The androgen-independent human prostate adenocarcinoma cell line DU-145 proliferates in serum-free medium and produces insulin-like growth factors (IGF)-I, IGF-II, and the IGF type-1 receptor (IGF-1R). They also secrete three IGF-binding proteins (IGFBP), IGFBP-2, -3, and -4. Of these, immunoblot analysis revealed selective proteolysis of IGFBP-3, yielding fragments of 31 and 19 kDa. By using an anti-IGF-I-specific monoclonal antibody (mAb), we detect surface receptor-bound IGF-I on serum-starved DU-145 cells, which activates IGF-1R and triggers a mitogenic signal. Incubation of DU-145 cells with blocking anti-IGF-I, anti-IGF-II, or anti-IGF-I plus anti-IGF-II mAb does not, however, inhibit serum-free growth of DU-145. Conversely, anti-IGF-1R mAb and IGFBP-3 inhibit DNA synthesis. IGFBP-3 also modifies the DU-145 cell cycle, decreases p34cdc2 levels, and IGF-1R autophosphorylation. The antiproliferative IGFBP-3 activity is not IGF-independent, since des-(1–3)-IGF-I, which does not bind to IGFBP-3, reverses its inhibitory effect. DU-145 also secretes the matrix metalloproteinase (MMP)-9, which can be detected in both a soluble and a membrane-bound form. Matrix metalloproteinase inhibitors, but not serpins, abrogate DNA synthesis in DU-145 associated with the blocking of IGFBP-3 proteolysis. Overexpression of an antisense cDNA for MMP-9 inhibits 80% of DU-145 cell proliferation that can be reversed by IGF-I in a dose-dependent manner. Inhibition of MMP-9 expression is also associated with a decrease in IGFBP-3 proteolysis and with reduced signaling through the IGF-1R. Our data indicate an IGF autocrine loop operating in DU-145 cells, specifically modulated by IGFBP-3, whose activity may in turn be regulated by IGFBP-3 proteases such as MMP-9.

In normal cells, proliferation is a coordinated process involving intercellular communication through soluble regulatory molecules known as polypeptide growth factors (1). In contrast, neoplastic cells are characterized by a relative autonomy of growth, a consequence of the constitutive expression of growth factors and receptors involved in autocrine loops (2). Examples of constitutive autocrine growth factor loops have been reported for different cancer cells and growth factors, such as transforming growth factor α, insulin-like growth factors (IGF-I and IGF-II), and platelet-derived growth factors, among others (3).

IGF-I and IGF-II are potent mitogens for several non-transformed and cancer cell types, and viral and nonviral oncopogenes appear capable of interfering with the IGF autocrine loop (4). Indeed, c-MYC increases IGF-I secretion and IGF type-1 receptor (IGF-1R) production. It has also been suggested that the IGF-1R is critical in the establishment and maintenance of the transformed phenotype. Mouse embryo cells with a targeted disruption of the IGF-1R gene (5, 6) cannot be transformed by SV40 large T antigen alone or in conjunction with Ha-ras (7, 8). In addition, antibodies to the IGF-1R (9), as well as antisense expression (10) and dominant negative mutants of this receptor (11), reverse the transformed phenotype and/or inhibit tumorigenesis.

In serum and in extracellular fluids, both IGF-I and -II are bound with high affinity to soluble IGF-binding proteins (IGFBP), seven of which have been identified to date (12). Many tumor cell types secrete one or more of these proteins (13). The relevance of the IGFBP lies in their potential to modify the metabolic and mitogenic effects of IGF. In fact, IGFBP may either inhibit and/or enhance IGF activity (14–17). The inhibitory effects of IGFBP have been attributed to competitive scavenging of IGF peptides away from the IGF receptors (18). The enhancer mechanism is poorly understood, however, and probably involves binding to the cell membrane or extracellular matrix and/or processing into smaller molecular weight species by limited proteolysis (15, 19–22). The result is a dramatic reduction in IGFBP affinity for IGF, which enhances the availability of the growth factors to the target cells (23). Possible direct effects have also recently been suggested for some IGFBP, independent of their IGF binding activity (12).

In this study, we characterize the role of IGF, IGF-1R, and IGFBP in tumor cell proliferation using DU-145 cells, a human androgen-independent prostate adenocarcinoma cell line (24). Earlier studies indicated that this cell line expresses several components of the IGF axis (25, 26). Our results suggest the existence of an IGF autocrine loop in DU-145 cells and its specific modulation by IGFBP-3 proteolysis. This proteolytic activity may be ascribed to the matrix metalloproteinase

The abbreviations used are: IGF, insulin-like growth factor; IGFBP, IGF-binding protein; IGF-1R, IGF type-1 receptor; 3T3-IGF-1R, 3T3 fibroblasts overexpressing the human IGF-1R AS antisense; CM, conditioned medium; DME, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; PO, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; SPM, serum-free medium; TIMP, tissue inhibitor of metalloproteinase; TdR, thymidine; BSA, bovine serum albumin; RT-PCR, reverse transcriptase-polymerase chain reaction; FACS, fluorescence-activated cell sorter; h, human.
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(MMP)-9, which is also produced by this cell line in an autocrine manner. Furthermore, we find MMP-9 both in soluble form and in a membrane-associated form on the DU-145 cell surface. The expression of an MMP-9 antisense cDNA leads to an 80% inhibition in DU-145 proliferation, which is reversed by the addition of exogenous IGF-I. This growth inhibition is associated with the abrogation of IGFBP-3 proteolysis as well as with a decrease in IGF-1R-promoted cell signals. MMP-9 therefore controls DU-145 cell proliferation by interacting, at least partially, with the IGF-I autocrine loop in this cell line.

EXPERIMENTAL PROCEDURES

Cell Culture—BALB/c 3T3 fibroblasts overexpressing the human IGF-1R (3T3-IGF-1R, a gift of Drs. A. Ullrich and R. Lammers) and DU-145 cells (ATCC HTB-81, American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal calf serum. Murine interleukin-3-dependent Ba/F3 cells (27) were cultured in RPMI, 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM l-glutamine, and 10% conditioned medium from the interleukin-3-producing cell line WEHI-3B.

Anti-IGF Monoclonal Antibodies—The monoclonal antibodies (mAb) KM5A1 and BB9E10 were raised in our laboratory by immunization with the IGF-I (55–70) synthetic peptide or recombinant human IGF-I, respectively (28, 29). KM5A1 is IGF-I-specific, with less than 0.1% cross-reactivity with IGF-II, and recognizes IGF-I when bound to IGFBP or the IGF-1R. BB9E10 has approximately 3% cross-reactivity with IGF-II and binds to an IGF-I epitope hidden by IGFBP and the IGF-1R. We also used an anti-IGF-II mAb raised in our laboratory, with an apparent affinity constant of 1011 M⁻¹ and less than 0.1% cross-reactivity with IGF-I, which is an IGF-II antagonist.

Amplification of IGF-I, -II, and IGF-1R mRNA by RT-PCR—Total RNA, derived from either starved or control cultured cells, was isolated by ultracentrifugation through a cesium chloride cushion (30). This material (5 µg) was then reverse-transcribed using a first strand cDNA synthesis kit (Pharmacia AB, Stockholm, Sweden). One-tenth of the transcription product was amplified for either 40 PCR cycles with hIGF-I-specific primers or for 30 cycles with primers specific for hIGF-II. The primers for hIGF-I were 5′-GGTGGATGCTCTTCAGTTCTGGTGTTG-3′ and 5′-GCAATACATCTCGCAGCTCTTGA-3′. The primers for hIGF-II were 5′-CTTACCGGCTCCGATGAGCCTGTG-3′ and 5′-CTTCCGACATTGGCAGGGAACCAC-3′. For amplification of the IGF-1R, the primers were used as described previously (31). Finally, 10 µl of the PCR products were resolved on 2% agarose gels.

Detection of IGFBP by Western Ligand and Immunoblotting—DU-145-conditioned medium (DU145-CM) was recovered from subconfluent cells cultured for 5 days in serum-free DMEM (SFM) and concentrated 10-fold using Centricon filters with a 3,000 molecular weight cut-off (Amicon, Danvers, MA). IGFBP species were detected with specific anti-IGFBP-1 mAb or anti-IGFBP-2, hIGFBP-4, anti-hIGFBP-5, or hIGFBP-6 (Austral Biologicals; San Ramon, CA) polyclonal antibodies, followed by PO-labeled goat anti-mouse or anti-rabbit IgG and ECL.

Detection of MMP-9 in DU145-CM—Either DU145-CM or conditioned medium from the phorbol 12-myristate 13-acetate-stimulated human fibrosarcoma HT-1080 cell line was recovered from subconfluent cultures after 2 days in serum-free DMEM supplemented with 0.5% BSA (RIA Grade; Sigma; SFM/BSA) and concentrated using Centricron filters. Zymography was performed in SDS-PAGE gels containing gelatin (1 mg/ml) as previously reported (35). The same samples were electrophoresed in SDS-PAGE under reducing conditions, blotted to nitrocellulose, and probed with anti-MMP-9 Ab3 antibody (Calbiochem), followed by PO-labeled goat anti-mouse antibody and ECL.

Growth Assays and Cell Cycle Analyses—DU-145 cells were detached using 0.05% trypsin, 0.02% EDTA (Life Technologies, Inc.) and plated at several cell densities in 96-well plates (for proliferation experiments) or in 24-well plates (for cell cycle analysis). Twenty-four hours later, the cells were washed extensively with PBS and cultured for 24 h in SFM/BSA. Thereafter, medium was renewed with SFM/BSA or without recombinant human IGF-I (rhIGF-1, Pharmacia & Upjohn), recombinant human des-(1–3)-IGF-I (kindly provided by Dr. Pär Gellerfors, Pharmacia & Upjohn, Stockholm, Sweden), IGFBP-3 (Calbiochem), blocking anti-hIGF-I and -II mAb, anti-IGF-1R mAb eir-3 (Oncogene Science, Uniondale, NY), aprotinin (Sigma), tissue inhibitor of metalloproteinases (TIMP)-2, or Batimastat (BB-94, kindly provided by Dr. F. Coletta, Pharmacia & Upjohn, Milan, Italy). Cell lysates from IGFBP-3- or anti-IGF-1R mAb-treated DU-145 cells were prepared and analyzed for IGF-1R autophosphorylation as described below.

For proliferation experiments, DU-145 cells were pulsed for 8 h with 0.5 µCi/well of [3H]thymidine ([3H]TdR, Amersham Pharmacia Biotech) at various times during the course of the experiment, and nuclei were harvested using a cell harvester (LKB-Wallac, Sweden). [3H]TdR incorporation was determined on a liquid scintillation counter. Ba/F3 cell proliferation assays were performed as described (28).

For cell cycle analysis, DU-145-treated cells were detached, washed with PBS, and stained with propidium iodide using the DNA-Prep Stain kit (Coulter Corp., Miami, FL). Cell cycle analysis was carried out in a flow cytometer equipped with a pulse processing facility to enable discrimination of cell doublets (Epics XL, Coulter). Cell number was...
FIG. 2. Flow cytometric detection of IGF-I binding to the DU-145 cell surface. A, cells were cultured in SFM, incubated with the biotinylated anti-IGF-I mAb KM5A1 or BB9E10 as indicated, and developed with phycoerythrin-labeled avidin (Av-PE). The cell-associated fluorescence intensity after incubation with an irrelevant antibody (gray area) was used as the negative control. KM5A1 binding specificity was assessed by preincubation of the mAb with IGF-I (KM5A1+IGF-I). B, cells were cultured as above, acid-washed before mAb incubation (see “Experimental Procedures”), and the cell-associated fluorescence after Av-PE development was determined. C, cells were treated as in B and then incubated with IGF-I. After removal of unbound IGF-I, biotinylated mAb were added and developed using Av-PE. D, cells were cultured in SFM/BSA supplemented with the indicated amounts of insulin for 72 h and then stained with the biotinylated antibodies as in A. Non Sp., nonspecific antibody.

**Detection of Cell Surface-bound IGF-I and MMP-9—DU-145 cells**

were washed and cultured in SFM/BSA alone or supplemented with different amounts of human insulin (Life Technologies, Inc.). Cells were detached after 72 h, washed twice in ice-cold PBS, and resuspended at 2 × 10⁶ cells/ml in PBS plus 0.5% BSA, 0.01% NaN₃. Biotinylated anti-IGF-I, anti-MMP-9 Ab3, or anti-MMP-2 (Calbiochem) mAb was added, followed by phycoerythrin-labeled avidin or fluorescein isothiocyanate-labeled goat anti-mouse IgG (Southern Biotechnologies, Birmingham, AL), respectively. An irrelevant isotype-matched mouse antibody was used as control. Cell-associated fluorescence was visualized by flow cytometry. To eliminate cell surface-bound IGF-I, detached cells received an acid wash (34) prior to staining as above.

**Autophosphorylation of the IGF-1R—Subconfluent DU-145 cells**

were cultured in SFM/BSA for 3 days and pulsed with IGFBP-3 for 24 h, with the anti-IGF-1R mAb αIR-3 for the times indicated, or with IGF-I (10 nM) for 5 min at 37 °C. After washing with ice-cold PBS, cells were lysed at 4 °C for 30 min using 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 1% Nonidet P-40, 10% glycerol, a proteinase inhibitor mixture, 1 mM sodium orthovanadate, and 10 mM NaF. Lysates were centrifuged for 25 min at 4 °C, and their protein concentration was determined using the micro-BCA kit (Pierce). Cell lysates (50 μg) were immunoprecipitated with αIR-3 or anti-IRS-1 mAb (Upstate Biotechnology Inc., Lake Placid, NY) for 3 h at 4 °C, followed by goat anti-mouse IgG agarose (Sigma), and then fractionated in 7.5% SDS-PAGE under reducing conditions. Tyrosine-phosphorylated proteins were developed with the PY-20 mAb (Santa Cruz Biotechnologies, Santa Cruz, CA), and IRS-1 specifically with anti-IRS-1 mAb, followed by peroxidase-labeled goat anti-rabbit Ig antibody (ICN, Costa Mesa, CA) and ECL. A similar protocol was followed for control 3T3-1R cells.

**Detection of pS6**—The level of cell division cycle 2 (Cdc-2) was estimated in DU-145 cell extracts treated with IGFBP-1, IGFBP-3, anti-IGF-I mAb, or BSA as described above. Samples containing equivalent amounts of protein were fractionated in 12.5% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and incubated for 2 h with anti-Cdc-2 (Transduction Laboratories, Lexington, KY) or anti-pS6 antibodies (PharMingen, San Diego, CA). After washing, the filter was incubated with a PO-labeled goat anti-mouse antibody and ECL.

**Transfection of MMP-9 Antisense cDNA**—The entire MMP-9 cDNA was cloned in the XbaI site of pEFBOS in the antisense direction, as determined by restriction analysis (pEFBOS-MMP-9AS). DU-145 cells were transfected with the pEFBOS-MMP-9AS or the pEFBOS empty vector using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Transfection efficiency was determined by cotransfecting an equal amount of green fluorescent protein-pEFBOS plasmid and subsequent FACs analysis determination of the percentage of cells expressing green fluorescent protein at 48 h. Expression was maximal between 48 and 96 h.

After 24 h, DU-145-transfected cells were detached and plated in 96- or 24-well plates, allowed to adhere, washed three times with PBS, and starved overnight in SFM/BSA. Cells were then washed extensively with PBS and cultured for 24 h in SFM/BSA alone or supplemented with different amounts of IGF-I or 20 nM IGFBP-3 and then processed for proliferation experiments as described above. DU-145-transfected cells in 24-well plates were maintained in SFM without BSA for an additional 48 h, after which conditioned medium was collected and analyzed for IGFBP-3 proteolysis by Western blot or for MMP-9 activity by zymography.

To analyze the effects of MMP-9 antisense expression on IGF-induced cell signaling, DU-145-transfected cells, either with the pEFBOS-MMP-9AS or the empty vector, were plated on 35-mm² dishes and treated as above. After 24 h starvation, dishes were incubated for 5 min in SFM/BSA with or without IGF-I (1 μg/ml). Cell lysates were obtained and immunoprecipitated with αIR-3 as described above and proteins
resolved in 7.5% SDS-PAGE gels, and after blotting, nitrocellulose membranes were incubated sequentially with the PY-20 mAb and anti-IGF-1R b-subunit rabbit polyclonal antiseraum (Santa Cruz Biotechnology) and ECL.

**Fluorometric Assay of DU-145 Cell-associated MMP Activity—**DU-145 cells, untransfected or transfected with pEFBOS-MMP-9AS or empty pEFBOS plasmid, were starved for 48 h, washed five times with PBS, and then incubated with the fluorogenic peptide 2,4-dinitrophenol-Pro-β-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys-(N-Methyl-aminobenzoyl) (Bachem, Bubendorf, Switzerland) at a final concentration of 5 μM, as described (33). The fluorescence increase after incubation at 37 °C was evaluated at 360 and 460 nm excitation and emission wavelengths, respectively. When inhibitors were tested, they were preincubated for 30 min with DU-145 before addition of the fluorogenic substrate.

**Cross-linking of DU-145 Membrane Proteins—**DU-145 cell monolayers were rinsed twice with minimal essential medium without amino acids plus 20 mM HEPES, pH 7.3, and cell-surface proteins were cross-linked by adding 0.5 mM disuccinimidyl suberate (Pierce). After 30 min at 4 °C, the cross-linking reagent was removed, and the reaction was terminated by rinsing and subsequent cell incubation with 37.5 mM Tris-HCl, pH 7.4, plus 150 mM NaCl for 10 min. The cells were then lysed with 100 mM n-octyl glucopyranoside (Calbiochem) in 37.5 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM benzamidine hydrochloride, and a protease inhibitor mixture as above. Cell lysates were resolved in 7.5% SDS-PAGE under reducing conditions, blotted, and probed with anti-MMP-9 mAb (Calbiochem) and, after stripping according to manufacturer’s specifications, with antibodies specific for different integrin chains.

**RESULTS**

**DU-145 Cells Express IGF-I, IGF-II, IGF-1R, and IGFBP—**To analyze DU-145 growth factor requirements, we tested the effect of several serum concentrations and cell densities in proliferation experiments. As reported previously, DU-145 cells grow in serum-free medium (36), determined both by [3H]Thr incorporation and the increase in the number of viable cells (data not shown). A sensitive, specific RT-PCR assay shows that DU-145 cells express mRNA coding for IGF-I and IGF-II and the IGF-1R (Fig. 1A).

IGFBP secreted by DU-145 cells were analyzed by Western ligand and immunoblot (Fig. 1B). Labeled IGFBP-2 binds to three proteins in the DU145-CM as follows: a protein of approximately 40 kDa, corresponding to the 39–42-kDa doublet characteristic of IGFBP-3, and two proteins of 34 and 25 kDa, identified in immunoblot as IGFBP-2 and IGFBP-4, respectively. A weak band of approximately 30 kDa is also occasionally visible, although no reactivity was observed with anti-IGFBP-1, -IGF-BP-5, or -IGFBP-6 antibodies. The anti-IGFBP-3 antibody confirmed that the 39–42-kDa doublet is IGFBP-3, but it also developed a 31-kDa protein and, weakly, another of 19 kDa, which are not detected in ligand blot. This suggests that IGFBP-3 is proteolyzed in DU145-CM, as it is in other biological fluids (37). This proteolytic processing was not observed for either IGFBP-2 or -4.

**Secreted IGF Binds to the Cell Surface and Activates the IGF-1R in DU-145 Cells—**To demonstrate that the IGF secreted by DU-145 cells binds to cell-surface receptors, we used the IGF-I-specific mAb KM5A1, which recognizes the growth factor complexed either to the IGF-1R or to IGFBP-1 or -3 (28). KM5A1 binds specifically to DU-145 cells that have been starved for 72 h (Fig. 2A); KM5A1 mAb binding to DU-145 cells is lost when the antibody is preincubated with IGF-I before being added to the cells (Fig. 2A). Other anti-IGF-I mAb (BB9E10) recognizing an epitope occult in the IGF-I/IGF-1R complex (28) do not bind to DU-145 cells. As an additional specificity control, we performed an acid wash of cells to remove any receptor-bound ligand; this wash completely abolishes KM5A1 reactivity with the cells (Fig. 2B), which can be restored by incubation of cells with IGF-I (Fig. 2C).

To determine whether KM5A1 recognizes IGF-I bound either to IGF-1R or to membrane-associated IGFBP, DU-145 cells were cultured for 72 h in serum-free medium supplemented with BSA and then pulsed for 24 h with different amounts of human insulin (Fig. 2D). Since insulin binds to the IGF-1R and to insulin receptor with different affinities and does not bind to IGFBP, the effect of insulin dose on KM5A1 mAb cell binding may indicate the receptor to which IGF-I ligates. KM5A1 mAb reactivity is completely abolished when the cells are cultured in 1 μM insulin and significantly reduced (but not lost) at 0.1 μM, indicating that autocrine-secreted IGF-I binds mainly to the IGF-1R. We were not able to analyze IGF-II binding in DU-145, although it probably is similar to that of IGF-I.

Since secreted IGF (or at least IGF-I) binds to IGF-1R, we examined IGF-1R tyrosine kinase activity in serum-starved DU-145 cells. After incubation of 3T3-IGF-1R cells with IGF-I, aIR-3 immunoprecipitates a 98-kDa phosphoprotein band that represents the autophosphorylated IGF-1R β-subunit and in B, the position of the 185-kDa band representing IRS-1.

**Fig. 3. IGF-1R is activated in DU-145 cells.** Cell lysates from IGF-I-treated and -starved cells were immunoprecipitated with aIR-3 mAb, and the blotted proteins were developed with either anti-phosphotyrosine (A) or anti-IRS-1 (B) mAb. As positive control, 3T3 fibroblasts overexpressing the human IGF-1R (3T3-IGF-1R) were used. Arrows in A indicate the position of the 98-kDa phosphoprotein representing the phosphorylated IGF-1R β-subunit and in B, the position of the 185-kDa band representing IRS-1.

**IGF-I and -II Antibodies, Block DU-145 Cell Growth—**We first used anti-IGF-I and anti-IGF-II mAb, which inhibit the binding of both ligands to the IGF-1R (29). Incubation of cells...
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with these mAb at concentrations as high as 100 μg/ml for 3 days does not inhibit DU-145 serum-free growth, as measured by [3H]TdR incorporation (Fig. 4A). The failure of anti-IGF-I mAb to inhibit DU-145 cell proliferation is not a consequence of lack of activity, since this mAb inhibits IGF-I-induced Ba/F3 cell proliferation at the same level as IGFBP-3 in Ba/F3 cells (Fig. 4C). The most probable explanation for the difference in the results with the IGF mAb in DU-145 and Ba/F3 cell lines is the presence of IGFBP in the DU145-CM, which may compete with antibodies for IGF binding.

The antagonistic anti-IGF-1R mAb αIR-3 inhibits cell proliferation by 40% at 10 nM (Fig. 4A). This contradicts earlier data showing that αIR-3 blocks IGF-II-dependent growth but not DU-145 serum-free proliferation (26). We also analyzed the effect of exogenous hIGFBP-3 on DU-145 cell proliferation. IGFBP-3 at 20 nM causes a 40% inhibition of DNA synthesis (Fig. 4A), and proliferation is partially restored when IGFBP-3 is preincubated with a molar excess of IGF-I (Fig. 4B). The des-(1-3)-IGF-I variant, which binds to the IGF-1R as does IGF-I, but does not bind to IGFBP, reverses IGFBP-3-induced DU-145 growth inhibition almost completely (Fig. 4B). This suggests that IGFBP-3 does not exert IGF-independent effects in these cells.

In accordance with the reduction in [3H]TdR incorporation, incubation with IGFBP-3 promotes a significant reduction in the percentage of cells in the G2/M peak (6 ± 0.5%) when compared with addition of anti-IGF-I plus anti-IGF-II mAb (17 ± 1.3%) or controls (20 ± 0.8%) (Fig. 5A). This is associated with an increase in the sub-G1 peak (20 ± 0.3% compared with 12.3 ± 2.1% in controls). Furthermore, IGFBP-3 promotes a drastic decrease in p34^<cyclicit>^<cyclicit>^<cyclicit>K^2^<cyclicit> levels compared with control or anti-IGF antibody-treated cells (Fig. 5B). This suggests that cell treatment with IGFBP-3 interferes with the pathway regulating Cdc-2 synthesis or stability. The same blot was re-probed with an anti-p83 mAb as an internal protein loading control, exploiting the constitutive overexpression of a mutant p53 form by DU-145 cells (39) (Fig. 5C).

To ascertain that αIR-3 and IGFBP-3 effects on DU-145 cell proliferation are mediated by interference with the IGF-1R signaling pathway, we measured the IGF-1R autophosphorylation level. Treatment of DU-145 cells with αIR-3 mAb promotes a biphasic effect on IGF-1R autophosphorylation; at short incubation times, αIR-3 significantly increases the IGF-1R β-subunit phosphorylation level, which is progressively reduced when incubation time is increased (Fig. 6A). The final balance is that, after 20 h of incubation, αIR-3 significantly reduces the IGF-1R-mediated signaling in DU-145. On the other hand, incubation of DU-145 cells with IGFBP-3 (20 nM) results in a decrease in IGF-1R β-subunit phosphorylation as compared with untreated cells; autophosphorylation can be recovered by co-addition of IGF-I with IGFBP-3 (Fig. 6B). As expected from cell growth assays, the anti-IGF-1r or anti-IGF-I plus anti-IGF-II mAb treatment does not induce changes in β-subunit autophosphorylation (data not shown).

MMP-9 Regulates DU-145 Proliferation by IGFBP-3 Proteolysis—To analyze the consequences of IGFBP-3 proteolysis on cell proliferation, we tested the effect of various protease inhibitors on serum-free DU-145 cell growth (Fig. 7A). Both BB-94 and tissue inhibitor of metalloproteinase (TIMP)-2, two MMP inhibitors, produce a significant reduction of DNA synthesis in

withdrawn from interleukin-3 and cultured for 20 h with IGF-I (250 ng/ml) alone (control, 100%) or in the presence of IGFBP-3 (●), IGFBP-1 (▲), or anti-IGF-I mAb BB9E10 (×) at the concentrations indicated and then pulse dusted with [3H]TdR. In all cases, data given are the percentage (n = 5) of [3H]TdR incorporated, divided by the control (absence of competitor).
Experimental Procedures. Cell number is depicted on the ordinate and DNA content, as measured by propidium iodide intensity after doublet exclusion, on the abscissa. The percentage of cells in each phase of the cycle is also indicated.

We thus examined whether MMP-9 secreted by DU-145 cells has been reported previously (40, 41). (Fig. 8A). Gelatinolytic activity in DU145-CM, identified with specific antibodies as MMP-9, has been designed such that its fluorescence remains 98% quenched in the uncleaved product (42); a fluorescence increase thus indicates MMP activity. Degradation of the substrates increases with time (Fig. 8B), and this MMP activity is partially inhibited by the specific MMP inhibitor BB-94 but not by aprotinin. Affinity cross-linking of cell-surface proteins was performed, and cell lysates were analyzed in Western blot using anti-MMP-9 mAb (Fig. 8C). A major band of 150 kDa and another minor 130-kDa band are observed in the cross-linked cells, in addition to the 92-kDa form that is detected in both cross-linked and non-cross-linked DU-145 cells. To establish further the association of MMP-9 to the cell surface, non-cross-linked cells were stained with anti-MMP-9 and analyzed by flow cytometry (Fig. 8D). The incubation of DU-145 with an anti-MMP-9 mAb promotes a shift in the fluorescence intensity associated to the cells compared with that obtained using either irrelevant or anti-MMP-2-specific antibodies. These results indicate the presence of a membrane-bound form of MMP-9.

To specifically inhibit MMP-9 activity in DU-145 cells, antisense expression studies were performed. pEFBOS-MMP-9AS-transfected DU-145 cells showed drastically reduced proliferation compared with untransfected cells or cells transfected with the empty vector; furthermore, the inhibition caused by antisense expression can be reversed by addition of exogenous IGF-I in a dose-dependent manner (Fig. 9A). Addition of IGFBP-3 to pEFBOS-MMP-9AS-transfected DU-145 cells does not result in increased growth inhibition (Fig. 9B), suggesting that IGFBP-3 inhibitory effects are dependent on MMP-9 activity. In fact, pEFBOS-MMP-9AS-transfected DU-145 cells show a drastic reduction in MMP membrane-associated activity (Fig. 9C), indicating that the MMP-9 antisense effectively inhibits MMP-9 expression. A reduction in gelatinolytic activity is also observed in MMP-9 antisense DU145-CM (data not shown).

The inhibition promoted by MMP-9 antisense expression correlates with a decrease in IGFBP-3 proteolysis (Fig. 9D). Furthermore, serum-starved pEFBOS-MMP-9AS-transfected cells show reduced IGF-triggered cell signaling, observed as a decrease in the tyrosine phosphorylation of the 98-kDa IGF-1R β-subunit as compared with empty vector-transfected cells (Fig. 9E). Densitometric analysis of Western blots demonstrates that, although the immunoprecipitated IGF-1R levels are comparable, pEFBOS-MMP-9AS DU-145 cells show a 4-fold decrease in tyrosine-phosphorylated IGF-1R β-subunit (Fig. 9F). Tyrosine phosphorylation at control levels is recovered in pEFBOS-MMP-9AS DU-145 cells by addition of exogenous IGF-I, indicating that MMP-9 antisense treatment interferes with the bioavailability of IGF to DU-145 cells.

A

B

C

D

E

F

Fig. 5. Exogenous IGFBP-3 modifies cell cycle parameters. A, analysis of the cell cycle distribution of DU-145 cells incubated with SFM/BSA (no additions) or SFM/BSA supplemented with anti-IGF-I plus anti-IGF-II antibodies or IGFBP-3 (20 nM). DU-145 cells were incubated for 72 h with additions as indicated in the upper right corner and then harvested and stained with propidium iodide as described under “Experimental Procedures.” Cell number is depicted on the ordinate and DNA content, as measured by propidium iodide intensity after doublet exclusion, on the abscissa. The percentage of cells in each phase of the cycle is also indicated. B, p53c/d expression in DU-145 cells. DU-145 cells cultured in serum-free medium were incubated for 72 h with IGFBP-3 (lane 1), anti-IGF-II mAb (lane 2), anti-IGF-I mAb BB9E10 (lane 3), anti-IGF-I plus anti-IGF-II mAb (lane 4), or an irrelevant antibody (lane 5). Cell lysates were prepared as described under “Experimental Procedures,” and Western blot analyses were performed. C, as an internal control, the filters in B were incubated with an anti-human p53 antibody. Similar results were obtained in three independent experiments.

a dose-dependent fashion, whereas the serine protease inhibitor aprotinin does not modify DU-145 cell proliferation. Addition of IGF-I together with the MMP inhibitors restores DU-145 cell proliferation (Fig. 7B). MMP inhibitor-promoted DU-145 growth abrogation correlates with the absence of IGFBP-3 proteolytic fragments detected in the conditioned medium of these cells (Fig. 7C), thus linking the antiproliferative effect of the MMP inhibitors and their interference in the IGF/IGF-1R axis by preventing IGFBP-3 proteolysis. Cell cycle analysis of DU-145 cultured with protease inhibitors revealed that, as in the case of IGFBP-3 addition, TIMP-2 and BB-94 treatment reduce the percentage of cells in G2/M compared with untreated or aprotinin-treated cells (Fig. 7D).

To identify the MMP activity responsible for DU-145 proliferation, DU145-CM was analyzed by gelatin zymography. Zymograms indicate the presence of a 92-kDa gelatinolytic activity in DU145-CM, identified with specific antibodies as MMP-9 (Fig. 8A). The localization of soluble MMP to the surface of normal and tumor cells has been reported previously (40, 41). We thus examined whether MMP-9 secreted by DU-145 cells can also be localized to the membrane. Fig. 7B shows the kinetics of a labeled MMP substrate degradation in starved DU-145 cells. This fluorogenic substrate, specific for several MMP, has been designed such that its fluorescence remains >98% quenched in the uncleaved product (42); a fluorescence increase thus indicates MMP activity. Degradation of the substrate increases with time (Fig. 8B), and this MMP activity is partially inhibited by the specific MMP inhibitor BB-94 but not by aprotinin. Affinity cross-linking of cell-surface proteins was performed, and cell lysates were analyzed in Western blot using anti-MMP-9 mAb (Fig. 8C). A major band of 150 kDa and another minor 130-kDa band are observed in the cross-linked cells, in addition to the 92-kDa form that is detected in both cross-linked and non-cross-linked DU-145 cells. To establish further the association of MMP-9 to the DU-145 cell surface, non-cross-linked cells were stained with anti-MMP-9 and analyzed by flow cytometry (Fig. 8D). The incubation of DU-145 with an anti-MMP-9 mAb promotes a shift in the fluorescence intensity associated to the cells compared with that obtained using either irrelevant or anti-MMP-2-specific antibodies. These results indicate the presence of a membrane-bound form of MMP-9.

To specifically inhibit MMP-9 activity in DU-145 cells, antisense expression studies were performed. pEFBOS-MMP-9AS-transfected DU-145 cells showed drastically reduced proliferation compared with untransfected cells or cells transfected with the empty vector; furthermore, the inhibition caused by antisense expression can be reversed by addition of exogenous IGF-I in a dose-dependent manner (Fig. 9A). Addition of IGFBP-3 to pEFBOS-MMP-9AS-transfected DU-145 cells does not result in increased growth inhibition (Fig. 9B), suggesting that IGFBP-3 inhibitory effects are dependent on MMP-9 activity. In fact, pEFBOS-MMP-9AS-transfected DU-145 cells show a drastic reduction in MMP membrane-associated activity (Fig. 9C), indicating that the MMP-9 antisense effectively inhibits MMP-9 expression. A reduction in gelatinolytic activity is also observed in MMP-9 antisense DU145-CM (data not shown).

The inhibition promoted by MMP-9 antisense expression correlates with a decrease in IGFBP-3 proteolysis (Fig. 9D). Furthermore, serum-starved pEFBOS-MMP-9AS-transfected cells show reduced IGF-triggered cell signaling, observed as a decrease in the tyrosine phosphorylation of the 98-kDa IGF-1R β-subunit as compared with empty vector-transfected cells (Fig. 9E). Densitometric analysis of Western blots demonstrates that, although the immunoprecipitated IGF-1R levels are comparable, pEFBOS-MMP-9AS DU-145 cells show a 4-fold decrease in tyrosine-phosphorylated IGF-1R β-subunit (Fig. 9F). Tyrosine phosphorylation at control levels is recovered in pEFBOS-MMP-9AS DU-145 cells by addition of exogenous IGF-I, indicating that MMP-9 antisense treatment interferes with the bioavailability of IGF to DU-145 cells.
DISCUSSION

Autocrine growth control has been considered to be a mechanism by which tumor cells proliferate autonomously (2). Recent reports have claimed a role for IGF ligands and receptors in the establishment and maintenance of the tumor phenotype (43), and studies dealing with IGF expression in human cancer cell lines have revealed that IGF autocrine loops may be operating in the majority of epithelial cancer cell lines (44). In addition to ligands and IGF-1R, the concurrent secretion of IGFBP, usually in a large excess over IGF ligands, has been also reported in the same cell lines (45); however, the physiological significance of IGFBP expression by cancer cells is still unclear.

Although several groups have studied the role of the IGF autocrine loop using neoplastic prostate cells, there are substantial contradictory results regarding the expression and mitogenic effects of IGF ligands and IGFBP in DU-145 cells. For example, Pietrzkowski et al. (36) reported both IGF-1R and IGF-I immunoreactivity in DU-145-CM. Conversely, Iwamura et al. (25) observed that IGF-I is a potent DU-145 mitogen but failed to establish the presence of IGF-I in conditioned medium from these cells. Connolly and Rose (46) found neither IGF-I nor IGF-II expression by DU-145 cells but demonstrated IGFBP production. With an RNase protection assay, Figueroa et al. (26) detected mRNA encoding IGF-II, IGF-1R, IGF-2R, and IGFBP-2-6 but not that coding for IGF-I. Employing a highly specific RT-PCR, we find mRNA expression for both IGF-I and IGF-II as well as for IGF-1R, and we also encounter the IGF-1R on the cell surface. In addition, the IGF-I D-domain-specific mAb KM5A1, which binds to IGF-I when complexed either with IGFBP or IGF-1R, detects IGF-I bound to the surface of starved DU-145 cells. This indicates that DU-145 cells secrete IGF-I. The loss of KM5A1 binding to cells cultured in the presence of insulin implies that autocrine IGF-I binds mainly to IGF-1R on DU-145. We have no similar IGF-II-specific antibody to monitor the binding of this growth factor to the DU-145 membrane, but it is assumed to be similar to IGF-I.

DU-145 cells are reported to grow in the absence of external growth factors (25, 26, 36, 46, 47). To ascertain whether the serum-free growth of DU-145 is due to the autocrine action of secreted IGF, these cells were exposed to the anti-IGF-1R mAb, αIR-3. This antibody is reported to mimic IGF activity in some cell types that overexpress the IGF-1R mAb, αIR-3. In agreement with this latter set of publications, incubation of DU-145 cells with αIR-3 significantly inhibited DNA synthesis.
To analyze further the inhibitory effect of aIR-3 on DU-145 cells, we examined IGF-1R b-subunit phosphorylation status. A short incubation of the cells with aIR-3 results in increased IGF-1R autophosphorylation, suggesting receptor activation. However, when the cells are exposed to the antibody for longer periods, aIR-3 reduces the IGF-1R autophosphorylation level compared with the basal conditions (i.e., absence of exogenous factors). aIR-3 is reported to stimulate IGF-1R autophosphorylation (49), although this “activation” does not necessarily lead to a cellular response (53). Moreover, Steele-Perkins et al. (48, 61) reported that aIR-3 alone induced cell proliferation and IGF-1R autophosphorylation, although when aIR-3 is added with IGF-I or IGF-II, the proliferation and IGF-1R autophosphorylation induced by these factors are blocked in this cell line. It is therefore possible that aIR-3 binding to the IGF-1R induces IGF-1R autophosphorylation by cross-linking the receptor at the cell surface but, as a consequence of this binding, a conformational change occurs that locks the receptor into a state that incapable of further signaling (61). This also may account for the result obtained in DU-145 cells, which constitutively produce both IGF-I and IGF-II. Nonetheless, further experiments are required to confirm this viewpoint.

Incubation of DU-145 cells with several anti-IGF-I mAb, alone or in combination with an anti-IGF-II mAb, failed to block the serum-free growth of DU-145 cells, although these mAb are effective inhibitors of IGF-induced cell survival and proliferation in several cell lines (28, 29); the interference by IGFBP in the DU145-CM might abolish the inhibitory capacity of these anti-IGF mAb.

Normal prostate epithelial cells secrete IGFBP-2 and IGFBP-4, whereas stromal fibroblasts produce IGFBP-2, -3, and -4 (62, 63). IGFBP-3 has been found, however, in human
prostate epithelial cell culture medium by others (64). Another androgen-independent prostate adenocarcinoma, PC-3, was reported to secrete IGFBP-3 and other IGFBP species (54). We observe secretion of at least three different IGFBP species (IGFBP-2, -3, and -4) in DU-145 cells, but only IGFBP-3 is proteolyzed. Moreover, the addition of exogenous hIGFBP-3 to DU-145 inhibits the serum-free growth of this cell line; co-addition with exogenous IGF-I partially reverses this effect. Concurring with the reduction in DNA synthesis, exogenous IGFBP-3 promotes a decline in the number of cells in G2/M phases and a decrease in p34\(^{\text{cdc2}}\) levels.

In vivo treatment of human rhabdomyosarcoma with an antagonist anti-IGF-1R antibody has been reported to down-regulate levels of p34\(^{\text{cdc2}}\) (9), a protein required for G2 transition to mitosis (65). The IGFBP-3 inhibitory effect is thus probably mediated by interference with IGF-induced IGF-1R activation. This view concurs with our data showing that the des-(1–3)IGF-I variant reverses IGFBP-3-induced DU-145 growth inhibition and that IGFBP-3 treatment reduces the level of IGF-1R \(\beta\)-subunit autophosphorylation.

Our data therefore support an inhibitory role for exogenous IGFBP-3 in DU-145 cell proliferation. The question that arises is why DU-145 secretes an inhibitory IGFBP. Autocrine production of IGFBP-3 by DU-145 cells may modulate ligand-receptor interactions at the cell surface, thus regulating cell responses to locally produced IGF. This modulation is probably more puzzling than simple interference with the IGF/IGF-1R interaction, however, since the anti-IGF-I and anti-IGF-II antibodies used here have no effect on DU-145 proliferation. IGF-independent effects of IGFBP-3 have been reported for other tumor cell lines (35, 66), and receptors for this binding protein are also found in some tumor cells (20). However, des-(1–3)IGF-I reverses IGFBP-3-induced DU-145 growth inhibition, suggesting that IGFBP-3 has no IGF-independent effect on this cell line.

The IGFBP enhancer mechanism is poorly understood but usually involves their proteolytic processing, leading to a diminished affinity for IGF ligands (22, 45). We found that DU-145 secretes MMP-9, a metalloproteinase that degrades IGFBP-3 (67). The control of DU-145 serum-free growth by MMP-9 is relevant, since overexpression of an antisense cDNA for MMP-9 blocks almost 80% of DNA synthesis, an effect also observed by the addition of nontoxic concentrations of MMP inhibitors such as BB-94 or TIMP-2. Our findings indicate that the antiproliferative effect obtained by inhibiting MMP-9 expression is related to the inaccessibility of IGF to the IGF-1R, since (i) addition of IGF-I to either pEFBOS-MMP-9AS-transfected or MMP inhibitor-treated cells restores DU-145 proliferation in a dose-dependent manner; (ii) MMP-9 antisense expression reduces autocrine IGF-triggered cell signaling through the IGF-1R, which is recovered by incubation with exogenous IGF-I; (iii) the growth inhibition correlates with the disappearance of IGFBP-3 proteolytic fragments, and (iv) IGFBP-3 does not promote an additive growth inhibitory effect on pEFBOS-MMP-9AS-transfected DU-145 cells.

By using PC-3 cells, Angelloz-Nicoud and Binoux (54) documented inhibition of cell proliferation by adding a serine pro-
tease inhibitor able to block urokinase-type plasminogen activator activity detected in the conditioned medium. In our study, incubation of DU-145 with aprotinin results neither in growth inhibition nor in the abolition of IGFBP-3 proteolysis. The involvement of kallikrein-type proteases in this process cannot be ruled out, but DU-145 does not produce prostate-specific antigen (68).

We demonstrate the existence of MMP-9 activity associated to the DU-145 cell surface using a fluorogenic substrate and FACS analysis. Affinity cross-linking also suggests the physical association of MMP-9 to a cellular receptor of about 60 kDa. Recent reports show cell-surface localization of proteinases, including urokinase-type plasminogen activator and MMP-2, on a variety of cell types both in vitro and in vivo (41, 69, 70). Little is known, however, regarding the biochemical interactions between MMP and cell-surface receptors. The association of MMP-2 with \( \alpha_\beta_3 \) integrin has been described (41), but reprobing of Western blots from DU-145 cross-linked cells with several anti-\( \alpha \)- and -\( \beta \) integrin chain antibodies failed to show this MMP-9/integrin association. A membrane-type MMP, called MT-MMP, has recently been identified (71) and has been implicated in the localization and activation of MMP-9 on the cell surface, in conjunction with TIMP-2 (40). MMP-9 binding to the DU-145 cell surface may thus be a step in MMP-9 activation, after which IGFBP-3 is degraded.

Our data support the idea that IGFBP-3 is an important regulator of prostate cancer cell growth. It can be conjectured that autocrine IGF-I and IGF-II are bound to IGFBP, which regulates liberation of growth factors in close proximity to the IGF-1R following MMP-9 proteolysis. The novelty of this report is the demonstration that optimal DU-145 growth is the result of a balance between autocrine secretion of IGF ligands, IGFBP, and MMP levels. When this equilibrium is upset, for example by addition of exogenous IGFBP-3 or inhibition of endogenous MMP-9, a reduction in cell proliferation results. Exogenous IGFBP-3-induced growth inhibition is thus not due to competitive scavenging of IGF ligands away from the IGF-1R but to the competition of MMP-9 activity with “unfilled” IGFBP-3.

This concurs with previous results in which androgen-induced growth inhibition of PC-3 cells transfected with a constitutively activated androgen receptor coincides with the complete absence of IGFBP-3 in the culture media of these cells (72). Treatment of these transfected PC-3 cells with IGFBP-3...
and IGF-I or IGF-II resulted in a proliferation rate greater than that observed with IGF-I or -II alone. Accumulated evidence indicates, however, that IGFBP-3 cell-surface association is required for the enhancement of IGF action, and factors increasing this membrane association also intensify the IGFBP-3 potentiating effect (28). Furthermore, IGFBP-3 that adhered to the cell surface was processed to lower molecular weight forms with decreased affinity for IGF (19). Membrane-bound MMP-9 may therefore act as an IGFBP-3 receptor enabling this enhancing mechanism.

In summary, we show compelling evidence for an autocrine loop, operative in DU-145 cells, for both IGF-I and IGF-II. This loop seems not to implicate both IGF ligands and IGR-I to IGFR-I but also IGFBP-3, which specifically cleave IGFBP-3. Anchorage of MMP-9 to the cell surface may thus provide a mechanism to coordinate IGFBP-3 proteolysis with increased IGF availability in close proximity to the IGF-IR. The study of this pathway could lead to the design of new anti-tumor agents for tumors resistant to other therapies and to a better understanding of complex processes such as tumor growth and metastasis.

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