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SPECIFIC REGIONS OF THE EXTRACELLULAR DOMAIN OF DLK, AN EGF-LIKE HOMEOTIC PROTEIN INVOLVED IN DIFFERENTIATION, PARTICIPATE IN INTRAMOLECULAR INTERACTIONS

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1. ABSTRACT

The level of expression of dlk, an EGF-like protein possessing six EGF-like repeats in its extracellular region, is critical for 3T3-L1 fibroblasts to differentiate into adipocytes in response to IGF1. The mechanism of action of dlk is not well understood, but its localization on the cell membrane suggests that dlk may function as a receptor, as a ligand or as a regulatory protein modulating the binding, the signaling, or the expression of other molecules involved in cell differentiation and growth. In this work, we demonstrate, by using the Yeast Two-Hybrid system, that dlk interacts with itself through specific regions of its extracellular domain. The strongest interactions were observed between specific EGF-like repeats and between a non EFG-like region where unknown proteases act to generate soluble forms of dlk. These observations suggest that the interaction between two membrane dlk molecules belonging to the same or to different cells, or the interaction between soluble and membrane dlk variants, may be important to regulate dlk function.

2. INTRODUCTION

The protein dlk is a member of the Epidermal Growth Factor (EGF)-like family of homeotic proteins, which includes Notch receptors and their ligands (1, 2). These proteins are transmembrane molecules that possess a variable number of EGF-like repeats at their extracellular domain. In many cases, the extracellular region of these proteins can be released to the extracellular medium by the action of specific proteases. EGF-like repeats are conserved structures, possessing six cysteines linked by disulfide bonds in a way similar to that found in the Epidermal Growth Factor (EGF). EGF-like motives mediate protein-protein interactions between receptors and ligands that lead to the activation of the receptors and the transmission of specific signals for cell differentiation and growth.

The mouse dlk gene (*Dlk1*) is composed of five exons separated by four introns of variable length. The gene is localized on chromosome band 12E-F1 in mice and chromosome 14q32 in humans (3, 4). dlk has been rediscovered six independent times by using differential screening hybridization techniques and for this reason it has received different names (5). dlk possesses six EGF-like repeats at the extracellular domain, a transmembrane domain and a short intracellular tail. Different spliced dlk mRNA variants, all of then occurring within the fifth exon of the gene, encode for proteins possessing or not an amino acid sequence susceptible to the action of unknown proteases able to release the glycosylated extracellular domain to the medium (6). Alternately spliced species of dlk mRNA have been observed in human neuroendocrine tumors (7) and preadipocytes (8).

dlk is present in animals from birds to mammals but it is absent from lower animals (7). *Dlk1* is expressed during development by several embryonic tissues, and it is also expressed by some adult tissues, including placenta, adrenal gland, preadipose tissue, pancreas, and bone marrow stroma cells (5). dlk participates in several differentiation processes, including adipogenesis, hematopoiesis, and adrenal gland differentiation. dlk plays also a role in the differentiation of neuroendocrine cells and perhaps in the increased malignance of undifferentiated tumors (7, 9, 10, 11, 12, 13, 14). A recent discovery indicates that dlk is an imprinted gene expressed exclusively from the paternally inherited allele (15). Changes in the imprinted expression status have been linked to the callipyge mutation in sheep (16). The fact that very few genes are imprinted, all of them involved in the control of fetal growth and differentiation, suggest that dlk may play an important role in the control of several differentiation or growth processes, including those mentioned above.

Adipogenesis still is one of the most studied differentiation processes (17, 18, 19). Several laboratories have studied the role of dlk in adipogenesis. *Dlk1* expression is down regulated during adipocyte differentiation (20, 21, 22). Over expression of the protein prevents, whereas inhibition of its expression increases, adipocyte differentiation of 3T3-L1 cells in response to IGF-1 (22, 23, 24). Addition of recombinant soluble dlk, or a synthetic peptide corresponding to the fifth EGF-like repeat of dlk to the differentiation medium, results in the inhibition of adipogenesis of 3T3-L1 fibroblasts in response to IGF-1. These observations suggested that dlk may function as inhibitor of differentiation in a juxtacrine or paracrine fashion. Hansen and coworkers (1998) (25) working with rat adypocites and Garcés and coworkers (1999) (22) working with 3T3-L1 cells observed an increase, rather than a decrease, in *Dlk1* mRNA and protein expression at earlier times after differentiation triggering. However, *Dlk1* mRNA variants encoding for potentially soluble dlk peptides, inhibitory for differentiation, were quickly down regulated. Antisense *Dlk1* transfected cells show an increase in the differentiation response, but cells mostly negative for membrane dlk expression show a decrease (22). Whereas *Dlk1* expression decreases at the end of the differentiation process in 3T3-L1 cells, NIH 3T3 cells or Balb/c 3T3 fibroblasts expresses *Dlk1* but are unable to down regulate it in response to adipogenic agents and are also unable to differentiate (22). These results suggested that the expression levels of *Dlk1* appear to play an important role in setting a cellular state permissive or not for differentiation in response to extracellular stimuli, and that the membrane-associated variants of the protein dlk might play a positive, rather than an inhibitory role, on differentiation.

A question that remains to be answered is whether dlk functions as a ligand, as receptor, or as a protein regulating the signaling outcome or the expression of other receptors and molecules that enable to transmit a differentiation signal. dlk lacks a DSL domain at the N-terminus of the protein, a domain present in the extracellular domains of Notch ligands. dlk lacks also any apparent functional intracellular domain capable to transmit a signal (5). However, on the basis of the structural homology of dlk with Delta, one of the ligands of Notch in *Drosophila*, and other members of the Notch family, it is reasonable to speculate that dlk may participate in the differentiation of specific cells and tissues through the interaction of its EGF-like repeats with specific domains present in members of this or other families of proteins. Furthermore, the interaction between two dlk molecules, through their EGF-repeats or through other regions of the protein, may be a way of self-regulation or a way of regulating other functional protein interactions occurring at the cell surface.

The protease-sensitive region of dlk is located at the carboxi terminus of the extracellular domain of the protein and possesses a structure similar to a leucine zipper. Leucine-zippers are protein-protein dimerization structures consisting of repeats of leucine residues separated by seven amino acid residues that thus form a coiled-coil structure (26). An increasing body of literature indicates that these motifs mediate homo- and heterodimerization in many kind of proteins. Therefore, the dlk leucine zipper-like structure could be involved in dlk dimerization.

In the present work, we show that dlk interacts with itself through specific regions of its extracellular domain. The interaction is stronger between specific EGF-like repeats and through the protease-sensitive region. These observations suggest that the interaction between two molecules in the same or two different cells, or the interaction between soluble and membrane dlk variants, may be important to regulate the function of dlk.

3. MATERIALS AND METHODS

3.1. Plasmid constructs

pAS2-1 and pACT2 basic vectors (Clontech, Palo Alto, CA, USA) were used to construct yeast *GAL4-Dlk1* fusion plasmids. pAS2-1 contains the *GAL4* DNA Binding Domain and the wild type *TRP1* gene as a yeast selectable marker. pACT2 contains the *GAL4* Activation Domain and the wild type *LEU2* gene as a yeast selectable marker.

Different fragments of *Dlk1* cDNA were amplified by PCR using native or recombinant pfu DNA Polymerase (Stratagene, La Jolla, CA, USA). Plasmid 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 at the 5' end and a *Bam*HI or a *Xho*I restriction site at the 3' end of the amplified fragments. The common 5´ and the two different 3´ restriction sites were used to clone the PCR DNA fragments into pAS2-1 or into pACT2, respectively, to generate in-frame GAL4 binding or activation domain-dlk fusion proteins.

Several positive or negative control plasmids were used in the two hybrid experiments. pCL1 encodes the full length GAL4 protein, pVA3-1 encodes the mouse p53 protein fused to the GAL4 binding domain, pTD1-1 encodes the SV40 large T-antigen fused to the activation domain of GAL4 and pLAM5'-1 encodes the human Lamin C protein fused to the GAL4 binding domain.

The oligonucleotides used to prepare the constructs were named according to the gene, mdlk, the position in the cDNA, its used as upper (u) or lower, (l) primers and the length in nucleotides. The oligonucleotides employed were the following: mDlk208u33 (1): 5'-ATG GGG GAA TTC GAA TGC GAC CCA CCC TGT GAC-3', mDlk1279l33 (2): 5'-GGG GAA CGC TGC GGA TCC TTA GAT CTC CTC ATC-3', mDlk856l33 (3): 5'-CTT CGC GGA TCC TTA GCA CGT GGG ACC CAT GAA-3', mDlk913u29 (4): 5'-GAG CAC GAA TTC CTG CCC AGC GGC TAT GG-3', mDlk1044l36 (5) : 5'-GAT GGC GGA TCC TTA CTC GGT GAG GAG AGG GGT ACT-3', mDlk1123u36 (6): 5'-ATC GTC GAA TTC AAC AAG TGC GAA ACC TGG GTG TCC-3', mDlk661u63 (7): 5'-TTA TTA GAA TTC GGT GGT GGT GGT GGT GGT GGT GGT TGT ACC CCT AAC CCA TGC GAG AAC GAT-3', mDlk742l31 (8): 5'-CGC CGG GAT CCT TAG

CAG GTC TTG TCG ACG A-3', mDlk661u65 (9): 5'-TTA TTA GAA TTC GAG GTG GTG GTG GTG GTG GTG GTG GTT GTA CCC CTA ACC CAT GCG AGA ACG AT-3', mDlk742l31 (10): 5'-CGC CGC TCG AGT TAG CAG GTC TTG TCG ACG A-3', mDlk208u35 (11): 5'-ATG GGG GAA TTC GAG AAT GCG ACC CAC CCT GTG AC-3', mDlk1279l33 (12): 5'-GGG GAA CGC TGC CTC GAG TTA GAT CTC CTC ATC-3', mDlk856l33 (13): 5'-CTT CGC CTC GAG TTA GCA CGT GGG ACC CAT GAA-3', mDlk208u66 (14): 5'-CCA ACA AGA ATT CGA GGA GGA GGA GGA GGA GGA GGA GGA TGC GAC CCA CCC TGT GAC CCC CAG TAT-3', mDlk 382l39 (15): 5'-CTA CTC CTC GAG TTA AGC CCG AAC GTC TAT TTC GCA GAA-3', mDlk295u66 (16): 5'-CCA CAA AGA ATT CGA GGA GGA GGA GGA GGA GGA GGA GGA GGA GAC AAG TGT GTA ACT GCC CCT GGC TGT-3', mDlk493l45 (17): 5'-CTA CTC CTC GAG TTA AGC CTT GTG CTG GCA GTC CTT TCC AGA GAA-3', mDlk388u64 (18): 5'-CCA CAA AGA ATT CGA GGA GGA GGA GGA GGA GGA GGA GGA GGA GAA ATA GAC GTT CGG GCT TGC ACC T-3', mDlk 634l39 (19): 5'-CTA CTC CTC GAG TTA GCT GTT GGT TGC GGC TAC GAT CTC-3', mDlk482u59 (20): 5'-CAA CAT GAA TTC GAG GTG GAG GAG GAG GAG GAG GAG GAC CCT GCG TGA TCA ATG GTT CT-3', mDlk734l39 (21): 5'-CTA CTC CTC GAG TTA GGC TGC AGG TCT TGT CGA CGA ATC-3', mDlk634u63 (22): 5'-CCA CAA AGA ATT CGA GGA GGA GGA GGA GGA GGA GGA GGA GAG ATC GTA GCC GCA ACC AAC AGC-3' and mDlk856l42 (23): 5'-CTA CTC CTC GAG TTA CTT CTT CGC GCA CGT GGG ACC CAT GAA-3'.

The constructs made are represented in figure 1. pASdlk and pACTdlk contain the entire dlk cDNA, lacking the N-terminus signal peptide, and were amplified with oligos 1 and 2 and 11 and 12, respectively; pASEGFs1-6 and pACTEGFs1-6 contain the six extracellular dlk EGF-like repeats, and were amplified with oligos 1 and 3; and 11 and 13, respectively; pASEGF5 and pACTEGF5 contain the fifth dlk EGF-like repeat, separated by a tail of 8 glycine codons from the GAL4 binding domain, and were amplified with oligos 7 and 8, and 9 and 10, respectively; pASINT contains the intracellular domain of dlk, and was amplified with oligos 6 and 2; and pASPRO, contains the protease-sensitive domain of dlk (nucleotides from +867 to +908, amino acid 260 to amino acid 304), and was amplified with oligos 4 and 5. Plasmids containing different pairs of dlk EGF-like repeats, considering EGF1 as the first repeat from the N-terminus of the protein, were also made. These EGF pairs were amplified also by PCR and cloned into the pAS2-1 or pACT2 vectors in frame with the GAL4 binding or activation domains coding regions, respectively. These plasmids are: pASdlk1/2 and pACTdlk1/2, amplified with oligos 14 and 15; pASdlk2/3 and pACTdlk2/3, amplified with oligos 16 and 17; pASdlk3/4 and pACT3/4, amplified with oligos 18 and 19; pASdlk4/5 and pACTdlk4/5, amplified with oligos 20 and 21 ;pASdlk5/6 and pACTdlk5/6, amplified with oligos 22 and 23. A plasmid derived from pACT2 vector by digestion with the restriction enzyme *Hind* III, lacking the GAL4 activation domain (pACTGAL4$^-$), was also made. All constructs were quality-controlled by restriction analysis and sequencing.

3.2. DNA sequencing

DNA sequencing was performed using the QuickLook Fluorescent Authomatic Sequencing kit (Bioserve Biotechnologies, MD, USA). For each sequencing reaction, two micrograms of plasmid DNA template and 100 nanograms of sequencing primer (GAL4 AD sequencing primer or GAL4 BD sequencing primer; sequence available in Clontech user manuals) were mixed in a final volume of 20 microliters and sent to Bioserve Biotechnologies Company (Laurel, MD, USA) for sequencing. The potential open reading frames of the sequences were analyzed by means of a Blast Search in a FASTA format.

3.3. Bacterial strains

The strain of *Escherichia coli* used was DH5 alpha. Liquid and solid LB and SOC media were prepared as described (27) and supplemented with 100 micrograms per ml of Penicillin or Carbenicilin, an Ampicilline derivative. Bacterial transformations were performed according to the manufacturer's indications (Gibco BRL, Life Technologies, Rockville, MD, USA).

3.4. Yeast Two Hybrid methods

Yeast media ingredients (Clontech) were used to prepare media according to the manufacturer's instructions. When solid media were needed, 1.5%-2% Bacto-Agar (Difco, Detroit, Michigan) was added.

Yeast transformations were performed using the high efficiency PEG/LiAc method of Giezt and Schiestl (1995) [28]. One microgram of plasmid DNA was used to transform the yeast cells. Three different strains of *Saccharomyces cerevisiae* were used: CG1945, PJ69-4A and Y187. CG1945 possesses two reporter genes: *HIS3* and Beta-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 (29), was used to eliminate background growth.

The strain PJ69-4A possesses three different reporter genes: *HIS3*, Beta-galactosidase, and *ADE2*, which permits a more stringent selection of the transformants and improved elimination of false positive clones (30). 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* as compared with strain CG1945.

Y187 is a mating partner of CG1945 and possesses only Beta-Galactosidase as a reporter gene. Mating assays are performed as a safer method to confirm the protein interactions found from transformations with only one strain. To perform a mating assay, one of those two strains was transformed with one of the DNA binding-domain plasmids and the other with one of the activation domain plasmids. The mating assay was performed

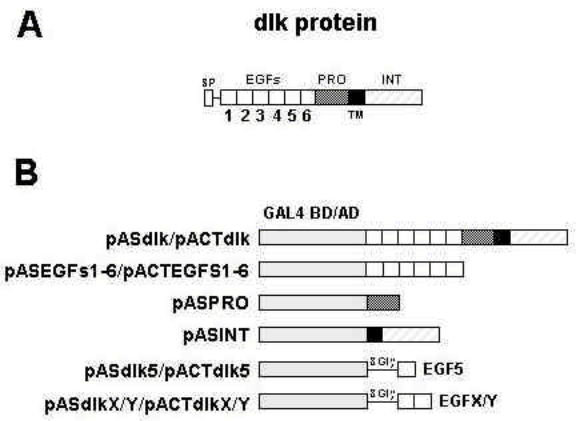

Figure 1. GAL4-dlk fusion protein used in the yeast two-hybrid library screenings. A) Schematic representation of dlk protein structure. SP: signal peptide, EGFs: EGF-like repeats, PRO: protease-sensitive signal, TM: transmembrane domain, INT: Intracellular domain. B) Schematic representation of different GAL4 binding domain and activation domain-dlk fusion proteins. X/Y represent the constructs made with different contiguous dlk EGF-like repeats.

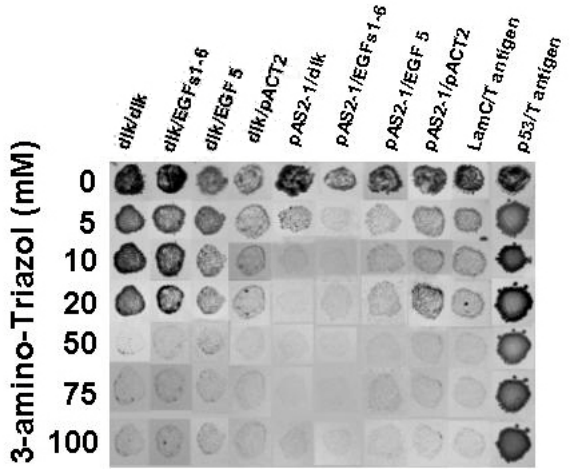

Figure 2. Mouse dlk interacts with itself in the yeast two-hybrid system. The figure shows CG1945 transformed colonies growing in a minimal medium lacking leucine, tryptophan and histidine plus concentrations of 3-Amino triazol ranging from 0 to 100 mM. pAS2-1: GAL4 binding domain plasmid. pACT2: GAL4 activation domain plasmid. LamC/T antigen and p53/T antigen are respectively the negative and positive controls for protein interaction.

incubating together CG1945 and Y187 transformants in a rich liquid medium (YPD) overnight at 37ºC (31).

The co-transformations and the 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 Beta-galactosidase gene expression, a filter assay of the transformant colonies was performed following the manufacturers' indications (Clontech).

4. RESULTS

4.1. Mouse dlk interacts with itself through different regions of its extracellular domain in the yeast two-hybrid system

We used different plasmids to investigate whether dlk interacts with itself in the yeast two-hybrid system. We first checked that the dlk fusion proteins we prepared could not activate the reporter genes by themselves. For this purpose, we cotransformed CG1945 with each one of dlk-fusion plasmids and the plasmid pACTGAL4$^-$. We selected the cotransformants in a solid minimal medium without histidine, leucine and tryptophan plus 10 mM 3-AT. None of the colonies that grew in the medium without 3-AT could grow when the drug was added in the same medium (data not shown). After this control experiment, we performed co transformations and mating assays of strains CG1945 and Y187 with dlk constructs in plasmids pAS2-1 and pACT2 (pASdlk, pACTdlk, pASEGFs1-6, pACTEGFs1-6, pASEGF5, pACTEGF5, pASINT and pASPRO; Figure 1). We selected the cotransformants that grew on a solid minimal medium lacking tryptophan and leucine and we made replicas of the colonies on a minimal medium lacking tryptophan, leucine and histidine plus concentrations of 3-AT ranging from 0 to 100 mM.

Figure 2 shows the growth of colonies of one of the different experiments we performed with the strain CG1945 co transformed with different pairs of plasmids. A strong interaction was observed between the fusion protein expressed by plasmid pACTdlk with the fusion proteins expressed by the plasmids pASdlk, pASEGFs1-6, and pASEGF5. The strongest interactions occurred between the entire dlk fusion proteins and between the entire dlk fusion protein and the fusion protein containing the six EGF-like repeats of dlk. Colonies from both cotransformations are able to tolerate concentrations of 3-AT up to 20 mM. Colonies containing the entire dlk fusion protein and the fusion protein containing the EGF5 repeat can only tolerate a maximum of 10 mM 3-AT. Different positive and negative interaction controls were used, as shown in figure 2. All together, these results indicate that dlk interacts with itself trough different regions of its extracellular region, which in turn suggest that dimerization of the protein, interactions between molecules on the surface of different cells, or interactions between membrane and soluble dlk variants are likely to occur *in vivo*.

The prior interactions were confirmed by performing mating assays with the strain CG1945 transformed with plasmids pACTdlk, pACTEGFs1-6 or

Table 1. Mouse dlk protein interacts with itself through different regions of the extracellular domain

Y187 Strain	CG1945 Strain			
	mdlk	mdlk EGFs1-6	mdlk EGF5	pACT2
mdlk	100/0	75/0	25/0	0/0
mdlk EGFs1-6	100/50	100/50	75/0	25/0
mdlk Intracellular	0/0	0/0	0/0	0/0
PAS2-1	0/0	0/0	0/0	0/0

Mouse dlk protein interacts with itself through different regions of the extracellular domain. The table shows the percentage of growth of CG1945/Y187 diploid transformed colonies in a minimal medium lacking leucine, tryptophan and histidine plus concentrations of 3-AT of 10/50 mM. pAS2-1: GAL4 binding domain plasmid. pACT2: GAL4 activation domain plasmid.

Table 2. Mouse dlk protein interacts with itself through specific extracellular EGF-like repeats

pAS2-1 Vector	**pACT2 Vector**						
	mdlk1/2	mdlk2/3	mdlk3/4	mdlk4/5	mdlk5/6	mdlk5	pACT2
mdlk1/2	20mM	20mM	10mM	20mM	20mM	ND	0mM
mdlk2/3	50mM	50mM	10mM	20mM	20mM	ND	0mM
mdlk3/4	50mM	20mM	50mM	10mM	10mM	ND	0mM
mdlk4/5	10mM	20mM	10mM	10mM	10mM	ND	0mM
mdlk5/6	10mM	20mM	10mM	10mM	10mM	ND	0mM
mdlk5	20mM	100mM	20mM	20mM	10mM	10mM	0mM
pAS2-1	0mM	0mM	0mM	0mM	0mM	0mM	0mM

Mouse dlk protein interacts with itself through specific extracellular EGF-like repeats. The table shows the concentration of 3-AT that PJ69-4A cotransformant colonies can tolerate in a medium lacking tryptophan, leucine and histidine. The yeast strain used in this experiment cannot tolerate 3-AT in the growth medium in the absence of protein-protein interactions. The dlk EGF-like repeats of the GAL4 binding or activation domain fusion proteins used for the cotransformation are indicated on the left and the top of the table. pAS2-1: GAL4 binding domain plasmids. pACT2: GAL4 activation domain plasmids.

pACTEGF5 and the strain Y187 transformed with plasmids pASdlk, pASEGFs1-6, pASEGF5, pASINT or pASPRO. Cotransformants that grew on a solid minimal medium lacking tryptophan and leucine were selected. As before, replicas of the colonies were made in a minimal medium lacking tryptophan, leucine and histidine plus concentrations of 3-AT ranging from 0 to 100 mM (Table 1). The results obtained were similar to those previously described. The strongest interactions occurred between two fusion proteins containing the entire dlk amino acid sequence and between the entire dlk fusion protein and the six-EGF-like fusion protein. We observed also an interaction between the entire dlk fusion protein and the fusion protein containing the dlk protease-sensitive region. This suggests that the protease-sensitive region, which possesses a leucine zipper-like structure, is one of the dlk regions involved in dlk intermolecular interactions. As expected, we did not observe any interaction between the extracellular and intracellular regions of dlk. Similarly, we did not observe interactions between two intracellular domains of dlk.

The 3-AT resistant colonies selected above became blue in a qualitative Beta-galactosidase filter assay, both in the co-transformation experiments and the mating experiments. This result represents an additional confirmation of the validity of the interactions described above (data not shown).

We decided to explore more specifically which EGF-like repeat or repeats of dlk mediate the interactions between the EGF-like regions of the dlk extracellular domain. We performed co-transformations of the strain JP69-4A with all the possible combinations of the following pAS2 and pACT2 vectors: pASdlk1/2, pACTdlk1/2, pASdlk2/3, pACTdlk2/3, pASdlk3/4, pACT3/4, pASdlk4/5, pACTdlk4/5, pASdlk5/6 and pACTdlk5/6. The co-transformants capable of growing on a solid minimal medium lacking tryptophan and leucine were selected, and replicas of the colonies both, in a minimal medium lacking leucine, tryptophan, histidine and adenine or on a minimal medium lacking tryptophan, leucine, histidine, plus concentrations of 3-AT ranging from 0 to 100 mM were made. As shown in table 2, all the co-transformants showed some interaction between the fusion proteins, but the interactions were stronger between specific dlk EGF-like repeats. As a control, we made similar experiments using a construct that expressed the 13 and 14 EGF-like repeats of Notch1 fused to GAL4. Co-transformant yeast colonies expressing this protein and any fusion protein of dlk and GAL4 were unable to grow in the presence of 3-AT (data not shown). This argues in favor of the specificity of the interactions observed between different dlk regions or EGF-like repeats. The cotransformants containing the fusion proteins showing the strongest interactions could tolerate concentrations of 3-AT up to 50 mM. As shown in table 2, some of the co-transformant colonies grew in a medium lacking histidine and adenine, nutrients finally synthesized by the enzymes expressed by two of the three reporter genes of the strain PJ69-4A.

The former results suggest that dlk interacts with itself. This interaction may lead to dimerization of molecules on the same cell, to interactions between membrane and soluble dlk forms, or to interactions between membrane dlk variants expressed by two

neighboring cells. All these interactions could be achieved in different ways, depending on what EGF-repeats are involved in each case.

5. DISCUSSION

Adipogenesis is one of the most studied differentiation processes (17, 18, 19). There are many genes whose expression is activated or inhibited during adipogenesis and that are critical for this process to occur. One of these genes is *Dlk1*. The level of expression of *Dlk1* and the rate and timing of the changes in expression of this gene are critical for preadipocyte cell lines, like 3T3-L1, to differentiate into adipocytes in response to extracelullar stimuli (6, 22).

dlk is a member of the Epidermal Growth Factor (EGF)-like homeotic family, which includes proteins like Notch receptors and their ligands (1, 2, 5). However, dlk lacks some properties that characterize the receptor or the ligand molecules of the family. All the proteins belonging to this family are also expressed either as integral transmembrane proteins or as proteins released to the extracellular medium. These proteins act as receptors or as ligands and participate in cell to cell interactions involved in cell differentiation decisions through the EGF-like repeats of their extracellular region (32, 33, 34, 35, 36). It is well known that the receptors of the EGF-like family can dimerize. The dimerization is produced through the interaction of different regions of the protein, including the EGF-repeat structures (37) It has been suggested that dimerization of these proteins can be important for its activity and it may be a way of regulating their function during cell interactions. In support for a role of dlk as an EGF-like receptor, ligand or regulatory molecule is the fact that a stoichiometric relationship between soluble and membrane dlk proteins during the triggering of the differentiation process by extracellular signals appears critical for the outcome of this process (22).

The results presented here suggest that dlk molecules expressed on the membrane of the same or neighboring cells, or membrane-associated and soluble dlk variants, may interact in different ways. This intramolecular interaction could play a role regulating the function of dlk. For example, the interaction between dlk molecules could modulate the interaction of dlk with other receptor/ligand or modulator molecules involved in the transmission and intensity of the differentiation signal.

The results from our experiments indicate that the interaction between two dlk proteins expressed on the membrane of a single or neighboring cells may involve the EGF-like repeats, and/or the leucine-zipper-like protease-sensitive region of the protein. Leucine zippers are protein-protein dimerization motifs consisting on repeat of leucine residues separated by seven amino acids. This positions the leucine at the same side of the alpha helix allowing the formation of coiled-coil dimers. The interaction between these motifs can generate both hetero and homodimers. The protease-sensitive region of dlk protein is composed of 22 amino acids and is situated at the extracellular region, next to the transmembrane domain of the protein. This sequence is rich in leucine residues possessing a structure similar to a leucine zipper domain, with a basic amino acid each four residues. The interaction observed between the entire dlk proteins, but not between the six dlk EGF-repeats, and the protease-sensitive region suggests that this interaction occurs between the protease domains of two dlk molecules. It also indicates that the interactions we observe are specific. The change of expression during adipogenesis of different dlk mRNA spliced variants lacking the region coding for the protease-sensitive sequence (7, 8) could influence the extent and even the type of interaction taking place between dlk molecules resulting in the observed increase of the membrane-associated variants known to be necessary at the beginning of the process to allow differentiation.

The interaction between two leucine zipper-like regions of dlk, and the expression of different dlk mRNA variants coding or not for this region, needs to be placed in the context of the interactions occurring between the EGF-like repeats of dlk. The data presented on table 2 allow speculating about whether or not the interaction between two dlk molecules favors a particular orientation. It is logical to think that interactions occurring between dlk molecules present on neighboring cells would more likely involve an interaction between the EGF-like repeats present at opposite ends of the dlk molecule. That is, the N-terminal EGF-like repeats of one molecule would interact with the C-terminal EGF-like repeats of the other. On the other hand, interactions between two dlk molecules occurring on the membrane of the same cell would more likely involve interaction between EGF-like repeats present at the same terminus of the molecules. The concentrations of 3-AT tolerated by the interactions supposed to take place in one or other situation (indicated in bold or bold cursive on table 2) do not allow to conclude that one type of interaction is favored respect the other. The participation of the leucine-zipper like region, however, could favor the interaction of dlk molecules present on the same cell and inhibit the interaction between dlk molecules of neighboring cells. In this regard, it is important to point out that during the first twenty-four hours of the differentiation process, dlk mRNA coding for protein variants lacking the protease sensitive region increase dramatically. This would result in an increase of membrane vs. released dlk and might result also in a change in the type of dlk interaction, favoring the interaction between molecules present on different cells. This interaction could be of importance to transmit a differentiation permissive state signal from cell to cell.

Further experiments are necessary to explore the interactions between the different variants of dlk that could be important for its function as a molecule modulating signals leading ort not to differentiation.

6. ACKNOWLEDGMENTS

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