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Dominant negative inhibition of tumorigenesis *in vivo* by human insulin-like growth factor I receptor mutant

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ABSTRACT **Although insulin-like growth factor I (IGF-I) is a mitogenic growth factor, its role in tumorigenesis is unclear. We therefore transfected wild-type and truncated β-subunit mutant (^{952}STOP) human IGF-I receptor cDNAs into Rat-1 fibroblasts. Rat-1 transfectants expressed 2.5- to 7-fold increased IGF-I receptor mass, while the K_d for IGF-I binding was unchanged. The Rat-1 cells transfected with wild-type receptor cDNA responded to *in vitro* IGF-I treatment by increased proliferation and DNA synthesis. Cells overexpressing wild-type receptors were also transformed as evidenced by ligand-dependent colony proliferation in soft agar. After injection into athymic nude mice, all wild-type transfectants formed solid sarcomas within 3 weeks, and *ex vivo* tumor cell assays confirmed continued overexpression of human IGF-I receptors. In contrast, both DNA synthesis and proliferation of ^{952}STOP-transfected cells were attenuated below that of untransfected cells. ^{952}STOP cells were nonresponsive to IGF-I *in vitro* and were unable to sustain anchorage-independent growth. No tumors were induced for up to 8 weeks after injection of ^{952}STOP transfectants into athymic mice, despite the presence of demonstrable endogenous IGF-I receptors on the ^{952}STOP-transfected cells. Therefore, ^{952}STOP behaves as a dominant negative inhibitor of endogenous IGF-I receptor function, probably by assembling nonfunctional hybrid rat/mutant human receptor tetramers.**

Peptide hormones may stimulate cell growth and have also been implicated in the transformation of normal cells by activating intracellular signaling pathways that are initiated by specific cell surface receptors (1, 2). Several potential mechanisms may allow tyrosine kinase receptors to transform cells. Certain viral oncogenes are highly homologous to growth factors or their receptors, as exemplified by expression of the v-*sis* protooncogene in platelet-derived growth factor receptor-containing cells leading to autocrine transformation (3). Constitutive activation of viral receptor homologues—e.g., v-ErbB and epidermal growth factor receptor—also leads to cellular transformation (4). Overexpression of normal receptors, including epidermal growth factor receptor (EGFR), colony-stimulating factor 1 receptor, and insulin-like growth factor I receptor (IGF-IR), causes ligand-dependent cellular transformation (5–7), suggesting that the absolute mass of growth factor receptor may be a determinant of cellular transformation.

Tumor cells expressing functional IGF-IRs may enhance their own growth by endocrine, paracrine, or autocrine-derived IGF ligands. Overexpression of normal IGF-IRs in NIH 3T3 mouse fibroblasts resulted in ligand-dependent cell transformation *in vitro* and in nude mice (7). In addition to promoting cellular growth, IGFs may also play a role in neoplastic transformation and metastasis. IGFs stimulate the motility of cultured melanoma cells, thereby enhancing the potential for local or distant tumor metastases (8).

Tyrosine kinase growth factor receptors are either covalently linked via disulfide bonds independent of ligand binding or form dimers in the presence of their respective ligands (9, 10). Previously, we have shown that a truncated human IGF-IR, lacking the cytosolic kinase domain, forms a nonfunctional (kinase-defective) heterodimer with wild-type (WT) IGF-IR, thereby blocking signal transduction (11). We now show that these hybrid IGF-IRs inhibit hormone-dependent cellular proliferation. This trans-dominant inhibition of endogenous IGF-IR function by an exogenous mutant half-receptor suggested strategies whereby physiological and pathological functions of the IGF-IR could be examined by using kinase-defective mutated forms of the receptor as a dominant negative phenotype in live animals. We show that a truncated form of IGF-IR can abrogate ligand-dependent cellular transformation and tumorigenesis mediated by WT IGF-IR both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Stable Transfection into Rat-1 Fibroblasts. The WT and ^{952}STOP mutant IGF-IRs and transfections have been described (11). We have designated parental nontransfected cells as Rat-1, cells transfected with the WT human IGF-IR as WT, and mutant transfectants expressing the kinase-deficient IGF-IR as ^{952}STOP. Clonal lines are designated by numbers after the respective cell descriptor. G418 (600 μg/ml)-resistant colonies WT.1 and WT.2 and ^{952}STOP.1 through ^{952}STOP.4 were subcloned and further characterized for ^{125}I-IGF-I binding (11).

Immunoprecipitation of Hybrid Receptors. Confluent cells were rinsed with phosphate-buffered saline and metabolically labeled at 37°C with 0.5 mCi of [^{35}S]methionine (1049 Ci/mmol; 1 Ci = 37 GBq) in 5 ml of serum-free methionine-free medium. After 16 hr, the cells were lysed in lysis buffer and centrifuged at 3000 rpm for 15 min at 4°C (11). Either αIR3 (1:500) (Oncogene Science) or Ab1-2 (1:300) [a monoclonal antibody which recognizes a C-terminal epitope in both human and rat IGF-IR β subunits, kindly provided by K. Siddle (University of Cambridge, Cambridge, England)] antibody was used. Immunocomplexes were precipitated and loaded onto nondenaturing 5% polyacrylamide gels with prestained protein markers (Bethesda Research Laboratories) (11).

Thymidine Incorporation. Cells (10^4) were seeded in 96-well tissue culture plates and deinduced in serum-free defined medium for 24 hr. IGF-I (3.25 nM) was added to the indicated wells for 24 hr. [^{3}H]Thymidine (1 μCi) (Amersham) was added to the culture medium (25 μl) for 5 hr at 37°C. Medium was aspirated and 100 μl of cold 10% (wt/vol) trichloroacetic

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Abbreviations: IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; WT, wild type.

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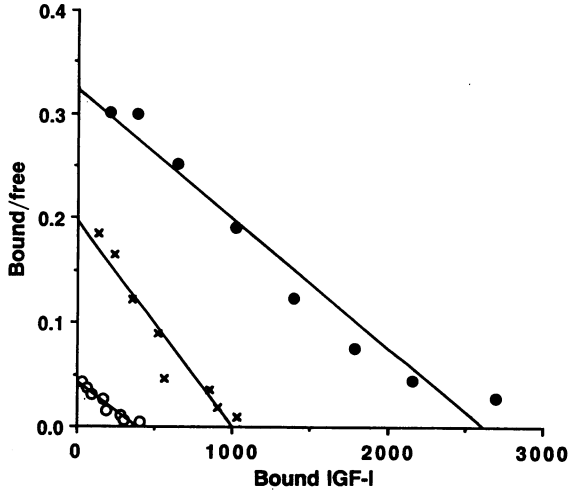

FIG. 1. Scatchard analysis of ^{125}I-IGF-I binding to Rat-1 (○), WT.1 (●), and ^{952}STOP.1 (X) cells. Each point represents the mean of three values of a representative experiment. The association constants and the numbers of binding sites per cell are as follows: Rat-1, K_d = 1.21 nM, 31,000 sites; WT.1, K_d = 1.08 nM, 210,000 sites; ^{952}STOP.1, K_d = 0.67 nM, 80,000 sites. Units for abscissa are pg/ml.

acid was added to each well for 20 min at 4°C. After aspiration, wells were washed twice with 10% trichloroacetic acid. Then 100 μl of 1 M NaOH was added to each well, plates incubated on a shaker for 15–30 min at room temperature, and 90 μl was removed from each well for liquid scintillation counting (12).

Crystal Violet Assay. Cells (10^4) were plated in 96-well plates. Following attachment, medium was changed to serum-free defined medium without or with IGF-I (3.25 nM) for the indicated times. The medium then was aspirated and the monolayer was washed twice with phosphate-buffered saline. Crystal violet (Sigma) made up to 0.5% in methanol at room temperature in 100 μl was added to each well for 15 min. Wells were destained with water and allowed to dry. The crystals were dissolved in 100 μl of 0.1 M sodium citrate, pH 4.2/50% ethanol for 30 min at room temperature. Plates were read on an ELISA plate reader at 540 nm (13). The growth stimulatory ratio (%) = $[(A_{sample} - A_{blank}/A_{control} - A_{blank})] \times 100$.

Soft-Agar Assays. Cell suspensions plated in semisolid medium consisting of α minimal essential medium with 0.3%

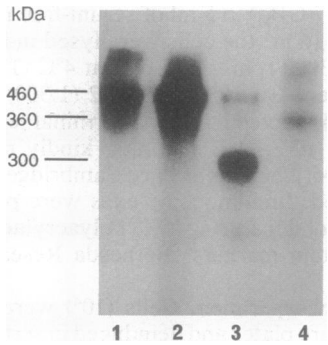

FIG. 2. Immunoprecipitation of IGF-IRs. Transfectants were metabolically labeled with [^{35}S]methionine and immunoprecipitated with either αIR3 or Ab1-2 monoclonal antibodies and electrophoresed in nondenaturing 5% polyacrylamide gels. The 460-kDa intact human or rat IGF-IR, 360-kDa rat/mutant human IGF-IR, and 300-kDa truncated human IGF-IR proteins are indicated. Lane 1, WT.1 plus αIR3; lane 2, WT.1 plus Ab1-2; lane 3, ^{952}STOP.1 plus αIR3; lane 4, ^{952}STOP.1 plus Ab1-2.

Table 1. Growth stimulatory ratio of fibroblasts

Cells	Growth stimulatory ratio, %	
	− IGF-I	+ IGF-I
Rat-1	8.9 ± 0.4	23.6 ± 0.7
^{952}STOP.1	7.4 ± 0.4	8.5 ± 0.2*

Cells (≈10^4) were seeded in serum-free defined medium for 24 hr. Medium was replenished without (−) or with (+) added IGF-I (3.25 nM) and incubation was continued for a further 48 hr. Cells were then stained with crystal violet and destained, and the absorbance at 540 nm was read on an ELISA plate reader. A representative experiment with six individual wells per point, representative of four similar experiments, is depicted. Similar data were obtained with ^{952}STOP clones 2, 3, and 4.
*$P < 0.005$ for Rat-1 vs. ^{952}STOP.1.

Bacto-Agar, 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and IGF-I (13 nM) were incubated at 37°C for 12–14 days.

Tumor Formation in Nude Mice. Rat-1, WT, and ^{952}STOP cells were tested for *in vivo* tumorigenesis by subcutaneous injection of 10^6 cells through a 22-gauge needle over the right or left flank of 5- to 6-week-old nude mice (Harlan Sprague–Dawley).

Statistics. Data are presented as mean ± SEM or SD, and differences were assessed by nonpaired *t* test.

RESULTS

Characterization of Hybrid Receptors. To examine the effects of overexpression of growth factor receptors, Rat-1 fibroblasts expressing ≈3 × 10^4 endogenous IGF-IRs per cell were stably transfected with either the intact human IGF-IR cDNA (WT), or a truncated receptor containing a synthetic stop codon at position 952 (^{952}STOP) (11). After G418 selection, clones were screened for expression of the human IGF-IR by ^{125}I-IGF-I binding. Fig. 1 shows Scatchard analysis of two of the cell clones selected for further study. Although the K_d values for the WT.1 clone and ^{952}STOP.1 mutant clone (1.08 and 0.67 nM, respectively) were similar to that for IGF-I binding to untransfected Rat-1 fibroblasts, all transfectants exhibited a 2.5- to 7-fold increase in IGF-I binding sites per cell. The other mutant clones, ^{952}STOP.2, -.3, and -.4, exhibited 22.6%, 15.3%, and 15% specific ^{125}I-IGF-I binding compared with 10% specific binding for Rat-1 cells. The second WT clone, WT.2, was chosen because it exhibited 47% specific ^{125}I-IGF-I binding, similar to that of the mutant clone ^{952}STOP.1, which had 49% specific binding for IGF-1. IGF-IR hybrid formation (rat IGF-IR/mutant human IGF-IR) was demonstrated by metabolically labeling the WT.1 and ^{952}STOP.1 transfectants with [^{35}S]methionine and then immunoprecipitating the proteins with both αIR3 and Ab1-2 monoclonal antibodies. αIR3 recognizes the

Table 2. [^{3}H]Thymidine incorporation into transfectants

Cells	^{3}H, cpm	
	− IGF-I	+ IGF-I
Rat-1	1519 ± 29	4282 ± 135*
^{952}STOP.1	117 ± 6†	189 ± 9.6†

Cells were cultured in serum-free defined medium for 24 hr. Medium was then replenished without (−) or with (+) added IGF-I (3.25 nM) for an additional 24 hr. [^{3}H]Thymidine was added to the monolayers for 5 hr at 37°C. After solubilization, radioactivity was measured in a liquid scintillation counter. Values represent the mean of six determinations. A representative experiment of four similar experiments is shown. Similar data were obtained with ^{952}STOP clones 2, 3, and 4.
*$P < 0.005$, IGF-I vs. no IGF-I.
†$P < 0.005$, Rat-1 vs. ^{952}STOP.1.

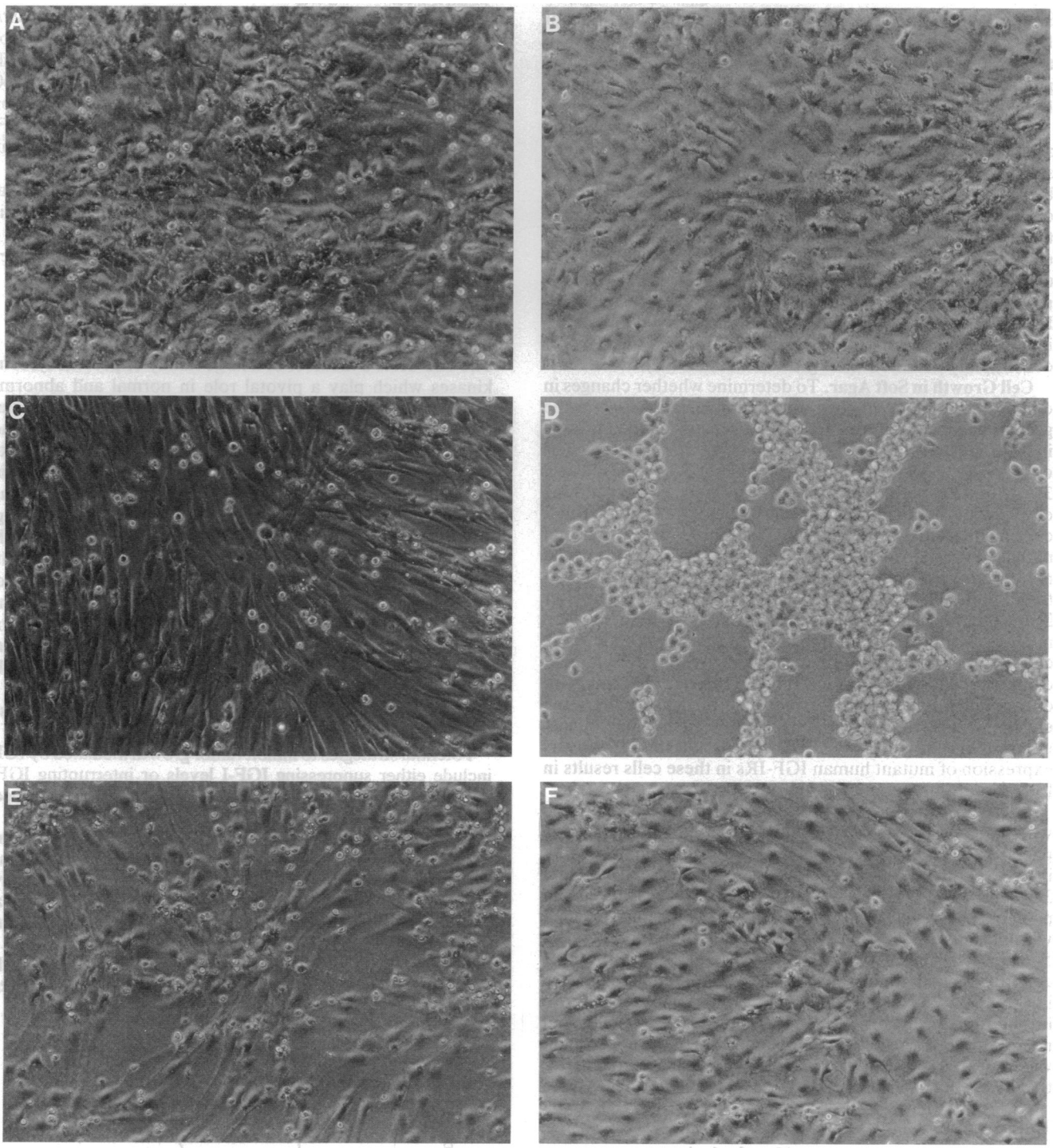

FIG. 3. Cell morphology in tissue culture dishes. Rat-1 (*A* and *B*), WT.1 (*C* and *D*), and ^{952}STOP.1 (*E* and *F*) cells were plated (10^5 cells per 100-mm plate) in serum-free defined medium without (*A*, *C*, and *E*) or with (*B*, *D*, and *F*) added IGF-I (50 nM) for 48 hr. WT.1 cells (*D*) always rounded up and became loosely adherent, whereas Rat-1 parental cells (*B*) and ^{952}STOP.1 transfectants (*F*) did not exhibit altered morphology in response to IGF-I treatment.

human IGF-IR α subunit visualized as 460- and 300-kDa bands from WT.1 and ^{952}STOP.1 cells, respectively. Ab1-2 recognizes a C-terminal β-subunit epitope in both rat and human IGF-IR and therefore identifies the 360-kDa hybrid (rat/mutant human) IGF-IR (Fig. 2). These hybrid receptors are kinase-defective (11).

Effect of IGF-I on Cell Growth. To determine the effects of rat/mutant human IGF-IR hybrids on cellular proliferation, Rat-1 cells or ^{952}STOP transfectants were incubated with IGF-I, and cell growth and DNA synthesis were measured. Table 1 depicts the growth stimulatory ratio for the Rat-1 cells and one of the mutant transfectants in response to IGF-I. Rat-1 cells grew faster than ^{952}STOP.1 cells both in the absence and in the presence of IGF-I. In addition, ^{952}STOP.1 cells did not respond to IGF-I. The blunted growth response of the IGF-IR transfectants was confirmed by measuring DNA synthesis as assessed by [^{3}H]thymidine uptake after IGF-I treatment. [^{3}H]Thymidine incorporated into Rat-1 cells more than doubled after the addition of IGF-I (3.25 nM) (Table 2). IGF-I, however, did not stimulate DNA synthesis

in ^{952}STOP.1 transfectants. Further, the absolute rate of DNA synthesis in ^{952}STOP.1 cells was markedly lower than that in Rat-1 cells. These results substantiate that IGF-I is a positive growth regulator in these cells and that an intact IGF-IR is required to facilitate this growth effect.

Effect of IGF-I on Cell Morphology. WT.1 cells grown in the absence of ligand had a normal phenotypic appearance similar to that of untransfected Rat-1 and ^{952}STOP.1 fibroblasts. In contrast, in the presence of ligand, IGF-I (50 nM) or insulin (650 nM), WT.1 cells overexpressing intact receptors rounded up and became loosely adherent (Fig. 3). Cell morphology was not changed in either Rat-1 or ^{952}STOP.1 cells after ligand addition. These growth factor-mediated phenotypic changes occur through the overexpressed IGF-IR, as IGF-I was 10-fold more potent than insulin in promoting this altered morphology. As previously described, WT.1 cells rapidly acidified their growth medium (12).

Cell Growth in Soft Agar. To determine whether changes in colony morphology implied a more universal change in the growth phenotype of transfectants, we analyzed their growth in soft agar. Rat-1 cells grow in soft agar; however, their colony size was much smaller than that of WT.2 cells (Table 3). After 12 days, colony growth was analyzed by counting only colonies containing >40 cells. The untransfected Rat-1 cells formed small colonies which increased in both size and number in response to IGF-I. However, WT.2 cells formed more numerous and larger colonies in the absence of IGF-I than Rat-1 cells in the presence of IGF-I ($P < 0.001$). After ligand treatment, WT.2 colonies increased in both size and number. A similar response to ligand was seen with the WT.1 cells (data not shown). In contrast, four ^{952}STOP mutant clones failed to grow in soft agar in the absence of ligand, with only minimal growth in the presence of ligand. Thus, the endogenous rat IGF-IRs were unable to sustain anchorage-independent growth of ^{952}STOP cells, suggesting that overexpression of mutant human IGF-IRs in these cells results in formation of nonfunctional hybrid IGF-IRs.

***In Vivo* Tumorigenicity of Transfectants.** To determine whether hybrid receptors would exert a dominant negative phenotype *in vivo*, three groups of athymic nude mice were injected with $\approx 10^6$ WT.1, Rat-1, or ^{952}STOP.1 cells. Within 3 weeks, all the mice injected with WT.1 cells developed palpable and visible tumors (Fig. 4). Six of nine mice injected with untransfected Rat-1 cells developed tumors only after 5–6 weeks, whereas none of the mice injected with ^{952}STOP.1 cells developed tumors by 8 weeks after injection. Mice injected with WT.1 cells developed larger tumors than those in mice injected with Rat-1 cells, which were smaller and necrotic. Similar results were obtained when the experiment was repeated with additional clonal receptor isolates. After the mice were sacrificed, Rat-1 and WT.1 solid tumors were enzymatically dispersed and their cells were cultured *in vitro* with G418. Both native and *ex vivo* Rat-1 cells failed to grow in the presence of G418, whereas all the *ex vivo* WT.1 cells derived from tumors proliferated *in vitro* with G418 at 600 μg/ml. Specific ^{125}I-IGF-I binding to these cells demonstrated that the *ex vivo* cells continued to overexpress IGF-I receptors (20–41% specific IGF-I binding), as compared with untransfected Rat-1 cells (5% specific IGF-I binding).

Pathological examination of formalin-fixed WT.1- and Rat-1-derived tumors was consistent with sarcoma differentiation, with the Rat-1-derived tumor having large areas of necrosis whereas the WT.1-derived tumor was extremely hard and had few necrotic areas.

Table 3. Colony formation in soft agar

Cells	No. of colonies	
	− IGF-I	+ IGF-I
Rat-1	7.3 ± 0.5	15.8 ± 2.5
WT.2	21.3 ± 5.0*	54.7 ± 5.7*
^{952}STOP.1	0.3	0.3
^{952}STOP.2	0	0.3
^{952}STOP.3	0.3	0
^{952}STOP.4	0	0.3

A total of 2000 cells were plated in 0.3% agar with 10% fetal bovine serum. In addition to parental Rat-1 cells and cells overexpressing the WT receptor, four different clones of ^{952}STOP transfectants were tested. IGF-I (13 nM) was added as indicated. Plates were scored at 12 days. Only colonies with at least 40 cells were counted. Values are means ± SD of six wells. Data are representative of six similar experiments. Similar results were obtained with the WT.1 clone.
*$P < 0.001$ vs. Rat-1.

DISCUSSION

The IGF-IR is a member of a large family of protein-tyrosine kinases which play a pivotal role in normal and abnormal proliferative processes (13–15). Enhanced tyrosine kinase expression has been directly correlated with several pathological states, as exemplified in chronic myelogenous leukemia, where the enhanced myeloid cell tyrosine kinase activity that underlies the disease results from activation of the c-*abl* protooncogene by fusion with the *bcr* element (16). Although IGF-I is essential for cell growth, the central role of the IGFs in tumor biology has only recently been appreciated. Numerous studies attest to the role of IGF-I in growth regulation of solid tumors, and of IGF-II in regulation of embryonal tumors (17, 18). However, addition of IGFs to cells *in vitro* does not promote their transformation. The rate-limiting factor for IGF-I-mediated cell transformation may, in fact, be receptor number. High levels of IGF-I, such as occur in acromegaly, cause hyperplasia of soft tissues with gigantism and/or soft tissue/organ overgrowth (19).

Potential strategies to inhibit tumor growth mediated by IGFs include either suppressing IGF-I levels or interrupting IGF-I action at the level of its cellular receptor. Drugs have been employed to reduce IGF synthesis and/or secretion (20).

Ligand–receptor function can be abrogated by utilizing monoclonal receptor antibodies such as αIR3 (21), the polyanionic compound suramin (22), or phosphorylation-defective analogues of the IGF-I ligand (23). We have employed an alternative approach to block IGF ligand signaling by utilizing a dominant negative IGF-IR mutant. There is a strong body of evidence that many neoplastic cells express or overexpress the IGF-IR. These studies were designed to characterize the *in vitro*

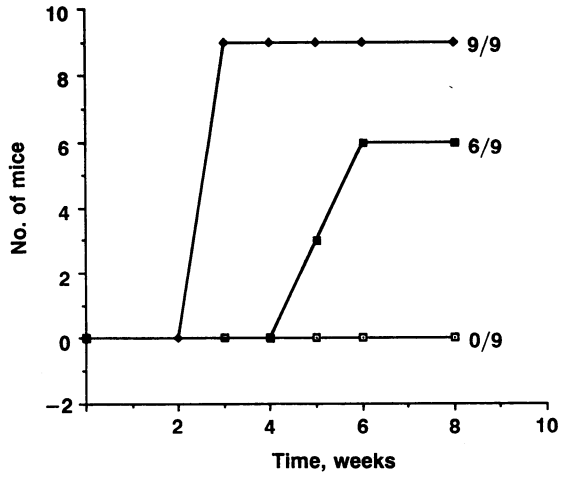

FIG. 4. Tumorigenesis *in vivo*. Groups of nine nude mice were injected subcutaneously with 10^6 Rat-1 (■), WT.1 (◆), or ^{952}STOP.1 (□) cells. Mice were observed for 8 weeks prior to sacrifice. Similar data were obtained in an identical experiment with different clonal isolates.

mechanism for inhibition of cellular proliferation and demonstrate the feasibility of such an approach *in vivo*.

Internalization of ligand is not a prerequisite for IGF-IR signaling (24). However, internalization of IGF-IRs requires the presence of intact tyrosine kinase activity (11). The failure of ^{952}STOP clones 1–4 to internalize ^{125}I-IGF-I compared with untransfected Rat-1 fibroblasts and WT.1 cells (data not shown) confirms that the majority of endogenous rat IGF-IRs are in hybrid formation with the truncated human IGF-IR lacking a functional kinase domain. This mutant human $\alpha\beta$ IGF-I half-receptor exerts a dominant negative action over the endogenous rat $\alpha\beta$ IGF-I half-receptor. To further demonstrate that assembly of rat $\alpha\beta$/mutant human $\alpha\beta$ IGF-I half-receptors abolishes the function of the receptor holotetramer, both cell growth and DNA synthesis were tested. ^{952}STOP.1 cells showed a significant reduction in proliferation in response to ligand compared with untransfected Rat-1 cells or transfectants overexpressing WT IGF-IR (data not shown), as assessed by crystal violet assay and [^{3}H]thymidine incorporation into DNA.

Ligand-mediated transformation of WT.2 transfectants as evidenced by changes in cellular morphology or enhanced growth in soft agar was not evident in four different ^{952}STOP transfectants, including clone 1, which has a similar number of IGF-IRs as the WT.2 transfectant. This confirms that ligand-mediated IGF-IR activation leading to cell transformation can be inhibited *in vitro* by a dominant negative IGF-IR mutant in a variety of clonal isolates. The definitive test that this strategy is relevant to control abnormal cellular growth is the demonstration that mutant receptors form nonfunctional hybrids *in vivo*. All mice injected with WT.1 cells developed tumors within 3 weeks of injection. These tumors increased progressively in size during the 8-week test period. This result is in agreement with an earlier study in which overexpression of the normal human IGF-IR in NIH 3T3 cells produced tumors in nude mice (7). Interestingly, six of nine mice injected with Rat-1 cells developed tumors by 5–6 weeks after injection. These tumors appeared later and were smaller and slower growing than those found in WT.1-injected mice. This occurrence was not unexpected, as Rat-1 cells contain $\approx 3 \times 10^4$ IGF-IRs per cell and do form colonies in soft agar. They therefore express a weakly transformed phenotype and are not primary cells. None of the mice injected with ^{952}STOP.1 cells developed tumors during 8 weeks of observation. This confirms that even *in vivo*, nonfunctional hybrid receptors which cannot mediate ligand signaling are assembled. Thus, the ^{952}STOP mutant human half-receptor failed to allow the WT endogenous rat half-receptor to function *in vivo*. Therefore, we infer that the mutant human IGF-IR produced a dominant negative phenotype *in vivo*. Interestingly, pathological examination of the tumors revealed that sarcomas overexpressing IGF-IR had very little tumor necrosis, whereas the small tumors developing from parental Rat-1 cells had large necrotic areas.

Documented defects in endocrine receptor function have been shown to underly clinical syndromes of thyroid hormone, androgen, vitamin D, and insulin resistance (25–29). These experiments of nature support the concept of utilizing targeted expression of IGF-IRs with a dominant negative function as a viable therapeutic intervention for pathophysiologic conditions in which a functional IGF-IR contributes to the altered cycle of cell growth.

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