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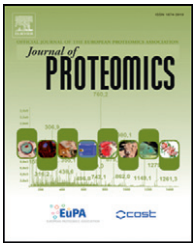

Proteomic profiling of ATM kinase proficient and deficient cell lines upon blockage of proteasome activity☆

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ABSTRACT

Ataxia Telangiectasia Mutated (ATM) protein kinase is a key effector in the modulation of the functionality of some important stress responses, including DNA damage and oxidative stress response, and its deficiency is the hallmark of Ataxia Telangiectasia (A-T), a rare genetic disorder. ATM modulates the activity of hundreds of target proteins, essential for the correct balance between proliferation and cell death. The aim of this study is to evaluate the phenotypic adaptation at the protein level both in basal condition and in presence of proteasome blockage in order to identify the molecules whose level and stability are modulated through ATM expression. We pursued a comparative analysis of ATM deficient and proficient lymphoblastoid cells by label-free shotgun proteomic experiments comparing the panel of proteins differentially expressed. Through a non-supervised comparative bioinformatic analysis these data provided an insight on the functional role of ATM deficiency in cellular carbohydrate metabolism's regulation. This hypothesis has been demonstrated by targeted metabolic fingerprint analysis SRM (Selected Reaction Monitoring) on specific thermodynamic checkpoints of glycolysis. This article is part of a Special Issue entitled: Translational Proteomics.

1. Introduction

Ataxia Telangiectasia (A-T) is an autosomal recessive disorder. The clinical hallmark of the disease is progressive neuromotor dysfunction resulting from gradual cerebellar cortical atrophy and Purkinje cells degeneration. Additional important features are telangiectasia in the eyes, thymic degeneration, immune deficiency, premature aging and higher predisposition to develop lymphomas and leukemia [1,2]. Moreover, A-T patients are highly sensitive to ionizing radiations (IR) [3]. A-T is linked to mutations of the ATM (Ataxia Telangiectasia Mutated) gene on both alleles, which ultimately impair the production of a functional ATM protein [4,5], a serine/threonine-PI3-kinase like protein originally identified as a central player of the DNA

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damage response (DDR) network [2]. ATM kinase mainly exerts its function through the phosphorylation of several protein substrates. These include proteins involved in cell cycle control, DNA repair and apoptosis, such as p53, Chk2, MRN complex, Rad51 and many more [3]. Following DNA damage, ATM molecule is (auto)phosphorylated on Ser 1981 and phosphorylation on this site has been proposed as a signature of an activation event [6]. ATM is activated mainly by DNA double strand breaks (DSBs), a cytotoxic lesion induced by IR, radiomimetic agents, such as neocarzinostatin (NCS), reactive oxygen species accompanying normal metabolism, and it triggers cell cycle arrest and DNA repair as well as, depending on the cellular context and on the severity of the damage, programmed cell death or apoptosis [7]. Increasing evidence suggests that the ubiquitin system plays a crucial role in the DDR providing a timed and coordinated degradation or relocalization or activation of regulatory proteins essential for the execution of the DDR [8]. In fact, cellular homeostasis maintenance and ability of adaptation to the environment depend on degradation of regulatory proteins. Furthermore, more recently non-degradative ubiquitylation of DNA repair proteins has been shown to play an essential role in the DDR. This post-translational modification of key DDR molecules provides direct and indirect routes to damage site recognition for DNA repair proteins [9]. Phosphorylation-dependent or independent ubiquitylation and deubiquitylation affect protein localization and pathway activation/inactivation and are signals regulating the multiple mechanisms allowing for DDR transient activity [10].

The ubiquitin–proteasome (Ub–P) system plays a key role in maintaining the integrity of cellular proteome and in protecting cells from protein damage. Accumulation of damaged proteins can interfere with normal cellular processes and may directly induce cell death. Under normal circumstances, ubiquitylation of proteins acts as a quality control mechanism, marking and destroying improperly manufactured proteins. Indeed upon cellular stresses such as metal and oxidants exposure or heat shock, there is a significant increase of ubiquitylated proteins level in the cell, and aberrations in this pathway are implicated in the pathogenesis of several diseases, including many neurodegenerative disorders [11,12].

In this scenario, it has been demonstrated that Ub–P is also induced in response to ATM kinase activation. NCS treatment endogenously increases ubiquitin conjugates in lymphoblastoid cells. A-T cells show an attenuated ability to mount the ubiquitylation response to stress, supporting a role of ATM in modulating the ubiquitylation machinery [13]. ATM modulates the activity of E3 ubiquitin ligases, affecting indirectly the stability of target proteins: for example the E3 ubiquitin ligases MDM2 and COP1 have been identified as ATM substrates and their ATM-dependent phosphorylation results in the inhibition of their enzymatic activity which in turn triggers p53 stabilization [14–16]. Recently, Stagni and colleagues have shown that ATM modulates the proteasome dependent down-regulation of c-FLIP therefore affecting death receptor induced apoptosis [17,18]. Furthermore it has been shown that ATM activity triggers NEMO ubiquitylation and NF-κB activation modulating the TNFα response [19]. A recent paper demonstrates how protein proteasome-mediated degradation is negatively affected in A-T cells due to the ATM impairment of ISG15 (an ubiquitin-like protein) pathway [20]. Importantly, proteomic approaches aimed to deciphering ATM substrates identified more than 700 proteins as novel ATM targets among which the Ub–P system is highly represented [21–23]. Moreover, these studies suggested that ATM may importantly contribute to several cellular functions beside DNA damage response. Indeed, DDR independent roles of ATM as cytoplasmic protein involved in different biochemical phenomena are starting to emerge linking ATM deficiency to increased oxidative stress, neurodegeneration, metabolic dysregulation and oncogenesis (reviewed in [24]). ATM participates in maintenance of cellular redox homeostasis by up-regulation of antioxidants, increasing production of reductive precursors and decreasing reactive oxygen species (ROS) production by mitochondria. Accordingly, the absence of a functional ATM results in a continuous state of oxidative stress causing adverse effects on particularly sensitive cells as neurons [25,26]. Moreover, an intrinsic up-regulation of ROS and mitochondria dysfunction are exhibited by ATM deficient lymphoblastoid cells [27] and Cheema and colleagues reported that ATM controls oxidative stress by regulating purine, pyrimidine and urea cycle pathways [28]. Interestingly, H_2O_2 dependent ATM Cys-2991 dimer formation was proposed as oxidation activation mechanism different from the classic Ser-1981 autophosphorylation occurring after the DBSs [29]. Other evidences supported ATM role in regulation of metabolic signaling pathways. ATM participates in insulin signaling through phosphorylation of eIF-4E-binding protein 1 [30] and glucose metabolism is affected by ATM activity as the levels of Insulin-like growth factor 1 receptor (IGFR1) are reduced in ATM-deficient cells [31] and translocation of the cell-surface Glucose transporter 4 (GLUT4) is regulated indirectly by ATM in response to insulin stimulation [32]. Moreover, a link between ATM and the pentose phosphate pathway has been provided [33] and ATM activity modulates metabolic syndrome [34,35].

Overall, these data confirm that ATM deficiency affects the cellular proteome composition resulting in multiple defective signaling pathways. Therefore, we developed a non-targeted proteomic investigation to analyze the profile of proteins whose levels change in response to ATM expression in order to elucidate the role of ATM in the control of protein quality and stability. To this aim, protein expression profiling was also assessed in the presence of the proteasome inhibitor MG132 to highlight those proteins whose expression is modulated by ATM most likely through the ubiquitin–proteasome system. Our investigation was pursued by the use of isotope-free shotgun proteomics approach that provides a relatively high-throughput assessment of changes in protein expression, which may act as a molecular remnant of ATM activity mechanism, and generates raw data for unsupervised data-mining of functional biological process. This approach allowed us to obtain an overview on the role of ATM in the modulation of the proteome, thereby offering a better understanding of its physiologic and pathologic implication.

2. Materials and methods

2.1. *Cell lines, antibodies and reagents*

L6pCDNA (L6), L6-Flag-ATM-wt (L6ATM), GM-03189 and HeLa cell lines were cultured as described previously [17,18,36].