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Overexpression of the Human Insulinlike Growth Factor I Receptor Promotes Ligand-Dependent Neoplastic Transformation

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The human insulinlike growth factor I receptor was overexpressed in NIH 3T3 cells as well as human and rat primary fibroblast strains. The NIH 3T3 cells displayed a ligand-dependent, highly transformed phenotype. When exposed to insulinlike growth factor I or supraphysiologic levels of insulin, NIH 3T3 cells that expressed high levels of receptors formed aggregates in tissue culture dishes, colonies in soft agar, and tumors in nude mice. Expression of 1 million receptors per cell, a 40-fold increase above the base-line level, was required for anchorage-independent growth. Primary fibroblasts that expressed high levels of receptors displayed a ligand-dependent change in morphology and an increase in saturation density but did not acquire a fully transformed phenotype. The results demonstrate that when amplified, this ubiquitous growth factor receptor behaves like an oncogenic protein and is capable of promoting neoplastic growth in vivo.

The orderly processes of cellular proliferation and differentiation are in part regulated by diffusible, extracellular growth factors. Their effects are mediated by specific receptors, many of which are membrane proteins with tyrosine kinase activity. Included in this category are the receptors for insulin, insulinlike growth factor I (IGF-I), platelet-derived growth factor (PDGF), colony-stimulating factor 1 (CSF-1), and epidermal growth factor (EGF) (reviewed in reference 15).

In some cases, alterations in growth factor or receptor activity can result in uncontrolled growth and neoplastic transformation. Therefore, it is not surprising that several viral oncogenes have been found to be homologous to growth factors or their receptors. There are several means by which uncontrolled growth may be triggered. Expression of a growth factor or homologous polypeptide in a cell that already expresses the receptor can result in autocrine stimulation. Such is the case with cells transformed by the PDGF homolog, v-*sis* (4, 10, 17, 21, 47). Alterations in receptor structure can lead to constitutive activation. This is seen with both the v-*erbB* oncogene, a modified, truncated version of the EGF receptor (11, 26, 43, 48), and the v-*fms* oncogene, a modified, truncated version of the CSF-1 receptor (8, 40). It has recently become clear that normal receptors, when overexpressed, can cause growth factor-dependent cellular transformation of NIH 3T3 cells. This has been shown for both the EGF (9, 45) and CSF-1 (38) receptors.

We were interested in determining whether the normal IGF-I receptor (IGF-IR) could also promote uncontrolled cell growth and neoplastic transformation. The role of IGF-I and its receptor in promoting tumor growth in vivo is unclear. Many tumors have been found to secrete IGF-I (18, 20, 27, 28, 32, 33), but whether the growth factor participates in tumorigenesis remains speculative. The addition of IGF-I to cultured cells does not result in transformation. Furthermore, when compared with other growth factors such as PDGF, IGF-I is found to be a relatively weak mitogen (reviewed in reference 14). As yet, no oncogene has been found to be closely homologous to IGF-I or its receptor.

Yet as a mitogen, IGF-I has widespread physiologic importance. In whole animals, deficiencies lead to small stature and excesses lead to gigantism (reviewed in references 14 and 35). In both cases, tissue distribution and body proportions remain normal. It seems likely that all cells respond, either directly or indirectly, to IGF-I stimulation. In vitro data also point to widespread cellular responsiveness. In defined medium, nearly all cells require insulin for growth (reviewed in references 1, 2, 42). However, the levels required are frequently supraphysiologic, suggesting that some of the insulin effects may be mediated through low-affinity cross-reactions with the IGF-IR. This has been substantiated for fibroblasts (13; reviewed in reference 42).

Our experimental design involved expressing high levels of the human IGF-IR in NIH 3T3 cells as well as human and rat primary fibroblast strains. The immortalized 3T3 cells acquired a highly transformed phenotype in response to added IGF-I or insulin. The primary cells grew to high saturation densities but failed to fully transform. The data demonstrate that enhanced IGF-IR activity can participate in neoplastic transformation in vivo.

MATERIALS AND METHODS

Cell culture and vector preparation. All cells were routinely cultured in Dulbecco modified Eagle medium (DME) supplemented with 10% serum (GIBCO Laboratories). The NIH 3T3 cells and their derivatives were usually grown in calf serum, whereas the PA317 (30) cells, their derivatives, and the primary human and rat fibroblasts were grown in fetal bovine serum (FBS). Sera were not routinely heat inactivated except for use in soft agar assays. For plating cells on a collagen substrate, 1 ml of Vitrogen (3.3 mg/ml; Collagen Corp.) was mixed with 1 ml of 2× medium in a 6-cm-diameter dish. After a 30-min period to allow the matrix to gel, cells were added in 4 ml of medium.

The cDNA encoding the IGF-IR (41) was 4.4 kilobase pairs in length, extending from an *Eco*RI site approximately 300 base pairs upstream from the start codon to a *Bam*HI site 6 base pairs downstream from the stop codon. It was ligated into the Moloney murine leukemia virus-based retroviral vector pLXSN (30a) at the polylinker site X. This polylinker

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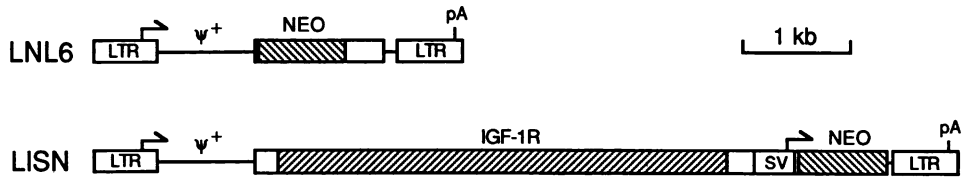

FIG. 1. Schematic representation of the two retroviral vectors. Abbreviations: LTR, long terminal repeat; ψ^+, extended retroviral packaging signal; NEO, neomycin phosphotransferase gene; IGF-IR, cDNA sequence encoding the IGF-IR; SV, simian virus 40 promoter; pA, polyadenylation site; kb, kilobase pairs. Hatched areas indicate protein coding regions.

includes *Eco*RI and *Bam*HI sites oriented 5′ to 3′. pLXSN has the extended packaging signal (ψ^+) for high virus titer as well as a mutated *gag* start codon and shortened envelope region to decrease the risk of helper virus generation. The resultant IGF-IR expression vector was termed pLISN (Fig. 1).

Virus-producing cells were prepared by a transient rescue procedure (31). pLISN was transfected into the ecotropic packaging line ψ2. Two days later, virus-containing culture supernatant was used to infect PA317 amphotropic packaging cells. One day after infection, the PA317 cells were split into medium containing G418 (0.5 mg/ml [active compound]). Producer clones, termed PA317/LISN, were collected and assayed for virus production. Genomic DNA was assayed by Southern blotting to ensure the integrity of the retroviral vector sequences. The generation of cells producing the control virus for this study, PA317/LNL6, has been described previously (3).

Virus-containing supernatants from PA317/LISN c4 and PA317/LNL6 c8 were used to infect NIH 3T3 cells and human and rat primary fibroblasts. A 100-μl sample of filtered (0.45-μm-pore-size filter) supernatant was added to 10^6 cells in the presence of Polybrene (4 μg/ml). The next day, the cells were split 1:10 into medium containing G418 (0.5 mg/ml [active compound]). This concentration was uniformly fatal to uninfected cells. The plates were grown to confluence to produce the nonclonal populations used in this study.

There was no evidence of ecotropic or amphotropic helper virus contamination as determined by a sensitive marker rescue assay. The murine and human cell populations were repeatedly passaged to allow any replication-competent retrovirus to spread through the population and rescue the LISN or LNL6 vector. After 2 months, 1-ml samples of culture supernatant were assayed for the ability to transfer G418 resistance to NIH 3T3 cells. No resistant 3T3 cells were obtained, ruling out helper virus contamination.

Soft agar assays. Cell suspensions were plated in semisolid medium containing 0.3% Bacto-Agar (Difco Laboratories), DME, 10% heat-inactivated (56°C for 30 min) FBS, penicillin, and streptomycin (GIBCO). Insulin (Sigma Chemical Co.), IGF-I (Boehringer Mannheim Biochemicals), and EGF (Collaborative Research, Inc.) were added at the indicated concentrations. The monoclonal antibody αIR3 (23) was kindly provided by Steven Jacobs (Burroughs Wellcome Co.). When used to inhibit colony formation, it was included at a final dilution of 1:400. The dishes were incubated at 37°C in a humidified 10% CO_2 atmosphere. Colonies were scored at 14 to 18 days.

Immunofluorescence and flow cytometry. Cells were harvested from confluent dishes in phosphate-buffered saline with 1 mM EDTA. All subsequent steps were done in phosphate-buffered saline with 0.1% bovine serum albumin at 0 to 4°C. The cells were washed twice and incubated for 30 min with αIR3 (1:250 dilution). They were again washed twice and incubated for 30 min with fluorescein isothiocyanate-conjugated goat $F(ab')_2$ anti-mouse immunoglobulin G (Tago). After two final washes, the cells were resuspended to approximately 10^6 cells per ml and analyzed with a Coulter flow cytometer. Histograms were plotted with fluorescence intensity on the abscissa and cell number on the ordinate. The fluorescence scale was a 256-channel/4-decade log scale with 64 channels per decade. To compare fluorescence with receptor number, this scale was linearized with the following transformation: relative brightness = $10^{(x/64)}$, where x represents the mean fluorescence of a cell population. Relative brightness was linearly correlated with receptor number as determined by IGF-I binding (see below).

IGF-I binding assay. The IGF-I binding assay has been described previously (41). Confluent 24-well plates of cells were exposed to radioiodinated IGF-I (100 pM; 2 μCi/pmol; Amersham Corp.) along with various concentrations of cold IGF-I (0 to 100 nM). The plates were incubated for 4 h at 4°C, rapidly washed, solubilized, and counted. The number of counts bound to the control cells at 100 nM IGF-I was taken as nonspecific binding and was subtracted from each datum point. The radiolabeled tracer had a threonine substitution at position 59. This increases the specificity but does not change the affinity for the IGF-IR (37).

Tumor formation in nude mice. 3T3/LISN clone 4 and 3T3/LNL6 cells were tested for tumorigenesis. Four-week-old male BALB/c nude mice (Simonsen) were injected subcutaneously over the right flank. For each of the two cell types, three mice were injected with 10^7 cells and three were injected with 10^5 cells. Mice bearing tumors were sacrificed when the tumor burden was approximately 0.5 g.

RESULTS

Transfer and expression of the IGF-IR. The IGF-IR is synthesized as a single precursor, with a molecular mass of 180 kilodaltons that is glycosylated and cleaved into α and β subunits. These are processed to form the heterotetramer structure $\alpha_2\beta_2$ (19). The α subunits contain the ligand-binding site, and the β subunits contain the transmembrane and tyrosine kinase domains. cDNA clones encoding the IGF-IR have been isolated from human placenta by Ullrich et al. (44) and Steele-Perkins et al. (41). Expression of the latter clone in Chinese hamster ovary cells confirmed that the protein product was processed properly. The receptor exhibited high-affinity binding of IGF-I as well as tyrosine kinase activity.

This 4.4-kb cDNA clone was inserted into a Moloney murine leukemia virus-based retroviral vector, pLXSN (30a), to form the IGF-IR expression vector pLISN (Fig. 1). The receptor cDNA is transcribed from the retroviral long terminal repeat. The neomycin phosphotransferase gene, which confers G418 resistance, is transcribed from an inter-

TABLE 1. Colony formation in soft agar[a]

Cell type	Growth factor, concn (ng/ml)	No. of colonies[b] (plating efficiency)[c]
3T3/LNL6	None	0 (0.0)
	IGF-I, 80	0 (0.0)
	Insulin, 5,000	0 (0.0)
3T3/LISN	None	30 (0.6)
	EGF, 25	41 (0.8)
	IGF-I, 4	32 (0.6)
	IGF-I, 20	138 (2.8)
	IGF-I, 50	163 (3.3)
	IGF-I, 100	123 (2.5)
	Insulin, 8	32 (0.6)
	Insulin, 40	30 (0.6)
	Insulin, 200	28 (0.6)
	Insulin, 1,000	180 (3.6)
	Insulin, 5,000	190 (3.8)
	αIR3 without growth factor	4 (0.1)
	αIR3 + IGF-I, 100	3 (0.1)
	αIR3 + insulin, 5,000	28 (0.6)

[a] A total of 5,000 cells were plated in 0.3% agar with 10% FBS. Growth factors were added at the indicated concentrations. When αIR3 was incorporated into the agar, a 1:400 dilution of ascitic fluid was used. Plates were scored at 14 days. Only colonies with at least 50 cells were counted.

[b] Mean of duplicate wells, which always agreed to within 10%.

[c] Percentage of plated cells that formed colonies.

nal simian virus 40 promoter. Amphotropic retrovirus was prepared in PA317 retrovirus packaging cells (30). The producer clone chosen, PA317/LISN c4, produced virus having a titer of 2×10^6 CFU/ml. The integrated viral sequences were unrearranged, and the retroviral preparations were devoid of helper virus (see Materials and Methods for details).

A second vector, LNL6 (3), was used for control infections (Fig. 1). It expresses only the neomycin phosphotransferase gene. The amphotropic producer clone used produced the vector at a titer of 10^7 CFU/ml and was helper free.

We initially sought to evaluate the transformation potential of IGF-IR overexpression in an immortalized cell line, NIH 3T3. These murine cells grow in a monolayer, are contact inhibited, and fail to grow in an anchorage-independent fashion in soft agar. They were infected with LISN and LNL6 viral supernatants and selected in G418. The resultant nonclonal populations were termed 3T3/LISN and 3T3/LNL6, respectively. It is expected that every cell possesses integrated viral DNA sequences but that transcription rates from these sequences may be highly variable from cell to cell.

IGF-IR overexpression promotes anchorage-independent growth. The cells were plated in soft agar with heat-inactivated FBS (10%) with or without added growth factors. Plates were examined 2 weeks later. Single cells, small colonies of ≈50 cells, and large colonies of several hundred cells were readily distinguishable. The results of a typical experiment are shown in Table 1. The 3T3/LNL6 cells failed to form colonies both in the absence of supplemental growth factors and in the presence of IGF-I or a supraphysiologic level of insulin. 3T3/LISN cells did form colonies of variable size in the absence of supplemental growth factors. In several experiments, this background number ranged from 8 to 35 colonies per 5×10^3 cells. Supplementation with either IGF-I or pharmacologic levels of insulin resulted in a five- to sixfold increase in colony number. IGF-I was approximately 50-fold more potent than insulin (Table 1). This ratio is consistent with the 50- to 100-fold difference in affinities of the two ligands for the IGF-IR (41, 49). EGF failed to significantly enhance colony formation (Table 1). The concentration chosen has previously been shown to promote colony formation in NIH 3T3 cells that overexpress the EGF receptor (9, 45).

To further demonstrate that colony formation was mediated through the IGF-IR, cells were plated in the presence of αIR3, a monoclonal antibody specific for the human IGF-IR (23). This antibody has been shown to attach at the ligand-binding site, prevent IGF-I binding, and block IGF-I-mediated receptor activation (6, 13, 36). The antibody can directly stimulate the receptor (41), but it did not do so in our hands at the concentration chosen. αIR3 nearly completely inhibited colony formation both in the absence of growth factors and in the presence of IGF-I (Table 1). αIR3 also blocked the insulin stimulation of colony formation, but not as completely.

These results demonstrate that expression of the human IGF-IR in NIH 3T3 cells can result in anchorage-independent growth. This effect is ligand dependent and is mediated through the IGF-IR. It should be stressed, however, that although every cell contained the LISN construct, only 3% could be induced to form colonies in soft agar.

IGF-IR overexpression promotes multilayered growth on a solid substrate. One feature of the transformed phenotype is the ability of cells to grow in three dimensions in a tissue culture dish. That is, growth no longer remains restricted to a monolayer. We tested our two cell lines for such unrestricted growth.

As expected, the 3T3/LNL6 cells exhibited a completely normal phenotype. The addition of supraphysiologic levels of insulin had no effect on their morphology. In contrast, the 3T3/LISN cells rearranged into aggregates (Fig. 2). The aggregates formed in 10% FBS without supplemental insulin but were much more numerous in the presence of this growth factor. First, the cells grew to confluence. Then chains of elongated cells, connected end to end, appeared to move toward central points, forming small papillae. Finally, thick cords of cells moved into these papillae to form large aggregates. Many cells failed to enter the aggregates and remained behind in the monolayer. It is noteworthy that aggregate formation occurred only on tissue culture plastic. When 3T3/LISN cells were plated on a collagen substrate, they piled up into a multilayered structure but did not rearrange into aggregates.

Furthermore, the morphology was also dependent on the type of serum used. The NIH 3T3 cells grew as well in 10% calf serum as in 10% FBS. When 3T3/LISN cells were grown in 10% calf serum, no aggregates formed. In the absence of supplemental insulin, the cells behaved exactly like the original NIH 3T3 cells. They exhibited contact inhibition and grew in a monolayer. When 5 μg of insulin per ml was added, the density of the monolayer increased, and round, loosely adherent cells began to pile up on it (Fig. 2). There was no evidence of aggregate formation. It is not known which serum components are necessary for aggregate formation.

Expression of the human IGF-IR in NIH 3T3 cells also resulted in obvious alterations in cellular metabolic activity. When insulin was added to a confluent or even subconfluent dish of 3T3/LISN cells, the tissue culture medium was rapidly acidified. The phenol red indicator dye turned bright yellow every 12 to 24 hours, mandating frequent medium changes. This effect was seen in both FBS and calf serum. It was never seen with the 3T3/LNL6 control cells, which, even when densely confluent, acidified medium slowly over several days.

Markedly elevated receptor levels are necessary for anchor-

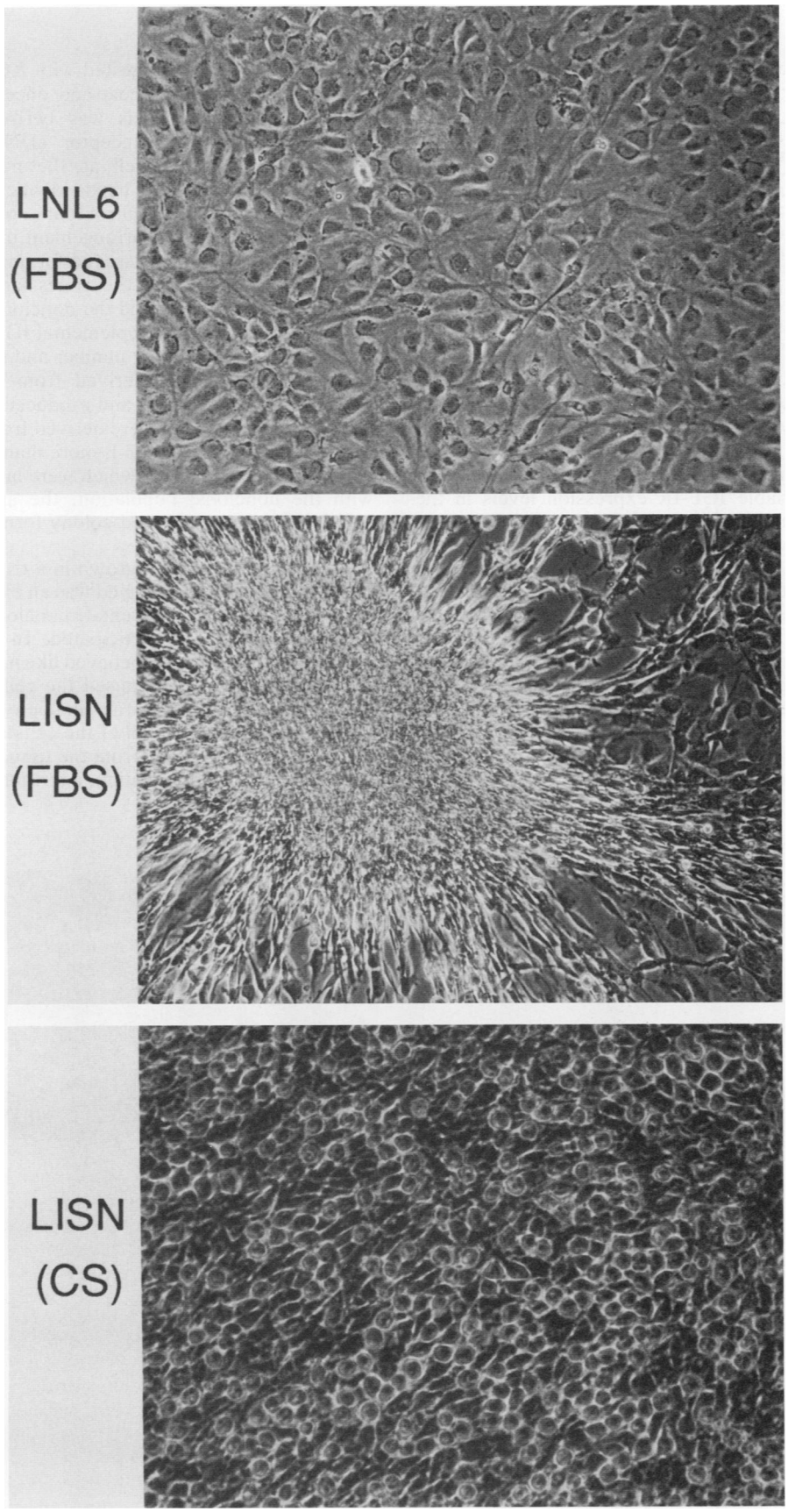

FIG. 2. Cellular morphology in tissue culture dishes. 3T3 cells were plated on tissue culture plastic in medium supplemented with insulin (5 μg/ml) and either 10% FBS or 10% calf serum (CS). Pictures were taken 2 days after the cells became confluent. 3T3/LNL6 cells always remained in a monolayer. 3T3/LISN cells formed aggregates in FBS but simply piled up in calf serum.

TABLE 2. Colony formation by 3T3/LISN clones in soft agar[a]

Clone	No. of colonies[b] (plating efficiency)[c]		
	No growth factor	IGF-I, 50 ng/ml	αIR3
2	71 (14)	255 (51)	5 (1)
4	240 (48)	411 (82)	52 (10)
5	54 (11)	173 (35)	1 (0)
8	138 (28)	337 (67)	4 (1)

[a] A total of 500 cells were plated in soft agar. IGF-I or αIR3 was added as indicated.
[b] Mean of duplicate wells.
[c] Percentage of plated cells that formed colonies.

age-independent growth. As noted above, not every LISN-infected cell displayed a transformed phenotype. Only a small fraction of the population formed colonies in soft agar or aggregates in submersion culture. We hypothesized that this was due to variable IGF-IR expression levels in the nonclonal population. Accordingly, only those cells expressing very high levels of the receptor would participate in the phenotypic changes. An alternate hypothesis is that viral gene transfer resulted in a low-frequency rearrangement that potentiated the activity of the receptor. These hypotheses were tested by cloning colonies from soft agar and assessing the clones for (i) gross vector rearrangement, (ii) plating efficiency in soft agar, and (iii) receptor number. 3T3/LISN cells were plated in soft agar with supplemental IGF-I, and individual colonies were harvested. Two small colonies (clones 2 and 5) and two large colonies (clones 4 and 8) were expanded and analyzed.

Genomic DNA was digested with *Kpn*I, which cuts once in each long terminal repeat and once within the receptor cDNA. Southern analysis was performed with a probe complementary to the receptor cDNA. All four of the 3T3/LISN clones, as well as the nonclonal population, yielded the two expected bands identical to those produced by the pLISN plasmid control (data not shown). There was no evidence of vector rearrangement in any clone.

The clones were then assayed for anchorage-independent growth in soft agar. All of the clones had substantially higher plating efficiencies than did the nonclonal population (Table 2). Each responded to supplemental IGF-I (50 ng/ml) with a further increase in colony number and size. Clones 4 and 8, which were originally derived from large colonies, had higher plating efficiencies and produced larger colonies than did the two clones that were derived from small colonies. In the presence of added IGF-I, more than 80% of clone 4 cells produced colonies, all of which were large. As was the case with the nonclonal population, the anti-IGF-IR antibody (αIR3) effectively inhibited colony formation, albeit incompletely (Table 2).

When the clones were grown in a tissue culture dish, the phenotype that they displayed was an exaggerated version of that displayed by the parental, nonclonal population. Furthermore, every cell participated. In calf serum without added insulin, the clones behaved like normal NIH 3T3 cells. The addition of insulin caused the cells to pile up but not aggregate. The medium was acidified every 12 h. In FBS with or without insulin, all of the cells rapidly entered large aggregates that detached from the tissue culture surface. As with the nonclonal population, aggregates did not form on a

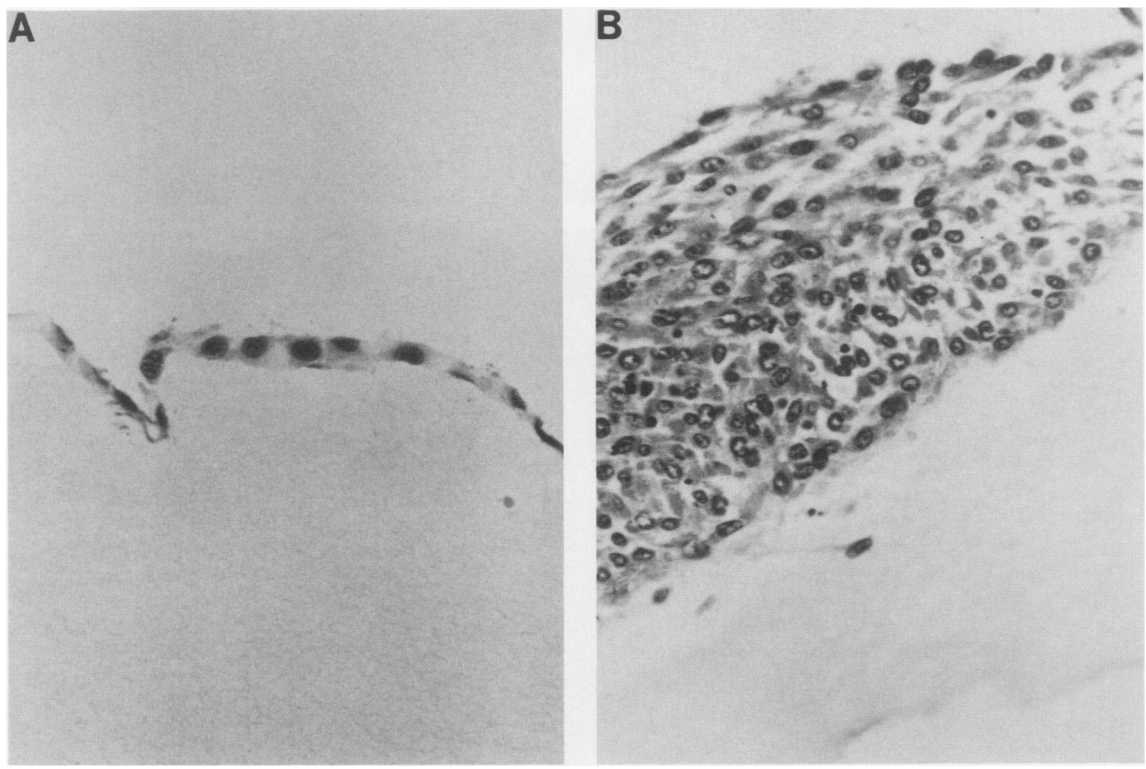

FIG. 3. Growth on a collagen substrate. 3T3/LNL6 (A) and 3T3/LISN clone 4 (B) cells were plated on a collagen substrate in medium supplemented with insulin (5 μg/ml) and 10% FBS. The medium was changed every other day. Three days after the cells became confluent, the substrate was fixed in Formalin, sectioned, and stained with hematoxylin and eosin. Control cells remained in a monolayer, whereas the 3T3/LISN clone 4 cells continued to grow into a multilayered structure.

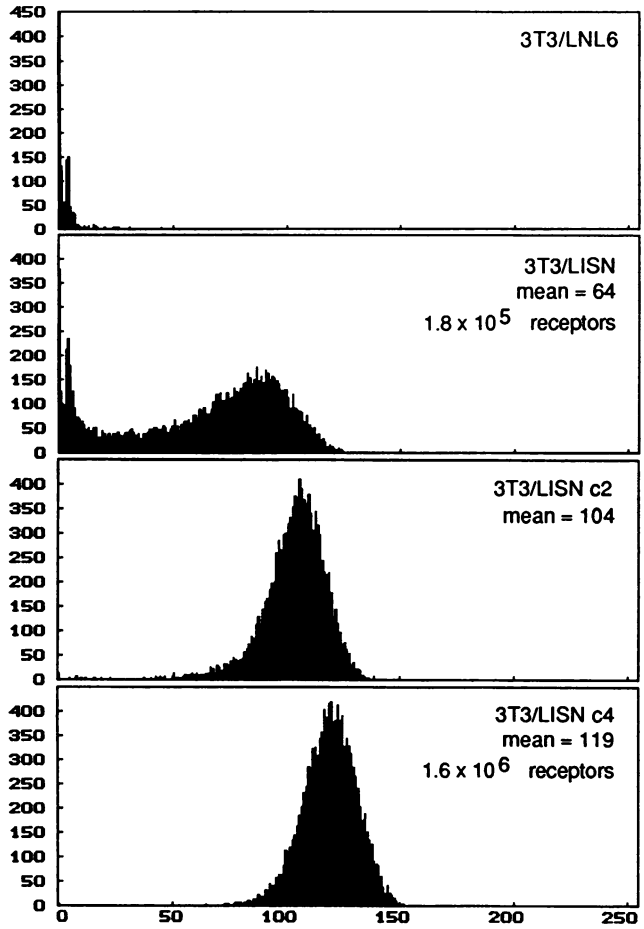

FIG. 4. Immunofluorescence histograms. Immunofluorescence, with αIR3, was done as described in the text. The cells were analyzed on a Coulter flow cytometer. The abscissa represents fluorescence intensity displayed on a 256-channel/4-decade log scale. The ordinate represents the number of cells in each channel. Mean refers to the average fluorescence for the entire population (see Table 3). Receptor number refers to the number of IGF-I receptors per cell (both human and murine) as measured by IGF-I binding (see Fig. 5 and text).

collagen substrate. However, 3T3/LISN cells did form a multilayered structure on collagen, in contrast to the single layer formed by 3T3/LNL6 cells (Fig. 3).

Each cell line was then assayed for receptor number by immunofluorescence with αIR3. The distribution of fluorescence intensity was assessed by flow cytometry. Histograms of the nonclonal populations and two of the clones are shown in Fig. 4. The 3T3/LNL6 control cells were nonfluorescent, since αIR3 does not bind to the rodent IGF-IR. The nonclonal population of 3T3/LISN cells displayed a wide variety of fluorescence; 15% of the cells had low fluorescence similar to that of the control 3T3/LNL6 cells. The mean fluorescence for the entire population was 64. In contrast, each of the four clones exhibited a gaussian distribution that overlapped the high end of the nonclonal population histogram. The mean fluorescence for each (Table 3) was greater than 100. Clones 4 and 8, which had the highest plating efficiencies in soft agar, were the most fluorescent.

Further evaluation of these histograms revealed a threshold effect for the number of receptors necessary to promote

TABLE 3. Summary of immunofluorescence data for 3T3/LISN populations[a]

Cell type	Mean fluorescence[b]	% of cells with fluorescence >110	Plating efficiency (%) in IGF-I-supplemented soft agar[c]
Nonclonal population	64	4	3
Clone 2	104	37	51
Clone 4	119	80	82
Clone 5	105	40	35
Clone 8	112	63	67

[a] Numerical data were obtained directly from the immunofluorescence histograms.
[b] Average for the entire population.
[c] Percentage of each population that formed colonies in soft agar.

anchorage-independent growth. For each of the 3T3/LISN populations, the percentage of cells whose fluorescence intensity was greater than 110 correlated well with the percentage that formed colonies in IGF-I-supplemented soft agar (Table 3). This correlation suggests that a high level of human receptors, sufficient to produce a fluorescence intensity greater than 110, is necessary for colony formation in soft agar.

Additional quantitative data were obtained with an IGF-I-binding assay for four of the cell populations: 3T3/LNL6, nonclonal 3T3/LISN, and 3T3/LISN clones 4 and 8. The amount of IGF-I bound at various ligand concentrations is shown in Fig. 5. For each cell population, binding displayed saturation kinetics. As expected, half-maximal binding occurred at approximately 1 nM IGF-I. The number of receptors per cell was calculated from the plateau levels. The 3T3/LNL6 control cells displayed 25,000 receptors per cell. Nonclonal 3T3/LISN cells displayed on average 180,000, whereas the two most aggressive clones, 4 and 8, displayed 1.6 million and 1.2 million, respectively. These results again show that colony formation in soft agar is associated with very high receptor numbers.

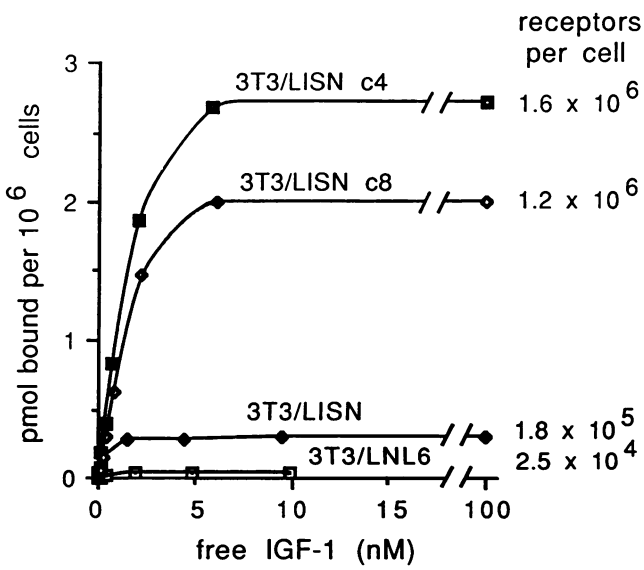

FIG. 5. IGF-I binding assay. The IGF-I binding assay was done as described in the text. Each datum point represents the mean of duplicate determinations, which always agreed to within 5%. Binding displayed saturation kinetics. The number of receptors per cell was calculated from the plateau levels.

Furthermore, for the three 3T3/LISN populations, the results of the prior immunofluorescence assay can be compared with those of the IGF-I binding assay. After linearization of the fluorescence scale (see Materials and Methods), the mean fluorescence of each population was linearly correlated with the number of human receptors measured by IGF-I binding. As noted above, for NIH 3T3 cells to form colonies in soft agar, a fluorescence intensity greater than 110 is necessary. This level of fluorescence corresponds to approximately 1.1 million IGF-I receptors per cell, representing an increase of more than 40-fold above the base-line level.

IGF-IR overexpression promotes tumor formation in nude mice. The data to this point indicate that high-level expression of the human IGF-IR in NIH 3T3 cells leads to transformation in vitro. The phenotypic changes are significantly enhanced by exposure to IGF-I or insulin. However, these data do not implicate the IGF-IR in any neoplastic change in vivo. It is not clear that the level of IGF-I present in vivo is sufficient to stimulate tumor development from these cells. To address this issue, nude mice were inoculated with either 3T3/LISN clone 4 cells or 3T3/LNL6 control cells. For each of the two cell populations, three mice were injected with 10^7 cells and three were injected with 10^5 cells.

The 3T3/LISN clone 4 cells rapidly formed tumors. Within 2 days, mice injected with 10^7 cells all developed visible, firm, subcutaneous nodules at the injection site. These grew progressively and were each 0.5 g at 3 weeks, when the mice were sacrificed. Pathologically, the tumors were fibrosarcomas with spindle-shaped cells arranged in fascicles. The nuclei were large, pleomorphic, and vesiculated. Mice injected with 10^5 clone 4 cells also developed progressively growing tumors that were first visible 10 to 14 days after injection. The control cells, on the other hand, failed to form tumors in any of the six mice monitored for 2.5 months.

The data demonstrate that overexpression of the IGF-I receptor can potentiate tumor growth in vivo.

IGF-IR overexpression alters the phenotype of primary diploid fibroblasts. All of the studies described above were done with immortalized murine fibroblasts. These aneuploid cells can transform spontaneously at low frequency and are readily transformed by single oncogenes. We therefore decided to further investigate the effects of IGF-IR overexpression in primary diploid fibroblasts, both human and rat.

Human fibroblasts were cultured from neonatal foreskin, and Wistar rat fibroblasts were cultured from the skins of 8-week-old animals. After approximately 10 population doublings, each cell type was infected with amphotropic LNL6 or LISN virus and selected in G418. The four resultant nonclonal cell lines were termed HF/LNL6, HF/LISN, RF/LNL6, and RF/LISN.

Receptor number and distribution on the human cells were analyzed by immunofluorescence and flow cytometry. LISN infection of these cells clearly altered their IGF-IR profiles. Only 28% of the HF/LNL6 control cells were fluorescent (>10 on the fluorescence scale), compared with 96% of the HF/LISN cells. The HF/LISN population had a mean fluorescence of 90, and values for 24% of the population were greater than 110. Despite this relatively high level of fluorescence, these cells did not form colonies in soft agar supplemented with 5 μg of insulin per ml.

They did, however, display ligand-dependent phenotypic changes when grown in a tissue culture dish. The HF/LNL6 control cells displayed a normal phenotype, both in the absence and in the presence of supplemental insulin (5 μg/ml). These cells grew in a monolayer forming a pattern of whorls (Fig. 6A). There were occasional, very small areas of overlapping cells at the centers of the whorls. The saturation density was 4×10^4 cells per cm^2. The HF/LISN cells, cultured without supplemental insulin, also displayed a normal phenotype and grew to the same saturation density. But when insulin was added to their medium, these cells became more refractile and spindle shaped. They continued in log-phase growth for one to two extra divisions, reaching a saturation density of 10^5 cells per cm^2. The cells grew in a more disorganized pattern and formed two overlapping layers that covered the entire tissue culture dish (Fig. 6B). As with the NIH 3T3 counterparts, confluent dishes of HF/LISN cells cultured with insulin rapidly acidified their medium. The indicator dye turned bright yellow every 48 h. This degree of acidification was never seen with the control cells, even after weeks in culture with insulin.

HF/LISN and HF/LNL6 cells were cultured for 4 months to determine whether IGF-IR overexpression could delay senescence. When passaged without insulin, the two populations had identical growth rates and life spans. With insulin (5 μg/ml), the HF/LISN cells initially grew to higher cell densities between transfers than did the HF/LNL6 cells. However, after undergoing extra divisions during the early passages, they ultimately ceased dividing several weeks earlier than the control cells. Hence, IGF-IR overexpression alters the morphology, saturation density, and metabolism of these cultured human fibroblasts but does not enable anchorage-independent growth or extend their life span in culture.

The primary rat fibroblasts differed in morphology from their human counterparts. These large, pleomorphic cells were flat and somewhat cuboidal. They were less refractile than the human cells and did not form a pattern of whorls. The RF/LNL6 control cells exhibited a completely normal phenotype that was not changed by the addition of insulin (Fig. 6C). In contrast, the RF/LISN cells had an altered phenotype, even in the absence of supplemental insulin. They were smaller, refractile, and spindle shaped. In the presence of insulin (5 μg/ml), these changes became even more obvious. The cells continued to grow but remained in a monolayer (Fig. 6D). Their saturation density was 3.5-fold higher than that of the RF/LNL6 cells. The rat fibroblasts were not tested for anchorage-independent growth or prolonged life span in culture.

DISCUSSION

We have shown that the native IGF-IR can behave like an oncogenic protein. The addition of ligand to cells that overexpress this receptor causes mitosis, overcoming the constraints of contact inhibition. NIH 3T3 cells obtain a fully transformed phenotype, including anchorage-independent growth, three-dimensional growth in a tissue culture dish, and rapid tumor formation in nude mice. As is the case with most oncogenes, the IGF-IR failed to fully transform primary fibroblasts (25). These cells displayed altered morphology and grew to high density but did not grow in soft agar or become immortalized.

It is clear that the growth factor-induced phenotypic changes were mediated through the overexpressed IGF-IR. IGF-I was 50-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 IGF-IR. Secondly, colony formation was inhibited in the presence of αIR3, an antibody that specifically blocks human IGF-IR activation. It is noteworthy that all of the soft agar experiments were done with heat-inactivated serum. This precau-

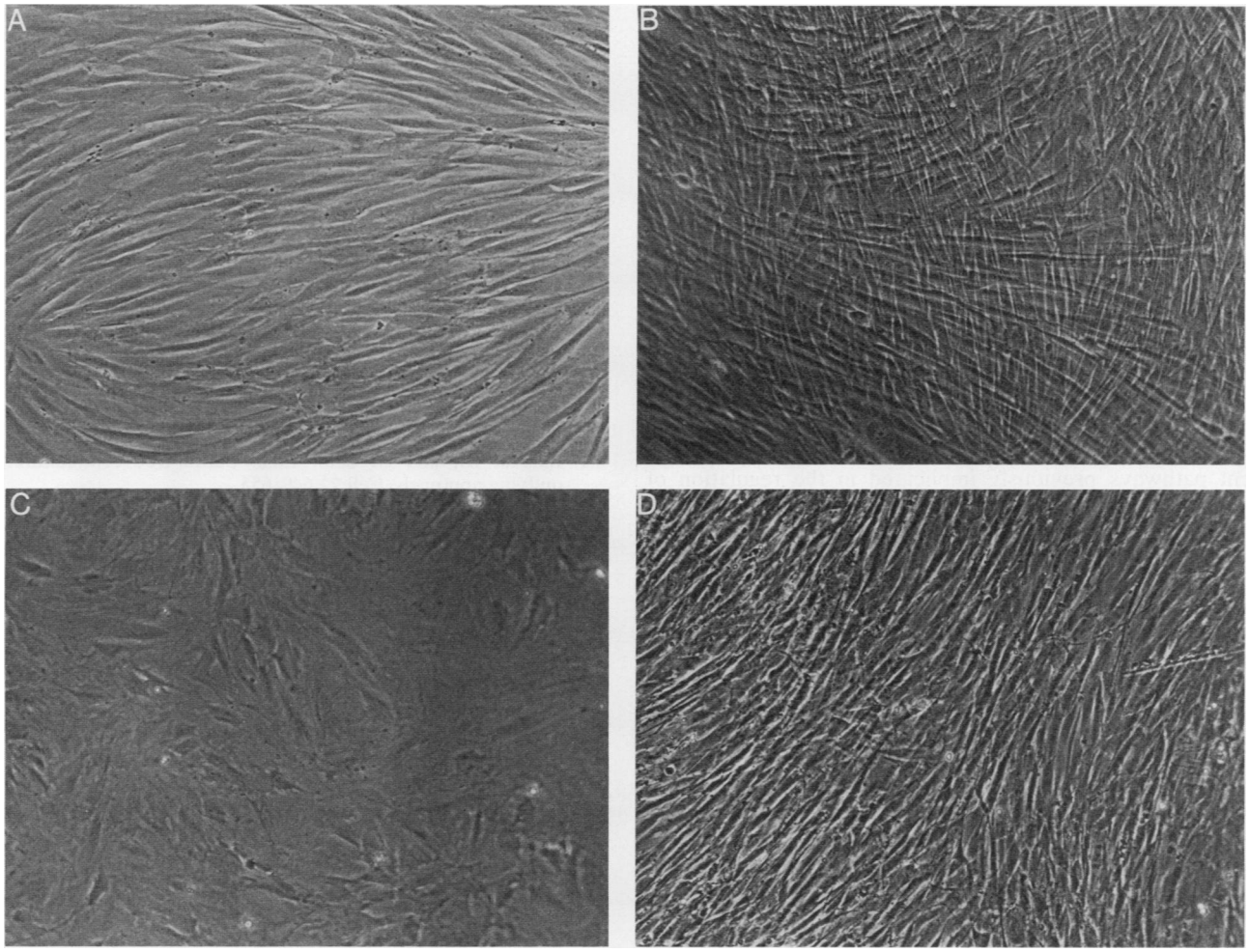

FIG. 6. Alteration of the morphology of primary fibroblasts by IGF-IR overexpression. HF/LNL6 (A), HF/LISN (B), RF/LNL6 (C), and RF/LISN cells (D) were cultured in medium supplemented with insulin (5 μg/ml). The medium was replaced every other day. Pictures were taken 3 days after the cells became confluent.

tion was taken to ensure that αIR3 was acting to block IGF-IR activation rather than to direct complement-mediated cell lysis.

The enhanced potency of many viral oncogenes relative to their cellular homologs stems from alterations that result in constitutive activation. Accordingly, we were concerned that the retroviral vector may, at some low frequency, transfer a rearranged, activated IGF-IR cDNA. This does not seem to be the case. Southern blotting of the 3T3/LISN nonclonal and clonal populations revealed no evidence of gross rearrangement. In addition, the functional response to ligand, ability to bind IGF-I, and ability to bind αIR3 suggest that the receptor-binding and catalytic domains were intact. NIH 3T3 cells that overexpressed the IGF-IR were indistinguishable from normal NIH 3T3 cells when grown in 10% calf serum without supplemental insulin. This finding indicates that retroviral gene transfer does not result in constitutive activation of the receptor, at least not at a level sufficient to promote the transformed phenotype. Therefore, it is reasonable to conclude that the native unrearranged receptor can transform cells, but only in a ligand-dependent manner.

The IGF-IR-transformed cells were tested for tumorigenesis in nude mice to determine whether ligand levels in vivo are sufficient to promote the transformed phenotype. This was not readily predictable from serum IGF-I levels, since the activity of this growth factor is substantially modulated by binding proteins produced both in the liver and locally in target tissues (5, 12, 14, 49). The finding that the IGF-IR-transformed cells rapidly formed tumors in nude mice gives credence to the hypothesis that overexpression of this receptor could trigger or at least potentiate neoplastic growth in vivo.

This study helps to shed some light on the role of the ligand, IGF-I, as an autocrine or paracrine mediator of tumor formation. IGF-I (or a supraphysiologic level of insulin) is a nearly ubiquitous requirement for cell growth in vitro. These growth factors can stimulate a small burst of DNA synthesis in confluent, quiescent murine 3T3 cells. When added in conjunction with PDGF, EGF, or fibroblast growth factor, the amount of DNA synthesis approaches that which results from 10% serum (7, 39). However, in general, IGF-I (and insulin)-mediated cellular proliferation has antineoplastic safeguards built in. In the absence of IGF-IR overexpression, the 3T3 cells and primary fibroblasts used in this study were completely unaffected by high levels of IGF-I and insulin. Although this growth factor is essential for cell growth, it does not promote transformation. Therefore, it

appears that the rate-limiting feature for transformation is receptor number. Furthermore, at least for NIH 3T3 cells, very substantial increases in IGF-IR number were necessary to produce a transformed phenotype. These cells exhibited anchorage-independent growth only if they expressed approximately 1 million receptors per cell, 40-fold above the base-line level. This may explain why high levels of IGF-I in vivo cause the hyperplastic diseases gigantism and acromegaly (14) but do not promote tumor development.

Implicit in the idea that receptor levels are the rate-limiting feature for transformation is the corollary that the second-messenger machinery is not rate limiting. It is logical to conclude that an amplified signal, transmitted via the IGF-IR second-messenger system, has the potential to transform cells. But this is not necessarily true. Second messengers for the IGF-IR have not been identified. When expressed at physiologic levels, the receptor does not appear to utilize either the cyclic AMP-dependent or protein kinase C-dependent pathways previously implicated in the regulation of mitosis (16, 34, 39). However, it is possible that overexpression of the IGF-IR may permit it to act promiscuously, to activate second messengers normally reserved for other growth factor receptors. So it remains unclear whether the usual IGF-IR second-messenger system can transmit a signal for transformation. In the case of the 3T3/LISN cells, the signal may be sent, in part, via an alternate pathway, perhaps involving cyclic AMP or protein kinase C. Definitive resolution of this issue awaits a better understanding of tyrosine kinase signal transduction.

We made the observation that cells overexpressing the IGF-IR rapidly acidified their tissue culture medium. One explanation comes from earlier studies using cloned cDNAs for both the IGF-I and insulin receptors (24, 41). Although these two receptors have a high level of sequence identity, it is generally believed that the insulin receptor is responsible for regulating short-term metabolic processes, whereas the IGF-IR regulates cellular proliferation. However, when overexpressed in tissue culture cells, the IGF-IR mediates many of the same short-term metabolic changes as does the insulin receptor. Accordingly, rapid acidification is likely to be due to an IGF-IR-mediated stimulation of glucose utilization and glycolysis.

Throughout this study, insulin was used at supraphysiologic levels capable of stimulating the IGF-IR. It was assumed that this growth factor was mediating its effects through the IGF-IR. Support for this assumption is discussed above. However, insulin, acting through its own receptor, has been shown to promote DNA synthesis as well as proliferation of some cell types (22, 29, 42). Furthermore, a truncated version of the insulin receptor enabled chicken embryo fibroblasts to form colonies in soft agar (46). To date, there is no evidence that the native insulin receptor is capable of fully transforming cells. It is possible, therefore, that although insulin mediated most of its transforming effects through the IGF-IR, insulin-exposed cells also received a synergistic, mitogenic boost through the insulin receptor. Clearly, this boost by itself was incapable of causing any phenotypic changes, since the control cells were not altered by insulin. However, synergism between the two receptors may explain why 3T3/LISN cells exposed to insulin formed more colonies in soft agar than did those exposed to IGF-I, both in the presence and in the absence of αIR3 (Table 1).

In conclusion, overexpression of the human IGF-IR in fibroblasts causes the receptor to function as a ligand-dependent oncogenic protein. It promotes unrestrained growth in vitro and may participate in tumor development in vivo. The retroviral construct LISN, which transfers the human IGF-IR cDNA, will be useful for studying the effects of overexpression of this receptor in a variety of cell types.

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