The upstream binding factor 1 (UBF1) is one of the proteins in a complex that regulates the activity of RNA polymerase I, which controls the rate of ribosomal RNA (rRNA) synthesis. We have shown previously that insulin receptor substrate-1 (IRS-1) can translocate to the nuclei and nucleoli of cells and bind UBF1. We report here that activation of the type I insulin-like growth factor receptor (IGF-IR) by IGF-I increases transcription from the ribosomal DNA (rDNA) promoter in both myeloid cells and mouse fibroblasts. The increased activity of the rDNA promoter is accompanied by increased phosphorylation of UBF1, a requirement for UBF1 activation. Phosphorylation occurs on a number of UBF1 peptides, most prominently on the highly acidic, serine-rich C terminus. In myeloid cells (but not in mouse embryo fibroblasts) IRS-1 signaling stabilizes the levels of UBF1 protein. These findings demonstrate that IGF-IR signaling can increase the activity of UBF1 and transcription from the rDNA promoter, providing one explanation for the reported effects of the IGF/IRS-1 axis on cell and body size in animals and cells in culture.

The upstream binding factor 1 (UBF1) is part of the complex of proteins that regulate the activity of RNA polymerase I at the ribosomal DNA (rDNA) promoter. RNA polymerase I controls the rate of rRNA synthesis and ribosome biogenesis (1–6). UBF1 must be activated to increase transcription from the rDNA promoter (1), and the general consensus is that activation of UBF1, resulting in increased transcription from the rDNA promoter, is largely regulated by phosphorylation, especially of its C terminus (8, 9). Other phosphorylated residues of UBF1 have been reported in the literature: serine 388 (9) and serine 484 (10) after serum, and threonine 117 and 201 (11) after EGF. UBF1 phosphorylation and activity increase in proliferating cells, where rRNA synthesis is known to be increased markedly over quiescent, resting cells (3, 10, and 12). Variations in UBF levels in cardiac myocytes and fibroblasts in culture have also been reported (13–15), suggesting that an alternative way of regulating UBF1 activity could be by decreasing or increasing its levels. We and others have reported recently that insulin receptor substrate-1 (IRS-1), a docking protein of the insulin and IGF-I receptors (16), can translocate to the nuclei and nucleoli of cells (17–20), where it binds UBF1 (21, 22). The binding of IRS-1 to UBF1 suggested a molecular explanation for the role of IRS-1 in regulating cell and body size (23, 24; see “Discussion”) because an activated UBF1 increases transcription from the rDNA promoter (1). However, binding to UBF1 does not necessarily lead to increased RNA polymerase I activity. There are at least three proteins that bind UBF1 and actually inhibit transcription from the rDNA promoter. These proteins are the retinoblastoma proteins (25–27), the p53 protein (28), and the interferon-inducible p204 nuclear protein (29). Growth-regulated phosphorylation of UBF has already been demonstrated (8), but the stimulatory factor in most cases was serum (10), and little is known of the mechanisms by which IGF-IR signaling may regulate UBF1 activity and rDNA transcription. IRS-1 has no known kinase activity, but we have reported recently that nuclear IRS-1 binds to the p85 regulatory subunit of PI3K, which directly phosphorylates UBF1 through its catalytic subunit (30). PI3K is strongly stimulated by IRS-1 (16, 31).

The purpose of this investigation was to study whether IGF-IR signaling can activate UBF1 and the rDNA promoter, and how it accomplishes an eventual activation. We used two different cell lines for the reasons given below: a myeloid cell line (32D cells) and mouse embryo fibroblasts (MEFs). We found that IGF-I up-regulates UBF1 and rDNA promoter activity and that the regulation is complex. In both myeloid cells and MEFs, IGF-I stimulation induces UBF1 phosphorylation. In MEFs, the C terminus and other peptides have been identified as phosphorylated by IGF-I. In myeloid cells (but not in MEFs), an activated IGF-IR and IRS-1 stabilize UBF1 protein levels. The importance of these studies resides in the fact that IRS-1/IRS-1 signaling controls cell and body size (about 50%) in animals and cells in culture (23, 32–36). Our findings provide new information on the molecular links between IRS-1 (21, 22) and cell growth (12).

**EXPERIMENTAL PROCEDURES**

**IRS-1 Mutants and Miniribosome Genes—** PHPTB IRS-1 comprises only the PH and phosphotyrosine binding (PTB) domains of IRS-1, approximately the first 300 amino acids (18). The miniribosome gene (see below) has been described by Voit and Grummt (9). A second reporter gene, in which the rDNA promoter drives the SV40 T antigen, has been described by Sturnaz and Col. (37).

**Cell Lines**—The original R gene (I) cells are 3T3-like fibroblasts (MEFs) generated from mouse embryos with a targeted disruption of the IGF-IR gene (38). R/T cells are R cells expressing the SV40 T antigen, whereas R cells are R cells expressing abundant copies of the human IGF-IR. These three cell lines were described in the original paper by Sell et al. (39). For experiments, MEFs are generally made quiescent in serum-free medium (SFM) and subsequently stimulated with IGF-I (50 ng/ml, unless otherwise indicated). In other experiments, we used 32D-
derived cells, 32D cells are murine myeloid cells that require Interleukin-3 (IL-3) for growth and undergo apoptosis when IL-3 is removed. The 32D-derived cells we used are described under “Results.”

Phosphorylation of UBF1—Western Blots—For Western blots, we used the following antibodies: anti-IRS-1 (Upstate Biotechnology, Inc., Lake Placid, NY), anti-IRS-1 (C-ter, Santa Cruz Biotechnology, Inc.), anti-UBF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-IRS-1 (Upstate Biotechnology, Inc., Lake Placid, NY), anti-IRS-1 (C-ter, Santa Cruz Biotechnology, Inc.), anti-UBF (Santa Cruz Biotechnology, Inc.), anti-IRS-1 (Upstate Biotechnology, Inc., Lake Placid, NY), anti-IRS-1 (C-ter, Santa Cruz Biotechnology, Inc.). Secondary antibodies were peroxidase goat anti-rabbit IgG (Oncogene Science Inc., Manhasset, NY) and peroxidase goat anti-mouse IgG (Oncogene). The Western blotting detection reagent was ECLTM (Amersham Biosciences).

RESULTS

The purpose of these experiments was to study the mechanisms by which IRS-1 regulates UBF1 activity. Because the IGF-IR itself is also involved in the control of cell and body size (see “Discussion”), it was necessary to determine the respective contributions of the receptor and IRS-1 in regulating UBF1 activity and the rDNA promoter. For this reason, we had to use two different cell culture systems. 32D-derived cells have the advantage that the parental cells do not express IRS-1 or IRS-2 (43, 44) and can therefore be used to distinguish between the effects of the IGF-IR and those of IRS-1 on UBF1 activity. 32D cells are myeloid cells that require IL-3 for growth and undergo apoptosis upon removal of IL-3 (45–47). When expressing the human IGF-IR (32D IGF-IR cells), they survive and grow for 48 h when shifted from IL-3 to IGF-I, then they stop growing and undergo granulocytic differentiation (44). Ectopic expression of IRS-1 in 32D IGF-IR cells (32D IGF-IR/IRS-1 cells) inhibits differentiation, and the cells grow indefinitely in the absence of IL-3 (35). Unfortunately, 32D cells are fastidious cells that grow in suspension, have very little cytoplasm, and present a number of problems when one wishes to purify nuclei or to collect large amounts of proteins. MEFS, like R- derived cells, are more suitable for these purposes. R- cells are 3T3-like MEFS (39) originating from mouse embryos with a targeted disruption of the IGF-IR genes (38). R- and R- cells are derived from R- cells. R- cells express the SV40 T antigen (39), whereas R- cells express high levels of a human IGF-IR (21). All three R- cell types express substantial levels of IRS-1 (48), which is largely nuclear in R- cells stimulated with IGF-I and in R-/T cells and cytoplasmic in R- cells (21, 22). It should be noted that nuclear translocation of IRS-1 and its binding to UBF1 have been shown to occur both in 32D-derived cells (18)
and in MEFs (21, 22). In the following experiments, we therefore switched from one model to the other, depending on the question asked.

Activation of the rDNA Promoter—Because activation of UBF1 is part of the mechanisms leading to the activation of the rDNA promoter (7, 9), the first question we asked was whether IGF-I increased transcription from the rDNA promoter. For this purpose, we used two different reporter systems. The first reporter was the miniribosome gene constructed by Grummt and co-workers (10), which is a good measure of rDNA transcription. The miniribosome gene was transiently transfected into the appropriate cell lines, and its activity was determined as described under “Experimental Procedures.” We transfected the miniribosome gene construct into a pool of R− cells that were then divided into two halves, unstimulated and stimulated with 50 ng/ml IGF-I. The results of a representative experiment are shown in Fig. 1A, where the determination was done on cells 24 h after stimulation. Transcription from the rDNA promoter increases after IGF-I stimulation. At this time after stimulation of R− cells, IRS-1 is still predominantly in the nuclei (21). We also compared the activity of the rDNA promoter in R− cells in SFM and 24 h after stimulation with IGF-I. This is also shown in Fig. 1A. The rDNA promoter is inactive in R− cells, and its activity does not increase after IGF-I. When quantitated by densitometry, the difference in rDNA promoter activity between stimulated and unstimulated R− cells was about 3-fold. A time course of rDNA promoter activity in R− after IGF-I stimulation is shown in Fig. 1B, where all points derive from a single pool of transfected R− cells. The activity of the rDNA promoter is already increased 8 h after IGF-I stimulation and reaches a peak at 20–24 h. These results are compatible with the time course of IRS-1 translocation to the nuclei of R− cells (21).

A qualitative confirmation of the ability of IGF-I to activate the rDNA promoter is shown in Fig. 2. For these experiments, we used R− and R+ cells transfected with a plasmid in which the rDNA promoter drives the coding sequence of the SV40 T antigen (37). The cells, either in SFM or after IGF-I stimulation, were fixed and stained with an antibody to T antigen. The R+ cells were transfected as a single pool, which was subsequently divided into two halves, stimulated with IGF-I, and unstimulated. No T antigen-positive cells are detectable in R− cells, whether in SFM (not shown) or in IGF-I (the blocks of stain are dead cells). T-positive cells are readily detectable in R+ cells after IGF-I stimulation (Fig. 2) or in 10% serum (not shown). Taken together, these results indicate that IGF-I activates the rDNA promoter, which suggests that the effect of IGF-I on UBF1 is activation, rather than inhibition as with pRB and other proteins (see the Introduction).

Phosphorylation of UBF in Mouse Embryo Fibroblasts Stimulated with IGF-I—As mentioned, it is generally agreed that activation of UBF1 depends largely on phosphorylation (8). We studied the extent of UBF phosphorylation in R−, R+/T, and R+ cells (where protein levels remain constant, see below) before and after stimulation with IGF-I. The results of one such experiment are shown in Fig. 3A. There is very little phosphorylation of UBF in R− cells, but there is phosphorylation in R+/T cells (where IRS-1 is nuclear). The extent of phosphorylation in R+/T cells does not increase with IGF-I, as expected, because R+/T cells do not express IGF-I receptors (39). In R+ cells, UBF phosphorylation is already evident in SFM, and it increases sharply after stimulation with IGF-I. This experiment was repeated with the same results. The comparison among these different cell lines is legitimate because they all have similar amounts of UBF (see below). The uptake of 32P is not the result of changes caused by IGF-I stimulation because the extent of UBF phosphorylation depends on the presence or absence of IRS-1 (30 and see also below).

We also identified by mass spectrometry the proteins in the immunoprecipitated UBF. The results of the peptide mapping were that the immunoprecipitated protein was mostly mouse UBF, although a small fraction of unrelated contaminating proteins was detected. In these cells, the ratio of UBF1 to UBF2 is about 20:1 (22), and it is reasonable to assume that about 90% of the UBF immunoprecipitated is UBF1 and not UBF2 (49). Although it is true that UBF2 is supposed to be inactive in the activation of the rDNA promoter (1), a recent paper had implicated both UBF1 and UBF2 in the activation of RNA polymerase II (50).

We have also compared 32D IGF-IR/IRS-1 cells with 32D IGF-IR PHPTB/IRS-1, comprising only the first 310 amino acids of IRS-1. In both cases, the IRS-1 (wild type or mutant) translocates to the nuclei, but the PHPTB mutant cannot sustain the proliferation induced by wild type IRS-1 (44, 51). The results, in Fig. 3B, show that UBF phosphorylation is higher in the 32D IGF-IR cells expressing wild type IRS-1 than in the same cells expressing the PHPTB mutant. This is in agreement with previous reports that PHPTB translocates to the nuclei (18) but fails to inhibit IGF-1-mediated differentiation (51). This experiment further confirms that phosphorylation of UBF is IRS-1 dependent and that the differences are not the result of changes in the pool of 32P induced by IGF-I stimulation.

These two cell lines have the same number of IGF-IRs and differ only in IRS-1 function.

Phosphopeptide analysis of UBF1 after Stimulation with IGF-I—We next set out to determine the tryptic peptides of UBF1 phosphorylated after stimulation of R− cells with IGF-I. Our first approach was to follow the procedures of Voit et al. (10) for the labeling of cells with 32P, immunoprecipitation with an antibody to UBF, tryptic digestion, and analysis by two-dimensional gel (see “Experimental Procedures”). One such experiment is shown in Fig. 4, repeated twice. There is a large, intensely labeled spot not far from the origin and three smaller spots. The largest spot is known to be the C terminus (8, 10), which is a large peptide, with more than 20 serines and many acidic amino acids (8, 52). We tried in three different experiments to identify phosphopeptides in tryptic digests of unstimulated R− cells. We were never able to detect any phosphopeptides in unstimulated R− cells. This is despite the fact that (Fig. 3) the UBF immunoprecipitated in unstimulated R− cells seemed to be phosphorylated to an extent about 30–40% that of stimulated R+ cells. Our failure to detect any phosphopeptide in unstimulated R− cells suggests that in these cells, UBF phosphorylation is below the levels of detection by the tryptic digestion technique.

Identification of the remaining tryptic phosphopeptides was difficult. The amount of material recovered by immunoprecipitation was not sufficient for peptide analysis of the three radioactive spots. We tried to increase the amount of UBF1 in R− cells by transfecting them with a plasmid expressing UBF1. Unfortunately, overexpressed UBF1 is somewhat toxic to MEFs, and the clones we obtained had very little additional expression of exogenous UBF1. After several attempts with other approaches, we eventually decided to identify phosphopeptides in UBF1 by using the procedure described under “Experimental Procedures,” a modification of the procedure of Knight et al. (42) and Guo et al. (41). This procedure combines digestion with an endoprotease with mass spectrometric analysis. The results are summarized in Table I. In IGF-I stimulated R− cells, we identified several peptides that were

--

2 A. Wu, X. Tu, M. Prisco, and Renato Baserga, unpublished data.
phosphorylated. Four of these peptides could be assigned to UBF1. Of these four peptides, one was also phosphorylated in unstimulated R– cells (peptide 318–338), and one peptide (108–126) was phosphorylated in unstimulated cells but not in stimulated cells. Two other peptides were found phosphorylated after IGF-I, but because of contaminating proteins in the immunoprecipitate, these other peptides could belong to contaminants and therefore have been excluded from Table I. Thus, we can say that Table I may not represent all of the peptides that are phosphorylated by IGF-I but that those listed are with all probability IGF-I-stimulated phosphorylated peptides. This experiment was repeated once.

Protein and mRNA Levels of UBF in 32D-derived Cells—R– derived cells have substantial amounts of IRS proteins. To
FIG. 2. Stimulation of the rDNA promoter in MEFs. R-derived cells were transiently transfected with a plasmid in which the SV40 T antigen is under the control of the rDNA promoter (37). R+ cells were transfected as a pool, which was then divided into two halves: one reincubated in SFM, the other stimulated with IGF-I. 24 h after transfection, the cells were stained with an antibody to the SV40 T antigen. The R- cells are shown only in 10% serum, but they were also negative after IGF-I stimulation. Magnification, ×40.

FIG. 3. Phosphorylation of UBF in R-derived and myeloid cell lines. A, R-derived cells were either in SFM (−) or stimulated with IGF-I for 24 h (+). The cells were labeled with 32P as described under “Experimental Procedures.” The lysates were immunoprecipitated with an antibody to UBF, and the blots were autoradiographed. The cell lines and the treatment are indicated below and above the panels. B, 32D IGF-IR cells expressing either the wild type IRS-1 or its mutant PHPTB IRS-1. The experiment was carried out in the same way as for the R-derived cells at zero time and 24 h after shifting the cells from IL-3 to IGF-I. The bands were counted, and the counts are shown below the autoradiographed blot. The bars show the mean of two observations (the ranges of the two observations are indicated).

distinguish between the effect of the receptor and that of IRS-1 on UBF1 activity, we investigated the UBF protein levels in 32D-derived cells. We first measured UBF1 protein levels in 32D IGF-IR cells (normal IGF-IR levels and no IRS-1) and in 32D IGF-IR/IRS-1 cells (expressing ectopic IRS-1). As mentioned, these two cell lines, when shifted from IL-3 to IGF-I, grow vigorously for 48 h. Then, 32D IGF-IR cells differentiate into granulocytes (44), whereas 32D IGF-IR/IRS-1 cells differentiate into granulocytes (day 9 after shifting from IL-3 to IGF-I). This is true of endogenous UBF1 or IRS-1 on UBF1 activity, we investigated the UBF protein levels in 32D-derived cells. We first measured UBF1 protein levels in 32D IGF-IR cells (normal IGF-IR levels and no IRS-1) and in 32D IGF-IR/IRS-1 cells (expressing ectopic IRS-1). As mentioned, these two cell lines, when shifted from IL-3 to IGF-I, grow vigorously for 48 h. Then, 32D IGF-IR cells differentiate into granulocytes (44), whereas 32D IGF-IR/IRS-1 cells maintain high levels of UBF1 protein in 32D cells stimulated by IGF-I. However, IRS-1 must be activated by the IGF-IR because 32D IGF-IR/IRS-1 cells, without IRS-1, simply die in the first 24 h after removal of IL-3 (44).

We next looked at the mRNA levels of UBF1 in the 32D-derived cell lines. The results (Fig. 5B) show little differences in mRNA levels of UBF1, certainly not comparable with the dramatic differences in protein levels. There are two isoforms of UBF1, certainly not comparable with the dramatic differences in protein levels. There are two isoforms of UBF1, certain isoforms of UBF1 and UBF2 (shorter because of the loss of an exon). The same panel (B) shows that 32D-derived cells express more UBF2 mRNA than UBF1 mRNA. All of these experiments were repeated twice. The mRNA levels from the pooled experiments were quantified by densitometry and subjected to statistical analysis (53). No significant differences were found in the mRNA levels of either cell line after shifting to IGF-I.

Protein and mRNA Levels in Differentiated Cells—The experiments described in Fig. 5 showed that in 32D IGF-IR cells, the UBF1 protein disappeared very quickly, even before the cells show morphological signs of differentiation (usually by day 4). At the same time, the mRNA remains high, although a careful observation may suggest that at 96 h, a slight decrease in mRNA may occur. We therefore extended our observation at later times after shifting 32D IGF-IR cells from IL-3 to IGF-I. We examined both 32D IGF-IR cells and the same cells expressing a FLAG-tagged UBF1 (54). The latter cell line was used to test whether the exogenous UBF1, being under the control of a viral promoter, would behave differently from the endogenous UBF. The results are shown in Fig. 6. The levels of UBF1 proteins decrease, and the protein disappears completely as 32D IGF-IR cells differentiate into granulocytes (day 9 after shifting from IL-3 to IGF-I). This is true of endogenous UBF1 or exogenous UBF1, whose transcription is under the control of a viral promoter. The endogenous mRNA decreases slightly, but not as much as the protein. Interestingly, the exogenous UBF1 mRNA remains high even in differentiated cells. These data
The phosphorylated peptides were identified as described under “Experimental Procedures.” The numbers in parentheses indicate the calculated number of phosphates in the peptide.

### TABLE I

| Residues | Peptides phosphorylated | Molecular weight |
|----------|-------------------------|------------------|
|          |                        | Expected | Observed |
| Unstimulated cells | HPDPKPKLTPTYRFMEK | 2634 | 2710 (1) |
| 107–126 | DVSPERMVCQSSQWKLSSQK | 2709 | 2850 (2) |
| 318–338 |                         |          |          |
| IGF-I-stimulated cells | (See above) |          |          |
| 318–338 | RYERELSMRAPPAATNSSK | 2309 | 2460 (2) |
| 540–559 | ERMVEIGSRWQRSQSKHEYK | 2936 | 3091 (2) |
| 595–616 | EYYSNKRK                | 1117 | 1190 (1) |
| 647–654 |                         |          |          |

**Fig. 5. Protein and mRNA levels of UBF in 32D-derived cells.**

A. UBF protein levels in 32D-derived cells at various times (in hours) after shifting the cells from IL-3 to IGF-I. Western blots using an antibody to UBF. The cell lines are indicated above the lanes. An antibody to Grb2 was used to monitor the amounts of protein in each lane. No bands of the same cell lines under the same conditions. The amount of RNA in each lane was monitored using amounts of rRNA (ethidium bromide staining).

B. Northern blots of the same cell lines under the same conditions. After shifting the cells from IL-3 to IGF-I. Western blots using an antibody to UBF. The cell lines are indicated above the lanes. An antibody to Grb2 was used to monitor the amounts of protein in each lane. Northern blots of the same cell lines under the same conditions.

**DISCUSSION**

The IGF-IR/IRS-1 axis controls, in a nonredundant way, about 50% of body size in animals from *Drosophila* to mice (see below). The binding of IRS-1 to UBF1 (a protein that, through RNA polymerase I, participates in the control of cell size) justifies an inquiry on the mechanism(s) by which IRS-1 and/or the IGF-IR modulates UBF1 activity and transcription from the rDNA promoter. Our novel findings on the IGF axis-UBF1 interaction can be summarized as follows: 1) IGF-I stimulation results in an increased activity of the rDNA promoter in both myeloid cells and MEFs. 2) IGF-I induces phosphorylation of UBF1, a requisite for activation. 3) IGF-I induces MEFs the phosphorylation of certain UBF1 peptides, most prominently the C terminus, which is required for activation of UBF1 (8, 9). 4) Increased phosphorylation of UBF1, however, is not the sole mode of UBF1 activity regulation by IGF-I. In myeloid cells, the activated IGF-IR and IRS-1 also maintain high levels of UBF1 protein, which are not sustained in cells that do not express IRS-1 (32D IGF-IR cells). The mRNAs for UBF are not affected. 5) The decreased levels of UBF1 in 32D IGF-IR cells are the
that the crucial sequence is in the C terminus because removal of the C terminus inactivates UBF1 in vitro (7) and in vivo. Two of the peptides (one of them also phosphorylated in unstimulated cells) are in high mobility group homology boxes (54). All three peptides identified in our experiments are highly conserved from Xenopus to humans (32). Unpublished data from our laboratory, using glutathione S-transferase constructs, have indicated that IRS-1 binds predominantly to high mobility group boxes in this region. One of the phosphorylated peptides, 318–338, is close to the pRb binding site, LXCRX, which is located at 307–311.

In certain cells, regulation of UBF activity occurs at the protein levels (13–15). In 32D IGF-IR cells, the UBF protein is unstable and decreases when the cells are shifted from IL-3 to IGF-I. The regulation of UBF protein levels in 32D-derived cells definitely does not depend on the levels of mRNA, which remain unchanged in 32D IGF-IR cells, at least for several days after shifting the cells to IGF-I. In 32D IGF-IR cells, UBF1 protein levels are already decreased markedly by 48 h. Eventually, as 32D IGF-IR cells differentiate (45), even the UBF mRNA tends to decrease, together with the disappearance of the nucleoli (56). UBF1 protein levels decrease even in cells expressing a FLAG-tagged UBF1, under the control of a viral promoter, whose mRNA remains stable even at day 9 of differentiation. This shows convincingly that regulation of UBF1 protein levels in differentiating myeloid cells occurs at the protein levels. Variations in UBF levels in cardiac myocytes and fibroblasts in culture have been reported (13–15). It seems therefore that both the amounts of protein and the extent of phosphorylation participate in regulating UBF1 activity.

UBF1 levels remain stable, however, in R- cells (no IGF-I receptor). 32D cells grow in suspension, whereas MEFs (R- derived cells in the present experiments) grow only in monolayer cultures. R- and R- derived cells have substantial levels of IRS-1 (57), but phosphorylation of IRS-1 in R- cells is not detectable unless one uses high concentrations of insulin (58). The fact that UBF1 remains stable in R- cells suggests that the mechanism for its stability in these cells may have something to do with cell adhesion. However, when MEFs were examined in suspension (polyHEMA plates), UBF1 levels still remained stable, even in R- cells. At the moment, we can only offer one alternative explanation for the discrepancy in UBF1 stability between 32D IGF-IR cells and MEFs. 32D IGF-IR cells differentiate into granulocytes, whereas MEFs are not prone to

result of both decreased synthesis and increased degradation of the protein. 6) UBF1 is stable, however, in MEFs, even in the absence of an IGF-IR (R- cells). The main conclusion of these experiments is that IGF-IR signaling regulates the activity of UBF1 and the rDNA promoter and that this regulation is complex, depending in part on phosphorylation and in part on the levels of UBF1.

Previous reports have shown that IRS-1 translocates to the nuclei and nucleoli and binds to UBF in MEFs (25, 26) and in 32D IGF-IR/IRS-1 cells (22) stimulated with IGF-I. The present experiments show that activation of the IGF-IR pathway leads to increased phosphorylation of the UBF1 protein, the phosphorylation of certain residues in UBF1, and the activation of the rDNA promoter. The phosphorylation of UBF1 observed in these experiments is likely because of PI3K, which has been shown to phosphorylate UBF1 in vivo and in vitro (36). This was demonstrated in nuclear extracts, indicating that PI3K, like IRS-1, can translocate to the nuclei (see below), a reasonable conclusion, because UBF1 is an exclusively nucleolar protein (41), and both IRS-1 and PI3K must translocate at least to the nuclei to interact with UBF1.

Thus, one way by which the IGF axis regulates UBF1 activity is through its phosphorylation by PI3K (36). The phosphorylation sites include the C terminus and other peptides. The C terminus of UBF is phosphorylated by IGF-I stimulation, an important consideration, because deletion of this C-terminal domain severely decreases UBF-directed activation of rDNA transcription in vitro (33), and in cells.2 There is a basal level of UBF phosphorylation, somewhat independent of IGF-I because we can detect UBF phosphorylation in R-/T cells. Apart from the C terminus, none of the peptides identified in our experiments corresponds to the phosphorylated peptides reported in the literature (9–11) and mentioned in the Introduction. Because, in most cases, the stimulation of growth was done by serum, which contains a variety of growth factors, it is not surprising that the peptides phosphorylated by serum may be different from those obtained by IGF-I stimulation. EGF does stimulate cell proliferation, but there is no evidence in the literature that deletion of the EGFR receptor causes a growth phenotype in mouse embryos (66). It is tempting to speculate that the residues phosphorylated by EGF may be related more to the cell cycle program than to cell size. At any rate, it seems that the crucial sequence is in the C terminus because removal

FIG. 6. Levels of UBF1 protein and mRNA in differentiated cells. 32D IGF-IR cells (32 GR15) were examined at various times (in days) after shifting from IL-3 to IGF-I. Under these conditions, 32D IGF-IR cells differentiate into granulocytes. On the left are the levels of UBF protein (upper blots) and RNA (lower blots) of 32D IGF-IR cells at zero time and at various days after shifting to IGF-I. On the right are the same levels but in the same cells stably transduced with a plasmid expressing the mouse UBF1 cDNA. The protein levels decrease and eventually disappear as cells differentiate (granulocytes begin to appear on day 4). The endogenous RNA (2187 bp) decreases slightly, but the exogenous mRNA (2320 bp) remains elevated, indicating that it is regulated differently than the endogenous mRNA. The amounts of proteins and RNA in each lane were monitored as in the Fig. 5.

UBF1 Regulation by IGF-I Receptor Signaling

2869

Downloaded from http://www.jbc.org/ by guest on February 23, 2020
differentiation. Perhaps it is the differentiation process that destabilizes the UBF1 protein, a possibility, given the fact that nucleoli have a tendency to disappear in some terminally differentiated cells (59).

As mentioned in the Introduction, activation of the rDNA promoter is not an unavoidable consequence of UBF1 binding (25–29, 60), but in the case of IGF-I stimulation, the binding of IRS-1 to UBF1 leads to increased transcription of the rDNA promoter, as demonstrated by two different procedures. The activation in R’ cells lasts for at least 24 h, by which time most of the IRS-1 is still nuclear (21). The increased phosphorylation of UBF1 in R’/T cells compared with R’ cells is not surprising. It is known that rRNA synthesis is stimulated by the SV40 T antigen (61–63). Interestingly, whereas IRS-1 binds to UBF1, T antigen binds to another protein involved in the regulation of RNA polymerase I activity, SL1 (63). It is possible that the phosphorylation of UBF1 in R’/T cells may still depend on its interaction with IRS-1. T antigen is known to interact with IRS-1, by coimmunoprecipitation (45, 64), requiring the N terminus of T antigen. For the moment, one can only speculate on the complex interrelationships among IRS-1, T antigen, and the proteins that regulate RNA polymerase I activity.

The significance of these findings is based on the reports in the literature that cell and body size are partially regulated by IGF-IR signaling, as first established by the pioneer work of Efstratiadis and collaborators, summarized by Ludwig et al. (32). Deletion of the IGF-IR genes reduces mouse embryo size by ~50%. Drosophila has a receptor that partakes both of the IGF-IR and the insulin receptor and homologs of the insulin receptor substrate (IRS) proteins, Akt and S6K1, all signal transducing molecules downstream of the IGF-IR (16). These homologs have been reported to regulate cell size and body growth in Drosophila (23, 33, 34). For instance, deletion of the Drosophila IRS homolog, called chico, reduces fly weight by 65% in females and 55% in males (23). These homologs regulate
both cell size and cell number, which combine to give a reduced body size of roughly 50%. The evidence accumulated in *Drosophila* can be extended to mice, to mammalian cells in culture (35), and probably to higher organisms (65). Mice with a targeted disruption of the IRS-1 (24) or S6K1 (36) genes are smaller than their wild type littermates. It is interesting that the reduction in body size, whether in *Drosophila* or mice, is 50%, as if the IGF axis were responsible, in a nonredundant way, for only 50% of growth, the other 50% being provided by alternative pathways. Although the insulin receptor may also play a role in regulation of body size, it should be noted that deletion of the insulin receptor genes in mice results in embryos of normal size at birth (66).

The report that IRS-1 can translocate to the nuclei and bind UBF1 established a first molecular connection between IGF-I signaling and RNA polymerase I activity. Because IRS-1 is not a kinase, the phosphorylation of UBF1 must depend on another molecule, and we have already reported that nuclear IRS-1 interacts with the p85 subunit and UBF1 can be phosphorylated directly by the p110 subunit of PI3K (30). Thus, a picture is emerging in which IGF-IR signaling causes the nuclear translocation of the IRS-1/PI3K complex (67), which causes the activation of UBF1 and the rDNA promoter, leading to an increase in cell size. Because Akt (68) and the p85 isoform of S6K1 (69) can also translocate to the nuclei, one is tempted to speculate that we are dealing here with a large complex moving to the nuclei, a kind of the signalosome proposed by Donahue and Fruman (70).

In conclusion, we have demonstrated that IGF-I, through the activation of the IGF-IR signaling pathway, up-regulates the activity of UBF1 and, consequently, of the rDNA promoter. In IGF-I regulation of UBF1 activity, the levels of protein can also be an important component, a finding that establishes the complexity of IGF-I regulation of UBF1 activation. IGF-I also induces the phosphorylation of a number of peptides that may be determinant in the activation of UBF1 and its effect on the rDNA promoter.

**FIG. 8. Levels of UBF protein and mRNA in R-derived cell lines.** The indicated cell lines (R⁻ and R⁺ cells) were made quiescent for 24 h in SFM, then kept in SFM or stimulated with 50 ng/ml IGF-I for the indicated times. A, whole cell extracts were prepared for this experiment, but UBF is an exclusively nucleolar protein (40). Western blots were performed with an antibody to UBF, and the amounts of proteins in each lane were monitored with an antibody to Grb2. B, levels of UBF mRNA in R⁻ and R⁺ cells. Lysates were prepared from the indicated cell lines (top), and Northern blots were carried out as described under “Experimental Procedures” using as a probe the full-length cDNA of UBF1. The cells were either in SFM (−) or in IGF-I (+). The levels of β-actin mRNA were used to monitor RNA amounts in each lane. C, statistical analysis of UBF1 mRNA levels in R-derived cells. The data of the experiments described in A and B were quantified by densitometry and subjected to statistical analysis (53). The bars are the mean ± S.D. of three separate experiments. In the upper panel are analyzed the data for R⁻ cells, in the lower panel those for R⁺ cells. In both instances, SFM is on the left and IGF-I stimulation on the right.
REFERENCES

1. Grummt, I. (1999) Progr. Nucleic Acids Res. Mol. Biol. 62, 109–153
2. Larson, D. E., Xie, W. Q., Gilbertic, M., O'Mahony, D., Sells, B. H., and Roth- blum, L. I. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7933–7936
3. Reeder, R. H. (1999) Progr. Nucleic Acids Res. Mol. Biol. 62, 293–327
4. Comai, L., Song, Y., Tan, C., and Bui, T. (2000) Cell Growth Differ. 11, 63–70
5. Moss, T., and Stefanovsky, V. Y. (1995) Progr. Nucleic Acids Res. Mol. Biol. 50, 25–66
6. Jorgensen, P., Nishiakawa, J. L., Breitkreutz, B. J., and Tyers, M. (2002) Science 297, 390–400
7. Grummt, I. (2003) Genes Dev. 17, 1691–1702
8. Voit, R., Schnapp, A., Kuhn, A., Jorgensen, P., Breitkreutz, B. J., and Tyers, M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 13631–13636
9. Voit, R., Hoffmann, M., and Grummt, I. (1999) EMBO J. 18, 1981–1989
10. Stefanovsky, V. Y., Pelletier, G., Hannan, R., Gagnon-Kugler, T., Rothblum, L. I., and Moss, T. (2001) Mol. Cell 8, 1063–1073
11. Wu, A., Sciacca, L., and Baserga, R. (2003) J. Biol. Chem. 278, 8290–8297
12. Hannan, K. M., Branderburger, Y., Jenskins, A., Farah, S., Sutcliffe, J. E., McLees, A., Alzuherri, H. M., and Hannan, R. D. (2003) Mol. Cell. Biol. 23, 17231–17238
13. Wu, A, Sciacca, L., and Baserga, R. (2003) J. Cell. Physiol. 195, 453–460
14. Tu, X., Batta, P., Innocent, N., Prisco, M., Casaburi, I., Belletti, B., and Baserga, R. (2002) J. Biol. Chem. 277, 44357–44405
15. Sun, H., Tu, X., Prisco, M., Wu, A., Casiburi, I., and Baserga, R. (2003) Mol. Endocri. 17, 48–56
16. Bohni, R., Riceo-Kevent, J., Oldham, S., Broglio, W., Stocker, H., Andrus, B. F., Beckingham, K., and Hafen, E. (1999) Cell 97, 865–875
17. Pete, G., Fuller, R. G., Oldham, J. M., Smith, D. R., D'Ercoli, A. J., Kuhn, C. R., and Lund, P. F. (1999) Endocrinology 141, 4178–4183
18. Carinci, O., Attisantini, P., Baffa, R., Liu, J., Draks, R., Wu, A., and Baserga, R. (2002) J. Biol. Chem. 277, 32078–32085
19. Boylan, J. M., and Grummt, I. (2002) J. Biol. Chem. 277, 17231–17238
20. Fujii, K., Attisantini, P., Baffa, R., Liu, J., Draks, R., Wu, A., and Baserga, R. (2002) J. Biol. Chem. 277, 32078–32085
21. Hannon, K. M., Branderburger, Y., Jenskins, A., Farah, S., Sutcliffe, J. E., McLees, A., Alzuherri, H. M., and Hannan, R. D. (2003) Mol. Cell. Biol. 23, 8862–8867
22. Ciarnatori, S., Scott, P. H., Stocillo, J. E., McLees, A., Alzuherri, H. M., Dannenberg, J. H., Riele, H., Grummt, I., Voit, R., and White, R. J. (2001) Mol. Cell. Biol. 21, 5806–5814
23. Budde, A., and Grummt, I. (1998) Oncogene 19, 1119–1124
24. Liu, C., Wang, H., and Lengyl, P. (1999) EMBO J. 18, 2845–2854
25. Draks, R., Tu, X., and Baserga, R. (2002) Proc. Natl. Acad. Sci. U. S. A. 101, 9272–9276
26. Sosic, L., Flechner, L., Gutkind, J. S., Wang, L., Baserga, R., Pierce, J. H., and Li, W. (1999) Mol. Cell. Biol. 19, 3816–3828
27. Ludwig, T., Egereschwiler, J., Fisher, P., D’Ercoli, J. P., Davenport, M. L., and Efstratiadis, A. (1996) Dev. Biol. 177, 517–535
28. Montagut, J., Stewart, M. J., Stocker, E., Hafen, E., Kozma, S. C., and Thomas, G. (1999) Science 285, 2126–2129
29. Verdu, J., Buravovich, M. A., Wilder, E. L., and Birnbaum, M. J. (1999) Nat. Cell Biol. 1, 500–506
30. Valentinis, B., Navarro, M., Zanocco-Marani, T., Edmonds, P., McCormick, J., Morrione, A., Sacchi, A., Romano, G., Reis, K., and Baserga, R. (2000) J. Biol. Chem. 275, 25451–25459
31. Wu, A., Sciacca, L., and Baserga, R. (2000) Science 290, 5172–5176
32. UBF1 Regulation by IGF-I Receptor Signaling

By guest on February 23, 2020http://www.jbc.org/Downloaded from
Regulation of Upstream Binding Factor 1 Activity by Insulin-like Growth Factor I Receptor Signaling
An Wu, Xiao Tu, Marco Prisco and Renato Baserga

J. Biol. Chem. 2005, 280:2863-2872.
doi: 10.1074/jbc.M406138200 originally published online November 8, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406138200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 67 references, 36 of which can be accessed free at http://www.jbc.org/content/280/4/2863.full.html#ref-list-1