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MUTATED *RAS* INDUCED *PLD1* GENE EXPRESSION THROUGH INCREASED Sp1 TRASCRIPTION FACTOR

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ABSTRACT

The underlying mechanisms of oncogene-induced phospholipase D (PLD) activation have not been fully elucidated. The effect of the mutated-*ras* on *PLD* mRNA was examined using colon cancer cell lines as well as mock- and mutated *ras*-transfected NIH3T3 cells. *Ras*-mutation and activation were correlated, and cells with enhanced ras-activation showed increased *PLD1* mRNA and protein. Analysis of the 5' *PLD1* promoter using a representative cell line, DLD-1 and also mutated *ras*-NIH3T3, showed one Sp1-site as the important *ras*-responsible motif. Sp1 inhibition with mithramycin A and Sp1 siRNA inhibited PLD1 protein expression and its promoter activity. Sp1 but not Sp3 protein level and increased Sp1-motif binding activity were correlated with *ras* ativation. Furthermore, overexpression of Sp1 in drosophila SL2 cells lacking Sp family proteins increased PLD1 promoter activity. EMSA and chromatin immunoprecipitation assay confirmed the importance of Sp1 protein binding to the Sp1-motif in *ras*-induced *PLD1* mRNA expression.

Key Words: Mutated *ras*, *PLD1* mRNA, Sp1 transcription factor, Promoter analysis, ChIP assay

INTRODUCTION

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline.[1)] Two PLD isoforms, PLD1 and PLD2, have been cloned.[2)] PLD plays a role in cell survival and proliferation[3)]. The regulation of PLD activity has been reported,[1,2)] and PLD activity is elevated in transformed cells by various oncogenes.[2)] Increased PLD activity was also observed in several clinical cancers.[4)] A mutation in *ras* gene has been shown to induce PLD enzyme activation.[5)] Although the increased gene expression leading to increased protein level might be one of the important causes of PLD activation observed in cancer cells, the mechanism of oncogene-induced *PLD* gene expression has not been elucidated before.

In the present study, we analyzed *PLD1* and *PLD2* mRNA levels and its regulatory mechanism in human colon cancer cell lines with or without mutated *ras.* NIH3T3 cells stably transfected

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with either mutated *Hras or* mock-expression vector were also analyzed to confirm the direct involvement of mutated *ras* on *PLD1* gene expression. Our results demonstrate, for the first time, that mutated *ras* increases *PLD1* but not *PLD2* gene transcription, mainly due to the increased Sp1 transcription factor that binds to the Sp1-motif of the 5' promoter of *PLD1* genome.

MATERIALD AND METHODS

Cell lines and reagents

NIH3T3 cells, mock-NIH3T3 cells and mutated *Hras* (V12G)-NIH3T3 cells were previously described.[6] Human colon cancer cell lines, HT-29 and HCT 116, DLD-1 and Caco-2 cells were from Dr. M. Kyogashima (Aichi Cancer Center, Nagoya, Japan). A drosophila cell line, SL2, supplied by Prof. T. Noguchi (Osaka Otani University, Osaka, Japan), was cultured in Schneider's medium (Invitrogen) with 10% FCS. Sp series expression vectors for SL2 cells, pPac, pPac-Sp1, and pPac-USp3 have been described previously.[7] pPac-RL[8] was used for the correction of transfection efficiency of SL2 cells. siRNA of Sp1 (pSilencer siRNA) and a control RNA (pSilencer) were the generous gifts from Dr. D.E. Vance[9], and were transfected using Lipofectin (Invitrogen). Mithramycin A was purchased from Fulka (Buchs, Switzerland). Schnieder's drosophila medium and Lipofectin was purchased from Invitrogen (Carlsbad, CA, USA).

Ras activation assay

To evaluate the cellular Ras activity, we used the EZ-Detect Ras activation kit (Pierce Biotechnology, Rockford, IL, USA). Western blotting analysis was performed to measure sepharose-bound activated Ras using anti-Ras antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Western blotting

Western blotting using anti-PLD1, anti-PLD2 and anti-β-actin antibodies was performed as described previously.[10] Anti-pan-Ras, anti-Sp1, and anti-Sp3 were from Santa Cruz.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR of human *PLD1, PLD2* and *GAPDH* was described previously.[10] Semi-quantitative RT-PCR of mouse *PLD1*, *PLD2* and *β-actin* was performed with the primer sets described below. In preliminary experiments, suitable amounts of cDNA and the range of PCR cycles that permit the linear amplification of *PLD1* and *PLD2* were determined. Mouse *PLD1* primers were forward, 5'-CGTCCCCGCCAAAGTGCAG-3'; and reverse, 5'-CCGATATCTCTG-GCCTTCCCTGT-3'. Mouse *PLD2* primers were forward, 5'-TGCTCCCTTTGGCTCGCTTT-3'; and reverse, 5'-GGATCACCCCTTCCAGTCCTTT-3'. Mouse *β-actin* primers were forward, 5'-CCGTGAAAAGATGACCCAGA-3'; and reverse, 5'-GTCTCCGGAGTCCATCACAA-3'. The PCR conditions for mouse *PLD1, PLD2* and *β-actin* were 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The numbers of cycles of mouse *PLD1*, *PLD2*, and *β-actin* were 26, 28 and 30; 28, 30 and 32; and 20, 22 and 24, respectively. Band intensities of mouse *PLD1* at 28 cycles, mouse *PLD2* at 30 cycles and mouse *β-actin* at 22 cycles were measured by NIH image version 6. The relative expression levels of mouse *PLD1* and *PLD2* mRNA were calculated as the ratio of *PLD/β-actin*.

5' RACE of PLD1 of DLD1 cells and PLD1 promoter construct preparation

The transcription initiation site of human *PLD1* of DLD-1 cells was determined with the RNA ligase-mediated rapid amplification methods of 5' cDNA ends (5' RACE) using a Gene Racer

kit (Invitrogen). The primer sets were described previously.[10] The luciferase vectors containing 5' promoter of human *PLD1* (originally: –674 bp/Luc, renamed –675 bp/Luc in the current study based on a 5' RACE study of DLD-1 cells), and their truncated forms were described previously.[10] Three additional *PLD1* promoter truncated mutants (–154 bp/luc, –109 bp/Luc and –80 bp/Luc) were obtained using the PCR-based method. To introduce a mutation to the two Sp1 motifs, the following primer sets were prepared, and PCR was performed using –154 bp/Luc as a template. The mutant designated as M1 contained a mutation in the distal Sp1 motif. M2 contained a mutation in the proximal Sp1 motif, whereas MD contained both distal and proximal Sp1 motives (Fig. 3b left). GL primer 2 was used as the lower primer. Primer sequences were M1 upper, 5'-TTTCTCGAGCGGGACCTGGCAaataacatCTCCACCCCTGCGAATCCGG-3'; M2 upper, 5'-TTTCTCGAGCGGGACCTGGCACCGCCTCGCTattagtaTGCGAATCCGGGGCGAAT-3'; and MD upper, 5'-TTTCTCGAGCGGGACCTGGCAaataacatCTattagtaTGCGAATCCGGGGCGAAT. The *Xho*1 enzyme site is underlined, and the mutated Sp1 motif is described in lowercase letters.

Promoter assay

Promoter analysis of *PLD1* using NIH3T3 series cells were performed using a calcium precipitation method. DLD-1 cells were transfected using Lipofectin (Invitrogen). In some experiments, mithramycin A was added 24 h before measuring the luciferase activity. Luciferase and β-gal activities were measured, and the promoter activity was normalized with β-gal activity.

Sp1 TransLucent Reporter Assay

Sp1 TransLucent reporter vector (Sp1(2)) designed to monitor the transcription-factor binding activity of the Sp family was from Panomics (Redwood City, CA). It contains two consensus Sp1 binding motifs as shown in Fig. 4b. Caco-2 and DLD-1 cells were transfected with 2 μg of either the control TransLucent vector or the Sp1(2) vector using Lipofectin, with 1 μg of *β-gal* expression vector being cotransfected to normalize the promoter activity. The reporter activity was normalized with β-gal activity.

Transfection to SL2 cells

pPac series expression vectors was transfected by the calcium precipitation method. Relative reporter activity was calculated as pPac-Sp/pPac-RL.

Electrophoresis mobility shift assay (EMSA) and chromatin immunoprecipitation assay (ChIP)

Nuclear extract was prepared from Caco-2 and DLD-1 cells. EMSA and CHIP assay were performed according to the method already described. [10] Sequences of wild-type oligo containing the distal Sp1 motif shown in Fig. 3b and mutated oligo used for EMSA were as follows. wild type oligo; CCTGGCACCGCCTCGCTCCA, mutated oligo; CCTGGCA*AATAACAT*CTCCA. Italic letters were mutated Sp1 motif. Supershift assay was conducted using anti-Sp1 and anti-Sp3 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

For ChIP assay, normal rabbit (control) IgG, anti-Sp1, or anti-Sp3 antibody (Santa Cruz, 1 μg/sample) were added and incubated overnight at 4°C. After DNA extraction, the *PLD1* promoter region containing the analyzed Sp1 motif was amplified by PCR using the following primers, 5'-TGGGAAAAGAGAACAAGGAACGC-3' (forward); and 5'-GCGGACTCTCAGGGCTCGG-3' (reverse). The size of the PCR product was 251 bp.

RESULTS

Correlation between Ras activity and PLD1 gene expression

Among the colon cancer cell lines analyzed, Ras activity correlated with cellular PLD1 protein level. DLD-1 and HCT 116 with high Ras activity were reported as mutated *ras* positive, whereas Caco-2 and HT-29 with low Ras activity possessed wild-type *ras* (Fig. 1a).[11,12] There was no significant relationship between Ras-activity and the PLD2 protein level. In further experiments, we focused mainly on DLD-1 and Caco-2 cell lines as the representative of cell lines with or without mutated-*ras*. Consistent with PLD protein levels, *PLD1* but not *PLD2* mRNA was higher in DLD-1 cells than Caco-2 cells (Fig. 1b). Furthermore, stable transfection of a mutated *Hras* into NIH3T3 cells induced PLD1 but not PLD2 protein nor its mRNA (Fig. 1a and 1b) compared with those of mock-transfected cells, providing additional support for the involvement of mutated *ras* in PLD1 induction.

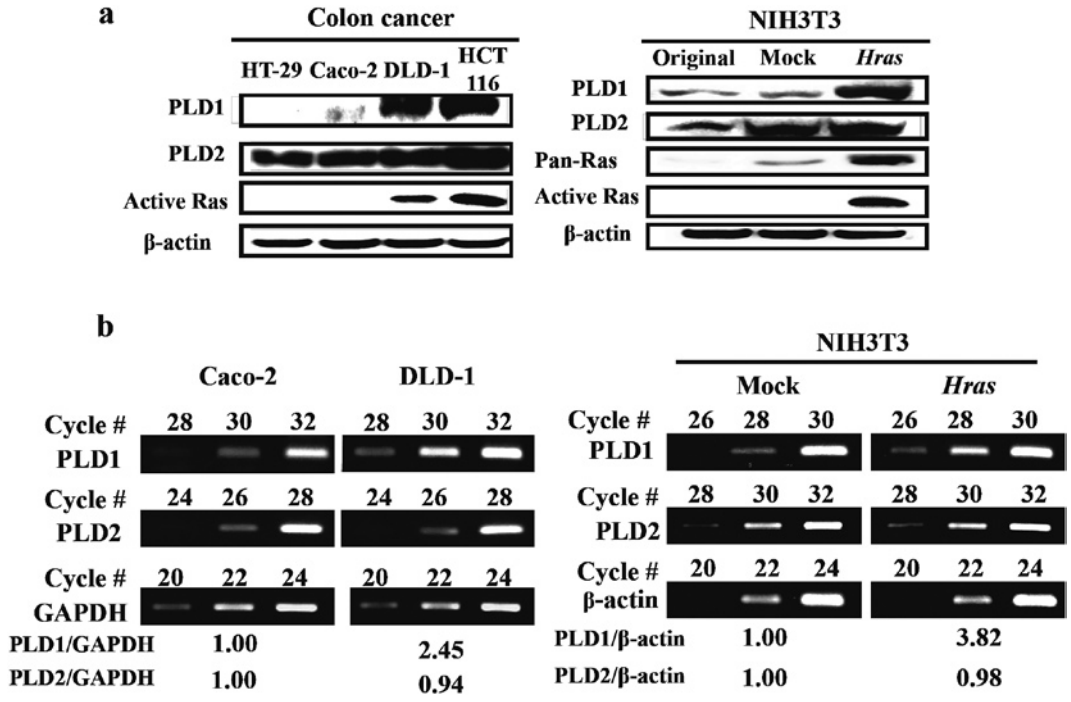

Fig. 1 Mutated *ras*-induced PLD1 expression.
PLD1, PLD2 and β-actin proteins of HT-29, Caco-2, DLD-1, and HCT 116 cells are shown. Results of ras activation assay performed according to Materials and Methods are also illustrated. (b) *PLD1* and *PLD2* mRNA levels were measured by the semi-quantitative RT-PCR as described in Materials and Methods. Cycle # denotes the number of PCR cycles. Relative *PLD1* and *PLD2* mRNA levels are described as the ratio of *PLD/GAPDH*. Relative mRNA levels of Caco-2 are regarded as 1.0, respectively. Results of PLD protein and *PLD* mRNA levels of original NIH3T3, mock-NIH3T3 and mutated *Hras*-NIH3T3 cells are also illustrated. Anti-pan Ras antibody was used to confirm the overexpression of mutated *Hras*. In the case of NIH3T3, the internal control of semi-quantitative RT-PCR was β-actin. Relative mRNA levels of mock-NIH3T3 are regarded as 1.0.

Promoter analysis of the 5'promoter of PLD1

Based on our 5'RACE of DLD-1 cells (Fig. 2a), we cloned the 5' promoter of human *PLD1*. The region between –154 bp and –109 bp of 5' promoter of *PLD1* was important for the *PLD1* gene expression of DLD-1 cells (Fig. 2b). Among two Sp1 sites located within this region, the distal Sp1 site is primarily important for PLD1 expression in DLD-1 cells. Direct comparison of the promoter activity between DLD-1 and Caco-2 was difficult mainly due to the low transfection efficiency in Caco-2 cells. Therefore, we examined mutated *Hras*-NIH3T3 and mock-transfected NIH3T3 cells to exclude the involvement of factors other than mutated-*ras*. Figures 3a and b also illustrate the importance of the same region in mutated *ras*-NIH3T3 cells as compared with mock-NIH3T3 cells. The proximal Sp1 motif was observed to be equally potent in *Hras*-NIH3T3 cells. Mutation of these two sites further inhibited promoter activity of *PLD1* in mutated *ras*-NIH3T3 cells.

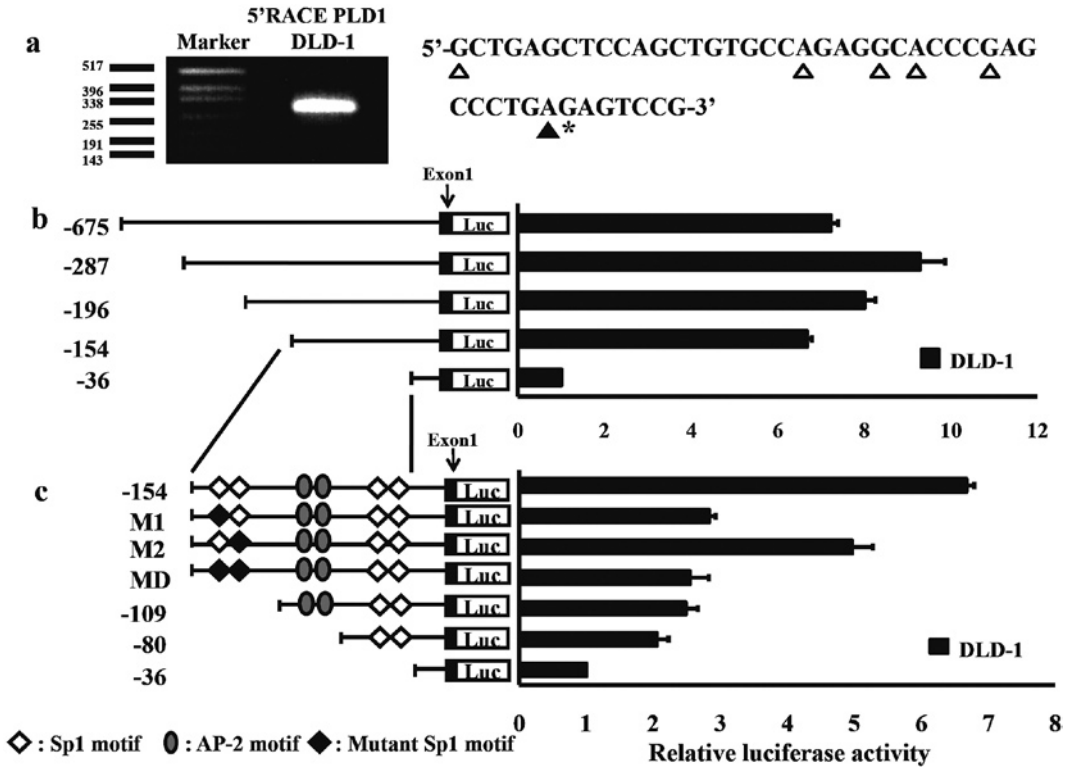

Fig. 2 5' RACE analysis and *PLD1* promoter analysis of DLD1 cells.
5' RACE was performed to determine the transcription start site of *PLD1* mRNA in DLD-1 cells as described in Materials and Methods. The right side illustrates several start sites (triangles) determined in our analysis by cloning and sequencing the band observed in the left side gel. An asterisk denotes the transcription start site available in the NCBI database. A solid triangle identifies the start site of exon 1 used in the present experiments. (b) and (c), *PLD1* promoter analysis performed using various truncated luciferase vectors as illustrated in the left part of the figure. Using DLD-1 cells, a reporter assay was performed as described in Materials and Methods. In the lower part (c), data on a further truncation and the introduction of mutations into Sp1 sites are shown. M1 denotes the distal Sp1 site mutation (solid square), while M2 shows the proximal Sp1 site mutation. MD means the mutation of both Sp1 sites. Other motifs were also illustrated in the figure.

Involvement of Sp1-motif and Sp family transcription factors in PLD1 gene expression

Mithramycin A, an inhibitor of Sp function, and siRNA of Sp1 (pSilencer siRNASp1) were used to analyze the involvement of Sp family transcription factors in *PLD1* gene expression (Fig. 3c and 3d). Results clearly show that these Sp1 motifs and Sp1 protein were necessary for mutated *ras*-induced *PLD1* gene expression. Among the cellular Sp family proteins analyzed, a marked increase of Sp1 but not Sp3 protein was observed in high PLD1-expressed colon cancer cell lines with mutated *ras*. Similarly, increased Sp1 protein was observed in mutated *Hras*-NIH3T3 but not in mock-NIH3T3 (Fig. 4a). In mutated *Hras*-NIH3T3, Sp1 mRNA measured by semi-quantitative RT-PCR was higher than those of original and mock-NIH3T3 cells (data not

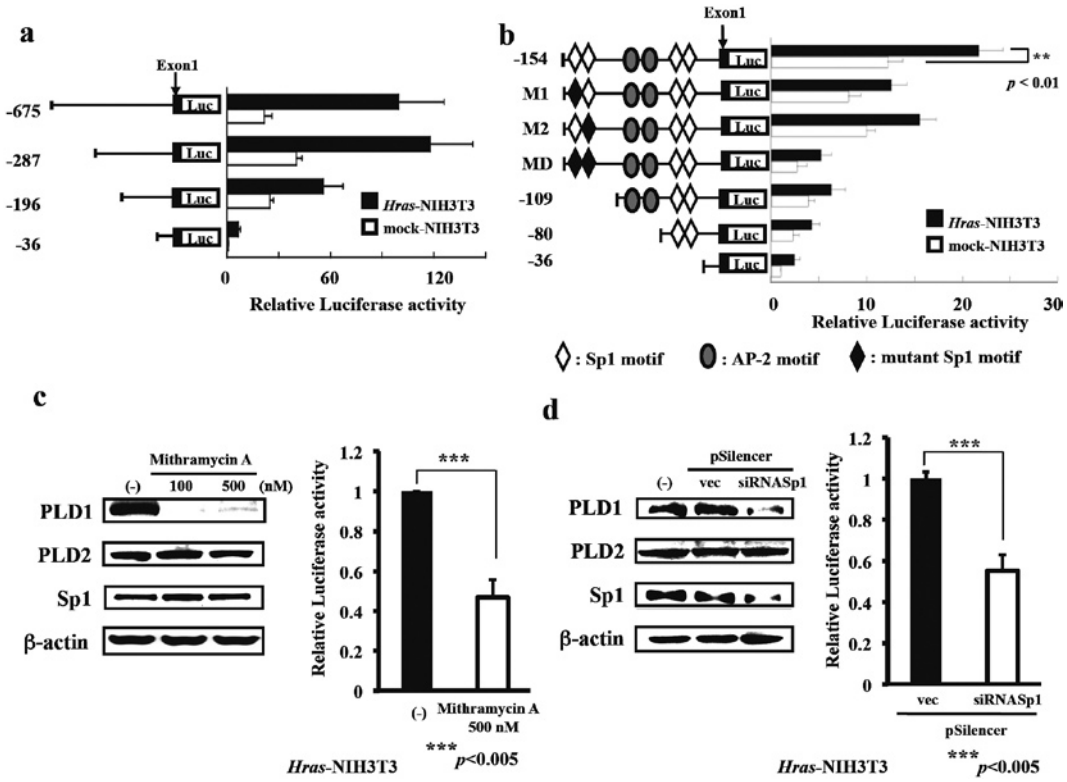

Fig. 3 *PLD1* reporter assay of mock- and mutated *Hras*-NIH3T3 cells and effects of mithramycin A and siRNA (pSilencer) of Sp1 on PLD protein level and promoter activity.
(a) and (b) Using mock- (open column) and mutated *Hras*-NIH3T3 cells (solid column), promoter analyses were performed using various luciferase vectors containing human *PLD1* 5' promoter shown at the left part of figure. Similar experiments using DLD-1 cells were performed (results are described in the text but data are not shown). Statistical significance was calculated by Student's *t* test. (c) Mutated *Hras*-NIH3T3 cells were treated for 24 h with mithramycin A (100 nM or 500 nM). Western blotting was performed using anti-PLD1, anti-PLD2 and anti-Sp1 antibodies, respectively. PLD1 promoter assay was performed using –154 bp PLD1/Luc. Five hundred nM of mithramycin A was added for 24 h after reporter vector transfection. The culture was performed in triplicate. Statistical significance was calculated by Student *t* test. *** denotes $p<0.005$ (d) Similar experiments were performed using pSilencer siRNA Sp1 (siRNASp1) and its negative control pSilencer (vec). Two μg of silencer vectors were used for 1 ml culture, and the transfection was accomplished using Lipofectin. Results of Western blotting and relative promoter activity are illustrated.

shown). Furthermore, we used Sp1 luciferase reporter vector containing the two consensus Sp1 motifs to evaluate the total functional activity of the Sp family proteins (Fig. 4b). Binding of transcription factor(s) to the Sp1 site was much greater in DLD-1 than in Caco-2 cells.

Since there has been reported difficulty in the analysis of Sp family protein overexpression in cells with high endogenous Sp family level, a drosophila cell line, SL2, lacking Sp family proteins was used to assess the involvement of Sp family proteins.[7] In Fig. 4c, transfection of either *Sp1* or *Sp3* to SL2 cells increased *PLD1* promoter activity, although the Sp3-induced increase was much smaller. The major increase was observed in –154 bp PLD1/luc, however, a mild increase was observed in –109 bp PLD1/luc, suggesting the presence of minor Sp-responsive elements between –109 bp and the first exon (Fig. 2c and Fig. 3b).

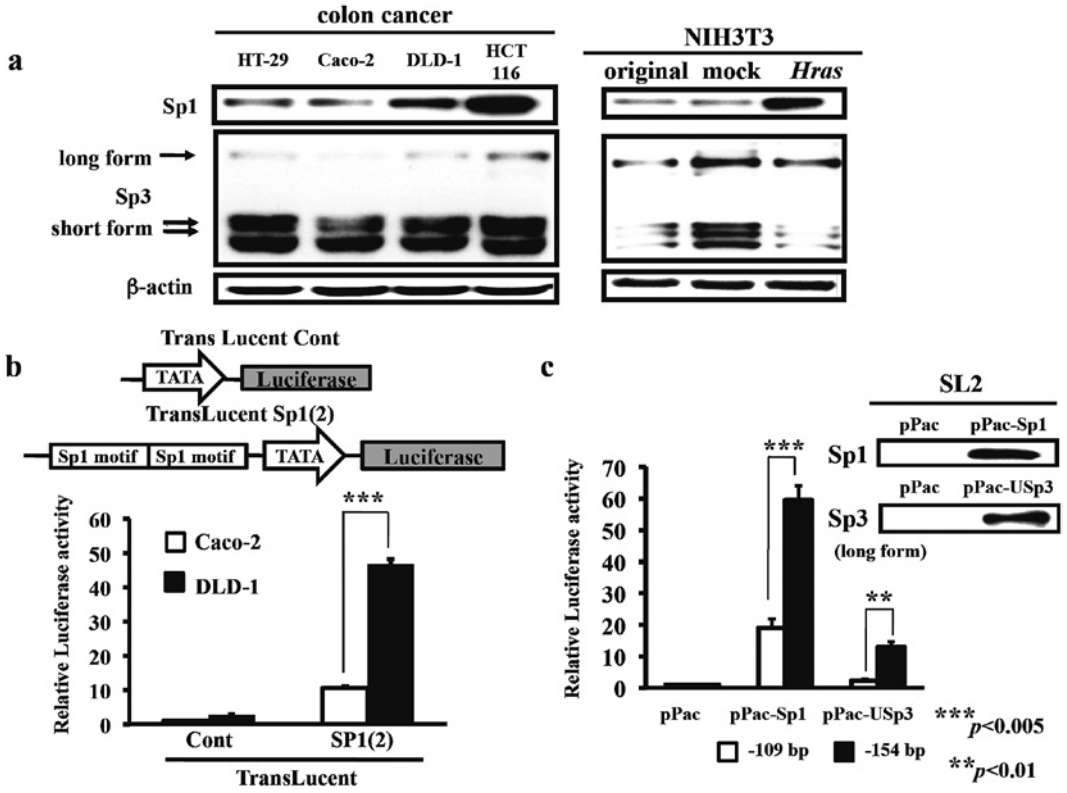

Fig. 4 Cellular Sp1 and Sp3 protein levels, a comparison of transcriptional activity bound to the consensus Sp1 site between Caco-2 and DLD-1, and a SL2 cell transfection experiment.
Cellular Sp1 and Sp3 proteins of various cell lines are shown. Long and short forms of Sp3 proteins are marked. (b) In the upper part, constructs of TransLucent vectors (Panomics Co.) with or without consensus Sp1 motifs are illustrated. With these vectors, the total transcriptional activities whose target was the consensus Sp1 motif were analyzed using Caco-2 and DLD-1 cells, respectively. Cell culture was performed in triplicate, and statistical significance was calculated by Student *t* test. *** denotes $p<0.005$. (c) Effects of Sp1 and Sp3 overexpression on *PLD1* promoter activity. SL2 cells lacking Sp family proteins were cultured in triplicate. Luciferase vectors of –154 bp PLD1/Luc and pPac-RL (as a control of transfection efficiency) were transfected together with either pPac-Sp1 or pPac-USp3 expression vector as described in the Materials and Methods. The relative luciferase activity of each sample was calculated as –154 bp PLD1/Luc /pPac-RL. Statistical significance is shown. ** means $p<0.01$ and *** denotes $p<0.005$.

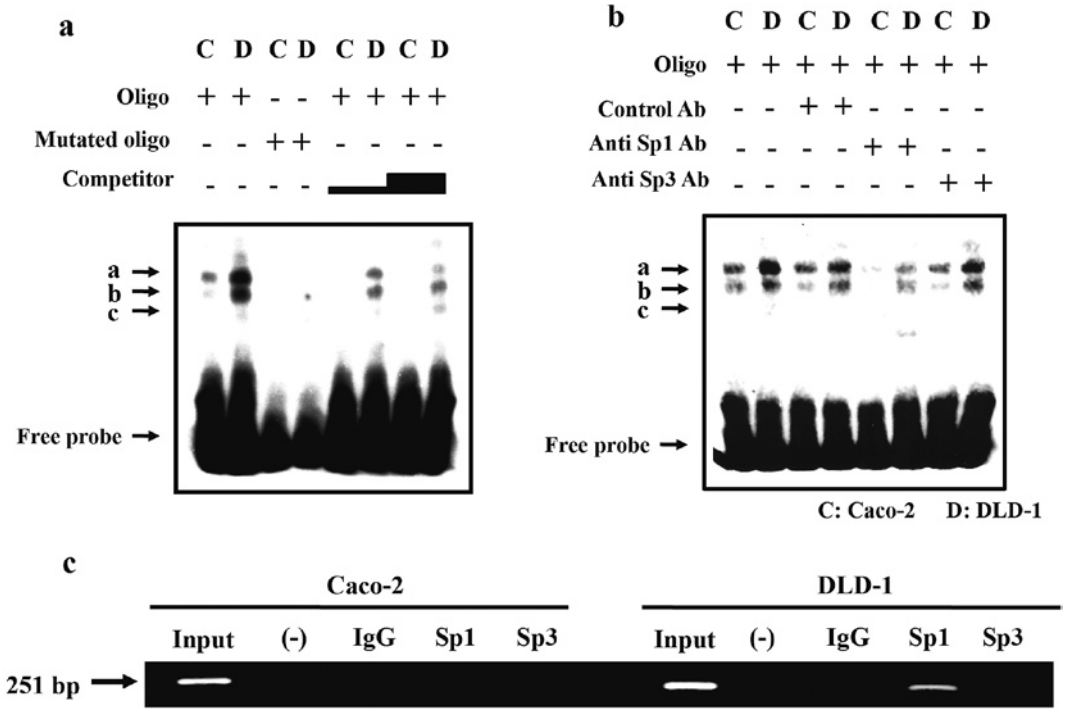

Fig. 5 Electrophoresis mobility shift assay and chromatin immunoprecipitation assay.
(a) Nuclear extracts (0.5 μg each) of Caco-2 (C) or DLD-1 (D) cells were mixed with 200 fmol of probes including a distal wild or mutated Sp1 site shown in Materials and Methods. EMSA was performed as described in Materials and Methods. Mutated oligo as shown in the Materials and Methods was used in some experiments. Cold oligo (x5 and x10) was used for the competition. (b) Supershift experiments using anti-Sp1 or anti-Sp3 antibody. Before mixing with a labeled probe, 2 μg/sample of non-specific antibody (control IgG), anti-Sp1 antibody or anti-Sp3 was added to nuclear extracts, and EMSA was performed. (c) ChIP assay was performed as described in Materials and Methods. Unrelated rabbit IgG, anti-Sp1 and anti-Sp3 antibodies were used to immunoprecipitate the DNA-protein complex. (–) denotes no antibody treatment. The size of the PCR product containing the distal Sp1 site was 251 bp.

EMSA using the distal Sp1 site of *PLD1* promoter as a probe illustrates the increased intensity of shifted bands (a and b) in DLD-1 as compared with Caco-2 cells (Fig. 5a and 5b). Experiments with mutated oligo and cold competitor showed the specificity of these bands. Pre-incubation of nuclear extracts with anti-Sp1 antibody and, somewhat less efficiently, anti-Sp3 antibody inhibited these bands. In the ChIP assay, anti-Sp1 antibody could immunoprecipitate the region containing the Sp1 site in DLD-1 but not in Caco-2 cells (Fig. 5c). However, anti-Sp3 antibody did not produce a positive band in these cell lines in our experimental conditions.

DISCUSSION

Although it has been reported that mutated *ras* increased PLD enzyme activity via various signaling pathways,[13,14] the mechanism of oncogene-induced *PLD* transcription has been largely unknown. In the present study using several human colon cancer cell lines with or without mutated *ras,* we showed that mutated *ras* selectively increased *PLD1* but not *PLD2* mRNA (Fig.1b),

suggesting that the *ras*-induced increase of PLD activity was by increased *PLD1* transcription. We further analyzed mutated *Hras*-NIH3T3 and mock-NIH3T3 cells (Fig. 1c and 1d), providing supportive evidence that the *PLD1* mRNA increase was attributed to mutated *ras*. However, due to heterogeneous genetic alterations within various cancer cells, we can completely neglect the presence of cells possessing highly activated Ras without elevated *PLD1* mRNA.

The promoter analysis of *PLD1* using DLD-1 cells showed the importance of the region between –154 bp and –109 bp from the first exon (Fig. 2b and 2c). The comparison using mutated *Hras*- and mock-NIH3T3 supported the results observed in DLD-1 cells. Mutated *Hras*-NIH3T3 showed higher promoter activity than mock-NIH3T3, and the similar region observed in DLD-1 proved to be critically important (Fig. 3). The mutation experiment of Sp1 sites confirmed the importance of these elements, however, the relative potential of two Sp1 sites was somewhat different between DLD1 and mutated *Hras*-NIH3T3 (Fig. 2 and Fig. 3). Mithramycin A and siRNA of Sp1 confirmed the involvement of Sp family protein in mutated *ras*-induced *PLD1* gene expression (Fig. 3c and 3d).

The Sp1 protein was more abundant in DLD-1 than in Caco-2, and Sp1 reporter vector clearly showed that positively functioning transcription factor(s) binding to the Sp1 site were more prevalent in DLD-1 compared to Caco-2 cells (Fig. 4a and 4b). Furthermore, using SL2 cells, we demonstrated that *Sp1* overexpression increased *PLD1* promoter activity (Fig. 4c). However, some involvement of factors other than Sp family in *ras*-induced *PLD1* gene expression cannot be excluded.

The intensity of shifted bands in EMSA was more distinct in DLD-1 than in Caco-2 cells. Both anti-Sp1 and anti-Sp3 antibodies, though somewhat less efficiently, reduced band intensity. The effect of anti-Sp1 antibody in the ChIP assay is consistent with the results of reporter assay and EMSA. However, the negative result of the anti-Sp3 antibody in the ChIP assay (Fig. 5c) suggests that Sp3 is not the main determinant in PLD1 transcription.

Taken together, we conclude that a mutated *ras* induces *PLD1* but not *PLD2* mRNA via an interaction between the Sp1 protein and the distal Sp1 site located between –154 bp and –109 bp of the 5' promoter of *PLD1* in DLD-1 cells and that it is involved in *ras*-induced PLD activation.

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