Identification of the p33ING1-regulated Genes that Include Cyclin B1 and Proto-oncogene DEK by Using cDNA Microarray in a Mouse Mammary Epithelial Cell Line NMuMG

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Abstract

The candidate tumor suppressor p33ING1 plays an important role in inducing growth arrest at G0-G1 phase of the cell cycle and/or promoting apoptosis in cancerous cells. p33ING1 is reported to act as a transcriptional cofactor by associating with tumor suppressor p53, HAT, or histone deacetyltransferase, suggesting that p33ING1 is involved in chromatin-mediated transcriptional regulation. However, the molecular mechanism of p33ING1-mediated transcriptional regulation is poorly understood. Here we analyzed expression profiles in mouse mammary epithelial cells (NMuMG) by using a cDNA microarray consisting of 2304 mouse cDNAs after induction with antisense inhibitor of growth 1 (ING1) in retrovirus vector. The subsequent confirmation of the altered expression levels of the selected genes by semiquantitative reverse transcription-PCR demonstrated that overexpression of the antisense ING1 stimulated expression of 14 genes, which included cyclin B1, 12-O-tetradecanoylphorbol-13-acetate-inducible sequence II, proto-oncogene DEK, and osteopontin, whereas we have detected transcriptional repression of 5 genes, including TPT1. In addition, adeno-virus-mediated overexpression of ING1 in NMuMG cells resulted in down-regulation of cyclin B1, 12-O-tetradecanoylphorbol-13-acetate-inducible sequence 11, DEK, and osteopontin, whereas the levels of TPT1 expression were increased. The further analysis using p33ING1 SAOS2 cells showed that the p33ING1-induced cyclin B1 down-regulation was p53 dependent. Our cDNA microarray analysis suggested that p33ING1 targets the multiple genes, including proto-oncogene DEK and cyclin B1, at least some of which are regulated in a p53-dependent manner, in the cells undergoing cell growth or apoptosis.

Introduction

A novel candidate tumor suppressor ING1 has been identified using a positive selection procedure that combined a PCR-mediated subtractive hybridization of cDNAs derived from normal and cancer cells with an in vivo selection assay (1). ING1 mRNA is expressed ubiquitously in adult mouse tissues, whereas its expression levels varied among them (2). ING1 is present in at least three variants (p47ING1a, p33ING1b, and p24ING1c) arising from the differential initiation and the splicing of mRNAs (2, 3). These ING1 variants share the nuclear localization signal, as well as the evolutionarily conserved PHD finger domain, which has been found in various transcription factors and proteins involved in chromatin-mediated transcriptional regulation (4). ING1 was also reported to be associated with HAT activity (5) or histone deacetyltransferase-dependent transcriptional repression, indicating that ING1 is involved in chromatin remodeling (6).

Inhibition of endogenous ING1 expression by antisense RNA results in the anchorage-independent growth in soft agar medium and promotes the tumor formation in nude mice (1). In addition, forced expression of antisense RNA prolongs the proliferative life span of normal human fibroblasts (7). Conversely, ectopic overexpression of ING1 leads to the G0-G1 arrest of the cell cycle or apoptosis in different experimental systems (1, 8). Accordingly, the levels of ING1 expression are regulated in a cell cycle-dependent manner and are also increased during the serum starvation-induced apoptosis in mouse teratocarcinoma cells (7, 8). In addition, decreased expression of ING1 was observed in cells of several breast carcinomas and gliomas (9, 10). Intriguingly, ING1 was physically associated with tumor suppressor p53 and activated p53-mediated transcriptional activation and growth suppression (10, 11). These observations suggest that ING1 contributes to the regulatory mechanism of cell cycle progression, cellular aging, and apoptosis.

ING1 has been mapped to human chromosome 13q33-34, which is the locus for the candidate tumor suppressor gene(s) of various human cancers (9, 12–15). As reported previously, the ING1 gene was rearranged in a neuroblastoma cell line, SK-N-SH, generating a truncated gene product (1). Several missense mutations have been reported in primary esophageal squamous cell cancers and head and neck squamous cell carcinomas (3, 16). Those mutations were detected within the nuclear localization signal or the PHD finger domain of ING1, indicating that normal function of ING1 may be abrogated in some of those cases. However, the frequency of ING1 mutation was quite low in these tumors, and no mutations were detected in breast and ovarian cancers (3, 9, 16). Thus, it is still unclear whether or not ING1 could act as a classic Knudson-type tumor suppressor.

In the present study, we analyzed expression profiles of 2304 genes by using cDNA microarray in mouse mammary epithelial cells (NMuMG) after antisense ING1-induced transformation. The subse-
quent confirmation of the candidate target genes by semiquantitative RT-PCR identified 14 and 5 genes whose expression levels were up- and down-regulated by overexpression of antisense ING1, respectively. Furthermore, adenovirus-mediated overexpression of sense ING1 in NMuMG cells confirmed the target genes that included cytogen B1 and proto-oncogene DEK.

Materials and Methods

Cell Culture. NMuMG (mouse epithelial cell line from mammary gland) cells, NIH3T3 cells, and human osteosarcoma SAOS2 cells were grown in DMEM in 10% (volume for volume) heat-inactivated fetal bovine serum in the presence of 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were maintained under an atmosphere of 5% CO2 at 37°C.

GSE Method. GSE assay was performed as described previously (1). In brief, a cDNA fragment encoding a COOH-terminal region of ING1 was amplified by PCR-based strategy, and subcloned into the pHoA restriction site of the plXSN vector in an antisense orientation to give pLSX-antisense ING1. pLSX or pLSX-antisense ING1 was transfected into the p2 packaging cells, and NMuMG cells (1 × 106 cells) infected with virus containing culture medium were injected into the lateral subcutis of athymic nude mice. The sequence of 388 bp for antisense ING1 was designed to block all isoforms at the region, including the PHD finger domain within exon 2 as follows: 5'-gattagttgcctcttcgcgaaggaaagcagcagtaattgtggctttacgcttctcctcacaccaggcgcctgtccacaaactac-ctggagagttcccttcgcgaaggaaagcagcagtaattgtggctttacgcttctcctcacaccaggcgcctgtccacaaactac-ctggagagttcccttcgcgaaggaaagcagcagtaattgtggctttacgcttctcctcacaccaggcgcctgtccacaaactac.

Growth-inhibitory Activity of ING1. Consistent with the previous report (1), NMuMG cells infected with virus-containing pLXSN-antisense ING1 TARGET GENES DETECTED BY cDNA MICROARRAY

ING1 was incubated with 4.5 μg of oligodeoxynucleotide primer (in a total volume of 15.4 μl) at 70°C for 10 min and chilled on ice. Then we added 6 μl of 5 × SuperScript II buffer, 3 μl of 100 mM DTT, 0.6 μl of deoxynucleotide triphosphate mixture (25 nem dATP, dCTP, dGTP, and 10 nem dTTP), 3 μl of 1 nem Cy3- (mRNA from pLXSN-antisense ING1, infected cells) or Cy5- (mRNA from empty vector-infected cells) dUTP, and 400 units of SuperScript II. After the incubation at 42°C for 1 h, template mRNAs were degraded at 65°C for 10 min in the presence of 1.5 μl of 1N NaOH/20 mM EDTA. The samples were neutralized with 270 μl of TE (pH 8.0) and 1.5 μl of 1N HCl. Hybridization was performed in a solution containing 2 mg/ml yeast RNA (Sigma Chemical Co., St. Louis, MO), 2 mg/ml poly(A) (Roche, Basel, Switzerland), 3.4 × SSC, and 0.3% SDS at 65°C overnight under humidified condition. After the hybridization, the arrays were washed twice for 5 min with 2 × SSC/0.1% SDS at room temperature, twice for 5 min with 0.2 × SSC/0.1% SDS at 40°C, and finally rinsed with 0.2 × SSC. The arrays were centrifuged at 1000 rpm for 1 min and then scanned with a fluorescence laser-scanning device (ScanArray4000; GSI Lumonics).

Western Analysis. Total cell lysates were separated on a 10% SDS-polyacrylamide gel and electrotransferred onto nitrocellulose membrane. The membrane was probed with a monoclonal antibody against ING1 (kindly provided by K. Riabowol). The membrane was visualized with an enhanced chemiluminescence detection system (enhanced chemiluminescence; Amer sham Pharmacia Biotech, Piscataway, NJ).

Results

Growth-inhibitory Activity of ING1. To identify genes involved closely in ING1-induced growth and/or tumor suppression, we decided to use the mouse breast epithelial cell line (NMuMG cells). Consistent with the previous report (1), NMuMG cells infected with the recombinant retrovirus vector for the antisense ING1 cDNA...
(pLXSN-antisense ING1) strongly inhibited expression of ING1 proteins (Fig. 1A) and promoted the anchorage-independent growth in soft agar medium, compared with cells infected with the empty vector (pLXSN; Fig. 1B). Similarly, down-regulation of ING1 expression by the antisense construct increased the frequency of focus formation in NIH3T3 cells (Fig. 1C). To examine the effect of antisense ING1 on the tumorigenicity, NMuMG cells infected with the pLXSN-antisense ING1 or the pLXSN were injected s.c. into nude mice. Eight weeks after the injection, four of the five animals injected with cells expressing antisense ING1 carried tumors, whereas only one of the five animals with control cells developed a small tumor (Fig. 1D). Thus, our system appeared to be suitable for searching the ING1-regulated genes.

**Gene Expression Analysis by cDNA Microarray.** We performed the expression analyses of 2304 genes in NMuMG cells infected with pLXSN-antisense ING1 by means of cDNA microarray. The cDNA probes derived from NMuMG cells infected with pLXSN-antisense ING1 or the empty vector were fluorescently labeled with Cy3-dUTP (red) or Cy5-dUTP (green), respectively. These probes were applied simultaneously onto the microarray, and the two fluorescent images were scanned with a fluorescence laser-scanning device. Each signal was normalized so that the Cy3:Cy5 intensity ratio of the housekeeping gene (H9252/α-actin) signal was 1. Red and green fluorescent signals indicated genes whose expression levels were relatively higher in cells infected with pLXSN-antisense ING1 and the backbone vector, respectively. Yellow signals indicate genes with equal expression in both cells. In the present study, we considered genes that exhibited a Cy3:Cy5 ratio > 1.5 as candidates of up-regulated genes and those with a Cy3:Cy5 ratio < 0.67 as candidates of down-regulated genes. Fig. 2A shows the scatter plots of the average mRNA expression of 2304 genes. Table 1 lists the genes that have shown a ≥1.5-fold increase or decrease in expression level in at least three of four independent experiments. Sixteen (0.7%) and 9 (0.4%) genes displayed relatively higher and lower expression levels during the antisense ING1-induced transformation, respectively. Each of the up- and down-regulated genes included two ESTs. Fig. 2B shows a representative result of microarray detection. Serum albumin, aldehyde dehydrogenase II, cyclin B1, TIS11, and proto-oncogene DEK displayed red signals, whereas RP S29 and TPT1 showed green signals. Serum albumin, AFP, and aldehyde dehydrogenase II were induced by the antisense ING1 to an extent that was 5.8, 5.4, and 2.8 times greater than the control infection, respectively, whereas IGF-II receptor, myosin light chain, SDR gene, and TDE1 were slightly activated.

**Semiquantitative RT-PCR Analysis.** To examine the reliability of the expression changes detected by the profiling analysis using the cDNA microarray, the semiquantitative RT-PCR analysis with the same RNA samples that had served for the microarray analysis was performed. In agreement with the microarray results, the expression

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**Fig. 1. Growth-inhibitory activity of ING1. A, decreased expression of the ING1 protein in NMuMG cells after stable infection by retrovirus vector carrying antisense-ING1. Western blot analysis. B, soft agar colony formation assay. A total of 5 × 10⁴ NMuMG cells was infected with pLXSN (a) or pLXSN-antisense ING1 (b). After growth for 5 weeks in the soft agar medium, the number of colonies formed in soft agar was counted. Representative results are shown. C, in vitro focus formation. NIH3T3 cells (5 × 10⁵ cells/10-cm dish) infected with pLXSN (a) or pLXSN-antisense ING1 (b) were grown in DMEM containing 5% fetal bovine serum for 4 weeks. Cells were then fixed in methanol and photographed, and the number of foci was scored. Representative results are shown. D, tumor formation in nude mice. Six-week-old nude mice (BALB/c-nu) were given injections of 1 × 10⁶ NMuMG cells infected with pLXSN (a) or pLXSN-antisense ING1 (b). Experiments were performed with five animals in each injection. Mice were examined for tumor formation over an 8-week period, and the representative cases are shown.
levels of 14 of 16 genes (87.5%), which were considered as up-regulated genes by the microarray analysis. The cDNA microarray was hybridized with Cy3- or Cy5-labeled probe prepared from cells infected with pLXSN-antisense ING1 or pLXSN, respectively. The expression levels were analyzed with the Quant Array computer program. The red spots in this graph represent genes whose expression levels changed >1.5-fold in at least three of four independent experiments. The axes scales are logarithmic.

B, a representative cDNA microarray analysis. Green and red color represent genes whose expression levels were significantly down- and up-regulated in cells infected with pLXSN-antisense ING1, respectively. Yellow spots show equal expression in both cells. Graduated color patterns indicate the degrees of expression changes.

Spots 1–7: RP S29, serum albumin, aldehyde dehydrogenase II, cyclin B1, TIS11, TPT1, and proto-oncogene DEK, respectively.

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Initiation of the \( p21^{\text{WAF1}} \) transcription was slightly delayed (at 12 h after the infection) compared with that of ING1. We subsequently examined whether the overexpression of ING1 could affect the expression pattern of the selected genes. Consistent with the results obtained by the cDNA microarray followed by the semiquantitative RT-PCR, the endogenous level of cyclin B1, TIS11, and DEK mRNAs was strikingly reduced in a time-dependent manner by the forced expression of ING1. Of interest, the initiation of the transcriptional down-regulation of these genes (at 48 h after the infection) was delayed significantly compared with that of ING1 accumulation. Similar delayed patterns of down-regulation were observed in osteopontin expression; however, the extent of the reduction was relatively low. On the other hand, the accumulation of TPT1 mRNA was detected at 18 h after the infection, slightly delayed compared with that of \( p21^{\text{WAF1}} \). These results verified the reliability of our cDNA microarray analysis to identify genes that could be involved in ING1-mediated transcriptional regulation.

**ING1-induced Suppression of Cyclin B1 Is p53 Dependent.** As described previously, ING1 cooperates directly with p53 by modulating p53-dependent transcription (10, 11). To examine whether the ING1-mediated down-regulation of cyclin B1 expression was dependent on p53, p53-deficient SAOS2 cells were infected with recombinant adenovirus encoding ING1 (Ad-ING1). Cells were infected with 10 MOI of Ad-ING1 and grown for the indicated time periods before RNA preparation. RT-PCR was carried out under linear amplification conditions. GAPDH expression is shown as a control. The expression level of ING1 was analyzed by Western blotting with a monoclonal anti-ING1 antibody.

### Table 1 Genes of up- or down-regulated by overexpression of antisense ING1

| Gene                        | Fold change
|-----------------------------|-------------
| Serum albumin               | 4.44        |
| AFP                         | 4.19        |
| Aldehyde dehydrogenase II   | 3.62        |
| Cyclin B1                   | 2.25        |
| TIS11                       | 2.11        |
| DEK                         | 1.70        |
| Osomotic stress protein     | 1.61        |
| EST-3                       | 1.94        |
| EST-4                       | 1.65        |
| γ actin                     | 1.90        |
| TDE 1                       | 1.53        |
| EST-1                       | 0.66        |
| RP S11                      | 0.66        |
| EF-2 (elongation factor-2)  | 0.59        |
| RP S7                       | 0.52        |
| EST-2                       | 0.54        |
| TPT1                        | 0.55        |
| RP S29                      | 0.66        |
| Int-6                       | 0.68        |
| RP L12                      | 0.67        |

a Genes with a >1.5-fold difference in signal intensity between NMuMG cells infected with pLXSN and those with pLXSN-antisense ING1 are presented. Fold change indicates a ratio of signal intensity (Cy3:Cy5).

**Fig. 3.** Semiquantitative RT-PCR analysis using RNA from NMuMG cells infected with an empty vector or pLXSN-antisense ING1. A, genes with up-regulated expression in cells infected with pLXSN-antisense ING1. B, genes with down-regulated expression in cells infected with pLXSN-antisense ING1. GAPDH expression is shown as a control. GenBank accession nos. of EST-1, EST-2, EST-3, and EST-4 are AI573918, X75312, AU080496, and AF111103, respectively.

**Fig. 4.** Time course of \( p21^{\text{WAF1}} \), TPT1, cyclin B1, TIS11, DEK, and osteopontin expression in NMuMG cells infected with recombinant adenovirus encoding ING1 (Ad-ING1). Cells were infected with 10 MOI of Ad-ING1 and grown for the indicated time periods before RNA preparation. RT-PCR was carried out under linear amplification conditions. GAPDH expression is shown as a control. The expression level of ING1 was analyzed by Western blotting with a monoclonal anti-ING1 antibody.

**Fig. 5.** Overexpression of ING1 alone caused no significant
change in cyclin B1 expression in SAOS2 cells. In contrast, a remarkable decrease in cyclin B1 expression level was observed 48 h after the infection with both Ad-ING1 and Ad-p53. Thus, it is likely that p53-dependent mechanism contributes to the down-regulation of cyclin B1 expression by ING1. DEK also had a tendency to be down-regulated when both Ad-ING1 and Ad-p53 were infected into SAOS2 cells (data not shown).

Discussion

A cDNA microarray technology has been shown to be a powerful as well as a convenient strategy for investigating complex changes in patterns of gene expression (20–22). A candidate tumor suppressor ING1 has a key role as a negative regulator of cell proliferation (1, 7, 8, 11). Recently, it has been shown that ING1 is associated with HAT activity or HDAC, indicating that ING1 might be involved closely in chromatin-mediated transcriptional regulation (5, 6). However, the ING1-mediated intracellular signaling pathways have not yet been elucidated clearly. To understand the genetic basis of the ING1-induced antiproliferative effect, we used a high-density microarray of 2304 mouse cDNAs to search for differences in gene expression associated with tumorigenicity induced by the antisense ING1 expression in NMuMG cells.

By means of the simultaneous two-color fluorescence hybridization, we have identified 16 genes (0.7% of 2304 transcripts) whose expression level was significantly increased and 9 genes (0.4% of 2304 transcripts) that were down-regulated on the overexpression of antisense ING1. Subsequent semiquantitative RT-PCR analysis revealed that 88% (14 of 16 genes) and 57% (5 of 9 genes) of the selected genes were confirmed to be up- and down-regulated, respectively. These results suggested that our cDNA microarray analysis was reliable. The recent cDNA microarray analysis showed that when the tumor suppressor PTE1 was overproduced in PTE1-deficient cells, 2.5 and 1.8% of 4009 genes were up- and down-regulated, respectively (23).

Overexpression of ING1 by adenovirus-mediated ING1 gene transfer to NMuMG cells significantly reduced the expression level of cyclin B1, TPT1, DEK, and osteopontin in a time-dependent manner. Among them, down-regulation of cyclin B1 and DEK was extremely intriguing. DEK was identified initially as a fusion partner of the putative oncogene product, CAN, in a subtype of acute myelogenous leukemia, and the DEK-CAN fusion protein appeared to be oncogenic (24). Recently, it has been shown that DEK is a nuclear protein that binds specifically to the HIV-2 enhancer or cooperates with histone H2A/H2B to change the topology of nucleosomal DNA (25, 26). It is of great interest to note that DEK is highly expressed in human hepatocellular carcinoma as compared with matched normal liver tissues (27), raising a possibility that ING1-mediated alteration of DEK expression is involved in the development of the malignant phenotypes.

Cyclin B1, which accumulates during G2-M phase of the cell cycle, is the regulatory subunit of the cdc2 protein kinase, and cdc2/cyclin B1 complex is required for mitotic initiation (28). In contrast, ING1 protein level starts to be increased at the late G1, reaching a maximum in S phase followed by a significant decrease in G2 (7). The significant reduction of cyclin B1 expression caused by the adenovirus-mediated overproduction of ING1 was in good agreement with the cell cycle-dependent expression patterns of cyclin B1 and ING1. However, it is conceivable that the effect of overexpression of p33ING1 or suppression of cyclin B1 expression could be secondary to the growth inhibition. This is because that ING1 protein itself has no DNA-binding activity and that it indirectly regulates transcriptional activities of p53 or HDAC by binding to either protein. In response to genotoxic stress induced by DNA damage, p53 is activated and inhibits G1-S transition in cells (29). In addition, p53 also plays an important role in regulating G2-M transition. Overexpression of p53 induced G2-M growth arrest in mammalian cells (30, 31). Recently, it has been shown that p53-dependent G2-M arrest was associated with a reduction in the rate of cyclin B1 transcription, followed by a decrease in intracellular cyclin B1 level (32–34). p53-/- mouse mammary adenocarcinomas express significantly higher levels of cyclin B1 as compared with those of p53+/+ counterparts (34). Consistent with the notion that p53 represses the transcription from a variety of promoters lacking the consensus p53-binding site (35), cyclin B1 promoter does not contain the putative p53-binding site (32, 33), suggesting that p53 down-regulates the transcription of cyclin B1 without binding to the cyclin B1 promoter. Intriguingly, ING1 was associated functionally with the HDAC1-mediated transcriptional repression (6). In addition, infection of p53 alone induced down-regulation of cyclin B1 expression, suggesting that endogenous ING1 cooperates with p53 to induce the down-regulation of cyclin B1 or that p53 may also act to inhibit cyclin B1 expression through the ING1-independent pathway. Furthermore, infection with ING1 alone in the p53-deficient SAOS cell line also resulted in down-regulation of cyclin B1 at 72 h, suggesting that ING1 also has a p53-independent effect on gene expression. Although the precise molecular mechanism by which ING1 represses the cyclin B1 transcription remains to be elucidated, it could be possible that p53 cooperates with ING1/HDAC1 complex in negative regulation of cyclin B1 expression. Our present results also supported this possibility.

Although ING1 was mutated rarely in primary breast and ovarian cancers, as well as breast cancer cell lines, the expression level of ING1 was reduced significantly in breast cancer cells as compared with that of normal mammary epithelial cells (9). Keyomarsi and Pardee (36) have reported that the majority of human breast cancer cell lines overexpressed cyclin B1. This inverse correlation between the expression levels of ING1 and cyclin B1 in breast cancer cells strongly supports the idea that ING1-mediated alteration of cyclin B1 expression may be responsible for the uncontrolled cell growth and tumor development.

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References


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