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ORIGINAL ARTICLE

Uncovering protein polyamination by the spermine-specific antiserum and mass spectrometric analysis

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Abstract The polyamines spermidine and spermine, and their precursor putrescine, have been shown to play an important role in cell migration, proliferation, and differentiation. Because of their polycationic property, polyamines are traditionally thought to be involved in DNA replication, gene expression, and protein translation. However, polyamines can also be covalently conjugated to proteins by transglutaminase 2 (TG2). This modification leads to an increase in positive charge in the polyamine-incorporated region which significantly alters the structure of proteins. It is anticipated that protein polyamine conjugation may affect the protein–protein interaction, protein localization, and protein function of the TG2 substrates. In order to investigate the roles of polyamine modification, we synthesized a spermine-conjugated antigen and generated an antiserum against spermine. In vitro TG2-catalyzed spermine incorporation assays were carried out to show that actin, tubulins, heat shock protein 70 and five types of histone proteins were modified with spermine, and modification sites were also identified by liquid chromatography and linear ion trap-orbitrap hybrid mass spectrometry. Subsequent mass spectrometry-based shotgun proteomic analysis also identified 254 polyaminated sites in 233 proteins from the HeLa cell lysate catalyzed by human TG2 with spermine, thus allowing, for the first time, a global appraisal of site-specific protein polyamination. Global analysis of mouse tissues showed that this modification really exists in vivo. Importantly, we have demonstrated that there is a new histone modification, polyamination, in cells. However, the functional significance of histone polyamination demands further investigations.

Keywords Polyamine · Spermine · Histones · Transglutaminase 2 · Polyamination · Post-translational modification

Abbreviations

TG2	Transglutaminase 2
PU	Putrescine
SPD	Spermidine
SPM	Spermine
GA	Glutaraldehyde
BSA	Bovine serum albumin
OVA	Ovalbumin
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
GP	Guinea pig
ACTB	Beta actin
TUBA1A	Alpha tubulin 1a
TUBB	Beta tubulin
DTT	Dithiothreitol
DNC	Monodansylcadaverine
PBS	Phosphate-buffered saline
TBS	Tris-buffered saline

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Introduction

The polyamines spermidine (SPD) and spermine (SPM), and their precursor putrescine (PUT), are found ubiquitously at millimolar concentrations in cells. They have been shown to play important roles in cell migration, cell proliferation, cell differentiation, apoptosis, tissue regeneration, and embryonic development (Igarashi and Kashiwagi 2010; Tabor and Tabor 1984). PUT is synthesized from ornithine by the rate-limiting enzyme, ornithine decarboxylase in the urea cycle. SPD and SPM are subsequently produced by SPD synthase and SPM synthase through sequential addition of propylamine to PUT and SPD, respectively. Owing to the cationic property, polyamines are first thought to be involved in nucleic acid-dependent processes. Alternatively, polyamines may also interact with other anionic macromolecules, such as cytoskeletal proteins. Depletion of polyamine pools by biosynthesis inhibitors induces significant change in cytoskeleton architecture, disappearance of actin stress fiber, and polymerized tubulin (Nedeva et al. 2013; Pohjanpelto et al. 1981). In addition, high levels of polyamines increase the levels of acetylation and ADP-ribosylation in proteins associated with chromatin (Estepa and Pestana 1981; Perrella and Lea 1978). Furthermore, SPD has an essential and unique role serving as the precursor in the hypusine modification of eukaryotic initiation factor 5A (eIF5A), a modified lysine with addition of the 4-aminobutyl moiety from SPD (Cooper et al. 1983). This modification is required for the essential functions of eIF5A (Park et al. 2010). Besides this unique covalent modification by SPD, polyamines can be covalently incorporated into proteins by transglutaminases (Folk et al. 1980).

Transglutaminase 2 (TG2) is a calcium-dependent enzyme which can catalyze the formation of an isopeptide bond between an amine acceptor (glutaminyl residues) and an amine donor (primary amine) (Folk 1980; Ientile et al. 2007). TG2 is expressed ubiquitously and is localized in the nucleus, cytosol, endoplasmic reticulum, and mitochondria (Gundemir et al. 2012). The amine donor can be a lysyl side chain in a polypeptide as well as free amines, such as serotonin, histamine, and polyamines (Fig. 1) (Folk et al. 1980; Walther et al. 2011), thus resulting in the formation of protein cross-linking or free amine conjugation. Among the substrates of TG2, RhoA is the most-studied protein modified with polyamines (Makitie et al. 2009; Shin et al. 2008; Singh et al. 2001). Polyamine-incorporated RhoA can function as a constitutively active G protein, leading to increased binding to its downstream protein, and RhoA-associated kinase-2 involved in formation of stress fiber. Other proteins, such as apolipoprotein B and phospholipase A2, were also regulated by polyamine modification (Cocuzzi et al. 1990; Cordella-Miele et al. 1993). The role of such modification on

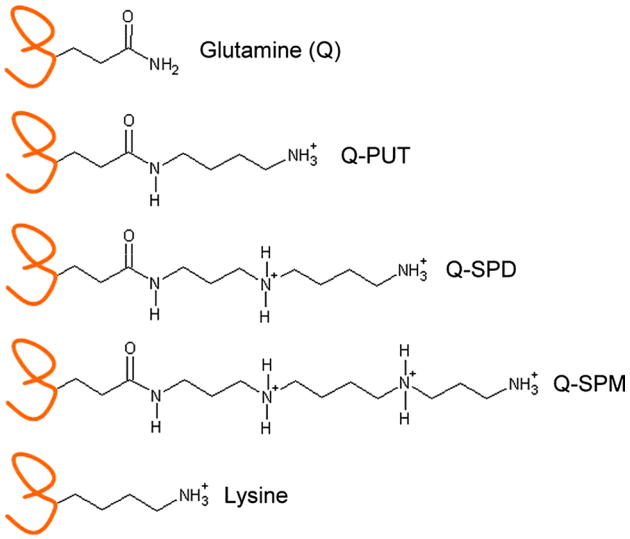

Fig. 1 Schematic representation of polyamine modifications. Transglutaminase 2 (TG2) catalyzes a calcium-dependent acyl transfer reaction to form an isopeptide bond between the γ-carboxamide group of a protein-bound glutamine and the primary amine group of a polyamine. Polyamine modification leads to an increase in positive charge in the polyamine-incorporated region as compared to the glutamine residue

apolipoprotein B was unknown, but a 2–3-fold increase in phospholipase A2 activity was observed upon polyamine modification. Some viral proteins, papillomavirus E7 and HIV-1 aspartyl protease, can also be incorporated with polyamines (Jeon et al. 2003a; Lentini et al. 2010). E7 binds and inactivates retinoblastoma protein (Rb) by causing the release of active E2F, while E7 binding of Rb is inhibited by polyamine incorporation. Activity of HIV-1 aspartyl protease is almost abolished by SPD which acts as a competitive inhibitor of intramolecular cross-linking catalyzed by TG2. Alternatively, TG2 is involved in autophagosome maturation, and SPD-induced autophagy is able to promote longevity of yeast (D'Eletto et al. 2009; Morselli et al. 2009). In addition, TG2 may contribute to neurodegeneration through cross-linking or polyamination of the brain proteins such as amyloid-β, tau, α-synuclein, and huntingtin (Jeitner et al. 2008, 2009, 2013). In particular, γ-glutamyl polyamines in human CSF are elevated in CSF from Huntington disease patients. Recently, we have demonstrated that TG2 acts on glyoxalase I (GlxI) leading to polyamine-conjugation or deamidation modification depending on the presence of polyamines or not, deamidation leading to activation and polyamine-conjugation to activation, and stabilization of GlxI. In this way, cancer cells overexpressing both GlxI and TG2 may gain growth advantage since the hazardous methylglyoxal formed spontaneously from the glycolytic pathway can be adequately disposed (Lee and Chang 2014). Therefore, it

is important to explore novel proteins modified with polyamines and the resulting functional impact imposed by polyamination.

Incorporation of radioactive isotope-labeled polyamines into proteins has been used for in vitro and in cellulo TG2 assay for decades (Folk and Chung 1985). However, the application of this classical method is limited in uncovering novel TG2 substrates. To date, 5-(biotinamido) pentylamine has been used as an alternative primary amine for the rapid and sensitive measurement of TG2 activity which allows detection and enrichment of amine acceptors (Wilhelm et al. 1996). However, the presence of endogenous biotin in cells is a major drawback to biotin-based detection systems. To investigate the function of polyamines, antibodies against natural polyamines have been used in ELISA and immunocytochemistry, rather than immunoblotting (Fujiwara 1994; Fujiwara et al. 1993). Thus, we raised an antiserum against SPM and used the antiserum to detect the in vitro SPM incorporation catalyzed by TG2. We confirmed some proteins can be modified with SPM; such as actin, tubulins, HSP70, and five histone proteins. Further, with mass spectrometric analysis, we unequivocally detected recombinant proteins and HeLa cell proteins modified with human TG2 and polyamines. Global analysis of mouse tissues showed that polyamine modification also occurred in vivo. This is the first report with detailed characterization of polyamination in cells and the data suggest polyamination as a novel histone post-translational modification.

Materials and methods

Materials

Polyamines, transglutaminase 2 from guinea pig liver (Sigma-Aldrich, St. Louis, MO); human transglutaminase 2 produced from insect cells (Zedira GmbH, Darmstadt); histone H2A, histone H2B, histone H3, histone H4, and histone H1 (New England Biolabs, Ipswich, MA); non-muscle actin protein from human platelets and tubulins from porcine brain (Cytoskeleton, Inc. Denver, CO); and heat shock protein 70 and heat shock protein 90 alpha/beta (BPS Bioscience, San Diego, CA) were purchased from manufacturers indicated in parentheses. Other chemicals were mostly from Sigma-Aldrich.

Cell culture

HeLa cells were obtained originally from American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), high glucose medium containing 10 % FBS within 5 % CO_2 atmosphere at 37 °C.

Production of antiserum against spermine

Antiserum against SPM was produced by immunizing the rabbit with SPM-conjugated ovalbumin dissolved in 0.3 ml phosphate-buffered saline (PBS) and emulsified with an equal volume of incomplete Freund adjuvant. Subcutaneous, multiple site injections were performed at biweekly intervals, and whole blood was collected from anesthetized animals 10 days after the sixth injection.

In vitro SPM incorporation reaction (transamidation reaction).

TG2 substrates such as purified proteins or HeLa cell lysate were incubated in 20 mM bicine solution, pH 8.0, containing 1 mM dithiothreitol (DTT), 1 mM $CaCl_2$, 1 mM SPM, and 50 mU/mL TG2 at 37 °C for 1 h. The HeLa cells were lysed with 2 % Triton X-100 in 20 mM Tris–HCl pH 7.4, and the cell lysate was obtained by centrifugation at 15,000×*g*. All the reactions were stopped by adding an equal volume of 2× sample buffer and heating at 100 °C for 3 min. For a peptide substrate, 10 μg synthetic peptide, SGQQQLGSSR, in 100 μL solution containing 20 mM bicine pH 8.0, 50 mU/mL human TG2, 1 mM $CaCl_2$, 1 mM DTT, and 1 mM putrescine, spermidine, or spermine was incubated at 37 °C for 1 h, and these reactions were stopped by heating at 100 °C for 3 min and the addition of 400 μL acetonitrile.

Extraction of histones

The cells were extracted in an isolation buffer containing 0.1 % CHAPS, 20 mM Tris–HCl pH 7.4, and a protease inhibitor cocktail (Sigma-Aldrich). The nuclei were pelleted by centrifugation at 1,000×*g* for 10 min and then washed once with the isolation buffer. The isolated nuclei then were suspended in 0.2 M HCl and kept overnight at −20 °C. On the next day, the solution was warmed to 4 °C and centrifuged at 6,500×*g* for 10 min at 4 °C to pull down the debris. The supernatant containing histones was added with 1/50 volume of 10 M NaOH and then with an equal volume of 2× sample buffer.

Immunoblotting

Proteins separated by PAGE were transferred to a poly (vinylidene difluoride) (PVDF) membrane with a wet Blotting System (Hoeffer Inc., Holliston, MA). The PVDF membrane was subsequently blocked with 5 % skim milk in Tris-buffered saline containing 0.05 % Tween (TBST) for 1 h. After incubation with anti-SPM antibody in TBST containing 0.3 % bovine serum albumin (BSA) for 1 h at room temperature, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody in TBST containing 0.3 % BSA for 1 h at room temperature.

The signals were developed with enhanced chemiluminescence system (Merck Millipore, Billerica, MA).

Immunofluorescence staining assay

HeLa cells were cultured on a glass cover slide for 24 h. After treatment of serum starvation and supplement of 10 % fetal calf serum, the specimens were obtained by fixing cells with 4 % formaldehyde in phosphate-buffered saline (PBS) for 30 min, permeabilization with 0.1 % Triton X-100 in PBS for 10 min, and blocking by 5 % skim milk in TBST for 1 h. Target proteins were recognized by anti-SPM antibody and anti-TG2 antibody, and nucleus was stained by 4′,6-diamidino-2-phenylindole (DAPI). Images were captured by using a fluorescence microscope (Leica DM 6000B, Leica Microsystems, Wetzlar, Germany).

In-solution digestion of recombinant histones and nucleosome

The solution of recombinant histones or nucleosome were reduced with 50 mM dithioerythreitol at 37 °C for 1 h first, and then alkylated with 100 mM iodoacetamide at room temperature for 1 h. The followed enzymatic hydrolysis was carried out with Arg-C (Roche, Basel) at an enzyme-to-substrate ratio of 1/300 at 37 °C for 20 h or with trypsin (Promega, Madison, WI) at an enzyme-to-substrate molar ratio of 1/50 at 37 °C for 16 h. The digested products were desalted using a C18 Zip-tip (Merck Millipore). The resulting peptides were evaporated to dryness using a SpeedVac, then diluted with 0.1 % formic acid and subjected to downstream MS analysis.

In-gel digestion of HeLa cell lysate for MS analysis

After the SDS-PAGE fractionation, the gel band was cut into small pieces and reduced with 50 mM dithioerythreitol at 37 °C for 1 h and alkylated with 100 mM iodoacetamide at room temperature for 1 h. The gel pieces were destained repeatedly with 25 mM ammonium bicarbonate in 50 % acetonitrile until they became colorless. Gel slices were dehydrated with 100 % acetonitrile for 5 min and with vacuum-dried for 5 min. The followed enzymatic hydrolysis was carried out with trypsin (Promega) or Asp-N (Promega) at an enzyme-to-substrate ratio of 1/40 at 37 °C for 16 h. The peptides were extracted twice with 50 % acetonitrile containing 5 % formic acid under moderate sonication for 10 min and dried completely under vacuum. The peptide mixtures were desalted by C18 Zip-tip (Merck Millipore) and subjected to downstream MS analysis.

Mass spectrometric analysis and data processing

The samples were reconstituted in 5 % acetonitrile and 0.1 % formic acid to give a volume of 4 μL, and loaded onto a C18 column of 75-μm × 250-mm (nanoACQUITY UPLC BEH130, Waters). The peptides mixtures were separated by online nanoflow liquid chromatography using nanoAcquity system (Waters, Milford, MA) with a linear gradient of 5–50 % acetonitrile (in 0.1 % formic acid) in 95 min, followed by a sharp increase to 85 % acetonitrile in 1 min and held for another 15 min at a constant flow rate of 300 nL min^{-1}. Peptides were detected in an LTQ-Orbitrap Velos hybrid mass spectrometer (Thermo Scientific) using a data-dependent CID Top20 method in positive ionization mode. For each cycle, full-scan MS spectra (m/z 300–2000) were acquired in the Orbitrap at 60,000 resolution (at m/z 400) after accumulation to a target intensity value of 5×10^6 ions in the linear ion trap. The 20 most intense ions with charge states ≥ 2 were sequentially isolated to a target value of 10,000 ions within a maximum injection time of 100 ms and fragmented in the high-pressure linear ion trap by low-energy CID with normalized collision energy of 35 %. The resulting fragment ions were scanned out in the low-pressure ion trap at the normal scan rate and recorded with the secondary electron multipliers. Ion selection threshold was 500 counts for MS/MS, and the selected ions were excluded from further analysis for 30 s. An activation $q = 0.25$ and activation time of 10 ms were used. Standard mass spectrometric conditions for all experiments were spray voltage, 2.0 kV; heated capillary temperature, 200 °C; predictive automatic gain control (AGC) enabled, and an S-lens RF level of 69 %. All MS and MS/MS raw data were processed with Proteome Discoverer version 1.4 (Thermo Scientific), and the peptides were identified from the MS/MS data searched against the Swiss-Prot (542782 sequences entries) database using the Mascot search engine 2.4.1 (Matrix Science). Search criteria used were as follows: trypsin/Asp-N/Arg-C digestion; considered variable modifications of glutamine spermine-modification (+185.1892 Da), spermidine-modification (+128.1314 Da), putrescine-modification (+71.0735 Da), methionine oxidation (+15.9949 Da), and cysteine carboxyamidomethylation (+57.0214 Da); up to three missed cleavages were allowed; and mass accuracy of 10 ppm for the parent ion and 0.6 Da for the fragment ions. The significant peptide hits defined as peptide score must be higher than Mascot significance threshold ($p < 0.05$) and therefore considered highly reliable, and that manual interpretation confirmed agreement between spectra and peptide sequence. False discovery rates were controlled using the target-decoy strategy to distinguish correct and incorrect identifications. For the identification, the false discovery rate was set to 0.01 for peptides, proteins, and sites. In no

case did this search retrieve more than a single peptide, and in all instances the peptide score was below the 0.05 significance level.

Extraction of proteins from mouse tissues

All mouse tissues were weighed and homogenized with ten volume of PBS buffer containing 8 M urea and 1 % SDS, followed by sonication and centrifugation. The supernatants were collected and used in immunoblotting assay.

Results

Production of antispermine antibody

We attempted to raise an antiserum against SPM and use the antibody in the detection of SPM modification in TG2 substrates. Antiserum against SPM was produced against SPM-ovalbumin coupled by glutaraldehyde (GA), and the specificity of this antiserum was analyzed by immunoblotting using BSA, BSA-GA, BSA-GA-PUT, BSA-GA-SPD, and BSA-GA-SPM. This antiserum clearly recognized BSA-GA-SPM instead of BSA-GA or BSA (Fig. 2a), but had slightly weaker reactivity with BSA-GA-SPD, possibly due to similar structure between SPD and SPM (Fig. 2b). Notice that cross-linked BSA-GA-SPM and BSA-GA-SPD were also detected. The cross-linker GA did not produce detectable signal in the immunoblotting assay. Thus, we have obtained a good antiserum against SPM and SPD, which can be used for rapid detection of TG2-catalyzed SPM incorporation.

Spermine incorporation into recombinant histone proteins

Histones were known as good glutaminyl substrates of TG2 when assayed with monodansylcadaverine (DNC) or putrescine as an amine donor (Ballestar et al. 1996; Cooper et al. 2000), and also TG2 catalyzed cross-linking of H2A-H2B and H2B-H4 (Kim et al. 2002; Shimizu et al. 1997). With the anti-SPM antibody, we wished to investigate whether TG2 can modify the recombinant histone proteins with SPM. Surprisingly, in the in vitro SPM incorporation assay, we found that the recombinant histone H2A, H2B, H3, and H4 were already SPM-modified (Fig. 3a, b). Cross-linking of histone H2A, H2B, H3, or H4 was observed in the presence of TG2 and further addition of SPM reduced the oligomerization. Only slight increase in SPM modification signals was observed in H2B and H4 in the presence of TG2 and SPM. However, no SPM modification was detected in the recombinant histone H1 monomers, either before or after TG2 catalyzation (Fig. 3a). We next isolated the nuclei from HeLa cells, and found that

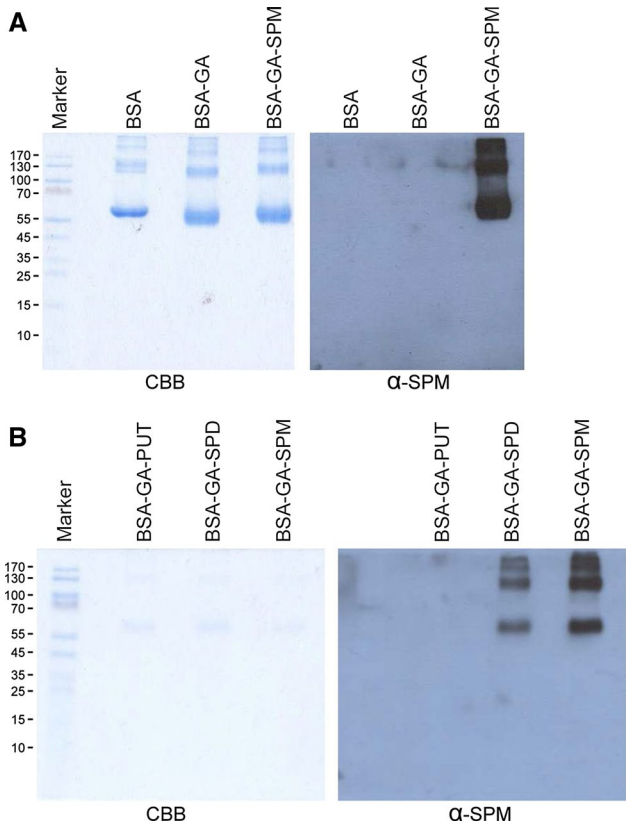

Fig. 2 Specificity of antispermine antiserum. **a** Bovine serum albumin (BSA) was modified with glutaraldehyde (GA) only or with GA-Spermine (SPM). BSA of 2.5 μg was loaded in each well (*left panel*, *CBB* Coomassie blue G-250 staining) and immunoblotted with an antiserum against spermine following SDS-PAGE (*right panel*). α-SPM, antispermine. 28 (**b**) BSA was conjugated with putrescine (PUT), spermidine (SPD), and SPM. Modified BSA of 1 μg was loaded in each well was stained with Coomassie blue G-250 (*left panel*), or immunoblotted with an antiserum against spermine following SDS-PAGE (*right panel*)

the purified histones had strong SPM modification signals, indicating that the nucleosome in cellulo had SPM incorporation (Fig. 3b).

We further used liquid chromatography and linear ion trap-orbitrap hybrid mass spectrometry to identify the modification sites in recombinant histone H2A, H2B, H3, H4, and H1, respectively, without the treatment of TG2 and spermine (annotation in black, Fig. 4a). We unexpectedly found that these five recombinant histones were modified with PUT, SPD, or SPM, indicating PUT, SPD, and SPM modifications actually occurred in *E. coli* cells. Mass spectrometry analysis was also performed on isolated histones from HeLa cells, excised from polyacrylamide gel (annotation in red, Fig. 4a). Undoubtedly, histone proteins from HeLa cells were also modified with three kinds of polyamines. We noticed that one given glutamine residue might be modified with more than one type of polyamine.

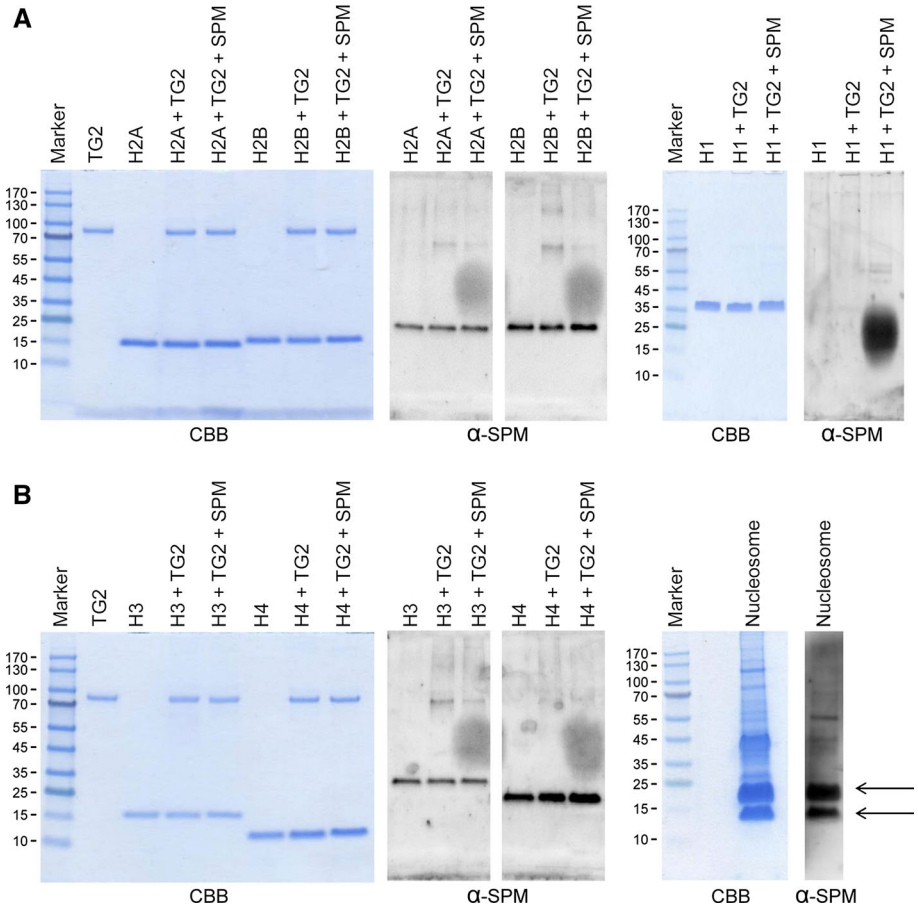

Fig. 3 In vitro spermine incorporation into recombinant histone proteins catalyzed by TG2. TG2-catalyzed spermine incorporation was carried out in vitro using TG2, spermine and one of the recombinant proteins: H2A, H2B, H1 (**a**), H3, and H4 (*left panel*, **b**). One μg of recombinant protein was used in the electrophoresis. Histone proteins (10 μg) from partially purified nucleosome were directly recognized by antiserum against spermine (*right panel*, **b**). TG2, recombinant human TG2 purified from insect cells. α-SPM, antispermine. *Arrows* indicate the position of histone H2, H3, and H4

Interestingly, histones were polyaminated at different sites in *E. coli* and HeLa cells. For example, histone H1 was modified at Q48, Q51, Q67, and Q83 in *E. coli,* while at Q37, Q48, and Q67 in HeLa cells. The data indicate that histone proteins expressed in *E. coli* or HeLa cells are constitutively modified with polyamines with some unique and some common sites.

Spermine incorporation into human platelet actin and porcine brain tubulins

Previous studies have demonstrated that polyamines can induce actin polymerization to form F-actin and actin bundling (Oriol-Audit 1978; Sowa et al. 2006), and depletion of polyamines in cells can cause reorganization of actin structure (McCormack et al. 1994; Pohjanpelto et al. 1981). Similarly, polyamines can interact with tubulins and induce formation of microtubule (Anderson et al. 1985; Mechulam et al. 2009), and polyamines play additional pivotal roles in microtubule assembly in cells (Savarin et al. 2010). In addition, transglutaminases are also involved in actin and microtubule functions (Del Duca et al. 2009; Nemes et al. 1997). Thus, we carried out the in vitro SPM incorporation assay on human platelet actin and porcine brain tubulins, and found that dramatic increases in SPM modification were observed in the presence of TG2 and SPM (Fig. 5a). The addition of 100 mM KCl or 1 mM $MgCl_2$ in the assay further increased the incorporation of SPM by about 20 % (data not shown). TG2 also lead to oligomerization of actin and tubulins (Fig. 5a). We also used mass spectrometry-based approach to identify the modification sites in human platelet actin and porcine brain tubulins treated with TG2 and SPM (Fig. 4b). Both proteins were SPM-modified as expected and PUT- and SPD-modified unexpectedly. Notice that SPM cannot be converted into PUT and SPD in vitro. Collectively, these data indicate that actin and tubulins are modified not only with SPM in vitro but also PUT and SPD in vivo.

Spermine incorporation into recombinant heat shock protein 70

Based on the previous reports that both TG2 and HSP70 were involved in epidermal growth factor-stimulated signaling pathway, leading to cancer cell migration and invasion (Antonyak et al. 2009; Boroughs et al. 2011), we examined whether TG2 can incorporate polyamines into HSP70. Thus, we performed the in vitro SPM incorporation assay

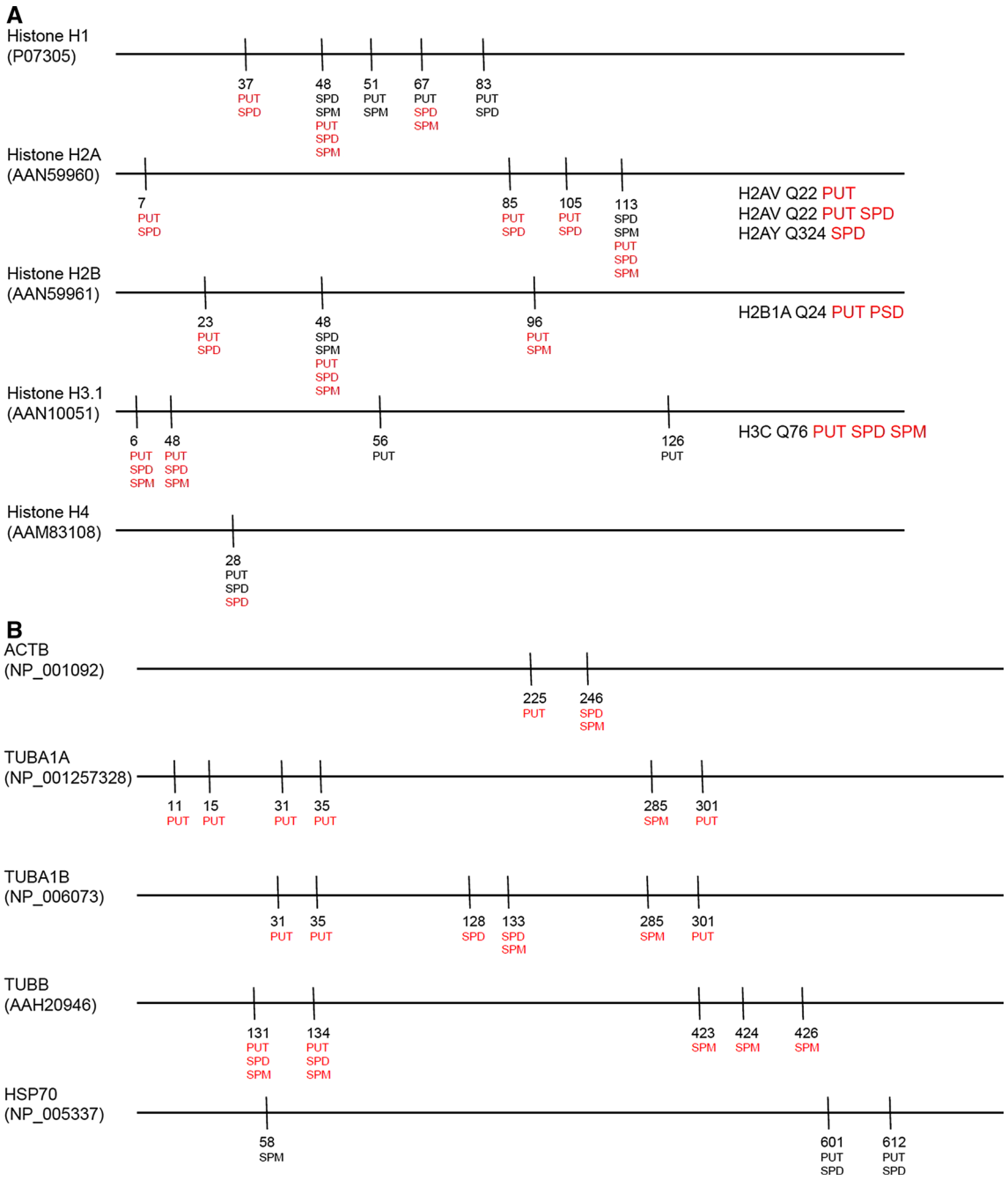

Fig. 4 Identified polyamine modification sites in recombinant proteins expressed in *E. coli* and purified native proteins. **a** Modification sites of recombinant histone proteins from *E. coli* (*black*) and isolated nucleosome from HeLa cells (*red*). **b** Modification sites of recombinant HSP70 (*black*), non-muscle actin from human platelets (*red*), and tubulins from porcine brain (*red*)

on HSP70 as well as HSP90. In this experiment, we found that HSP70 was recognized by anti-SPM antibody, indicating HSP70 can be modified by SPM (Fig. 5b). The addition of 100 mM KCl or 1 mM $MgCl_2$ in the assay further increased the incorporation of SPM by about 20 % (data not shown). In contrast, HSP90A and HSP90B were not recognized by anti-SPM antibody. In our immunofluorescence staining assay, TG2 and SPM modification signals were predominantly merged in the perinuclear region and cytosol in untreated HeLa cells, but can be detected along the leading edges in response to serum (EGF) stimulation (Fig. 6a). We next identify the modification sites in recombinant HSP70 treated with TG2 and SPM (Fig. 4b). Similarly, HSP70 was modified with three types of polyamines, while only SPM was provided in the reaction solution. Therefore, HSP70 is already PUT- and SPD-modified when synthesized in *E. coli* cells.

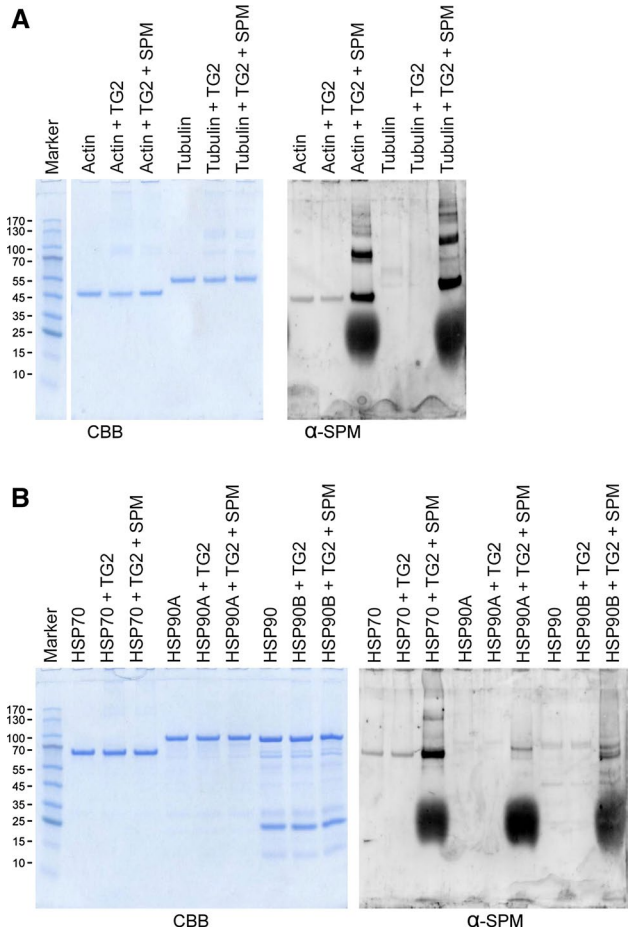

Fig. 5 In vitro spermine incorporation into actin, tubulins, heat shock protein 70, and heat shock protein 90 catalyzed by TG2. TG2-catalyzed spermine incorporation was carried out in vitro using TG2, spermine and one of the proteins: human platelet non-muscle actin, porcine brain tubulins (**a**), 29 recombinant HSP70, and recombinant HSP90A/B (**b**). TG2, recombinant human TG2 purified from insect cells. α-SPM, antispermine. Actin or tubulin of 1 μg was added in each well in (**a**). HSP70 or HSP90A/B of 1.5 μg was added in each well in (**b**)

Spermine incorporation into HeLa Cell lysate proteins

SPM incorporation into HeLa cell lysate proteins was then examined. We used TG2 isolated from guinea pig liver (Sigma-Aldrich) and recombinant human TG2 expressed in insect cells (Zedira) to catalyze the transamidation reaction. The SPM modification signals increased in a dose-dependent manner and showed distinct patterns catalyzed by the two TG2 preparations (Fig. 6b). We further identified the modification sites of HeLa cell proteins treated with recombinant human TG2 and 1 mM SPM (Supplementary Table 1); 254 polyaminated sites in 233 proteins. Among the sites, PUT occupied 168 of them, SPD 75 and SPM 25, with some sites modified by more than one polyamine. Interestingly, HSP90B was found to be modified with PUT, indicating HSP90 could be affected by polyamination. As expected, beta-tubulin was found to be modified with PUT and SPD at ^{131}Q and ^{134}Q (EAESCD-CL^{131}QGF^{134}QLTHSLGGGTGSGMGTLLISK, Fig. 4b and Supplementary Table 1). Unfortunately, HSP70 and actin were not found in the polyaminated proteins. Polyamine-modified proteins include constituents of cytoskeletons (tubulins, myosin, filamin, talin, and laminin, microtubule-actin cross-linking factor 1, microtubule-associated protein RP/EB family member 1, microtubule-associated protein 4, drebrin, syntabulin, and spectrin beta chain), signaling molecules (DNA-dependent protein kinase catalytic subunit, guanine nucleotide-binding protein subunit beta-2-like 1, casein kinase II subunit beta, rho guanine nucleotide exchange factor 2, Atlastin-3, Abelson tyrosine-protein kinase 2, mitogen-activated protein kinase kinase kinase 11, rho guanine nucleotide exchange factor 10, cyclin-dependent kinase 11A, platelet-derived growth factor receptor alpha, guanine nucleotide-binding protein subunit gamma-11, G kinase-anchoring protein 1, protein phosphatase 1J, mitogen-activated protein kinase kinase kinase 14, serine/threonine-protein kinase tousled-like 1, cyclin-dependent kinase 13, Arf-GAP with coiled coil, ANK repeat and PH domain-containing protein 2, non-receptor tyrosine-protein kinase TYK2, death-associated protein kinase 1, ras-like protein family member 10B, tumor necrosis factor receptor superfamily member 11B, Netrin-receptor UNC5C, and neuronal acetylcholine receptor subunit alpha-10), ribosomal proteins (ribosomal protein L5, ribosomal protein SA, Ribosome-binding protein 1, ribosomal protein L8, mitochondria ribosomal protein S34, and ribosomal protein S8), proteins associated with nucleic acids (DNA-dependent protein kinase catalytic subunit, nuclease-sensitive element-binding protein 1, splicing factor 3B subunit 2, cytoplasmic cysteine-tRNA ligase, ATP-dependent RNA helicase DDX42, spliceosome RNA helicase DDX39B, splicing factor 3B subunit 1, double-strand-break repair protein rad21 homolog, ATP-dependent RNA helicase DDX39A, mitochondrial nuclease EXOG, DNA-binding protein SMUBP-2, far upstream element-binding protein 2, and 5′→3′ exoribonuclease 1), ubiquitin protein ligases (E3 HUWE1, E3 UBR4, E3 HECTD4, and E2 O), and many metabolic enzymes. Meanwhile, we collected ten different tissue extracts from one male mouse to examine whether polyamine modification really occurs in vivo (Fig. 7). This global analysis showed many signals of SPM or SPD modifications in many tissues with strong TG2 signals, indicating that polyamine modification indeed occurs in vivo, and the heart shows the strongest signal. Subsequent mass spectrometry-based shotgun proteomic analysis identified six polyaminated sites in six proteins from the mouse heart tissue proteins (Supplementary Table 2).

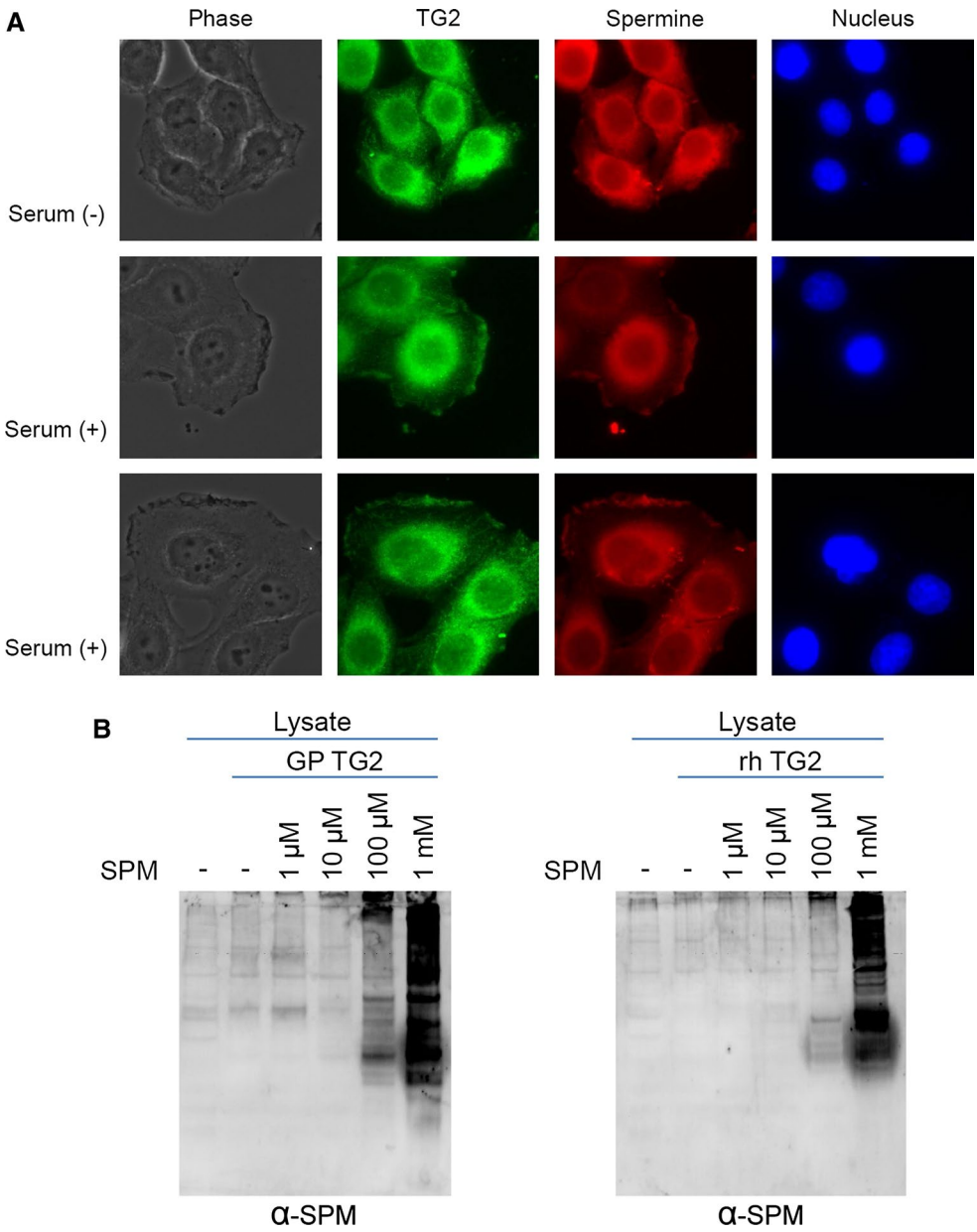

Fig. 6 Localization of TG2 and spermine in cultured HeLa cells and spermine incorporation into HeLa cell lysate proteins. **a** HeLa cells were cultured in medium with (+) or without (−) 10 % fetal calf serum. Immunofluorescence was performed using an antiserum against TG2 or spermine. Nuclei were stained with DAPI. **b** SPM in vitro incorporation assay was performed on HeLa cell lysate with 50 mU/ml TG and different concentrations of spermine. Ten μl of cell lysate collected from 10-cm dish confluent cells lysed with 1 ml lysis buffer was added in each well

Discussion

Transglutaminase is a Ca^{2+}-dependent enzyme catalyzing the acyl transfer from glutamine residues via two steps. In the first step, the enzyme binds to a glutamine-containing substrate. Nucleophilic attack by the active site cysteine of the enzyme on the gamma-carboxamide group of a glutaminyl residue in the substrate protein leads to formation of a substrate-enzyme acyl-thioester intermediate with the release of ammonia. The amine donor substrate then attacks the acyl-thioester, resulting in the formation of isopeptide bond product. In the reaction, the enzymes are more stringent to the structure spanning the glutamine residues in proteins than the amine donor (Keillor et al. 2014). A few non-radioactive assays for transglutaminase activity with synthetic primary amines have been used, but the extent of transamidation catalyzed by TG2 depends on the substrate affinity and the type of primary amine used (Jeon et al. 2003b; McConoughey et al. 2009; Stamnaes et al. 2008). Here, we have successfully raised an anti-SPM antiserum which could be used to recognize the SPM modification on target proteins. The antiserum can be used for in vitro SPM incorporation assay as well as detection of SPM conjugation in proteins isolated from cultured cells and animal tissues. In addition, polyamine modification sites in recombinant histone proteins and HSP70 as well as native proteins isolated from mammalian cells or tissues have been determined by liquid chromatography and linear

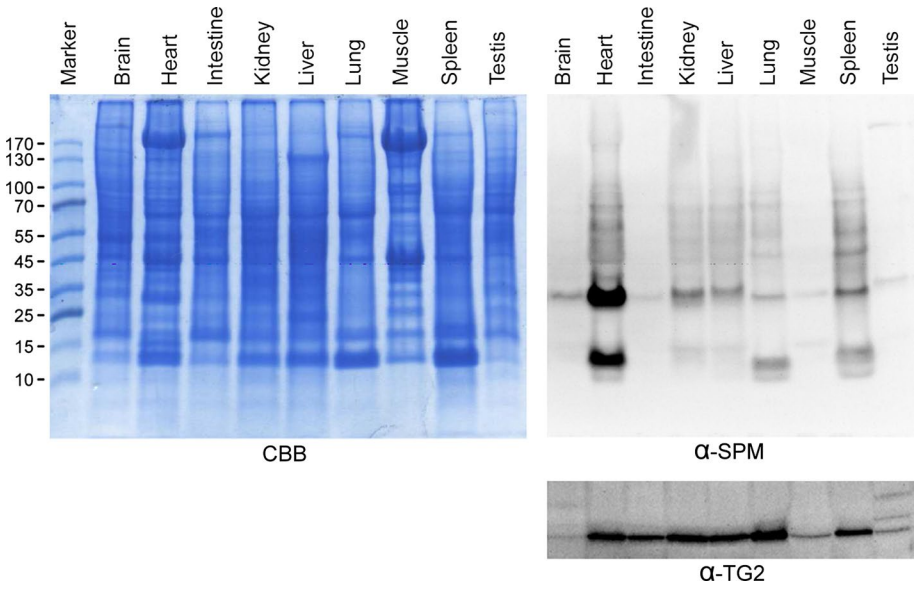

Fig. 7 Global analysis of polyamine modification in mouse tissue extracts. All mouse tissues were weighed and homogenized in the ten volume of PBS with 8 M urea and 1 % SDS, followed by sonication and centrifugation. The proteins were examined by Coomassie blue G-250 staining (*left panel*), immunoblotting with antispermine antibody and TG2 antibody following SDS-PAGE (*right panel*). Tissue protein extract of 50 μg was added in each well

ion trap-orbitrap hybrid mass spectrometry; nine sites in recombinant histone proteins, three sites in recombinant HSP70, thirteen sites in human histone proteins, two sites in human actin, thirteen sites in porcine tubulins (Fig. 4), 254 polyaminated sites in 233 proteins from the HeLa cell lysate (Supplementary Table 1), and seven sites in six mouse heart proteins (Supplementary Table 2). Surprisingly we found that histone proteins and HSP70 expressed and purified from *E. coli* already had PUT, SPD, and SPM modification. In these six proteins, twelve sites have been identified with PUT occupying 8, SPD 7, and SPM 5 sites of them. Transglutaminase activity exerted by *Bordetella* dermonecrotizing toxin (DNT) from *Bordetella bronchiseptica* and cytotoxic necrotizing factor 1 (CNF1) from *E. coli* is responsible for deamidation or polyamination of rho GTPases, inhibition of GTP hydrolysis, and persistent activation of rho GTPases in host cells (Boquet 2001; Caprioli et al. 1987; Horiguchi et al. 1997; Schmidt et al. 1997, 1998). CNF1 shares a catalytic dyad of cysteine and histidine residues with eukaryotic transglutaminases, but the catalytic activity does not require calcium (Schmidt et al. 1998). However, CNF1 is only present in pathogenic *E. coli* strains, but not in the non-pathogenic strain which is used for recombinant protein expression. These toxins have not been tested for their transglutaminase activity toward bacterial proteins. Our data strongly suggest that additional factors present in non-pathogenic *E. coli* strains possess transglutaminase-like activity.

We have identified 254 polyaminated sites in 233 proteins isolated from HeLa cells. Among the sites, PUT occupied 168 of them, SPD 75, and SPM 25, with some sites modified by more than one polyamine. Given the fact that the concentration of PUT is the lowest among polyamines within HeLa cells (Gallo et al. 1986; Goyns 1980), TG2 seems to have a strong substrate preference for PUT (Tsai et al. 1998). In support of this, we examined the incorporation of PUT, SPD, and SPM into a synthetic peptide, SGQQQLGSSR, in three separated TG2-catalyzed reactions under similar assay conditions. We found 180 PUT-modified, 48 SPD-modified, and 2 SPM-modified peptides out of 319, 197, and 173 total peptide counts, respectively, using mass spectrometric analysis. Unfortunately, our antiserum against SPM does not recognize PUT modification rendering the immunological detection of polyamines by anti-SPM antibody less attractive. However, immunoblotting using the anti-SPM antiserum can complement the elaborated analysis with LC–MS in uncovering potential substrates of TG2.

It is interesting to point out that basic proteins such as histones are modified with highly cationic polyamines, resulting in further increases in positive charge. Histone H2A, H2B, H3, and H4 purified from chicken erythrocyte were shown to be good substrates of TG2. Nine out of 18 glutamine residues in these histone proteins were modified with DNC (Ballestar et al. 1996). Importantly, five out of the nine known modification sites were confirmed in the nucleosomes isolated from HeLa cells; Q105 (104 in chicken) and Q113 (112) in H2A, Q96 (95) in H2B, Q6 (5) in H3 and Q28 (27) in H4 (Fig. 4a). Polyamines caused chromatin compaction in an array made of positioning nucleosomes with 177 bp in length (Korolev et al. 2010). Further, increasing concentrations of SPM and SPD caused an increase in chromatin compaction in permeabilized HeLa cells with maximum compaction seen at 1.5 mM of SPD and at 0.4 mM of SPM (Visvanathan et al. 2013). Therefore, conjugation of polyamines in histone proteins catalyzed by TG2 may cause chromatin compaction nearby and possibly modulate chromatin remodeling. Interestingly,

TG2 can also function as a protein kinase phosphorylating H1, H2A, H2B, H3, and H4 in vitro, especially histone H3 serine 10 phosphorylation modulates H3 acetylation levels (Mishra et al. 2006). The exact mechanism of how phosphorylation, acetylation, and polyamination interact with each other and the consequence of each modification still remains elusive and demands further investigation.

Cationic molecules including polyamines induce actin bundles (Muhlrad et al. 2011) and specific growth of actin-enriched lamellipodia in cells (Nedeva et al. 2013) and promote the bundling of microtubules (Hamon et al. 2011). In addition, transglutaminase-catalyzed polyamination of tubulins stabilizes microtubules in vitro and in vivo (Song et al. 2013). All these findings suggest that binding or modification of polyamines in actin and tubulins may reorganize the cytoskeleton and affect cell morphology and function. Importantly, known modified residues on α-tubulins (Q31, Q128, Q133, and Q285) were also identified in this report (Song et al. 2013); TUBA1B in Fig. 4). Further, C-terminal region of tubulins is involved in the binding with positive regulators Tau and SPM, and negative regulator calcium (Lefevre et al. 2011). In addition, calcium can compete with the binding of Tau and SPM and with the positive effect of these two molecules on microtubule assembly in vitro. Q423, Q424, and Q426 residues of β-tubulin C-terminal tail were modified with SPM (TUBB in Fig. 4) suggesting that some non-covalent binding of SPM may be converted into covalent modification by TG2.

Conceivably, cellular effects of polyamines may be mediated by either non-covalent or covalent binding to the target proteins leading to alteration of protein function. For example, non-covalent binding of polyamines activates cytosolic NADP-isocitrate dehydrogenase (Murakami et al. 2012), attenuates the physical interaction between Src and activated EGFR (Ray et al. 2012), enhances tubulins to microtubule assembly (Lefevre et al. 2011), accelerates codon recognition by transfer RNAs on the ribosome (Hetrick et al. 2010), blocks outward current in the inwardly rectifying potassium channels (Osawa et al. 2009), and activates the capsaicin receptor TRPV1 (Ahern et al. 2006). On the other hand, covalent conjugation of polyamines catalyzed by transglutaminases hinders the interaction of high-risk human papillomavirus (HPV) E7 with tumor suppressor Rb (Jeon et al. 2006), reduces the GTPase activity of RhoA leading to constitutive activation (Masuda et al. 2000), and increases stability and activity of glyoxalase 1 (Lee and Chang 2014). Most of the known effects of polyamines are not unique to SPM, but shared by other polyamines with a different potency. However, SPM may still play important, although not vital, roles in higher organisms because lack of SPM in mammals results in severe abnormality in brain and muscle development. Snyder-Robinson syndrome characterized by intellectual disability, muscle, and bone abnormalities results from mutations in the spermine synthase gene located on the X chromosome (Cason et al. 2003; Peron et al. 2013). The male Gyro (Gy) mouse has a partial chromosomal deletion in the X chromosome spanning most of SpmS and Phex leading to symptoms of hypophosphatemic rickets, circling behavior, hyperactivity, head shaking, inner ear abnormalities, deafness, sterility, and post-natal growth retardation (Lorenz et al. 1998; Meyer et al. 1998). All symptoms of Gy mouse can be corrected except hypophosphatemic disorder by transgenic expression of SPM synthase (Wang et al. 2004). It has been suggested that possible functions of SPM include serving as storage pool from which SPD and PUT can be back converted, increasing resistance to oxidative stress, modulating activities of channels such as inwardly rectifying potassium channels and glutamate receptors where SPM is more potent than SPD (Pegg 2014). We noticed that some of the polyamination sites are occupied by PUT, SPD, and SPM. It is possible that PUT, SPD, and SPM modification induce different physiological effects or the same effect to different extents.

Conclusions

In this report, we used an in vitro spermine incorporation assay to reveal that five histone proteins, actin, tubulins, and HSP70 were incorporated with spermine by TG2, and modification sites of these proteins, either recombinant proteins expressed in *E. coli* or native proteins, were identified. We also showed 254 polyaminylated sites in 233 proteins from HeLa cell lysate catalyzed by TG2 with SPM. Thus, polyamine incorporation by TG2 as one post-translational modification probably plays an important role in both prokaryotic and eukaryotic cells. Immunoblotting using the anti-SPM antiserum can complement the elaborated analysis with LC–MS in uncovering potential substrates of TG2.

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Conflict of interest The authors have no conflicts of interest to disclose.

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