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Mouse polyomavirus large T antigen inhibits cell growth and alters cell and colony morphology in *Saccharomyces cerevisiae*

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Abstract The gene for mouse polyomavirus large tumor (LT) antigen, a potent oncoprotein, was expressed in *Saccharomyces cerevisiae* from the inducible *GAL1* promoter. Substantial cell growth inhibition as well as colony and cell morphology changes dependent on cyclic adenosine monophosphate (cAMP) were observed. In contrast to cell and colony morphology alterations, the growth inhibition appeared to be transient, thus indicating the existence of an active adaptation of yeast cells to the LT antigen presence.

Key words: Polyomavirus large tumor antigen; Expression in *Saccharomyces cerevisiae*; Growth inhibition; Cyclic adenosine monophosphate

1. Introduction

Budding yeast *Saccharomyces cerevisiae* with the fully sequenced genome, extensive molecular biology tools and a series of well-defined mutants available from EUROSCARF (http://www.uni-frankfurt.de/fb15/mikro/euroscarf) proved to be a good and relatively simple model system for an investigation of functions of viral proteins of higher eukaryotes.

Because of the evolutionary conservation of many cellular mechanisms (e.g. gene expression and its regulation or cell cycle regulation), yeast cells might provide an opportunity to identify interactions of viral proteins with cellular structures and connect them with molecular processes and their control machinery [1–6]. Thus, evidence has been obtained that several targets of the adenovirus E1A regulatory protein are conserved between mammalian and budding yeast cells: the expression of the adenovirus E1A protein in yeast cells led to the finding that E1A specifically blocks SWI/SNF-dependent transcriptional activation [7]. Miller et al. discovered that the inhibition effect of E1A on yeast cell growth is dependent on the cyclic adenosine monophosphate (cAMP) signalling pathway [8]. This appeared to be in parallel with the E1A function in gene activation/repression in mammalian cells as stimulation of cAMP-dependent protein kinase is absolutely required for activation of the mouse c-fos and jun B genes by the E1A protein [9].

Another viral regulatory protein – oncoprotein Tax of human T-lymphotropic virus type 1 (HTLV-1) has two major functions during the HTLV-1 life cycle: it activates viral transcription and deregulates the cellular mechanisms critical for cell growth and division. Its expression in *S. cerevisiae* resulted in a G2/M slow down or arrest and a loss of cell viability. The results support the notion that Tax aberrantly targets and activates APC^{Cdc20p}, leading to unscheduled degradation of Pds1p/securin and Clb2p/cyclin B1 connected with severe chromosome aneuploidy in yeast or human cells. These results provided a molecular explanation for the frequent chromosomal abnormalities in HTLV-1 infected T cells and the highly aneuploid nature of adult T-cell leukemia cells [10].

Our studies have been focused on a multifunctional regulatory protein, large tumor (LT) antigen of mouse polyomavirus (PyV), a member of the *Polyomaviridae* family. The genome of this small non-enveloped DNA tumor virus encodes only six gene products. Three non-structural proteins (LT, middle tumor (MT) and small tumor (ST) antigens) were shown to play roles in cell immortalization and transformation processes. LT antigen is an 88.1 kDa oncoprotein that interacts with multiple host factors (at least two subunits of DNA polymerase α primase, single-stranded DNA binding protein, replication protein A, DNA topoisomerase I) to coordinate the expression of viral genes and initiate replication of the viral genome [11–15]. Besides affecting viral transcription and DNA replication, it influences the cell cycle, especially the entry into the mitotic cycle. In addition to other cellular factors (CBP/p300 transcriptional co-activators [16,17], hsc70 [18]), LT antigen interacts with one of the key cell cycle regulators, tumor suppressor protein ($p105^{Rb}$) as well as with related proteins p107 and p130 [19–22]. Interaction of LT antigen with the pRb protein family causes E2F transcription factor release from the pRb–E2F complex and leads to cell cycle progression from G0 or G1 into S phase [23–25].

In contrast to PyV LT antigen, the LT antigen of a related simian virus, SV40, interacts with another tumor suppressor protein, p53 [26–31]. For SV40, studies using tsA mutants demonstrated that LT antigen was required for both the initiation and maintenance of the transformed state [32,33]. PyV LT antigen can immortalize cells; however, the major transforming protein is the membrane bound MT antigen (absent in polyomaviruses of primates), which possesses the ability to transform cells through its interactions with c-Src tyrosine kinase and other proteins of the signal transduction pathway [34]. Expression of SV40 LT in yeast cells led to the findings that SV40 LT antigen interacts with an important cell cycle regulator, $p34^{CDC28}$, highly conserved protein kinase that controls, together with the cyclins, different steps of progression

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of eukaryotic cells through the cell cycle (for review see [35]). Fewer studies have been performed to understand immortalization by PyV LT antigen, which does not bind p53. An essential role in immortalization for the $p105^{Rb}$ binding activity of polyoma LT has been demonstrated. However, it is likely that additional activities are also required. The *S. cerevisiae* model system could help us to elucidate the pleiotropic effect of PyV LT antigen, to detect other protein–protein interactions and to determine their functional consequences.

2. Materials and methods

2.1. Media and strains

S. cerevisiae strains BY4742 (*MATα*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*) from EUROSCARF, BY4741-TUB3-GFP (*MATa*; *TUB3-GFP*, *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) kindly provided by L. Synek (Charles University, Prague, Czech Republic), LRA85 (*MATα*; *cdc35-11*; *leu2Δ0*; *ura3Δ52*; *his4Δ0*) and JC482 (*MATα*; *leu2Δ0*; *ura3Δ52*; *his4Δ0*) kindly provided by K. Tatchell (Louisiana State University, USA) were used. *Escherichia coli* XL1-blue strain: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′proAB lacI q ZDM15 Tn10 (Tet^r)] (Stratagene) was used for propagation of plasmids.

Yeast cells were grown in liquid minimal medium (MM) (0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.5% $(NH_4)_2SO_4$, 0.1% Wickerham's solution [36] with appropriate supplements (50 mg/l amino acids and 40 mg/l bases) and either 2% glucose (MMGlc) or 2% galactose (MMGal). Alternatively, solid media MAGlc (MMGlc, 2% agar) and MAGal (MMGal, 2% agar) were used.

2.2. Preparation of the recombinant plasmid

The recombinant p425GAL1-LT plasmid was constructed by ligation of the *Bam*HI–*Bam*HI fragment containing the intronless LT gene cut from p423GAL1-LT and yeast shuttle expression vector p425GAL1 [37] cut with *Bam*HI. The recombinant p423GAL1-LT plasmid was constructed by ligation of the *Eco*RI–*Eco*RI and *Eco*RI–*Sal*I fragments containing the intronless LT gene cut from pGBT9LT [38] together with yeast shuttle expression vector p423GAL1 [37] cut with *Eco*RI and *Sal*I. DNA recombination techniques and agarose gel electrophoresis were performed according to [39] and *E. coli* electroporation as described in [40].

2.3. Yeast cell transformation

Transformation of yeast cells was performed using the lithium acetate method according to [41].

2.4. Preparation of yeast cell lysates

The MMGalHisUraLys (500 ml) was inoculated at starting concentration 2×10^7 cells/ml and cell suspension was cultivated for 48 h. Then, the cell culture was centrifuged, the cell sediment transferred into a stirring dish and the cells disrupted by stirring under liquid nitrogen. The RIPA buffer (150 mM NaCl, 5 mM ethylenediamine tetraacetic acid (EDTA) pH 8.0, 50 mM Tris–HCl pH 7.4, 0.05% NP-40, 1% deoxycholic acid, 1% Triton X-100) with 0.01% sodium dodecyl sulfate (SDS) was added (approximately 0.5 ml) and the mixture was stirred again into a snow-like consistency. After subsequent thawing, the lysate was transferred into a tube and cell debris was removed by centrifugation.

2.5. Western blot analysis

Proteins resolved on Laemmli SDS–polyacrylamide gel electrophoresis (PAGE) were electrotransferred onto a nitrocellulose filter using blotting buffer (1.44% glycine, 0.3% Tris, 20% methanol) with 0.1% SDS according to [39]. For detection of LT antigen, a mix of C1 and C4 (1:1) rat monoclonal antibodies against the common region of PyV T antigens was applied as primary antibody and anti-rat IgG antibody conjugated with peroxidase as secondary antibody. Anti-LT antibodies were kindly provided by Prof. Beverly Griffin (St. Mary's Hospital, London, UK).

2.6. Indirect immunofluorescence and 4,6-diamidino-2-phenyl-indole (DAPI) staining of yeast cells

Immunodetection was performed as described in [42]. Briefly, *S. cerevisiae* BY4742/p425GAL1-LT-induced cell suspension was incubated for 5 min with 30 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (pH 7.0) and pepstatin (20 μg/ml) at 28°C. Yeast cells were fixed with paraformaldehyde and spheroplasts were prepared by digestion with zymolyase (200 μg/ml) in KPC (33 mM citric acid, 0.13 M K_2HPO_4, pH 5.9) and pepstatin (20 μg/ml) for 60 min. Cells were washed twice in PEMI (0.1 M PIPES, 5 mM EGTA, 5 mM $MgCl_2$, pH 6.9 and pepstatin (10 μg/ml)). Cell membranes were permeabilized with 1% Triton X-100 in PEMI. After washing in PEMI and pre-incubation in 1% bovine serum albumin in PEMI for 10 min, a mix of C1 and C4 (1:1) rat monoclonal antibodies against the common region of PyV T antigens (in 1% bovine serum albumin (BSA) in PEMI) was applied as primary antibody for 1 h at room temperature. Cells were washed twice in PEMI and the bound antibodies were detected by 45-min incubation with goat anti-rat IgG conjugated with Alexa Fluor 488 (green, Molecular Probes). After the final wash in PEMI, cells were resuspended in mounting medium (0.1% (w/v) *p*-phenylenediamine in 100 mM Tris–HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5 and 0.5 ug/ml of DAPI freshly prepared).

2.7. Detection of LT antigen production in yeast cells within a colony

A colony was resuspended in distilled water, plated on MAGal and incubated for 11 days for monocolony development. A replica nitrocellulose filter was prepared and the immunodetection of LT antigen (using antibodies described in Section 2.5) was performed as described [43].

3. Results and discussion

3.1. Production of PyV LT antigen in yeast cells

PyV LT gene was cloned under the yeast inducible *GAL1* promoter in the high-copy 2μ-based plasmid p425GAL1, as described in Section 2.2. *S. cerevisiae* strain BY4742 was transformed by recombinant p425GAL1-LT (p425GLT) or by the 'empty' p425GAL1 (p425G) plasmid. Transformants were selected on MAGlcHisUraLys. The first colonies formed by BY4742/p425GLT (BY/p425GLT) or by BY4742/p425G (BY/p425G) appeared approximately 2–3 days after the transformation. The LT antigen gene expression from the *GAL1* promoter was induced by the transfer of yeast cells from glucose- to galactose-containing MMHisUraLys as described in

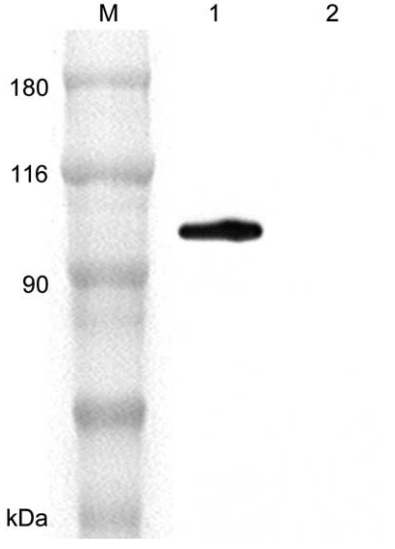

Fig. 1. Western blot of PyV LT antigen produced in *S. cerevisiae* visualized by a mix of C1 and C4 (1:1) anti-LT antibodies and secondary anti-rat antibody conjugated with peroxidase. Molecular weight marker (lane M), lysate of BY/p425GLT (lane 1), lysate of BY/p425G (lane 2).

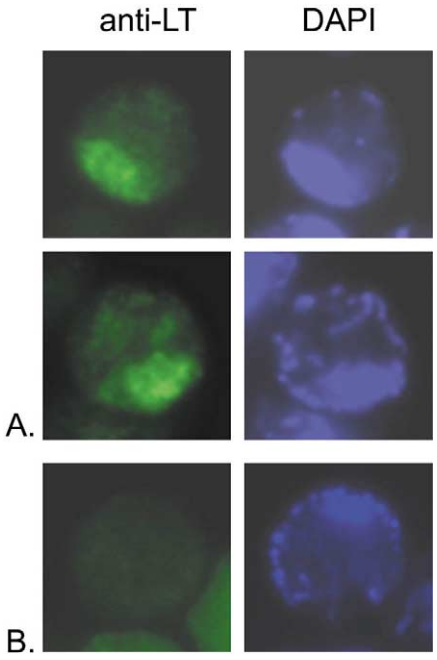

Fig. 2. Indirect immunofluorescence of yeast cells expressing LT antigen (BY4742/p425GLT) (A) and control (BY4742/p425G) (B). LT was visualized with anti-LT antibody and anti-rat antibody conjugated with Alexa Fluor 488 (green). Whole cell DNA was stained by DAPI (blue).

Section 2.4. The amount of 3×10^9 cells was harvested and the presence of LT antigen confirmed by Western blot using a mix of rat monoclonal anti-PyV LT antibodies, as described in Section 2.5 (Fig. 1).

3.2. Subcellular localization of PyV LT antigen using indirect immunofluorescence in yeast

For the indirect immunofluorescence procedure, the cells were harvested 8 h post-induction. LT antigen was visualized with anti-LT antibody (green), whole cell DNA was stained by DAPI (blue). Fig. 2 clearly shows nuclear localization of LT antigen within yeast cells and its accumulation in, yet non-identified, subnuclear foci. Interestingly, similar non-random LT antigen distribution was also observed in mammalian cells during the late phase of PyV infection (Plevka et al., our unpublished results).

3.3. Growth inhibition of yeast cells expressing the PyV LT antigen

On the galactose agar medium, the colonies of the strain expressing LT antigen (BY/p425GLT) appeared with a delay of approximately 6 days in comparison with the colonies of the strain containing the control BY/p425G plasmid (without the LT gene). Both strains grew with similar efficiency on the glucose-containing agar medium (Fig. 3). The inhibition was also observed when LT-expressing cells were grown in liquid culture (data not shown). This observation implicates that the expression of LT antigen inhibits growth of the yeast cells.

However, more detailed analyses surprisingly revealed that this growth inhibition had only a temporary character: The 'distinction' (presumably caused by the LT growth inhibition) between colonies formed by BY/p425GLT and those formed by BY/p425G gradually diminished. After a prolonged time of incubation (approximately 16 days), the BY/p425GLT colonies reached the size of colonies formed by BY/p425G (Fig. 4A). About 98–100% of monocolonies formed by cells taken from a 16-day-old BY/p425GLT colony still produced LT antigen, thus indicating that the escape from the growth arrest was not due to the loss of LT antigen (Fig. 5). This implies that BY/p425GLT cells are able to overcome the inhibition effect of LT antigen. Despite the fact that the mechanism is unknown, there are basically two possible explanations: either the decrease of the plasmid copy number inside the cells causes the decrease of LT antigen below the inhibitory level, or the yeast cells are somehow able to adapt and to neutralize a toxic effect of LT antigen. Minimal appearance of cells with no detectable LT antigen (0–2% of cells per colony) makes the adaptation hypothesis more probable.

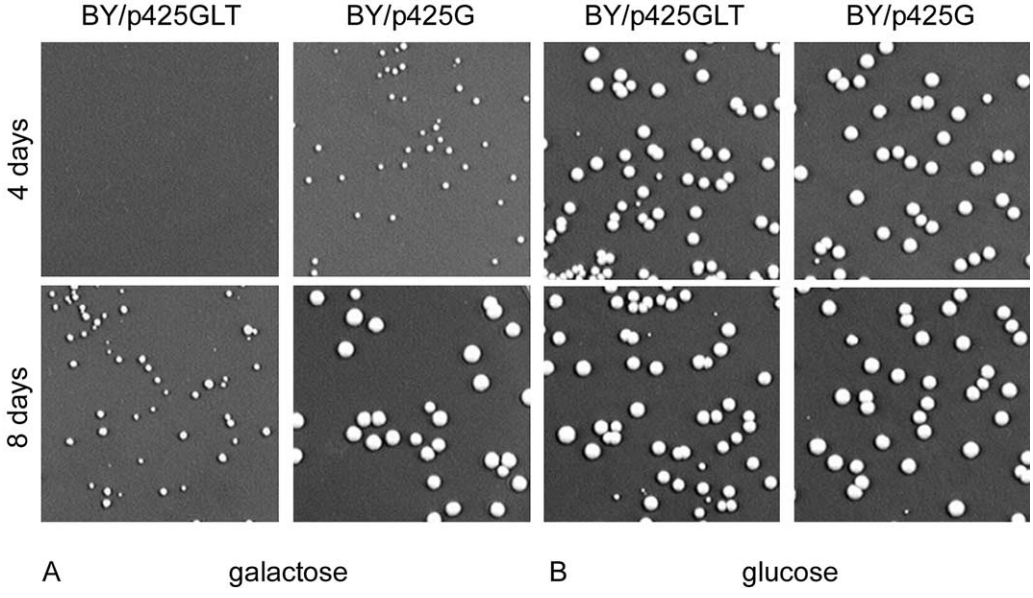

Fig. 3. Expression of the LT protein inhibits growth of yeast cells. Cells were plated on the agar medium with the same efficiency. BY/p425GLT and BY/p425G on induction medium (MAGal) (A) and on non-induction medium (MAGlc) (B).

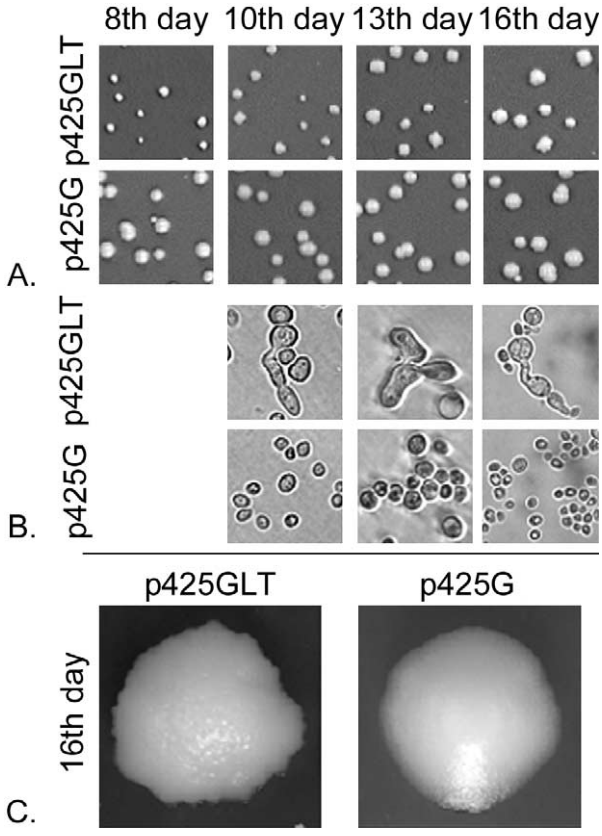

Fig. 4. Transient growth inhibition of yeasts expressing LT antigen (BY/p425GLT). The LT antigen inhibition of yeast cells is only transient (A). There is altered morphology of yeast cells during the entire examined time period (B), LT antigen producing monocolonies grown up to the size of the control ones have different morphology (C).

The intensity of LT signals of individual colonies in Fig. 5B does not reflect the amount of the LT protein, due to unequal replica plating and autolysis efficiency of colonies of different sizes.

Similarly, effective adaptation of yeast cells to the inhibitory effect of the VP1 protein of PyV was observed [44]. However, in comparison to VP1 (subcloned into the same plasmid as LT antigen), LT antigen exhibits more efficient inhibition of yeast cells at an early time of colony development (data not shown). This could be caused by the fact that, in contrast to VP1, which is the major structural protein of PyV capsid, the LT antigen is a regulatory protein, which can affect various cellular processes of higher eukaryotes, including cell cycle regulation. Experiments are underway to identify LT interaction(s) responsible for the observed inhibition.

3.4. *Expression of mouse PyV LT antigen causes morphological changes in* S. cerevisiae *cells*

The production of LT antigen causes alterations of the morphology of monocolonies formed by BY/p425GLT. These monocolonies are heterogeneous in size and 'shrunken' in comparison with 'smooth' monocolonies formed by BY/p425G cells (Fig. 6). The 'shrunken' monocolony morphology is not observed when cells grow on non-induction glucose medium (Fig. 6). The effect of LT antigen on the morphology of 'giant' colonies (i.e. colonies arising from a cell suspension spotted on agar [45]) is also visible. 'Giant' colonies formed by cells producing LT antigen have a dentate rim and are more flat in comparison with the BY/p425G 'giant' colonies (data not shown).

The cells of monocolonies growing on induction medium MAGalHisUraLys as well as cells from liquid MMGalHisUraLys were observed under a light microscope. The distinct morphology changes of the subpopulation of BY/p425GLT cells were observed in comparison to BY/p425G cells, thus indicating the effect of LT antigen on the cell morphology and cell cycle (Fig. 7). These 'monstrous' cells also exhibited modifications in microtubule arrangement as indicated in Fig. 8.

In contrast to the gradual escape of older colonies from the inhibition effect of LT antigen (see above), the colony morphology of BY/p425GLT differed from that of BY/p425G during the entire estimated period (Fig. 4C). Also, the morphologically changed cells were present in colonies of different age (Fig. 4B). These observations suggest that despite possible BY/p425GLT colony growth adaptation, the LT antigen present within most of the cells might affect the cell division, possibly influencing also their budding pattern and cell separation.

A very similar effect on cell morphology was observed when the LT antigen of SV40 was expressed in *S. cerevisiae* [46].

An SV40 LT antigen deletion mutant, lacking 150 amino acids at the carboxy-end, did not cause the aberrant cell morphology. This indicates that the carboxy-end region of SV40 LT antigen, referred to as the host range/helper function domain, was responsible for yeast cell cycle disruption [47].

However, SV40 LT antigen contains approximately 70 amino acids at its carboxy-end that have no homology with any sequences in PyV LT antigen.

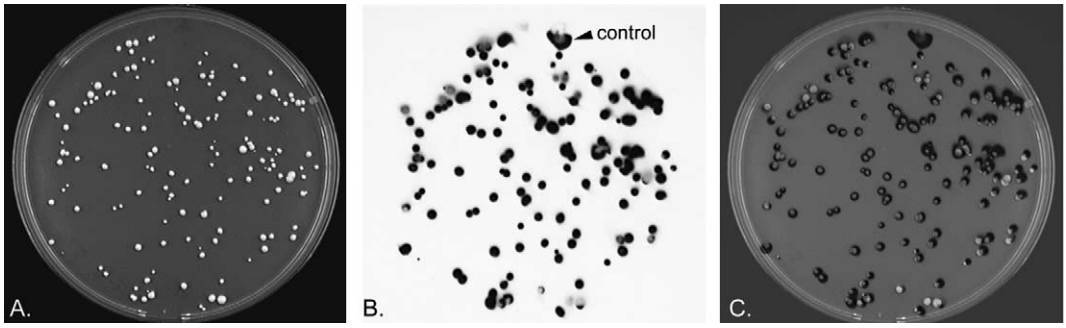

Fig. 5. Monocolonies formed by cells taken from a 16-day-old BY/p425GLT colony still produce LT antigen. 11-day colonies grown on MAGalHisUraLys (A) transferred by replica plating on nitrocellulose filters, lysed and immunostained with LT specific antibody (B). A and B merge (C).

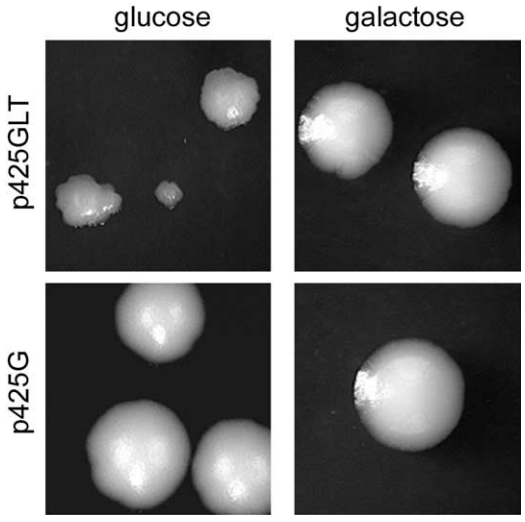

Fig. 6. Expression of the LT antigen alters the morphology of BY4742 monocolonies. Induction medium (galactose) and non-induction medium (glucose). 8th day.

3.5. *Yeast growth inhibition by PyV LT antigen depends on the level of intracellular cAMP*

The p425GLT plasmid was introduced by a standard procedure (with omitted heat shock step) into the cdc35-11 (adenylate cyclase) thermosensitive LRA85 mutant strain. Transformants carrying the plasmid were selected on MAGlc-HisUra at a permissive temperature, 24°C. Selected transformants were plated on MAGalHisUra agar and parallel plates were incubated at three different temperatures: (i) permissive temperature, 24°C, (ii) semi-permissive temperature, 28°C and (iii) non-permissive temperature, 37°C. After 6 days, substantial inhibition of cell growth was evident at the permissive temperature. On the contrary, at semi-permissive temperature of 28°C, where the level of intracellular cAMP was decreased, the cells carrying the p425GLT plasmid and producing LT antigen grew faster than p425GLT cells growing at the permissive temperature (Fig. 9A). Also, the morphology of colonies growing at the semi-permissive temperature (Fig. 9B) is less 'shrunken' than the morphology of colonies at the permissive temperature. On the contrary, the

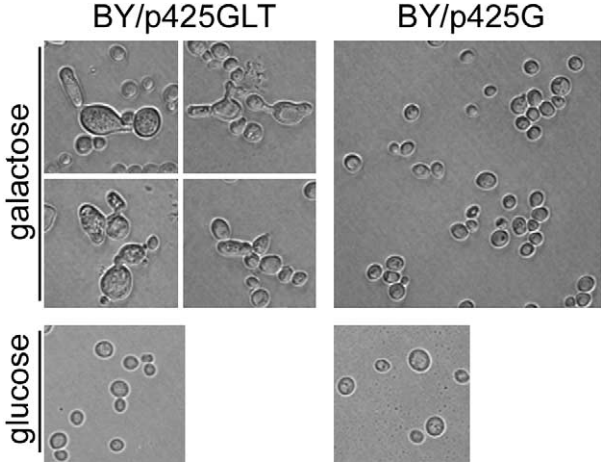

Fig. 7. Expression of the LT antigen alters the morphology of yeast cells. Cultivation of BY/p425GLT and BY/p425G in liquid induction MMGalHisUraLys.

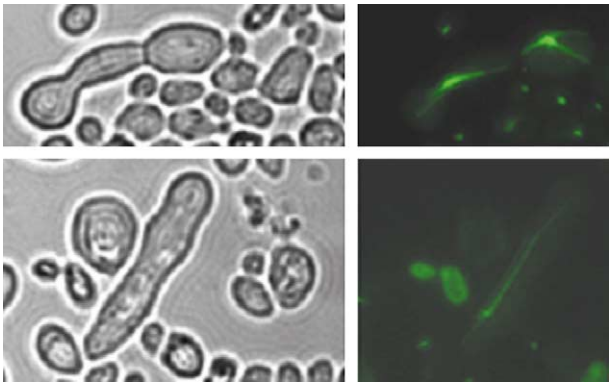

Fig. 8. 'Monstrous' cells producing LT antigen with the Tub3p-GFP marker (BYGFP/p425GLT) exhibit modification in microtubules. Light (left) and fluorescence (right) microscopy.

growth inhibition and shrunken colony morphology of the isogenic parental strain, JC482, containing the p425GLT plasmid was the same at 24 and 28°C (data not shown). These observations imply that the inhibition effect of LT antigen is dependent on the intracellular cAMP level. The level of intracellular cAMP can be influenced by at least three different signalling pathways, i.e. RAS, GPA2, and MAP kinases. Very similar results of cAMP dependence were observed when the adenoviral E1A protein was expressed in *S. cerevisiae* cdc35 (adenylate cyclase) and cdc25 (RAS guanyl-nucleotide exchange factor) thermosensitive mutants [8]. Further studies using defined mutants in these three aforementioned pathways should determine the signalling pathway involved.

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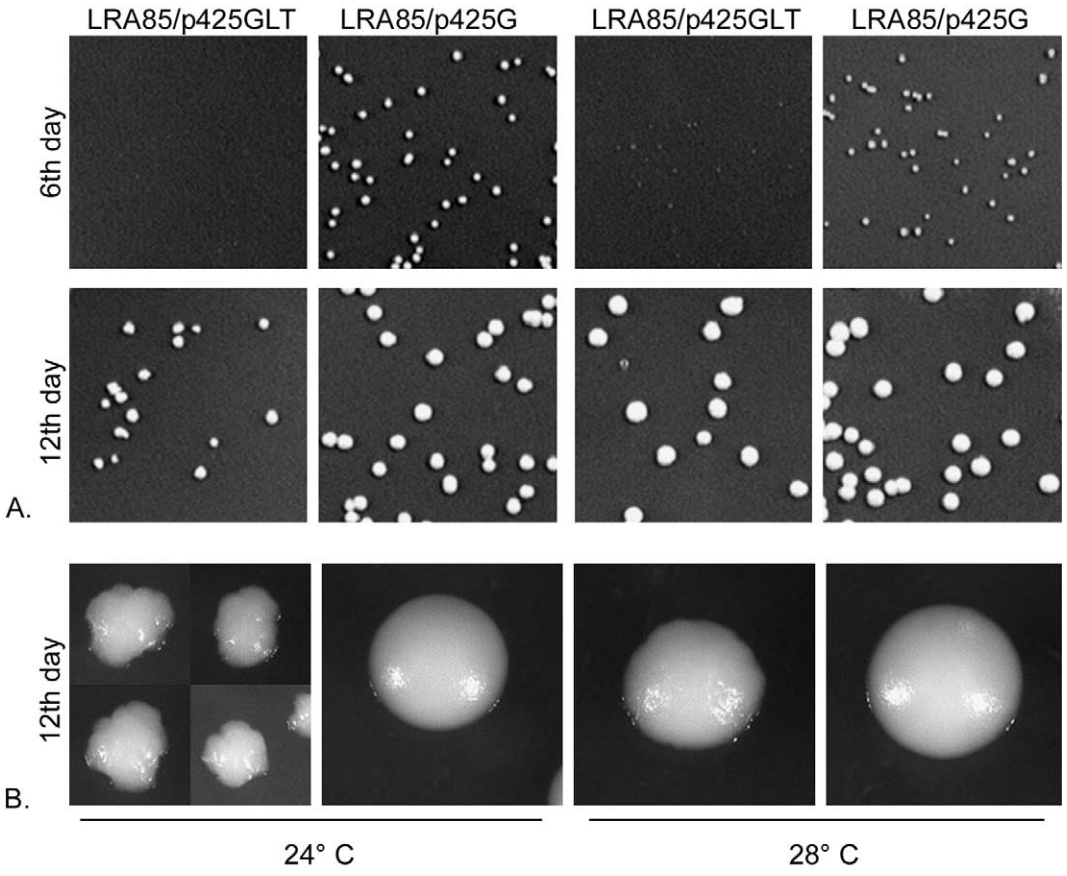

Fig. 9. The inhibition effect of the LT protein is cAMP-dependent. LRA85/p425GLT and LRA85/p425G at permissive (24°C) and semi-permissive (28°C) temperature (A) and a detail of a monocolony (B).

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