Insulin Receptor Substrate-1 Regulates the Transformed Phenotype of BT-20 Human Mammary Cancer Cells

Ozlem Dalmizrak,1 An Wu,1 Jia Chen,1 Hongzhi Sun,1 Fransiscus E. Utama,1 Diana Zambelli,2,3 Thai H. Tran,1 Hallgeir Rui,1 and Renato Baserga1

1Department of Cancer Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania and 2Istituto Ortopedici Rizzoli, Bologna, Italy

Abstract

Although originating from a human breast cancer, BT-20 cells do not form colonies in soft agar. BT-20 cells do not express insulin receptor substrate-1 (IRS-1), which is known to promote both normal and abnormal growth and to inhibit differentiation. Stable expression of IRS-1 confers to BT-20 cells the ability to form colonies in soft agar. BT-20 cells form tumors in xenografts in mice, but expression of IRS-1 is low or absent when the cells express IRS-1. The increased transformed phenotype is characterized by occupancy of the rDNA and cyclin D1 promoters by IRS-1 and the activation of the cyclin D1, c-myc, and rDNA promoters. In addition, the retinoblastoma protein, which is detectable in the rDNA promoter of quiescent BT-20/IRS-1 cells, is replaced by IRS-1 after insulin-like growth factor-I stimulation. Our results indicate that in BT-20 human mammary cancer cells, expression of IRS-1 increases colony formation in soft agar and tumorigenicity in mice. The increased transformation is accompanied by IRS-1 occupancy of the rDNA and cyclin D1 promoters; the increased transformed phenotype of colonies in soft agar (20) and xenografts in mice. Using these criteria, we show that IRS-1 expression is often increased in human cancer (3), and overexpression or ectopic expression of IRS-1 causes cell transformation, including the ability to form colonies in soft agar and tumors in mice (4, 5).

Introduction

Insulin receptor substrate-1 (IRS-1) is a docking protein for both the insulin receptor and type I insulin-like growth factor receptor (IGF-IR). It binds to and activates phosphatidylinositol 3-kinase (PI3K) and, thus, plays an important role in signal transduction from the two receptors (1). IRS-1, especially when activated by the IGF-IR, sends a strong mitogenic, antiapoptotic, and antidermatization signal (1, 2). IRS-1 expression is increased in human cancer (3), and overexpression or ectopic expression of IRS-1 causes cell transformation, including the ability to form colonies in soft agar and tumors in mice (4, 5). When IRS-1 levels are decreased by experimental procedures (antisense or small interfering RNA), cancer cells lose their transformed phenotype (6–8). IRS-1 levels are low or even absent in differentiating cells (2, 9, 10), and ectopic expression of IRS-1 inhibits differentiation (4, 10). IRS-1 translocates to nuclei and nucleoli (11, 12), where it binds the upstream binding factor 1 (UBF1; refs. 13, 14), a protein that participates in the regulation of rRNA synthesis (15). Nuclear translocation of IRS-1 has been reported in tissue sections of human breast cancer (16, 17) and human medulloblastoma (11).

BT-20 cells originated from a human breast cancer, but they do not form colonies in soft agar or form only a few small ones (5, 18). BT-20 cells do not express IRS-1 (5) although they do express IRS-2 (see below). The absence of IRS-1 expression makes BT-20 cells an attractive model to test the mechanisms by which IRS-1 promotes cell transformation in mammary cancer cells. We have compared parental BT-20 cells to three BT-20–derived cell lines, all expressing IRS-1 by stable transfection with plasmids or retroviruses. BT-20/103 cells express modest amounts of IRS-1; BT-20/159 cells express high levels; and BT-20/NLS/IRS1 express an IRS-1 with a nuclear localization signal (19). We have used as criteria for transformation formation of colonies in soft agar (20) and xenografts in mice. Using these criteria, we show that, regardless of levels or localization, expression of IRS-1 in BT-20 cells increases colony formation in soft agar and tumorigenicity in mice. The increased transformation is accompanied by IRS-1 occupancy of the rDNA and cyclin D1 promoters; the dramatic activation of the rDNA, cyclin D1, and c-myc promoters; and the displacement by IRS-1 of the retinoblastoma tumor suppressor protein (pRb) from the rDNA promoter.

Materials and Methods

Cell lines and cell culture. BT-20 breast cancer cells were obtained from Eva Surfmac (Temple University, Philadelphia, PA) and cultured routinely in DMEM/Nutrient Mixture F-12 (Ham) 1:1 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% calf serum, glutamine, and antibiotics at 37°C in 10% CO2 atmosphere. Media were supplemented with 2 μg/ml ampicillin for BT-20/NLS/IRS1 cells, 20 μg/ml hygromycin for BT-20/103 cells, and 0.5 μg/ml puromycin for BT-20/159 cells.

Stable transfection of BT-20 cells with the NLS-IRS-1 plasmid. Cells were transfected with pCMV/myc/nuc plasmid (Invitrogen, Carlsbad, CA) carrying a wild-type mouse IRS-1 cDNA at the Xho1/Not1 restriction site by electroporation. Nucleofector device and solutions were used (Amaxa, Gaithersburg, MD) using the program recommended by the manufacturer for this cell type.

Retroviral transduction of BT-20 cells with pGR103 and pGR159 plasmids. pGR103 and pGR159 plasmids were used for retroviral transduction of BT-20 cells. Plasmid pGR103 is a pMSCVhyg plasmid carrying wild-type mouse IRS-1 cDNA with its 3′ untranslated region (3′-UTR). Plasmid pGR159 is based on a self-inactivated form of the MSCV retroviral vector system and contains an internal cytomegalovirus promoter, a puromycin resistance gene, and wild-type mouse IRS-1 cDNA lacking the 3′-UTR (21). Transduction was carried out as previously described (21).

Cell growth and colony formation in soft agar. For monolayer growth, quiescent BT-20, BT-20/NLS/IRS1, BT-20/103, and BT-20/159 cells were stimulated with serum or with 20 ng/ml IGF-I (Invitrogen). Cell numbers were determined in triplicate after 24, 48, and 72 h by counting only the cells able to exclude trypan blue. Anchorage-independent growth was determined as previously described (5).

Xenografts. Animal experiments were done under approved Institutional Animal Care and Use Committee protocols. Intact female NCR athymic
nude mice (N = 21; 7 mice per group; 8–9 weeks of age; obtained from Taconic, Hudson, NY) were inoculated s.c. with human BT-20, BT-20/159, or BT-20/IGF-IR/IRS1 cells (10^6 suspended in 200 μL of 50% Matrigel (BD Biosciences, Bedford, MA) and 50% DMEM/F-12 (Invitrogen)) into each of two dorsolateral sites. Tumor growth was measured twice a week using a caliper and volume was calculated as 0.5 x (length x width x height), as described in detail by Tomayko and Reynolds (22).

Western blots. Western blots were carried out by standard procedures, as detailed in previous articles from this laboratory (4, 5, 13, 23).

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation assays were carried out according to the manufacturer's instructions (Upstate, Lake Placid, NY) and the methods described by Chen et al. (19). The primers used were as follows: rDNA promoter forward, 5'-TGCTCTGTTGGGTACGAC-3'; reverse, 5'-TGGCAGAGGACCAGGTG-3'. Cyclin D1 forward, 5'-CGGACTACAGGGGAGTTTGTC-3'; reverse, 5'-TCCAGCATCACGGTGGCAGAT-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-TACTAGCGTTTTACGGGCG-3'; reverse, 5'-TCCAGCATCACGGTGGCAGAT-3'. For chromatin immunoprecipitation of pRb, we followed the procedure of Jackson and Pereira-Smith (24). Epitope Tag Safe PCR System (Epicentre, Madison, WI) was used for PCR. The amplification products were analyzed in a 2% agarose gel and visualized by SYBR Gold (Molecular Probes, Eugene, OR) staining. Gels stained with SYBR Gold were scanned by using Typhoon Laser scanner (Typhoon 9400 Variable Mode Imager, Amersham Biosciences, Piscataway, NJ).

Luciferase reporter assay. To determine the activation of cyclin D1 [25] and c-myc [26] promoters by IGF-I, full-length p3A-Luc (obtained from Dr. Richard G. Pestell, Thomas Jefferson University, Kimmel Cancer Center, Philadelphia, PA) and pBv-Luc (a kind gift of Dr. Bert Vogelstein, Howard Hughes Medical Institute and Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins Medical Institutions, Baltimore, MD) constructs were used, respectively. Cells (2 x 10^5) were transiently transfected with 1 μg/well reporter plasmid by using nucleofector (Amaxa). To test transfection efficiency, cells were cotransfected with 50 ng/well of pRL-TKLuc, a plasmid encoding Renilla luciferase (Promega, Madison, WI). The values were normalized to pRL-TKLuc to generate relative luciferase unit.

rDNA promoter activity. The activity of the rDNA promoter was measured as described by Wu et al. (23).

Glutathione S-transferase pulldown assays. Glutathione S-transferase (GST) pulldown assays were carried out as previously described (19) For IRS-1, we synthesized GST constructs containing the following sequences: 1 to 300; 301 to 700; 701 to 1,000; and 1,000 to 1,234. A similar approach was used to generate UBF1 GST constructs. All plasmid constructs were confirmed by DNA sequencing and protein expression to guarantee accuracy. The proteins that coprecipitated with the GST fragments were analyzed by SDS-PAGE followed by immunoblotting with the appropriate antibodies.

Immunoprecipitation. Five hundred micrograms of total protein extract were used for immunoprecipitation. IRS-1 was immunoprecipitated with anti-IRS-1 polyclonal antibody (Upstate) overnight. Antibody-IRS-1 complexes were collected with Protein G plus/Protein A agarose suspension and washed thrice with HNTG buffer [20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 0.1% Triton X-100, 10 mmol/L EDTA, 1 mmol/L EGTA, 10 μg/mL Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride], then resuspended in Laemmli sample buffer (Bio-Rad, Hercules, CA) and denatured for 10 min. Proteins were separated on 4% to 15% polyacrylamide gradient gels (Bio-Rad) and electrobolotted onto nitrocellulose filters. IRS-1 tyrosine phosphorylation was detected with anti-PY20 horseradish peroxidase–conjugated antibody (Transduction Laboratories, Lexington, KY). Membranes were incubated with stripping buffer (Pierce, Rockford, IL) and reprobed with anti-IRS-1 antibody (Upstate). Further details used in our laboratory are given in ref. 23.

Antibodies. Antibodies used were anti-IRS-1 and anti-IRS-2 [rabbit immunopositivity-purified immunoglobulin G (IgG); Upstate], anti-actin (Sigma, Sigma-Aldrich Co., St. Louis, MO), antihuman Rb, anti–SV40 large T antigen (BD Biosciences Pharmingen), peroxidase goat anti-rabbit IgG (Oncogene, La Jolla, CA), and donkey anti-rabbit IgG-FITC sc-2090 for confocal microscopy (Santa Cruz Biotechnology, Santa Cruz, CA).

Results

IRS-1 expression. The expression of IRS-1 in BT-20 and BT-20–derived cell lines is shown in Fig. 1. Parental BT-20 cells do not express IRS-1, confirming previous results from Castles et al. (18) and DeAngelis et al. (5). BT-20/103 cells are transduced with a retrovirus expressing an IRS-1 cDNA with its 3'-UTR, whereas BT-20/159 cells express an IRS-1 cDNA without its 3'-UTR. The presence of the normal 3'-UTR causes a marked reduction in the expression of IRS-1 (almost 50-fold), suggesting the possibility of microRNAs regulating the expression levels of IRS-1. This possibility is being explored but will not be considered further in this article, in which the difference in expression between the two cell lines is the relevant finding. BT-20 cells expressing an IRS-1 with a NLS show low levels of expression, a finding we had already noted before (19). IGF-I clearly causes tyrosine phosphorylation of IRS-1 (Fig. 1B), Fig. 1B also shows that IRS-2 is expressed in parental BT-20 cells (bottom).

Growth of BT-20–derived cells. The cell lines of Fig. 1 were examined for their ability to grow in monolayer, in 1% or 10% serum, or in IGF-I. Serum definitely increases growth in all cell lines, with 10% serum being slightly better than 1% serum (Fig. 2A). The cells respond less to IGF-I probably because the levels of IGF-IR in parental BT-20 cells are low. IRS-1 levels do not seem to affect the response to serum, which is determined by IGF-IR levels (27) and the presence of other growth factors. Figure 2B shows that colony formation in soft agar is dramatically different from monolayer growth. Whereas parental BT-20 cells form only a
few colonies in soft agar, the BT-20 cells expressing IRS-1 form a significant number of colonies, which are increased by IGF-I supplementation. The levels or subcellular localization of IRS-1 are unimportant; provided the cells express IRS-1, they form colonies in soft agar. The method we use for colony formation in soft agar is very stringent because we seed 2,000 cells per plate and only count colonies >125 \( \mu \text{m} \) in diameter after 3 weeks, as proposed in the original article (28). Parental BT-20 cells actually form very small colonies in soft agar, <125 \( \mu \text{m} \) in diameter, which are excluded from our stringent criteria. It is interesting that IGF-I, which is so weak in monolayer growth, can actually double the number of colonies in soft agar cells expressing IRS-1.

**Tumor formation in nude mice.** We next determined the growth of BT-20–derived cells in nude mice. Parental BT-20 and BT-20 cells expressing IRS-1 (two clones) were tested for their ability to form tumors in nude mice (22). Tumor volumes were measured at various times after s.c. injection and the results are summarized in Fig. 3. BT-20 cells expressing IRS-1 form tumors that are twice as large as the tumors formed by parental BT-20 cells.

Because of the differences between monolayer growth and growth in soft agar or nude mice, we set out to investigate the mechanisms by which IRS-1 increases the transformed phenotype of BT-20 mammary cancer cells.

**Chromatin immunoprecipitation of the rDNA promoter.** Chen et al. (19) have shown that IGF-I can cause occupancy of the rDNA promoter by IRS-1. We have confirmed these results in BT-20–derived cells. Figure 4A shows a time course of IGF-I stimulation on the occupancy of the rDNA promoter. UBF is always present in the rDNA promoter (even in quiescent cells, there is always a moderate rRNA synthesis), whereas IRS-1 occupancy is detectable only after 8 h of stimulation with IGF-I. This result is compatible with the original data by Tu et al. (13) that IRS-1 appears in the nuclei 8 h after stimulation, peaking at ~16 h. Figure 4 also shows the negative controls (we have omitted from the figure the control with GAPDH).

**Chromatin immunoprecipitation of the cyclin D1 promoter.** A similar experiment to the one described above for the rDNA promoter was carried out for the cyclin D1 promoter (Fig. 4B). Interestingly, UBF and IRS-1 are not detectable in the cyclin D1 promoter of BT-20–derived cells unless stimulated with IGF-I. Both proteins appear in the cyclin D1 promoter 8 h after stimulation with IGF-I. IRS-1 is absent from the cyclin D1 promoter of parental BT-20 cells, as expected.

**Effect of nuclear IRS-1 on the activity of the cyclin D1, c-myc, and rDNA promoters.** We next wanted to determine whether occupancy by IRS-1 of the rDNA and cyclin D1 promoters also had a functional effect on their activity. We added in this experiment the c-myc promoter. Parental BT-20 cells, BT-20 NLS-IRS1 cells, and BT-20/159 cells were transiently and separately transfected with two plasmids. The first has the cyclin D1 promoter, and the second has the c-myc and rDNA promoters.

![Figure 2](image-url)
driving luciferase (25); in the second plasmid (26), luciferase is driven by the c-myc promoter (see Materials and Methods). The presence of a nuclear IRS-1 markedly increases the activity of both cyclin D1 (Fig. 5A) and c-myc (Fig. 5A) promoters. The activity of the c-myc promoter peaks earlier than that of the cyclin D1 promoter, at 16 versus 24 h (Fig. 5).

For the activity of the rDNA promoter, we used the reporter gene described by Wu et al. (23), in which the rDNA promoter drives the expression of an SV40 T antigen. The plasmid was transiently transfected into parental BT-20 cells, BT-20/159 cells, and R+ cells that served as a positive control. IGF-I induces expression from the rDNA promoter in BT-20/159 cells but not in parental BT-20 cells (Fig. 5B). The levels of expression are comparable to those of R+ cells, a line of mouse embryonic fibroblasts in which IGF-I causes nuclear translocation of IRS-1 and increased RNA synthesis (13).

Activation of the cyclin D1 and c-myc promoters by serum. The experiments in Fig. 5A and B were done with cells stimulated by IGF-I. In the colony-forming assay, cells are in 10% serum, supplemented or not with IGF-I (Fig. 2). We therefore repeated the experiments by stimulating the cells with 10% serum. The results (Fig. 5C) were essentially the same. Serum activated both the cyclin D1 and c-myc promoters in BT-20/159 cells (expressing IRS-1) much more than in parental BT-20 cells.

IRS-1 and pRb in the rDNA promoter. It has been reported that pRb can be found in the nucleus of quiescent or differentiating cells, where it binds UBF1 and causes a decrease in transcription from the rDNA promoter (29–31). It has also been reported that pRb binds to the pRb binding motif (LxCxE) of UBF1 (29), but subsequent reports have indicated that pRb binds to a different sequence of UBF1 (30). We first determined whether IRS-1 and pRb competed directly for binding to UBF1, and then we tested whether the expression of IRS-1 inhibited the occupancy of the rDNA promoter by pRb. We already knew indirectly that IRS-1 binds to UBF1 by its PHPTB domain (14). Figure 6A confirms directly that the PHPTB domain of IRS-1 is sufficient to immunoprecipitate UBF1 from cell lysates. We used the GST method (see Materials and Methods) to identify the UBF1 sequences required for IRS-1 binding. The NH2-terminal sequences were not necessary for binding of IRS-1 to UBF1 (not shown), which required instead the sequences located between residues 301 and 388 (Fig. 6B). Notice that in Fig. 6B, we also tried to identify the UBF1 sequences that bind PI3K, which phosphorylates UBF1 (32). PI3K binds the UBF1 sequences between residues 389 and 475. The GST experiments with pRb were not clear-cut as with IRS-1, but Fig. 6C shows that pRb binds preferentially to UBF1 sequences between 1 and 280, different therefore from the sequences binding IRS-1. We then proceeded to test the hypothesis that IRS-1 expression inhibits rDNA promoter occupancy by pRb.

The results (Fig. 6D) show that pRb is detectable in the rDNA promoter in parental BT-20 cells even after stimulation with IGF-I. In BT-20/159 and BT-20/NLS/IRS1 cells, pRb is detectable in the
The transformed phenotype of cell lines is characterized by a number of features of graded importance. According to the literature (20), the decreased requirement for growth factors (or serum) is the first step in transformation, followed by loss of contact inhibition (foci in monolayer cultures), acquisition of anchorage independence (colony formation in soft agar), and finally the ability to form tumors in experimental animals. We have used colony formation in soft agar and xenografts in mice to test whether the expression of IRS-1 could modulate the transformed phenotype of BT-20 human mammary cancer cells. In addition, we have investigated some of the mechanisms by which IRS-1 can increase the transformed phenotype. We have used BT-20 cells because parental cells do not express IRS-1 (although they express IRS-2). The use of BT-20 cells is therefore very convenient for studying how IRS-1 expression affects the transformed phenotype.

Our results can be briefly summarized as follows: (a) expression of IRS-1 in BT-20 cells has very little effect on their ability to grow in monolayers, whether in serum or IGF-I, but it has a profound effect on their ability to form colonies in soft agar; (b) expression of IRS-1 increases the growth of BT-20 tumors in xenografts in mice; (c) IRS-1 in these cells translocate to the nuclei and can be found, by chromatin immunoprecipitation, in the rDNA and cyclin D1 promoters; (d) nuclear IRS-1 causes a marked activation of the rDNA, cyclin D1, and c-myc promoters, genes that are known to be involved in cellular proliferation; (e) the pRb protein, which binds and inhibits UBF1 (see above), is found in the rDNA promoter of quiescent cells, but it disappears from the promoter on stimulation by IGF-I and occupancy of the promoter by IRS-1; (f) although both pRb and IRS-1 bind to UBF1, they use different binding sites on the UBF protein. Whereas the effect of IRS-1 on the transformed phenotype is purely confirmatory, the mechanism involved (occupancy and activation of selected promoters) is novel.

There is an extensive literature on IRS-1 and its ability to promote growth (1, 2). IRS-1, activated by either the insulin

Discussion

Figure 5. Activation of the cyclin D1, c-myc, and rDNA promoters in BT-20 cell lines. The indicated cell lines were transiently transfected (see Materials and Methods) with reporter plasmids driving luciferase. A, top row, the plasmid was pA3-Luc, where the cyclin D1 promoter drives luciferase; bottom row, the luciferase-driving promoter was the c-myc promoter pBV. For each cell line, the columns are zero time, 8, 16, and 24 h after IGF-I. Luciferase activity was measured at the indicated hour after transfection and stimulation with IGF-I (50 ng/mL). B, activation of the rDNA promoter. Parental BT-20 cells (lanes 1–4), BT-20/159 cells (lanes 5–8), and R+ cells (lane 9) were transfected in transient with the appropriate plasmid (see text) and were left unstimulated or were stimulated with IGF-I for the times (in hours) indicated above the lanes. Western blot with an antibody to SV40 T antigen, driven by the rDNA promoter. The cell lines and the times (in hours) after stimulation with IGF-I are indicated above the lanes. C, activation of the cyclin D1 and c-myc promoters by serum. Parental BT-20 cells and BT-20/159 cells were made quiescent in serum-free medium and were then stimulated with 10% serum for the indicated times. The activity of the cyclin D1 (top) and c-myc (bottom) promoters was then determined by the luciferase assay as in (A).

rDNA promoter at zero time, but disappears after stimulation with IGF-I, when IRS-1 is detectable in the rDNA promoter.
The IRS-signalling system: a network of cell proliferation

Abrogation of the transformed phenotype (6–8). IRS-1 plays a major role in the regulation of cell size (2, 4, 37), and deletion of IRS-1 genes in mice (38) or *Drosophila* (39) causes a 50% reduction in body size. The reduction in body size is due both to decreased cell size and decreased cell number (40). Ectopic expression of IRS-1 doubles the size of myeloid cells (4, 37). This effect on cell size is explained by the finding that IRS-1 activates UBF1 (13, 14, 23), a protein that regulates the activity of RNA polymerase I and, therefore, cell size (15).

Our communication, however, uncovers another aspect of IRS-1, specifically its ability to promote growth by translocating to the nuclei and acting directly on the rDNA, c-myc, and cyclin D1 promoters. Nuclear translocation of IRS-1 has been reported in cells in culture (12–14) and *in vivo* in medulloblastomas (11), human breast cancer (16, 17), and rat liver (41). We show here that IRS-1 can actually be found in the rDNA and cyclin D1 promoters. Its presence there, and presumably in the c-myc promoter, causes a marked activation of the three promoters, whether by IGF-1 or by serum.

There are many reports of growth factors, growth factor receptors, and signal transduction molecules accumulating in the nuclei. Some of them, such as the epidermal growth factor receptor, fibroblast growth factor (FGF)-2, and FGF receptor-1, may act as transcriptional cofactors [refs. 42–45; reviewed by Massie and Mills (46)]. In the experiments of Sheng et al. (47), FGF-2 was shown to activate the rDNA promoter in a way similar to our demonstration that IRS-1 activates it. Linggi et al. (48) found a nuclear fragment of ErbB-4 with an active tyrosine kinase activity. The finding that IRS-1 binds to UBF1 and stimulates rRNA synthesis (13, 14) is in itself a strong suggestion of IRS-1 acting as a transcriptional cofactor. In fact, it is not surprising that IRS-1 moves to the promoters of genes that it activates. A recent report by Pokholok et al. (49) has indicated that, in yeast, activated signal transduction kinases can be found in the promoters of genes they regulate. This pattern may be more common than it is at present realized.

In conclusion, we have shown that in BT-20 mammary cancer cells, IRS-1 expression markedly increases the transformed phenotype, and that this effect is accompanied by occupancy and activation of IRS-1. From our data, IRS-1 seems to be a good candidate for targeting if one wishes to decrease cell proliferation and increase differentiation. Whereas IRS-1 acts usually through PI3K, the advantage of targeting IRS-1 is that abrogation of IRS-1 expression inhibits proliferation and favors differentiation of normal cells but does not necessarily kill them, whereas abrogation of the catalytic subunit of PI3K is a lethal phenotype (50).

The significance of these findings is that IRS-1 seems to be an important role of IRS-1 in growth and transformation, especially of a nuclear IRS-1. The presence of IRS-1 in the rDNA and cyclin D1 promoters (and also in the c-myc promoter) suggests the possibility that IRS-1 in the nuclei may act as a cotranscriptional cofactor for both RNA polymerase I– and RNA polymerase II–directed transcripts. The results of Fig. 6 suggest an explanation. The literature has shown that pRb can bind UBF1 and inhibit it (see above) whereas IRS-1 binds also to UBF1 but activates it (13, 14). We show here that, as expected, pRb can be found in the rDNA promoter of BT-20 cells. In BT-20 cells expressing IRS-1, pRb is present in the rDNA promoter only at zero time, and disappears after IGF-I stimulation (which causes occupancy by IRS-1; see Fig. 4).

The significance of these findings is that IRS-1 seems to be a good candidate for targeting if one wishes to decrease cell proliferation and increase differentiation. Whereas IRS-1 acts usually through PI3K, the advantage of targeting IRS-1 is that abrogation of IRS-1 expression inhibits proliferation and favors differentiation of normal cells but does not necessarily kill them, whereas abrogation of the catalytic subunit of PI3K is a lethal phenotype (50).

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