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The Role of mGrb10α in Insulin-like Growth Factor I-mediated Growth*

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Several isoforms of Grb10 are known to interact with either the insulin receptor or the insulin-like growth factor I (IGF-I) receptor, or both. Inasmuch as the data in the literature on the function of Grb10 are not always concordant, we have investigated the role of one of these isoforms, mGrb10α, in cell proliferation. For this purpose, a plasmid expressing mGrb10α was stably transfected into p6 cells and other mouse embryo fibroblast cell lines. An overexpressed mGrb10α inhibits IGF-I-mediated growth, causes a delay in the S and G_2 phases of the cell cycle, and partially reverses the transformed phenotype. In contrast, it has no effect on insulin stimulation of cell proliferation. These studies indicate that this isoform of Grb10 has an inhibitory effect on IGF-I signaling of cell proliferation.

The insulin-like growth factor I receptor (IGF-IR)[1] activated by its ligands plays an important role in growth regulation, both *in vivo* and *in vitro* in at least three different ways: 1) it is mitogenic; 2) it is quasi-obligatory for the establishment and maintenance of the transformed phenotype; and 3) it protects cells from apoptosis (1). The mitogenicity of the activated IGF-IR *in vitro* has been known for several years (2, 3), and confirmed *in vivo* by the elegant genetic experiments of Efstratiadis and co-workers (4–6). IGF-IR signaling probably takes place through more than one pathway (7), but one of the pathways is certainly the Ras pathway, that has been described in detail in several reviews (8). The IR and the IGF-IR also interact with several substrates, of which the best known are IRS-1 (9–11), IRS-2 (12), phosphatidylinositol 3-kinase (13, 14), and the Shc proteins (15).

More recently, another family of interacting proteins has been identified, Grb10 (16), now mGrb10α,[2] with its homologues. At least seven laboratories have reported Grb10 proteins interacting with either the Insulin or the IGF-I receptors. The first report was by Liu and Roth (17), who identified a Grb10, which they called Grb-IR (now hGrb10β), lacking, in respect to mGrb10α, a 46-amino acid sequence in the PH domain. hGrb10β inhibited insulin signaling, in particular tyrosyl phosphorylation of IRS-1 and phosphatidylinositol 3-kinase activity (17). Hansen *et al.* (18) identified Grb10 (the same as the original one) as interacting with the C terminus of the IR, apparently at residue tyrosine 1322, and as noninteracting with IRS-1. Five other papers appeared almost simultaneously, describing an interaction of Grb10 (or similar molecules) with the IGF-IR (19, 20), the IR (21, 22), or both (23). The first of these papers reported the identification of the original mGrb10α (16), while in the case of Dey *et al.* (20), only fragments of a human sequence were identified. O'Neill *et al.* (23) identified a splice variant, which they called Grb10/IR-SV1 (now hGrb10γ1). A peptide (presumably acting as a dominant negative) of this splice variant inhibited insulin- and IGF-I-mediated DNA synthesis. The Grb10 identified by Frantz *et al.* (21) is very similar to hGrb10γ1, and has been called hGrb10γ2, while the peculiarity of mGrb10ϵ (22) is that it interacts with the IR, but not at all with the IGF-IR.

Very little has been published on the function of the Grb10 proteins, and that little is discordant, inasmuch as Liu and Roth (17) found an inhibitory action while O'Neill *et al.* (23) gave opposite results in terms of IR or IGF-IR signaling, albeit with a different isoform. We therefore decided to investigate in some detail the effect of the original mGrb10α (16) on IGF-I-mediated cell proliferation. For this purpose, we stably transfected a plasmid expressing the full-length Grb10 (mGrb10α) into mouse embryo fibroblasts. The results indicate that mGrb10α has an inhibitory effect on IGF-I signaling for cell proliferation. The most intriguing finding, however, is that it does not inhibit entry into S phase, but that, instead, it seems to inhibit progression through the S and G_2 phases of the cell cycle.

MATERIALS AND METHODS

Cell Cultures—p6, TC4 and R^-/IR cells are all mouse embryo fibroblasts, generated by a 3T3-like protocol, and have all been described in detail previously (24–26). p6 and TC4 cells have about 5,000 insulin receptors/cell (26); TC4 and R^-/IR cells have no IGF-IRs (25, 26). Each cell line was generated by the single transfection of the appropriate plasmid. All Grb10-expressing cell lines were generated by cotransfecting the plasmid pMJ30/Grb10, expressing the full-length mouse Grb10α fused to a Myc tag, (a kind gift from Dr. Ben Margolis and James Ooi, Howard Hughes Medical Institute, The University of Michigen Medical Center, Ann Arbor, MI) with a plasmid carrying the hygromycin resistance gene (27). Clones were selected in medium containing 800 μg/ml (p6 and TC4) or 300 μg/ml (R^-/IR) amounts of hygromycin, using the Transfectam® reagent (Promega Corp.).

Determination of Cell Growth—For all experiments assessing proliferative effects, cells were seeded at a density of $2.5 \times 10^3/cm^2$ in medium containing 10% fetal bovine serum and allowed to attach for 24 h. The cultures were then placed in SFM supplemented with 0.1% bovine serum albumin and 50 μg/ml transferrin, with or without purified growth factors (Sigma, Life Technologies, Inc.), and cell numbers were determined after 3 days in culture.

Transformation Assay—Anchorage-independent growth and focus formation were carried out as described previously (7, 28). For the soft agar assay, 10^3 cells/plate were seeded and the colonies (diameter >125 μm) counted after 2 weeks in culture. For focus formation, cells were plated at a concentration of 5×10^5; after 14 days, the cultures were fixed in methanol containing 0.5% crystal violet.

Immunoprecipitation and Immunoblotting—Cell lysates (500 μg of

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[1] The abbreviations used are: IGF-IR, type I insulin-like growth factor receptor; IGF-I, insulin-like growth factor I; IR, insulin receptor; SFM, serum-free medium; PBS, phosphate-buffered saline; IRS, insulin-like receptor substrate.

[2] The nomenclature of the various Grb10 isoforms is the one recently agreed upon by the authors.

protein) were immunoprecipitated with the indicated antibody in HNTG buffer (20 mM Hepes (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.2 mM sodium orthovanadate, 0.2 mM phenylymethysulfonyl fluoride, 2 mg/ml aprotinin). The immunoprecipitates were resolved on a 4–15% gradient gel by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. For immunoblotting, membranes were blocked with 5% bovine serum albumin or 5% dry nonfat milk in TBST buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 0.1% Tween 20) overnight at 4 °C and probed with the indicated first antibodies, followed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Oncogene Science). Blots were developed with the ECL system (Amersham Corp.) according to the manufacturer's instructions.

To detect Grb10 expression in the transfected clones, cell lysates from exponentially growing cells were immunoprecipitated with a monoclonal antibody against the Myc tag (Oncogene Science) and membranes were probed with an anti-Grb10 polyclonal antibody (309; a kind gift of Dr. Ben Margolis). For IGF-IR, IRS-1, and Shc analysis, cells were incubated in SFM for 48 h and then stimulated with 20 ng/ml IGF-I (Life Technologies, Inc.). Cell lysates were immunoprecipitated, respectively, with an anti-IGF-IR antibody (Ab1, Oncogene Science), an anti-IRS-1 antibody (Upstate Biotechnology), or a polyclonal anti-Shc antibody (Transduction Laboratories).

To detect phosphorylation of immunoprecipitated proteins, we used an anti-phosphotyrosine horseradish peroxidase-conjugated antibody (PY20, Transduction Laboratories). After stripping on the same membranes, the amount of the immunoprecipitated protein was detected with an anti-IGF-IR β-subunit polyclonal antibody (Santa Cruz), an anti-IRS-1 antibody (Upstate Biotechnology), or a monoclonal anti-Shc antibody (Transduction Laboratories). Grb2 was immunoblotted with a monoclonal anti-Grb2 antibody (Transduction Laboratories).

Cytofluorimetric Analysis—p6 and p6/Grb10 cells were grown in serum-free medium with or without IGF-I. Incubation was stopped by washing with PBS and trypsinizing the cells after 72 h. The analysis by flow cytometry was performed as described by Sell *et al.* (29). Briefly, trypsinized cells were washed in cold PBS and fixed in 70% ethanol at −20 °C. After a wash in cold PBS, cells were incubated in PBS with RNase A (75 units/ml) at 37 °C for 30 min, washed in PBS, and resuspended in PBS containing 15 μg/ml propidium iodide. A minimum of 20,000 cells/sample were analyzed with a Coulter Epics Profile II (Coulter Electronics, Inc., Hialeah, FL). Cell cycle distribution was calculated by the Multi-Cycle Program (Phoenix Flow Systems, San Diego, CA).

DNA Synthesis—Cells were seeded at 2.5×10^4/35-mm dish on coverslips in medium containing 10% fetal bovine serum and allowed to attach for 24 h. The cultures were then placed in SFM supplemented with 0.1% bovine serum albumin and 50 μg/ml transferrin for 96 h, to become quiescent before the addition of growth factors. Tritiated thymidine (0.5 μCi/ml) was added at the same time as the growth factors, and the incubations were continued for the times indicated. The cells were then fixed in cold methanol and autoradiographed. The percentage of labeled cells was determined by scoring a total of 1,000 cells.

Determination of Apoptosis in Vivo—This has been described in detail previously by Resnicoff *et al.* (30). At the times indicated, the cells recovered from the chambers were counted and examined for the presence of apoptotic cells.

RESULTS

As a starting cell line, we selected p6 cells (24), which are Balb/c 3T3 cells stably transfected with a plasmid expressing the full-length human IGF-IR cDNA (31). p6 cells express about 5×10^5 IGF-IRs/cell, grow very well in SFM supplemented solely with IGF-I, and are transformed because they form colonies in soft agar (7) and grow in the subcutaneous tissue of animals (30).

Effect of mGrb10α Expression on p6 Cells—p6 cells were transfected with the Grb10 plasmid, and clones were selected as described under "Materials and Methods." Three clones (clones J, 5, and 6) were found to express substantial amounts of Grb10 (Fig. 1, *lower panel*), while three other clones (4, E, and H) expressed lower amounts, although still more than the parental cell line (see *lanes 3–5* and *1* in Fig. 1). We define as overexpressing those clones that have substantial amounts of Grb10 (clones J, 5, and 6). The various cell lines were tested for their ability to grow in SFM supplemented solely with IGF-I.

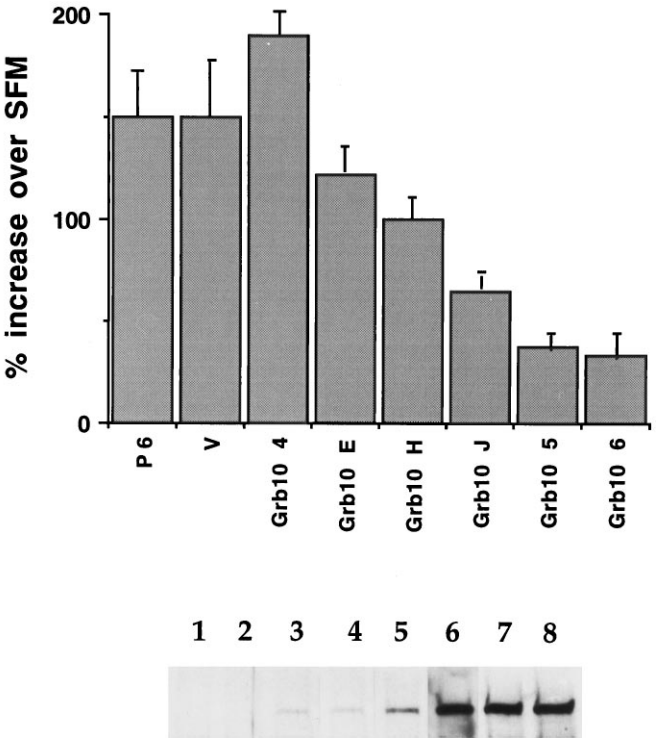

FIG. 1. **Effect of mGrb10α overexpression on the growth of p6 cells.** The *upper panel* gives the growth of p6 cells and derivatives (expressed as increase over serum-free medium) after 3 days in IGF-I (20 ng/ml). *V* are p6 cells transfected with an empty vector, and the others are clones of p6 stably transfected with the mGrb10 plasmid. The *lower panel* gives the levels of expression of mGrb10α in the eight cell lines, in the same order as in the *upper panel*.

As Fig. 1 (*upper panel*) shows, the growth of the three clones J, 5, and 6 was inhibited markedly, in respect to the parental cell line, the V cell line transfected with an empty vector, and the three cell lines expressing only moderate amounts of Grb10. Significantly, clone H, the highest of the low expressors, was inhibited moderately.

mGrb10α Does Not Inhibit R⁻ Cells Expressing a Truncated IGF-I Receptor—TC4 cells are R^- cells (28) stably transfected with a plasmid expressing a human IGF-R cDNA lacking the last 108 amino acids at the C terminus. These cells grow in IGF-I only, but are not transformed (25). Inasmuch as two laboratories have reported that isoforms of Grb10 interact with the C terminus of either the IR (18) or of the IGF-IR (19), we transfected TC4 cells with the Grb10 plasmid and selected three clones. These three clones express substantial amounts of Grb10 (Fig. 2, *lower panel*, *lanes 3–5*); *lane 6* gives the level of expression of p6/Grb10 clone 5, showing that the TC4 cells express at least as much Grb10 as the p6/Grb10 clones, whose growth in IGF-I is inhibited. However, the Grb10-expressing TC4 cells are not inhibited in the test for IGF-I-mediated growth (Fig. 2, *upper panel*), indirectly confirming that mGrb10α interacts with the C terminus of the IGF-IR.

mGrb10α Does Not Inhibit Insulin-mediated Growth—To clarify the discordant results of the literature (see Introduction), we tested the ability of mGrb10α to inhibit the growth of R^-/IR cells (26, 32), which are R^- cells (no IGF-IRs) expressing 5×10^5 IRs/cell. Four clones expressing substantial amounts of Grb10 (Fig. 3, *lower panel*, *lanes 3–6*) were analyzed. As in Fig. 2, the last lane (*lane 7* in this case) shows lysate of p6/Grb10 clone 5 cells, to offer a comparison of expression levels. The various cell lines were then tested for growth in SFM supplemented with 100 ng/ml insulin. Fig. 3 (*upper panel*) shows that the Grb10-expressing clones grow just as well as the parental

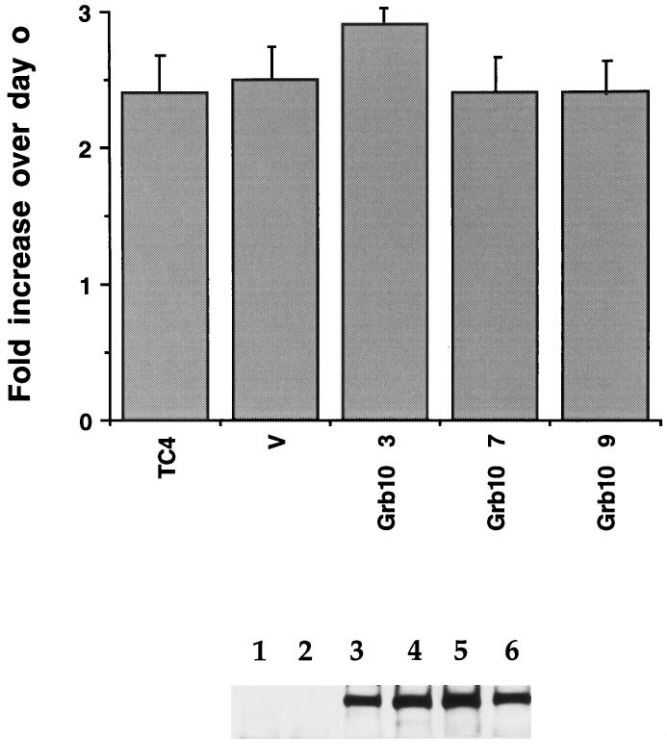

FIG. 2. **Lack of effect of mGrb10α overexpression on the growth of R⁻ cells expressing a truncated IGF-I receptor.** TC4 cells are R⁻ cells expressing a truncated IGF-IR (see "Materials and Methods"). One clone was stably transfected with an empty vector; the other three clones were derived from TC4 cells stably transfected with mGrb10 plasmid. The cells were grown in SFM supplemented with IGF-I (20 ng/ml) for 3 days, and their growth (*upper panel*) is expressed as -fold increase over day 0. The levels of expression of mGrb10 are given, in the same order, in the *lower panel*. *Lane 6*, however, is the level of expression of p6/Grb10 clone 5 of Fig. 1.

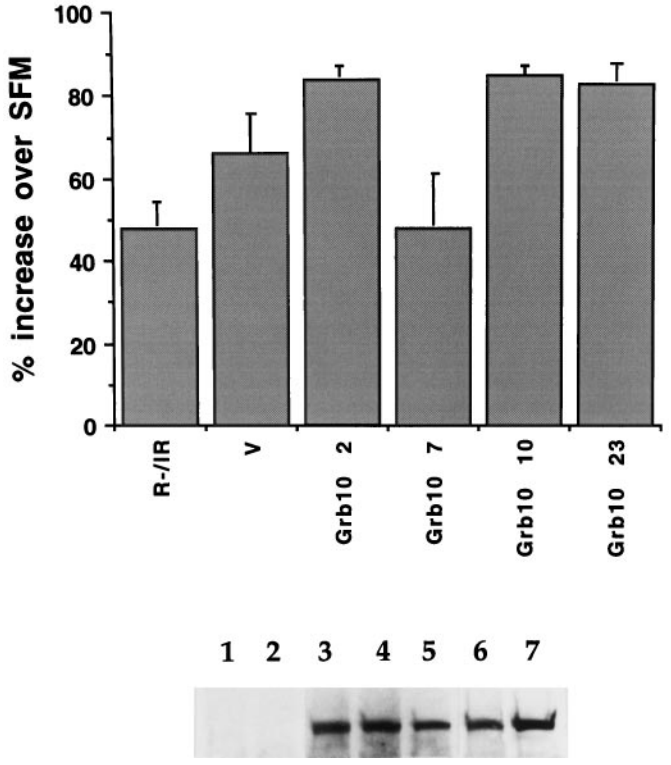

FIG. 3. **Lack of effect of an overexpressed mGrb10α on the growth of R⁻ cells overexpressing the insulin receptor.** R⁻/IR cells (no IGF-IRs, and about 5×10^5 insulin receptors/cell) were transfected with either an empty vector (*V*) or with a mGrb10α plasmid. The growth of these cells in SFM supplemented with 100 ng/ml insulin is given in the *upper panel*, and the expression levels of the individual clones in the *lower panel* (*lane 7* gives, for comparison, the expression level of p6/Grb10 clone 5 of Fig. 1).

cell line, R⁻/IR, and parental cells stably transfected with an empty vector (V cells).

mGrb10α Delays Cells in the S/G_2 Phases of the Cell Cycle—Fig. 4 shows a FACS analysis of p6 cells (*A* and *B*) and p6/Grb10 clone 5 cells (*C* and *D*) in either SFM or in SFM supplemented with IGF-I (20 ng/ml). The p6 cells stably transfected with an empty vector gave the same result as the parental p6 cells, while p6/Grb10 clone 6 cells were undistinguishable from clone 5 (data not shown). The Grb10-expressing cells clearly accumulated in the S and G_2 phases of the cell cycle, in SFM 70% against 28.2% in the parental p6 cells. After the cells were stimulated to grow by IGF-I (*right panels*), 86.8% of p6/Grb10.5 cells were in S/G_2 *versus* 60% in the parental p6 cells.

Because of the results obtained by O'Neill *et al.* (23), we then determined whether an overexpressed mGrb10α inhibited the entry of quiescent cells into S phase. For this purpose, cells were made quiescent in SFM and were subsequently stimulated with IGF-I. Fig. 5 shows the number of cells labeled by [^{3}H]thymidine, as determined by autoradiography. There is no significant difference between the parental p6 cells and the two clones overexpressing Grb10 in their ability to enter S phase after stimulation with IGF-I.

mGrb10α Partially Reverses the Transformed Phenotype—Foci formation and colony formation in soft agar are a characteristic of transformed cells, like p6 cells (7). p6 and clones 5 and 6 of p6/Grb10 cells were tested for foci formation in monolayers (Fig. 6). While p6 cells (*panel A*) formed distinct foci, p6/Grb10 clones 5 and 6 (*panels B* and *C*), although growing to high saturation density, did not form foci.

Several cell lines were then tested for colony formation in soft agar (Table I). p6 cells, V cells (empty vector), and p6/Grb10 clone 4 cells (low level of Grb10 expression) formed essentially the same number of colonies in soft agar. The p6/Grb10 clones 5 and 6, by contrast, especially clone 5, had a reduced number of colonies, and the size of the colonies was also smaller. These experiments were repeated at least three times.

However, mGrb10α does not cause apoptosis of p6 cells. This is shown in Table II, where the ability to induce apoptosis was determined by a very sensitive assay, the diffusion chamber described by Resnicoff *et al.* (30, 33). p6 cells overexpressing mGrb10α do not undergo apoptosis in this system; in fact, they double in number in 24 h. Clone 5 showed a lower recovery of cells, but still well above the initial number of cells loaded into the chamber. This lower recovery is probably simply due to a slowed growth rate. It should be pointed out that, when the IGF-IR is targeted (30, 33, 34) recovery of cells in this assay is between 1 and 5%.

Phosphorylation of the IGF-I Receptor, IRS-1, and Shc Proteins—Because the G_1 phase of the cell cycle does not seem to be affected (Figs. 4 and 5), we did not expect gross alteration in IGF-IR autophosphorylation and in tyrosyl phosphorylation of either IRS-1 or Shc (see "Discussion"). However, we wanted to confirm our prediction by comparing p6 and V cells to clones 5 and 6 of p6/Grb10. Fig. 7 shows the results for the IGF-IR (*panels A* and *B*) and for IRS-1 (*panels C* and *D*). An overexpressed mGrb10α does not seem to affect IGF-IR autophosphorylation (*panel A*). Similarly, tyrosyl phosphorylation of IRS-1 is the same in all the 4 cell lines tested (*panel C*). Fig. 8 shows the same experiment for the expression and tyrosyl phosphorylation of the Shc proteins. In each cell line, an additional band is tyrosyl-phosphorylated after stimulation with IGF-I,

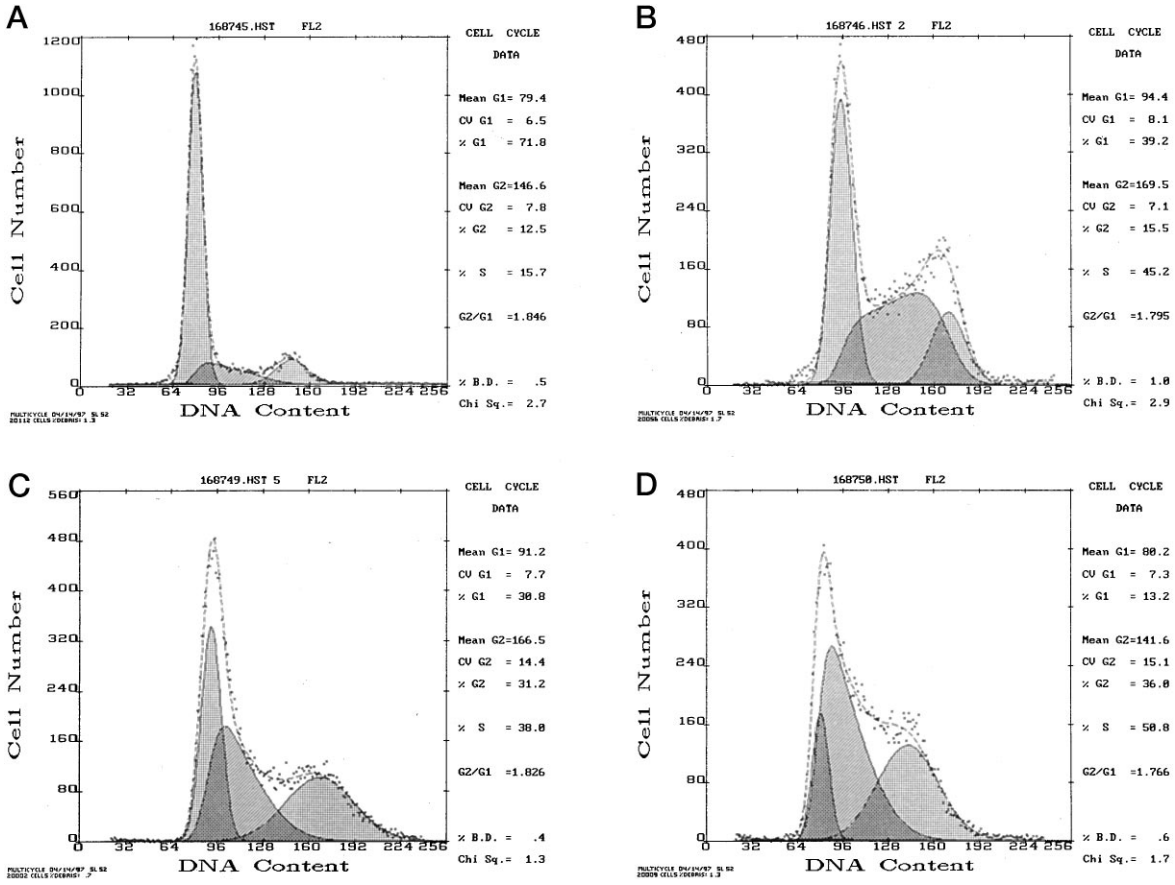

FIG. 4. **Effect of an overexpressed mGrb10α on the cell cycle phases of p6 cells.** FACS analysis of p6 cells (*panels A* and *B*) and p6/Grb10 clone 5 cells (*panels C* and *D*) in serum-free medium (*left*) and after stimulation with IGF-I (*right*).

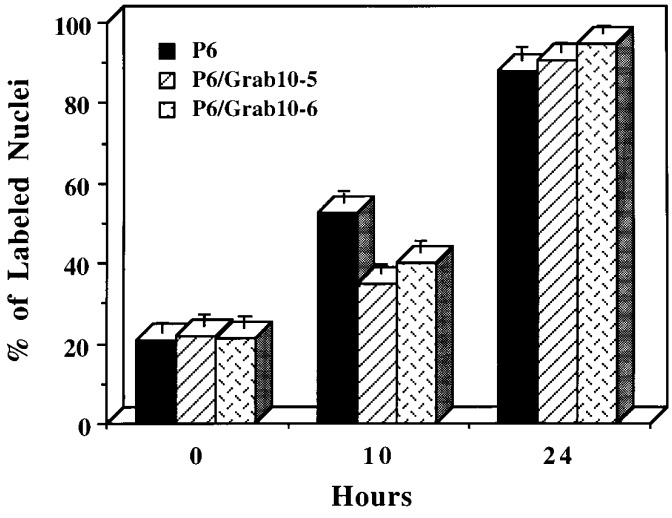

FIG. 5. **An overexpressed mGrb10α does not inhibit entry into S phase.** p6 cells and clones 5 and 6 of p6/Grb10 cells were made quiescent in serum-free medium and then stimulated with IGF-I (20 ng/ml) for the indicated times. [^{3}H]Thymidine was added to the medium with IGF-I, and the number of labeled cells determined by autoradiography.

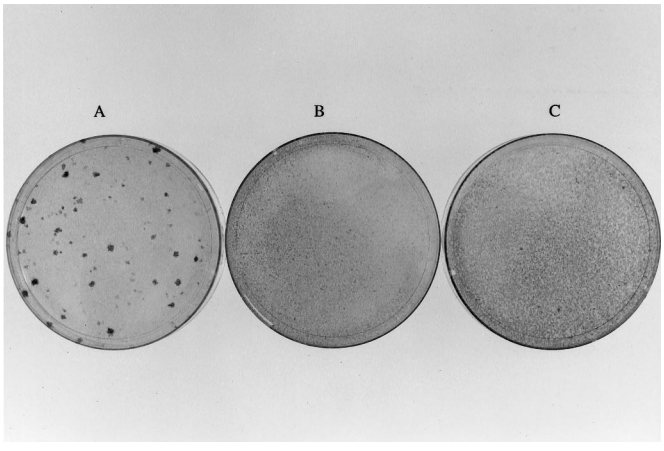

FIG. 6. **p6 cells overexpressing mGrb10α do not form foci in monolayers.** p6 cells (*panel A*) and p6/Grb10 clones 5 (*panel B*) and 6 (*panel C*) were plated in 10% serum. The photographs of the plates stained with crystal violet were taken after 14 days in culture.

but the three cell lines are indistinguishable from each other (*panels A* and *B*). In Fig. 8 (*panel C*), we tested for the possibility that Grb10 may compete with Grb2 for interaction with Shc (35). For this purpose, the blot of lysates immunoprecipitated with anti-Shc antibody, was stripped and reblotted with an antibody to Grb2. The amount of Grb2 bound to Shc is the same in the three cell lines. We also tested for a direct interaction of mGrb10α with Shc proteins on the same blot; no Grb10 band was detectable (data not shown).

DISCUSSION

The results of our experiments can be summarized as follows. 1) An overexpressed mGrb10α inhibits IGF-I-mediated growth; 2) partially reverses the transformed phenotype, but does not cause cells to undergo apoptosis; 3) has no effect on the growth of cells expressing a C-terminally truncated IGF-IR (lacking the last 108 amino acids); 4) has no effect on insulin-stimulated cell proliferation in cells overexpressing the IR; and 5) causes delays in the second half of the cell cycle, suggesting

TABLE I
Colony formation in soft agar of p6 and p6-derived cells

Cell line	No. of colonies[a]	Maximum size
		μm
p6	252/243	250
p6/V (empty vector)	253/260	200
p6/Grb10 clone 4	255/261	250
p6/Grb10 clone 5	13/10	125
p6/Grb10 clone 6	153/189	200

[a] The number of colonies (equal to or greater than 125 μm) was determined after 2 weeks.

TABLE II
Overexpression of mGrb10α does not cause apoptosis in p6 cells

Cells (5×10^5) were loaded into a diffusion chamber as described by Resnicoff *et al.* (30), and then implanted for 42 h in the subcutaneous tissues of rats. At that time, the chambers were removed from the animals and the cells were quantitatively recovered and counted. The number of recovered cells is expressed as percent of cells loaded into the chambers.

Cell line	Percentage of cells recovered
p6	350
p6/V2 (empty vector)	371
p6/Grb10 clone 4	355
p6/Grb10 clone 5	278
p6/Grb10 clone 6	340

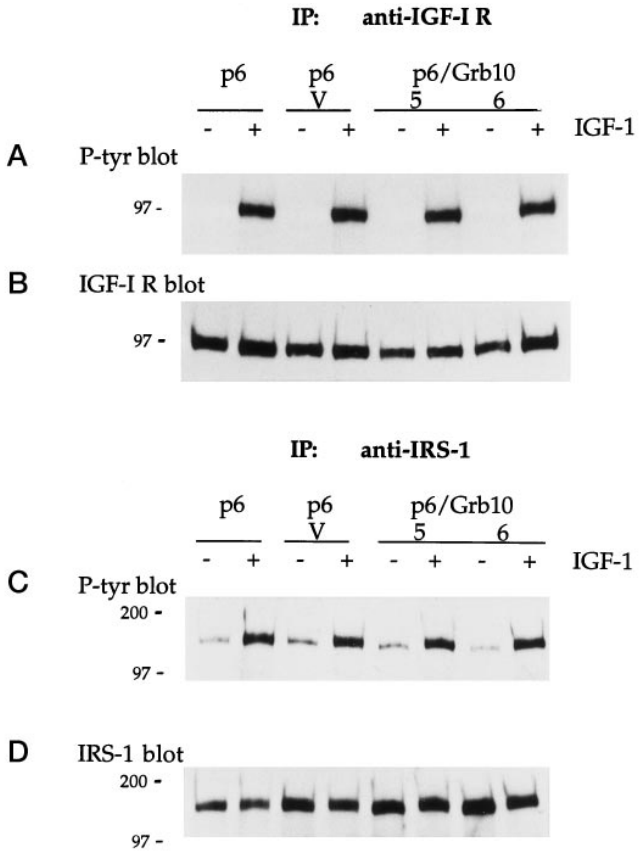

FIG. 7. **The phosphorylation of the IGF-I receptor and IRS-1 is not affected by an overexpressed mGrb10α.** Lysates of cells were immunoprecipitated with an antibody against the IGF-I receptor (*panels A* and *B*) or against IRS-1 (*panels C* and *D*), and blotted with a phosphotyrosine antibody or, after stripping the membrane, with the respective antibody. − or + indicate either serum-free medium or IGF-I (20 ng/ml) for 10 min. *P-tyr*, phosphotyrosine

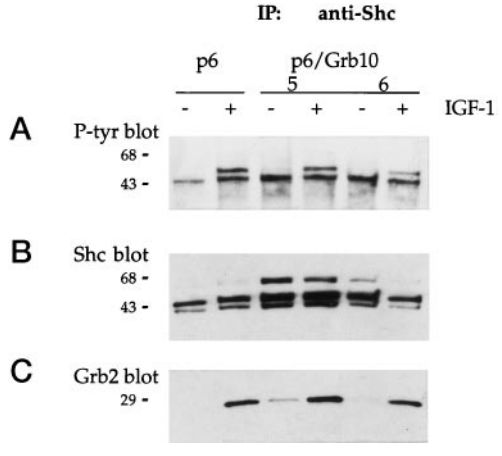

FIG. 8. **Tyrosyl phosphorylation of Shc proteins in p6 cells overexpressing mGrb10α.** Figure shows the same lysates as in Fig. 7, except that the antibody used for immunoprecipitation and Western was an anti-Shc antibody. The membrane was stripped and blotted again with an antibody anti-Grb2 (*panel C*) as described under "Materials and Methods." *P-tyr*, phosphotyrosine.

that its inhibitory action may be due to interference with progression through S phase and G_2. These findings are discussed separately.

Inasmuch as several reports have now appeared on the interaction of Grb10 proteins with either the IR or the IGF-IR, it may help the reader to summarize the Grb10 proteins so far published in Table III. The original Grb10 (16) is now called mGrb10α; it is known to interact both with the IR (18) and with the IGF-IR (19), and it is the one used in the present experiments. Of the others, hGrb10γ1 (23) also interacted with both receptors, whereas mGrb10ε (22) interacted only with the IR. Grb10 function has been investigated very little, in fact only with hGrb10β (17), hGrb10γ1 (23), and in the present paper. hGrb10β seemed to interfere with IRS-1 phosphorylation (17), but cell proliferation function was studied only by O'Neill *et al.* (23), and their findings are relevant to our paper. These authors found that a microinjected peptide acted as a dominant negative of hGRb10γ1, by inhibiting both insulin and IGF-I-mediated DNA synthesis, but not DNA synthesis stimulated by either epidermal growth factor or serum. The corollary was that wild type hGrb10γ1 must have a stimulatory effect on IGF-induced DNA synthesis. Our data with mGrb10α indicate an opposite conclusion. We have used several stably transfected clones, not only, but when Grb10 was poorly expressed, growth was not inhibited. Indeed, there is fair correlation between expression levels and inhibition of IGF-I-mediated cell proliferation. We have also repeated, substantially, the experiment of O'Neill *et al.* (23), and, under our conditions, an overexpressed mGrb10α does not inhibit entry into S phase after IGF-I stimulation. On the other hand, the experiments of O'Neill *et al.* (23) were also carefully controlled, the data were quite convincing, and the only reasonable conclusion is that these two Grb10, mGrb10α and hGrb10γ1, have different, in fact, opposite functions.

Although mGrb10α is known to interact both with the IR (18) and the IGF-IR (19), it inhibits only IGF-I-mediated mitogenesis, and has no effect on insulin-mediated mitogenesis (Fig. 3). It should be remarked that p6 and R^-/IR cells have approximately the same number of IGF-IRs and IRs, respectively, and expressed similar levels of Grb10 protein. It seems that mGrb10α signals differently from the two receptors. This may be due to the fact that, although mGrb10α seems to interact with the C terminus of either receptor (18, 19), these C termini have substantially different sequences (31), and also differ functionally (36, 37). The role of the C terminus is confirmed in the present experiments by the finding that mGrb10α does not inhibit the growth of 3T3-like cells expressing a truncated form of the IGF-IR (25), lacking the last 108 amino acids.

TABLE III
Interactions of Grb10 family of proteins

The nomenclature is the one agreed upon by the investigators in this field. ND, not done; m stands for mouse, h for human.

Nomenclature	Reference	Interaction with		Function
		IR	IGF-IR	
mGrb10α	Ooi *et al.* (16)	+	+	See this paper
hGrb10β	Liu and Roth (17)	+	ND	Inhibitory
hGrb10γ1	O'Neill *et al.* (23)	+	+	Stimulatory
hGrb10γ2	Frantz *et al.* (21)	+	ND	ND
mGrb10ε	Laviola *et al.* (22)	+	−	ND

Overexpression of mGrb10α partially reverses the transformed phenotype of p6 cells. We use the expression "partial reversion," because, although foci formation is abrogated, colony formation in soft agar is only partially inhibited, especially when compared with the effect of inhibition of the IGF-IR function, either by antisense strategies (38) or dominant negatives (34, 39), which result in a much higher inhibition of colony formation in soft agar. Compatible with this partial inhibition is our finding that overexpression of mGrb10α does not induce apoptosis, even in a very sensitive assay. Our previous findings with the IGF-IR have indicated that several mutants of the receptor (acting as dominant negatives) that reduce colony formation in soft agar are not sufficiently impaired to cause apoptosis (30, 33, 34).

Our results show that the inhibitory effect of mGrb10α is not the effect of a block in G_1, but, instead, of a delay in the progression of cells through S phase and G_2. This should not be surprising, because Sell *et al.* (7) have shown that the activated IGF-IR is required for cell cycle progression in each phase of the cell cycle, and not just in G_1. Accordingly, we found no inhibition of IRS-1 or Shc tyrosyl phosphorylation, which are usually associated with the transition from G_0 to S phase (40, 41). Because IGF-IR signaling in p6/Grb10 cells does not seem to be impaired in G_1, we have not insisted in extending our studies to transducing molecules downstream of these two IGF-IR substrates, IRS-1 and Shc. The delay in S phase/G_2 progression suggests to us that we must look at a different pathway, connecting the IGF-IR to the later phases of the cell cycle. This is not an easy task, inasmuch as very few clues are available on the identity of such a pathway; the only clue, in fact, comes from the observation that one of the cyclin-associated proteins, cdc2, is induced by IGF-I (42). Studies are in progress on the functional status of cyclins A and B and their associated proteins in p6/Grb10 cells *versus* the parental cell line.

In conclusion, we report here that overexpression of mGrb10α has an inhibitory effect on IGF-mediated mitogenesis and transformation. The discrepancy with the opposite results obtained by O'Neill *et al.* (23) and the differential interaction of another Grb10 with the IR and the IGF-IR (22) suggest an important conclusion, that different Grb10 proteins may have different functions, in fact, that some isoforms may be competitive inhibitors of other isoforms.

REFERENCES

1. Baserga, R., Resnicoff, M., D'Ambrosio, C., and Valentinis, B. (1997) *Vitam. Horm.* **53,** 65–98
2. Scher, C. D., Shephard, R. C., Antoniades, H. N., and Stiles, C. D. (1979) *Biochim. Biophys. Acta* **560,** 217–241
3. Stiles, C. D., Capone, G. T., Scher, C. D., Antoniades, N. H., Van Wyk, J. J., and Pledger, W. J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 1279–1283
4. Liu, J.-P., Baker, J., Perkins, A. S., Robertson, E. J., and Efstratiadis, A. (1993) *Cell* **75,** 59–72
5. Baker, J., Liu, J.-P., Robertson, E. J., and Efstratiadis, A. (1993) *Cell* **75,** 73–82
6. Ludwig, T., Eggenschwiler, J., Fisher, P., D'Ercole, J. P., Davenport, M. L., and Efstratiadis, A. (1996) *Dev. Biol.* **177,** 517–535
7. Sell, C., Dumenil, G., Deveaud, C., Miura, M., Coppola, D., DeAngelis, T., Rubin, R., Efstratiadis, A., and Baserga, R. (1994) *Mol. Cell. Biol.* **14,** 3604–3612
8. White, M. F., and Kahn, C. R. (1994) *J. Biol. Chem.* **269,** 1–4
9. Myers, M. J., Sun, X. J., Cheatham, B., Jachna, B. R., Glasheen, E. M., Backer, J. M., and White, M. F. (1993) *Endocrinol.* **132,** 1421–1430
10. Keller, S., and Lienhard, G. (1994) *Trends Cell Biol.* **4,** 115–119
11. Craparo, A., O'Neill, T. J., and Gustafson, T. A. (1995) *J. Biol. Chem.* **270,** 15639–15643
12. Araki, E., Lipes, M. A., Patti, M. E., Bruning, J. C., Haag, B., III, Johnson, R. S., and Kahn, C. R. (1994) *Nature* **372,** 186–190
13. Staubs, P. A., Reichart, D. R., Saltiel, A. R., Milarski, K. L., Maegawa, H., Berhanu, P., Olefsky, J. M., and Seely, B. L. (1994) *J. Biol. Chem.* **269,** 27186–27192
14. Seely, B. L., Reichart, D. R., Staubs, P. A., Jhun, B. H. Hsu, D., Maegawa, H., Milarski, K. L, Saltiel, A. R., and Olefsky, J. M. (1995) *J. Biol. Chem.* **270,** 19151–19157
15. Pronk, G. J., McGlade, J., Pelicci, G., Pawson, T., and Bos, J. L. (1993) *J. Biol. Chem.* **268,** 5748–5753
16. Ooi, J., Yajnik, V., Immanuel, D., Gordon, M., Moskow, J. J., Buchberg, A. M., and Margolis, B. (1995) *Oncogene* **10,** 1621–1630
17. Liu, F., and Roth, R. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10287–10291
18. Hansen, H., Svensson, U., Zhu, J., Laviola, L., Giorgino, F., Wolf, G., Smith, R. J., and Riedel, H. (1996) *J. Biol. Chem.* **271,** 8882–8886
19. Morrione, A., Valentinis, B., Li, S., Ooi, J. Y. T., Margolis, B., and Baserga, R. (1996) *Cancer Res.* **56,** 3165–3167
20. Dey, B. R., Frick, K., Lopaczynski, W., Nissley, S. P., and Furlanetto, R. W. (1996) *Mol. Endocrinol.* **10,** 631–641
21. Frantz, J. D., Giorgietti-Peraldi, S., Ottinger, E. A., and Shoelson, S. E. (1997) *J. Biol. Chem.* **272,** 2659–2667
22. Laviola, L., Giorgino, F., Chow, J. C., Baquero, J. A., Hansen, H., Ooi, J., Zhu, J., Riedel, H., and Smith, R. J. (1997) *J. Clin. Invest.* **99,** 830–837
23. O'Neill, T. J., Rose, D. W., Pillay, T. S., Hotta, K., Olefsky, J. M., and Gustafson, T. A. (1996) *J. Biol. Chem.* **271,** 22506–22513
24. Pietrzkowski, Z., Lammers, R., Carpenter, G., Soderquist, A. M., Limardo, M., Phillips, P. D., Ullrich, A., and Baserga, R. (1992) *Cell Growth Diff.* **3,** 199–205
25. Surmacz, E., Sell, C., Swantek, J., Kato, H., Roberts, C. T., Jr., LeRoith, D., and Baserga, R. (1995) *Exp. Cell Res.* **218,** 370–380
26. Miura, M., Surmacz, E., Burgaud, J.-L., and Baserga, R. (1995) *J. Biol. Chem.* **270,** 22639–22644
27. Gritz, L., and Davies, J. (1983) *Gene* (*Amst.*) **25,** 179–188
28. Sell, C., Rubini, M., Rubin, R., Liu, J.-P., Efstratiadis, A., and Baserga, R. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11217–11221
29. Sell, C., Baserga, R., and Rubin, R. (1995) *Cancer Res.* **55,** 303–306
30. Resnicoff, M., Abraham, D., Yutanawiboonchai, W., Rotman, H. L., Kajstura, J., Rubin, R., Zoltick, P., and Baserga, R. (1995) *Cancer Res.* **55,** 2463–2469
31. Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kahuria, S., Chen, E., Jakobs, S., Francke, U., Ramachandran, J., and Fujita-Yamaguchi, Y. (1986) *EMBO J.* **5,** 503–512
32. Morrione, A., Valentinis, B., Xu, S., Yumet, G., Louvi, A., Efstratiadis, A., and Baserga, R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3777–3782
33. Resnicoff, M., Burgaud, J.-L., Rotman, H. L., Abraham, D., and Baserga, R. (1995) *Cancer Res.* **55,** 3739–3741
34. D'Ambrosio, C., Ferber, A., Resnicoff, M., and Baserga, R. (1996) *Cancer Res.* **56,** 4013–4020
35. Sasaoka, T., Draznin, B., Leitner, J. W., Langlois, W. J., and Olefsky, J. M. (1994) *J. Biol. Chem.* **269,** 10734–10738
36. Lammers, R., Gray, A., Schlessinger, J., and Ullrich, A. (1989) *EMBO J.* **8,** 1369–1375
37. Hongo, A., D'Ambrosio, C., Miura, M., Morrione, A., and Baserga, R. (1996) *Oncogene* **12,** 1231–1238
38. Resnicoff, M., Sell, C., Rubin, R., Rubini, M., Coppola, D., Ambrose, D., Baserga, R., and Rubin, R. (1994) *Cancer Res.* **54,** 2218–2222
39. Burgaud, J.-L., Resnicoff, M., and Baserga, R. (1995) *Biophys. Biochem. Res. Commun.* **214,** 475–481
40. Waters, S. B., Yamauchi, K., and Pessin, J. E. (1993) *J. Biol. Chem.* **268,** 22231–22234
41. Rose, D. W., Satiel, A. R., Majumdar, M., Decker, S. J., and Olefsky, J. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 797–801
42. Surmacz, E., Nugent, P., Pietrzkosky, Z., and Baserga, R. (1992) *Exp. Cell Res.* **199,** 275–278