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Downregulation of the Upstream Binding Factor1 by Glycogen Synthase Kinase3β in Myeloid Cells Induced to Differentiate

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Abstract The upstream binding factor 1 (UBF1), one of the proteins that regulate the activity of RNA polymerase I, is downregulated in 32D myeloid cells induced to differentiate into granulocytes, either by the type 1 insulin-like growth factor (IGF-1) or the granulocytic colony stimulating factor (G-CSF). Downregulation of UBF1 is largely due to protein degradation, while mRNA levels are not affected. Inhibition of UBF1 degradation by lithium chloride (LiCl)and lactacystin suggest a role of glycogen synthase kinase β (GSK3β) in a proteasome-dependent degradation of UBF. GSK3β phosphorylates in vitro and in vivo the UBF protein, which has five putative motifs for phosphorylation by GSK3β. Elimination and/or mutations of these motifs stabilize the UBF1 protein even in cells induced to differentiate. Conversely, a stably transfected, constitutively active GSK3β accelerates the downregulation of UBF1. We show further that activation of the differentiating protein C/EPBα in 32D cells transformed by the oncogenic BCR/ABL protein causes downregulation of UBF1. Finally, inhibition of differentiation of myeloid cells by a dominant negative mutant of Stat3 stabilizes the UBF1 protein, while rapamycin-induced differentiation of myeloid cells downregulates UBF1 levels. Taken together, our results indicate that the induction of granulocytic differentiation in 32D murine myeloid cells causes the degradation of UBF1, via GSK3β and the proteasome pathway. J. Cell. Biochem. 100: 1154–1169, 2007.

Key words: IGF-1 receptor; IRS-1; differentiation; myeloid cells

The upstream binding factor 1 (UBF1) is one of the proteins that regulate the activity of RNA polymerase I, which regulates, in turn, ribosomal RNA (rRNA) synthesis [Grummt, 2003]. The activity of UBF1 is increased by growth signals that phosphorylate its C-terminus [Voit et al., 1992, 1995, 1999; Voit and Grummt, 2001], and other amino acid residues outside the C-terminus [Voit et al., 1995, 1999; Grummt, 1999; Stefanovsky et al., 2001; Wu et al., 2005]. Overall activity of UBF1 depends also on its levels. UBF1 levels are increased in hypertrophic cells [Hannan et al., 1996, 1998; Kabler et al., 1996; Brandenburger et al., 2001; Poortinga et al., 2004] and overexpression of UBF1 increases cell size [Prisco et al., 2004]. The insulin receptor substrate-1 (IRS-1), a docking protein for both the IGF-1 and insulin receptors, interacts with UBF1 [Tu et al., 2002; Sun et al., 2003] and the C-terminus of UBF1 is phosphorylated by stimulation of cells with IGF-1 [Drakas et al., 2004], confirming the results obtained with other growth factors. IGF-1 also regulates UBF1 levels.

32D murine myeloid precursor cells are a cell line that behaves in culture like myeloid precursor cells in the bone marrow of animals, that is with the appropriate growth factors they differentiate into granulocytes. 32D cells do not express IRS-1 or IRS-2 [Wang et al., 1993; Valentinis et al., 1999]. The lack of IRS-1 expression in 32D cells is in agreement with several findings that cells prone to differentiation do not express IRS-1, or express very low levels, or downregulate it when induced to differentiate [Sun et al., 1991; Baserga, 2000].

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32D IGF-IR cells (17×10^3 receptors/cell and no IRS-1) survive when shifted from Interleukin-3 (IL-3) to IGF-1, and grow vigorously for about 48 h, after which time they begin to differentiate along the granulocytic pathway [Valentinis et al., 1999]. Ectopic expression of IRS-1 in 32D IGF-IR cells (32D IGF-IR/IRS1 cells), inhibits IGF-1-induced differentiation, the cells grow indefinitely in the absence of IL-3 and form tumors in mice [Valentinis et al., 2000].

When 32D IGF-IR cells are induced to differentiate by IGF-1, the UBF1 protein (but not its mRNA) is downregulated [Wu et al., 2005]. Most of the decrease is due to increased degradation, although there is also a small decrease in synthesis. IGF-I causes the downregulation of UBF1 in differentiating 32D IGF-IR cells, but not in proliferating 32D IGF-IR/IRS-1 cells [Wu et al., 2005]. These findings raise the question of whether the UBF1 downregulation in 32D IGF-IR cells may be due to the induction in these cells of the differentiation program. It is known that terminal differentiation of hemopoietic cells causes nucleolar involution [Ringertz and Savage, 1976; Likovsky and Smetana, 2000], and a decrease in ribosomal RNA synthesis [Larson et al., 1993; Comai et al., 2000]. UBF1 decreases and, together with the whole nucleolus, eventually disappears during granulocytic differentiation of 32D cells [Tu et al., 2003].

We have investigated the stability of UBF1 in 32D IGF-IR cells induced to differentiate by IGF-1 and in parental 32D cells induced to differentiate by the granulocytic-colony stimulating factor (G-CSF). The G-CSF receptor sends signals that are overlapping and similar to those sent by the IGF-IR [Avalos, 1996; Hunter and Avalos, 1998; Ward et al., 1999; and see Discussion].

Our results confirm the downregulation of the UBF1 protein in 32D IGF-IR cells induced to differentiate by IGF-1. Glycogen synthase kinase 3β (GSK3β) phosphorylates UBF1, presumably marking it for proteasome-dependent degradation. Mutation or elimination of the GSK3β phosphorylation sites stabilizes the UBF protein in differentiating cells. UBF1 degradation also occurs in parental 32D cells induced to differentiate by G-CSF. The role of GSK3β in UBF1 degradation is confirmed by the finding that expression of a constitutively active GSK3β causes accelerated degradation of UBF1, while its downregulation is suppressed by LiCl, an inhibitor of GSK3β.

Downregulation of UBF1 in 32D myeloid cells correlates with the induction of differentiation, even in a derivative 32D cell line transformed by the oncogenic BCR/ABL protein. Inhibition of differentiation stabilizes the UBF1 protein and, conversely, induction of differentiation in proliferating cells causes UBF1 degradation. Taken together, our results show that phosphorylation by GSK3β modulates the downregulation of UBF1 in 32D and 32D-derived cells, and this downregulation is related to the differentiation program.

METHODS AND MATERIALS

Cells and Cell Cultures

The cell lines used were 32D parental cells [Valtieri et al., 1987], 32D IGF-IR, 32D IGF-IR/DN Stat3, and 32D IGF-IR/IRS-1 [Valentinis et al., 1999; Prisco et al., 2001]. 32D and 32D-derived cells are grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Inc.), 10% WEHI cell-conditioned medium as a source of IL-3, 2 mM L-glutamine (Life Technologies, Inc.), and the antibiotic required for selection: 250 μg/ml of G418 (Life Technologies), 1.5 μg/ml of puromycin or 250 μg/ml of hygromycin (Calbiochem, La Jolla, CA). The 32D BCR-ABL cells carrying the plasmid conditionally expressing C/EBPα (or the corresponding empty plasmid) have been described by Ferrari-Amorotti et al. [2006].

Plasmids

A cDNA fragment coding for the C-terminal deletion of the UBF1 protein was amplified by PCR from the plasmid template MSCVpuroUBF1. The PCR products was subcloned into pMSCVpuro using *Eco*RI and *Xho*I restriction sites to generate the C-terminus truncated UBF1 tagged with FLAG (MSCVpuro FLAG/C-terminus truncated UBF1). A cDNA fragment coding for UBF2 protein, which lacks a 37-amino-acid sequence in HMG box2, was amplified by PCR from the plasmid template MSCVpuroUBF1. The PCR product was then subcloned into pMSCVpuro using *Eco*RI and *Xho*I restriction sites to generate the FLAG-tagged MSCVpuro FLAG/UBF2 [Chen et al., 2005]. To generate *Mig*RI GSK3ß-S9A-HA plasmid, pCR 3.1 S9A GSK3ß-HA was

digested by the *Eco*RI restriction enzyme, and the released fragment was subcloned into a dephosphorylated *Eco*RI-digested *Mig*RI which contain the humanized GFP [Corradini et al., 2005].

Site-Directed Mutagenesis

The C-terminus truncated UBF1 was generated by mutation of a glutamine codon at residue 672 to a stop codon, using the Quickchange site-directed mutagenesis kit (Stratagene, LaJolla, CA). The oligonucleotide primers used to introduce the desired mutation were 5′-AGC CGG ACC ACC CTG TAG TCC AAG TCG GAG TCC-3′ and 5′-GGA CTC CGA CTT GGA CTA CAG GGT GGT CCG GCT. The codon change at residue 672 eliminates the C-terminus, arbitrarily located from 673 to 765. For mutations of the GSK3β phosphorylation sites, MSCVpuro FLAG/C-terminus truncated UBF1 was used as template to generate the MSCVpuro FLAG/UBF1 triple mutants, again using the Quickchange site-directed mutagenesis kit. They were generated by changing serines 40, 43, and 44 into alanines in one of the potential GSK3ß phosphorylation sites in UBF1 and serines 392 and 396 and proline 395 into alanine in another potential GSK3ß phosphorylation site. LxCxE plasmid was generated by changing leucine 307 into alanine using plasmid MSCVpuroUBF1 as template. All mutations were sequenced.

Transfection and Retroviral Infection

32D IGF-IR cells were transduced with the MSCVpuro FLAG/C-terminus truncated UBF1, MSCVpuro FLAG/UBF1 triple mutants (see above), LxCxE mutant, and MSCVpuro DN Stat3 Y705F, respectively [Prisco et al., 2004]. 32D cells were transduced with the MSCVpuro FLAG/UBF2. 32D IGF-IR and 32D IGF-IR/IRS-1 cells were transduced with *Mig*RI GSK3β-S9A-HA (also tagged with the GFP reporter gene) respectively. Fluorescent-activated cell sorting (FACS, MoFlo DAKOCYTOMATION) was used to measure the mean GFP fluorescence of *Mig*RI-transfected cells.

Northern blotting. Exponentially growing cells were washed three times with HBSS and seeded at 5×10^4 cells/ml in RPMI 1640 medium supplemented with 10% FBS, 10% WEHI cell-conditioned medium, and G-CSF at 25 ng/ml (BD Biosciences Pharmingen, Franklin Lakes, NJ). At various times, cells were collected and total RNA for each sample was run on a 1% agarose formaldehyde gel, blotted onto a nylon membrane, and hybridized with UBF1 cDNA [Wu et al., 2005].

Measuring ubiquitin conjugation. To detect ubiquitination of UBF1 at endogenous levels of ubiquitin, IGF-1 or G-CSF-stimulated cell (in the presence of MG132 or lactacystin, Staub et al., 1997) were used. Lysates were boiled in 1% sodium dodecyl sulfate (SDS) for 5 min and diluted 11 times with HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 0.2 mM Na_3VO_4) to dilute the SDS prior to immunoprecipitation with anti-UBF1 antibodies and 25 μl of protein A + G beads (Amersham Biosciences, Buckinghamshire, England). After washing, the ubiquitinated proteins were resolved by standard single-dimensional SDS–PAGE using 7.5% polyacrylamide gels at a constant voltage of 80 V for approx 6–7 h. To enhance the electroelution of ubiquitinated proteins from SDS–polyacrylamide gels, the gels were soaked in 2.3% SDS, 5% 2-mercaptoethanol, 63 mM Tris-HCl, pH 6.8 for approx 30 min prior to electrotransferring. Then the gels were electrotransferred overnight at 40–50 V. To promote ubiquitin epitope exposure and increase the sensitivity of anti-ubiquitin immunoblots, the membranes were immersed in 6 M guanidine-HCl, 20 mM Tris-HCl, pH 7.5, 1 mM PMSF, and 5 mM DTT, for 30 min at room temperature before blocking. After blocking, the proteins were stained with anti-ubiquitin (Covance) and anti-UBF antibodies, respectively.

Western Blotting

Exponentially growing 32D-derived cells (in IL-3) were washed three times with Hanks balanced salt solution (HBSS) and seeded at a density of 5×10^4 cells/ml in RPMI 1640 medium supplemented with 10% FBS, IGF-I at 50 ng/ml, 10% WEHI cell-conditioned medium, or G-CSF at 25 ng/ml (BD Biosciences). Cells harvested at various times after shifting to IGF-1, were washed with cold phosphate-buffered saline (PBS), and lysed in 50 mM HEPES (pH 7.5)-150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% NP-40-100 mM NaF-10 mM sodium pyrophosphate-0.2 mM sodium orthovanadate-1 mM phenylmethylsulfonyl fluoride-10 μg of aprotinin/ml. Aliquots of lysates were resolved by 4–15% SDS–polyacrylamide gels and transferred to nitrocellulose

membranes. They were probed with the appropriate antibodies and developed using the ECL system (Amersham Biosciences). The rest of the procedure was the same as that described in detail in previous reports [Navarro et al., 2001; Prisco et al., 2001].

GSK3β kinase assays. GSK3β assays were performed as described previously [Ito et al., 2006]. UBF1 was immunoprecipitated from quiescent cells, and the immune complexes were washed with RIPA and the kinase (20 mM Tris-HCl, pH 7.5, 10 mm $MgCl_2$, 5 mM dithiothreitol) buffers. Kinase reactions were carried out in 30 μl of kinase buffer containing 1 μl of rabbit GSK3β (500,000 units/ml, New England Biolabs, Inc., Beverly, MA) and 20 μCi of [γ-32P]ATP(1Ci = 37GBq) (Perkin-Elmer Life and Analytical Sciences, Boston, MA) for 30 min at 30°C and stopped by the addition of Laemmli sample buffer and heating for 5 min at 95°C. Proteins were separated on 4–15% PAGE (Bio-Rad Ready Gel, Bio-Rad Laboratories, Hercules, CA). The gel was fixed in Fixing solution (11.5% trichloroacetic acid, 3.45% sulfosalicylic acid) for 30 min, and destained in 30%MeOH/10% acetic acid three times for 10 min, dried, and exposed to X-ray film (Hyperfilm, Amersham Biosciences). Densitometric analysis was performed with the ImageQuant program, and results are expressed in arbitrary units.

Cell labeling. 32DIGF-IR/IRS-1 and 32DIGF-1/IRS-1GSK3ß S9A cells were seeded at a density of 5×10^4 cells/ml in RPMI 1640 medium supplemented with heat-inactivated 10% fetal bovine serum and 50 ng/ml IGF-1 (Invitrogen, Carlsbad, CA) for 24 h. The cells were shifted to phosphate-free RPMI 1640 medium (Invitrogen) for 1 h, labeled with [^{32}P]orthophosphate for 4 h) (Perkin-Elmer Life and Analytical Sciences). After labeling, the cells were lysed and UBF was immunoprecipitated. The samples were then divided into two halves after addition of Laemmli sample buffer and heating for 5 min. After separating on 4–15% PAGE, one set of gel was fixed, destained, dried, and exposed to X-ray film. The other gel was transferred to a nitrocellulous membrane and blotted with UBF1 antibody. A densitomeric analysis of the bands in autoradiograph was also performed with the ImageQuant program.

Confocal microscopy. 32D IGF-IR UBF-triple mutant cells (32DIGF-IR UBF triple mut) were seeded at a density of 5×10^4 cells/ml and grown in RPMI 1640 medium supplement with 10% heat-inactivated fetal bovine serum (Life Technologies) containing IL-3 (3 ng/ml) for 24 h. Cell samples were prepared on glass slides using the cytospin method. Slides were washed with PBS and cells fixed with 3.0% formaldehyde in PBS for 20 min at room temperature, followed by permeabilization with 0.2% Triton X-100 in PBS for 5 min at room temperature. After a PBS wash, the slides were digested with RNase (1 μg/ml) for 30 min, washed again with PBS, and blocked in 10% normal donkey serum (sc-2044; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in PBS for 30 min at room temperature. The slides were incubated for 1 h at room temperature with an anti-FLAG M2 F1804 (Sigma, St. Louis, MO) antibody, washed with PBS, and then incubated for 1 h at room temperature with anti mouse IgG-fluorescein isothiocyanate-conjugated (sc-2078) antibody. After being washed with PBS, the slides then stained with propidium iodide (2.5 μg/ml; P-3566; Molecular Probes) for 5 min. Finally, the glass slides were mounted with Vectashield mounting medium (H-100; Vector Laboratories, Inc.). Fluorescent images were collected by using a Zeiss Axiovert 100 confocal microscope with a Zeissx 40 objective.

Histochemistry. To assess the effect of LiCl on IGF-1-induced differentiation, 32D IGF-IR cells were seeded at a density of 5×10^4 cells/ml and allowed to grow/differentiate in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) containing IGF-1 (50 ng/ml) only or IGF-1 and LiCl (10 mM) respectively for 5 days. Replacement of half the volume of the medium and growth factors was done once every 48 h. Cell samples were then prepared on glass slides using the cytospin method and stained with Wright-Giemsa (Sigma). Bands and polymorphonuclear cells were considered differentiated cells.

Antibodies

The following antibodies were used: mouse monoclonal anti-UBF (Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-ubiquitin (Convance, Berkeley, CA), mouse monoclonal anti-GSK-3β (Santa Cruz), rabbit polyclonal anti-phopho-GSK3β (Ser 9), mouse monoclonal anti-FLAG conjugated with peroxidase (Sigma-Aldrich, St. Louis, MO), rabbit polyclonal anti-Pescadillo (Bethyl, Montgomery, TX),

mouse monoclonal anti-Grb2 (Transduction Laboratories), and rabbit polyclonal anti-β actin (Sigma). 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 ECL (Amersham Bioscience).

Materials

Rapamycin, lithium chloride (LiCl), and 4-hydroxytamoxifen were purchased from Sigma Chemical Company (St. Louis, MO). Recombinant mouse IL-3 (IL-3) was purchased from BD Biosciences Pharmingen, recombinant human G-CSF was purchased from Chemicon International, Inc.(Temeculla, CA), and recombinant human IGF-I from Invitrogen Life Technologies. Lactacystin and MG-132 were purchased from Calbiochem.

RESULTS

Inhibitors of the Proteasome Pathway Prevent the Degradation of UBF1

We have previously reported [Wu et al., 2005] that the levels of UBF1 protein (but not UBF1 mRNA) decrease sharply when 32D IGF-IR cells are induced to differentiate by IGF-1. The decrease is largely (85%) due to increased degradation rather than decreased synthesis (15%). We have confirmed downregulation of the UBF protein in 32D IGF-IR cells induced to differentiate by IGF-1 (Fig. 1, panel A). Panel A also shows that pescadillo (Pes1), another nucleolar protein [Lerch-Gaggl et al., 2002] involved in ribosome biogenesis [Oeffinger et al., 2002], is downregulated in 32D IGF-IR cells shifted to IGF-1. Because of the rapid downregulation of UBF1 in 32D IGF-IR cells shifted to IGF-1, we asked whether ubiquitination was involved. We inhibited the proteasome pathway with lactacystin or MG132. Figure 1, panel B, shows that lactacystin (10 μM) inhibits the degradation of UBF1, quite evident at 72 h, when UBF1 has practically disappeared from untreated cells, but is still well expressed in lactacystin-treated cells. The same results were obtained (not shown) with MG132, another potent inhibitor of the proteasome pathway [Vecchione et al., 2003]. Interestingly, lactacystin did not inhibit the degradation of Pes1 (Fig. 1, panel C). Lactacystin does not alter the levels of UBF1 in 32D IGF-IR/IRS1 cells, where the shift to IGF-1 has no effect on the stability of the UBF1 protein (Fig. 1, panel D).

Levels of UBF1 Protein in Parental 32D Cells Induced to Differentiate by G-CSF

Since IGF-1 induces granulocytic differentiation of 32D IGF-IR cells [Valentinis et al., 1999],

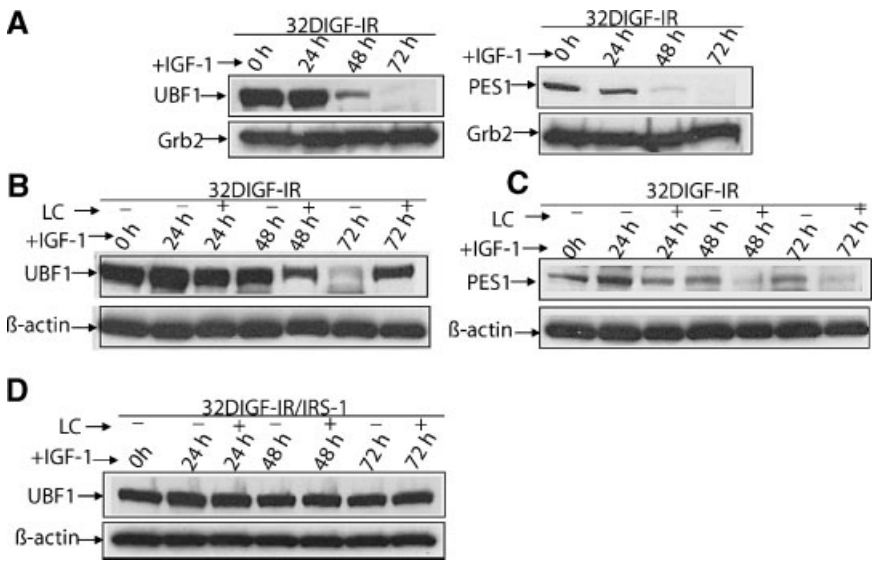

Fig. 1. Lactacystin inhibits the downregulation of UBF1 in 32D IGF-IR cells. 32D IGF-IR cells were shifted from IL-3 to IGF-1, and lysates were obtained at the indicated intervals (in h) after shifting (0 h are cells in IL-3). **Panel A**: Western blots were developed with antibodies to either UBF or Pes1 (an antibody to Grb2 was used to monitor protein amounts/lane). In **panels B** and **C**, the cells were divided into two groups, treated with IGF-1 only or treated with IGF-1 plus lactacystin (LC-10 μM). Panel B: Western blot with antibodies to UBF and to actin (the latter one to monitor protein amounts/lane). Panel C: Western blot with antibodies to Pes1 and actin. Panel D: 32D IGF-IR/IRS1 cells were shifted to IGF-1 and left untreated or were treated with lactacystin (LC).

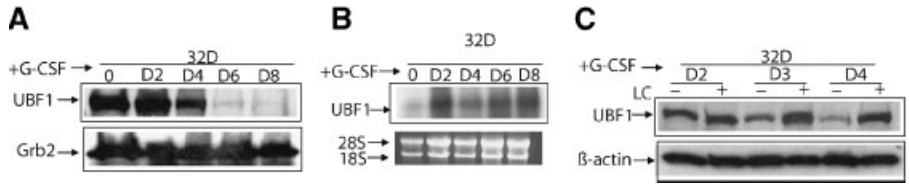

Fig. 2. Lactacystin inhibits the downregulation of UBF1 in parental 32D cells induced to differentiate by G-CSF. Parental 32D cells were shifted from IL-3 to G-CSF (25 ng/ml) and lysates obtained at the indicated intervals in days. Western blots with antibodies to UBF, Grb2 and actin. **Panel A**: Western blot of 32D cells treated with G-CSF. **Panel B**: Northern blot of the same cells. **Panel C**: 32D cells treated with G-CSF only or G-CSF plus lactacystin (LC, 10 μM).

we asked whether G-CSF, another inducer of granulocytic differentiation in 32D cells [Valtieri et al., 1987; Tu et al., 2003], could destabilize UBF1 in 32D cells. Figure 2 panel A shows that G-CSF downregulates UBF1, the levels being decreased on Day 4 of G-CSF treatment and essentially non-detectable on Days 6 and 8. When compared to 32D IGF-IR cells induced to differentiate by IGF-1, it seems that degradation of UBF1 is somewhat faster with IGF-1 (Fig. 1, panel B) than with G-CSF. The stability of UBF1 mRNA, previously reported for IGF-1-induced differentiation, was confirmed in G-CSF-induced 32D cells. There was no decrease in the levels of UBF mRNA, at least as late as 8 days after G-CSF treatment (Fig. 2, panel B). As with the 32D IGF-IR cells, lactacystin inhibited the degradation of UBF1 in parental 32D cells treated with G-CSF (Fig. 2, panel C).

We directly confirmed the ubiquitination of UBF1 under these conditions. After immmunoprecipitation, UBF can be stained with an antibody to ubiquitin. The ubiquitination of UBF1 is detectable 4 h after 32D IGF-IR cells are induced to differentiate by IGF-1, in the presence of a proteasome inhibitor (Fig. 3, panel A). The ubiquitination of UBF, confirmed by a shift in mobility, occurs also in parental 32D cells after induction of differentiation with G-CSF (Fig. 3, panel B). Ubiquitination is detectable as early as 15 min, and is prominent in cells treated with MG132.

Downregulation of UBF1 Is Mediated by an Active GSK3β

GSK 3β is a ubiquitously expressed serine/threonine kinase active in resting cells, which undergoes inhibition through phosphorylation at serine 9 [Zhou et al., 2004] in response to different stimuli, including IGF-1 and insulin [Desbois-Mouthon et al., 2001]. It is a substrate of Akt [Pap and Cooper, 1998], which is strongly activated by PI3K [Myers et al., 1994]. PI3K activity in myeloid cells is markedly increased by phosphorylation of IRS-1 [Soon et al., 1999], whose expression stabilizes UBF1. GSK3β is also known to phosphorylate certain substrates and mark them for ubiquitination and degradation via the proteasome pathway [Diehl et al.,

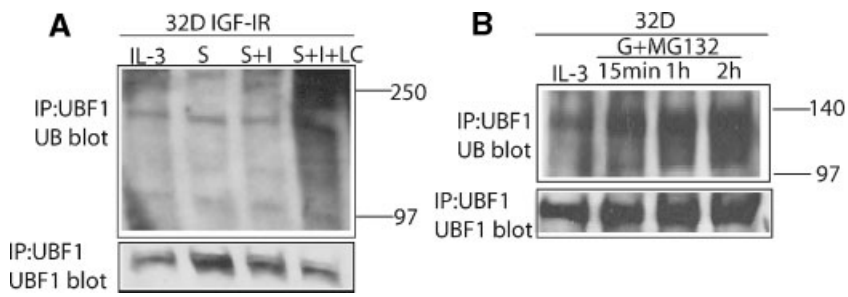

Fig. 3. Ubiquitination of UBF. 32D IGF-IR cells were kept in IL-3 or shifted from IL-3 to other culture media. Lysates were made and immuno-precipitated with an antibody to UBF1 (**Panel A**). Western blots were developed with an antibody to ubiquitin (UB, **upper row**) or UBF (**lower row**). Lanes are: IL-3; 10% serum without IGF-1 for 4 h (S); serum plus IGF-1 for 4 h (S + I); serum, IGF-1 and lactacystin (S + I + LC, 10 μM) for 4 h. **Panel B**: Parental 32D cells. Immuno-precipitation with an antibody to UBF, Western blots with antibodies to ubiquitin (UB, **upper row**) or UBF (**lower row**). All cells were treated with MG132, an inhibitor of the proteasome pathway. Lanes: IL-3, G-CSF (G), and MG132 for 15 min, 1 h, and 2 h, respectively.

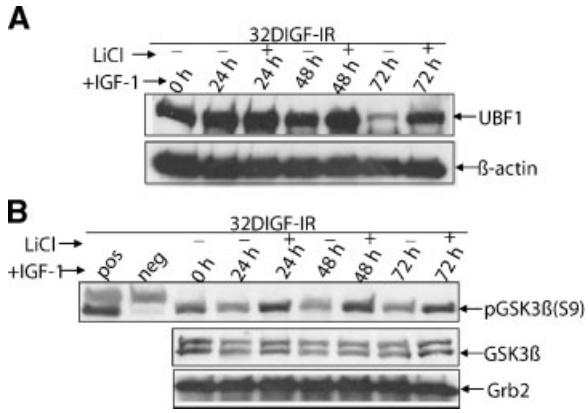

Fig. 4. Inhibition of UBF1 degradation by lithium chloride. In **panels A** and **B**, 32D IGF-IR cells were induced to differentiate with IGF-1 or treated with IGF-1 and LiCl (10 mM) at various times, as indicated. Western blot with antibodies to UBF1 and actin in panel A, antibodies to phospho-GSK3β (specific for phosphorylation at serine 9), total GSK3 and Grb2 in panel B. Panels A and B; lanes from left to right: IL-3; IGF-1, 24 h; IGF-1 and LiCl 24 h; IGF-1, 48 h; IGF-I and LiCl 48 h; IGF-1, 72 h; IGF-1 and LiCl 72 h. Panel B, the first two lanes are the positive (pos) and negative (neg) controls, respectively.

1998; Litovchick et al., 2004; Viatour et al., 2004]. GSK3β activity is said to be specifically inhibited by LiCl or Lithium acetate [Beurel et al., 2004]. Figure 4, panel A, shows that LiCl (10 mM) effectively inhibits the degradation of UBF1 in 32D IGF-IR cells induced to differentiate by IGF-1. It is at least as effective as lactacystin. Figure 4, panel B, shows that serine 9 phosphorylation of GSK3β is detectable in 32D IGF-IR cells in IGF-1, and that its phosphorylation (inactivation) is increased by treatment with LiCl.

In Vitro and In Vivo Phosphorylation of UBF1 by GSK3β

To test whether UBF1 is a substrate for GSK3β, we used purified GSK3β and UBF immuno-precipitated from cell lysates for an in vitro reaction. The results in Figure 5, panel A, show that purified GSK3β phosphorylates UBF in vitro. The reaction is ATP-dependent (lane 3) and requires both the enzyme (lane 2) and the substrate (lane 4). We also investigated whether the expression of a constitutively active GSK3β could increase the phosphorylation of UBF1 in vivo. GSK3β in this construct is constitutively active because of a serine to alanine mutation at amino acid 9. The cells used were 32D IGF-IR/IRS1 cells, stably transfected with the plasmid expressing the mutant GSK3β. Figure 5, panel B shows that the expression of the constitutively active GSK3β increased UBF1 phosphorylation.

Mutation or Elimination of UBF1 Residues Recognized by GSK3β Stabilizes UBF1

GSK3β recognizes an S/TxxxS motif, and it phosphorylates a serine or threonine at position −4 from a serine residue [Park et al., 2004; Viatour et al., 2004]. There are five such motifs in the UBF1 sequence, three in the C-terminus (nor surprisingly as the C-terminus contains 20 serines), and two more outside the C-terminus, at residues 40–44, and 392–396. To confirm the role of GSK3β in the degradation of UBF1, we introduced into 32D IGF-IR cells the C-terminus mutant (tagged with FLAG) of UBF1. The construct (see Methods and Materials) has a termination codon in lieu of an amino acid coding triplet, thus giving a normal length mRNA but a truncated UBF1 protein, lacking three of the motifs putatively recognized by GSK3β. The C-terminus truncated UBF1 can be recognized both by its size (90 amino acids shorter) and by its 5′ FLAG tag (Fig. 6). When the C-terminus UBF mutant is stably expressed in 32D IGF-IR cells (Fig. 6, panel A), it is definitely more stable than the wild type UBF in the same cells. This is especially interesting as the C-terminus mutant is functionally inactive (Grummt, 1999, and unpublished data from our laboratory). The protection conferred by the truncation at the C-terminus is not complete, and we therefore decided to mutate the other two GSK3β motifs of UBF1. The resultant mutant was a truncated UBF1 (C-terminus truncated) with serine to alanine mutations at the other two sites (see Experimental Procedures). It was introduced into 32D IGF-IR cells, and the stable cell line was then induced with IGF-1 (Fig. 6, panel B). The endogenous wild type UBF1 is again degraded, while the mutant UBF remains stable, whether stained with an antibody to UBF1 or an antibody to the FLAG tag. This experiment has been repeated twice. At $t = 96$ h, endogenous UBF1 was 10% of the 0 time value (by densitometry), while the 5-motifs mutant was 74%. Since about 15–20% of the UBF1 decrease in 32D IGF-IR cells can be attributed to decreased synthesis [Wu et al.,

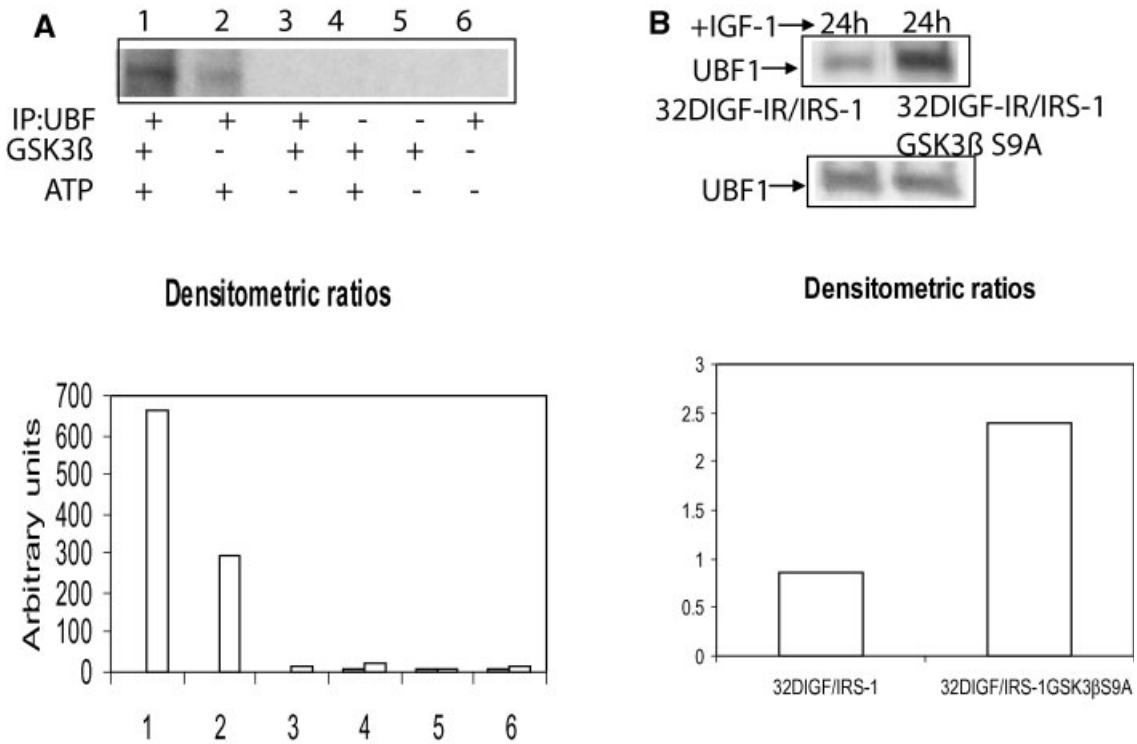

Fig. 5. Phosphorylation of UBF1 in vitro and in vivo by GSK3β. **Left**: UBF1 was immunoprecipitated from quiescent cells and phosphorylated in immune complexes prepared with the UBF1 antibody (IP: UBF1) and incubated with GSK3β (**lane 1**). There is slight UBF phosphorylation even when GSK3β is not added, but it is much less than with GSK3β (**lane 2**). As a negative control, GSK3β was incubated without immunoprecipitated UBF, and no phosphorylation was detected (**lane 4**). Beneath the autoradiograph is a densitomeric analysis of the autoradiographic bands performed with the ImageQuant program. The data represent one of two experiments performed with similar results. **Right**: Phosphorylation of UBF in parental 32D IGF-IR/IRS1 cells and in the same cells expressing a constitutively active GSK3β (mutation at serine 9). 32DIGF-IR/IRS-1 and 32DIGF-IR/IRS-1 GSK3 βS9A cells were shifted from IL-3 to IGF-1(50 ng/ml) for 24 h, and labeled with [^{32}P] orthophosphate for 4 h (The methodology is described under Methods and Materials). Beneath the autoradiograph is a Western blot of UBF from both cell lines. A densitomeric analysis of the bands was also performed with the ImageQuant program.

2005], the 74% figure is an acceptable limit for stabilization. A mutation at the pRb-binding motif LxCxE at residue 307 did not increase the stability of UBF1 in 32D IGF-IR cells in IGF-1. This mutant is degraded at the same rate as the wild type UBF1 (Fig. 6, panel C), indicating that mutations per se do not affect UBF1 stability.

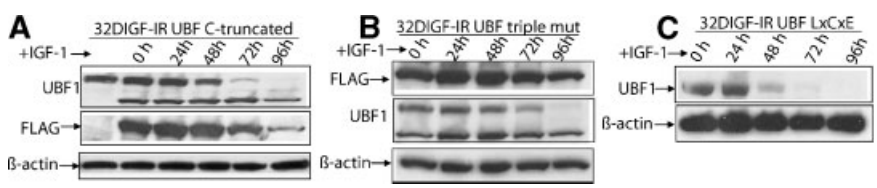

Fig. 6. Mutant UBF1 proteins lacking the GSK3β phosphorylation sites are more stable than the endogenous wild type UBF1 in 32D IGF-IR cells induced to differentiate by IGF-1. The C-terminus truncated UBF1, generated as described in Methods and Materials, was introduced into 32D IGF-IR cells, and a stable mixed population was developed. Lysates were made at the times after shifting to IGF-1 indicated above the lanes. Western blots with antibodies to UBF, FLAG, and β-actin. **Panel A** is a composite Western blot developed with an antibody to UBF1 (**upper rows**) or an antibody to FLAG (arrow), of lysates of cells expressing the C-terminus truncated UBF1, with a FLAG tag at the 5′ end. The blot was first stained with an antibody to FLAG, then, after stripping, with an antibody to UBF. The truncated UBF protein is visible as a shorter protein in the blot stained with an antibody to UBF, and also in the blot developed with an antibody to FLAG. **Panel B**: 32D IGF-IR cells were stably transfected with a UBF1 truncated at its C-terminus and with mutations at the serines of the other two phosphorylation sites for GSK3β (serines 40, 43, and 44, serines 392 and 396 and praline 395, all mutated to alanines). Whether by anti-FLAG (**upper row**) or by antibody to UBF1 (**middle rows**), the mutant UBF1 is definitely more stable than the endogenous wild type UBF1. **Panel C**: A mutant at the putative pRb binding site (LxCxE) is unstable when transfected into 32D IGF-IR cells.

Effect of a Constitutively Active GSK3β on the Levels of UBF1

To confirm the role of GSK3β in the downregulation of UBF1, we stably transfected 32D IGF-IR and 32D IGF-IR/IRS1 cells with a plasmid expressing a constitutively active GSK3β (see above). This plasmid has a GFP marker, and the expression was readily demonstrated by FACS analysis (Fig. 7, panel A). We determined the effect of the constitutively active GSK3β on the levels of UBF1 at various times after the cells were shifted from IL-3 to IGF-1. When compared to the nontransfected cell lines, the serine 9 mutant of GSK3β induces degradation of UBF1 in 32D IGF-IR/IRS-1 cells and accelerates the downregulation of UBF1 in 32D IGF-IR cells (Fig. 7, panels B,C).

Subcellular Localization of Mutant UBF

We asked next whether the mutant UBF (truncated and mutated at the other 2 serine sites) still localized to the nuclei. The result is shown in Figure 8, where the cells were stained with an anti-FLAG antibody and counterstained with Propidium Iodide. The mutant UBF still localizes to the nuclei of cells.

UBF1 Protein Stability in 32D-Derived Cell Lines

The above experiments show that both the activated IGF-IR and G-CSF, in the absence of IRS-1 expression, cause the downregulation of UBF1 in 32D myeloid cells, and that the degradation is mediated through GSK3β and the subsequent ubiquitination of the protein. One explanation for our results is that IRS-1, by

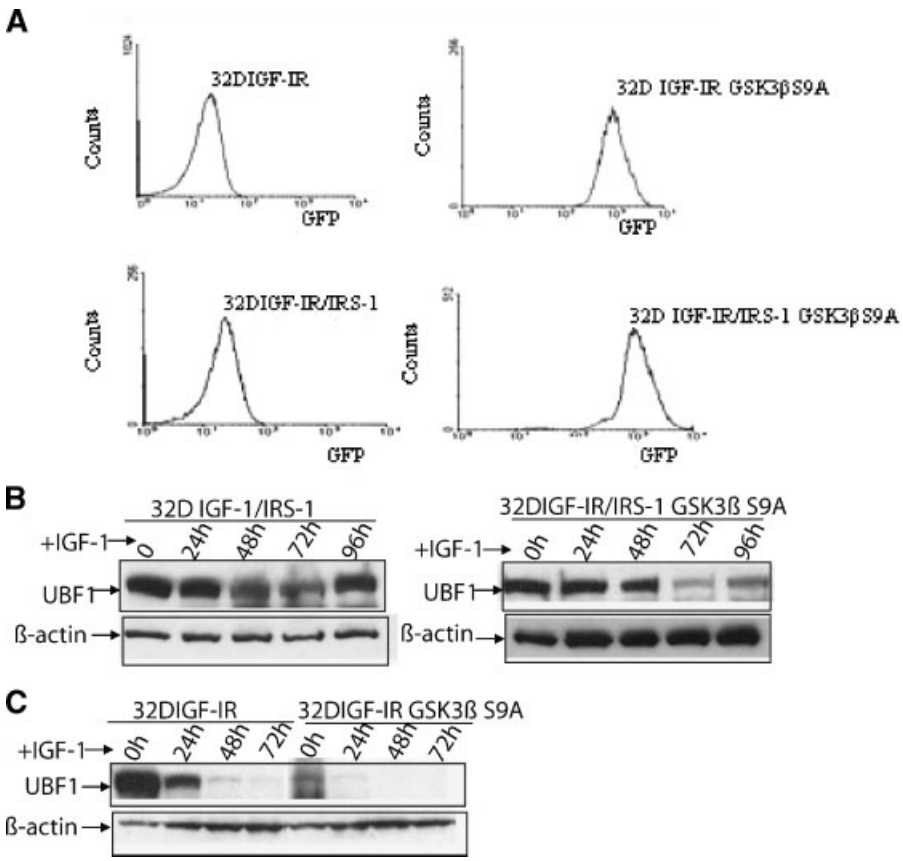

Fig. 7. A constitutively active GSK3β accelerates the degradation of UBF1. This plasmid expresses a GFP marker, and **panel A** shows that the transduced cells strongly express the serine-9 mutant of GSK3β. **Panel B** shows UBF1 levels by Western blots, at different hours after shifting from IL-3 to IGF-1. On the left are the parental 32 IGF-IR/IRS1 cells, on the right, the cells with the serine 9 mutant of GSK3β. Lysates were made and blots stained with antibodies to UBF1 and β-actin. Note that the protein amounts are higher in the panel of cells with the mutant GSK3β. **Panel C** shows a similar experiment with 32D IGF-IR cells, untransduced (**left**) or retrovirally transduced with the serine-9 mutant of GSK3β.

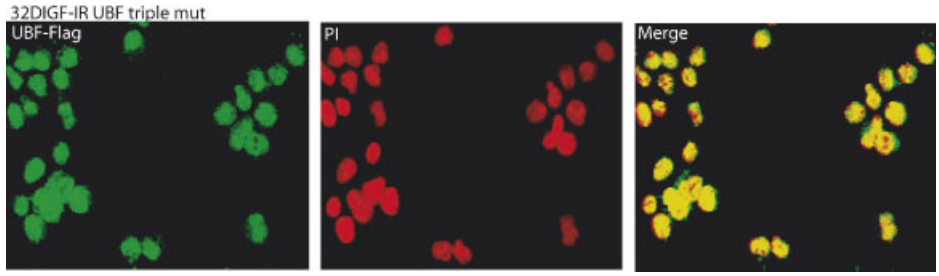

Fig. 8. Confocal microscopy of cells expressing the mutant UBF1. 32D IGF-IR cells expressing the triple UBF1 mutant were examined by confocal microscopy as described in Experimental Procedures. The cells were stained with an antibody to FLAG and counterstained with Propidium Iodide. The 3rd panel is the merged picture. The mutant UBF is localized to the nucleus. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

inactivating GSK3β (see above), prevents the degradation of UBF1. IRS-1, however, is also known to inhibit the differentiation of 32D IGF-IR cells [Valentinis et al., 1999], through the induction of ID2 [Prisco et al., 2001], a powerful inhibitor of the differentiation program [Perk et al., 2005]. An alternative explanation could therefore be that UBF1 is degraded as part of the involution of the nucleolus that occurs when a terminal differentiation signal is operating in hemopoietic cells (se above). In the following experiments, we tested the possibility that the induction of differentiation may be the trigger for the degradation of UBF1 by GSK3β.

C/EBPα is a key regulator of differentiation in many cell types [Zhang et al., 1997; Radonska et al., 1998; Ross et al., 1999], including 32D myeloid cells [Keeshan et al., 2003], where it promotes granulocytic differentiation. In addition, it has been reported that the GSK3 isoforms can induce conformational changes in C/EBPα [Ross et al., 1999]. We took advantage of a 32D BCR-ABL cell line (undifferentiated and transformed) in which C/EBPα can be conditionally activated by treatment with tamoxifen [Ferrari-Amorotti et al., 2006]. Activation of C/EBPα in these cells causes molecular and morphological aspects of terminal differentiation. Figure 9 shows the levels of UBF1 in BCR-ABL cells, one transfected with an empty vector, the other with the plasmid expressing the tamoxifen-dependent C/EBPα. Both cell

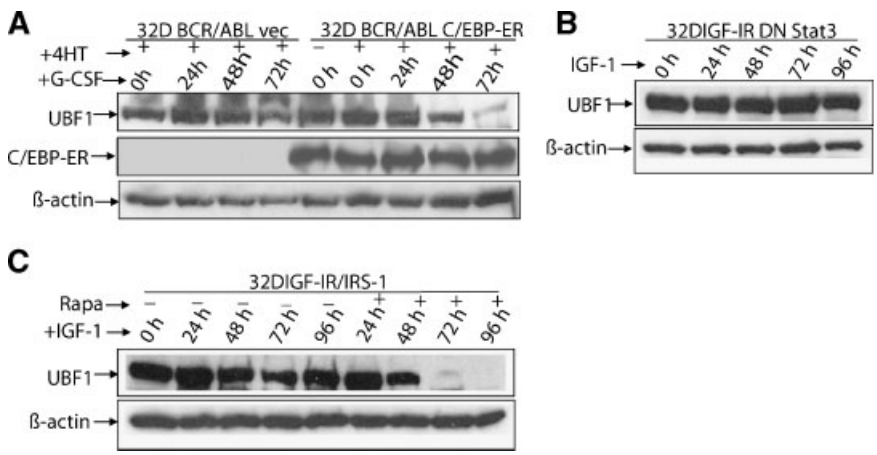

Fig. 9. Differentiation and UBF1 Stability. **Panel A**: Activation of C/EBPα in 32D BCR/ABL cells causes the downregulation of UBF1. BCR/ABL cells carrying the activated C/EBPα plasmid or an empty vector were treated with tamoxifen (see text). **Upper row**: In cells with the empty vector (transformed), UBF1 is stable as in 32D IGF-IR/IRS1 cells (also transformed). UBF1 is downregulated in cells with the tamoxifen-activated C/EBPα (differentiating). **Middle row**: Expression of C/EBPα, absent in cells transfected with the empty vector. β-actin is used to monitor protein amounts in each lane. **Panel B**: 32D IGF-IR cells stably expressing a dominant negative mutant of Stat3 were used in this experiment. The cells were shifted from IL-3 to IGF-1 and lysates were prepared at various times afterwards. The levels of UBF1 remain stable in these cells that no longer differentiate [Prisco et al., 2001]. **Panel C**: 32D IGF-IR/IRS1 cells were induced to differentiate with 10 ng/ml of rapamycin (Rapa). Control cells remained untreated. All cells were shifted to IGF-1, and UBF levels determined as usual at the indicated times.

lines were treated with tamoxifen (100 nM). In cells expressing C/EBPα (upper row), treatment with tamoxifen induced the downregulation of UBF1, while there was no effect in cells stably transfected with the empty vector.

In 32D cells, Stat3 regulates differentiation, and a dominant negative mutant (DN) of Stat3 inhibits G-CSF- or IGF-1-induced differentiation [Bromberg et al., 1998; Prisco et al., 2001]. We tested the stability of UBF1 in 32D IGF-IR cells stably expressing a DN mutant of Stat3 [Prisco et al., 2001]. In 32D IGF-IR cells expressing the DN mutant of Stat3, which inhibits their differentiation, UBF1 remained stable after IGF-1 treatment (Fig. 9, panel B).

32D IGF-IR/IRS-1 cells, as mentioned above, do not differentiate and in fact form tumors in mice [Valentinis et al., 2000], and UBF1 in these cells is stable (Wu et al., 2005 and this paper). 32D IGF-IR/IRS-1 cells can be induced to differentiate by rapamycin [Valentinis et al., 2000; Tu et al., 2003]. We treated 32D IGF-IR/IRS-1 cells with rapamycin and determined the stability of UBF1, as usual, by Western blots. Figure 8, panel C, shows that treatment with rapamycin causes downregulation of UBF1.

Effect of Lithium Chloride and a Mutant GSK3β on the Morphology of 32D-Derived Cells

We have shown above that treatment with LiCl (which inhibits GSK3β) stabilizes UBF1 in 32D IGF-IR cells. We have also shown that other forms of induced differentiation can downregulate UBF1 expression. We asked next whether inhibitors of GSK3β could inhibit differentiation or, conversely, whether a constitutively active GSK3β could induce differentiation. We examined first the morphology of LiCl-treated cells (Fig. 10, panel A). As usual, 32D IGF-IR cells differentiate into granulocytes after shifting to IGF-1 (5 days in this experiment). Cells treated with LiCl show little evidence of differentiation. There are many dead or dying cells in this population, also an occasional differentiated one, but many cells show no sign of differentiation, and are quite large, as one would expect of cells that maintain higher levels of UBF1 [Prisco et al., 2004]. To appreciate the size of the 32D IGF-IR cells in LiCl, compare them with the cells in panel B, taken at the same magnification, where we examined the 32D IGF-R/IRS1 cells expressing

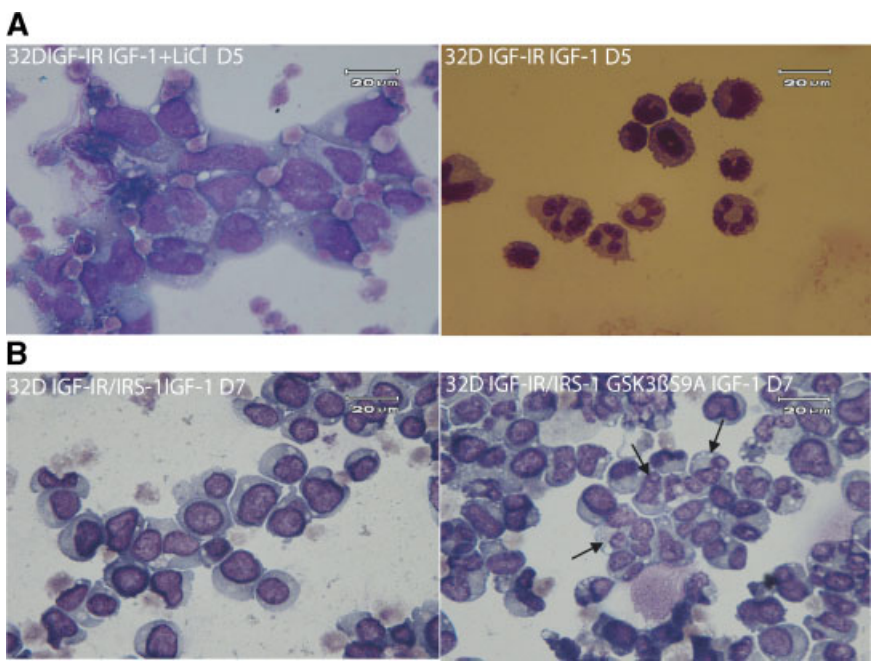

Fig. 10. Morphology of 32D-derived cells treated with Lithium chloride or expressing a constitutively active GSK3β. In **panel A**, 32D IGF-IR cells were shifted to IGF-1 and either left untreated or treated with LiCl (10 mM). These pictures were taken 5 days after shifting to IGF-1 at the same magnification. There are many dead cells in the LiCl-treated cells, but many of the surviving cells are undifferentiated and very large. In untreated cells, most of the cells are already differentiated into granulocytes. In **panel B**, the morphology of parental 32D IGF-IR/IRS1 cells and cells expressing the serine-9 mutant of GSK3β is compared. Cells expressing the mutant GSK3β show signs of differentiation, with segmented nuclei, some actually looking like granulocytes (arrows). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the serine-9 mutant of GSK3β (Fig. 10, panel B). We show only the picture of cells on Day 7 after shifting to IGF-1. The untransfected cells maintain their blast morphology, while in the cells expressing the constitutively active GSK3β, there are quite a few cells showing a differentiated morphology, with granulocyte-type nuclei (arrows). We do not show the 32D IGF-IR cells transfected with the serine-9 mutant, because those cells already differentiate when shifted to IGF-1 [Valentinis et al., 1999, and this paper].

UBF2 Degradation During 32D Cells Differentiation

There are two isoforms of UBF that differ by only 37 amino acids located between residues 221 and 257 and deleted in UBF2 [Jantzen et al., 1990; O'Mahony and Rothblum, 1991]. The commercially available antibody does not distinguish between the two isoforms, although they can be distinguished by their slightly different sizes on Western blots. UBF2 is detectable in MEFs [Tu et al., 2002], but it is very poorly expressed in 32D and 32D-derived cells. To test the stability of UBF2 in granulocytic differentiation, we used a UBF2 cDNA tagged with Flag [Chen et al., 2005] and generated from 32D IGF-IR cells a stable cell line, expressing it. We then determined its fate after induction of differentiation with IGF-1. The results (Fig. 11, panel A) show that UBF2 is also downregulated, approximately with the same kinetics of UBF1. Similar results were obtained when the UBF2-Flag cDNA was stably expressed in parental 32D cells. G-CSF differentiation caused disappearance of UBF2 with roughly the same kinetics as 32D IGF-IR cells treated with IGF-1 (Fig. 11, panel B). These experiments are an indirect confirmation of the role of GSK3β in the degradation of the UBF proteins, as all GSK3β phosphorylation sites are equally present in both isoforms.

DISCUSSION

Our results can be summarized as follows: (1) the UBF1 protein is downregulated in 32D IGF-IR cells induced to differentiate by IGF-1 and in parental 32D cells induced to differentiate by G-CSF; (2) the use of inhibitors suggest that UBF1 is ubiquitinated, after being marked for ubiquitination by GSK3β; (3) GSK3β phospohorylates UBF1 in vitro and in vivo; (4) elimination and/or mutations of the five putative phosphorylation sites of GSK3β stabilize the UBF1 protein in differentiating 32D cells; (4) stable expression of a constitutively active GSK3β accelerates the degradation of UBF1; (5) inhibitors of GSK3β inhibit the differentiation of the 32D-derived cells, while a constitutively active GSK3β promotes differentiation; (6) there is a correlation between the downregulation of UBF1 and the induction of the differentiation process. These points will be considered separately.

UBF1 activity depends on its phosphorylation and on its levels (see Introduction). When 32D IGF-IR cells are induced to differentiate by IGF-1, UBF1 protein levels (but not mRNA levels) decrease and the protein becomes eventually undetectable [Tu et al., 2003; Wu et al., 2005 and this paper]. Most of the UBF1 decrease is due to increased degradation of the protein [Wu et al., 2005]. Induction of differentiation by G-CSF in parental 32D cells also causes downregulation of UBF1, without decreasing mRNA levels. Both G-CSF in parental 32D cells and IGF-1 in 32D IGF-IR cells induce differentiation along the granulocytic pathway. Under these conditions, UBF1 is ubiquitinated. Ubiquitination is sometimes preceded by phosphorylation of the

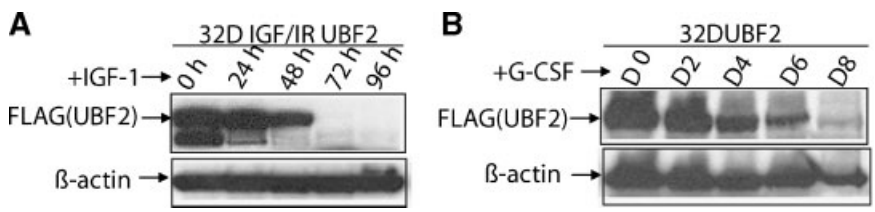

Fig. 11. UBF2 is also downregulated in 32D IGF-IR cells grown in IGF-1 or parental 32D cells induced to differentiate by G-CSF. **Panel A**: UBF2 protein with a FLAG-tag [Chen et al., 2005] was stably expressed in a mixed population of 32D IGF-IR cells. The cells were shifted from IL-3 to IGF-1, and the Western blots were stained with antibodies to FLAG and to actin (to monitor protein amounts). UBF2 is degraded at roughly the same rate as UBF1 (compare with Fig. 1). **Panel B**: The experiment was repeated with parental 32D cells, expressing a FLAG-tagged UBF2. Treatment with G-CSF (25 ng/ml) caused the degradation of UBF2.

protein by GSK3β. GSK3β phosphorylates and downregulates cyclin D1 [Diehl et al., 1998], β-catenin [Dong et al., 2005], the oncoprotein BCL-3 [Viatour et al., 2004], and RBL2/p130 during cell quiescence [Litovchick et al., 2004]. GSK3β preferentially phosphorylates sites that have a SxxxS/T sequence (where S is serine and T is threonine) [Aberle et al., 1997; Patel et al., 2004]. Degradation of UBF1 was inhibited by LiCl, an inhibitor of GSK3β activity [Beurel et al., 2004]. Purified GSK3β phosphorylates UBF in vitro, and a constitutively active GSK3β increases UBF phosphorylation in vivo. Because UBF1 has five motifs for GSK3β phosphorylation, we tested directly the possibility that UBF is directly phosphorylated by GSK3β. Mutations or elimination of the five GSK3β motifs stabilize UBF1 even when the process of differentiation is induced. These GSK3β phosphorylation sites on UBF1 are located at serines 40 and 392 and in the C-terminus. The C-terminus of UBF1 (arbitrarily the last 90 amino acids or so) has 20 serines, and it is not surprising that it has 3 GSK3β phosphorylation motifs. They are also preferred sites, because the rest of the amino acids in the UBF1 C-terminus are acidic, and the presence of a D (glutamic acid) re-enforces the site for targeting by GSK3β [Patel et al., 2004]. The other two sites (extra C-terminus) instead have adjacent prolines, important in directing GSK3β [Diehl et al., 1998].

The importance of GSK3β in the regulation of UBF1 protein levels is confirmed by the effects of a retrovirally transduced constitutively active GSK3β [Corradini et al., 2005]. Under these conditions, UBF1 degradation is accelerated, whether in differentiating or proliferating cells. Less direct support for a role of GSK3β and ubiquitination in the degradation of UBF1 is provided by inhibitors of either GSK3β or the proteasomal pathway. Thus both LiCl (an inhibitor of GSK3β-62) and lactacystin (an inhibitor of the proteasomal pathway) inhibit the degradation of UBF1 in cells induced to differentiate along the granulocytic pathway. Another indirect confirmation of the role of GSK3β in the downregulation of UBF1 is given by rapamycin. Rapamycin leads to activation of GSK3β [Dong et al., 2005], and to the downregulation of UBF1 (this paper). The disappearance of UBF proteins in 32D-derived cells occurs in the nucleolus; at variance with other proteins, UBF1 is not exported to the cytoplasm for degradation by the differentiation process [Tu et al., 2003].

Our results with the conditionally activated C/EBPα-ER^{TAM} chimeric protein, with the DN mutant of Stat3, with rapamycin, and with G-CSF in parental 32D cells, strongly suggest that the degradation of UBF1 in 32D myeloid cells is tied to the induction of the differentiation process. For the experiments with C/EBPα, we used a plasmid described by Ferrari-Amorotti et al. [2006], in which the chimera C/EBPα/ER is activated by tamoxifen. Tamoxifen-dependent activation of C/EBPα (by nuclear translocation) induces differentiation in 32D BCR-ABL cells. In our experiments, activation of C/EBPα/ER causes downregulation of UBF1. The importance of differentiation in the downregulation of UBF1 has been confirmed in 32D IGF-IR cells expressing the DN mutant of STAT3, which no longer differentiate, although they do not express IRS-1 [Prisco et al., 2001]. Conversely, 32D IGF-IR/IRS-1 cells treated with rapamycin still express IRS-1, but differentiate [Tu et al., 2003] and UBF1 is degraded. The degradation of UBF1 in parental 32D cells induced to differentiate by G-CSF is also consistent with the role of granulocytic differentiation in this process, as the G-CSF receptor sends signals that overlap with those originating from the IGF-IR. According to Avalos [1996], Hunter and Avalos [1998], and Ward et al. [1999], the cytoplasmic domain of the G-CSF receptor has three boxes, and elimination of Box 3 (last two tyrosines at 744 and 764) results in a receptor that causes proliferation but no differentiation (G-CSF stimulation, like IGF-I in 32D IGF-IR cells, first causes a burst of cell proliferation, followed by the differentiation process). This truncated G-CSF receptor fails to phosphorylate Shc, which is phosphorylated by the wild type, class I receptor [Avalos, 1996; Dong and Larner, 2000]. The JAK and STAT proteins are still activated by this receptor [Avalos, 1996]. These findings are consistent with our own findings that expression of a dominant negative mutant of Shc inhibits IGF-1-mediated differentiation of 32D IGF-IR cells [Valentinis et al., 1999].

If differentiation is important in triggering UBF1 degradation, the question is whether GSK3β, which targets UBF1, can modulate differentiation. GSK3β plays a role in differentiation in several models from Xenopus to hematopoietic cells in culture [He et al., 1995;

Pierce and Kimelman, 1995; Zhou et al., 2004; Corradini et al., 2005]. According to Park et al. [2004] and Tang et al. [2005] phosphorylation of C/EBPβ by GSK3β is required for the activation of C/EBPα, a powerful inducer of differentiation in either adipocytes or hematopoietic cells. A connection between GSK3β and C/EBP proteins has also been reported by Ross et al. [1999] and by Zhao et al. [2005]. Our experiments provide additional evidence, as LiCl, an inhibitor of GSK3β, inhibits the differentiation of 32D IGF-IR cells, while a constitutively active GSK3β promotes the differentiation of 32D IGF-IR cells. This is especially remarkable as IRS-1 is a potent inhibitor of the differentiation program through the induction of Id proteins. A role of GSK3β in differentiation is indirectly confirmed by its negative role in cell proliferation. GSK3β marks for degradation via the proteasomal pathway cyclin D1 [Diehl et al., 1998], β-catenin [Dong and Larner, 2000], and c-Myb [Corradini et al., 2005] that play important roles in cell cycle progression. One could formulate the hypothesis that the induction of differentiation activates GSK3β (dephosphorylated serine 9), which, in turn, leads to the activation of differentiation-promoting transcription factors, like C/EBPα (see above) and to the phosphorylation and degradation of proliferation-promoting factors, like cyclin D1, β-catenin, c-Myb, and UBF1. In this hypothesis, IRS-1 could stop the whole process by simply inactivating GSK3β (which is inactivated by IGF-1 and PI3K).

The process of nucleolar involution in differentiation is not limited to myeloid cells only, but it is not generalized. While differentiating keratinocytes lose not only the nucleolus, but also the nucleus, there are differentiated cells like neurons and muscle cells that preserve nuclear and nucleolar integrity. These latter cells, however, although differentiated, usually persist for a long time, whereas differentiated myeloid cells have a short life span [Likovsky and Smetana, 2000]. Interestingly, the nucleolus is absent in nucleated terminally differentiated chick erythrocytes, and when these cells are re-activated by cell fusion, production of specific chick proteins requires the previous reconstitution of the nucleolus [Ringertz and Savage, 1976]. Perhaps, nucleolar disappearance may be a sine qua non for the terminal differentiation of hemopoietic cells. This interpretation would be compatible with the limited life span of differentiated hematopoietic cells, in comparison to other differentiated cells, like neurons and muscle cells, which maintain their nucleoli. The mechanism we have described for the degradation of UBF1, however, ought to be valid for hematopoietic cells, and 32D cells in this respect are bona fide myeloid cells that differentiate into granulocytes, like myeloid cells in the bone marrow.

In conclusion, we have demonstrated that the induction of granulocytic differentiation in 32D myeloid cells causes downregulation of the UBF1 protein, through phosphorylation by GSK3β and proteasome-dependent degradation. Our results do not imply that this is the only mechanism for the regulation of UBF1 (or UBF2) levels. We suspect in fact that degradation of UBF1 by GSK3β is a special case for hematopoietic cells induced to differentiate, as inhibition of the PI3K pathway in MEFs does not de-stabilize UBF1 [Wu et al., 2005]. However, the data we have described may throw some light on the mechanisms by which the nucleolus (and with it rRNA synthesis) is dismantled in hematopoietic cells induced to terminally differentiate.

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