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Mass spectrometry-based quantification of the cellular response to methyl methanesulfonate treatment in human cells

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ABSTRACT

Faithful transmission of genetic material is essential for cell viability and organism health. The occurrence of DNA damage, due to either spontaneous events or environmental agents, threatens the integrity of the genome. The consequences of these insults, if allowed to perpetuate and accumulate over time, are mutations that can lead to the development of diseases such as cancer. Alkylation is a relevant DNA lesion produced endogenously as well as by exogenous agents including certain chemotherapeutics. We sought to better understand the cellular response to this form of DNA damage using mass spectrometry-based proteomics. For this purpose, we performed sub-cellular fractionation to monitor the effect of methyl methanesulfonate (MMS) treatment on protein localization to chromatin. The levels of over 500 proteins were increased in the chromatin-enriched nuclear lysate including histone chaperones. Levels of ubiquitin and subunits of the proteasome were also increased within this fraction, suggesting that ubiquitin-mediated degradation by the proteasome has an important role in the chromatin response to MMS treatment. Finally, the levels of some proteins were decreased within the chromatin-enriched lysate including components of the nuclear pore complex. Our spatial proteomics data demonstrate that many proteins that influence chromatin organization are regulated in response to MMS treatment, presumably to open the DNA to allow access by other DNA damage response proteins. To gain further insight into the cellular response to MMS-induced DNA damage, we also performed phosphorylation enrichment on total cell lysates to identify proteins regulated via post-translational modification. Phosphoproteomic analysis demonstrated that many nuclear phosphorylation events were decreased in response to MMS treatment. This reflected changes in protein kinase and/or phosphatase activity in response to DNA damage rather than changes in total protein abundance. Using these two mass spectrometry-based approaches, we have identified a novel set of MMS-responsive proteins that will expand our understanding of DNA damage signaling.

1. Introduction

Maintaining genome integrity over the course of each cell cycle requires fidelity in DNA replication and segregation as well as a robust DNA damage response (DDR) [1,2]. The lattermost is particularly challenging because of the number and scope of potential DNA lesions that may be encountered by the cell. Sources of DNA damage can be endogenous and exogenous. It has been estimated that each cell experiences approximately 20,000 spontaneous DNA damage events each day. If not repaired properly, DNA damage can be mutagenic, possibly leading to diseases such as cancer. Therefore, the DDR co-ordinates cellular proteins and pathways to regulate cell cycle progression, DNA repair and apoptosis. Many cancer therapeutic agents utilize the DDR to induce cytotoxicity [3].

Alkylating agents represent an important source of DNA damage. For example, S-adenosyl methionine (SAM), which serves as a methyl donor in various metabolic reactions, can also target DNA in a potentially mutagenic reaction. *N*-nitrosamine, a carcinogen found in tobacco, is an exogenous source of DNA alkylation. Finally, cancer chemotherapeutics such as nitrogen mustards including cyclophosphamide and bis-chloroethylnitrosourea (BCNU) are alkylating agents. In the laboratory, methyl methanesulfonate (MMS) and methylnitronitrosoguanidine (MNNG) have been used frequently to study the consequences of DNA alkylation [4]. The major adduct formed by treatment with the above agents is 7-methylguanine (N7-MeG). Methylation at this site frequently leads to generation of an abasic site via hydrolysis of the modified base. The base excision repair (BER) pathway is frequently called upon to remedy lesions caused by alkylation [5,6].

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The effect of MMS treatment on cellular signaling pathways has been primarily explored in yeast. Initially, microarray analysis was used to determine the transcriptional response to MMS [7]. A library of GFP-tagged proteins was screened by flow cytometry and led to the identification of 157 MMS-induced gene products [8]. Screens performed on haploid deletion libraries have identified many non-essential genes whose deletion renders cells MMS sensitive [9–11]. More recently, epistatic miniarray profiles (E-MAPs) have been assembled to identify genetic interactions among 418 yeast genes that display differential interactions upon MMS treatment [12]. DNA damage signaling events have also been examined by comparing phosphorylation in wild-type and checkpoint kinase null cells, leading to the identification of 62 DDR-regulated phosphorylation sites [13]. Systematic examination of the proteins involved in response to MMS treatment has not been extensively performed in higher eukaryotes, although an RNAi screen has been carried out in *Drosophila* cells [14].

Mass spectrometry-based proteomics is a powerful tool for identifying and quantifying protein expression, protein modifications and protein interactions that is widely used in biological investigation of cellular processes [15]. Multi-dimensional protein identification technology (MudPIT), which utilizes orthogonal liquid chromatography (LC) separations of peptides prior to tandem MS analysis, is routinely used to interrogate the protein constituents of complex biological samples [16]. Stable isotope labeling with amino acids in cell culture (SILAC) can be used to perform relative quantification of protein and protein modifications in combination with mass spectrometry [17]. The use of mass spectrometry-based proteomics in studies of the DDR has led to a significant leap forward in our understanding of the cellular signaling pathways engaged by human cells in response to DNA damage. In particular, spatial proteomics and phosphoproteomics have been performed in several studies.

DNA damage occurs in the context of chromatin and must be repaired in this environment as well [18,19]. This means that nucleosome positioning, histone modifications and variants as well as other DNA binding proteins are all features of chromatin that must be regulated during DNA repair. Numerous specialized structures are present within the nucleus and it is likely that they also influence how DNA repair proceeds. Recently, several efforts have been made to understand the chromatin landscape in response to DNA damage. Biochemical sub-cellular fractionation into nuclear or chromatin-enriched fractions that were subsequently probed using mass spectrometry has been used to identify DNA-binding proteins sensitive to treatments such as etoposide, ultraviolet (UV) light or ionizing radiation (IR) [20–22].

Reversible protein post-translational modification (PTM) is a dynamic regulatory mechanism widely used in cellular signaling pathways including the DDR. PTMs may influence protein stability, protein activity, protein localization and protein interactions. Phosphorylation, ubiquitylation and sumoylation are PTMs frequently utilized as part of the DDR [23]. In particular, checkpoint kinases such as ATM and ATR are activated in response to multiple forms of DNA damage to phosphorylate and regulate their substrates. Several studies have used "substrate" antibodies that recognize phosphorylated S/T-Q sites to identify hundreds of targets of these kinases in the context of hydroxyurea, UV light or IR [24–26]. However, the activities of other protein kinases are also affected by DNA damage [27–29]. Unbiased phosphoproteomics studies performed using etoposide, neocarzinostatin (NCS) or IR have identified additional phosphorylation sites regulated by the DDR [30–32].

Although many of the proteins that comprise the initial response to MMS have been identified, the complete repertoire of downstream DDR events induced by MMS remains poorly understood. Mass spectrometry-based proteomics is well-suited to discover additional MMS responsive proteins. This approach was applied to two themes of the DDR, protein recruitment to chromatin and protein post-translational modification, focusing on phosphorylation.

2. Materials and methods

2.1. Cell culture

HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. SILAC was performed for more than 6 passages to differentially label proteins. HeLa cells were cultured in either "light" SILAC media (unlabeled lysine and arginine) or "heavy" SILAC media ($^{13}C_6$-lysine and $^{13}C_6$, $^{15}N_4$-arginine) (Invitrogen). For DNA damage treatment, separate populations of HeLa cells grown in either "light" or "heavy" SILAC media were seeded in equal cell numbers onto 150 mm dishes. The following day the cells were treated with thymidine (Sigma-Aldrich) at a concentration of 2 mM for approximately 16 h. HeLa cells grown in "light" SILAC media were simultaneously treated with MMS (Sigma-Aldrich) diluted to a final concentration of 0.05% during the final hour of thymidine treatment. Cells were then harvested for the acute time point. For the S-phase time point, both cell populations were washed twice with phosphate buffered saline (PBS) and then replaced with fresh SILAC media. Cells were grown for an additional 4 h prior to harvest.

2.2. Cell lysis

Sub-cellular fractionation was performed as previously described with minor modifications [33]. Cells were lysed in Buffer A (1 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol (DTT) and protease inhibitors) supplemented with 0.1% TritonX-100 for 10 min at 4 °C. Cells were centrifuged at 4000 rpm for 5 min to pellet the nuclei. The supernatant was clarified by re-centrifuging at full speed and this lysate was used as the cytosol-enriched fraction. The nuclei were washed in Buffer A for 10 min at 4 °C followed by centrifugation at 4000 rpm for 5 min; the supernatant was then discarded. The nuclei were lysed in Buffer B (3 mM ethylenediaminetetraacetic acid (EDTA), 0.2 M ethyleneglycoltetraacetic acid (EGTA), 1 mM dithiothreitol (DTT) and protease inhibitors) for 30 min at 4 °C and then centrifuged at 4000 rpm for 5 min. The supernatant was removed and the pellet was re-suspended in Buffer C (50 mM Tris–HCl pH 8, 250 mM NaCl, 1% NP-40, 1 mM dithiothreitol (DTT) and protease inhibitors) for 20 min at 4 °C. Following centrifugation at full speed for 5 min, the resulting supernatant was used as the chromatin-enriched nuclear fraction.

Whole cell protein lysates were prepared by incubating cells in 50 mM Hepes pH 7.0, 250 mM NaCl, 0.1% NP-40, PhosSTOP phosphatase inhibitor cocktail (Roche Applied Science) and protease inhibitors for 30 min at 4 °C. Cells were centrifuged at full speed for 10 min and the resulting supernatant was saved for either direct analysis or phosphorylation enrichment.

2.3. Sample preparation for mass spectrometry

Samples were first denatured in 8 M urea and then reduced and alkylated with 10 mM Tris(2-carboxyethyl)phosphine hydrochloride (Roche Applied Science) and 55 mM iodoacetamide (Sigma-Aldrich) respectively. The urea concentration was diluted to 2 M with 100 mM Tris pH 8.5 and then the samples were incubated overnight at 37 °C with trypsin (Promega) at a final protease to protein ratio of 1:50.

For phosphorylation analysis, phosphopeptide enrichment was performed using titanium dioxide magnetic beads (Thermo Scientific) according to the manufacturer's specifications with only the