Functional Quantification of DNA-binding Proteins p53 and Estrogen Receptor in Cells and Tumor Tissues by DNA Affinity Immunoblotting

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ABSTRACT

Functional assays of proteins can monitor the consequences of defects attributable to posttranslational activating or inhibitory events as well as to genetic mutations. Such assays promise to permit evaluation of cooperating oncogenic or tumor suppressor pathways in cells and tumors. As a step toward realizing this promise, we designed the DNA affinity immunoblotting (DAI) method to measure the activities of multiple sequence-specific DNA-binding proteins simultaneously [initially p53 and estrogen receptor (ER)] in lysates of cells or frozen tumor tissues. DAI is a novel application of biotin/streptavidin affinity chromatography and immunoblotting. The p53 and ER proteins in cell or tissue lysates were bound to biotinylated, specific DNA probes, retrieved using a streptavidin-conjugated matrix, and then quantified in parallel with total protein by immunoblotting. The assay results were reproducible and specifically correlated with the known functional status of p53 in mouse and human cells of known p53 genotype, including those with low levels of p53 protein. ER immunohistochemistry of human breast samples, which is highly correlated with functional status and prognosis in human breast cancer, was also highly correlated with DNA binding activity results by DAI. In contrast, the p53 protein in cells is frequently expressed but inactive, potentially accounting for the lack of strict correlation of p53 immunohistochemical or mutational status with tumor response to chemotherapy. DAI offers a new means of molecular profiling and monitoring of p53 and other DNA-binding protein activities in cells and tumors. DAI has applications in the detection and identification of covalently modified forms of DNA-binding proteins and in the identification of their interacting proteins in complex with DNA.

INTRODUCTION

DNA-binding proteins play pivotal roles as transcription factors that govern gene expression, such as steroid hormone receptors that confer specialized functions and DNA-repair proteins that maintain the integrity of the genome. Altered DNA-binding proteins can contribute significantly to tumorigenesis as oncogenes and tumor suppressor genes (such as myc, jun/fos, myb, NFkB, and p53) or as reliable prognostic tumor markers such as ER. 3

p53 is the most frequently affected gene in human cancer. More than 90% of p53 mutations occur in the central DNA-binding domain, affecting its DNA binding activity and ability to transactivate (1). Although the NH2- and COOH-terminal domains are not frequently mutated, they are subjected to regulation through covalent modifications and interactions with other cellular proteins in response to genotoxic stress. Defects in p53 regulation can inactivate wild-type p53 protein, which is activated by phosphorylation at the NH2-terminus Ser 15 through DNA-PK (2) and/or ATM in response to DNA damage (3, 4). Defects in p53 phosphorylation (for example, mutation of the ATM gene in AT cells) can result in a p53 null phenotype. p53 protein is also subjected to activation through the COOH-terminus (5) by acetylation (6). Viral oncogene E1A can block p53 acetylation by displacing PCAF from the p300/CBP complex (7). Besides covalent modifications, association with cellular factors can alter p53 activity. Mdm2 association with p53 blocks its transactivation activity (8) and targets it for proteolytic degradation (9, 10). The presence of Mdm2 also diminishes the DNA binding activity of p53 (11). Mdm2 is found to be overexpressed in 30% of sarcomas (12) and a subset of breast carcinomas, almost all of which contain wild-type p53 (13). Wild-type p53 protein is inactivated by a number of viral oncogenes such as E6 protein from HPV, which targets ubiquitin-mediated protein degradation (14). Hepatitis B viruses, involved in 90% of hepatocarcinomas, encode HBXAg that binds to p53 in the cytoplasm and blocks p53 entry into the nucleus (15). p53 can also be inactivated by murine viral oncoproteins, such as SV40 Large T antigen, which interact with and block the central DNA binding domain (16). Thus, diverse pathways of p53 activation and inactivation, in addition to mutation, determine p53 protein functional status. Functional assays are needed for evaluation of p53 and other proteins that are subject to defects in diverse regulatory pathways.

p53-mediated apoptosis has been demonstrated to be a common mechanism of tumor response to chemotherapeutic agents and radiation therapy. Nevertheless, p53 mutation as a reliable prognostic marker or predictor of response to chemotherapy in human cancer is controversial. Reported mutation frequencies of p53 in human breast cancer range from 20 to 50% (17, 18). However, more than one-third of patients with clinically wild-type p53 breast cancer are not responsive to chemotherapy or radiation therapy (19). This discordance can be explained by lack of sensitivity and specificity for accurate assessment of p53 status. IHC detects antigenicity but does not necessarily reflect functionality of the protein (20). Functional assays of p53 include single-strand conformational polymorphism analysis combined with DNA sequencing, which detects p53 mutations precisely but needs additional information to distinguish between silent and functional mutations (21). A yeast-based assay has the ability to detect p53 mutations at a functional level (22) but has narrower application because p53 can be inactivated without mutation. In addition to mutation, wild-type p53 protein can be activated/inactivated through multiple mechanisms (23, 24, 25). A functional assay based not only on genotype and protein levels but also on the integrity of the pathways should be more reliable for evaluating p53 activation in cells and for functional testing of p53 and other DNA-binding proteins for prognosis and treatment plan.

In this report, we describe an assay applicable to multiparameter analysis of DNA-binding proteins from lysates of culture cells and tumor specimens. It is more sensitive than EMSA, offers two independent assurances of specificity, the DNA binding sequence and the specific antibodies, and is quantitative relative to the total steady-state levels of the DNA-binding protein in the sample. The assay is applicable to clinical specimens and to basic research on posttranslational
or alternative splice variants of the DNA-binding protein and on partner proteins in complex with the DNA-binding protein and DNA.

MATERIALS AND METHODS

Cell Culture and Nuclear Extract. Human adenocarcinoma cell lines SW837 (248, Arg→Trp) and SW480 (273, Arg→His) and cervical carcinoma cell C-33 (273, Arg→Cys) were obtained from the American Type Culture Collection. Cells were cultured in Eagle’s MEM plus 10% fetal bovine serum. Human embryonic kidney cell line 293 (Microbiex Biosystems, Inc.) was maintained in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic (Life Technologies, Inc.). Mouse nontransformed keratinocyte cell strain 291 was maintained in Eagle’s MEM supplemented with 0.04 mM Ca²⁺, nonessential amino acids, 5% chex-laced FBS, and 1% antibiotic-antimycotic (Life Technologies, Inc.; Ref. 26), and initiated keratinocyte clone 03C was maintained similarly except for a Ca²⁺ concentration of 1.4 mM. Mouse mammary tumor cells TM3, TM4, TM9, and TM10 were maintained in DMEM/F12 medium supplemented with 2% FBS. 0.8% antibiotic-antimycotic, 5 μg/ml endosomal growth factor (EGF), and 10 μg/ml bovine insulin (27). To induce p53 expression, cell cultures were treated with 400 rads of X-ray using a Siemen’s Therapy X-ray tube. Nuclear extracts were prepared using a modified protocol as described previously (28). Cells were lysed in lysis buffer (20 mM HEPES (pH 7.5), 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 μl of leupeptin, and 1 μg/ml Pepstatin A). After 5-min centrifugation at 800 x g, the pellet was suspended in lysis buffer with 500 mM NaCl, then rocked on a rotating platform for 30 min at 4°C. The extracts were collected by centrifugation in a microfuge, aliquoted, and then stored at ~80°C.

Tissue Lysate Preparation. Frozen breast tumor tissues stored at −70°C for ≤2 years were pulverized in liquid nitrogen and rinsed in wash buffer [100 mM KCl, 20 mM HEPES (pH 7.9), 10 mM EDTA, 20% glycerol, 1 mM PMSF, 50 μl of leupeptin, and 1 μg/ml Pepstatin A]. The tissue suspension was centrifuged at 2000 rpm in a microcentrifuge. The pellet was resuspended in lysis buffer with 500 mM NaCl and then rocked on a rotating platform for 30 min at 4°C. The extracts were centrifuged, and the supernatants were aliquoted and stored as above.

DAI. Oligonucleotides of a consensus p53 binding sequence, 5′-TCAGAGGCGATGTCAGGCATGTC-3′ (29) and ER element DNA, 5′-GTCTAAATCAGGATCATGTCATG-3′ (30) were synthesized and 5′-end-labeled with biotin at the Biopolymer Facility at Roswell Park Cancer Institute. Double-stranded forms were prepared by incorporating equal amounts of oligonucleotides in 10 mM Tris-HCl (pH 8.0) at 100°C and then cooling to room temperature. Equal protein concentrations of lysate (200 μg) were incubated with 50 μM of leupeptin, and 1 μg/ml Pepstatin A]. The mixture was preincubated with preincubated with 80°C. The reaction was stopped by heating the samples at 80°C for 3 min. The samples were then incubated at 70°C for 30 min.

The DAI assay was developed for the quantification of specific DNA-protein interactions at a level of sensitivity beyond that of standard DNA binding assays like EMSA. The DAI assay combines biotin/streptavidin affinity chromatography (31) and immunoblotting (32) to retrieve and specifically detect endogenous DNA-binding proteins in specific complexes with biotinylated target DNA molecules. The reaction conditions for DAI were based on EMSA protocols for p53 DNA binding (33). The amount of radiolabeled DNA used in standard EMSA is ~0.2 pmol in a 20-μl reaction (10 nm). We tested the influence of target DNA concentration on the functional index measurement, using lysate proteins from γ-irradiated 03C mouse keratinocytes because of their competent p53 response to DNA damage. There was no significant change in p53 DNA binding dependent on biotinylated hup53 DNA concentration in the range of 6.25–50 nm (Fig. 1A). On the basis of these results, 25 nm biotinylated DNA was chosen for the DAI protocol to ensure that the DNA probe was not rate-limiting in these reactions. With 25 nm biotinylated hup53 DNA, no significant difference in p53 DNA was observed in reactions incubated for increasing reaction times from 30 to 120 min (Fig. 1B). Because salt concentration affects protein-DNA interactions, NaCl concentration was titrated as shown in Fig. 1C. DNA binding at lower Na⁺ (50–75 mM) was higher overall and included a nonspecific DNA binding component, as evident by p53 binding to a mutated p53 DNA binding sequence. Binding to hup53 DNA was specific between 100 and 150 mM Na⁺ (low) and dropped dramatically at 200 mM Na⁺. On the basis of lack of detectable nonsequence-specific binding and stability of DNA binding, 100 mM Na⁺ salt concentration was selected for standardization of the DAI reactions.

By increasing the volume of nuclear extract 2- to 4-fold of that used in EMSA, DAI was able to detect DNA binding activity of p53 protein in untreated, nontransformed epidermal 291 cells (Fig. 2A). The signal intensity of DAI with 120 μg of nuclear extract was comparable with that of direct immunoblotting with 30 μg of nuclear extract, which indicated that one-quarter of the total endogenous p53 protein in these uninduced cells was active in sequence-specific DNA binding. In comparison, active p53 forms were undetectable in the 30 μg aliquot typically used in EMSA. Specificity of the DAI assay is ensured at two levels: specific DNA templates and specific antibodies applied in distinct steps. As shown in Fig. 2B, DNA binding by p53 proteins was specific to the biotinylated p53 consensus sequence but not to a distinct sequence.
mutated sequence. The p53 signal was abrogated by an unbiotinylated p53 consensus sequence but not by poly(dl-dC) or by mutant p53 DNA competitors. The specificity of DAI was established in addition by using nuclear extracts from cells with different p53 genotypes (Fig. 2C). Significant DNA binding activity, representing approximately one-third of the total p53 protein, was detected in human 293 cells, consistent with their wild-type p53 genotype. The p53 proteins from human adenocarcinoma cell line SW837 and cervical carcinoma cell line C-33 exhibited negligible DNA binding, consistent with their mutant genotypes (248 Arg→Trp in SW837; 273 Arg→Cys in C-33).

However, residual DNA binding activity was detected in human adenocarcinoma cell line 480 with the p53 273 Arg→His mutation, although weaker than that for wild-type p53 in 293 cells (functional index of 0.3 and 1.5, respectively). This is consistent with previous observations of DNA binding of the 273 Arg→His mutant detected by EMSA (34).

Activities of p53 wild-type and mutant proteins were then measured in cells of a mouse mammary model (27). The p53 gene is wild type in weakly tumorigenic cell lines TM9 and TM10 and mutant in weakly tumorigenic line TM3 and highly tumorigenic line TM4 (35). DAI results indicate that p53 proteins from TM3 and TM4 bound at negligible levels (functional index of 0 and 0.2, respectively) consistent with their mutant genotypes (Fig. 3). The p53 protein in TM10 cells exhibited strong DNA binding activity (functional index of 2, 40% of total p53 protein), consistent with their wild-type p53 genotype. However, p53 protein from TM9 cells, which also contain only wild-type p53, failed to bind DNA. Thus, DAI could distinguish between transformed mammary cells with a wild-type p53 genotype containing functional p53 protein and those containing nonfunctional p53 protein. This is consistent with evidence that p53 can be inactivated by defects other than p53 mutation (36). The DAI results mirrored EMSA results in these cells and correlated with the induction of the p53 downstream target gene p21 by DNA damaging agents, yet have the advantage of providing total protein controls and more quantitative information for comparison among samples.

An additional advantage of DAI is simultaneous measurement of multiple DNA-binding proteins of different molecular weights. This is particularly significant because other DNA-binding proteins have been identified as prognostic, including steroid receptors for estrogen, progesterone, and androgen factors (e.g., in breast, ovarian, and prostate cancers). The ER protein was detected bound to its specific DNA probe in all but TM4, the most aggressively tumorigenic of the mammary cell lines (Fig. 3). Essentially all of the ER protein in mammary line TM10 was active for binding to the DNA probe containing the estrogen responsive element.

Because the DAI technique as presented relies on immunoblotting to detect the proteins, quantification of signal intensity is subject to a number of variables including transfer efficiency, antibody incubation, wash stringency, and exposure time. Quantification results could vary dramatically among experiments even with the same starting material. Calculation of the functional index mitigates these effects by measuring the intrinsic properties of DNA binding with a standard reference for the same protein (total protein by direct immunoblotting) subjected to the same conditions and variables. To test the reproducibility of DAI, DNA binding activities of ER and p53 were measured using three independently prepared MFC-7 cellular lysates at four different amounts of lysate input (Fig. 4). Although the absolute signal intensity was highly variable among the experiments, there is no

Fig. 2. Sensitivity and specificity of DNA binding activities of p53 by DAI. A. Quantification of p53 protein active in DNA binding by DAI. Indicated amounts of lysate from nontransformed mouse epidermal 291 cells were subjected to DAI. A 30 μg aliquot of lysate was loaded as reference for total p53 protein. B. Competition for sequence-specific p53 DNA binding. Aliquots of 200 μg of 291 lysate were subjected to DAI with indicated biotinylated competitor DNAs. C. Measurement of functional p53 proteins in cells with different p53 genotypes. Nuclear extracts (200 μg) from cell lines with functional wild-type (293), or mutant p53 (480), or nonfunctional mutant p53 (837, C-33), see Text, were subjected to DAI (+) and compared with total proteins (−), using PAb122 for immunoblotting. Functional index was calculated by dividing the signal intensity of DNA bound protein (+) by total protein (−).

Fig. 3. Functional analysis of p53 and ER in the mouse mammary model. Nuclear extracts (200 μg) from each of the cell lines were reacted with a biotinylated DNA mixture containing a p53 consensus sequence and an ER response element DNA sequence. The gel was blotted with ER antibody (MC-20) and p53 antibody (PAb122). The functional index was calculated by dividing the signal intensity of DNA bound protein (+) by that of 40 μg of total protein (−). kD, molecular weight in thousands.

Fig. 4. Reliability of DAI in analysis of DNA binding activities of p53 and ER. Cell lysates were collected from 70% confluent MFC-7 cells, and independent aliquots were subjected to DAI analysis at the lysate input indicated. The signal intensities of total (−) ER and p53 protein versus DNA bound (+) ER and p53 protein were quantified and expressed as the functional index as summarized in the bottom panel. The mean functional index of ER and p53 are shown from three DAI reactions with cell lysates from three independent harvests of MFC-7 cultures.
significant variation in functional index among reactions using different lysate input or among the different experiments (summarized in Fig. 4, bottom panel). Thus, functional index appears to be a reliable measure of the DNA binding activity of ER and p53 in complex cell lysates.

As a prerequisite to applications in evaluation of DNA-binding protein functional status in patient tumors, DAI was applied to measurement of DNA binding activities of p53 and ER proteins in lysates prepared from cryopreserved human breast cancer tissues (Fig. 5). DNA binding activity of ER was detected in five of nine tumor tissue samples, with functional indices ranging from 0.5 to 1.1. The specimens with an ER functional index of 0.5 or above were highly correlated with ER-positive results by IHC with one exception: Br-7. DNA binding activities of p53 protein were detected in four of nine of these tumor tissue samples, with functional indices ranging from 0.5 to 1.4. To compare with conventional approaches for detection of p53 mutations, these tumor specimens were examined by IHC with DO-1, the same antibody used in DAI. The IHC staining pattern was heterogeneous among the tumors, with either nuclear staining or diffuse cytoplasmic staining (data not shown). The discordance of IHC antigenic and DAI functional assessment was greater for p53 than for ER, implying that IHC for p53 protein is particularly prone to error in predicting prognostic or treatment outcomes related to p53 protein function. DNA binding was observed in two cases with IHC-positive p53: Br-1 and Br-7. In three other cases, IHC failed to detect nonfunctional p53 proteins: Br-4, Br-5, and Br-9. It is possible that p53 protein is functional but elevated in certain breast tumors (e.g., in response to genomic damage or physiological stimuli such as ER-mediated proliferation), leading to false positives by IHC. Conversely, p53 proteins could be nonfunctional without being overly expressed, leading to false negatives by IHC.

**DISCUSSION**

The specificity and sensitivity of the DAI assay permitted functional assessment of p53 and other DNA-binding proteins in normal and nontransformed cells in culture and in solid tumor specimens. Because DAI directly measures the sequence-specific DNA-binding property, rather than expression of antigen or presence of the protein, it can monitor abnormalities in activating and inactivating pathways, including gene mutations, posttranslational modifications of the protein, and protein-protein interactions, although these are not yet fully known. The functional index facilitates quantification of partial inactivation, e.g., by heterozygous mutation of the gene encoding the DNA-binding protein or by abnormalities in the activities of activating or inactivating enzymes targeting the DNA-binding protein.

The DNA binding activity of ER correlated highly with the ER protein level detected by IHC. However, not all ER proteins detected by IHC or immunoblotting are capable of binding to DNA. This is one potential explanation of tamoxifen therapy failure in some patients with positive ER. Although DNA binding activity of p53 was in agreement with p53 genotype in the human tumor cell lines, the results from tumor tissues suggest that p53 protein level is not closely correlated with p53 protein function. On one hand, the accumulation of functional wild-type p53 protein can result from DNA damage (37), hypoxia (38), and oncogene expression. On the other hand, not all p53 alterations cause p53 overexpression, particularly nonsense mutations or frameshift mutations. Analysis of p53 protein by DAI has the advantage of detecting the consequences of various mechanisms of p53 inactivation. Although outside the scope of this study, the DNA binding activity of p53 and ER proteins tended to be correlated in the cell lines and tumor specimens. This correlation, if confirmed in a greater number of tumor samples, may suggest common elements in the regulatory pathway of these two critical proteins in breast cancer. Because DAI can be used not only to assess functionality but also to retrieve p53 and ER proteins and interacting proteins in complexes with DNA, it has the potential for identifying putative common elements in parallel pathways.

Although EMSA has been widely used in p53 DNA binding studies, it has not been successfully applied to samples from solid tumor specimens. Tissue lysate derives from a complex mass containing extracellular matrix, vascular networks, and various cell subpopulations that may be quiescent and may contain latent p53 protein. Consequently, the relative p53 activity in tissue lysate can be much lower than in cell culture lysate. Unlike EMSA, in which sample volume is restricted by the capacity of the electrophoresis gel well, DAI sensitivity can be improved by increasing input lysate in the reactions. DAI detected p53-DNA binding in tumor tissue lysates at five times the standard sample volume in EMSA. Furthermore, DAI detected the DNA binding activities of wild-type p53 and the various p53 mutants found in human tumors consistent with their known activities, and results could be more precisely measured and compared among samples. DAI may be applied to human tumor molecular profiling for comparisons among tumor samples and testing of relationships to prognosis and outcome of chemotherapy. Such functional profiling may be of particular usefulness in initial evaluation and monitoring of effectiveness of molecular therapies targeting the DNA-binding protein of interest, such as p53 gene therapy to reintroduce function or by lytic adenoviruses activated by a lack of p53 function. As with any biochemical or molecular assessment of tissue lysate, tissue handling must be optimized and standardized, e.g., by minimizing the time from surgical dissection to sample freezing and by optimizing homogenization and extract preparation and cryopreservation. Optimization of freezer storage conditions may minimize potential decreases in DNA binding activity, for example, by protein oxidation. Cysteine, an amino acid critical for DNA binding is particularly susceptible, and it has been reported that wild-type p53 can...
acquire a mutant conformation by exposure to the oxidant diamide (39). An internal control DNA-binding protein (such as the general transcription factor TATA-binding protein) could be included to normalize the functional index among samples based on the quality of tissue lysate.

Basic research applications of DAI include functional assessment of upstream activating pathways in p53 or other DNA proteins, the activity of which depends on posttranslational modification. The DAI assay permits comparison of activities and specificity of DNA-binding proteins for different template sequences. It lends itself to characterization of DNA-binding protein activities in cells at different stages of response to DNA damage or other activators. It not only permits assessment of activity but retrieval of the DNA-binding protein and associated proteins in the complex that may contribute to its activities. Clinical application of DAI will depend on an extensive longitudinal survey to determine the correlation between active DNA binding and clinical response to therapy. Functional quantitative analysis has potential for molecular profiling of individual cancers to predict prognosis and treatment response. In view of the complex nature of cancer, the accurate prediction of a clinical course will require multiple biomarkers. Multiparameter analysis in the same samples is critical to understanding connections among pathway defects and will provide a more accurate profile for predicting prognosis. The DAI assay lends itself to functional multiparameter analysis because it can simultaneously detect multiple DNA-binding proteins that differ in molecular weight in the same reactions, using specific biotinylated DNA templates and antibodies. Because most DNA-binding proteins are transcription factors that govern a variety of downstream events, it is conceivable that a global view of gene expression can be evaluated by measuring DNA binding activities of a few “sentinel” DNA-binding proteins.

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