Epifluorescence, confocal laser microscopy and colocalization analyses in the study of protein glutathionylation in primary cultured fibroblasts and in their in situ extracted matrix

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Fluorescence microscopy is an important tool in the investigation of protein modifications. Glutathione provides a mean of regulating protein function by glutathionylation, consisting in the formation of mixed disulfides linking cysteines with glutathione. The glutathionylation of proteins represents for the cell a mechanism to bond physiological processes, and/or adaptive stress responses, to changes in the intracellular redox states. Despite the role of oxidative stress biomarkers assumed recently by glutathionylated proteins in human diseases, so far no information is available on the intracellular distribution of glutathionylated proteins in human cell lines in steady state conditions. Using confocal laser microscopy and colocalization analysis, we report the compartmentalization of constitutively glutathionylated proteins in different intracellular districts, such as cytoskeleton, nuclear lamina, endoplasmic reticulum and mitochondria, in primary cultured fibroblasts. The tight association between glutathione and our interest proteins was clearly confirmed by the persistence of its distribution in in situ extracted matrix samples. Fluorescence imaging will provide an additional important tool to obtain a detailed subcellular visualization of this critical marker of protein oxidation, thus potentially allowing the identification of new pathogenic pathways in human diseases

Keywords: immunofluorescence; confocal laser microscopy; colocalization analysis; in situ extracted matrix; glutathionylated proteins

1. Introduction

S-glutathionylation is a mechanism of signal transduction by which cells respond effectively and reversibly to redox inputs. Glutathione is the most abundant non enzymatic antioxidant in cells, where it plays an important role against oxidative stress-induced cell injury [1]. Intracellularly, total glutathione can be free or bonded to proteins. Free glutathione is present mainly in its reduced form (GSH), which can be converted to the oxidized form (GSSG) during oxidative stress. In mammalian cells, the redox status depends on relative amounts of the reduced and oxidised glutathione (GSH/GSSG), with GSH normally exceeding GSSG, but the oxidation of a small amount of GSH to GSSG can dramatically alter this ratio, causing the thiol groups of some intracellular proteins to be modified by a process known as S-glutathionylation [2, 3]. Recently, protein glutathionylation has attracted increasing attention as a key molecular event in the process of redox regulation of protein functions, and the growing evidence of proteins glutathionylated both in vitro and in vivo supports the role of glutathione in signal transduction [4-7].

A number of functionally critical proteins possess accessible cysteine residues, liable to undergo redox changes depending on variations of the intra- as well as extracellular conditions [4, 6-8]. Many glutathionylated proteins are enzymes involved in various pathways of carbohydrate/energy metabolism. Some glutathionylated proteins belong to the class of cytoskeletal proteins, and some are involved in transcription, translation and degradation [5, 9]. Among the cytoskeletal proteins, actin has been found to be glutathionylated in human platelets [10], erythrocytes [11], hepatocytes and in T lymphocytes [12], after exposure to oxidants. The glutathionylation of tubulin has also been demonstrated in vitro by using the purified protein [13].

The glutathionylation of proteins may constitute a sensor of tissue oxidative stress and its extent, in pathophysiological conditions, could help to define a threshold of basal antioxidant status. Indeed, in the last years, glutathionylated proteins have also been investigated as possible biomarkers of oxidative stress in human diseases and the extent of protein glutathionylation may indicate the evolution of the disease, thus acquiring a diagnostic/prognostic value. Significant increases of glutathionylated proteins, for instance, have been found in hyperlipidemia, chronic renal failure, and diabetes mellitus [7]. Furthermore, evidence for a dysfunction of glutathione metabolism has been proposed for the pathogenesis of several neurodegenerative diseases, such as Parkinson’s disease, Alzheimer’s disease, Friedreich’s ataxia and Amyotrophic lateral sclerosis [14-16].

However, protein glutathionylation also occurs under physiological conditions and the presence of constitutively glutathionylated proteins strongly supports a role for glutathionylation in physiological signaling [5]. Indeed, growing evidence indicates glutathionylation as a modulatory mechanism of many cellular pathways, able to regulate the activity of several transcription factors (Nrf2 and NFκB) and to interfere with the phosphorylation/dephosphorylation mechanism by interacting with kinases (PKA, CK) and/or phosphatases (PP2A, PTEN) [17].
Furthermore, protein glutathionylation seems to be involved in cell proliferation and differentiation by contributing to the mitotic spindle formation during cell division. In fact, the supra-molecular organisation of microfilaments and microtubules depends on the presence of exposed –SH residues and it is potentially susceptible to glutathionylation. Actin, for instance, is a target for S-thiolation in several cells under different oxidative stress conditions, and its glutathionylation is considered a physiological regulatory mechanism of G-actin polymerisation [18-21]. Also tubulin, with its 20 free sulphhydryl groups, can be readily oxidised in vitro, and the extent of tubulin cysteine oxidation has been shown to correlate with inhibition of microtubule polymerisation [7, 22]. Using immunohistochemical techniques, Sparaco et al. [23] demonstrated the constitutive glutathionylation of neuronal cytoskeleton in human CNS, and we recently demonstrated that actin glutathionylation caused impairment of microfilaments dynamics and cytoskeletal functions in fibroblasts of patients with a neurodegenerative disease [8].

In the last two decades, the use of confocal laser scanning microscopy has progressively allowed to obtain detailed morphological and functional informations in the analysis of localization and distribution of proteins within cells. In the meanwhile, the rapid development of a large spectral range of fluorescent molecules, coupled with advances in digital imaging, has provided powerful tools to investigate submicroscopic structures in cells and tissues. It is recognized that the location and distribution of a protein are crucial aspects to understanding its physiological function and its role in biological processes. To this end, the optical sectioning performed by confocal microscopy provides the means to localize molecules with high spatial resolution. In particular, the colocalization analysis allows to correlate the spatial localization of two proteins occupying the same volume (or voxel) of interest.

In view of the important patho-physiological implications of glutathione distribution among cellular organelles, we describe the compartmentalization of constitutively glutathionylated proteins in different subcellular structures in human dermal fibroblasts using widefield microscopy, confocal laser scanning microscopy and colocalization analysis, making use of a monoclonal antibody that specifically reacts with glutathione bound to proteins as well as antibodies directed against cytoskeleton, endoplasmic reticulum (ER), mitochondria and nuclear lamina proteins.

2. Materials

2.1 Lectins and vital probes

The lectin Concanavalin A (Con A) selectively binds to α-mannopyranosyl and α-glucopyranosyl residues, primarily in the ER. In this study we used Alexa Fluor 633 conjugated to Con A, as shown in Table 1, with excitation/emission peaks about 632/647 nm. Rhodamine Phalloidin was used to label filamentous F-actin, while nuclear counterstaining was obtained by Hoechst 33342 (H3569, Invitrogen).

The mitochondrion selective-vital probe Mito Traker passively diffuses across the plasma membrane and accumulates in mitochondria. We preferentially used Mito Traker Red because able to resist to paraformaldehyde fixation and to permeabilization with some detergents (for example, Triton X100), so it may be employed before applying the immunocytochemistry protocol.

<table>
<thead>
<tr>
<th>Alexa Fluor 633 Concanavalin A</th>
<th>C21402</th>
<th>Invitrogen</th>
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<tbody>
<tr>
<td>Mito Traker Red</td>
<td>M7512</td>
<td>Invitrogen</td>
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<tr>
<td>Rhodamine Phalloidin</td>
<td>R415</td>
<td>Invitrogen</td>
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<tr>
<td>Beta-Tubulin</td>
<td>MAB1864</td>
<td>Chemicon</td>
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<td>GS-Pro</td>
<td>A-101</td>
<td>Virogen</td>
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<tr>
<td>Lamin B</td>
<td>SC-6216</td>
<td>Santa Cruz Biotechnology</td>
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<tr>
<td>PDI (protein disulfide isomerase)</td>
<td>S34200</td>
<td>Invitrogen</td>
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<tr>
<td>Vimentin</td>
<td>SC-7557</td>
<td>Santa Cruz Biotechnology</td>
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2.2 Antibodies

In this study we employed a monoclonal antibody that specifically reacts with glutathione bond to proteins (GS-Pro) as reported in Table 1. We performed single and double immunostaining following the indirect immunofluorescence method, and we studied the subcellular glutathionylation using antibodies against microtubules and intermediate filaments (beta-tubulin and vimentin, respectively), nuclear lamina (lamin B), and ER (protein disulfide isomerase, PDI).

Goat anti-lamin B or anti-vimentin antibodies were revealed by Alexa Fluor 555 donkey anti-goat IgG, whereas Alexa Fluor 488 or 555 conjugated goat anti-mouse or anti-rat immunoglobulins (Invitrogen) were used to detect the immunoreaction of anti GS-Pro and anti-beta-tubulin antibodies, respectively. In addition, goat anti-mouse IgG2a conjugated to fluorescein and goat anti-mouse IgG2b conjugated to rhodamine (Mitosciences) were used to perform GS-Pro/PDI double staining (see details in the immunocytochemistry procedure).
3. Experimental procedures

3.1 Immunocytochemistry

To study the distribution of glutathionylated proteins, primary dermal fibroblasts from normal human skin biopsies were grown on glass coverslips in Dulbecco’s modified minimum essential medium (D-MEM) supplemented with 20% fetal calf serum (Life Technologies), penicillin and streptomycin.

Subconfluent cells were processed for indirect immunofluorescence using the following incubation steps: after a wash in phosphate buffered saline (PBS), cells were fixed in ice cold 4% paraformaldehyde in PBS (10 min), washed in PBS, and permeabilized with 0.15% Triton X-100 in PBS (10 min).

Samples were saturated in 5% normal goat serum in PBS with added 1% bovine albumin serum (BSA) for 30 min, when we used conjugated goat IgG as secondary antibody, or alternatively with PBS/BSA 5% when secondary antibodies produced by different species were used. Then, cells were incubated overnight at 4° C, with the mouse anti GS-Pro antibody at 1:100 dilution in PBS/BSA 1%, subsequently washed with PBS and incubated with Alexa Fluor 488 conjugated goat anti-mouse IgG (1:500, at room temperature) in PBS/BSA 1%.

In double labeling experiments, samples were incubated with a second primary antibody for 1 hr (rat anti beta-tubulin or goat anti-vimentin) or overnight (goat anti-lamin B). After washes in PBS, the immunoreactions was revealed by incubation with Alexa Fluor 555 donkey anti-goat IgG, or Alexa Fluor 555 goat anti-rat IgG in PBS/BSA 1%. Negative controls were performed using PBS/BSA without the primary antibody.

The antibody directed against glutathionylated proteins consists on mouse IgG2a, while the antibody against protein disulfide isomerase is represented by mouse IgG2b; in this way, we can use two different subclasses of goat anti-mouse IgG2a and 2b (Mitosciences) conjugated to two different fluorophores (fluorescein and rhodamine, respectively), to perform GS-Pro/PDI double staining.

Further, some samples were labeled with rhodamine phalloidin (2 units/ml) or with Alexa Fluor 633 Con A (200 µg/ml dilution) to detect actin cytoskeleton and ER, respectively.

Hoechst 33342 (H3569, Invitrogen) was employed at dilution of 10 µg/ml to stain nuclei. Finally, slides were mounted with Prolong antifade reagent (Invitrogen).

3.2 In situ matrix extraction procedure

Primary dermal fibroblasts near to confluence were processed to obtain their in situ extracted matrix owing to a previously reported protocol [24], with some modifications:

a) subconfluent cells are washed in D-MEM w/o serum, then in PBS;

b) permeabilization with TBS-5 buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl and 5 mM MgCl2), 1%

NP40, 2mM sodium tetrathionate, 1mM phenylmethylsulfonyl fluoride, 10 µg/mg leupeptin and 10 µg/mg aprotinin (for 15m, at RT);

c) washes in TBS-5, and digestion with 0.01 mg/ml DNase I for 1h at RT;

d) TBS-5 washes, and double extraction in 2M NaCl in TBS-5 (5m);

e) washes in TBS-5, followed by fixation in ice cold 4% paraformaldehyde.

Then, in situ extrated matrix samples processed for immunocytochemistry following the procedure reported above, and labelled with anti GS-Pro and lamina B antibodies, and the immunoreaction was revealed by Alexa Fluor 488 and 555 conjugated secondary antibodies. Nuclei were counterstained with Hoechst 33342.

Wide-field microscopy fluorescence images were collected with a Zeiss Axioskope2 microscope equipped with a 75 Watt mercury arc lamp using a 40x objective (0.75 N.A.).

Images were recorded with a digital Diagnostic Instruments charge-coupled device (CCD) camera.

3.3 Confocal laser scanning microscopy

The confocal imaging was performed on Olympus Fluoview FV1000 confocal IX81 inverted microscope (Olympus Europa GMBH), equipped with FV10-ASW software version 2.0, Multi Ar (458-488 and 515 nm), 2X He/Ne (543 and 633 nm) lasers, as well as a 405-nanometer diode laser, using 60x objective (1.42 N.A. oil).

Optical single sections were acquired with a scanning mode format of 1024 x 1024 pixels with a pixel sixe of 0,21 µm, sampling speed of 40 µs/pixel, and 12 bits/pixel images were obtained.

Fluorochromes unmixing was performed by acquisition of automated-sequential collection of multi-channel images, in order to reduce spectral crosstalk between channels.

Colocalization analysis for dual stained samples was carried out using the FV10-ASW software colocalization function, and images were processed with Photoshop software version 9.0 (Adobe Systems Inc., San Jose, CA).
4. Results and Discussion

The confocal laser scanning microscopy analysis of subconfluent dermal fibroblasts reveals two patterns of GS-Pro immunostaining, granular and microfilamentous, as shown in Figure 1. The granular distribution of glutathionylated proteins is concentrated in the cytoplasm, and shows a high colocalization with markers of the ER, as Con A. The microfilamentous staining underlines components of cytoskeleton and microtubules. In addition, a clear concentration of GS-Pro labeling was reported around nuclei, that significantly colocalized with markers of the nuclear lamina.

![Fig. 1 Patterns of distribution of glutathionylated proteins in human dermal fibroblasts and colocalization analysis with markers of the nuclear lamina (lamin b in red, in b) and endoplasmic reticulum (Con A, pseudocolored in blue, in c). GS-Pro antibody (a) stained the perinuclear rim (arrows) and cytoskeletal filaments, and showed a granular distribution in the cytoplasm and near nuclei (arrowheads). Colocalization masks of GS-Pro and lamin B (white areas in e) or Con A (white areas in f), revealed a significant degree of overlap. Merge image in d. Magnification bar = 40 µm.](image)

Whereas the granular pattern may be observed in several cells, the cytoskeletal one is generally founded in all cells, along with the characteristic perinuclear distribution.

We used the threshold-based approach to perform the colocalization analysis. In this way, we considered only pixels above a certain threshold in the calculation of the image correlation. To obtain colocalization masks (visualized as white areas/spots), we used the colocalization function by FV10-ASW Olympus software version 2.0.

We confirmed, in Figure 2, the ER-like distribution of glutathionylated proteins by double incubation of GS-Pro with PDI, an antibody directed against the protein disulphide-isomerase localized in the ER that is responsible of –S-S– bonds formation.

In addition, we examined the immunoexpression of GS-Pro in relation to mitochondria distribution using the mitotraker vital staining. A significant pattern of colocalization was detected between GS-Pro and PDI; besides, mitochondria showed a complete colocalization with GS-Pro, in correspondence to areas where glutathione displayed the granular and punctate staining.
Further, we characterized the cytoskeletal distribution of glutathionylated proteins with markers of the cytoskeleton, like F-actin, beta-tubulin and vimentin, as displayed in Figure 3.

Colocalization analysis showed a clear overlap of GS-Pro with filamentous actin, particularly in correspondence to stress fibers. Microtubules stained by beta-tubulin exhibited a complete and significant colocalization pattern in all distribution points, whereas vimentin intermediate filaments showed a partial degree of colocalization limited to central and perinuclear areas.

The association between glutathione and nuclear lamina was in-depth analyzed by the analysis of samples obtained after in situ extraction of matrix, as demonstrate in Figure 4. The extraction procedure allowed to verify if there was a tight association of glutathione with nuclear lamina and cytoskeleton.

The immunofluorescence analysis of extracted matrix samples detected a more brilliant signal by glutathionylated proteins in correspondence to the cytoskeleton and the nuclear lamina, as the extraction procedure allowed to discard cytosol, organeli, ER cisternae, nucleoplasms and DNA. By this method it is possible to reveal antigenic sites and to amplify the intensity of GS-Pro immunoexpression.

Epifluorescence images were deconvolved by Image-Pro Plus software version 6.0, to reduce out-of-focus fluorescence in widefield microscopy, improving the quality of images.

Our results obtained in matrix extracted samples confirm the tight binding between glutathione and components of nuclear lamina and cytoskeleton. The glutathionylation of these districts was also suggested by the analysis of colocalization images of GS-Pro with lamin B, beta-tubulin and filamentous actin in particular in correspondence to stress fibres. Conversely, vimentin intermediate filaments resulted partially involved in the glutathionylation process.
Fig. 3 Glutathionylation of cytoskeleton elements. GS-Pro (green) and filamentous actin (red in b) showed several colocalization areas (d), clearly visible in correspondence to stress fibers (arrows). Beta-tubulin (f) and GS-Pro exhibited a complete and significant pattern of colocalization (g and h), whereas vimentin (j) overlapped with glutathione in some areas concentrated in perinuclear and central districts (k and l). Magnification bar: 40 µm.

These data underlined that the glutathionylation of microtubules and F-actin may carry out an important role in processes of cellular proliferation, migration and differentiation. On the other hand, the clear association of glutathione and nuclear lamina strongly suggest that glutathionylation process may be involved in DNA replication and transcription and chromatin-remodelling. Indeed, accumulating evidence demonstrates that functions of the nuclear envelope are much beyond the simple barrier separating the nuclear and cytoplasmic compartments [25, 26]. Besides, the nuclear membrane increasingly represents a “node” of regulating signal transduction pathways in eukaryotic cells. In this context, the glutathionylation of nuclear lamina becomes a critical factor in redox signaling, and nuclear envelope acquires a crucial role in maintaining the cellular redox compartmentalization.

In addition, we have revealed a tight association between glutathione-ER and glutathione-mitochondria in non-extracted fibroblasts, in those subcellular districts where glutathione shows a granular and punctate distribution. The ER glutathionylation detected by our colocalization study, confirms previous data demonstrating that the ER lumen contains relatively higher concentrations of GSSG [27] allowing the formation of native disulphide bonds, and involving the participation of PDI and other oxidoreductases as catalysts.

Our study highlights another important site of colocalization of glutathionylated proteins in human fibroblasts: the mitochondria. Mitochondria are the most redox-active compartment of mammalian cells and represents the major source of reactive oxygen species. Therefore, the mitochondrial redox regulation is critical in maintaining the molecular machinery “active” which is required for oxidative phosphorylation, and protein glutathionylation may intervene in modulating the cysteine residues of the electron transport chain enzymes.
Fig. 4  Epifluorescence analysis of control fibroblasts (a-c) and extracted cells (d-f) with anti GS-Pro (green) and lamin B (red) antibodies. GS-Pro was normally distributed around nuclei, in the cytoplasm and in correspondence to cytoskeletal filaments (a). In extracted matrix samples, GS-Pro labelling was particularly enhanced in correspondence to nuclei and cytoskeleton (b). Lamin B staining allowed detecting the coincidence of the perinuclear rim positive to GS-Pro and the nuclear lamina, in control (b) and, significantly, in extracted cells (e). Nuclei were stained with Hoechst in control unextracted cells (c), but extracted samples were negative owing to DNA digestion by DNAse (f). Magnification bar: 50 µm.

The co-localization of glutathione with mitochondria was already suggested by Soderdhal et al [28], in a human lung carcinoma cell line. Furthermore, we previously reported the association of glutathione with subcellular organuli like mitochondria in normal and pathological liver, by ultrastructural immunocytochemical analysis [29], and recently we identified, by biochemical techniques, the respiratory chain enzyme Complex I as a sensitive target of glutathionylation in isolated cardiac mitochondria [30].

Thus, the ability to visualize an abnormal compartmentalization of glutathionylated proteins in cultured cells and/or in tissue sections, will represent an additional tool to understand the mechanisms underlying the pathogenesis in diseases where oxidative stress is presumed to be the cause, as we showed before in Friedreich’s Ataxia [16] and patients with non-alcoholic fatty liver disease [29].

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References


