Human Lymphoblastoid Proteome Analysis Reveals a Role for the Inhibitor of Acetyltransferases Complex in DNA Double-Strand Break Response

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Abstract

A DNA double-strand break (DSB) is highly cytotoxic; it emerges as the type of DNA damage that most severely affects the genomic integrity of the cell. It is essential that DNA DSBs are recognized and repaired efficiently, in particular, prior to mitosis, to prevent genomic instability and eventually, the development of cancer. To assess the pathways that are induced on DNA DSBs, 14 human lymphoblastoid cell lines were challenged with bleomycin for 30 and 240 minutes to establish the fast and more prolonged response, respectively. The proteomes of 14 lymphoblastoid cell lines were investigated to account for the variation among individuals. The primary DNA DSB response was expected to occur within the nucleus; therefore, the nuclear extracts were considered. Differential analysis was done using two-dimensional difference in gel electrophoresis; paired ANOVA statistics were used to recognize significant changes in time. Many proteins whose nuclear levels changed statistically significantly showed a fast response, i.e., within 30 minutes after bleomycin challenge. A significant number of these proteins could be assigned to known DNA DSB response processes, such as sensing DSBs (Ku70), DNA repair through effectors (high-mobility group protein 1), or cell cycle arrest at the G2-M phase checkpoint (14-3-3ζ). Interestingly, the nuclear levels of all three proteins in the INHAT complex were reduced after 30 minutes of bleomycin challenge, suggesting that this complex may have a role in changing the chromatin structure, allowing the DNA repair enzymes to gain access to the DNA lesions. (Cancer Res 2006; 66(3): 1473-80)

Introduction

Decreased DNA stability is a key factor in the development of cancer. An impaired response to DNA damage, in particular to double-strand breaks (DSB), plays a role in the earliest stages of carcinogenesis (1). In addition, in inherited genetic syndromes such as ataxia telangiectasia and Fanconi anemia, genes that play a role in the maintenance of DNA stability were affected, and these syndromes are associated with a predisposition to cancer (2). Specific polymorphisms or low pathogenic mutations in these genes may subtly affect the function of the encoded enzymes, causing intermediate phenotypes associated with a moderate increase in cancer risk (2–4). Nevertheless, not only the genotype, but also the exposure to environmental carcinogens is an important determinant for cancer risk. In particular, the epithelial cells lining the body surfaces are continuously challenged by exogenous DNA-damaging agents. A typical example in this respect is head and neck squamous cell carcinoma (HNSCC). These tumors arise in the mucosal linings of the upper aerodigestive tract in the 6th and 7th decade of life. The main etiologic factors are smoking and alcohol drinking, which together, have a synergistic effect. The risk of developing HNSCC is related to carcinogen exposure, and for heavy smokers and drinkers, the relative risk is as large as 20 (5). Notwithstanding, the large majority of smokers and alcohol drinkers do not develop HNSCC, indicating that the individual cancer risk does not depend solely on exposure, but that this acts in concert with the capacity of individuals to deal with the DNA damage induced by these exogenous agents (6–8).

Of the numerous DNA damage types occurring, cellular genome integrity is most severely affected by DSBs. Cells respond to DSBs by activating complex response pathways, including cell cycle arrest, DNA repair activation, and—in case of extensive damage—apoptosis (9, 10). There are two distinct and complementary mechanisms known for DNA DSB repair, i.e., homologous recombination and nonhomologous end-joining (10). Much is already known about the signaling pathways that are induced in the formation of DNA DSBs. The involvement of tumor suppressor gene products such as p53, ATM, BRCA1, BRCA2, and of many other genes involved in the cell cycle checkpoint, DNA repair, or apoptosis, has thus far predominantly been investigated at the level of single proteins or separate signaling pathways. These signaling pathways have been studied at the mRNA level to establish the effect of DSB induction, like the transcriptional response of lymphoblastoid cells to ionizing radiation (11–13). However, an integrative analysis of the proteomic response to DNA DSB induction in cells, involving several human samples to enable appropriate statistics, has never been reported.

The proteomic response of DNA DSB induction in human lymphoblastoid cells was determined at two time points, to identify both the fast and more prolonged changes. The induction of DNA damage was accomplished by bleomycin, which was selected because its mode of action resembles that of environmental carcinogens. Moreover, we are particularly interested in the response to DSBs, and bleomycin is more efficient in producing DSBs than the often used ionizing radiation because it binds to the DNA and can cleave the DNA at two opposing strands, resulting in a DSB (14). The ratio between double- and single-strand breaks is 1:9 for bleomycin, and 1:100 for ionizing radiation (15). The
samples were analyzed using two-dimensional difference in gel electrophoresis (DiGE), allowing the simultaneous analysis of many samples which could not be easily done using any of the alternative approaches in quantitative proteomics. The use of fluorescent dyes permitted the analysis of small changes in protein expression both for relatively low and high protein levels, and the use of an internal standard to normalize the gel-to-gel variation. Proteins whose nuclear levels changed statistically significantly upon DNA DSB induction were identified using (tandem) mass spectrometric techniques. Our results showed that many of the identified proteins are part of known DNA DSB response signaling pathways and that these proteins showed a relatively fast response. Surprisingly, a fast decrease was detected in the relative levels of the three proteins forming the so-called “inhibitor of acetyltransferases” or INHAT complex, of which involvement in DNA DSB response has not been previously described.

Materials and Methods

Donor characteristics, cell culture, and stimulation of cells. Blood was drawn from 14 individuals who took part in an epidemiologic study evaluating risk factors for HNSCC. The study was approved by the Institutional Review Board of the VU University Medical Center, and written informed consent was obtained from all individuals. For each individual, a lymphoblastoid cell line was produced by transformation with Epstein-Barr virus, after which the phenotype was checked (7). These cell lines were cultured for a rather short time (no more than 20 passages) in RPMI 1640 (with glutamine) supplemented with 15% fetal bovine serum (Bio Whittaker, Verviers, Belgium), 1% penicillin and streptomycin (Life Technologies, Paisley, United Kingdom), and 0.1% 1 mol/L pyruvic acid (Sigma, Zwijndrecht, the Netherlands). Cells were incubated in a humidified 5% CO2 in cultured flasks with filter caps (Nalgene-Nunc, Roskilde, Denmark). Cells were challenged using bleomycin (Dagra Pharma, Diemen, the Netherlands) at a final concentration of 10 µmol/L in 0.1% hypertonic buffer containing 20 mmol/L HEPES (pH 7.9), 420 mmol/L KCl, 1 mmol/L DTT, and 20% glycerol. Nuclei were purified using the protocol of Busch and Daskal (17). Subsequently, nuclear extracts were prepared basically as described by Valcarcel and Stunnenberg (18). In short, purified nuclei were lysed in a humified incubator at 37°C in 5% CO2 in cultured flasks with filter caps (Nalgene-Nunc, Roskilde, Denmark). Cells were challenged using bleomycin (Dagra Pharma, Diemen, the Netherlands) at a final concentration of 10 µmol/L in H2O to induce DSBs (16), and were harvested after 30 and 240 minutes, separately. Control cells were harvested without any challenge.

Sample preparation and protein labeling. For harvesting, cells were pelleted and washed on ice using PBS containing one tablet/25 ml of complete protease inhibitor cocktail (Roche Diagnostics, Almere, the Netherlands) and 1:100 (v/v) of phosphatase inhibitor cocktails 1 and 2 (Sigma). Nuclei were purified using the protocol of Busch and Daskal (17). Subsequently, nuclear extracts were prepared basically as described by Valcarcel and Stunnenberg (18). In short, purified nuclei were lysed in hypertonic buffer containing 20 mmol/L HEPES (pH 7.9), 420 mmol/L KCl, 5 mol/L MgCl2, 0.1 mmol/L EDTA, 20% glycerol, 1 mmol/L DTT, and protease and phosphatase inhibitors as mentioned above. After continuous stirring at 4°C for 1 hour, samples were centrifuged at 40,000 × g. The supernatants, i.e., the nuclear extracts, were collected and stored at −80°C.

Nuclear extract protein concentrations were determined using the PlusOne 2D Quant Kit (Amersham Biosciences, Uppsala, Sweden). For each two-dimensional gel, 50 µg of nuclear extract proteins were precipitated using the PlusOne 2D Clean-up kit (Amersham Biosciences) and was redissolved in 5 to 10 µl labeling buffer containing 30 mmol/L Tris (pH 8.5), 7 mol/L urea, 2 mol/L thiourea, and 4% CHAPS.

Nuclear protein samples were labeled as previously described (19). In brief, 50 µg of precipitated protein sample was labeled with 400 pmol of one of the cyanine dyes (Cy3 or Cy5; see Table 1 for labeling scheme) during 30 minutes in the dark. The internal standard, consisting of equal amounts of all 42 samples, was labeled with Cy2. The cyanine dyes contain an NHS-ester reactive group that covalently attaches to the epsilon amino group of the protein lysine residues. Because the cyanine dyes are limiting in the reaction, on average, only one lysine residue is labeled per protein. The labeling reaction was quenched by adding 1 µl of a 10 mol/L lysine solution. Both steps were done at 4°C. CyDye labeling efficiency was verified by comparing the total density of a one-dimensional SDS-PAGE image of a nuclear extract lysate with that of an Escherichia coli lysate, prepared according to the description of the manufacturer.

Two-dimensional DiGE. After pooling the Cy2-labeled internal standard, Cy3-, and Cy5-labeled samples according to Table 1, the proteins were resolved over two dimensions according to their charge and size via two-dimensional gel electrophoresis. Conditions for two-dimensional gel electrophoresis were as previously described (19). The cyanine dyes are designed such that dye-labeled lysines still contain a single positive charge and possess an added Mf of ~500 Da. This combination of size- and charge-matching ensures an exact overlay of the protein patterns from the three differently labeled samples in a single two-dimensional gel.

Image acquisition and statistical analysis. Because the three fluorescent Cy-dyes are spectrally distinct, each sample in a two-dimensional gel image was independently scanned at the appropriate label-specific excitation and emission wavelengths using narrow bandpass filters with a Typhoon 9400 imager (Amersham Biosciences). DeCyder software (version 5.01.01, Amersham Biosciences) was used for image analysis as described before (19). The number of estimated spots was set to 2,500. Detection and matching of protein spots required manual intervention to set landmarks on the gels to increase cross-gel matching accuracy. Data concerning the spot volume (with background subtracted) were collected for each spot, and then standardized abundance ratios were calculated for each pair of images (Cy3/Cy2 and Cy5/Cy2). These ratios were normalized to correct for differences in dye intensities, and subjected to statistical analysis, which was all done within the DeCyder software. A paired ANOVA method was used, which assigned statistical significance to the differences in normalized protein abundance between the three different time points of bleomycin stimulation. First, a change in protein spot intensity was only further regarded as relevant when it was detected in at least 80% of the 84 images. Second, the nuclear protein abundances were considered to be significantly changed when the paired ANOVA was P < 0.05.

To account for genetic variations among individuals, 14 human lymphoblastoid cell lines were explored, which served as replicates. To establish the effect of considering less than 14 individuals, data sets from 2 to 14 randomly chosen dissimilar samples were evaluated with paired ANOVA statistics, using the same criteria as described for the evaluation of 14 samples, i.e., detection of the spot in >80% of the gels (P < 0.05).

![Table 1. Setup of the two-dimensional DiGE experiment to analyze 3 × 14 human nuclear extracts in a paired manner](https://www.aacrjournals.org)

<table>
<thead>
<tr>
<th>Two-dimensional gel no.</th>
<th>Cy2-labeled sample</th>
<th>Cy3-labeled sample</th>
<th>Cy5-labeled sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>internal standard</td>
<td>nuclear extract (t = 0)</td>
<td>nuclear extract (t = 30')</td>
</tr>
<tr>
<td>2</td>
<td>internal standard</td>
<td>nuclear extract (t = 0)</td>
<td>nuclear extract (t = 240')</td>
</tr>
</tbody>
</table>

NOTE: The lymphoblastoid cells were challenged with bleomycin for 0, 30, and 240 minutes, indicated with (t = 0), (t = 30'), and (t = 240'), respectively. For each of the 28 gels, the Cy2-labeled internal standard consisted of an equal amount of all 42 samples that were analyzed. Other samples whose differential nuclear protein abundances had to be established were labeled with Cy3 or Cy5, as indicated.
Staining and protein identification using mass spectrometry. As previously described (19), gels were silver stained after fluorescence detection, which enabled manual spot excision, and subsequently protein digestion was done. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS peptide mass fingerprints were acquired on a Voyager DE-STR MALDI TOF mass spectrometer (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) in the positive reflectron mode with delayed extraction, using the following settings: accelerating voltage, 20 kV; grid voltage, 72%; guide wire, 0.01% delay time, 150 ns. Internal calibration was done using trypsin autodigest peaks. After baseline correction and noise filtering, spectra were de-isotoped and the resulting peak lists were searched against the Swiss-Prot and National Center for Biotechnology Information databases for protein identity using the Mascot search engine. Tandem mass spectra were acquired on a 4700 proteomics analyzer MALDI-TOF/TOF mass spectrometer (AB 4700 proteomics analyzer, Applied Biosystems). This instrument is equipped with a 200 Hz Nd/YAG laser operating at 355 nm. Experiments were done in a reflectron-positive ion mode using delayed extraction. Typically, 2,000 shots per spectrum were acquired in the MS mode and 15,000 shots per spectrum in the MS/MS mode. Details of the peptide sequence coverage and Mascot score are given in the Supplementary Table.

Biochemical analysis of INHAT proteins. A core histone NH2-terminal consensus peptide, with sequence SGRKAGKGRKGKTRQC, was immobilized onto Sulfolink beads (Pierce Biotechnology) at a concentration of 1 mg/mL. Affinity purifications were done as described by Schneider et al. (20). The immobilized peptide was fully acetylated during 1 hour at room temperature using NHS-acetyl in 200 mmol/L Na2CO3 (pH 8). After washing the beads, the experiment was repeated as described above. Eluted proteins were analyzed using one-dimensional SDS-PAGE and visualized using silver staining.

For Western blotting, nuclear proteins were resolved by SDS-PAGE and subsequently transferred to nitrocellulose (Bio-Rad, Veenendaal, the Netherlands), and probed with the primary anti–acidic nuclear phosphoprotein pp32 (pp32A) antibody (Abcam, Cambridge, United Kingdom) in block buffer (3% bovine serum albumin, 0.1% Tween 20, v/v in PBS) and incubated overnight at 4°C. A Cy5-linked secondary antibody (Abcam) was used to visualize the pp32A bands, fluorescence intensities were determined using the Typhoon 9400 fluorescence scanner (Amersham Biosciences), and subsequently normalized using the total protein intensity as detected with Coomassie blue.

Figure 1. Representative example of a two-dimensional gel image from the analysis set. Note that each spot intensity appears as the sum of three individual sample spot intensities because each gel contains three samples, labeled with separate Cy-Dyes. On average, 2,100 spots were separated on a nonlinear IPG strip (pH 3-10) in the first dimension and on a 12.5% SDS-PAGE gel in the second dimension. The selected subset of proteins, as listed in Table 2, are shown.

Results

Two-dimensional DiGE. The nuclear proteomic response to DNA DSB induction was investigated in 14 human lymphoblastoid cell lines, using two-dimensional DiGE. This approach permitted a simultaneous paired comparison of 56 samples, which were normalized against 28 internal standards (Table 1). Image analysis revealed that typically 2,100 protein spots were detected per gel, and that differences in both low and high expression levels could be quantified over a linear dynamic range of four to five orders of magnitude. Moreover, the set of 84 gel images was highly reproducible because >60% of the spots were detected in all gels. Figure 1 shows a representative example of a two-dimensional DiGE gel image in which spot positions are indicated for the proteins that will be discussed. Further characteristics of these indicated proteins are summarized in Table 2. The complete data set of identified proteins can be found in the Supplementary Table. Although we restricted the paired ANOVA to P < 0.05, the majority of P values were much smaller than 0.05; i.e., for 50 proteins, the P values ranged from 9 × 10^-18 to 0.01; for 16 proteins, the P values were 0.01 to 0.025; and for 22 proteins, the P values were 0.025 to 0.05. Hence, in total, 88 nuclear extract proteins showed a significant change in protein abundance, and remarkably, significant changes in protein abundance occurred rather fast, i.e., after 30 minutes of bleomycin challenge. Moreover, the number of lymphoblastoid cell lines necessary was estimated for the study described here, i.e., a study in which it is essential to account for genetic variations among individuals. As shown in Fig. 2, it seems that for this data set of 2,100 detected spots, a more or less constant number of significantly changed protein levels is found when 9 to 14 cell lines are considered. Figure 2 also shows an evaluation of the consistency of the data sets, i.e., how many proteins are found in common with the final set for 9 to 14 cell lines, whereas ≥92% proteins are in common for 11 to 14 cell lines.
**Table 2. Selected subset of proteins that showed significantly changed nuclear levels upon DSB induction**

<table>
<thead>
<tr>
<th>Protein classification</th>
<th>Protein name</th>
<th>Regulation*</th>
<th>Accession no.</th>
<th>Mass (Da)</th>
<th>p/i</th>
<th>Sequence coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensors of DNA damage</strong></td>
<td>ATP-dependent DNA helicase II, 70 kDa (Ku70)</td>
<td>▲ ▲</td>
<td>P12956</td>
<td>70,084</td>
<td>6.23</td>
<td>31</td>
</tr>
<tr>
<td><strong>Effectors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibitor of acetyltransferases complex</td>
<td>TAF 1α</td>
<td>▼ ▼</td>
<td>Q01105-1</td>
<td>33,469</td>
<td>4.23</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>TAF 1β</td>
<td>▼ ▼</td>
<td>Q01105-2</td>
<td>32,084</td>
<td>4.12</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>pp32A</td>
<td>▼ ▼</td>
<td>P39687</td>
<td>28,585</td>
<td>4.50</td>
<td>41</td>
</tr>
<tr>
<td>DNA-interacting proteins</td>
<td>HMG1</td>
<td>▼ ▼</td>
<td>P09429</td>
<td>25,049</td>
<td>5.62</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>HMG1</td>
<td>▼ ▼</td>
<td>P09429</td>
<td>25,049</td>
<td>5.62</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>HCC1</td>
<td>▲ ▲</td>
<td>P82979</td>
<td>23,713</td>
<td>4.10</td>
<td>44</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>Eukaryotic translation initiation factor 5A (eIF5A)</td>
<td>▼ ▼</td>
<td>P63241</td>
<td>17,049</td>
<td>4.08</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>14-3-3 ζ/θ (14-3-3)</td>
<td>▼ ▼</td>
<td>P63104</td>
<td>27,899</td>
<td>4.73</td>
<td>28</td>
</tr>
</tbody>
</table>

NOTE: The relation with DNA DSBs is discussed in the text. The regulation is either indicated by arrows (red and green to point out up-regulation or down-regulation, respectively) or by an equal sign (for "no change"). The complete list of identified differential proteins, and further relevant data are summarized in the Supplementary Table. All proteins were identified using MALDI-TOF peptide mass fingerprinting and MALDI-TOF/TOF peptide sequencing.

*Regulation of protein expression levels; A, regulation between 0 and 30 minutes of bleomycin stimulation; B, regulation between 30 and 240 minutes of bleomycin stimulation.

1Accession number according to entries in the Swiss-Prot database.
2Theoretical values, mass in Daltons.
3Theoretical values.
4Obtained by a combination of peptide mass fingerprinting and ToF/ToF peptide sequencing.

**Protein identification.** Using MALDI peptide mass fingerprinting and MALDI-TOF/TOF sequence analysis, 56 significantly changed proteins (which are summarized in Table 2 and the Supplementary Table) could be identified. The low expression level of the 32 other significantly changed proteins hampered their identification, even when analyzing spots from preparative gels. This shows that the sensitivity of fluorescent detection exceeds that of MS detection. An example of the protein identification of high-mobility group protein 1 (HMG1) using mass spectrometry is depicted in Fig. 3, showing a typical flow scheme for the identification of HMG1 (the spot of the most basic isoform in Fig. 1). Paired ANOVA analysis showed that for this spot, the nuclear protein level significantly changed over three time points in all samples. The protein was identified through the peptide mass fingerprint, and identification was confirmed using the sequence information in the MS/MS spectra, of which one example is shown in Fig. 3B and C, respectively.

**Response to DNA DSB induction.** Following bleomycin challenge, proteins whose levels in the nuclei changed significantly were classified according to their function, which was based on the information present in the Swiss-Prot and the National Center for Biotechnology Information nonredundant databases. Table 2 shows the proteins classified as involved in DNA repair (ATP-dependent DNA helicase II or Ku70), in DNA interaction [template-activating factor 1α (TAF 1α), pp32A, HMG1, and nuclear protein Hcc-1 (HCC1)], and in cell cycle regulation (14-3-3 ζ/θ, eukaryotic translation initiation factor 5A). Significant changes were also found for protein levels known to be involved in several other processes such as oxidative stress response, these were, however, not directly related to DNA DSBs (see Supplementary Table). HMG1 is an example of a protein that was identified in more than one distinct protein spot, differing in the isoelectric point, which may implicate that posttranslational modification occurred. Unfortunately, the mass spectra of these samples did not reveal the type of posttranslational modification.

**Response of INHAT complex to DNA DSBs.** A remarkable response to the DNA DSB damage was that of the decreased levels of the TAF 1α, TAF 1β, and pp32A, proteins that form the INHAT complex (21, 22). Decreased nuclear levels of this complex suggest that it may be involved in the response to DNA DSB induction through the regulation of nucleosome assembly. Even though this complex is known to be involved in HAT-dependent transcriptional regulation (23, 24), the role of DNA DSB damage response has not been previously described. In particular, it has been found that the NH2-terminal tails of the histones are central to the regulation of the chromatin structure, as they form a binding platform for multiple protein complexes such as the INHAT complex. Furthermore, it has been shown that the binding was differentially affected by various modifications within the histone NH2-terminal tail (20). Therefore, our results concerning the INHAT complex proteins were further investigated using the same lymphoblastoid nuclear extracts. To establish if the INHAT complex binds preferably to nonacetylated histones, an immobilized peptide with the

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sequence SGRGKAGKGRGAKTRQC was used as bait, which is a sequence considered as a histone NH2-terminal "consensus" peptide. Both the nonacetylated and the acetylated forms of this histone NH2-terminal consensus peptide were used as bait in separate experiments. Specifically bound proteins were purified and identified using one-dimensional SDS-PAGE and mass spectrometry, respectively. As can be concluded from Fig. 4A, the INHAT complex proteins were detected in the eluate and not in the flow-through for the nonacetylated histone NH2-terminal consensus peptide. Binding to this peptide was indeed specific because the INHAT complex proteins did not bind to the acetylated histone NH2-terminal consensus peptide. This is shown in Fig. 4B, in which the INHAT complex proteins were detected in the flow-through and not in the eluate fraction. This is in agreement with results obtained by Schneider et al. (20) who found reduced binding of INHAT proteins to histone NH2-terminal tails upon (partial) modification of amino acids in the NH2 terminus. The protein band that appeared in the eluate fraction was identified as the NAD-dependent deacetylase sirtuin-1, which is known to deacetylate (and bind) acetylated histones in vitro. This protein was not identified in the eluate from the nonacetylated immobilized peptide. In addition, the two-dimensional DiGE quantification of the INHAT complex proteins in the nuclear extracts were validated for the protein pp32A using Western blotting. The example depicted in Fig. 4C, showing the densitograms, and in Fig. 4D, showing the relative quantities, confirmed that the nuclear levels of pp32A decreased after 30 and 240 minutes, respectively, of bleomycin challenge compared with the reference (t = 0).

Discussion

DNA DSBs are highly cytotoxic, and must be recognized and repaired efficiently to prevent genomic instability, and eventually, the development of cancer. The signaling pathways that are

Figure 2. Assessment of the effect of the number of lymphoblastoid cell lines taken into consideration on the number of protein levels that are significantly changed, and the consistency of the data sets. The 14 cell lines used for this study were taken as reference. For this analysis, 2 to 14 independent randomly chosen cell lines were subjected to paired one-way ANOVA statistics. White columns, the percentage of proteins that were significantly changing compared with the reference, which is relatively constant when 9 to 14 cell lines are considered. Black columns, the consistency of the data sets, i.e., the number of proteins in common with the reference, showing that 80% or more of proteins are in common with the final set for 9 to 14 cell lines, whereas 92% or more proteins are in common for 11 to 14 cell lines.

Figure 3. Flow scheme for the mass spectrometric identification of a differential protein, HMG1 protein, from a two-dimensional gel. A, statistical analysis, i.e., paired one-way ANOVA, showed that the protein significantly changed in abundance over three time points in all samples. The corresponding protein spot was excised from the gel and subsequently digested using trypsin. B, the peptide mass fingerprint was used to identify the protein. C, to confirm the identification, a peptide was selected for collision-induced dissociation (CID), from which sequence information was retrieved.

activated on DNA DSB induction were investigated by challenging 14 human lymphoblastoid cell lines with bleomycin, and subsequent monitoring of the proteomic response in time. In the relatively small time span of 30 to 240 minutes of bleomycin challenge, it may be expected that transcriptional or translational control plays a minor role because processes such as correct splicing and folding are time-consuming, in particular, in higher eukaryotes. Instead, the signaling at these time intervals will be mainly controlled by, for example, phosphorylation, formation of protein-protein interactions, or shuttling of proteins from the nucleus to the cytoplasm. The latter mechanism is described as an example for the regulation of BRCA1, which has been found to be regulated through nuclear export in response to DNA damage induced by ionizing irradiation (25).
Response of proteins known to be involved in DNA DSBs.

A substantial fraction of the differential nuclear proteins was recognized as being involved in the DNA DSB response pathways. The first step in the DNA damage response is the recognition of lesions in the DNA by specific sensor proteins (9, 10). The DNA repair machinery started relatively quickly because the ATP-dependent DNA helicase II or Ku70 was one of the proteins which the nuclear level increased within 30 minutes of DSB induction. This important sensor and initiator of DNA repair is part of a heterodimer consisting of Ku70 and Ku80, of which Ku70 is known to bind DNA ends in a non–sequence-specific manner and starts DNA repair through nonhomologous end joining (26). In the subsequent step, the sensor proteins recruit signal transducers such as the ataxia telangiectasia–mutated kinase (27), its homologue ataxia telangiectasia and Rad3–related protein (28), the DNA-dependent protein kinase (29), p53 (30), BRCA1 (31), and Chk1 and Chk2 (32). These signal transducer proteins were either not found to be differentially present in the nuclei or they were not detected, the latter because these regulatory proteins have a high molecular weight (ataxia telangiectasia–mutated/ataxia telangiectasia and Rad3–related) or are very low abundant and unstable (p53), and thus hard to visualize on a two-dimensional gel.

In turn, the signal transducers activate a broad range of effector proteins, for example, those involved in cell cycle arrest (33, 34). A relatively fast response was found for the effector protein isoforms 14-3-3ζ/δ, of which the 14-3-3 protein ζ is known to be involved in cell cycle regulation through binding to Cdc25 (35, 36). The latter protein is a phosphatase that dephosphorylates Cdc2, thereby positively regulating entry into mitosis. However, upon induction of DNA damage, Cdc25 is phosphorylated to create a 14-3-3 binding site. After binding, 14-3-3ζ-bound Cdc25 is sequestered from the DNA damage, Cdc25 is phosphorylated to create a 14-3-3 binding site. After binding, 14-3-3ζ-bound Cdc25 is sequestered from the cytosol, where it cannot act on Cdc2, thus, mitosis is prevented, and as a result, the cell cycle is halted at the G2-M checkpoint (35, 36). Also, a reduction in the levels of eIF5A, a protein whose function is related to the checkpoint at the G1-S transition, was detected after 30 minutes. This might indicate an arrest of the cell cycle prior to the start of the S phase in order to prevent damaged DNA from being replicated (37, 38). Recently, it was found that a complex of eIF5A and syntenin has a function in the regulation of p53 and p53-dependent apoptosis (39).

Another category of effector proteins consists of those involved in DNA repair. The HMG1 protein was found to be reduced in abundance in the nuclear extract after bleomycin stimulation. HMG1 has been described to enhance intramolecular and intermolecular ligations of cohesive-ended and blunt-ended DNA through its DNA-binding properties, and it has been suggested that HMG1 is involved in the final ligation step in DNA end-joining processes (40, 41). It has also been found that HMG1 is a direct activator of the DNA-binding capacities of p53. This interaction has been found to be independent of the COOH-terminal tail of p53, a region known to interact with other activators (42). Another DNA-binding protein is the nuclear protein, Hec-1, that showed an increase in abundance only after 240 minutes of bleomycin challenge. Not much is known about the exact function of this protein, although a PSI-BLAST search showed that the first 42 amino acids of the protein forms a SAP domain (43). This type of domain is a putative DNA-binding motif that is known to be involved in transcription regulation and DNA repair (44).

Response of INHAT complex to bleomycin challenge.

Strikingly, it was established that the nuclear levels of the tripartite INHAT complex proteins were reduced after 30 minutes of bleomycin challenge. It has been suggested that this complex inhibits p300/CBP and PCAF-mediated acetylation of histones, most likely by masking the accessibility of the histone lysines (22–24). When the levels of the INHAT complex proteins are reduced, the basic NH2-terminal ends of the core histones...
become accessible to p300/CBP. Histone acetylation by p300/CBP is an important mechanism in chromatin dynamics during transcription because this results in a decreased density of packed DNA, which allows transcription factors to enter the DNA and the genes to be transcribed (45). The fact that the nuclear levels of all these three proteins of the INHAT complex were reduced after 30 minutes of DNA damage induction may indicate that this complex is not only involved in transcriptional regulation, but plays a similar role in response to DNA DSBs, as illustrated in Fig. 5.

**Comparison with other proteomics studies.** Even though the effect of DNA damage has been studied extensively, the response to DNA DSB–inducing agents as efficient as bleomycin has never been investigated using a proteomics approach. In contrast, the proteomic responses to UV irradiation of HeLa cells (46), and to ionizing radiation induction of L929 cells (47) have already been investigated. Some of the proteins that were found to be differentially regulated on UV irradiation over a time course of 24 hours were also found in our study, like eIF5A, lactate dehydrogenase, and a proteasome subunit, but the main effect was detected to occur on DNA replication at the G1-S boundary (46). The L929 cells were challenged with 6 Gy ionizing radiation, and after 72 hours, differential expression of 47 proteins was detected. The identified proteins participate either in protective and reparative cell responses or induction of apoptosis. Like in our study, eIF5A and several subunits of the proteasome, 14-3-3ζ, transketolase, and protein disulfide isomerase were found to be differentially regulated (47).

In conclusion, the use of two-dimensional DiGE permitted investigation of the proteomic response of 14 independent human biological replicates to DNA DSBs induced by bleomycin. It was established that for this study of human samples, at least nine independent replicates are needed to find a rather constant and consistent number of significant changes in nuclear protein levels. Many of the proteins whose nuclear levels changed could be said to be involved in the repair of DNA DSBs. Although it is known to a large extent which pathways are involved, it is still not known how DNA repair enzymes could gain access to DNA lesions within the chromatin, prior to the repair of the damaged DNA. The significance of chromatin remodeling in DNA repair has been reported in several studies (48–50). Intriguingly, the tripartite INHAT complex protein levels were detected to be significantly reduced upon DNA DSB induction, suggesting that these inhibitors of chromatin modifiers not only play a role in transcription, but also play a role in DNA repair.

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