A Gel-electrophoretic Analysis for Improved Sensitivity and Specificity of DNA-dependent Protein Kinase Activity

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DNA-dependent protein kinase (DNA-PK) is considered a critical enzyme in the repair and/or signal transduction of DNA double-strand breaks. DNA-PK activity has been mostly measured through “DNA-plus-minus” or “DNA-pull-down” procedures using synthetic peptide as substrate followed by filter-binding analysis, i.e. liquid scintillation counting of acid-insoluble radioactivity bound to phosphocellulose filter. Considering that non-specific phosphorylation of other cellular proteins in filter-bound acid-insoluble count could interfere with the detection of specific phosphorylation of peptide substrate, we examined the specificity and characteristics of these assay procedures by SDS gel-electrophoresis of the reaction mixture. The electrophoretic pattern showed phosphorylation in wide range of non-specific protein bands other than the specific substrate. The very low DNA-PK activity shown by murine L5178Y or FSA1233 cells was unambiguously detectable as the count in substrate band. Even following DNA-pull-down procedure, which would separate DNA-PK from most of other protein kinases, substantial amount of phosphorylation of other cellular proteins were still contaminated. Thus by selectively counting the particular bands, small amount of specific phosphorylation of peptide substrate was reliably quantified. These results indicated that the DNA-PK activity through filter-binding analysis was, as suspected, contaminated by non-specific phosphorylation of other cellular proteins and also that the gel-electrophoretic analysis would improve detectability of specific phosphorylation by DNA-PK of synthetic peptide substrate and, therefore, would improve the kinase assay in both sensitivity and specificity.

INTRODUCTION

DNA-dependent protein kinase (DNA-PK) is a serine/threonine kinase, composed of 470...

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Abbreviations: DNA-PK; DNA-dependent protein kinase, DNA-PKcs; DNA-PK catalytic subunit, scid; severe combined immunodeficiency, SDS-PAGE; sodium dodesylsulfate-polyacrylamide gel electrophoresis
kDa catalytic subunit (DNA-PKcs) and 86 kDa and 70 kDa heterodimer of Ku antigen (Ku86 and Ku70, respectively) \(^1\)-\(^3\). DNA-PK binds to and requires ends of double-strand DNA to phos- 
phorylate a number of nuclear proteins including p53 and replication protein A \(^1\)-\(^3\). DNA-PKcs and 
Ku86 subunits are product of murine \textit{scid} (severe combined immunodeficiency) causative gene 
and XRCC5 (X-ray repair cross-complementing) gene, respectively, that are defective in a num-
ber of ionizing radiation-sensitive mammalian cells with defective DNA double-strand break 
repair \(^4\)-\(^10\). DNA-PK activity is lost by radiosensitizing agent such as hyperthermia or 
wortmannin \(^3\),\(^11\)-\(^14\). Thus DNA-PK is considered a critical enzyme in the repair and/or the signal 
transduction of double-strand breaks induced by ionizing radiation. Additionally, DNA-PK might 
play essential roles in V(D)J recombination \(^4\)-\(^9\).

The widely used protocols to quantify DNA-PK activity were “DNA-plus-minus” and 
“DNA-pull-down” assays and synthetic peptide as substrate with modified sequence around one 
of DNA-PK phosphorylation site, Ser 15, of p53 (Fig. 1 and Refs. 15–17). Since the peptides 
were inefficiently phosphorylated in lysates from DNA-PK deficient cells or in the absence 
of DNA, they have been considered selective substrates for DNA-PK \(^15\)-\(^17\). In “DNA-plus-minus” 
assay protocol, the crude cell lysate is incubated with these peptides and [\(\gamma\)-\(^32\)P] ATP as sub-
strates in the presence or absence of DNA, absorbed onto phosphocellulose filters and washed in 
acetic acid and ethanol followed by liquid scintillation counting and DNA-dependent phosphory-
lation of the peptide is interpreted as DNA-PK activity \(^15\). However, this protocol has difficulty in 
detecting DNA-PK activity in rodent cells, which is at least 10- to 50-fold lower than that in 
human cells, probably due to lower expression of DNA-PKcs and/or Ku subunits \(^15\)-\(^17\). “DNA-
pull-down” assay protocol differs from the above protocol in that crude cell lysate was mixed 
with DNA-cellulose and precipitated prior to kinase reaction \(^16\). This procedure, possibly separat-
ing DNA-PK from most of other kinases, phosphatases and possible inhibitors through specific 
binding of DNA-PK to DNA, enabled detection of low DNA-PK activity in rodent cells \(^16\).

Here we analyzed these DNA-PK assay procedures by fractionating the reaction mixture 
through gel electrophoresis, instead of binding to phosphocellulose filter, in order to separate 
peptide substrate from cellular proteins. The results showed that non-specific phosphorylation on 
cellular proteins interfered with the detection of the specific phosphorylation of peptide substrate 
in murine cell lines and that SDS gel-electrophoretic analysis substantially improved both sensi-
tivity and specificity of the previous procedures.

**MATERIALS AND METHODS**

**Cell culture and lysate preparation**

Human leukemic MOLT-4 cells \(^1\),\(^18\),\(^19\), murine leukemic L5178Y cells \(^20\) and murine fibro-
sarcoma FSA1233 cells \(^21\) have been used in our laboratory. These cells were cultured at 37°C in 
RPMI medium with 10% bovine calf serum, in Fischer’s medium with 10% equine serum and in 
McCoy’s 5A medium with 10% bovine calf serum, respectively. All media and sera were pur-
chased from Life Technologies (Chagrin Falls, Ohio, USA). Ten to twenty million cells were 
recovered either by treatment with 0.25% trypsin (Boehringer Mannheim, Mannheim, Germany)
for 5 min at room temperature followed by centrifugation at 1,000 rpm (200 \( \mu \)g) for 5 min at 4°C for FSA1233 cells or by centrifugation alone for MOLT-4 or L5178Y cells, rinsed with ice-cold Tris-buffered saline (10 mM Tris-HCl (pH 7.4), 140 mM NaCl, 2 mM MgCl_2) and resuspended in 200 \( \mu l \) of ice-cold high salt buffer (20 mM HEPES-NaOH (pH 7.9), 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% v/v glycerol, 0.02% v/v Tween 20, 1 mM DTT, 1 mM PMSF, 1 \( \mu g/ml \) of leupeptin, pepstatin and antipain, respectively). The cell suspension was lysed by three rounds of freeze-thaw cycle, i.e. repeated freezing in liquid nitrogen bath followed by thawing in water bath at 30°C, and was clarified by centrifugation at 15,000 rpm (18,000 \( \mu g \)) for 7 min at 4°C.

In experiments assaying DNA-PK activity in mouse tissue, crude lysate was prepared from liver of wild-type C3H/HeJ or C.B.17\textit{scid} mice (Nippon Clea, Tokyo, Japan). Pieces of liver, weighing 0.1 to 0.3 g, were rinsed with ice-cold Tris-buffered saline and resuspended in 0.5 to 1.5 ml of ice-cold high salt buffer. The suspension was homogenized using glass-teflon homogenizer (2 ml capacity, Ikemoto Scientific Technology, Tokyo, Japan) followed by freeze-thaw cycle and clarified by centrifugation at 15,000 rpm (18,000 \( \mu g \)) for 7 min at 4°C.

Protein concentration was assayed using BCA protein assay kit (Pierce, Rockford, Illinois, USA) with bovine serum albumin as the standard.

Kinase assay

The kinase assay procedures are outlined in Figs. 1 or 2 and detailed below.

DNA-plus-minus assay

Crude cell lysate (150 \( \mu l \), 10–20 mg protein/ml) was passed through DEAE Bio-Gel A column (150 \( \mu l \) of bed volume, Bio-Rad, Richmond, California, USA) in order to remove nucleic acids and diluted serially with the high salt buffer. Five \( \mu l \) of the diluted lysate was mixed with 15 \( \mu l \) of 1.33 \( \times \) kinase assay buffer (contents of 1 \( \times \) kinase assay buffer: 20 mM HEPES-NaOH (pH 7.2), 5 mM MgCl_2, 150 mM KCl, 50 \( \mu M \) \( \gamma^{32}P \)-ATP, 1 mM DTT and 0.5 mM each of NaF and \( \beta \)-sodium glycerophosphate) with or without 0.25 \( \mu g/\mu l \) synthetic peptide hp53-S15 (sequence: EPPLSQEAFADLWKK) and/or 20 ng/\( \mu l \) sonicated salmon sperm DNA (Fig. 1C and Refs. 11, 15) was incubated at 37°C for 10 min. The reaction was stopped by the addition of 20 \( \mu l \) of either 30% acetic acid or 2 \( \times \) SDS-PAGE sample buffer (contents of 1 \( \times \) SDS-PAGE sample buffer: 62.5 mM Tris-HCl (pH 6.8), 2% sodium lauryl sulfate, 10% glycerol, 2.5% \( \beta \)-mercaptoethanol, 0.01% bromophenolblue, 0.005% crystalviolet) and analyzed through filter-binding or gel-electrophoresis, respectively (see below). DNA-dependent portion of hp53-S15 phosphorylation is regarded as the DNA-PK activity.

DNA-pull-down assay

Crude cell lysate (150 \( \mu l \), 10–20 mg protein/ml) was diluted serially with the high salt buffer and a fraction of 15 \( \mu l \) was mixed with 5 mg of native DNA-cellulose (Pharmacia, Uppsala, Sweden) suspended in 35 \( \mu l \) of binding buffer (20 mM HEPES-NaOH (pH 7.2), 1 mM MgCl_2, 50 mM KCl, 15% v/v glycerol, 1 mM DTT). After 30 min at 4°C, the mixture was centrifuged at 15,000 rpm for 3 min and the precipitate was rinsed with 1 ml of binding buffer, resuspended in the 20 \( \mu l \) of 1 \( \times \) kinase assay buffer containing 150 mM KCl, instead of 50 mM KCl, with or
Figure 1. “DNA-plus-minus” and “DNA-pull-down” assays for DNA-PK activity. A and B show the outline of DNA-plus-minus assay and DNA-pull-down assay, respectively. These procedures should be followed either by filter-binding analysis or by gel-electrophoretic analysis (Fig. 2). C shows the amino acid sequence of synthetic peptide hp53-S15, as DNA-PK substrate, compared with the original human p53 protein between Glu11 and Lys24. The DNA-PK phosphorylation site, Ser15, is underlined and modified residues are shown in italics. Thr18 and Ser20 in human p53 protein were changed to alanine in order to minimize phosphorylation by other kinases and an extra lysine residue was appended to the carboxy-terminal in order to strengthen binding to phosphocellulose filter.

Figure 2. Filter binding and gel-electrophoretic analysis in DNA-PK assay.
without 0.25 μg/μl hp53-S15 and incubated at 37°C for 10 min. Reaction was stopped by the addition of 20 μl of either 30% acetic acid or 2% SDS-PAGE sample buffer and the supernatant following centrifugation at 10,000 rpm (8,000 × g) for 10 sec was either transferred to phosphocellulose filter or separated by gel electrophoresis, respectively (see below). The whole phosphorylation of hp53-S15 in this assay is regarded as the DNA-PK activity.

Filter-binding analysis

Acetic acid-added reaction mixture (40 μl) was absorbed onto a P81 phosphocellulose filter (2.3 cm of diameter, Whatman, Maidstone, UK) and was washed in 15% acetic acid and in 99% ethanol followed by counting in a liquid scintillation counter11,15). The net phosphorylation of hp53-S15 was calculated as phosphate incorporation in reaction with hp53-S15 minus that in reaction without hp53-S15 divided by the specific radioactivity of ATP.

Gel-electrophoretic analysis

SDS-PAGE sample buffer-added reaction mixture (40 μl) was heated at 95°C for 5 min and separated by SDS-PAGE, through 15% polyacrylamide gel (acrylamide : bisacrylamide = 292 : 8) in Tris-glycine buffer system according to Laemmli22), followed by scanning of dried gel using phosphor imager BAS2000 (Fuji, Tokyo, Japan). The front edge of the gel, which might contain ATP or free phosphate, was cut away. The phosphorylation of hp53-S15 was estimated from the phosphorescence of the spot with known amount of ATP as the standard. The reaction mixture without cell lysate served as the negative control and the amount was subtracted as non specific background count.

RESULTS

Gel-electrophoretic analysis of “DNA-plus-minus” assay for DNA-PK activity

DNA-PK activity in murine L5178Y or FSA1233 cells was much lower than that in human MOLT-4 cells and was difficult to detect through DNA-plus-minus/filter-binding assay (Fig. 3). Additionally, the quantification of DNA-PK activity in L5178Y or FSA1233 cells might be affected by overwhelming non-specific phosphorylation, which was dependent neither on hp53-S15 nor on DNA (data not shown). These results are agreeable with previous reports showing that DNA-PK activity in rodent cells, being much lower than that in human cells, could not be quantified reliably through DNA-plus-minus/filter-binding procedure, possibly buried in non-specific phosphorylation of cellular proteins15–17).

Regarding that hp53-S15 is much smaller than most of cellular proteins, we fractionated the reaction mixture by SDS-PAGE through high percentage, i.e. 15%, of polyacrylamide gel followed by phosphor image analysis, instead of binding to phosphocellulose filter (Fig. 4). The spots indicated with arrowheads would most likely be phosphorylated hp53-S15, since it migrated nearly to the front edge of the gel, as expected from the molecular mass of 1.8 kDa, and was absent in the reaction mixture without the peptide. The spots appearing above hp53-S15 might be phosphorylated cellular proteins. In the presence of DNA, the phosphorylation of hp53-
S15, as estimated from the phosphorescence of spot, was enhanced by 20 to 40-fold in MOLT-4 and by 2 to 3-fold in L5178Y or FSA1233 cells. DNA-PK activity was 40- to 50-fold and 100- to 150-fold lower in L5178Y cells and in FSA1233 cells, respectively, than in MOLT-4 cells (Figs. 4 and 5). DNA-independent phophorylation might not be solely due to contaminated cellular DNA, since it would be removed through DEAE-column, but more likely due to kinase(s) other than DNA-PK (see below). DNA-PK activity tended to saturate with increasing protein concentration, possibly due to inhibitors. Additionally, non-specific phosphorylation of cellular proteins were comparable among three cell lines and, as a result, overwhelmed the specific phosphorylation of hp53-S15 in L5178Y and FSA1233 cells (Fig. 4). The conventional filter-binding assay and the gel-electrophoretic assay gave similar result for MOLT-4 cells with high DNA-PK activity.

**Figure 3.** DNA-plus-minus/filter-binding kinase assay. Crude cell lysate was passed through DEAE-column, diluted and incubated at 37°C for 10 min in kinase assay buffer with or without hp53-S15 and/or sonicated salmon sperm DNA. The reaction mixture was transferred to phosphocellulose filter and acid-insoluble radioactivity bound to filter was counted by liquid scintillation counter. The net phosphorylation of hp53-S15, either in the presence or absence of DNA, was calculated as the filter-binding radioactivity resulted in reaction with hp53-S15 minus that resulted in reaction without hp53-S15. The DNA-PK activity was then calculated as the net phosphorylation of hp53-S15 in the presence of DNA minus that in the absence of DNA divided by the specific radioactivity of ATP.
Figure 4. The gel-electrophoretic analysis of DNA-plus-minus kinase assay. The reaction mixture following the DNA-plus-minus kinase assay (Fig. 3) was separated through SDS-PAGE. The phosphor images of the gels are shown with arrowheads indicating hp53-S15. The spots appearing above hp53-S15 are cellular proteins. Molecular mass markers were myosin (220 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (46 kDa), Carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa).
but the latter assay gave much more reliable result for L5178Y or FSA1233 cells with low DNA-PK activity within a wider range of protein concentration (Figs. 3 and 5).

**Gel-electrophoretic analysis of “DNA-pull-down” assay for DNA-PK activity**

Low DNA-PK activity in murine L5178Y or FSA1233 cells were detectable through DNA-pull-down procedure, separating DNA-PK from most of other protein kinases, followed by filter-binding analysis. However, 30 to 50% of filter-bound radioactivity was non-specific, i.e. independent of hp53-S15 (data not shown). Gel-electrophoretic analysis of the reaction mixture after DNA-pull-down procedure showed that phosphorylation of non-specific cellular protein was still significant (Fig. 6). DNA-PK activity could be estimated from the phosphorescence of hp53-S15 spot (Fig. 7). Compared to DNA-plus-minus assay, DNA-PK activity was linear within a wide range of protein concentration, possibly due to removal of inhibitors (Figs. 5 and 7).
Figure 6. The gel-electrophoretic analysis of DNA-pull-down assay. Crude cell lysate was diluted, mixed with DNA-cellulose and precipitated. The precipitate was then incubated at 37°C for 10 min in kinase assay buffer with or without hp53-S15 as indicated. The reaction mixture was separated by SDS-PAGE and the phosphor images of the gels are shown with arrowheads indicating hp53-S15. The spots appearing above hp53-S15 are cellular proteins.
Kinase activity in scid mouse

Lysate homogenate preparations from the liver of DNA-PKcs deficient scid mouse or wild-type mouse were examined for kinase activity using hp53-S15 as substrate. Using DNA-plus-minus and electrophoretic analysis, presence of DNA enhanced the phosphorylation of hp53-S15 two-fold in normal mouse liver lysate but not in scid mouse liver lysate (Fig. 8A and B). Background amount of phosphorylation in the absence of DNA, which was similar between wild-type and scid mouse, was considered non-specific including those catalyzed by kinase(s) other than DNA-PK. In DNA-pull-down and electrophoretic analysis, peptide phosphorylation was evident in wild-type mouse liver lysate, but not in scid mouse liver lysate (Fig. 8C and D). DNA-PK activity per mg protein of crude liver lysate was less than one tenth of that of cultured murine cell lines L5178Y or FSA1233 (compare Fig. 8 with Figs. 3 and 5).
In the present study, we investigated the specificity and characteristics of widely used “DNA-plus-minus” and “DNA-pull-down” methods for DNA-PK assay using gel-electrophoretic analysis to clarify the problems, especially, in measuring very low DNA-PK activity in rodent cells. The problems may be attributed partly to extremely low activity and to overwhelming non-specific phosphorylation of cellular proteins. Gel-electrophoretic phosphor image analysis could demonstrate unambiguously the specific but low level phosphorylation of hp53-S15 in the preparations from murine cell lines with enhancing effect of DNA in spite of that the amount of non-specific phosphorylation of cellular proteins was substantial against the specific phosphorylation of the peptide substrate. Although DNA-pull-down assay was found less affected by other kinases than DNA-plus-minus assay, phosphorylated bands of other cellular proteins still existed. These results indicated that electrophoretic separation improved detectability, both in sensitivity

**Figure 8.** Kinase activity toward hp53-S15 in scid and wild-type mouse liver lysate. In A and B, crude liver lysate from either scid or wild-type mouse was passed through DEAE-column, and a fraction containing 5 μg of protein was incubated at 37°C for 10 min in kinase assay buffer with or without hp53-S15 and/or sonicated salmon sperm DNA as indicated. In C and D, a fraction of crude liver lysate containing 75 μg of protein was mixed with DNA-cellulose and the precipitate was incubated at 37°C for 10 min in kinase assay buffer with or without hp53-S15 as indicated. A and C are the phosphor images of the gel after electrophoresis of the reaction mixture, with arrowheads indicating phosphorylated hp53-S15. B and D show the phosphorylation of hp53-S15, estimated from the phosphorescence of spots with known amount of ATP as the standard. Two bars in a pair represent results from repeated or duplicate experiments.

**DISCUSSION**

In the present study, we investigated the specificity and characteristics of widely used “DNA-plus-minus” and “DNA-pull-down” methods for DNA-PK assay using gel-electrophoretic analysis to clarify the problems, especially, in measuring very low DNA-PK activity in rodent cells. The problems may be attributed partly to extremely low activity and to overwhelming non-specific phosphorylation of cellular proteins. Gel-electrophoretic phosphor image analysis could demonstrate unambiguously the specific but low level phosphorylation of hp53-S15 in the preparations from murine cell lines with enhancing effect of DNA in spite of that the amount of non-specific phosphorylation of cellular proteins was substantial against the specific phosphorylation of the peptide substrate. Although DNA-pull-down assay was found less affected by other kinases than DNA-plus-minus assay, phosphorylated bands of other cellular proteins still existed. These results indicated that electrophoretic separation improved detectability, both in sensitivity.
and in specificity, of the phosphorylation by DNA-PK, because of separability of synthetic peptide substrate from most of cellular proteins due to the difference in molecular weight. Additionally, DNA-PK activity determined by DNA-plus-minus assay tended to saturate with increasing cell lysate preparation, while that determined by DNA-pull-down assay was linear within a wide range of protein concentration.

DNA-PKcs was considered the causative gene for scid mice and the mutation on DNA-PKcs gene was found at codon 4046 in carboxy terminal catalytic domain, with 83 amino acids missing. Although Blunt et al reported the absence of DNA-PK activity in scid cell lines using DNA-pull-down assay without electrophoretic analysis, recently Woo et al reported that primary embryonic fibroblast, but not established cell lines, from scid mice had DNA-PK activity about a half of control Balb/c fibroblast. Considering these discrepant results, it was obvious that further studies were necessary. In the present DNA-pull-down and electrophoretic analysis, hp53-S15 phosphorylation was undetectable in scid mouse tissue. In our DNA-plus-minus and electrophoretic analysis, there was no DNA-dependent hp53-S15 phosphorylation in scid liver lysate, although DNA-independent background phosphorylation was comparable to wild-type. Since the contamination of DNA in lysate preparation was minimized by passing through DEAE-column, these results indicate that DNA-dependent phosphorylation of hp53-S15 was mainly due to DNA-PKcs, while DNA-independent phosphorylation might be due to other kinases. In this regard, ATM and ATR, with sequence similarity to DNA-PKcs in carboxy-terminal catalytic domain, have been recently reported to phosphorylate Ser15 of p53 in response to DNA damage but without enhancing effect of DNA. Priestley et al, in a very recent publication, also used successfully a similar procedure to our DNA-pull-down with electrophoretic analysis to demonstrate the absence of DNA-PK activity in chinese hamster ovary-derived DNA-PKcs-deficient cell line, irs.

As described above, the electrophoretic kinase assay was useful because of its high specificity and sensitivity and enabled us to reliably examine DNA-PK, in detail, of low activity such as murine cells and tissues.

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