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Biochemical and Biophysical Research Communications 293 (2002) 55–60

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The amino-terminal region of insulin-like growth factor binding protein-3, $^{1–95}$IGFBP-3, induces apoptosis of MCF-7 breast carcinoma cells

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Received 25 February 2002

Abstract

In an earlier study, we reported that an N-terminal proteolytic fragment ($^{1–95}$IGFBP-3) corresponding to the first 95 residues of human insulin-like growth factor binding protein-3 (IGFBP-3) inhibits proliferation in a variety of fibroblasts. With a view to investigating its cytostatic capacity in carcinoma cells, we transiently transfected MCF-7 breast adenocarcinoma cells with an expression vector containing $^{1–95}$IGFBP-3 cDNA. The transfected cells secreted a hyper-glycosylated form of $^{1–95}$IGFBP-3. Twenty-four hours after transfection, cell morphology and viability were similar in control and $^{1–95}$IGFBP-3-secreting cells. However, after 48 h, $^{1–95}$IGFBP-3-secreting cells were apoptotic, with marked cytoplasmic vacuolation and increased free histones in the cytoplasm. Culture media conditioned by $^{1–95}$IGFBP-3-secreting cells also induced morphological changes and apoptosis in wild-type MCF-7 cells, indicating that $^{1–95}$IGFBP-3 was responsible for the effects observed. These results provide further evidence that the N-terminal proteolytic fragment of IGFBP-3 has a functional role.

Keywords: IGFBP-3; $^{1–95}$IGFBP-3; MCF-7 breast carcinoma cells; Apoptosis

At the cellular level, the IGFs (insulin-like growth factors I and II) are mitogenic and anti-apoptotic. These effects are modulated by specific high-affinity binding proteins, the IGFBPs. IGFBP-3, the predominant IGFBP in serum, is produced in almost all tissues [1,2] and, both in vivo and in culture medium, undergoes limited proteolysis, via which IGFs are released and their bioavailability is enhanced [3–6]. Characterization of the proteolytic fragments of IGFBP-3 in biological fluids and following plasmin treatment of recombinant human IGFBP-3 in vitro has shown that proteolysis of the 264-amino acid protein generates a major glycosylated fragment of 30 kDa (in SDS–PAGE under non-reducing conditions). This fragment, $^{1–160}$IGFBP-3, which corresponds to the first 160 residues, can itself be cleaved further to yield a 20-kDa (glycosylated) or 16-kDa (non-glycosylated) fragment comprising the first 95 residues [7–9]. The latter, $^{1–95}$IGFBP-3, has virtually no affinity for IGFs either in western ligand blotting or in solution assay under equilibrium binding conditions [8,9]. Nevertheless, it blocks IGF-I-induced DNA synthesis in a chick embryo fibroblast assay [8] and inhibits the mitogenic action of FGFs in mouse fibroblasts with a targeted disruption of the type I IGF receptor (IGF-IR) gene [10]. These data indicate that $^{1–95}$IGFBP-3 may act via a signalling pathway other than that involving IGF-IR. Breast adenocarcinomas and cell lines derived from them are known to secrete proteases that are capable of degrading IGFBP-3 in the bloodstream [11]. We used such a cell line, MCF-7 cells, transiently transfected with an expression vector encoding $^{1–95}$IGFBP-3 to investigate the biological effects of this peptide.

Materials and methods

Materials. Specific polyclonal anti-IGFBP-3 antibodies raised against recombinant human-IGFBP-3 in rabbits [7] were used at 1/1000 dilution for immunoblotting experiments. Non-glycosylated

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0006-291X/02/$ - see front matter
PII: S0006-291X(02)00181-X

(rh)-IGFBP-3 (*coli*) and glycosylated rhIGFBP-3 (CHO) were generous gifts from Celtrix Pharmaceuticals (Santa Clara, CA). Lipofectamine and Opti-MEM medium were purchased from Life Technologies SARL (Cergy Pontoise, France), the pcDNA-3 expression vector from Clontech Laboratories (Palo Alto, CA), and bFGF from R&D Systems (Minneapolis, MN). All other biochemicals were obtained from Sigma (Saint-Quentin Fallavier, France) or ICN (Orsay, France).

Cell culture. The MCF-7 human breast cancer cell line was grown in 35 or 100 mm dishes to 50–60% confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For 1 h before each transient transfection experiment, cells were washed and incubated in serum-free Opti-MEM.

Construction of the pcDNA-3 vector encoding $^{1-95}$IGFBP-3(pcDNA/1–95). The human $^{1-95}$IGFBP-3 cDNA encoding sequence was PCR-amplified from human IGFBP-3 cDNA (a gift from D.R. Clemmons (Chapel Hill, NC) using the following primers: forward primer = 5′ CGC GGA TCC GGC GTC ATG CAG CGG GCG; reverse primer = 5′ CGA ATT CCT AGC GGC TGA CGG CAC TAG. The amplified sequence was inserted into the pcDNA-3 expression vector following digestion with *Eco*RI and *Bam*HI. The orientation of the insert was confirmed by restriction mapping and sequencing was carried out to confirm that no error had been introduced during the PCR steps.

Transient transfection of MCF-7 cells. The expression vector (0.3 μg or 3 μg) was diluted in 100 μl or 750 μl serum-free Opti-MEM for further transfection in 35- or 100-mm dishes, respectively, and 6 or 20 μl PLUS reagent was added. Lipofectamine was diluted (1/25) in serum-free Opti-MEM (100 or 750 μl) and mixed with the plasmid/Opti-MEM/PLUS reagent, then incubated for 15 min at room temperature. Thereafter the mixture was carefully added to the cells preincubated in 800 μl or 3.5 ml Opti-MEM (35- or 100-mm dishes, respectively).

Western immunoblotting. Conditioned culture media from transiently transfected cells were collected and centrifuged for 10 min at 3000 rpm to remove the floating cells. Proteins were precipitated with TCA and then separated on denaturing (SDS)–12.5% polyacrylamide gels under non-reducing conditions. Proteins were electrotransferred onto PVDF sheets which were incubated with anti-IGFBP-3 antibodies and were revealed by chemiluminescence.

Deglycosylation experiment. Cells transiently transfected with either pcDNA-3 or pcDNA/1–95 expression vector were treated with or without tunicamycin (1–10 μg/ml) for 24 h. Proteins secreted into the conditioned media were analysed by western immunoblotting as described above.

Trypan blue dye exclusion. Cells were resuspended in PBS and loaded onto a haemocytometer with trypan blue dye (1/1). Live (capable of excluding the dye) and dead cells were counted, from which the percentage of dead cells was calculated.

Apoptosis measurement. Cells transiently transfected with either pcDNA-3 or pcDNA/1–95 expression vector were collected and apoptosis was measured using the Cell Death Detection ELISA kit (Roche) that measures the release of histones into the cytoplasm during the apoptotic steps. As a positive control for the induction of apoptosis, wild-type cells were treated with camptothecin (4 μg/ml) for 4 h before apoptosis measurement.

Results

MCF-7 cells secrete a hyper-glycosylated form of $^{1-95}$IGFBP-3

First, we investigated the nature of the protein secreted by MCF-7 cells transiently transfected with pcDNA-3 expression vector containing $^{1-95}$IGFBP-3 cDNA (pcDNA/1–95). Cells were transfected with either pcDNA-3 or pcDNA/1–95 as described in Materials and methods. After 24 h, conditioned media were collected, proteins were precipitated with TCA, separated by SDS–PAGE, transferred to PVDF sheets, and analysed by western immunobloting using anti-IGFBP-3 antibodies. No immunoreactive proteins were detected in cells transfected with pcDNA-3, indicating no secretion of intact IGFBP-3 or any proteolytic fragments (Fig. 1). Cells transfected with the pcDNA/1–95 secreted an immunoreactive protein migrating as several bands between 20 and 25 kDa (Fig. 1). The apparent molecular mass of this protein was higher than that of glycosylated $^{1-95}$IGFBP-3 (20 kDa), but was lower than that of glycosylated $^{1-160}$IGFBP-3 (30 kDa) in human serum (Fig. 1).

To check that the post-translational alterations of the secreted $^{1-95}$IGFBP-3 were related to glycosylation, which would account for its slower electrophoretic migration, transiently transfected MCF-7 cells were treated with increasing concentrations of tunicamycin for 24 h. The proteins in the conditioned media were analysed as described above. Tunicamycin treatment dose-dependently provoked the appearance of an immunoreactive protein migrating with an apparent molecular mass of 16 kDa (similar to that of non-glycosylated $^{1-95}$IGFBP-3 in human serum) and, at the highest dosage, the disappearance of the 20–25-kDa protein (Fig. 1). These results indicated that MCF-7 cells transiently transfected with pcDNA/1–95 secreted a hyper-glycosylated form of $^{1-95}$IGFBP-3.

$^{1-95}$IGFBP-3 induces morphological changes in MCF-7 cells

The next step was to analyse the biological effects of $^{1-95}$IGFBP-3 on MCF-7 cells. As shown in Fig. 2, 48 h after transfection there were dramatic morphological changes in the $^{1-95}$IGFBP-3-secreting cells as compared with control cells (transfected with pcDNA-3). These changes were characterized by a strong vacuolation of the cytoplasm. To assess the role of secreted $^{1-95}$IGFBP-3 in this process, wild-type cells were incubated with media conditioned by cells transfected with either pcDNA/1–95 (and secreting $^{1-95}$IGFBP-3) or pcDNA-3. As shown in Fig. 3, 48 h after addition of $^{1-95}$IGFBP-3-containing conditioned media, these cells contained numerous small vacuoles compared with control cells (wild-type cells incubated with media conditioned by pcDNA-3-transfected cells). These findings indicated that $^{1-95}$IGFBP-3 induced the morphological changes in MCF-7 cells.

$^{1-95}$IGFBP-3 induces apoptosis in MCF-7 cells

Since such morphological changes may reflect apoptosis, we measured cell death 48 h after transfection,

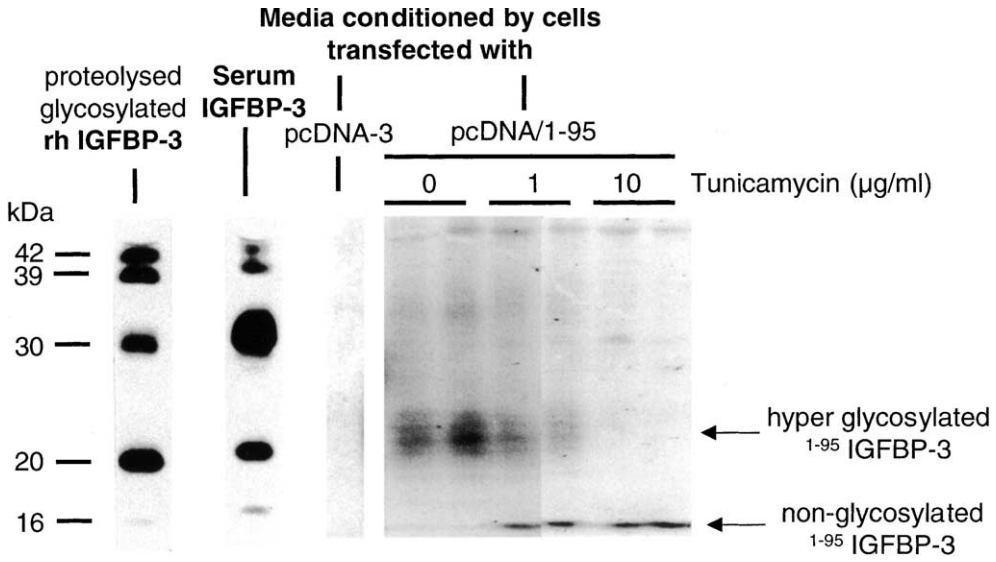

Fig. 1. Hyper-glycosylation of the $^{1-95}$IGFBP-3 form secreted by MCF-7 cells. The cells were transiently transfected with either pcDNA-3 vector or pcDNA/1–95 (pcDNA-3 vector containing $^{1-95}$IGFBP-3 cDNA). After 24 h, conditioned media were collected and proteins were precipitated with TCA. The proteins were separated by SDS–PAGE, transferred to PVDF sheets, and immunoblotted with anti-IGFBP-3 antibodies as described in Materials and methods. In some experiments, cells were treated for 24 h with or without tunicamycin (1 or 10 μg/ml). The results shown are those of a typical experiment. As controls for migration, the two proteolytic fragments obtained after incubating rh-IGFBP-3 with plasmin and those in human serum (3 μl) were analysed simultaneously (left panel). The different fragments secreted by MCF-7 cells were identified according to Lassarre and Binoux [22], i.e., intact glycosylated rh-IGFBP-3: 42–39 kDa; glycosylated $^{1-160}$IGFBP-3: 30 kDa; glycosylated $^{1-95}$IGFBP-3: 20 kDa; non-glycosylated $^{1-95}$IGFBP-3: 16 kDa.

using two experimental approaches: (1) Using trypan blue dye exclusion (Fig. 4A), the percentage of dead cells was approximately 10% in controls (wild-type and pcDNA-3-transfected cells) and was significantly greater (~29%; $p < 0.001$) in cells transfected with pcDNA/1–95. In addition, conditioned media containing $^{1-95}$IGFBP-3 induced cell death in ~20% wild-type cells. (2) Using the apoptosis assay (Fig. 4B), histone concentrations (reflecting the number of nucleosomes in the cytoplasm) in cells transfected with pcDNA/1–95 were twice those in controls (transfected with pcDNA-3) and those in wild-type cells treated with the apoptotic agent, camptothecin, five times the control levels.

Discussion

We previously demonstrated that the amino-terminal region corresponding to the first 95 amino acids of recombinant human IGFBP-3, $^{1-95}$IGFBP-3, which has no affinity for IGFs, inhibits the mitogenic signals resulting from the activation of either IGF-IR [8] or FGF-R [10]. In the present study, we have shown that transfection of MCF-7 breast carcinoma cells with a pcDNA-3 vector coding for $^{1-95}$IGFBP-3 induces apoptosis. It was clear that the effect was provoked by $^{1-95}$IGFBP-3 secreted into the culture media, since media conditioned by $^{1-95}$IGFBP-3-secreting cells also induced apoptosis in wild-type cells.

More than one hypothesis may account for the mechanisms by which $^{1-95}$IGFBP-3 induces cell death. The first would entail a direct mechanism where $^{1-95}$IGFBP-3 binds to a membrane receptor, resulting either in activation of apoptotic pathways or inhibition of anti-apoptotic pathways. Such binding to the cell surface has been observed for intact IGFBP-3 [12–15], but the nature of the putative $^{1-95}$IGFBP-3 receptor remains to be determined. It could be different from the intact IGFBP-3 receptor, since $^{1-95}$IGFBP-3 inhibits proliferation in the fibroblasts of mice with a targeted disruption of IGF-IR gene, whereas IGFBP-3 does not [10,16]. Nevertheless, IGFBP-3 is also capable of inducing apoptosis [17–19] independently of its ability to bind IGFs [20] and further study is needed to determine whether the same signalling pathways are activated by IGFBP-3 and $^{1-95}$IGFBP-3. Another hypothesis would involve $^{1-95}$IGFBP-3 secreted into the conditioned media inducing production of a pro-apoptotic factor which in turn would be responsible for the apoptosis observed.

As regards the $^{1-95}$IGFBP-3 secreted by MCF-7 cells transfected with $^{1-95}$IGFBP-3-encoding vector, its apparent molecular mass of 20–25 kDa was larger than that (20 kDa) of the glycosylated $^{1-95}$IGFBP form found in serum and resulting from cleavage of native IGFBP-3. The latter comprises three carbohydrate moieties of approximately 4, 5 and 6 kDa, located at Asn89, 109, and 172, respectively [21,22]. The experiments using tunicamycin treatment clearly demonstrated that the

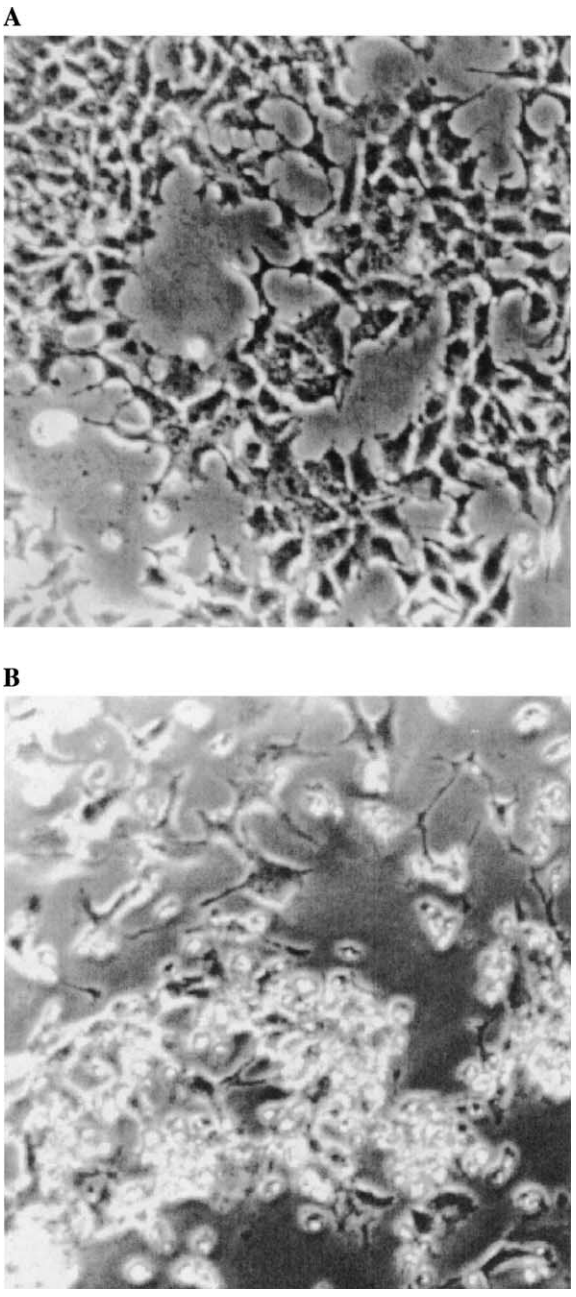

Fig. 2. Morphological alteration of MCF-7 cells transfected with pcDNA/1–95. Cells were transfected with either pcDNA-3 vector (A) or pcDNA/1–95 (B) as described in Materials and methods. After 48 h, cells were examined microscopically (×400). The results shown are those of a typical experiment.

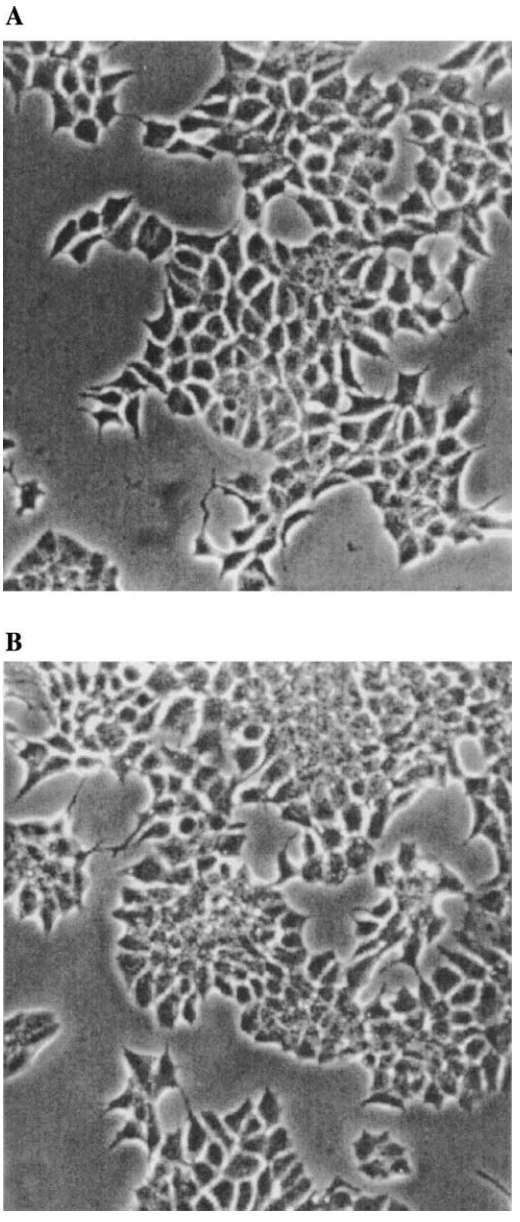

Fig. 3. Morphological alteration of wild-type MCF-7 cells treated with media conditioned by $^{1-95}$IGFBP-3-secreting cells. Wild-type cells were treated for 48 h with medium conditioned for 24 h by MCF-7 cells transfected with either pcDNA-3 (A) or pcDNA/1–95 ($^{1-95}$IGFBP-3-expressing) vector (B). The results shown are those of a typical experiment.

peptide secreted by MCF-7 cells corresponded to a hyper-glycosylated form of $^{1-95}$IGFBP-3. Similar hyper-glycosylation has been observed in MCF-7 cells transfected with an expression vector encoding intact IGFBP-3 (unpublished data). Abnormal protein glycosylation has been found in other tumour cell models, such as HL-60 (promyelocyte leukaemia cells) [23], choriocarcinoma, and hydatid mole cells which secrete hCG hormone with abnormal glycosylation in both subunits [24]. Anomalies of protein glycosylation appear to contribute towards the invasive tumour phenotype of these cells, by virtue of the suspected role of certain sugars in cell mobility and, more generally, in cell communication with the extra-cellular medium [25].

The results of the current study therefore demonstrate the apoptotic action in MCF-7 cells of $^{1-95}$IGFBP-3, a proteolytic fragment of IGFBP-3. Since this fragment is generated physiologically in the bloodstream, identification of its target cells and the signalling

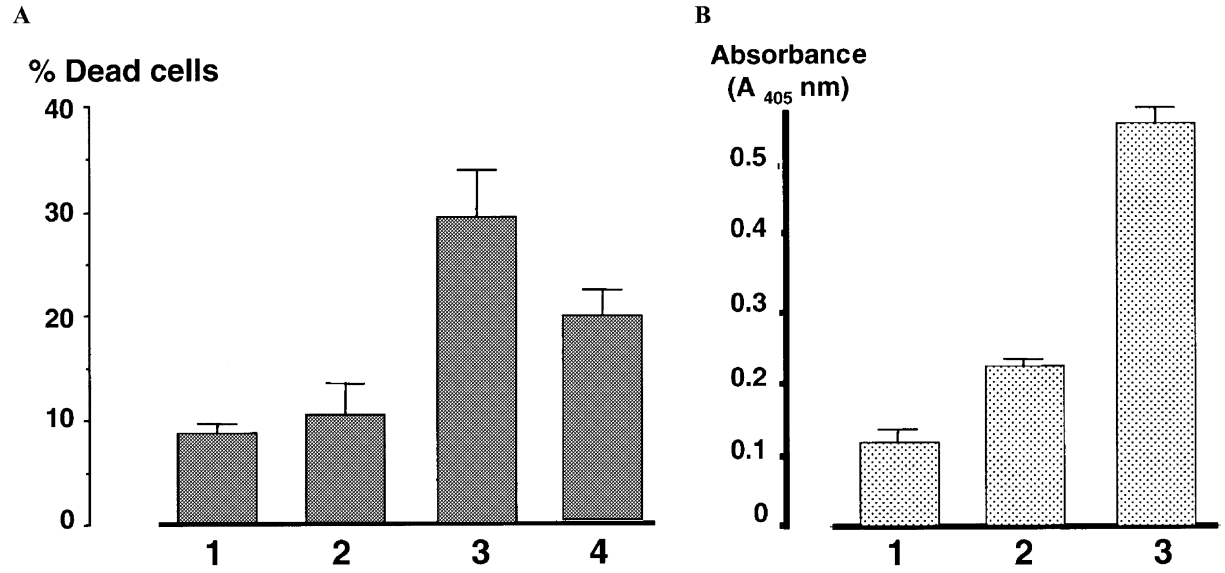

Fig. 4. IGFBP-3$^{1-95}$-induced apoptosis in MCF-7 cells. Cells were or were not transfected with pcDNA-3 or pcDNA/1–95 vector as described above and were analysed 48 h later. (A) Graphs represent the percentages of dead cells measured by trypan blue dye exclusion: (1) wild-type (untransfected) cells; (2) pcDNA-3-transfected cells; (3) pcDNA/1–95-transfected cells; (4) wild-type cells incubated with media conditioned for 48 h by pcDNA/1–95-transfected cells. Results are the means ± SEM of three independent experiments. (B) Cytoplasmic histone concentrations measured as described in Materials and methods. Results are the means of two independent experiments: (1) pcDNA-3 transfected cells; (2) pcDNA/1–95-transfected cells; (3) camptothecin-treated cells.

pathways involved in its effects represent important aspects in understanding the multiple roles of the elements of the IGF system and the interactions between them.

Acknowledgments

The work was supported by the Institut National de la Santé et de la Recherche Médicale and the University of Paris VI. Jean-Marc Ricort is a fellow of the Association pour la Recherche contre le Cancer and Laurence Bernard is a fellow of the Fondation pour la Recherche Médicale.

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