Loss of Protein Phosphatase 2A Expression Correlates with Phosphorylation of DP-1 and Reversal of Dysplasia through Differentiation in a Conditional Mouse Model of Cancer Progression

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ABSTRACT

A conditional mouse model of time-dependent dysplasia reversal demonstrated that reversal and differentiation of dysplastic salivary gland tissue at the 4-month reversible stage was characterized by the appearance of a phosphorylated slower mobility form of Differentiation Related Transcription Factor 1-polypeptide-1 that was correlated with cellular differentiation. The phosphorylated form of DP-1 was not found at the 7-month irreversible stage or in adenocarcinomas. At the 4-month reversible stage, protein phosphatase 2A expression was down-regulated coincident with loss of oncogene expression, whereas PP2A expression persisted at the 7-month irreversible stage. Results are consistent with the hypothesis that persistent PP2A expression prevented the appearance of the phosphorylated form of DP-1 required for cellular differentiation and reversal of dysplasia after loss of oncogene expression.

INTRODUCTION

The tTA/TAg⁵ transgenic mouse model of cancer progression exhibits clearly defined stages of reversible and irreversible dysplasia after down-regulation of TAg expression at 4- and 7-months, respectively (1). Salivary adenocarcinomas and metastases develop when TAg expression is not interrupted (2). The presence of both reversible and irreversible dysplasia in this model is relatively unique in comparison with other conditional mouse models of cancer progression.

Recently, conditional transgenic models have been adapted to examine the requirement of oncogene expression for cancer maintenance. Studies in transgenic mice expressing conditionally regulated oncogenes such as H-Ras in a melanoma model (3), K-Ras in a lung adenocarcinoma model (4), and BCR-ABL in a leukemia model (5) demonstrate reversibility of malignancy when oncogene expression is down-regulated. Additionally, reversibility of c-myc-induced carcinogenesis is reported in such diverse cancer models as mammary adenocarcinoma (6), lymphoma and leukemia (7), osteogenic sarcoma (8), and pancreatic β-cell islet cell tumors (9). In these models, cancer initiation and maintenance remains largely dependent on the expression of the initial oncogenic trigger. This reversibility is due either to rapid apoptosis of tumor cells (3–5, 7) or to redifferentiation of cells causing arrest of cellular proliferation and loss of malignant potential (7–9). These results are important because they indicate that ablation of signaling from one oncogenic pathway can be sufficient to reverse a malignant phenotype in multiple different tissues.

Nevertheless, oncogene-independent recurrent growth of tumors in animals with regressed primary tumors is reported in a small percentage of cases in the H-Ras-induced melanoma (3) and c-myc-induced lymphoma and leukemia (7). Accumulation of additional tumorigenic mutations that bypass reliance on the initial transforming event is postulated to be the driving force behind these recurrences (5), as has been postulated for the tTA/TAg mouse model used in the present study (1). Additional examination of the possible mechanisms and secondary pathways involved in driving recurrent tumor emergence after initiation by a specific oncogenic event has not been performed in any of the above studies because this phenomenon occurred rarely. It is, however, important to identify mechanisms that govern non-reversal and oncogene-independent tumor regrowth because many human cancers are able to bypass growth restraints imposed by loss of initial oncogenic event. To this end, conditional transgenic models of c-myc-, Neu-, and Wnt1-initiated mammary tumors have been used (6, 10, 11). Two mechanisms that govern the regression and oncogene-independent growth of tumors and metastases have been identified from these studies. In the c-myc model, mutations in k-ras prevent tumor regression (6), whereas in the Wnt model, loss of p53 is dispensable for mammary adenocarcinoma regression but increases the probability of tumor recurrence and progression to Wnt-independent growth (11).

Reversibility of oncogenesis is not necessarily inevitable as has been shown in the literature (12). The current study was initiated to examine the molecular mechanisms responsible for the time-dependent reversibility of the dysplasia found in the tTA/TAg model to test the hypothesis that genetic changes control reversibility and irreversibility. Unlike other models, the adenocarcinomas that develop in this model do not regress with loss of oncogene expression. Instead, the ability to regress is lost in the preneoplastic cells in a time-dependent manner. Results from the present study demonstrate that the mechanism regulating reversal of early-stage dysplasia was cellular differentiation mediated by phosphorylation of DP-1. Irreversible dysplasia resulted when persistent expression of the PP2A phosphatase blocked the emergence of the phosphorylated form of DP-1 that appeared to be required for cellular differentiation.

MATERIALS AND METHODS

tTA/TAg Double Transgenic Mice, Doxycycline Administration, and Animal Care. Mouse mammary tumor virus long terminal repeat-driven expression of a tTA and tetracycline responsive promoter (teto)-SV40 Tag double transgenic mice (tTA/TAg) were identified using the PCR after DNA extraction from either ear or tail tissue (1). Doxycycline (200 µg/ml) was administered in the drinking water to selected groups of mice for between 2 and 14 days to down-regulate TAg oncogene expression. Dysplastic submandibular salivary gland tissue and adenocarcinomas were harvested from mice after euthanasia. All procedures involving animals were performed in accordance with current Federal (NIH Guide for the Care and Use of Laboratory Animals, December 1996) and institutional guidelines.
Animals) and university guidelines and were reviewed and approved by the Georgetown University Institutional Animal Use and Care Committee.

RNA Isolation, cDNA Microarray Analysis, and Analysis of DP-1 Expression by Reverse Transcriptase-PCR. Salivary gland specimens were snap frozen at the time of dissection. Total RNA was isolated from salivary gland tissue samples by Trizol extraction (Invitrogen, Carlsbad, CA), quantified on a spectrophotometer, and cDNA synthesis performed. cDNAs from salivary gland tissue from tTA/TAg mice were synthesized, purified, and labeled with colored fluors, green (Cy3) and red (Cy5). Individually, green and red fluoro-labeled cDNA probes from three separate pairs of 4- and 7-month-old salivary gland tissue were hybridized to a mouse onchocerch printed with 2640 cDNA identified by expressed sequence tags involved in cancer pathways (National Cancer Institute, NIH, Bethesda, Maryland; Ref. 13). Each individually labeled cDNA pair was hybridized to two separate onchocers with reversal of the red-green fluoro labeling to test for reproducibility of results. Samples were purified and concentrated with YM30 Microcon columns (Millipore, Bedford, MA). Slides were prehybridized for 1 h, then hybridized overnight at 42°C in 25% formamide, washed sequentially to dispose of unbound probe for four min each in 2× SSC/0.1% SDS, 1× SSC, 0.2× SSC, and 0.05× SSC for 1 min, dried by centrifugation, scanned on a GenePix 4000A scanner (Axon Instruments, Inc., Foster City, CA) and images analyzed by GenePix Pro 3.0 software (Axon Instruments, Inc.). The normalized relative ratio of gene expression was calculated for each spot by measuring the ratio of Cy5 signal to Cy3 signal (Cy5/Cy3) at each spot. Array data were then formatted and uploaded to the National Cancer Institute mKdb bioinformatics web page4 for analysis of specific intensity ratios and gene clusters and links to external data sources. For semiquantitative DP-1 reverse transcription-PCR, RNA was isolated and cDNA synthesis performed. Primers used to detect DP-1 mRNA expression by reverse transcription-PCR at 24 cycles were as follows: forward GGT GGC TGA CGA GCA GGTC GGC and reverse CTG CAC CAA GGT CTT GAA GCC, which yielded a 304-bp PCR fragment. Reverse transcriptase-PCR for β-actin was performed as a control for RNA integrity and equal loading of cDNA.

Histological Analyses, in situ Detection of Apoptosis, Determination of Mitotic Index, and DP-1 Immunohistochemistry. Salivary gland specimens were fixed in 10% buffered formalin overnight at 4°C and embedded in paraffin using standard techniques. Sections (5 μm) were cut for H&E staining, quantification of mitotic figures/10×40 high power fields, in situ detection of apoptosis, and DP-1 immunohistochemistry. At the 4-month stage, the numbers of mitotic figures were compared between 9 untreated mice and 32 mice that received doxycycline treatment for 10 to 14 days. At the 7-month stage, the numbers of mitotic figures were compared between 15 untreated mice and 7 mice that received doxycycline for 10–14 days. In situ detection of apoptotic cell nuclei was performed using the ApopTag Apoptosis Detection Kit (Intergen Company, Purchase, NY), and the percentage of apoptotic cells (apoptotic index) within a minimum of 1000 submandibular striated ductal cells was determined (14) for 9 untreated and 18 doxycycline-treated mice at the 4-month stage and 9 untreated and 3 doxycycline-treated mice at the 7-month stage. DP-1 immunohistochemistry was developed using the DP-1 rabbit polyclonal antibody (K-20, sc-610; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as follows. Slides with paraffin-embedded tissue sections were deparaffinized, rehydrated, and quenched with 3% hydrogen peroxide. Antibodies were retrieved by immersing the slides in preheated 1× High pH Target Retrieval Solution (S3307; Dako Cytomation, Carpinteria, CA) for 20 min. Slides were allowed to cool in the retrieval solution for an additional 20 min, blocked with buffers from the Zymed Histomouse SP kit (95-9541; Zymed Laboratories, Inc., South San Francisco, CA), incubated with a 1:150 dilution of the DP-1 antibody (K-20), and then with a biotinylated secondary antibody and streptavidin-peroxidase conjugate. DP-1 expression was detected with aminothiols carbazole chromagen solution. Slides were counterstained with hematoxylin and mounted with glycerol vinyl alcohol. A slide with a tTA/TAg adenocarcinoma treated with doxycycline in which exposure to the DP-1 primary antibody was omitted was included as a negative control. Digital photographs were taken using the Nikon Eclipse E800M microscope setup with Nikon DMX1200 software (Nikon Instruments, Inc., Melville, NY).

Statistical analyses were performed using the univariate ANOVA by the Scheffé posthoc test (SPSS, version 11.0.0; SPSS, Inc., Chicago, IL).

Protein Extraction, Western Blot Analysis, and in Vitro Phosphatase Exposure. Frozen salivary gland tissue was homogenized in protein lysis buffer to extract whole proteins. Protein concentration was quantified by bicinchoninic acid protein assay (Pierce, Rockford, IL). Proteins (20 μg) were fractionated on precast 8% Tris-glycine gels (EC6015; Invitrogen Life Technologies, Inc. Carlsbad, CA) and transferred onto polyvinylidene difluoride membranes for Western blot analysis. Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline and 1% Tween overnight at 4°C and exposed to a 1:150 dilution of DP-1 rabbit polyclonal antibody (K-20, sc-610), a 1:500 dilution of SV40 large TAg mouse monoclonal (Pab 101, sc-147), a 1:500 dilution of SV40 small and large TAg mouse monoclonal (Pab 108, sc-148; Santa Cruz Biotechnology), or a 1:500 dilution of PP2A c-subunit mouse monoclonal (05-545; Upstate Biotechnology, Inc., Charlottesville, VA). Membranes were exposed to a 1:5000 dilution of the appropriate secondary antibody (Santa Cruz Biotechnology) and protein expression was visualized using the ECL Plus Western Blotting Detection Kit (RP2133; Amersham Biosciences, Piscataway, NJ). Membranes were exposed to the large TAg antibody (Pab 101) as a control for TAg protein expression during doxycycline treatment. Membranes were exposed to the SV40 antibody that recognizes both small and large TAg (Pab 108) to rule out the presence of small TAg. For in vitro dephosphorylation of proteins, proteins (20 μg) were treated with 0, 0.25, 0.5, or 1.0 μl of the serine-threonine phosphatase PP2A purified from human RBCs (t14-111, Upstate Biotechnology, Inc.) at 30°C for 30 min, then fractionated on precast 8% Tris-glycine gel and transferred to a polyvinylidene difluoride membrane and visualized as above.

RESULTS

Reversal Was Completed within 10 Days of Initiating Down-Regulation of Oncogene Expression. Western blot analysis of whole protein extracts from tTA/TAg mice treated with doxycycline for 2, 4, 6, 8, 10, 12, and 14 days to abrogate TAg expression demonstrated that in all mice, regardless of stage, down-regulation of TAg expression was seen as early as 2 days and was always complete by 6 days of doxycycline administration (Fig. 1C). No significant difference in timing of TAg down-regulation was observed between the reversible and irreversible stages. Similar mouse-to-mouse variation in the extent of down-regulation after 2–4 days of doxycycline treatment was found at both stages (data not shown). Small TAg is not expressed from the expression vector in the tTA/TAg mouse model. The absence of small TAg was confirmed by Western blot analysis (data not shown); therefore, all results are attributable to the presence of large TAg alone. Reversal of dysplasia through differentiation of the submandibular striated ductal cells was indicated by the appearance of striations within the ductal cells after 8 days of doxycycline administration at the 4-month stage (Fig. 1A, arrowheads and insert). In contrast, no differentiation or reversal was observed in dysplastic salivary gland tissue at the 7-month stage (Fig. 1B) or in salivary adenocarcinomas (data not shown) even 14 (Fig. 1B, right panel) or 21 days (1) after loss of oncogene expression. Results indicated that differentiation is an important mechanism whereby dysplasia is reversed at the 4-month reversible stage and implied that a block in differentiation prevented glandular remodeling after loss of oncogene expression at the 7-month irreversible stage.

There was no indication that changes in either rates of cell proliferation or apoptosis were responsible for the differences in reversibility at the two stages. The number of mitotic figures fell significantly after down-regulation of TAg expression at both stages (from 69 ± 11 to <1 at the 4-month reversible stage after 10–14 days of doxycycline treatment (P < 0.0001, Scheffe) and from 60 ± 7 to <1 at the 7-month irreversible stage after 10–14 days of doxycycline treatment (P = 0.0001, Scheffe)). Although the apoptotic index was signifi-
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DP-1 mRNA Expression Levels Were Increased 1.7-Fold at the Irreversible as Compared with Reversible Stage and Protein Expression Was Localized to the Nuclei of Striated Ductal Cells in the Submandibular Salivary Gland. A microarray study was initiated to investigate the hypothesis that changes in gene expression patterns may be responsible for the lack of differentiation and reversibility at the 7-month irreversible stage. Labeling of the cDNA from the submandibular salivary glands of 4- and 7-month-old TAg mice with uninterrupted TAg expression and hybridization of the cDNA to the mouse oncochip (Fig. 2A) revealed that mRNA expression levels of the DP-1 gene, transcription factor dimerization partner (Image: 576112), a mandatory dimerization partner of the E2F family of transcription factors, were increased an average of 1.7-fold above the 4-month reversible stage at the 7-month irreversible stage (Fig. 2A, insert). Semiquantitative reverse transcriptase-PCR confirmed this expression difference (Fig. 2B). In this mouse model, TAg expression is localized to the striated ductal cells of the submandibular salivary gland (1). Immunohistochemistry was performed to determine

Fig. 1. Time-dependent phenotypic reversal of dysplasia was complete after 10 days of doxycycline treatment at the 4-month reversible stage. A, phenotypic reversal after 8–14 days of doxycycline treatment at the 4-month reversible stage as indicated by the appearance of striations (arrowheads) consistent with cellular differentiation. Insert at 8 days of doxycycline treatment shows a close-up of ductal cells to highlight the striations (pink) with two nuclei (blue). B, there was no significant phenotypic reversal at the 7-month irreversible stage after doxycycline treatment. C, Western blot analysis demonstrated that TAg expression was down-regulated by 4 days of doxycycline treatment at both the 4- and 7-month stages. Thin arrows indicate dysplastic submandibular striated duct cells without striations; thick arrows indicate phenotypically reversed cells. Digital photographs taken at ×40 and ×60 (insert). Bars indicate 20-μm scale. Doxy, doxycycline; −, no doxycycline treatment; D, days of doxycycline treatment; R, reversible stage; I, irreversible stage.
whether DP-1 expression was localized to the same cell type. Results demonstrated nuclear localization of DP-1 in striated ductal cells at all stages of cancer progression in both untreated and doxycycline-treated mice (Fig. 2). Differently Migrating Forms of DP-1 Protein Were Found at the Reversible as Compared with the Irreversible Stage after Down-Regulation of Oncogene Expression. Western blot analyses of DP-1 expression levels after TAg down-regulation at the 4-month reversible stage revealed few differences between the stages. Insert is a close-up of the spot for the DP-1 gene, transcription factor dimerization partner (Image: 576112). B, semiquantitative reverse transcriptase-PCR analysis of DP-1 mRNA expression in the submandibular salivary gland demonstrated higher expression levels at the 7-month irreversible stage as compared with the 4-month reversible stage. Reverse transcriptase-PCR for β-actin expression levels was performed as a control. C, localization of DP-1 expression to the striated ductal cells of the submandibular salivary gland by immunohistochemistry in 4-month reversible, 7-month irreversible salivary glands and 8–10-month-old salivary adenocarcinomas in the absence (top) and presence (bottom) of doxycycline. Localization of DP-1 in the nuclei of striated ductal cells of the submandibular salivary gland was observed at all stages. Salivary gland from a wild-type nontransgenic mouse treated with doxycycline was exposed to DP-1 antibody and included as a positive control. A TAg adenocarcinoma treated with doxycycline in which exposure to the DP-1 primary antibody was omitted was included as a negative control. Arrows indicate representative cells demonstrating nuclear-localized DP-1. Digital photographs taken at ×40. Bars indicate 10-μm scale. Doxy, doxycycline; D, days of doxycycline treatment; R, reversible stage; I, irreversible stage.

The Slowest Mobility Form of DP-1 Was Sensitive to in Vitro PP2A Phosphatase Treatment. Exposure of salivary gland protein extracts from the 4-month reversible stage to the serine-threonine phosphatase

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PP2A in vitro demonstrated loss of the slowest mobility form in a dose-dependent manner (Fig. 3D). In contrast, protein levels of the faster migrating middle form of DP-1 were unchanged after in vitro PP2A phosphatase exposure at the 4-month reversible (Fig. 3D, top panel), 7-month irreversible stage (Fig. 3D, bottom panel) and adenocarcinoma stages (data not shown).

**PP2Ac Expression Persisted at the 7-Month Irreversible Stage but Was Down-Regulated at the 4-Month Reversible Stage with Loss of Oncogene Expression.** Western blot analysis demonstrated that expression of PP2Ac, the catalytic subunit of PP2A, was present before down-regulation of TAg at both stages but was selectively lost at the 4-month reversible stage with loss of TAg expression (Fig. 4, top panel). Loss of PP2Ac expression was correlated with the appearance of a slower migrating form of DP-1 at the 4-month reversible stage and persistent PP2Ac expression was correlated with the absence of this form of DP-1 at the 7-month irreversible stage (Fig. 4, middle panel).

**DISCUSSION**

The E2F family of transcription factors plays important roles in regulating cell cycle progression, apoptosis, and differentiation (16–18). DP-1 is a mandatory binding partner of E2F family members. Heterodimerization of DP and E2F is essential for both high-affinity DNA binding and efficient transcriptional activation (16, 17). Binding of pRb to the E2F/DP heterodimer results in the silencing of E2F/DP activity. pRb phosphorylation or sequestration by TAg releases the E2F/DP transcription factor, resulting in its conversion to the transcriptionally active free form of E2F/DP and progression of the cell cycle (17). DP-1 is required for development and differentiation of the extraembryonic tissues required for embryonic survival (18). It is hypothesized that DP-1 also possesses E2F-independent proto-oncogenic properties as exemplified by cooperation of DP-1 with activated H-Ras in the transformation of rat embryo fibroblasts (19). Transgenic mice expressing DP-1 under the control of a keratin 5 promoter develop epidermal hyperplasia and more extensive skin carcinogenesis in a two-stage chemical carcinogenesis study (20).

DP-1 is differentially phosphorylated during cell cycle progression as evident from its migration as a doublet and its phosphorylation-dependent mobility shift during the cell cycle (16). DP-1 binds to DNA in the hypophosphorylated state. During differentiation of adipocytes, activation of peroxisome proliferator-activated receptor γ, a nuclear hormone receptor, has been shown to cause cell cycle arrest associated with an increase in DP-1 phosphorylation and loss of E2F/DP DNA binding and transcriptional activity (15). Moreover, the serine/threonine PP2A has been implicated in the maintenance of E2F/DP complex in an underphosphorylated form (15).

PP2A is a trimeric complex composed of the scaffolding A subunit, the regulatory B subunit, and the catalytic C subunit (21, 22). PP2A itself plays a role in the control of cellular signal transduction by regulation of protein kinase cascades controlled through reversible phosphorylation. Through a complex regulatory system that involves diversity in subunit expression and cellular localization, PP2A has been implicated in the regulation of such diverse cellular functions as cell cycle progression, differentiation, oncogenic transformation, apoptosis, angiogenesis, and cell adhesion (21, 22). As a consequence, in addition to DP-1, PP2A targets for control are many and varied, including the cyclin B-cdc2 protein kinase, which controls G2-M transition, the Wnt pathway, β-catenin, E-cadherin, the G1-specific cyclin-dependent kinases, the transcription factor SP1, signal transducers and activators of transcription 3, the mitogen-activated protein kinase pathway, Mdm2, Akt, Bcl-2, c-fos and c-jun, Raf-1, and telomerase (22). Genetic and biochemical experiments using this mouse model can be used to test for the individual contributions of the different down stream targets to reversal of neoplasia.
Results from this study are consistent with the hypothesis that persistent PP2Ac expression blocked the appearance of the phosphorylated form of DP-1. Phosphorylated DP-1 may be required for cellular differentiation and reversal of dysplasia after loss of oncogene expression. More broadly, the investigations support the development of therapeutic approaches that target regulation of PP2Ac expression and activity in reversal of preneoplasia.

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REFERENCES


