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Reversible protein phosphorylation plays a crucial role in the circuitry controlling diverse cellular processes, and the activities of phosphorylating and dephosphorylating enzymes must be carefully balanced in normal cells. It is well documented that many of these enzymes are constitutively expressed, but their activities are tightly regulated by a variety of post translational mechanisms. In the case of the serine/threonine-specific protein phosphatase 2A (PP2A), a catalytic subunit is bound by two regulatory subunits designated A and B. We have established a functional complementation assay in the yeast *S. cerevisiae* and have used this assay to isolate two dominant-defective mutations in a human PP2A catalytic subunit gene, via oligonucleotide-mediated site-directed mutagenesis. We have expressed the mutant and wild-type catalytic subunit genes in mammalian cells, and have initiated our characterization of the effects of these proteins on endogenous PP2A activity, as well as cellular physiology. These studies will thus increase our understanding of PP2A function in the regulation of proliferation and malignant transformation, and will yield important information about structure-function relationships in the PP2A catalytic and regulatory subunit proteins.

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Alison DeLong 6/12/98

PI - Signature Date

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INTRODUCTION

Protein phosphatase 2A (PP2A) comprises catalytic (C) and regulatory (A and B) subunits, and a heterotrimeric (ABC) holoenzyme is thought to predominate *in vivo*, although a heterodimeric (AC) form also has been purified (reviewed in Walter and Mumby 1993). The catalytic subunit exhibits strong similarity to protein phosphatase 1 (PP1) in regions that constitute the catalytic active site. The reaction mechanism of PP1 and PP2A has not yet been defined, but catalysis is thought to involve an invariant pair of aspartate and histidine residues (Goldberg et al. 1995). Although the total number of PP2A substrates is not known, it is likely that regulation of PP2A activity is necessary for the correct functioning of signal transduction cascades in which PP2A participates.

PP2A has not been identified as an oncogene per se, but its interactions with proteins responsible for tumorigenesis are of demonstrated functional significance. Evidence for the role of PP2A in mammalian cell proliferation has originated in studies showing that PP2A is a target for the small-T antigen of SV40 and the small- and medium-T antigens of polyoma virus. These tumor antigens bind the AC heterodimeric PP2A complex (Pallas et al. 1990; Walter et al. 1990) and exclude the B subunit. Interaction with SV40 small t antigen reduces PP2A activity toward the mitogen-activated protein kinases (MAPKs) ERK1 and MEK1, causing deregulation of the MAPK cascade and induction of cell growth (Sontag et al. 1993). Middle T antigen mutants that fail to bind PP2A also are defective in transformation (Campbell et al. 1995). Furthermore, the tumor-promoting agent okadaic acid (OKA; Suganuma et al. 1988) is a potent and specific inhibitor of PP2A and PP1, with PP2A showing approximately 100-fold higher sensitivity (Cohen et al. 1989). The targeting of PP2A by viral T antigens and the tumor-promoting effect of OKA at low concentrations both support the hypothesis that PP2A activity normally suppresses proliferation, and that antagonism of PP2A may be important in tumorigenesis.

Essential PP2A subunit functions are strongly conserved throughout evolution. We have previously shown that the *Arabidopsis thaliana* PP2A-A subunit gene *RCN1* complements the cold- and temperature-sensitive phenotypes of the *tpd3* mutation, which affects the single *S. cerevisiae* PP2A-A subunit gene (Garbers et al. 1996). Complementation of *tpd3* by the *RCN1* cDNA indicates that the *Arabidopsis* Rcn1 protein is capable of all essential interactions with endogenous yeast proteins. Similarly, the *Drosophila* PPV complements the temperature-sensitive phenotype of the yeast *sit4* catalytic subunit mutation (Mann et al. 1993).

Dominant gain-of-function alleles have revealed the identities and functions of many proteins involved in growth control. We proposed to isolate dominant-defective mutants of human PP2A as a tool for investigating potential growth control functions of PP2A in normal and breast cancer cells. Possible causes of dominance for such mutants include competition with the wild-type protein for substrates or for positive regulatory subunits. The PP2A catalytic subunit (PP2A-C) provides a good target for mutagenesis aimed at generation of dominant alleles, because putative catalytic residues have been identified and interactions with positive regulatory subunits are known to be important for normal activity.

We have established a functional complementation assay for PP2A-C in the yeast *S. cerevisiae* and have used this system to isolate two dominant-defective mutants in the human PP2A-Ca gene. We have performed preliminary experiments to elucidate the basis for the dominant-defective phenotype of the mutants. We have expressed mutant and wild-type PP2A-C alleles in mammalian tissue culture cells and have begun to characterize the resulting effects on PP2A enzyme activity and cell physiology.

BODY

Development of a functional complementation assay for human PP2A subunits in S. cerevisiae
We have shown that the human PP2A catalytic subunit alpha isoform (PP2A-Cα) provides sufficient PP2A-C activity to allow the growth of yeast. The coding sequences of the PP2A-C α and β isoform cDNAs were fused to the galactose-inducible GAL1 promoter in the low- and high-copy number yeast shuttle vectors YCp22GAL and YEp112GAL. These PP2A-C constructs (GAL-Cα and GAL-Cβ) were transformed into yeast strain CY3007 (Lin and Arndt, 1995) and screened for their ability to complement the strain's temperature sensitive phenotype. The chromosomal copies of the endogenous yeast PP2A-C genes (PPH21 and PPH22) are disrupted in CY3007 cell, and PP2A-C activity is supplied by a plasmid-borne temperature sensitive allele of the pph21 gene. This strain is viable at 25°C or 30°C, but fails to grow at 35°C. CY3007 transformants carrying GAL-Cα fusions also grow at 25°C or 30°C on media containing either glucose or galactose as carbon source, but are viable at 35°C only when grown on galactose-containing medium. (Fig. 1) Transformants carrying either shuttle vector (without the PP2A-C coding sequence) or carrying anti-sense orientation GAL-Cα fusions fail to grow on galactose-containing medium at 35°C. These data demonstrate that human PP2A-Cα expressed in yeast cells is functional, indicating that interactions with essential substrate molecules and/or regulatory subunits are conserved.

In contrast, the GAL-Cβ construct fails to complement the temperature-sensitivity of CY3007 (data not shown). We have assayed the levels of expression of the human PP2A-C subunits in yeast via western blotting. After growth in galactose-containing medium at 30°C, yeast cells carrying the empty vector or GAL-PP2A-C fusion constructs were harvested, and total cellular proteins were extracted. Preliminary western blot analysis of the resulting extracts indicates that the GAL-Cα and GAL-Cβ fusions are expressed at equivalent levels in yeast. Therefore, poor complementation by the GAL-Cβ fusion may reflect functional differences between the two isoforms. We are performing further experiments to test this hypothesis.

Isolation of dominant-defective mutations in human PP2A-C
Functional complementation by PP2A-Cα provided us with an assay for dominant defective mutations in human PP2A-C. We used a standard oligonucleotide-mediated mutagenesis procedure (Deng and Nickoloff, 1992) to introduce a single point mutation in the PP2A-Cα coding sequence that results in the substitution of an asparagine for histidine at position 118 in the PP2A-Cα protein (Cα-H118N). (Proper incorporation of the mutation was verified by DNA sequence analysis of the region spanning the mutagenized site.) This histidine residue belongs to the invariant aspartic acid/histidine pair thought to be involved in PP2A catalysis (Goldberg et al. 1995). The corresponding mutation in the lambda phosphatase causes a 10^5-fold decrease in catalytic activity, but does not affect the enzyme's ability to bind a synthetic substrate (Zhuo et al. 1994). The mutant allele was transformed into strain CY3007 under control of the GAL1 promoter, and transformants were tested for growth on glucose and galactose at 25°C, and 35°C. When grown on glucose-containing medium, cells carrying GAL-Cα-H118N constructs are viable at 25°C, but are inviable at 35°C. When grown on galactose-containing medium these transformants are inviable at 25°C and 35°C (Figures 1 and 2). These data show that the H118N mutation inactivates PP2A-Cα, eliminating complementing activity at 35°C. Furthermore, the mutation creates a dominant-defective enzyme that prevents growth of cells expressing the mutant protein.

Two alternative hypotheses that can be invoked to explain the dominance of the H118N mutant

are 1) the mutant enzyme binds substrate efficiently and prevents the pph21-ts gene product from interacting with one or more essential substrate(s) or 2) the mutant enzyme interacts normally with PP2A regulatory subunits (A and/or B) that are required for normal activity of the pph21 gene product. To test the idea that the dominant-defective phenotype may require substrate binding, we designed a second mutation that converts arginine at position 89 to alanine (R89A). The corresponding mutant of lambda phosphatase shows a 20-fold reduction in its affinity for a synthetic substrate, and a 500-fold decrease in catalytic activity (Zhuo et al. 1994). This change was introduced into the wild-type and H118N mutant genes, yielding plasmids carrying Cα-R89A and Cα-R89A H118N mutant alleles. Mutations were verified by DNA sequence analysis, and their effects were assayed in strain CY3007. Like the H118N mutation, the R89A mutation eliminated complementation by PP2A-Cα; cells carrying GAL-Cα-R89A fusion failed to grow at 35°C on either galactose (Fig. 2) or glucose (data not shown). Like H118N, the R89A mutation also inhibited growth of galactose-grown cells at permissive temperatures, although less strongly than the H118N allele. The phenotype of the Cα-R89A H118N double mutant was identical to that of the R89A mutant (Fig. 2). Thus both the R89A and R89A H118N mutants exhibit dominant-defective phenotypes, albeit weaker than that of the H118N mutant. These data suggest that while interaction with the A and B regulatory subunits probably is the primary determinant required for expression of the dominant-defective phenotype, the ability of the enzyme to bind substrate enhances that phenotype. We are currently establishing PP2A assay conditions that will allow us to test our predictions about the activities of the mutant enzymes and to quantitate the effect of galactose induction on overall PP2A activity in yeast. We will also microscopically characterize the phenotypes of yeast cells in which Ca-H118N expression has been induced.

It is important to note that initially the R89A mutation was introduced only into low-copy number constructs, and the resulting weak phenotype is due in part to copy number effects. The high-copy H118N construct clearly shows a stronger dominant-defective phenotype than does low-copy H118N, and high-copy R89A mutants of an arabidopsis PP2A-C gene also show stronger phenotypes than do the corresponding mutants of human PP2A-Cα (Fig 2). We are now generating high-copy number constructs carrying the human PP2A-Cα R89A and R89A H118N alleles to verify this hypothesis.

Expression of wild type and dominant-defective PP2A -C alleles in mammalian cells
To assess the feasibility of our plan to express PP2A alleles in normal and breast cancer cell lines, we have initially focused on expression of Cα+ and Cα-H118N in the well-characterized normal fibroblastic cell line TGR1 (Prouty et al. 1993). We chose the Cα-H118N mutant because of its stronger dominant-defective phenotype. We have placed the wild-type and dominant-defective PP2A-C genes under control of viral LTR promoters in the mammalian retroviral shuttle vectors pWZLneo and pLXSHyg (Miller et al 1993). Each of these vectors allows us to isolate stable cells lines expres-sing the mutant and wild-type proteins, and to assay the effects PP2A alleles. Retroviral lysates were harvested from ψ2 cells (a standard ecotropic retroviral packaging cell line) electroporated with the empty vector, the Cα+ construct or the Cα-H118N construct, according to a standard packaging protocol. Lysates were infected into TGR 1 cells, and clonal cell lines were isolated by ring-cloning drug-resistant colonies.

We also performed colony formation assays to test for gross effects of PP2A-C expression. With both retroviral vectors, we expect to see a correlation between expression of the drug resistance gene and the Ca gene. In the case of pWZLneo, translation of Ca and the neomycin phosphotransferase (Neo) protein are coupled by an IRES site. In the case of pLXSHyg, the correlation arises simply because position effects usually modulate the expression of both genes, resulting in strong or weak expression of both the hygromycin resistance (Hyg) and PP2A-C

cassettes. In the colony formation assay, cells infected with the retroviral construct are plated in increasing drug concentrations; the resulting drug-resistant colonies are counted after 10-14 days. At high drug concentrations colony formation is reduced in cells infected with the pLXSHyg/Cα+ construct, and is further reduced in cells infected with the pLXSHyg/Cα-H118N construct (Fig. 3A), suggesting that high-level expression of these genes is deleterious. The effect is less than two-fold for Cα+ and two- to four-fold for Cα-H118N. We do not see a negative effect of expressing PP2A-C in the pWZLneo colony formation assay (Fig. 3B), however, G418 is known to be less effective than hygromycin in selecting for cells expressing high levels of drug resistance (Hanson and Sedivy, 1995). Cells expressing a relatively low level of Neo protein are resistant to high levels of G418, thus the selection for high-level PP2A-C expression is less effective with pWZLneo constructs. We are currently testing these hypotheses by assaying PP2A expression levels in cell lines selected at high drug concentrations in each of these experiments.

Our preliminary assays have used western blotting with a PP2A-C antibody to establish the C subunit levels in stable cells lines. Several clones carrying the pLXSHyg vector alone were analyzed to establish the background level of endogenous PP2A-C expression in TGR1 cells. In addition, 9 lines carrying Cα+ and 11 carrying Cα-H118N were assayed. When equal amounts of total protein are analyzed, 2 Cα+ lines and 6 Cα-H118N lines show PP2A-C expression greater than the endogenous level. However, the Cα+ cell lines show only a 2- to 4-fold increase in PP2A-C levels, while several Cα-H118N show 7- to 25-fold increases. Thus we have not recovered any cell lines expressing high levels of Cα+. Taken in conjunction with our colony formation assay data, this suggests that overexpression of Cα+ may have growth-inhibiting effects. This would be consistent with the hypothesis that PP2A activity normally suppresses proliferation. Our stable cell lines show no striking morphological alterations or changes in growth rate, and do not exhibit highly transformed phenotypes. However, we hypothesize that expressing higher levels of wild-type PP2A will cause more marked inhibition of growth, and higher levels of mutant alleles also may produce more dramatic effects.

These experiments have demonstrated that we can express exogenous PP2A-C constructs in mammalian cells, and that a dominant-defective allele can easily be expressed at a 20-fold higher level than endogenous PP2A. Our next steps will include assaying for biological and biochemical effects of expressing mutant and wild-type PP2A alleles in cultured cells, and optimization of our PP2A expression strategy. Currently we are assaying phosphorylation of MAP kinase in response to serum and growth factor stimulation in several of these cell lines to test for changes in activity in the MAPK signal transduction pathway. We also are constructing PP2A expression vectors that will provide higher expression levels of epitope-tagged PP2A alleles, to facilitate biochemical and molecular analyses. These second-generation constructs will also be tested in breast cancer cell lines for effects on growth and signal transduction.

CONCLUSIONS

We have shown that *S. cerevisiae* can be used as a rapid in vivo assay system for human PP2A-C activity. Mutation of a conserved histidine residue in the Cα sequence confers a strong dominant-defective phenotype; mutation of a conserved arginine residue results in a weaker dominant-defective phenotype. Expression of relatively high levels of a dominant-defective PP2A allele (up to 20- to 25-fold) is tolerated by a normal fibroblastic cell line, however, overexpression of wild-type PP2A may have deleterious effects. This latter observation is consistent with the hypothesis that PP2A normally exerts a negative or inhibitory role in growth control.

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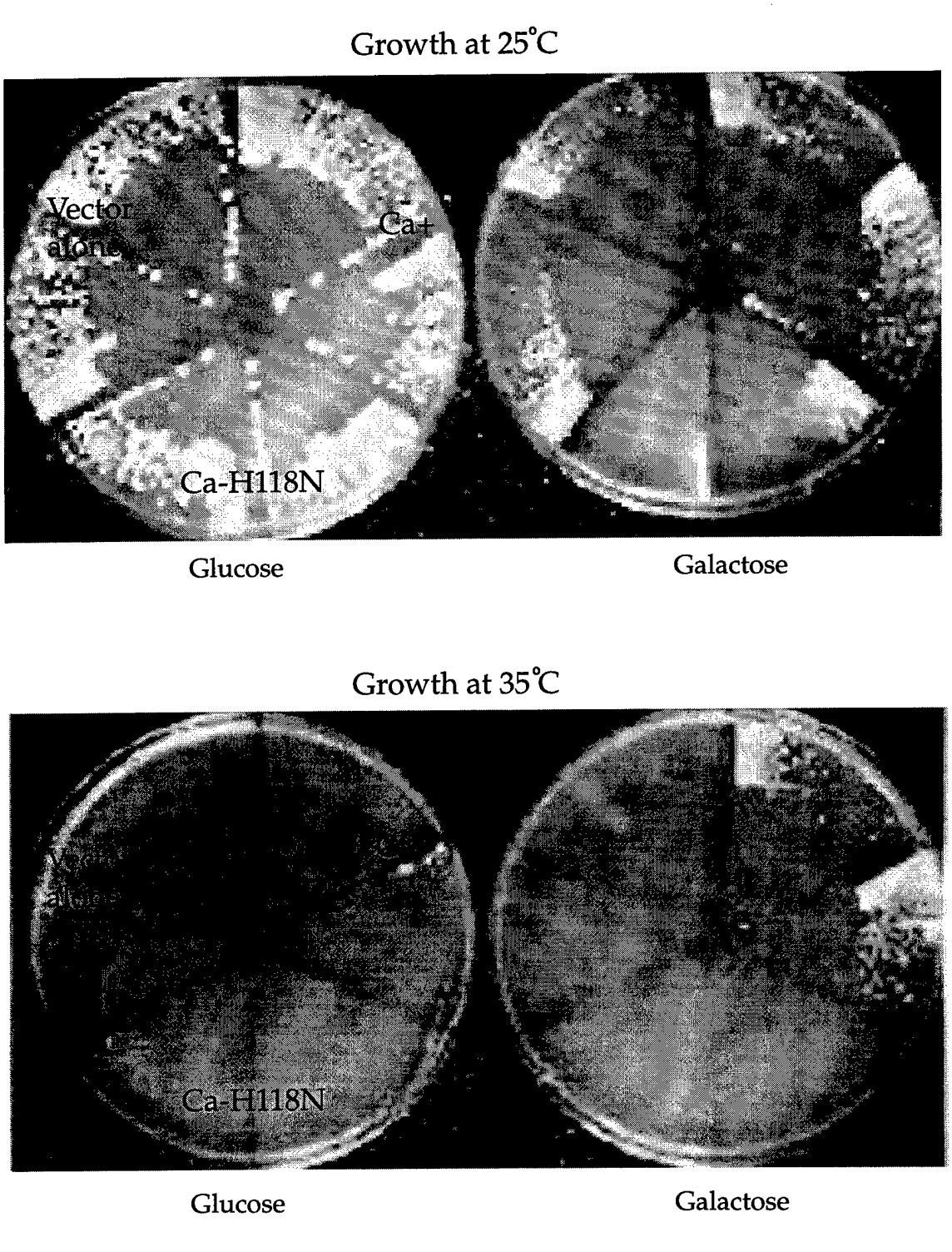

Figure 1. Human PP2A-Cα complements the temperature sensitive phenotype of a yeast PP2A-C-ts yeast strain. Replica plates grown at 25°C (upper panel) and 35°C (lower panel) are shown. Pairs of sectors show growth of CY3007 transformants carrying the empty vector, the wild-type GAL-Cα+ fusion, and the mutant GAL-CαH118N fusion were streaked on plates containing glucose (left) or (galactose) as indicated.

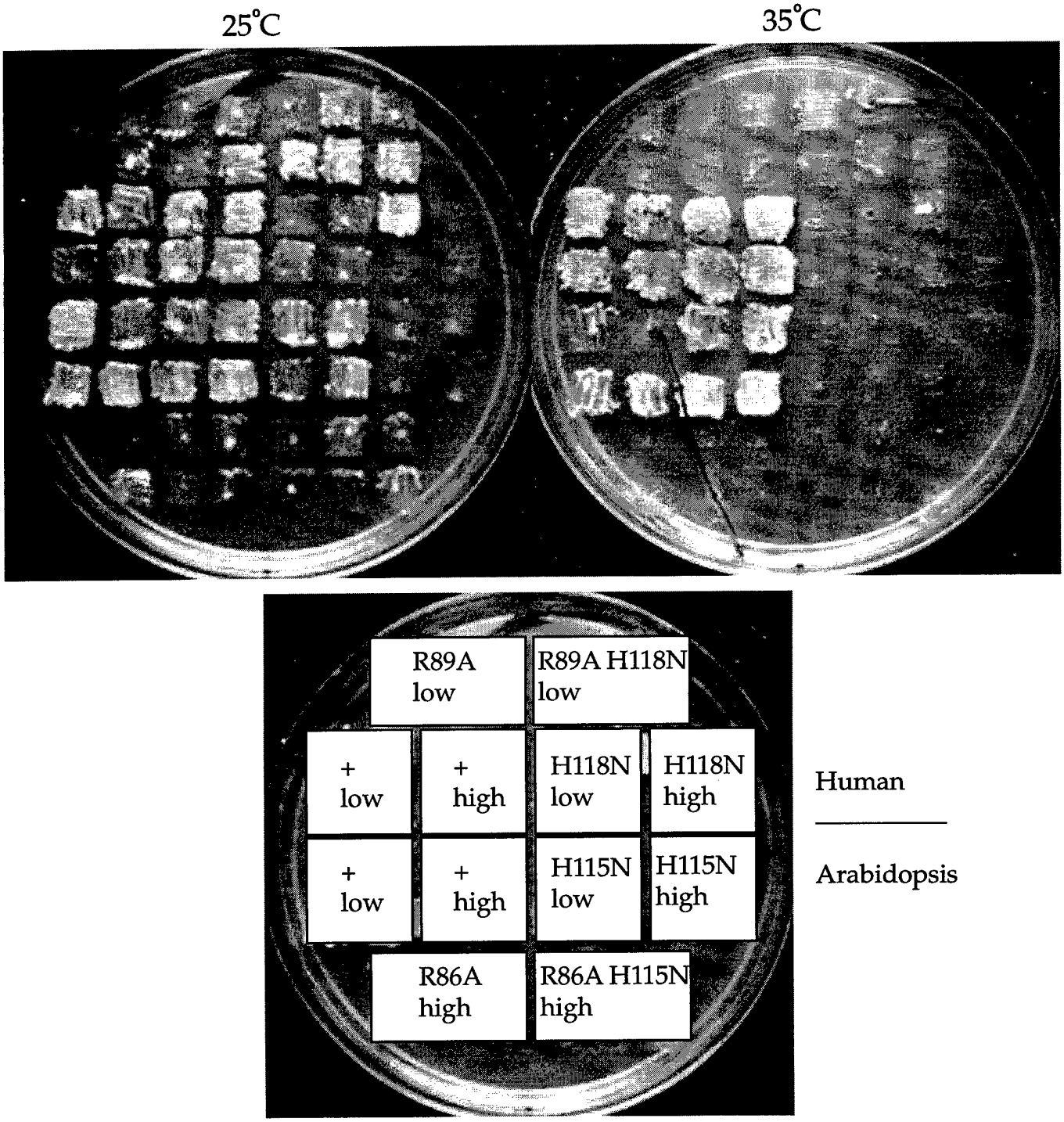

Figure 2. Phenotypes of human and arabidopsis dominant-defective mutants. Galactose-containing replica plates grown at 25°C (left) and 35°C (right) are shown above. CY3007 transformants carrying the PP2A constructs shown were patched in the locations indicated below. Replica patches on a glucose-containing plate grown at 25°C show that all transformants were viable (not shown).

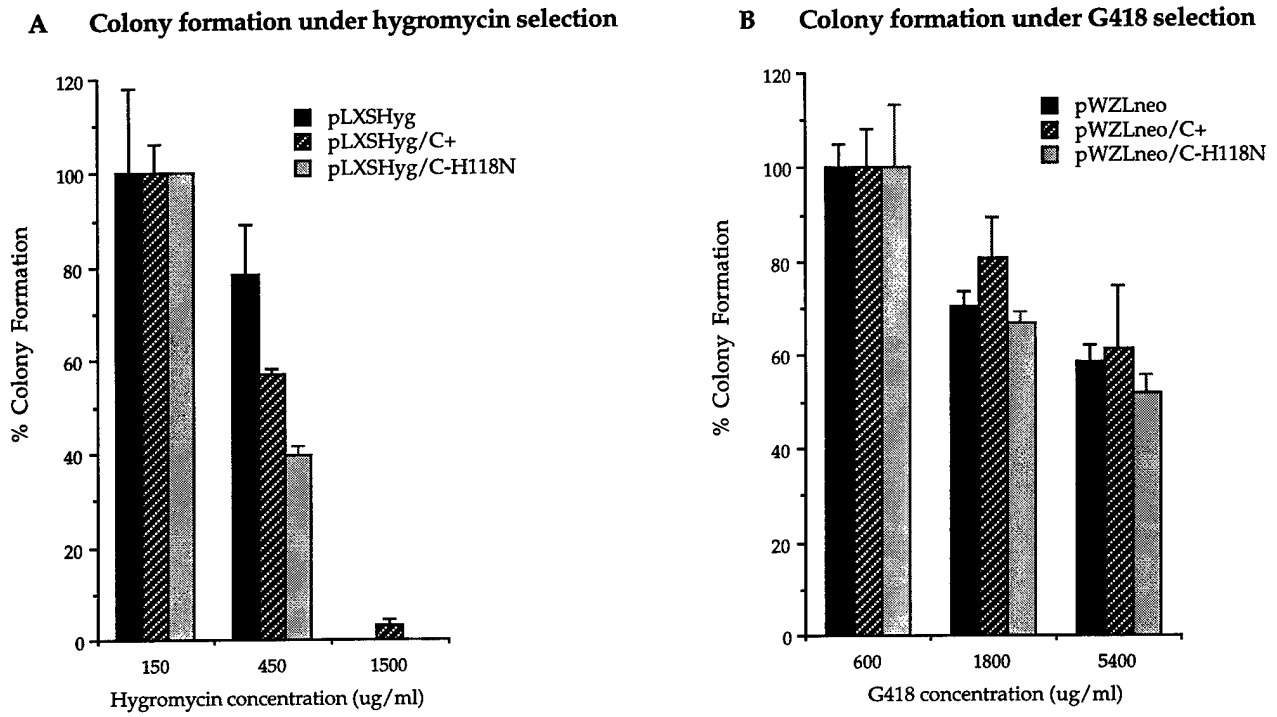

Figure 3. Colony formation assays for pLXSHyg/PP2A-C constructs. Retroviral lysates were infected into TGR1 fibroblasts; infected cells were later diluted into medium containing increasing drug concentrations. After approximately two weeks selective growth, colonies were stained with crystal violet and counted. For each construct, colony formation is normalized to the number of colonies formed at the normal selective concentration of A) hygromycin (150μg/ml) or B) G418 (600 μg/ml). At high G418 concentrations, medium was buffered with HEPES pH 7.1. Values shown represent the average of at least two (A) or three (B) replicates.