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Expression of delta-lactoferrin induces cell cycle arrest

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Abstract

Delta-lactoferrin (ΔLf) mRNA is the product of alternative splicing of the Lf gene. It has been found in normal tissues and was reported to be absent from their malignant counterparts. Our recent investigations have shown that ΔLf expression is a good prognostic indicator in human breast cancer. However, ΔLf has up till now only been identified as a transcript, and in order to characterize the ΔLf protein and determine its function we have used a ΔLf cDNA construct to produce the protein *in vitro* and *in vivo*.

A 73 kDa protein was immunoprecipitated from *in vitro* translation products and this molecular weight is in accordance with the use of the first in frame AUG start codon located in exon 2. We also produced a cell line expressing ΔLf under doxycycline induction. Using this model we have been able to show that ΔLf is mainly distributed in the cytoplasm. Its expression induces cell cycle arrest and inhibits cell proliferation. Our results suggest that ΔLf may play an important role in the regulation of normal cell growth.

Introduction

The Lf gene was mapped to human chromosome 3 (Mc Combs *et al.* 1988) and two transcripts are produced, Lf and delta-Lf (ΔLf) mRNAs which are products of alternative splicing of the Lf gene determined by the use of alternative promoters P1 and P2 (Siebert & Huang 1997, Liu *et al.* 2003). Transcription from promoter P1 leads to the Lf messenger and from the P2 promoter, present in the first intron of the Lf gene, to ΔLf. These promoters are used differentially and P2 is very active in some lymphoid cell lines and strongly upregulated by the transcription factor Ets (Liu *et al.* 2003).

Alternative splicing concerns exon 1 which characterizes the Lf mRNA and is replaced by exon 1β in the ΔLf messenger. Exon 1β contains a start codon, but this is immediately followed by a stop codon, suggesting that translation occurs in exon 2. Apart from the 5′end, the ΔLf messenger is identical to the Lf mRNA and translation from exon 2 would lead to a protein devoid of a leader sequence and the first 25 amino acids. Thus, the intracellular distribution of the two Lf isoforms would be different, leading to a secreted Lf (Lf) and a cytosolic ΔLf. Since both Lf forms contain consensus nuclear targeting signals, cytoplasmic ΔLF may locate to the nucleus (Liu *et al.* 2003). The presence of four putative *O*-*N*-acetylglucosaminylation sites might suggest a control of the nuclear traffic *via* the balance between *O*-*N*-acetylglucosaminylation and phosphorylation (review: Hart *et al.* 1996). Moreover, specific interactions between Lf and DNA have also been described suggesting that it may function as a transcription factor (He & Furmanski 1995).

The only available data on the role of ΔLf are from Siebert & Huang (1997) who detected ΔLf mRNA at various levels in all human tissues and did not find it in any of the tumor-derived cell lines they checked. Most of the data available in the literature refer to Lf and do not discriminate between the two Lf isoforms. We therefore investigated the level of ΔLf expression in parallel to those of Lf in human breast cancer cell lines and biopsies (Benaïssa *et al.* 2004). We found that ΔLf was highly expressed by normal human mammary gland epithelial cells compared to breast cancer cell lines. We also investigated ΔLf mRNA expression levels in 99 biopsies of human primary breast cancer. ΔLf transcripts were found in all of the tumor biopsies. Statistical evaluation showed that ΔLf expression was related to histoprognostic grading and in Cox univariate analyses, ΔLf was a prognosis parameter, high concentrations being associated with a longer overall survival (Benaïssa *et al.* 2004). These findings suggest that this isoform may play an important role in the regulation of normal cell growth and demonstrated the need for a better characterization of the protein and its role.

Materials and methods

Plasmid construction of the pBS-ΔLf

Construction of pBS-ΔLf consisted in an exchange between exon 1 and exon 1β in the pBS-Lf which was a kind gift of Dr D. Legrand (UGSF, UMR 8576 CNRS-UST Lille1, Villeneuve d'Ascq cedex, F). Exon 1β was generated by PCR using placenta total RNA and appropriate primer pairs (S:5′-CCCTCGAGCGCCTACTGAGGTCTGAAGTTT-3′; AS:5′-GACACAGTCCCAACTTCTGTCA-3′). The construction was verified by sequencing.

RNA preparation

Placenta total RNA was prepared as described (Benaïssa *et al.* 2004). ΔLf and Lf messengers were produced from the *Xba* I linearized pBS-ΔLf and pBS-Lf, respectively. Both capped messengers were synthesized from the T7 promoter using the mMESSAGE mMACHINE Kit (Ambion).

In vitro *translation of ΔLf mRNA*

In vitro translation using rabbit reticulocyte lysate (Promega) has been performed with 2 μg of Lf and ΔLf capped mRNAs in the presence of [^{35}S]-methionine (Amersham). Luciferase was included as a control. Labeled cell free translation products were then resolved classically by SDS-PAGE electrophoresis and autoradiography.

Translation of ΔLf mRNA in Xenopus laevis *oocytes*

Oocytes (Dumont, 1972) were microinjected with 60 nl of ΔLf messengers and labeled with [^{35}S]-methionine (300 μCi/ml; Amersham) as described (Pierce *et al.* 1985). Controls referred to oocytes injected with saline. Homogenization, TCA precipitation and counting as well as immunoprecipitation of lysates were performed as described (Pierce *et al.* 1985) using the M90 rabbit polyclonal antibodies against lactoferrin (1/1000). The half life of ΔLf was studied using a pulse chase technique. mRNA stability was investigated by RT-PCR (Benaïssa *et al.* 2004).

Establishment of a stable inducible cell lines for ΔLf

This was realized using the tetracyclin inducible Tet-on system (Clontech). HEK 293 cells which was already stably expressing the transactivator were a kind gift from Dr J.-C. Dhalluin (INSERM U 524, Lille, F). These cells were further stably transfected with the pRev responder construct containing ΔLf cDNA. Isolated clones were expanded and induced with doxycyclin (2 μg/ml). The presence of ΔLf messengers was detected by PCR (Benaïssa *et al.* 2004). Subcellular localization of ΔLf was observed by confocal microscopy. Cells were grown on cover glasses, induced or not with doxycyclin for 24 h before fixation. Immunostaining was realized as described (Fillebeen *et al.* 2001). Visualization was done using a Zeiss confocal microscope equipped with an argon-krypton laser using a 40× objective lens. [^{3}H]-thymidine uptake and cell cycle analyses were performed as previously described (Damiens *et al.* 1999).

Results and discussion

Characterization of ΔLf

Figure 1A shows the *in vitro* synthesis of both the Lf isoforms in reticulocyte lysates. Whilst the same amount of messengers was used, translation efficiency was not equal for the two Lf mRNA isoforms and in consequence the amount of protein loaded on the

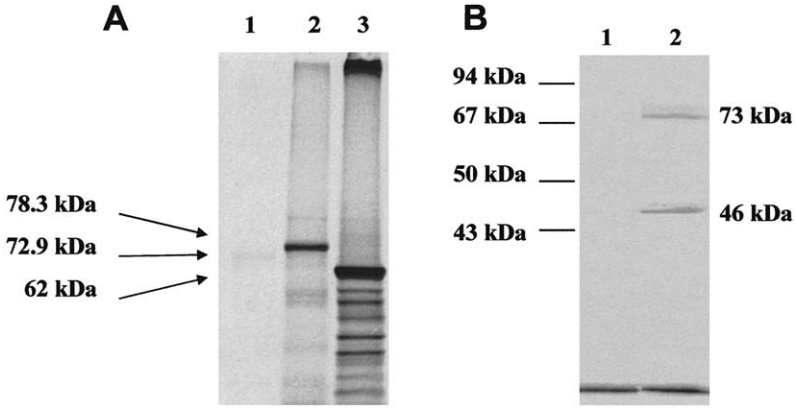

Fig. 1. Translation products of the ΔLf messenger resolved by SDS-PAGE electrophoresis. (A) cell free system: 1 ΔLf mRNA (2 μg); 2 Lf mRNA (2 μg) ; 3 luciferase (1 μg). (B) *X. laevis* oocytes injected with 3 μg/μl of ΔLf mRNA (2) versus control (1). Immunoprecipitation with M90 anti-Lf antibodies (1/1000) corresponds to a 20 oocyte lysate.

SDS-PAGE was different suggesting that ΔLf was extremely poorly expressed compared to Lf. The electrophoresis profile showed also that their molecular weights differ. Unglycosylated Lf has a calculated molecular weight of 78.3 kDa and luciferase, used as a control, 62 kDa. ΔLf is visible with a molecular weight which corresponds to that calculated of 72.9 kDa, based on the use of the first in-frame AUG codon in exon 2. Figure 1B shows the results of immunoprecipitation with anti-Lf antibodies both on a control *Xenopus* oocyte lysate and on a lysate corresponding to 20 oocytes injected with ΔLf at a concentration of 3 μg/μl or with saline. ΔLf again appears as a faint band at 73 kDa. There is also a band at 46 kDa which may correspond to a degradation fragment. Thus both translation systems showed a 73 kDa ΔLf, compatible with a translation initiation site in exon 2. Both experiments also showed a poor level of ΔLf synthesis which may be due to the difficulties encounted by ribosomes in finding the initiation site. On the other hand, ΔLf might have a short half-life or its messenger may be very suceptible to degradation.

RNA and protein stability

The translation of ΔLf mRNA in *X. laevis* oocytes was followed during 24 hours. Figure 2A shows that 24 h after injection, 60% of the injected ΔLf mRNA is still present as measured by RT-PCR. Since ΔLf messengers were not totally degraded during experiment we measured the half life of the ΔLf protein (Figure 2B). After a 2 h incubation with radiolabeled methionine, a pulse chase experiment with an excess of cold methionine was conducted. In this experiment

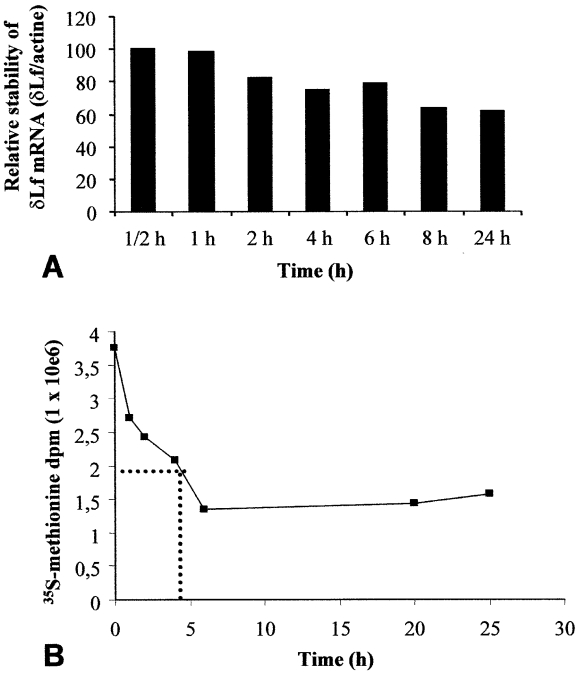

Fig. 2. ΔLf mRNA stability and half life of the ΔLf protein in *X. laevis* oocytes. (A) ΔLf mRNA stability was followed during 24 h using RT-PCR. (B) After a 2 h incubation with [^{35}S]-methionine, pulse chase was performed with 5 mM methionine for 24 h.

each point corresponds to 20 oocytes, and we determined an approximate half-life of about 4 h. Thus, it is more likely that the weak ΔLf expression is due to an unusual ribosome scanning than to a rapid protein or mRNA degradation. Nevertheless, if the N-terminal methionine is removed, destabilizing amino acids such as arginine and lysine might be uncovered, which

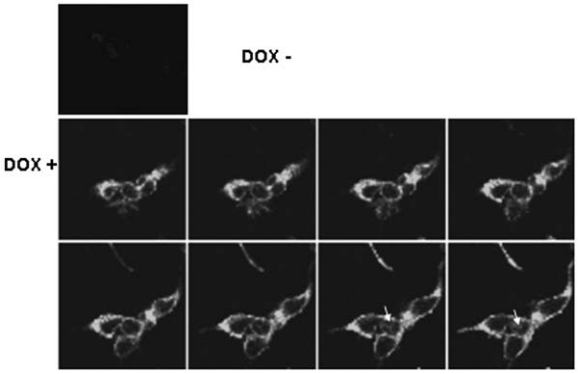

Fig. 3. Subcellular localization of ΔLf visualized by immunostaining using a confocal microscope. HEK 293 Δ18 cells are induced (Dox +) or non induced (Dox −). Fixed cells were stained with anti-Lf (FITC). Original magnification : X 40. Arrows : nuclear staining

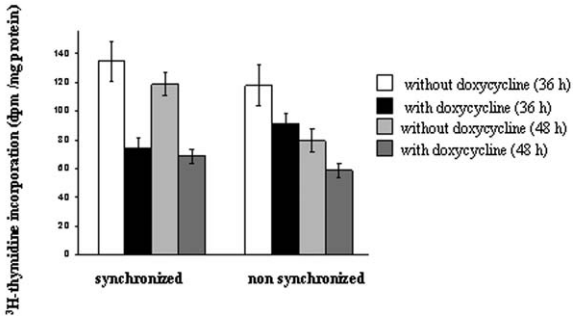

Fig. 4. Effect of ΔLf on [^{3}H]-thymidine uptake by HEK 293 Δ18 cells. Cells were induced or not by doxycyclin for 36 and 48 h. Prior to incubation with [^{3}H]-thymidine for 4 h, cells were synchronized by serum privation (2.5% FCS) for 24 h or not (10% FCS). After incubation the cells were harvested, TCA precipitated, solubilized in 500 μl 0.5 M NaOH. Protein content was assayed on one twenty fifth of the material, the rest was counted. Data are expressed as a ratio of dpm per mg of protein. Each point represents the mean of three experiments.

according to Varshavsky's N-end rules (Varshavsky 1996) would lead to a rapid uptake of the protein by the ubiquitin system.

ΔLf subcellular distribution

In order to evaluate the impact of ΔLf overexpression on cell proliferation we developped an inducible established stable cell line. HEK 293 cells, which do not synthesize Lf transcripts, were stably transfected for ΔLf. Expression was obtained in most of the clones tested but background levels were sometimes substantial and clone 18 was chosen for its zero background expression. This clone was periodically checked for leakiness. ΔLf cellular distribution was further observed by confocal microscopy (Figure 3). HEK293 Δ18 cells were grown in the presence or absence of doxycyclin. Control cells expressed no ΔLf but ΔLf was detected mainly in the cytoplasm of induced HEK 293 Δ18 cells as previuosly observed (Liu *et al.* 2003). However, a sight nuclear labeling was also visible (Figure 3).

Table 1. Cell cycle analysis by FACSscan cytometer using the CellFIT software of propidium iodine stained HEK 293 Δ18 cells.

	G0G1*	S*	G2M*
without doxycyclin	44	40	16
with doxycyclin (15 h)	32	65	2
with doxycyclin (24 h)	29	67	4
with doxycyclin (48 h)	36	56	8

*Results are expressed as a percentage of cells in each cell cycle phase with 100% corresponding to the total cell number.

ΔLf overexpression leads to an antiproliferative effect

Using this cellular model we next investigated ΔLf function. In order to verify that doxycyclin was not responsible for the reported activities, all the following experiments were also conducted in parallel on control HEK cells, negative for the production of ΔLf. Results are not shown but no effect of doxycyclin induction was detectable. ΔLf expression reduced cell proliferation as measured by [^{3}H]-thymidine uptake and this effect was most marked between 36 to 48 h after induction (Figure 4). Compared to controls when the cells were synchronized by growing in 2.5% fetal calf serum, thymidine uptake was decreased by about 50% in ΔLf producing cells.

ΔLf overexpression leads to an arrest of cell cycle progression

The effects of ΔLf expression on cell cycle progression were next studied. Table 1 summarizes the data from 3 different experiments. Induced HEK 293 Δ18 cells were analysed at various times after induction and compared to non-induced cells. Expression of ΔLf resulted in an accumulation of cells in S phase, consistent with an S phase arrest detectable as early as 15 h. After 48 h we observed a resumption of cell cycle progression consistent with a diminution of doxycyclin concentration in the medium since the half-life of doxycyclin is around 2 days. These results confirm that ΔLf modulates cell proliferation of HEK 293

Δ18 cells by an arrest at the S phase. However, more work has to be done in order to characterize the mechanism by which ΔLf modulates the expression and the activity of key S phase regulatory proteins.

Conclusion

In conclusion we have shown that ΔLf is translated from the first in frame start codon present in exon 2. Its subcellular location is mainly cytoplasmic, but cytoplasmic ΔLf could enter the nucleus, and carry out some of the cellular functions that were previously reported for the secreted form of Lf. Its expression is a good prognosis indicator in human breast cancer and in keeping with this observation, we showed that ΔLf has an antiproliferative activity and blocks the progression of the cell cycle in S phase.

Acknowledgements

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