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The EGF-like Homeotic Protein dlk Affects Cell Growth and Interacts with Growth-Modulating Molecules in the Yeast Two-Hybrid System[1]

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Levels of dlk, an EGF-like homeotic protein, are critical for several differentiation processes. Because growth and differentiation are, in general, exclusive of each other, and increasing evidence indicates that *Dlk1* expression changes in tumorigenic processes, we studied whether dlk could also affect cell growth. We found that, in response to glucocorticoids, Balb/c 3T3 cells with diminished levels of dlk expression develop foci-like cells that have lost contact inhibition, display altered morphology, and grow faster than control cell lines. Balb/c 3T3 cells spontaneously growing more rapidly are also dlk-negative cells. Moreover, screening by the yeast two-hybrid system, using *Dlk1* constructs as baits, resulted in the isolation of GAS1 and acrogranin cDNAs. Interestingly, these proteins are cysteine-rich molecules involved in the control of cell growth. Taken together, these observations suggest that dlk may participate in a network of interactions controlling how the cells respond to growth or differentiation signals.

Key Words: **dlk; cell growth; tumoral transformation; yeast two-hybrid system; growth factor; growth arrest; GAS1; acrogranin.**

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The protein dlk (delta-like) is a member of the epidermal growth factor (EGF)-like homeotic family, which includes proteins like the Notch receptors and their ligands (1, 2). These proteins are involved in cell differentiation decisions leading to a variety of mature tissues like the nervous or the immune systems (3–7).

These proteins possess extracellular regions with several EGF-like repeats, which are characterized by an arrangement of cysteines similar to that found in the epidermal growth factor (EGF). EGF-like proteins are expressed either as integral transmembrane proteins or soluble proteins released to the extracellular medium by the action of specific proteases. Generally, these proteins act as receptors or ligands and participate in cell to cell interactions through their EGF-like repeats and related motifs of their extracellular regions.

The mouse dlk gene (*Dlk1*) possesses five exons separated by introns of variable length (8), and it is localized on chromosome 12E-F1 in mice and on chromosome 14q32 in humans (9, 10). dlk possesses six EGF-like repeats in the extracellular domain, a transmembrane domain and a short intracellular tail. Differently spliced dlk mRNA variants encode for proteins possessing or not an amino acid sequence susceptible to the action of unknown proteases capable of releasing the extracellular domain to the medium (8, 11–13). Alternately spliced species of dlk mRNA have been observed in preadipocytes (8) and in human neuroendocrine tumors (11).

Dlk1 is absent from lower animals but is present in animals from birds to mammals and is expressed in both embryonic and adult tissues (12). It is known that dlk participates in several differentiation processes including adipogenesis, hematopoiesis, and adrenal gland differentiation (2). In addition, lack of dlk expression correlates with increased malignance of undifferentiated tumors (12, 14–17).

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The role of dlk in adipogenesis has been extensively studied by several laboratories. *Dlk1* expression is downregulated during adipocyte differentiation of 3T3-L1 cells (18–20). NIH 3T3 and Balb/c 3T3 fibroblasts express dlk but are unable to downregulate it and unable to differentiate in response to IGF1 (20). Forced overexpression of the protein or the addition of recombinant soluble dlk, or a synthetic peptide corresponding to the fifth EGF dlk repeat (EGF5), to the differentiation medium results in the inhibition of adipogenesis of 3T3-L1 fibroblasts (13, 20). Partial inhibition of *Dlk1* expression increases adipocyte differentiation of 3T3-L1 or Balb/c 3T3 cells in response to IGF1 (20–22). However, cells negative for dlk expression have lost also their ability to differentiate (20). These results indicate that the expression levels of dlk are critical in setting a cellular state permissive or not to differentiation in response to extracellular stimuli.

In the present work, we present evidence indicating that dlk protein may play a role in cell growth and tumoral transformation. In response to glucocorticoids, cells with diminished levels of *Dlk1* expression develop foci displaying growth and morphological features of pretumoral cells. Moreover, we demonstrate, by the yeast GAL4 two-hybrid system, that dlk strongly interacts with two cysteine-rich proteins also involved in the control of cell growth and tumoral transformation. These proteins are growth-arrest specific protein 1 (GAS1) and the epithelin/granulin precursor. GAS1 is a membrane GPI anchor-linked protein, the expression of which causes growth arrest. GAS1 is downregulated under proliferative or differentiative conditions (23–26). The effects of GAS1 depend on p53, in particular, the proline-rich domain of p53 is required for the function of GAS1 (27, 28). Acrogranin is the precursor of epithelins and granulins, small peptides involved in the control of cell proliferation and growth of several tumor types (29, 30).

These observations suggest that dlk may participate in a network of protein interactions involved in the control of the mechanisms directing the cells to respond specifically to extracellular stimuli as either growth or differentiation signals.

MATERIALS AND METHODS

Cell culture. Cells were cultured at 37°C in a 5% CO_2 atmosphere, in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Rockville, MD) containing 10% or 5% fetal bovine serum (FBS). The cell lines used were: Balb/c 3T3 (clone A31, ATCC CCL 163), 3T3-L1 (clone-ATCC CCL-92.1) and Balb/c 14, clone obtained in our laboratory by selection of Balb/c 3T3 subclones negative for dlk expression. The stable transfected cell-lines used were established and cultured as described previously (20).

For induction of adipocyte differentiation, 1-day confluent cells were treated with 0.5 mM methylisobutyl-xanthine (IBMX) and 1 mM dexamethasone (Sigma, St. Louis, MO). Two days later, the medium was replaced and 1 mM insulin was added. The differentiated phenotype was determined by direct observation in a microscope with or without red oil O staining (31). In some experiments, cells were treated with only one or a combination of two of the adipogenic agents mentioned above.

Analysis of growth and morphology. One million transfected or control cell lines were plated on 10-cm culture plates with DMEM culture medium plus 10% FBS and 750 μg/ml G418. When cells reached confluence, cells were counted under the microscope before and after harvesting them. Morphology of the transfectant and control cell lines was examined under phase contrast microscopy before or following fixation with methanol and staining with 4% Giemsa. All experiments were repeated three times.

Flow cytometry analysis of membrane dlk expression. Cells were detached from culture plates by incubation with 0.5 mM EDTA in PBS at 37°C for 10 min. Then, cells were washed twice with cold PBS, resuspended in 100 μl of complete DMEM and incubated for 30 min at 4°C with a rabbit anti-dlk antiserum, (generously provided by Dr. Bronek Pytowski, Imclone Inc., NY) directed against the extracellular domain of the dlk molecule. Cells were then washed with complete medium and incubated for 30 min at 4°C with a goat-anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC). After washing three times with complete medium, cells were fixed with 1 ml of 0.1% formalin in PBS and analyzed in a flow cytometer (Becton–Dickinson).

Soft agar cloning assay. Transfected and control cells lines were mixed with 2 ml plating agar (0.33% agar in 1× DMEM/10% FBS) and overlaid onto 2 ml of 0.5% base agar in 1× DMEM/10%/FBS using six-well culture plates. Plates were incubated for 14 days at 37°C in 5% CO_2 atmosphere and 100% humidity. The number of colonies per plate with a diameter of 0.1 mm or greater was determined.

Plasmid constructs and cDNA libraries. FLdlkS and FldlkAS constructs contain the full-length mouse dlk cDNA in sense or antisense orientation, respectively, cloned in the *Eco*RI site of the pCD2 vector (a gift from Dr. J. Battey). They were used to establish stable transfected cell lines, as described elsewhere (20). pAS2-1 and pACT2 basic vectors (Clontech, Palo Alto, CA) were used to construct yeast *GAL4-Dlk1* fusion plasmids. pAS2-1 contains the *GAL4* DNA binding domain and the wild-type *TRP1* gene as the yeast selectable marker. pACT2 contains the *GAL4* activation domain and the wild-type *LEU2* gene as the yeast selectable marker.

Different fragments of *Dlk1* cDNA were amplified by PCR using native or recombinant Pfu DNA Polymerase (Stratagene, La Jolla, CA). Plasmidic DNA, containing the entire cDNA of the mouse *Dlk1* gene, was used as a template. Oligonucleotide primers were designed to contain an *Eco*RI restriction site in the 5′ end and a *Bam*HI restriction site in the 3′ end of the amplified fragments. These restriction sites were used to clone the PCR DNA fragments into the pAS2-1 vector and to generate in frame yeast GAL4 binding domain-dlk fusion proteins. pASdlk contains the entire dlk cDNA, except the N-terminus signal peptide, and it was amplified with oligo mdlk208u33, 5′-ATG GGG GAA TTC GAA TGC GAC CCA CCC TGT GAC-3′, as upper primer and oligo mdlk1279l33, 5′-GGG GAA CGC TGC GGA TCC TTA GAT CTC CTC ATC-3′, as lower primer. pASEGFs1-6 contains the six extracellular EGF-like repeats of dlk and it was amplified with oligo mdlk208u33 (see above), as upper primer and oligo mdlk856l33, 5′-CTT CGC GGA TCC TTA GCA CGT GGG ACC CAT GAA-3′, as lower primer. pASINT contains the intracellular domain of dlk and it was amplified with oligo mdlk1123u36, 5′-ATC GTC GAA TTC AAC AAG TGC GAA ACC TGG GTG TCC-3′, as upper primer and oligo mdlk1279l33 (see above), as lower primer. Finally, pASPRO contains the protease-sensitive domain of dlk (nucleotides +867 to +908, amino acid 260 to 304) and it was amplified with oligo mdlk913u29, 5′-GAG CAC GAA TTC CTG CCC AGC GGC TAT GG-3′, as upper primer and oligo mdlk1044l36, 5′-GAT GGC GGA TCC TTA CTC GGT GAG GAG AGG GGT ACT-3′, as lower primer. Plasmids containing cDNA fragments encoding different contiguous pairs of dlk EGF-like repeats or only the fifth EGF-like repeat, considering the EGF1 as the first repeat

from the N-terminus of the protein, were also made. These cDNA fragments were amplified also by PCR and cloned into pAS2-1. The primers used were engineered to add eight glycin codons, intended to add a bridge of eight glycins to separate the EGF repeats from yeast *GAL4* cDNA binding domain in the fusion protein produced. The resulting plasmids were pASdlk1/2 (first and second EGF-like repeats), amplified with oligo mdlk208u63, 5′-CAC ACA GAA TTC GGA GGA GGA GGA GGA GGA GGA GGA TGC GAC CCA CCC TGT GAC CCC CAG TAT-3′, as upper primer and oligo mdlk382l39, 5′-CTA CAC GGA TCC TTA AGC CCG AAC GTC TAT TTC GCA GAA-3′, as lower primer; pASdlk2/3 (second and third EGF-like repeats), amplified with oligo mdlk295u63, 5′-CAC CAC GAA TTC GGA GGA GGA GGA GGA GGA GGA GGA GAC AAG TGT GTA ACT GCC CCT GGC TGT-3′, as upper primer and oligo mlk493l45, 5′-CTC CAC GGA TCC TTA AGC CTT GTG CTG GCA GTC CTT TCC AGA GAA-3′, as lower primer; pASdlk3/4 (third and forth EGF-like repeats), amplified with oligo mdlk388u61, 5′-CAA CAT GAA TTC GGT GGA GGA GGA GGA GGA GGA GGA GGA GAA ATA GAC GTT CGG GCT TGC ACC T-3′, as upper primer and oligo mdlk635l39, 5′-CTA CTC GGA TCC TTA GCT GTT GGT TGC GGC TAC GAT CTC-3′, as lower primer; pASdlk4/5 (forth and fifth EGF-like repeats), amplified with oligo mDlk505u63, 5′-CAA CAT GAA TTC GGT GGA GGA GGA GGA GGA GGA GGA GAA CAC AAG GCT GGG CCC TGC GTG ATC-3′, as upper primer and oligo mdlk748l39, 5′-CTA CTC GGA TCC TTA GTT GCT CAC CGG GCG GCT GCA GGT-3′, as lower primer; pASdlk5 (fifth EGF-like repeat), amplified with oligo mdlk661u63, 5′-TTA TTA GAA TTC GGT GGT GGT GGT GGT GGT GGT GGT TGT ACC CCT AAC CCA TGC GAG AAC GAT-3′, as upper primer and oligo mdlk742l31, 5′-CGC CGG GAT CCT TAG CAG GTC TTG TCG ACG A-3′, as lower primer; and pASdlk5/6 (fifth and sixth EGF-like repeats), amplified with oligo mdlk634u63, 5′-CAA CAT GAA TTC GGT GGA GGA GGA GGA GGA GGA GGA GAA GAG ATC GTA GCC GCA ACC AAC AGC-3′, as upper primer and oligo mdlk865L42, 5′-CTA CTC GGA TCC TTA CTT CTT CGC GCA CGT GGG ACC CAT GAA-3′, as lower primer. All constructs were quality controlled by restriction analysis and sequencing.

Three different cDNA premade MATCHMAKER libraries (Clontech), cloned in the vector pACT2, were amplified in *Escherichia coli* and used in the screening. These were a 17-day mouse embryo, an embryonic mouse fibroblast (NIH 3T3 cell line), and a human bone marrow library. Each of the libraries contained 3.5×10^6 independent clones and a cDNA insert size range between 0.4 and 4 kb.

DNA sequencing. DNA sequencing was performed by using the QuickLook Fluorescent Automatic Sequencing kit (Bioserve Biotechnologies, Bethesda, MD). For each sequencing reaction, 2 μg of pure plasmid DNA template and 100 ng of sequencing primer (*GAL4* activation domain or *GAL4* binding domain sequencing primers; sequence information can be found in the user manuals provided by Clontech) were mixed in a final volume of 20 μl. The solution was sent to Bioserve Biotechnologies Co. (Laurel, MD) for sequencing. The potential open reading frames of the different sequences found were analyzed by BLAST search in a FASTA format.

Bacterial strains. DH5α *E. coli* competent cells were used for plasmid amplification (Gibco BRL, Life Technologies, Rockville, MD). Liquid and solid LB and SOC media were prepared as described (32) and supplemented with 100 μg/ml of penicillin or carbenicilin, an ampicillin derivative. Bacterial transformations were performed following the manufacturer's indications (Gibco BRL, Life Technologies, Rockville, MD).

Yeast strains and yeast two-hybrid methods. Yeast media ingredients were purchased from Clontech. Liquid or solid media (containing 1.5–2% Bacto-Agar; Difco, Detroit, MI) were prepared according to the manufacturer's specifications.

Yeast transformations were done by using a high-efficiency PEG/LiAc method, as previously described (33). One microgram of DNA was used when the yeast cells were transformed with an isolated plasmid. Ten to 50 μg of DNA was used for yeast transformation with the premade MATCHMAKER cDNA libraries.

Three different strains of *Saccharomyces cerevisiae* were used: CG1945, PJ69-4A, and Y187. CG1945 possesses two reporter genes: *HIS3* and β-galactosidase. The expression of *HIS3* in this strain is not tightly controlled and, even in the absence of protein interactions, cells can grow slowly in a minimal medium lacking histidine. For this reason, 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the yeast His3p protein (34, 35), was used to eliminate background growth.

Strain PJ69-4A possesses three different reporter genes: *HIS3*, β-galactosidase, and *ADE2*, which permits a more stringent selection of the transformants and improved elimination of false positive clones (36). PJ69-4A can hardly grow in a medium lacking histidine and cannot tolerate the presence of even low concentrations of 3-AT due to a tighter control of the expression of HIS3 compared with strain CG1945.

Y187 is a mating partner of CG1945 and possesses only β-galactosidase as reporter gene. To perform a mating assay, one of these two strains was transformed with the DNA binding-domain plasmids and the other with the activation domain plasmids selected from a prior transformation. The mating assay was performed incubating together CG1945 and Y187 transformants in a rich liquid medium (YPD) overnight at 37°C (37). These mating assays are performed to confirm or not the protein interactions that appeared from transformations with only one strain.

Transformants, cotransformants, and mating cultures were plated in different selective media lacking specific amino acids to search for potential protein-protein interactions. The selective culture media used (for details, see Clontech Matchmaker GAL4 manuals) consisted in minimal medium lacking tryptophan, leucine, histidine and adenine, or minimal medium lacking tryptophan, leucine and histidine plus different concentrations of 3-AT depending on the strain of *Saccharomyces cerevisiae* used. The strength of the interactions, which is proportional to the expression of the *HIS3* gene, was estimated by using concentrations of 3-AT ranging from 0 to 100 mM in the appropriate culture media. When analyzing β-galactosidase gene expression, a filter assay of the transformant colonies was performed following the manufacturer's indications (Clontech).

pACT2 derived plasmids were isolated after the elimination of pAS2-1 derivatives from the yeast cells. To induce the loss of the pAS2-1 plasmids, retaining the different pACT2 derivatives, the cotransformed colonies were plated in a selective medium lacking leucine and containing Cycloheximide, according to the manufacturer's recommendations (Clontech). Once the pAS2-1 plasmids were eliminated, pACT2-derived plasmids were isolated using a simple and highly efficient procedure for rescuing autonomous plasmids from yeast (38). The plasmids were then amplified by transforming and growing DH5α *E. coli.* A suitable amount of DNA was isolated for its analysis using the QIAfilter Plasmid Maxi kit (Quiagen Inc., Valencia, CA).

RNA preparation and Northern blot analysis. Cell monolayers were washed with PBS, detached by treatment with 0.5 mM EDTA in PBS, collected by centrifugation (3000*g*, 5 min at 4°C) and washed twice with PBS. Extraction of total RNA was performed using the Rneasy kit, purchased from Qiagen (Chatsworth, CA). To perform Northern blot assays, ten micrograms of RNA were electrophoresed in a 1% formaldehyde-agarose gel in 1× Mops buffer, stained with ethidium bromide and transferred to Hybond-N nylon membranes (Amersham Corp., Arlington Heights, IL), using standard protocols. After RNA-UV crosslinking, random primed ^{32}[P] DNA probes were prepared, using ^{32}[P]dCTP. Filters were prehybridized and then hybridized to the probes for at least 18 h and finally washed according to the protocols provided by Amersham (Arlington Heights, IL). Finally, autoradiographies were developed and scanned. To study dlk expression, an *Eco*RI fragment containing the entire *Dlk1* cDNA was used as a probe. To study GAS1 expression, a *Bam*HI fragment isolated from the cDNA of GAS1 was used as a probe. The amount of ribosomal RNA stained by the ethidium bromide was used as a loading control.

RT-PCR analysis. cDNAs were obtained by using a cDNA kit according to the manufacturer's recommendations (Clontech). To perform RT-PCR analysis, cDNAs were amplified by PCR using AmpliTaq DNA Polymerase (Perkin–Elmer Applied Biosystems, Branchburg, NJ). PCRs were conducted for 40 cycles using normalized amounts of cDNAs. To check for the expression of *Dlk1*, a temperature of 70°C for 2 min was used for annealing and extension, using mdlk208u33 as the upper primer, and mdlk856l33 as the lower primer to obtain a 648-bp fragment. The expression of the housekeeping gene *G3PDH* (glycerol-3-phosphate dehydrogenase) was used as a quality control. In this case, an annealing temperature of 55°C for 45 s and an extension temperature of 72°C for 2.5 min was used. PCRs products were electrophoresed on a 1% agarose gel and visualized by staining with ethidium bromide.

RESULTS

Balb/c 3T3 Cells Expressing Low Levels of dlk Display Altered Growth and Morphology

Upon induction of 3T3-L1 preadipocyte differentiation with glucocorticoids, IBMX and IGF1, the increase, and subsequent decrease, of membrane dlk expression is coincident with the clonal expansion and growth arrest stages of the differentiation process (20, 39). There are increasing evidences indicating that the change in the level of Dlk1 expression correlates with increased malignance of undifferentiated tumors (12, 14–17, 40). Previous data obtained in our laboratory indicate that the level of *Dlk1* expression affects the level and kinetics of ERK/MAPK activation in response to insulin/IGF1 (41). Moreover, recent data demonstrate that *Dlk1* functions as an imprinted gene (42–44) that is paternally expressed, as are many genes involved in cell growth regulation during development. All these observations suggest that, under certain conditions, dlk could play a role in the control of cell growth.

To test this hypothesis, we used Balb/c 3T3 cell lines stably transfected with antisense *Dlk1* expression constructs. Compared to nontransfected controls, these transfected cells display consistently diminished levels of dlk expression and show increased levels of adipocyte differentiation in response to insulin/IGF1 (20, 45). In fact, ERK/MAPK was differently activated in response to insulin/IGF1 depending on the levels of *Dlk1* expression (41), and the activation levels correlated with the differentiation response. In contrast to 3T3-L1 preadipocyte cell line, glucocorticoids were not required by Balb/c 3T3 cells to undergo adipogenesis (20, 45) and they appear to decrease, not increase, their differentiation in response to IGF1. Moreover, it has been shown that glucocorticoids collaborate with ras oncogene to prevent contact inhibition and induce cell transformation (46–50) by acting through the ERK/MAPK cascade. For these reasons, we decided to culture our cell lines in a dexamethasone-containing media for up to 4 weeks. Cells transfected with antisense dlk formed foci 2 weeks after initiation of the culture in presence of this glucocorticoid-derivative. Foci formation did not occur in control non-transfected cells or in cells transfected with dlk expression constructs in sense orientation when treated under the same conditions. Treatment with other agents commonly used to induce adipogenesis, such as IBMX or insulin, had no effect on foci formation (Fig. 1).

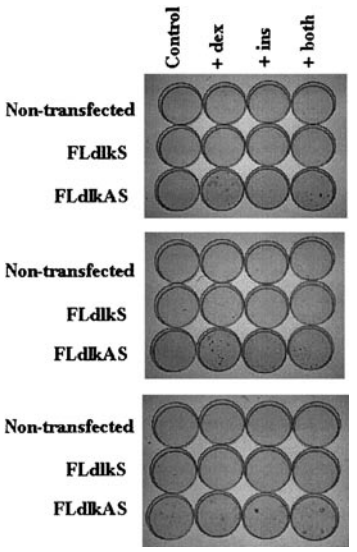

FIG. 1. Foci formation in Balb/c 3T3 antisense dlk transfected cells. The different Balb/c 3T3 transfectants were incubated for up to a month with several combinations of adipocytic hormones and then stained with 4% Giemsa to check for foci formation. (Top) Cells incubated in complete DMEM medium during the entire treatment. (Middle) Cells incubated in complete DMEM medium supplemented with IBMX during all the treatment. (Bottom) Cells incubated in DMEM/IBMX for 2 days and then transferred to complete DMEM for the rest of the treatment. IBMX, isobutylmethyl-xanthine; dex, dexamethasone; ins, insulin; FLdlkS, Balb/c 3T3 cells transfected with full-length dlk cDNA in sense orientation; FLdlkAS, Balb/c 3T3 cells transfected with full-length dlk cDNA in antisense orientation.

We isolated several foci-like colonies and analyzed their cell growth characteristics and morphology. These cells were able to grow significantly faster than control cells (data not shown). The levels of *Dlk1* expression displayed by these selected colonies were analyzed by flow cytometry. The results showed that *Dlk1* expression was lower than that of the full-length antisense dlk transfected cell line from which they were derived (FLdlkAS; Fig. 2). The cells were also smaller in size, more elongated in shape, and grew tightly packed, reaching greater densities at confluence than control cells (Fig. 2). Subcloned foci-forming cells could be maintained in culture and they continued to display their modified morphology, growth kinetics, and low *Dlk1* expression even in the absence of externally added glucocorticoids. Thus, once acquired upon glucocorticoid treatment, this phenotype remained stable under our culture conditions for at least 4 months.

We analyzed whether the morphologically transformed isolated cells possessed a decreased or had lost contact inhibition. When cultured over a monolayer of

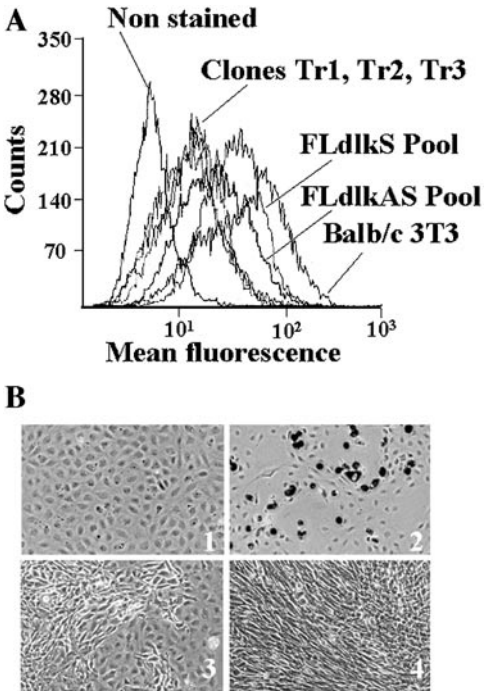

FIG. 2. Morphologically transformed Balb/c 3T3 cells show very low levels of dlk. (A) Flow cytometry analysis of membrane-associated dlk expression in Balb/c 3T3 cell lines transfected with FLdlkS and FLdlkAS constructs (20, 33), and three rapidly growing clones, Tr1, Tr2, and Tr3, isolated from the pool of FLdlkAS transfected cells. (B) Microphotographs of FLdlkAS-transfected cells induced to differentiate to adipocytes following a standard protocol or treated with dexamethasone (see Materials and Methods): 1, control cells noninduced to differentiate; 2, cells induced to differentiate to adipocytes after 7–10 days of treatment with insulin/IGF1. Differentiation outcome was determined by staining the cells with Oil red O and examination under a phase contrast microscope; 3, photography showing the development of a transformed colony upon treatment either with differentiating agents (insulin + dexamethasone, standard protocol) or dexamethasone alone for 3 weeks. Surrounding normal cells are also shown to facilitate the evaluation of the morphology change. 4, the morphologically transformed cells could be isolated and maintained in culture under standard conditions with no effect on their transformed phenotype.

normal Balb/c 3T3 cells, these cells were still able to develop foci. However, the same cells were unable to form colonies in soft agar, indicating that their growth was not anchorage-independent. These results indicate that these cells are not fully transformed into tumoral cells.

Pools of antisense dlk-transfected Balb/c 3T3 fibroblast cells that had been selected by cell sorting for low levels of dlk expression (20) and that corresponded to the 5% of the population with lower dlk levels were also analyzed for their capacity to develop foci in response to glucocorticoids. The incidence of foci formation significantly increased compared with that of nonsorted antisense dlk transfected cells. Whereas non-sorted cells formed an average of only five to ten foci in a 10-cm plate, a plate of sorted cells developed hundreds of foci. The morphology and growth of these foci was virtually identical to that of the foci previously described. These data support the notion that low levels of dlk expression may be associated with pretumoral transformation in response to glucocorticoids.

Another approach used to decrease the level of dlk expression in a population of Balb/c 3T3 cells was to transfect cells with a dlk gene targeting construct to knockout dlk and to select for dlk-negative cells by sorting. This genomic *Dlk1* constructs was engineered to eliminate the first three exons of the *Dlk1* gene. After three rounds of sorting, we observed a progressive decrease in the amount of genomic DNA able to hybridize with a dlk cDNA probe (data not shown). These data indicated that the population of cells was being enriched in a stable manner for cells negative for dlk expression. When we analyzed the ability of these cells to develop foci-forming cells, we observed that they behaved similarly to our sorted antisense dlk transfected cells, also showing very low dlk expression. Growth and foci-forming ability appeared again to correlate with a low level of dlk expression.

Finally, we established and characterized a cell line derived from Balb/c 3T3 cells that had been selected for its ability to grow under culture conditions favoring the growth of fast dividing cells, which included extended periods of culture at confluence. Using this strategy, we developed a cell line, named Balb/c 14, displaying approximately half the doubling time that of parental Balb/c 3T3 cells (data not shown). Analysis of dlk expression demonstrated that this cell line is negative for dlk expression, as determined by both Northern blot or RT-PCR analysis (Figs. 3A and 3B). Moreover, this cell line also shows decreased levels of GAS1 expression compared with the parental cell line (Fig. 3C) (see below). Finally, the morphology of these cells was highly similar to that of the cells derived from the foci generated by treating Balb/c 3T3 antisense dlk cells with glucocorticoids (Fig. 2), although Balb/c 14 cells were unable to form foci when grown over a monolayer of Balb/c 3T3 cells. Taken together, all these data suggest that dlk expression is negatively correlated with cell growth.

GAS1 and Epithelin/Granulin Precursor Interact with the Extracellular Domain of Mouse dlk in the Yeast GAL4 Two-Hybrid System

We searched for proteins that potentially interact with dlk by using the yeast two-hybrid system. For this purpose, we constructed pAS2-1-derived plasmids to drive the expression of different dlk domains fused to the yeast GAL4 binding domain. We used the plasmids pASdlk, pASEGFs1-6, pASINT, and pASPRO (Fig. 4) to perform the yeast two-hybrid screening of one human and two mouse cDNA libraries. We demonstrated also that the different dlk-GAL4 binding domain fusion

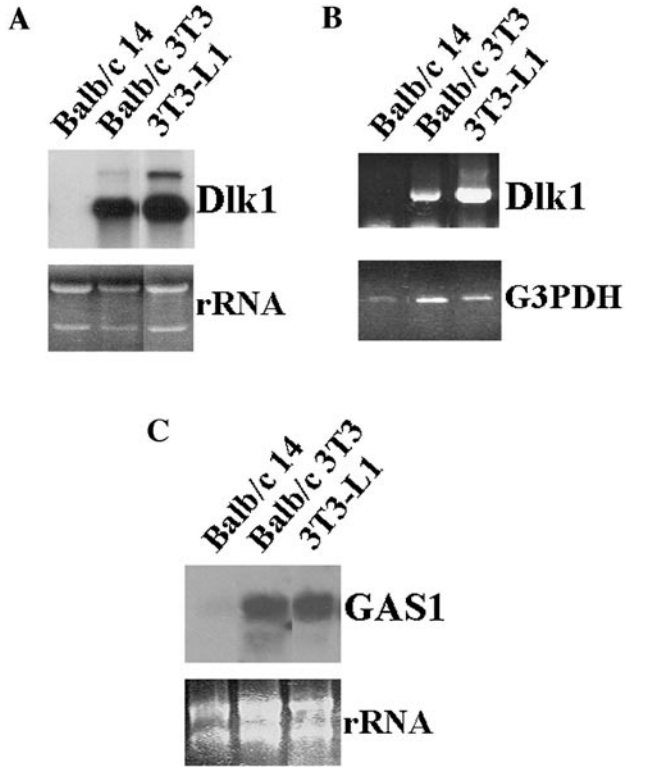

FIG. 3. Expression of Dlk1 and GAS1 by the Balb/c 14 cell line. dlk expression was analyzed by Northern blot (A) or RT-PCR (B) in Balb/c 14, Balb/c 3T3, and 3T3L1 cell lines. (C) Analysis of GAS1 expression by Northern blot in the same cell lines. *G3PDH* was used as a sample loading control for RT-PCR. rRNA corresponds to ribosomal RNA used as a sample loading control for Northern blot assays.

proteins were not able to transactivate the yeast strains reporter genes by themselves.

We obtained transformed yeast colonies expressing proteins potentially interacting with the fusion proteins expressed by all the major dlk domain constructs, except with that possessing the dlk intracellular domain (plasmid pASINT) (Table 1). We selected one thousand colonies (50–60 colonies from each different transformation) and performed a 3-AT assay using concentrations of the drug ranging between 0 and 50 mM. The number of potential positive colonies found with each library and their level of resistance to 3-AT is shown in Table 1. A great number of colonies, capable of growing in 50 mM 3-AT, were obtained from the three libraries when we used the EGF5 construct of dlk as a bait (data not shown).

The insert size of the isolated plasmids, containing full-length cDNAs or cDNA fragments encoding for potential dlk-interacting proteins, ranged from 100 bp to 2.5 kpb. Table 2 shows the characteristics of the different cDNA clones isolated. Some of them were confirmed to be false positives. Among the genes that appeared to encode proteins truly interacting most strongly with the extracellular region of dlk, we found two cystein-rich proteins: mouse acrogranin, also known as the epithelin/granulin precursor, which is involved in cell growth control (30), and mouse GAS1, involved in growth arrest (25). Both clones were obtained twice when screening the NIH3T3 cDNA library, and their yeast colonies were able to tolerate a high concentration of 3-AT in the culture medium.

GAS1 is an integral membrane protein that appears to lack an intracellular region (25). Interestingly, we have discovered that mouse GAS1 possesses, within a larger cysteine-rich sequence, two regions whose structure is highly similar to the structure of an EGF-like repeat (one from Cys 112 to Cys 174 and the other from Cys 227 to Cys 269) (Fig. 5A). The analysis of the DNA sequence of the GAS1 cDNA clone obtained in the screening indicated an insert size of 1.6 kbp. The insert lacks the 5′ nontranslated sequence and 315 bp of the complete open reading frame, but contains most of the GAS1 protein sequence (269 amino acids of a total of 384), and most of the 3′ nontranslated region (1011 bp of a total of 1485) (Fig. 5B). In particular, this insert encodes for the entire sequence of one of the EGF-like-like structures and most of the sequence of the other (Fig. 5B). This truncated GAS1 protein also contains a RGD sequence presumably involved in potential protein–protein interactions (51).

Analysis of the cDNA sequence of the acrogranine clone isolated indicated an insert size of 2.4 kbp. The insert contains the entire open reading frame of the epithelin/granulin precursor encoding for 590 amino

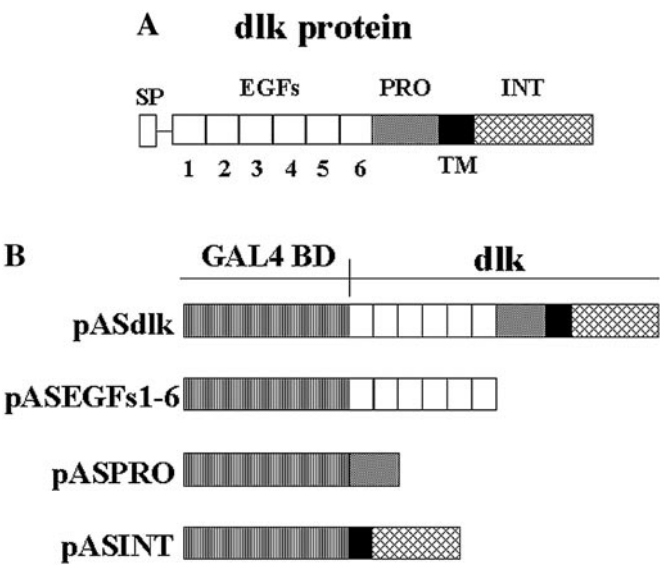

FIG. 4. dlk constructs used in the GAL4 yeast two-hybrid screening. (A) Cartoon indicating the different dlk domains used in the yeast two-hybrid bait constructs. SP, signal peptide; EGFs 1, 2, 3, 4, 5, and 6, epidermal growth factor-like motives; PRO, protease-sensitive signal; TM, transmembrane domain; INT, intracellular domain. (B) GAL4-dlk fusion proteins obtained with the vector pAS2-1 (see Materials and Methods for specific details).

TABLE 1

Number of 3-AT-Resistant Colonies Obtained in the Screening of Two Mouse and One Human cDNA Library

	m*Dlk1* constructs								
	m*Dlk1* EGFs region			m*Dlk1* protease-sensitive region			Entire m*Dlk1* cDNA		
Concn of 3-AT	10	20	50	10	20	50	10	20	50
	Number of clones obtained with each cDNA library								
HBM	2	8	0	0	4	1	0	0	0
NIH3T3	1	10	12	0	2	2	0	0	0
17-Day ME	2	6	7	0	3	0	0	1	1

Note. The number of colonies obtained in the screening with the different *GAL4–Dlk1* constructs is shown. The cDNA libraries used were a human bone marrow cDNA library, indicated as HBM, a mouse NIH 3T3 cDNA library, indicated as NIH3T3, and a 17-day mouse embryo cDNA library, indicated as 17-day ME. The concentration of 3-AT that the clones were able to tolerate is indicated at the top. The maximal concentration of 3-AT these clones can tolerate in a minimal medium lacking tryptophan, leucine, and histidine is 50 mM.

acids, the entire 3′ nontranslated region and 23 bp of the 5′ nontranslated region.

To confirm the interaction of GAS1 and acrogranin with dlk, we performed cotransformations of the yeast strain CG1945 with several *Dlk1-GAL4* fusion constructs (pASdlk, pASEGFs1-6, pASPRO, or pASINT) and the plasmids containing GAS1 or acrogranin cDNAs. Moreover, we performed mating experiments between the yeast strain CG1945 transformed with the GAS1 or acrogranin plasmids, and the yeast strain Y187 transformed with the plasmids pASdlk, pASEGFs1-6, pASPRO or pASINT. In all the cases, we used the empty vectors pAS2-1 and pACT2 as negative controls. As shown in Table 3, GAS1 and acrogranin fusion proteins interacted with the fusion proteins containing the entire dlk protein and the six-EGF-like repeat dlk region. The colonies transformed with the plasmid containing the six dlk-EGF-like repeats and the plasmid containing GAS1 or acrogranin isolated

TABLE 2

cDNA Clones Obtained in the Yeast Two-Hybrid Screening of the Mouse and Human cDNA Libraries

cDNA clone	m*Dlk1* construct	cDNA library	[3-AT] (mM)	Insert size (kbp)
	Potential true positives			
m-GAS1	pASEGFs 1-6	NIH 3T3	50	1.6
m-Acrogranin	pASEGFs 1-6	NIH 3T3	50	2.5
m-AM2 receptor	pASEGFs 1-6	NIH 3T3	20	2.6
m-Serum albumin	pASEGFs 1-6	E17	50	0.7
	False positives			
m-Zyxin	pASEGFs 1-6/ pASPRO	NIH 3T3/E17	50	2.2
h-Jun binding protein	pASPRO	Human bone marrow	20	1.3
m-Ubiquinol-cytochrome *c* reductase	pASdlk	E17	10	0.3
m-ATP syntase (mitocondrial)	pASEGFs 1-6	NIH3T3/E17	20	0.9
h-Hemoglobin	pASEGFs 1-6	Human bone marrow	10	0.5
m-60S ribosomal protein	pASEGFs 1-6	E17	10	0.3

Note. The cDNA clones identified in the screening, *Dlk1* constructs and cDNA libraries used, the maximal concentrations of 3-AT that the clones can tolerate, and the size of each clone are shown. False-positive clones, considered as such because of their intracellular localization, are also indicated.

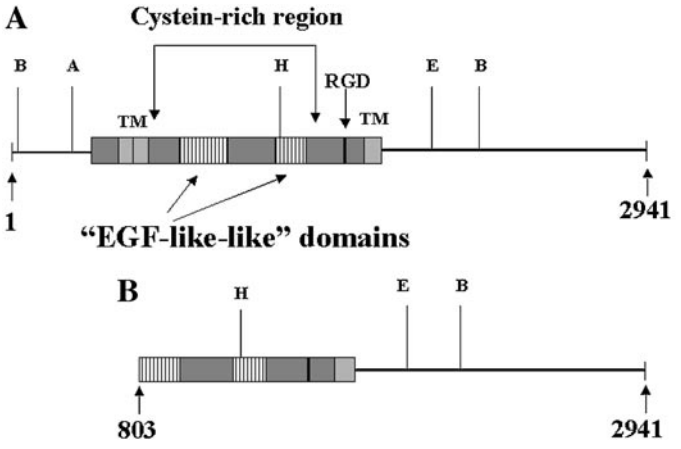

FIG. 5. Scheme of the longest GAS1 cDNA clone obtained in the yeast two-hybrid screening. The figure compares the structure of the entire GAS1 cDNA with the longest GAS1 cDNA clone obtained in the yeast two hybrid system screening. (A) Complete ORF of GAS1 cDNA and partial endonuclease restriction map. B (*Bam*HI), A (*Apa*I), H (*Hin*dIII), and E (*Eco*RI). The regions possessing similar structure to EGF-like repeats are indicated: Cystein-rich region and EGF-like-like domains. RGD, Arg-Gly-Asp tripeptide; TM, transmembrane domain. Numbers indicate sizes of the GAS1 cDNA. (B) GAS1 cDNA clone isolated from screening of the mouse NIH 3T3 cDNA library by the two-hybrid system.

TABLE 3

The Extracellular Region of Mouse dlk Interacts with Mouse GAS1

Y187 + GAL4 BD-dlk fusion protein	CG1945 + GAL4 AD fusion proteins		
	m-GAS1	m-Acrogranin	pACT2
mdlk	100/0	75/0	0/0
m-dlk EGFs 1-6	100/50	100/50	0/0
m-dlk intrac	0/0	0/0	0/0
m-dlk protease	100/0	0/0	0/0
pAS2-1	0/0	0/0	0/0

Note. The percentage of growth of the yeast diploid colonies obtained from a mating assay between Y187 and CG1945 yeast transformants is shown. Y187 was transformed with the *Dlk1* constructs indicated in the left column. CG1945 was transformed with mouse GAS1 or acrogranin cDNA plasmids. As a control, diploid colonies containing the pAS2-1 and pACT2 plasmids were used. pAS2-1, *GAL4* binding domain plasmid; pACT2, *GAL4* activation domain plasmid.

from the libraries tolerated a concentration of 50 mM 3-AT. Moreover, all 3-AT resistant colonies became blue in a qualitative β-galactosidase filter assay, which confirmed the protein interactions by checking for the activity of the β-galactosidase reporter gene (data not shown).

As shown in Table 3, we did not observe interactions between GAS1 or acrogranin and the dlk intracellular domain when we used concentrations of 3-AT of 10 mM or greater. However, we did detect a weak interaction between GAS1 and the protease-sensitive region of dlk.

GAS1 Interacts with dlk through Specific EGF-like Repeats in the Yeast Two-Hybrid System

A literature survey indicated potentially important functional and structural links between GAS1 and dlk proteins. To explore further the interaction between dlk and GAS1, we analyzed whether GAS1 specifically interacts with particular EGF-like repeats of dlk. Some reports indicate that the interactions between EGF-like homeotic proteins involve a contiguous pair of EGF-like repeats. As we indicated before, the protein GAS1 possess two regions similar to EGF-like structures that could be important for the interaction with specific EGF-like domains of dlk (46). For these reasons, we performed both nutritional and 3-AT assays with the strain PJ69-4A cotransformed with GAS1 and each one of the following dlk constructs: pASdlk1/2, pASdlk2/3, pASdlk3/4, pASdlk4/5, pASdlk5, and pASdlk5/6 (see Materials and Methods). We used also the construct containing only the fifth dlk EGF-like repeat, because this repeat has been shown to posses biological activity by itself in the control of adipogenesis (20).

Table 4 shows that GAS1 interacts with the proteins expressed by pASdlk5/6, pASdlk1/2, and pASdlk4/5. However, only in the case of the protein expressed by pASdlk5/6, the yeast colonies were able to grow in the presence of 50 mM 3-AT, indicating a strong interaction between GAS1 and the protein expressed by this construct. The interaction of GAS1 with the protein encoded by the dlk EGF5 construct was weaker and the yeast colonies grew only if the concentration of 3-AT was lower than 10 mM.

These results suggest, therefore, that dlk and GAS1 interact through their extracellular domains and that the interaction involves specific EGF-repeats and perhaps the protease-sensitive region of dlk.

DISCUSSION

The first effects of dlk expression on the control of cell growth were observed in pre-B cells. These cells

TABLE 4

The Extracellular Domain of Mouse dlk Interacts with GAS1 through Specific EGF-like Repeats

m-*Dlk1*–GAL4 *BD* constructs	m-GAS1–GAL4 AD construct (pACTGAS1)					pACT2	
	−TLHD	−TLH	−TLH (1 mM)	−TLH (10 mM)	−TLH (50 mM)	−TLHD	−TLH
pASdlk1/2	0	100	100	0	0	0	0
pASdlk2/3	0	0	0	0	0	0	0
pASdlk3/4	0	0	0	0	0	0	0
pASdlk4/5	0	100	100	0	0	0	0
pASdlk5/6	100	100	100	100	50	0	0
pASdlk5	0	100	100	100	0	0	0
pAS2-1	0	0	0	0	0	0	0

Note. The percentage of growth of different GAS1/*Dlk1* cotransformant colonies of the yeast strain PJ69-4A is shown. The GAS1 cDNA clone is a pACT2 derivative. *Dlk1*-EGF-like repeat constructs (pAS2-1 derivatives) are indicated on the left. Empty vectors, pAS2-1 and pACT2, were used as controls. The figures inside parentheses indicate the concentrations 3-AT added. The minimum medium used is indicated below the construct or control plasmid used. −TLHD, minimal medium lacking tryptophan, leucine, histidine, and adenine. −TLH, minimal medium lacking tryptophan, leucine, and histidine.

usually require cell to cell interactions with stroma cells and soluble factors, mainly IL-7, to grow in culture and maintain their state of differentiation. However, when pre-B cells are grown over antisense dlk transfected Balb/c 3T3 cells expressing diminished levels of dlk (the same cell lines that were used in the present study), pre-B cells can grow in culture and maintain their differentiation state in the absence of IL-7 (52). Thus, lower dlk expression levels could affect the growth of cells in need of cellular interactions with stromal cells expressing dlk. Effects of dlk on in T cell development and cell growth were observed also by other investigators (53, 54). On the other hand, recent works indicate that *Dlk1* is an imprinted gene (42–44) that is paternally expressed, which suggests that *Dlk1* is involved in the control of embryonic growth. Moreover, we have shown that dlk may modulate the kinetics and level of activation of ERK/MAPK in response to IGF1 (41). All these evidences together with recent observations that correlate the level of *Dlk1* expression to tumorigenic transformation and the fact that adipocyte differentiation requires prior clonal expansion and growth arrest steps (54), prompted us to further study the involvement of dlk in the control of cell growth.

In this report, we show that, together with glucocorticoids, dlk is involved in the first stages of *in vitro* tumor cell formation, in Balb/c 3T3 fibroblasts. This is a novel biological property of dlk that raises important questions about the mechanism of dlk action, a mechanism that must reconcile the effects of dlk on growth and differentiation in a paracrine or autocrine fashion. A relationship between the state of malignant transformation, tumoral differentiation and dlk expression has been observed in tumors of neuroendocrine origin, including pheochromocytomas, neuroblastomas and small cell carcinomas (11, 12, 40). Our results support that the levels of dlk expression may be correlated with tumor development and growth. This effect, in turn, must be related to the effect of dlk on the control of cell differentiation in response to IGF1. In this regard, and taking into account that IGF1 activates the ERK/MAPK pathway, it is worth mentioning that glucocorticoids have been shown to collaborate with the *K-ras* oncogene in increasing both adipogenesis and tumorigenesis of human skin fibroblasts. Glucocorticoids appear then to increase both differentiation of preadipocytes and malignant transformations (46, 47). Our data indicate that, in response to glucocorticoids, both biological effects can be obtained in Balb/c 3T3 cells, a fibroblast cell line, in which dlk expression has been decreased. This, in turn, is consistent with the effect of dlk on the MAPK pathway recently observed (40).

What is known so far about the biology of dlk indicates that this protein must function trough the interaction with yet unknown molecular partners present on the membrane of neighboring cells of the same or different lineage. (12, 13, 21, 53). However, no molecular counterparts for dlk have been reported yet. This article is the first to report the nature of two molecules that may potentially interact with dlk at the cell surface.

Because the majority of the EGF-like proteins interact with other members of the same family, it was speculated that the ligand/receptor for dlk must also belong to this family of proteins. However, our screening of three different libraries by the yeast two-hybrid system has failed to identify any interactions between dlk and another EGF-like molecule of the family. Instead, the molecules that displayed a strong interaction with dlk were acrogranin, and GAS1, which are molecules involved in the control of cell growth.

We did not have access to antibodies specific for these molecules, and therefore we could not confirm the interaction of dlk with either acrogranin or GAS1 by coimmunoprecipitation followed by Western blot. We are, however, of the opinion that this *in vitro* assay is not scientifically sound if used with the purpose to confirm the protein interactions revealed by the yeast two-hybrid system, because the conditions used for this assay (protein concentration, specific buffers, etc.) are even more artificial than the ones present in the yeast nucleus. For that reason, we used contiguous dlk EGF-like repeats to show whether the interaction with GAS1 occurred specifically with some of them. The data shown in Table 4 indicate that this is the case. Experiments performed to explore the interaction between GAS1 and EGF-like repeats of other proteins, like Notch-1, further confirm the absence of non-specific interactions (data not shown). These results rule out a non-specific interaction between GAS1 and EGF-like structures. In any case, we cannot conclude that dlk interacts with either acrogranin or GAS1 in mammalian cells. However, the biological and some of the structural properties of these molecules are interestingly related to dlk.

The amino acid sequence of both epithelin/granulin and GAS1, indicate that, although in a strict sense these proteins do not possess EGF-like repeat sequences, they possess highly related structures. The interaction of Granulin/Epithelin or GAS1 with the EGF-like repeats of dlk does not appear then as an exception to the general rule of how EGF-like molecules appear to interact with each other. Mature epithelin/granulins are small peptides that originate from a common precursor containing seven repeats of a similar structure characterized by a specific spacing and arrangement of cysteines that participate in disulfide bonds folding the peptide in a secondary structure similar to EGF/TGFα (29). Processing of the precursor originate biologically active peptides possessing agonistic or antagonistic properties on cell growth, depending on the cell type. These molecules have been implicated in the control of cell proliferation in hematopoiesis and growth of several tumor types, including

breast cancer, adenocarcinoma and epidermoid carcinoma (29). A recent report indicates that inhibition by antisense expression constructs of the Granulin precursor is able to inhibit tumorigenicity of the human breast carcinoma cell line MDA-MB-468 (55). Thus, epithelin/granulins may participate in autocrine loops affecting cell growth and tumorigenicity. Therefore, the potential interaction of these molecules with dlk may play a role in modulating how they interact with their receptors. Results of crosslinking experiments indicate that at least one of these acrogranin receptors possesses an apparent molecular mass of 120 kDa (56), but very little is known about the receptors for most members of the EGF-like family. dlk could be a component with receptor function, or it could modulate the interaction of one or more of the Epithelins with their receptors.

The biological properties of GAS1 appear to relate more closely to dlk. GAS1 encodes an integral plasma membrane glycoprotein rich in cysteine residues and with two putative transmembrane domains flanking a large extracellular domain (25, 26). This produces an inverse U-shaped molecule on the cell surface. The GAS genes were isolated by virtue of their accumulation in serum-starved growth-arrested NIH 3T3 cells (25). One of these genes, GAS1, is expressed in several human, murine and rat tissues, including skeletal muscle, kidney, heart, placenta, and fetal brain (26). Despite lacking any intracellular amino acids, GAS1 induces growth arrest in the G0 to S phase of the cell cycle. GAS1-mediated growth suppression requires endogenous p53 but does not require Rb (28).

Within a larger cystein-rich region, GAS1 contains two "EGF-like-like" regions, one of which possesses six cysteines in a pattern which only lacks one amino acid to produce a perfect match with the accepted consensus sequence for an EGF-like repeat (our own observations). Ruaro and co-workers (24) were able to restrict the minimal functional domain of GAS1 between amino acids 182 and 234, a region situated between the two EGF-like-like motives we have defined. This region retains a growth inhibitory effect, although smaller than the one achieved with the entire protein, or a truncated GAS1 protein from amino acids 1 to 229. This observation suggested to the authors that the amino terminal region of the protein (amino acids 1–229) is necessary for the inhibitory effect of the minimal functional domain between amino acids 182 and 234. Interestingly, one of the GAS1 EGF-like-like motives we have defined comprises the region between amino acids 112 and 174. These observations suggest that the GAS1 amino terminal region that includes one of the EGF-like-like structures may interact with dlk and be important for the growth inhibitory effect of GAS1.

The first step in the differentiation of preadipocytes is a clonal expansion followed by growth arrest and withdrawal from the cell cycle. Addition of adipogenic agents to preadipocytes stimulates the growth-arrested, postconfluent cells, to reenter the mitotic cell cycle. Once completed, cells enter again in growth arrest and continue the adipogenic differentiation program. To better understand the relationship between cellular growth and adipocyte differentiation, Shugart and co-workers (57) characterized the expression of growth-arrest-specific genes (GAS) and DNA damage-inducible genes (GADD) during adipose conversion of 3T3-L1 cells. They observed a strong expression of GAS1 during the first days of the adipogenic process. GAS1 expression decreased later upon completion of differentiation. A similar pattern of expression is observed for dlk (20). Therefore, both molecules are present at the membrane of differentiating cells at the same moment of the differentiation process. Their potential interaction could modulate the clonal expansion and growth phase arrest of this process. The release of soluble variants of dlk (13, 20) could also modulate GAS1 action.

There is a large body of experimental evidence involving GAS1 in different tumorigenic processes. NIH3T3 cells transfected with GAS1 cDNA, under the control of a dexamethasone inducible promoter, resulted in normal numbers of transfected cells. If then GAS1 expression was induced with dexamethasone, the growth rate was greatly inhibited and morphological changes were observed (58, 59). Overexpression of the mouse GAS1 gene by microinjection of a GAS1-expressing plasmid into normal and oncogene-transformed NIH3T3 cells (with the exception of SV40-transformed NIH3T3 cells) blocks proliferation of actively growing cells and also inhibits entry of quiescent cells into S phase when activated by serum (25). In contrast, NIH3T3 cells transfected with antisense GAS1 cDNA construct displayed altered morphology and grew, with loss of contact inhibition, to a much higher saturation at confluence than control cell lines. This effect, however, was insufficient to fully transform the cells, suggesting that additional genetic events are required for a fully malignant phenotype (58).

Similarly, when A549 cells were transfected with a dexamethasone-inducible GAS1 expression plasmid, expression of GAS1 inhibited cell growth *in vitro,* and fewer slow-growing tumors arose in nude mice, which indicates that GAS1 suppresses proliferation in the A549 lung adenocarcinoma cell line (58). Human GAS1 exhibits 82% protein sequence homology with mouse GAS1. The microinjection of plasmids expressing human GAS1 also inhibits proliferation of both normal fibroblasts and tumor cell lines (26).

The data pertinent to GAS1 discussed above are strikingly similar to those we report here for dlk: loss contact inhibition, altered morphology and faster growth for cells with diminished levels of dlk in response to dexamethasone. As for GAS1, the cells are

not fully transformed. This similar behavior is consistent with what it should be expected after decreasing the expression of an interacting counterpart of GAS1 on the cell surface. In addition to preadipocytes, other tissues express both dlk and GAS1, including the placenta (12, 26). This observation suggests that in some tissues during development or during some cell differentiation processes in the adult, the interaction between dlk and GAS1 could be of importance.

The data reported here indicate that dlk plays an important role, not only in differentiation processes, but also in the regulation of cell proliferation in response to extracellular stimuli. The specific interactions we report between dlk and GAS1 or acrogranin, by using the yeast two-hybrid system, also suggests that the effects of dlk on cell growth may occur through a functional interaction *in vivo* with these proteins. Despite the lack of additional *in vitro* data to confirm these interactions, the differences in strength demonstrated in the interactions between GAS1 or acrogranin and specific dlk EGF-like repeats, as well as the lack of interactions with other dlk regions, or EGF-like domains of other proteins, strongly suggest that the interaction between dlk and these proteins is specific.

The interaction of dlk with growth-modulating molecules and the accelerated growth of cells with very low levels of dlk in response to glucocorticoids indicate a new role for dlk in cell to cell contact and cell proliferation. Further studies are needed to elucidate the nature of this interactions and the mechanism by with they lead to cellular proliferation or differentiation.

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