Transcriptional Activation Capacity of the Novel PLAG Family of Zinc Finger Proteins*

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We recently discovered PLAG1, a novel developmentally regulated C_{2}H_{2} zinc finger gene at chromosome 8q12 as the main target for pleomorphic adenomas of the salivary gland (1, 2). The largest cytogenetic subgroup of pleomorphic adenomas of the salivary gland carries chromosome 8q12 aberrations with 3p21 as preferential translocation partner. The t(3;8)(p21;q12) results in promoter swapping between PLAG1 and the constitutively expressed gene for β-catenin (CTNNB1), a protein interface functioning in adherens junctions and the W/G/WNT signaling pathway (3). Fusions occur in the 5′-noncoding regions of both genes, exchanging regulatory control elements while preserving the coding sequences (4). These data suggest that the activation in pleomorphic adenomas of PLAG1 most likely results in uncontrolled activation of downstream target genes.

We have isolated and characterized two novel cDNAs encoding C_{2}H_{2} zinc finger proteins showing high sequence homology to PLAG1, a protein ectopically activated by promoter swapping or promoter substitution in pleomorphic adenomas with chromosomal abnormalities at chromosome 8q12. PLAG1 and the two new PLAG1 family members (PLAGL1 and PLAGL2) constitute a novel subfamily of zinc finger proteins that recognize DNA and/or RNA. To examine the potential of the three human proteins to modulate transcription, we constructed several PLAG/GAL4 DNA binding domain fusion proteins and measured their ability to activate transcription of a reporter gene construct in different mammalian cell lines and in yeast. Although the carboxy-terminal part of PLAG1 shows strong overall transcriptional activity in mesenchymal (COS-1) and epithelial cells (293), both PLAG1 and PLAG2 transactivate in mesenchymal cells only if depleted from a repressing effect. This effect is less profound in epithelial cells. These data suggest that the activation in pleomorphic adenomas of PLAG1 most likely results in uncontrolled activation of downstream target genes.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U83992 (PLAGL1), AF006005 (PLAGL2).

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EXPERIMENTAL PROCEDURES
Identification of PLAG1-related ESTs—The data base of ESTs was screened for PLAG1 homologous sequences using the tBLASTN program at the NCBI (6). Clones for ESTs from the Merck/Washington University project were provided by Research Genetics.

cDNA Libraries—cDNA clones were isolated from a mixed poly(dT)/random-primed library in agt11 constructed from human fetal kidney (CLONTECH) or from a 12.5-days post-coitum mouse total embryo cDNA library in pACT2 (CLONTECH). Library screening was performed by plaque or colony hybridization using various DNA probes (inserts of two EST clones, a PLAG1 ORF probe, or subsequently isolated cDNA clones), according to the manufacturer’s instructions.

Northern Blot Analysis—Expression of PLAG1L1 and PLAGL2 was examined by Northern hybridization using a DNA fragment of the respective coding regions as a probe. Human tissue RNA blots (2 µg of poly(A)^+ RNA loaded/lane) were purchased from CLONTECH, hybridized, and analyzed by autoradiography according to standard procedures in Kas et al. (1).

DNA Sequencing and Computer Analyses—Nucleotide sequences were determined according to the dideoxy chain termination method using the T7 polymerase sequencing kit of Amersham Pharmacia Biotech/LKB or the double-stranded DNA Cycle Sequencing System (Life Technologies, Inc.). Sequencing results were analyzed using an A.L.F. DNA sequencer/TM (Amersham Pharmacia Biotech) on standard 30-cm, 6% HydroLynK, Long Range™ gels (AT Biochem). Sequence analysis utilized Lasergene (DNASTAR) and BLAST and BEAUTY searches (National Center for Biotechnology Information; Ref. 6). All oligonucleotides were purchased from Amersham Pharmacia Biotech.

* The abbreviations used are: EST, expressed sequence tag; GBD, GAL4 DNA binding domain; ORF, open reading frame; PLAG1, pleomorphic adenoma gene; PLAGL1/PLAGL2, PLAG-like/PLAG-like 2; kb, kilobases(s).
Plasmid Constructions—The PLAG constructs used in mammalian cells were generated by polymerase chain reaction with primers containing restriction sites for cloning. Every construct was sequenced fully to verify fidelity of the polymerase chain reaction. PLAG cDNAs (P1 is PLAG1, PL1 is PLAGL1, PL2 is PLAG2) were fused in-frame to the DNA binding domain of GAL4 using the PM1 or PM3 expression vector (7). PITAD contains residues 243–500 cloned in the EcoRI and SalI sites, P1del2 contains residues Asp385–Gln500 cloned into EcoRI and BamHI sites, and P1del2B contains residues Asp385–Pro466 cloned into the BamHI site of pM3. P1del1 contains residues Val243–Leu384 and is derived from PITAD by digestion with BamHI and HindIII and religation. PITAD contains residues 214–463, P1del1 contains residues Gly214–Pro366, P1del2 contains residues Pro366–Arg463, all cloned in the EcoRI and HindIII sites of pM3. P1del3 (Ser293–Gly408) and P1del4 (Ser266–Met429) are cloned in the EcoRI and BamHI sites of pM1. P1del5 (Ser266–Arg463) is cloned in the EcoRI and HindIII sites of pM1. P1del6 (Glu241–Gly408), and P1del7 (Glu241–Met429) are cloned in the EcoRI and BamHI sites of pM3. Finally, P2TAD contains residues Ile249–Gln496, P2del1 contains residues Gly214–Pro382, and P2del2 contains residues Pro382–Gln496, all cloned in the EcoRI and BamHI sites of pM3. As a positive control for transactivation, we use pM3-SPF1, an in-frame fusion between the DNA binding domain of GAL4 (amino acids 1–147) and the activation domain of STF1 (8). All PLAG clones used in yeast were generated by polymerase chain reaction with primers containing restriction sites for cloning into the yeast fusion vectors pGBT9 and pGADT7 (CLONTECH). Each construct was sequenced fully. These constructs include full-length PLAG1 (500 amino acids (P1ORFHYB)) and the following portions of PLAG1 or PLAGL1: PLAG1 residues His236–Gln500 (PITADHYB), PLAG1 residues Asp385–Pro466 (P1del2BHYB), and PLAG1 residues Gln500 (P1TADHYB), PLAG1 residues Asp385–Pro466 (P1dlet2HYB), and PLAG1 residues Asp385–Pro466 (P1del2HYB). Site-directed Mutagenesis—Two aforementioned trans-activating constructs, PITAD and P1TAD1, containing the conserved STS box were used to mutate the middle two residues to alanine. For this we applied the QuikChange site-directed mutagenesis kit (Stratagene) according to the instructions of the supplier. With the following sequences for PLAG1 (PL1muta1, 5'-ATCCGCTTTGATCGCATGCA-3'); PL1muta2, 5'-AGACCGCCATACGTCAGGAG-3'); PLAG1 (PL1muta1, 5'-ACAAACACACCTGCGCCCATAC-3'); PLAG1 (PL1muta2, 5'-AATGACACACCTGCGCCCATAC-3'); and PLAG1 (PL1muta3, 5'-AATGACACACCTGCGCCCATAC-3'). The cycling conditions consisted of 95 °C for 30 s and 16 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 9.5 min. Constructs were sequenced to confirm the site-specific mutagenesis. Cell Culture, Transfections, and Luciferase Assay—COS-1 kidney fibroblasts (ATCC, CRL 1650) and 293 fetal kidney epithelial cells (ATCC, CRL 1573) were cultured according to the suppliers’ protocols. DNAs were purified using anion exchange chromatography (NucleoBond AX, Machery-Nagel, Germany). Cells were propagated in the prescribed media supplemented with 10% fetal calf serum. Cells (6-well plates) were transiently cotransfected with 200 ng of construct DNA and 1 μg of reporter plasmid, together, using cationic liposomes (Lipofectamine, Life Technologies, Inc.) according to the manufacturer’s protocol. For each experiment, luciferase activity was determined in triplicate wells. The results are expressed as the mean of three individual transfection experiments. Cells were harvested 24 h after the start of transfection, and luciferase reporter enzyme activity driven by the various GALA/DDB-PLAG/TAD constructs was determined with the luciferase assay system (Promega) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory) and performing end point assays.

Yeast Strain and Assays—The yeast strain Y190 as provided in the CLONTECH Matchmaker two-hybrid system contains two reporter genes, HIS3 and LacZ, under the control of promoters containing the GAL4 binding sites. The yeast was grown according to the instructions of the supplier. The pAS2 or pGBT9 constructs were transformed into the yeast strain Y190 as provided in the Genexpress cDNA program with notice-
inating from various fetal and adult human tissues (Fig. 2). A
PLAGL1 ORF probe identified prominent messengers of 4.5
and 2 kb in adult (heart, brain, placenta, lung, liver, skeletal
muscle, kidney, pancreas) human tissues. In fetal tissues (kid-
ey, liver, lung, brain) as well as in placenta, the most promi-
nent mRNAs are approximately 3 and 4 kb in size. A number of
weaker bands might represent less prevalent alternative splice
forms or alternative polyadenylation sites. A PLAGL2 probe
identified a 7.5-kb messenger RNA that is highly expressed and
a 2.5-kb mRNA that is expressed only at low levels in fetal
kidney, liver, lung, and brain but not in adult tissues (Fig. 2).
The latter pattern resembles the expression profile as seen for
PLAG1. PLAG1, however, shows a single band of 7.5 kb in fetal
tissue but is not detectable by Northern blot in fetal brain (1).

The PLAG Family of Zinc Finger Transcription Factors

Plag1, Plagl1, and Plagl2 Show Transcriptional Activ-
ity in Mammalian Cells—To establish whether the three PLAG
proteins exhibit transcription activation in mammalian cells,
sequences encoding the carboxyl-terminal regions of all three
proteins were cloned into the mammalian GAL4-DNA binding (GBD) fusion protein expression vector pM3 (7). These constructs encoded residues 243–500 of PLAG1, residues 214–463 of PLAGL1, and residues 249–496 of PLAGL2. They were co-transfected into the mesenchymal kidney cell line COS-1 and the epithelial fetal kidney 293 cells together with a luciferase reporter gene construct that contained five tandemly repeated GAL4 binding sites in its promoter region, preceding the luciferase gene. The results of these transfection assays are shown in Fig. 3. Transfection of the reporter plus pM3 (the GAL4 cloning vector) alone failed to induce luciferase activity, whereas GBD linked to the potent transcription activation domain of STF1 (8) caused a strong stimulation of luciferase expression (1144-fold in COS, 3096-fold in 293 cells). The carboxyl terminus of PLAG1 (Val-243–Gln-500) and PLAGL2 (Ile-249–Gln-496) fused to GBD showed 6-fold more luciferase activity as compared with the GBD only when COS cells were transfected. This increase in transcriptional activity was low but was consistently observed in four different experiments. In contrast, in 293 cells, the carboxyl terminus of PLAG1 showed more than 100-fold luciferase activity as compared with the GBD vector. The activity of PLAGL2 in these cells remained low (3-fold luciferase activation). The same set of experiments was performed using the carboxyl termini of PLAG1 (Glu-214–Arg-463) fused to GBD. Although PLAG1 is a strong transactivator in COS cells (55-fold luciferase activation), the effect in 293 cells is impressive, with a 880-fold luciferase activation (Fig. 3). Hence, the transfections of GBD-PLAG fusion constructs revealed profound differences between the cell lines tested as well as between the three PLAG family members.

**Transcriptional Activation Capacity Is Confined to the Second Part of the Carboxyl-terminal Region of PLAG1 and PLAGL2**—To sublocalize the critical transactivation domain within the aforementioned carboxyl-terminal region of the three PLAG proteins, a series of deletion constructs was generated (Fig. 4). Three constructs were generated for PLAG1: P1del1 (Val-243–Leu-384), P1del2 (Asp-385–Gln-500), and P1del2B (Asp-385–Pro-466). Seven constructs were generated for PLAGL1: PL1del1 (Glu-214–Pro-366), PL1del2 (Pro-366–Arg-463), PL1del3 (Ser-293–Gly-408), PL1del4 (Ser-266–Met-429), PL1del5 (Ser-266–Arg-463), PL1del6 (Glu-214–Gly-408), and PL1del7 (Glu-214–Met-429). Two constructs were generated for PLAGL2: PL2del1 (Ile-249–Pro-382), and PL2del2 (Pro-382–Gln-496). These constructs were co-transfected into the mesenchymal kidney cell line COS-1 and the epithelial fetal kidney 293 cells together with the luciferase reporter gene construct as described above. P1del2 shows 775- and 723-fold activation in COS and 293 cells, respectively, whereas P1del1 does not or just very weakly transactivates. PL2del2 shows 282- and 816-fold activation in COS and 293 cells, respectively. These assays indicate that the second part of the carboxyl terminus in PLAG1 (Asp-385–Gln-500) and in PLAGL2 (Pro-382–Gln-496) harbors the essential transcriptional activation domain. A GBD-PLAG1 construct retaining only residues 385–466 (P1del2B) is as effective as the construct containing the very carboxyl-terminal 34 residues, too, in 293 cells (726-fold versus 723-fold), but activates less in COS cells (87-fold). In contrast, the first parts of the PLAG1 and PLAGL2 carboxy termini (P1del1/PL2del1 series) display very little or no ability to stimulate transcription of the luciferase reporter construct (Fig. 4). Since the overall activity of the carboxyl termini is much lower than the combined effect of the first and second part, the first part might have an inhibitory function. The PLAG1 deletion constructs show a more complex picture. Not only does the arbitrary split of the carboxyl-terminal region disable the transactivation domain (PL1del1 and PL1del2), constructs covering the central region of the carboxyl-terminal region also hardly transactivate (PL1del3 and PL1del4). Besides a central core (Ser-293–Gly-408), the presence of the very carboxyl terminus in the absence of the first 51 amino acids of the carboxyl terminus (Ser-266–Arg-463, PL1del5) or to a smaller extent, the presence of the amino terminus of the carboxyl-terminal region in the absence of the last 55 amino acids (Glu-214–Gly-408, PL1del6), retains high activation capacity. In comparison with PL1del6, PL1del7 shows that the presence of only 21 amino acids (Gly-408–Met-429) almost fully restores transactivation capacity. This might be due to the presence of four glutamine residues in a row.

The Conserved STSY Box Has No Role in Transactivation Regulation—The first part of the carboxyl terminus contains four consecutive residues conserved among the three PLAG proteins (STSY, position 338–341 in PLAG1, position 317–310 in PLAG1, position 346–349 in PLAGL2, Fig. 1), all being putative targets for phosphorylation by tyrosine or serine/threonine kinases. Cross-species alignment indicates that only the middle two residues are conserved among the PLAG homologs in mice (data not shown). To explore whether these residues are important in transactivation potential, Thr-339 and Ser-340 in PLAG1 and Thr-308 and Ser-309 in PLAGL1 were changed to alanine by site-directed mutagenesis of GBD-PLAG1/PLAGL1 fusion constructs. As shown in Fig. 4, substi-
tution of these two residues has little effect on the transactivation capacity of the carboxyl terminus of the respective protein: 5- versus 6-fold activation in COS cells, 105- versus 113-fold activation in 293 for PLAG1, and 59- versus 55-fold activation in COS and 730- versus 880-fold activation in 293 cells for PLAGL1. The inhibitory capacity of the first parts of the respective PLAG carboxyl termini is not released.

FIG. 3. PLAG1, PLAGL1, and PLAGL2 show transcriptional activity in mammalian cells. PLAG transcription reporter assays using GBD-PLAG carboxyl-terminal domain fusion proteins. 293 cells and COS-1 cells were co-transfected with luciferase reporter and each GBD-PLAG fusion construct as indicated. Luciferase activity was measured as described. Stimulation relative to the control (pM3 with the GBD only) is given in the table, and values are the mean and standard deviations of at least two independent series of three transfections. Schematic representations of the GBD-PLAG fusion proteins (P1 is PLAG1, PL1 is PLAGL1, PL2 is PLAGL2) are shown.

FIG. 4. Dissection of the transcriptional activation capacity of the PLAG proteins. Transcription reporter assays using GBD-PLAG carboxyl-terminal domain deletion and mutagenesis constructs. 293 cells and COS-1 cells were co-transfected with luciferase reporter and each GBD-PLAG fusion construct as indicated. P1MUTA is identical to P1TAD except two substitutions: T339A and S309A. COS-1 and 293 cells were co-transfected with luciferase reporter and the GBD-P1/PL1MUTA fusion construct and compared with the original GBD-P1/PL1TAD fusion construct. Luciferase activity was measured as described. Stimulation relative to the control is given in the table, and values are means and standard deviations of at least two independent series of three transfections. Schematic representations of the GBD-PLAG fusion proteins are shown.

The C2H2-type zinc finger motif defines an extremely large family of DNA/RNA-binding proteins with probably more than 1000 structurally distinct members present in a vertebrate genome. Apart from transcriptional modulation and control of the LacZ gene. However, when fused to the carboxyl-terminal domain of PLAG1 (residues His-236–Gln-500 (P1TADHYB) or residues Asp-385–Pro-466 (P1del2BHYB)) or PLAG1 (residues Glu-214–Arg-463 (PL1TADHYB)), the expression clone causes activation of β-galactosidase activity, consistent with the presence of an activation activity mediated through PLAG1 or PLAGL1. On the contrary, expression constructs containing full-length PLAG1 (P1ORFHYB) and PLAG1 residues Val-243–Leu-384 (P1del1HYB) do not activate β-galactosidase activity (Fig. 5). The latter is in accordance with the lack of activation in mammalian cells (P1del1, Fig. 4).

DISCUSSION
RNA metabolism, chromatin packaging might also constitute an important activity through which zinc finger proteins exert their regulatory roles (5). To investigate whether PLAG1, the major target for pleomorphic adenomas of the salivary gland (1), is a member of a C$_2$H$_2$ zinc finger subfamily, we screened cDNA libraries to identify cDNAs homologous to PLAG1. Identification of new members may be of general relevance to our understanding of tumor predisposition.

Starting from two EST entries that showed significant sequence homology to PLAG1 and by screening a mouse total embryo and a human fetal kidney cDNA library under low stringency using the PLAG1 ORF, we were able to identify two cDNAs coding for proteins highly homologous to PLAG1. The predicted primary structure of these proteins shows very high sequence identity in the amino-terminal zinc finger region and still significant sequence similarity in the second part of the protein, especially between the last 30 amino acids of PLAG1 and PLAGL2 (Fig. 1). A strikingly conserved stretch of four amino acids (STS$N$) is present in the less conserved region (first part of the carboxyl terminus) of all three proteins. This is an obvious target site for phosphorylation by tyrosine and serine/threonine kinases serving an important regulatory function.

Using YAC clone/computer-aided chromosomal localization, PLAG1 could be assigned to chromosome 6q24-q25 (data not shown), a locus preferentially affected in malignant salivary gland tumors (12). This chromosomal region has also been implicated in the genesis of ovarian, breast, kidney, and pleural mesothelial cancers. Very recently, PLAG1 was also identified by different means and referred to as LOT1 and ZAC1. LOT1 is the human homologue of a rat gene that shows decreased or lost expression in independently transformed rat ovarian surface epithelial cell lines compared with the normal progenitor cells (13, 14). ZAC1 is a mouse protein that regulates apoptosis and cell cycle arrest. The induction of apoptosis and G$_1$ arrest was shown to proceed independently of retinoblastoma protein and of regulation of p21$^{WAF1/Cip1}$, p27$^{Kip1}$, p57$^{Kip2}$, and p16$^{INK4a}$ expression (15). ZAC1/PLAGL1 is thus the first gene besides the p53 tumor suppressor gene that concurrently induces apoptosis and cell cycle arrest. It should be noted, however, that the homology between mouse ZAC1 and human PLAG1 does not include 34 PLE, PMQ, and PML repeat sequences present in ZAC1 nor the very carboxyl-terminal end of both proteins (15), and thus, ZAC1 might represent a fourth member of the PLAG protein family.

The mRNA expression pattern of PLAGL1 is unique compared with those of PLAG1 and PLAGL2. PLAG1 is ubiquitously expressed in all fetal and adult human tissues tested and generates multiple transcripts. The expression of PLAG1 and PLAGL2 is restricted to fetal tissues. The different expression patterns probably reflect different needs during different stages of development. However, PLAG1 shows no expression in fetal brain, in contrast to PLAGL2 (Fig. 2). Hence it will be interesting to find out whether PLAGL2 is involved in any cancer in a similar way as PLAG1, i.e., overexpression as a result of a chromosomal aberration. Currently, we have no evidence yet for aberrant expression of PLAG1 or PLAGL2 in any type of human tumor.

Most oncogenes activated by chromosomal translocations in leukemias, lymphomas, or solid tumors are transcription factors (16). The presence of six or seven canonical C$_2$H$_2$ zinc fingers in the three PLAG1 family members suggests they are DNA and/or RNA-binding proteins. Studies on the eukaryotic transcription factors indicate that, as opposed to DNA binding domains, the transcription activation domains of these proteins are less well defined. If the PLAG proteins encode DNA-binding proteins, as the presence of the C$_2$H$_2$ zinc fingers suggests, the serine- and proline-rich carboxyl-terminal region might represent the transactivation domain. At least four different primary sequence motifs that characterize the activation domains are identified thus far, i.e., acidic, glutamine-rich, proline-rich, and serine/threonine-rich. Transactivation sequences presumably function by direct interaction with elements of the transcription initiation complex (e.g., TFIID) or by interaction with intermediary proteins (e.g., VP16) that in turn recognize the initiation complex (17). To examine whether the three members of the PLAG protein family are able to modulate transcription, we constructed carboxyl-terminal PLAG/GAL4 DNA binding domain fusion proteins and measured their ability to activate transcription of a reporter gene construct in mammalian cells as well as in yeast.

Our transfection assays in COS-1 and 293 cells show that the three PLAG proteins function as transcriptional activators in mammalian cells. PLAG1 and PLAGL2 show similar activation capacities. PLAG1 is a much stronger transcriptional activator, both in COS and 293 cells (Fig. 3). The essential activation domain is confined to the 115 carboxyl-terminal amino acids in PLAG1 (Asp-385–Gln-500) and PLAGL2 (Pro-382–Gln-496). The essential activation domain in PLAG1 is spread over a larger region, and not one single domain is responsible for its activity (Fig. 4). We found that the overall transcriptional activity of PLAG1 was much higher than that of PLAGL2 in the 293 cell line, whereas the dissected parts behaved identically in both cell lines. This might indicate that the function of PLAG1 is cell type-specific and is in accordance with the salivary gland epithelial cells as the target tissue for PLAG1 mutations as observed in pleomorphic adenomas (1, 2). However, the differential activity of PLAG1 in different cell types may also reflect different repertoires of interacting factors without the implica-

![Expression Construct](image)

**Fig. 5. PLAG1 and PLAGL1 have transcriptional activity in yeast.** Transcriptional activity of GBD/PLAG1/2 fusion proteins in yeast. Schematic representation of the regions of PLAG1 and PLAGL1 cloned in the yeast expression vector pGBKT7 and summary of the transcription activity of each fusion protein when transformed in yeast Y190. The topmost figure depicts full-length PLAG1, whereas those below indicate the portions of PLAG1 or PLAGL1 tested for transcription activity in yeast. Transcription activity of each construct was determined by the ability of transformants: 1) to produce histidine and grow in the presence of histidine-free medium; 2) to generate blue color (LacZ activity) in filter lift assays. In the LacZ filter lift assays, we scored for the presence (indicated with + or ++) or absence of blue colonies (indicated with −). The difference between P1TADHYB and PL1TADHYB (+) and P1del2BH1YB (+++) represents the level of enzyme activity (and hence the strength of the transcriptional activation).
tion of a different primary function for the PLAG1 protein itself. The activity of the entire carboxyl-terminal region of PLAG1 and PLAG2 was lower than that of the dissected part of the carboxyl terminus, suggesting the presence of a repressor domain in the first 150 amino acids after the zinc finger domain. In contrast, the (arbitrary) division of the carboxyl terminus of PLAG1 led to the loss of transcriptional activity. The results with the PLAG1 deletion constructs show that the activation capacity is confined to a larger region as in PLAG1 or PLAG2 and that there is no repressor domain. The similar behavior of PLAG1 and PLAG2 is in agreement with their relatively high sequence identity (35% in the carboxyl terminus) (Fig. 1), whereas the carboxyl terminus of PLAG1 shows lower homology (19% identity) to PLAG1. The transcriptional activity of the carboxyl termini of PLAG1 and PLAG1 was also tested in a yeast assay and shown to be similar as in mammalian cells (Fig. 5). The presence of one or more activation and repression domains seems to become a recurrent theme in transcription factors. In the LIM domain protein Rhombotin 2 for instance, the tandemly repeated cysteine-rich zinc binding LIM motifs confer the repressor domains (Ref. 18 and references therein). In the breast cancer gene BRCA2, a region at the amino terminus shows sequence similarity with the activation domain of c-jun. This region is flanked by two inhibitory regions (19). The protein structure or distribution of amino acids in the repressor and activation domain of PLAG1 and PLAG2 does not reveal an obvious feature explaining the different activation capacity (both are proline- and serine-rich).

The three zinc finger proteins described in this paper show, besides their C_{2}H_{2} zinc finger domain, a conserved box of four amino acids (STSY) in their carboxyl-terminal region. Mutation of the middle two residues of this putative target for phosphorylation by tyrosine or serine/threonine kinases has little effect on the transactivation capacity of the carboxyl terminus of the respective protein (Fig. 4) nor does it release the repression capacity of the first part of the respective PLAG carboxyl terminus. However, the evolutionary conservation of these four amino acids may have a function in DNA binding specificity or subcellular compartmentalization.

The presence of a transcriptional transactivation domain in PLAG1 is further evidence for transcription being the major target for translocations in cancer (both solid tumors and leukemias) (16). The data presented here suggest that the activation of PLAG1 due to promoter swapping/substitution in pleomorphic adenomas most likely results in uncontrolled transcriptional activation of a downstream target gene(s). We are currently trying to identify such preferential target sequences for the three family members using cyclic amplification and selection of target sequences and representational differential analysis. Although the conserved zinc finger domains between the three PLAG proteins suggest a same target, it is hard to speculate whether these proteins act in the same pathway and/or whether they act in concert. Identification of such target genes is critical for understanding the function and reason for diversity of the PLAG family members.

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