in Ind myoblasts, but is constitutive in Per myoblasts) and myogenin (as an early differentiation marker). Their expression was analyzed during the time course of spontaneous differentiation by RT-PCR and Northern blot for MyoD and myogenin, respectively (Fig. 2). At the time of differentiation medium switch, Ind-Neo and Ind-R cells did not express MyoD or myogenin. The two transcription factors appeared in Ind-R cells from 24 h onward, whereas they were not expressed in Ind-Neo cells even after 72 h in differentiation medium. As expected, MyoD was present at all times in Per cells, while myogenin appeared within 24 h in differentiation medium.

Myotube formation in Ind-Neo, Ind-R, and Per cell cultures was assessed by analysis of troponin T expression as a marker of biochemical differentiation (Fig. 3). This protein was detected in Ind-R and Per cells after 48 h in differentiation medium (Fig. 3A). In contrast, Ind-Neo cells failed to exhibit any positive staining for this protein during the entire differentiation time course. Hoechst staining showed that cell density was similar in the three cell lines and that the cells expressing troponin T were fused and multinucleated cells (Fig. 3A). Consistent with the expression of troponin T, a large number of fused and terminally differentiated muscle

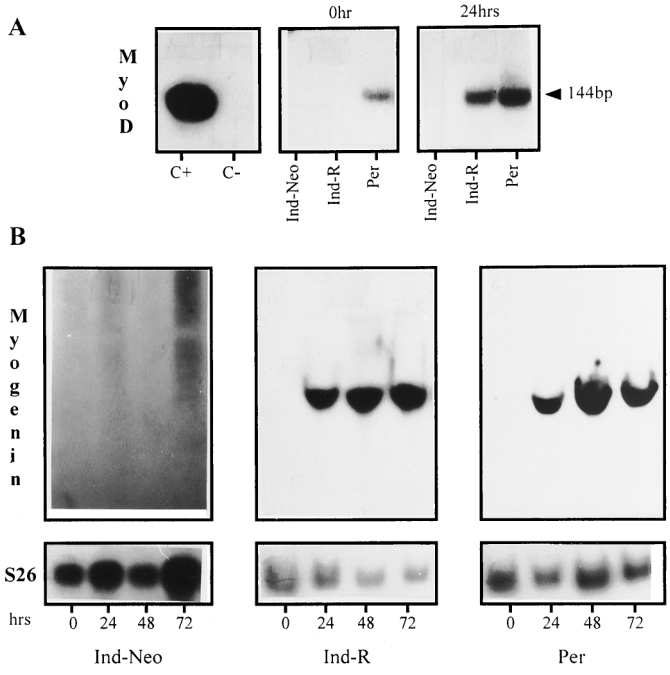

FIG. 2. Expression of myogenin and MyoD, in differentiating Ind-Neo, Ind-R, and Per cells. A, PCR amplification of MyoD cDNA. Total RNA of Ind-Neo, Ind-R, and Per cells 0 and 24 h after the switch to differentiation medium were extracted and subjected to RT-PCR as described in *Materials and Methods*. Amplified fragments were detected by hybridization with a MyoD-specific, random primed, ^{32}P-labeled probe. cDNA samples were replaced by water or the plasmid containing the MyoD cDNA as negative (C−) and positive (C+) controls, respectively. B, Northern blot analysis of myogenin expression during a time course of spontaneous differentiation of Ind-Neo, Ind-R, and Per cells. Twenty micrograms of total RNA (corresponding to 0, 24, 48, and 72 h after the switch to differentiation medium) were hybridized with a myogenin-specific, random primed, ^{32}P-labeled probe (54). The ribosomal S26 protein transcript was probed onto the same blot as a control for the amount of RNA loaded on the gel.

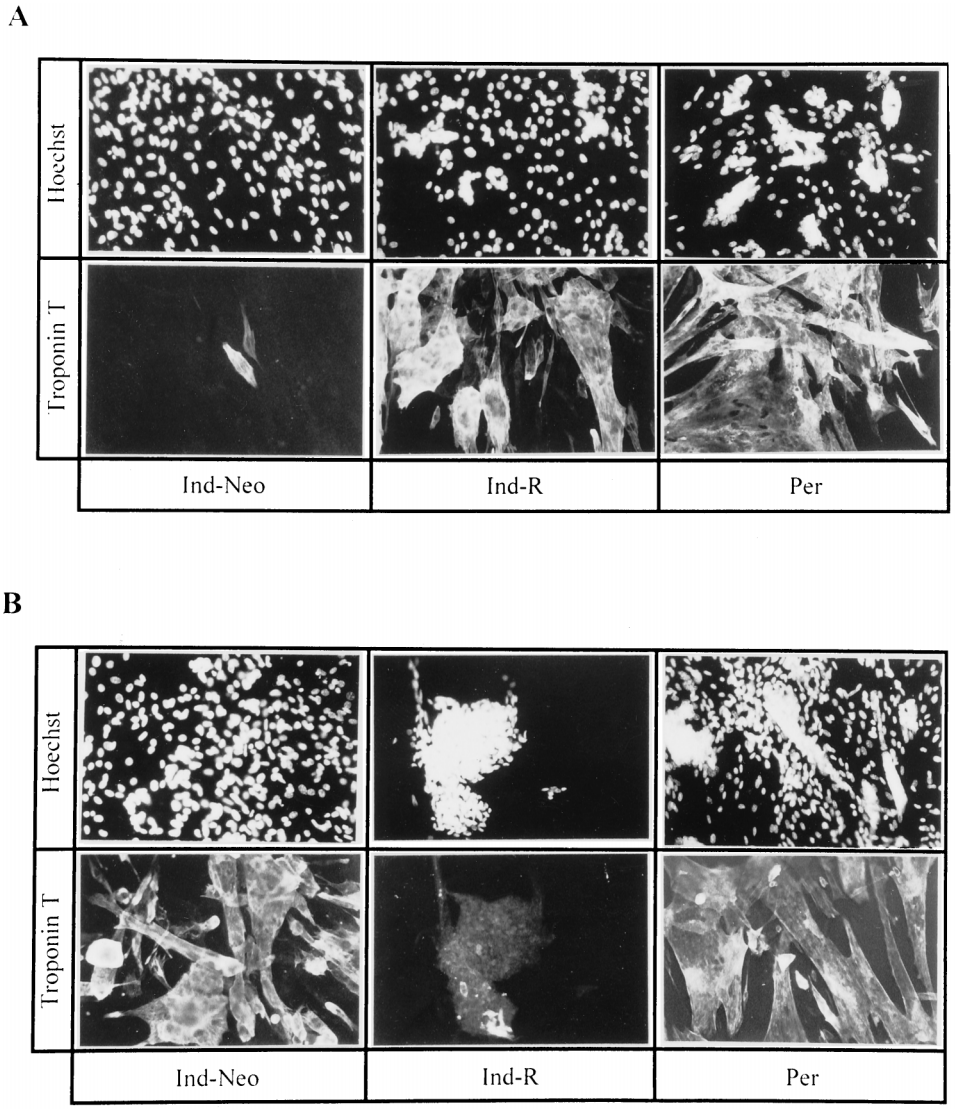

FIG. 3. Detection of troponin T in differentiating Ind-Neo, Ind-R, and Per cells. Cells were grown for 3 days in proliferation medium, then switched to low serum differentiation medium in the absence (A) or presence (B) of IGF-I (10^{-8} M). Immunofluorescence analysis of troponin T expression was carried out after 72 h in differentiation medium. Nuclei were stained with Hoechst dye B2883 to determine cell density. Results are typical of experiments performed four times. Micrographs are shown at ×200 original magnification.

cells appeared in Ind-R and Per cell cultures after 72 h in differentiation medium. In contrast, no myotubes were formed in Ind-Neo cell cultures (data not shown).

The appearance of effectors of the myogenic program and muscle-specific proteins, following the switch to differentiation medium lacking IGF-I, indicates that type I IGF-R overexpression permits the spontaneous differentiation of Ind-R cells. Therefore, the overexpressed type I IGF-R confers one of the phenotypic characteristics of permissive cells to the inducible cell line.

To further characterize the phenotype of Ind-R cells with regard to differentiation, cells were stimulated with 10^{-8} M IGF-I, a concentration inducing optimal differentiation of C2 cells (26). Under these conditions, instead of differentiating, Ind-R cells continued to proliferate and formed cellular aggregates that failed to express troponin T (Fig. 3B). Using lower concentrations of IGF-I (10^{-9} and 10^{-10} M), Ind-R cells differentiated normally.

These data suggest that type I IGF-R signals may induce differentiation or proliferation depending on the level of activation of the receptor.

Overexpression of type I IGF-R promotes a ligand-dependent transformation of Ind-R cells

Type I IGF-R overexpression has been shown to promote neoplastic transformation of fibroblasts (4). Likewise, Quinn *et al*. (37, 38) have shown that myoblasts overexpressing type I IGF-R exhibit some properties similar to those of transformed cells. Therefore, we studied the proliferation of Ind-R cells and evaluated the transformation potential of type I IGF-R overexpression in inducible C2 myoblasts.

In proliferation medium (20% FCS) supplemented or not with IGF-I, Ind-R and Ind-Neo cells grew at the same rate until they became confluent. This shows that the growth rate of Ind cells in proliferation medium is not affected by type I IGF-R overexpression (data not shown).

Next, we evaluated the effect of type I IGF-R overexpression on the three common characteristics of transformed cells, *i.e.* loss of contact inhibition, growth in the presence of low level of growth factors, and ability to grow and form colonies in soft agar.

Under proliferation conditions (20% FCS) and in the ab-

sence of IGF-I, Ind-R cells continued to proliferate after reaching confluence, and occasional areas of multilayered cells were observed (data not shown). In the presence of IGF-I (10^{-8} M), the loss of contact inhibition was further stimulated, and multilayering became uniform in the culture (Fig. 4A). This was not observed in Ind-Neo cells, which grew as a monolayer and stopped proliferating when they reached confluence in the presence or absence of IGF-I. In low serum differentiation medium supplemented with IGF-I (10^{-8} M), as described above, Ind-R cells failed to differentiate. In contrast, they proliferated and lost contact inhibition, forming large multilayered focal aggregates (Fig. 4B). This was not observed with Ind-Neo cells, which differentiated normally.

To estimate the proliferation of Ind-R cells in the presence of a low level of growth factors, Ind-R and Ind-Neo cells were plated and grown in medium containing only 0.5% FCS, with or without IGF-I (10^{-8} M). Under these conditions, Ind-R cells proliferated, and addition of IGF-I stimulated their growth rate 4- to 6-fold (Fig. 5). In contrast, Ind-Neo cells did not grow in low serum in the presence or absence of IGF-I.

Colony formation in soft agar was assessed to determine whether Ind-R cells acquire the property of anchorage-independent cell growth, which is characteristic of an advanced transformed phenotype (Fig. 6). Cells were plated in soft agar in the presence of 20% FCS, with (Fig. 6B) or without (Fig. 6A) IGF-I, and plates were examined 2 weeks later. Under these conditions, we were able to clearly distinguish small colonies containing less than 100 cells from large colonies of several hundred cells. Ind-R cells exhibited anchorage-independent growth that was strongly stimulated by IGF-I. Indeed, in the absence of IGF-I about 5% of seeded Ind-R cells formed colonies, but the proportion increased to 40% with IGF-I stimulation. Moreover, large size colonies predominated in the presence of IGF-I, whereas most colonies were small in its absence. In contrast, Ind-Neo cells failed to form colonies even in the presence of IGF-I.

Taken together, these data provide evidence that overexpression of type I IGF-R promotes an IGF-I-dependent transformed phenotype in Ind-R cells.

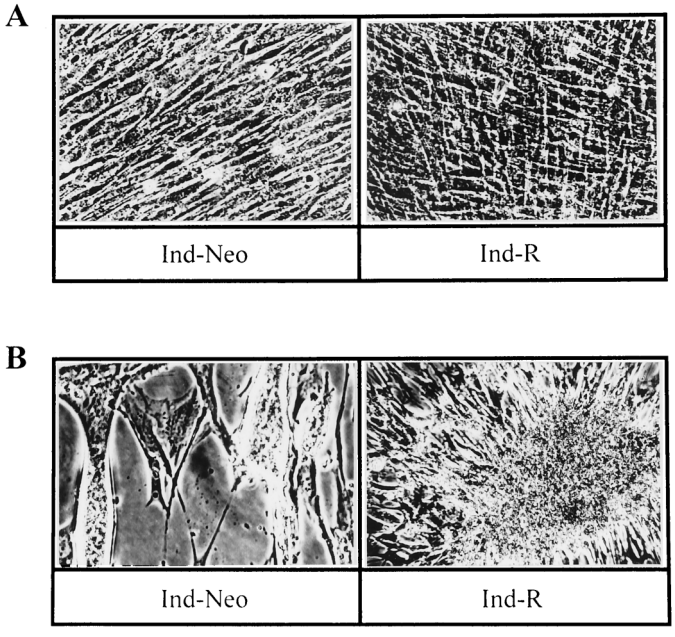

FIG. 4. Effects of IGF-I on Ind-R cells cellular morphology. A, Morphology of Ind-Neo and Ind-R cells under proliferation conditions. Cells were grown in proliferation medium (20% FCS) supplemented with IGF-I (10^{-8} M). Phase contrast photomicrographs were taken 2 days after confluence. B, Morphology of Ind-Neo and Ind-R cells in differentiation conditions. Cells were grown 3 days in proliferation medium, then switched to low serum (0.5% FCS) differentiation medium supplemented with IGF-I (10^{-8} M). Phase contrast photomicrographs were taken 3 days after the switch to differentiation medium. Results shown in A and B are typical of experiments performed four times. Micrographs are shown at ×250 original magnification.

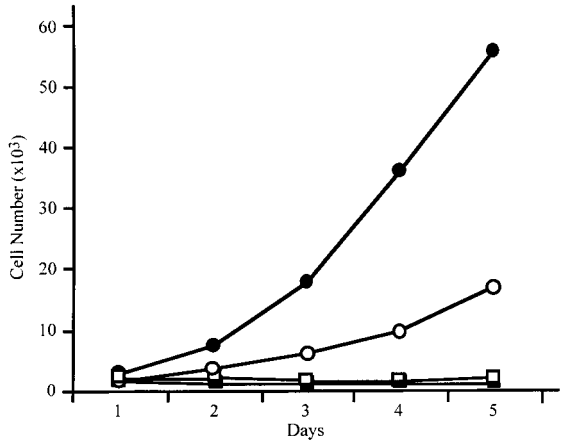

FIG. 5. Effects of IGF-I on Ind-Neo and Ind-R cells proliferation in 0.5% FCS. Ind-Neo (□ and ■) and Ind-R (○ and ●) cells were grown in low serum (0.5% FCS) medium supplemented (● and ■) or not (○ and □) with IGF-I (10^{-8} M). Counting was performed with a Coulter counter (Coulter Electronics, Hialeah, FL). Data represent the mean number of cells from three separate wells.

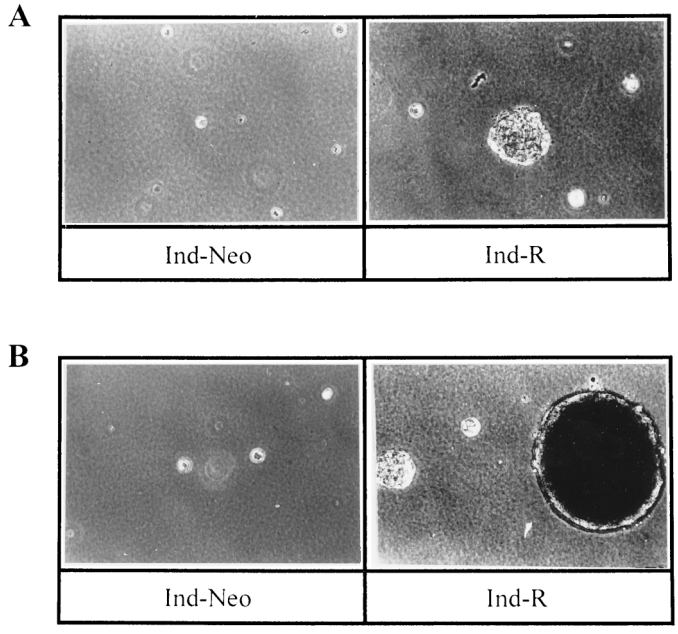

FIG. 6. Colony formation in soft agar of Ind-R cells. Cells were plated in proliferation medium containing 0.2% agar as described in *Materials and Methods*. A, Without IGF-I. B, With IGF-I (10^{-8} M). Photographs of typical colonies in soft agar cultures were taken 14 days after plating. Results are typical of experiments performed three times. Micrographs are shown at ×250 original magnification.

Antibody αIR3 inhibits the effects of human type I IGF-R overexpression

To make sure that the phenotypic characteristics of Ind-R cells are due to the forced expression of human type I IGF-R, we incubated the cells with αIR3, a monoclonal antibody specific for the human type I IGF-R. This antibody recognizes the α-subunit of human type I IGF-R, but not the murine type, prevents IGF-I binding, and thus blocks IGF-I-mediated receptor activation (39, 40). The effects of αIR3 were studied in differentiation and transformation assays (Fig. 7).

Under differentiation conditions, αIR3 strongly inhibited spontaneous formation of myotubes in Ind-R cell cultures (Fig. 7A, *upper right*). Furthermore, the addition of αIR3 abolished the IGF-I-induced Ind-R cell transformation. Indeed, Ind-R cells differentiated in differentiation medium containing IGF-I plus αIR3 (Fig. 7A, *lower right*), whereas in the absence of αIR3, they proliferated and lost contact inhibition (Fig. 7A, *lower left*). Colony formation of Ind-R cells in soft agar was nearly completely inhibited by αIR3 in both the presence and absence of IGF-I (Fig. 7B). Control Ind-Neo cells were not affected by the presence of antibody αIR3, which did not bind to murine type I IGF-R.

Thus, antibody αIR3 strongly inhibits the phenotypic changes observed in Ind-R cells, indicating that these modifications are mediated through the transfected human type I IGF-R.

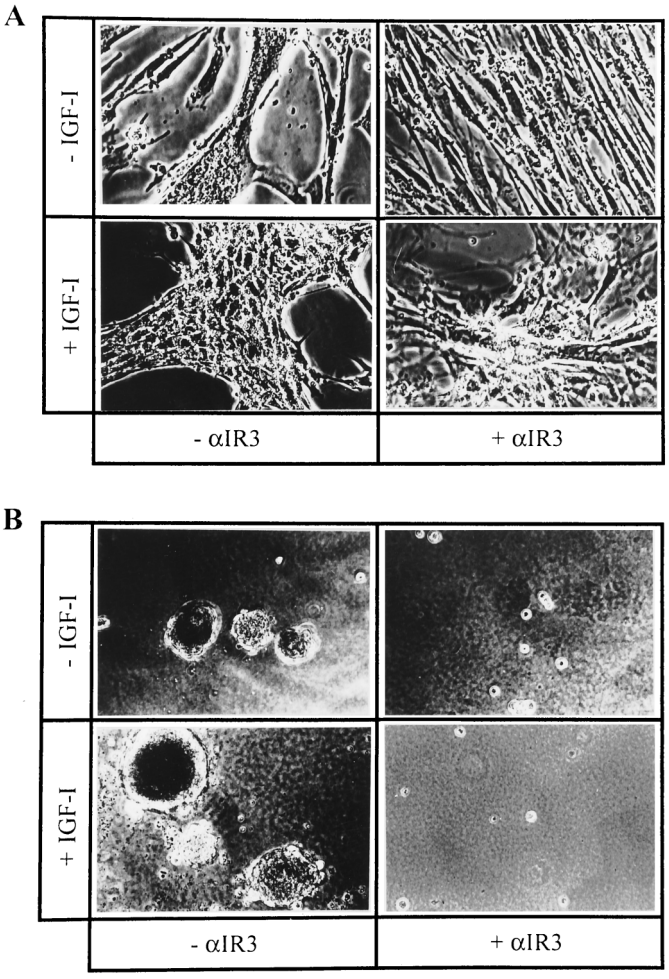

FIG. 7. Effects of the antibody αIR3 on differentiation and transformation of Ind-R cells. A, Effects on cell differentiation. Cells were grown for 3 days in proliferation medium, then switched to low serum differentiation medium in the absence or presence of IGF-I (10^{-8} M). The antibody αIR3 was added to both proliferation and differentiation medium at 1 μg/ml. Photographs were taken after 3 days in differentiation medium. B, Effects on cell transformation. Cells were plated in proliferation medium containing 0.2% agar supplemented or not with IGF-I (10^{-8} M) in the presence or absence of 1 μg/ml αIR3. Photographs were taken 14 days after plating. Micrographs are shown at ×250 original magnification.

Discussion

It is well established that IGFs play a major role in skeletal muscle development through the control of both proliferation and differentiation of myoblasts. These effects have been shown to be mediated through the type I IGF-R *in vivo* and *in vitro* (21). It has recently been suggested that the type I IGF-R may trigger differentiation and proliferation, which are mutually exclusive cellular programs, by selective discrimination of their related signaling pathways (41). To gain further insights into the role of type I IGF-R, we investigated the effects of human type I IGF-R overexpression on differentiation and proliferation of C2 inducible myoblasts. Our results show that 1) forced expression of the type I IGF-R in Ind-R cells bypasses their IGF requirement for differentiation; and 2) overexpression of type I IGF-R promotes a ligand-dependent transformed phenotype in Ind-R cells.

The type I IGF-R overexpression level in Ind-R cells indicates that the thymidine kinase promotor of the pNTK expression vector is active in the C2 myogenic cell line and provides a transcription rate similar to that observed with other promoters used in different muscle cell lines (38, 42). Binding experiments and autophosphorylation assays indicate that the transfected human type I IGF-R is fully functional in Ind-R cells and exhibits affinities for IGF-I and IGF-II that agree with values usually reported for the type I IGF-R (43). We have previously demonstrated that inducible cells exhibit an atypical $\alpha_2\beta_2$ type I IGF-R that differs from the classical type I IGF-R by its higher affinity for IGF-II compared with IGF-I and the higher mol wt of its β-subunits (29, 30). This atypical type I IGF-R may result from specific posttranslational processing of the type I IGF-R in inducible cells. Therefore, we looked at the molecular size of the β-subunit of the transfected receptor in Ind-R cells. Ind-Neo and Ind-R cells exhibit a 106-kDa β-subunit corresponding to the endogenous atypical type I IGF-R previously found in inducible cells (30). In addition, Ind-R cells exhibit a 101-kDa band that represents the β-subunit of the transfected receptor. This 101-kDa moiety may correspond to one of the type I IGF-R β-subunits previously described in C2 permissive cells (30). Taken together, these results and the higher affinity of the transfected receptor for IGF-I than for IGF-II indicate that the human type I IGF-R is processed as a classical type I IGF-R in murine C2 inducible cells. Further transfection experiments using the murine type I IGF-R cDNA will be performed to definitively determine whether posttranslational processing is responsible for the properties of the atypical type I IGF-R in these cells. However, Ind-R cells overexpressing a fully functional human type I IGF-R constitute a

suitable tool to study the role of the type I IGF-R on the control of differentiation and proliferation of muscle cells.

Forced expression of the classical human type I IGF-R allows C2 inducible myoblasts to differentiate spontaneously as do parental C2 permissive myoblasts. The inhibitory effect of the monoclonal antibody αIR3 confirms that spontaneous differentiation is mediated through the transfected human type I IGF-R and that activation of this receptor by IGF-I binding is necessary for induction of cell differentiation. Quinn *et al*. (38) previously observed that differentiation of C2 cells overexpressing human type I IGF-R was faster than that in control cells. The present study provides evidence that the type I IGF-R is more than a modulator and is a major effector of myoblast differentiation. However, Ind-R myoblasts do not differentiate until they are switched to low serum differentiation medium. This suggests that overexpression of type I IGF-R is not sufficient to abrogate the effects of some serum growth factors inhibiting myoblast differentiation (2).

The spontaneous differentiation of Ind-R cells may be closely related to the level of type I IGF-R activation. Indeed, the IGFs present in the medium bind to the overexpressed human type I IGF-R and thus contribute to its high endogenous kinase activity. Consequently, the signaling provided by the human type I IGF-R in response to endogenously produced IGFs may be sufficient to promote cell differentiation in the absence of exogenous IGF when Ind-R cells are switched to low serum differentiation medium. In addition, the molecular form of the transfected IGF-R may be also involved in supporting spontaneous differentiation of myoblasts. Indeed, overexpression of the human type I IGF-R introduces into inducible myoblasts a classical type I IGF-R, whereas these cells endogenously express an atypical one (29, 30). Thus, to accurately determine the relative importance of the molecular form and the number of type I IGF-R for inducible myoblast differentiation, the classical murine type I IGF-R should be transfected into inducible cells in which the gene of the endogenous type I IGF-R has been disrupted.

Although Ind-R myoblasts undergo spontaneous differentiation, they do not exhibit all the characteristics of the permissive phenotype. In particular, MyoD mRNA is not detectable at the myoblast stage in Ind-R cells. Moreover, Ind-R myoblasts produce 200 and 10 times less IGF-I and IGF-II than Per myoblasts, respectively (not shown). The same difference in IGF production has been observed between Ind and Per myoblasts (27). Thus, overexpression of the human type I IGF-R is not sufficient to induce MyoD expression and increase the secretion of IGFs. This suggests that the expression of the MyoD gene and the production of IGFs are not regulated solely by the type I IGF-R (24), but require other pathways that are active in permissive, but not in inducible or Ind-R, myoblasts.

We observed that Ind-R cell differentiation is stimulated by IGF-I concentrations up to 10^{-9} M, whereas it is inhibited by higher concentrations. This is in agreement with previous observations reporting that low concentrations of IGF-I stimulate whereas higher concentrations cause progressive inhibition of myoblast differentiation (2). However, compared with Per and Ind-Neo cells, Ind-R cells display an increased sensitivity to the ligand. Indeed, at the same dose of IGF-I (10^{-8} M), differentiation is inhibited in Ind-R cells, whereas it is stimulated in Per and Ind-Neo cells. This increased sensitivity to the ligand in Ind-R cells is probably due to amplified signaling through much more abundant type I IGF-R. Indeed, we find that Ind-R cells express about 10 times as much type I IGF-R as Ind-Neo cells. The number of type I IGF-R in Ind-R cells is also 10 times higher than that reported in Per cells (29).

Previous studies have shown that overexpression of tyrosine kinase receptors such as the epidermal growth factor receptor, insulin receptor, and type I IGF-R induces a ligand-dependent transformed phenotype in fibroblasts (4, 44, 45). The involvement of the type I IGF-R in the expression of the transformed phenotype was clearly demonstrated by Sell *et al.* (46) using a fibroblast cell line generated from mouse embryos homozygous for a targeted disruption of the *Igf1r* gene. These findings led us to evaluate the transformation potential of type I IGF-R overexpression in C2 inducible myoblasts. We found that in the presence of IGF-I (10^{-8} M), Ind-R cells exhibit the characteristics of a cellular transformed phenotype, *i.e.* growth in 0.5% FCS medium, loss of contact inhibition, and anchorage-independent growth. We also observed that IGF-I is 100-fold more potent than insulin in promoting colony formation in soft agar, a ratio that parallels the difference in affinities of the two ligands for the type I IGF-R. This and the fact that αIR3 antibody blocks colony formation indicate clearly that IGF-I-induced transformation is mediated through the overexpressed human type I IGF-R. Thus, overexpression causes the type I IGF-R to function as a ligand-dependent transforming protein in C2 inducible myoblasts. Furthermore, we observed that in the absence of type I IGF-R overexpression, high levels of IGFs do not promote transformation of inducible or permissive cells. This suggests that a sufficient type I IGF-R density is required to invoke transformation-related signaling pathways. Thus, in the present Ind-R cells as well as in murine fibroblasts (47), the rate-limiting feature for transformation seems to be the number of type I IGF-Rs and the associated level of kinase activation. It is of interest that Ind-R cells lose contact inhibition and form colonies in soft agar in the presence of 20% FCS and IGF-I (10^{-8} M), although they exhibit no change in their rate of growth. This result further suggests that the control of cellular proliferation and transformation may involve distinct signaling pathways (46, 48).

We cannot positively exclude that the human receptor overexpressed in murine cells behaves differently from the murine receptor. Therefore, the effects observed in transfected cells could be due to the specific properties of the human type I IGF-R rather than to its overexpression *per se*. However, our conclusions agree with previous statements obtained in several cell types and species on the role of the type I IGF-R in cellular differentiation, proliferation, and transformation (4, 43, 49–51). In addition, we found that the two C-terminus regions of the human type I IGF-R that were proven to be specifically implicated in its transforming activity (*i.e.* Y1251 and the residues around H1293) (52) are identical in the human and murine receptors (personal data). Therefore, we can reasonably consider that the effects observed in transfected cells are not artifacts due to species

specificity, but are actually due to overexpression of the type I IGF-R.

We conclude from this study that 1) the type I IGF-R is strongly involved in the phenotypic differences between inducible and permissive C2 cells with respect to the differentiation program; and 2) the type I IGF-R can mediate signals for both differentiation and transformation of muscle cells. It seems that discrimination among IGF-I-induced differentiation, proliferation, or transformation is dependent upon IGF-R number and the corresponding level of kinase activation. Discrimination among these cellular programs may also be related to the molecular form of the type I IGF-R. Thus, these quantitative and qualitative characteristics may direct the interaction of the type I IGF-R with different intracellular substrates to trigger specific pathways for cellular differentiation, proliferation, or transformation. Further studies with the Ind-R cell line may help to distinguish the different signal transduction pathways activated in response to type I IGF-R stimulation in muscle cells.

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