Journal of Immunological Methods 382 (2012) 189–195

Contents lists available at SciVerse ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Defining TNF-α and IL-1β induced nascent proteins: Combining bio-orthogonal non-canonical amino acid tagging and proteomics

Ka-Yee (Grace) Choi [a,b], Dustin N.D. Lippert [a], Peyman Ezzatti [a], Neeloffer Mookherjee [a,b,*]

[a] Manitoba Centre for Proteomics and Systems Biology, Department of Internal Medicine, Canada
[b] Department of Immunology, University of Manitoba, Winnipeg, MB, Canada

ARTICLE INFO

Article history:
Received 4 April 2012
Received in revised form 4 June 2012
Accepted 5 June 2012
Available online 12 June 2012

Keywords:
L-Azidohomoalanine
TNF-α
IL-1β
Inflammation
Proteomics
Monocytes

ABSTRACT

An impediment in the development of new therapeutic strategies for chronic inflammatory diseases is the limited understanding of underlying molecular mechanisms. The objective of this study was to identify newly synthesized (nascent) proteins induced by critical inflammatory cytokines TNF-α and IL-1β in human monocytic THP-1 cells. We optimized methods to combine two different approaches, bio-orthogonal non-canonical amino acid tagging (BONCAT) along with proteomics using isobaric tags (iTRAQ). BONCAT employed the incorporation of L-azidohomoalanine (AHA), an analog of methionine, into TNF-α or IL-1β induced nascent proteins. The AHA-containing nascent proteins were tagged with alkyne-biotin to allow enrichment using avidin affinity purification. The differential expressions of the enriched proteins were further determined using iTRAQ reagents and mass spectrometry (MS). The combination of BONCAT and proteomics represents a unique approach that has uncovered the nascent proteome induced by inflammatory cytokines TNF-α and IL-1β.

1. Introduction

Molecular indicators of a disease state or activity (biomarkers) can provide insight into disease pathogenesis, new therapeutic targets and molecules that may be used to predict the responsiveness of candidate therapeutics. Biomarker identification continues to be an important goal in biomedical research. Gene expression monitoring or cytokine profiling from serum or other body fluids are common approaches for biomarker discovery that have been used in recent years (O'Hara et al., 2006). However, gene expression is not a robust indicator of disease phenotype and multi-analyte cytokine profiling is a hypothesis-driven, surrogate endpoint approach that does not differentiate between the wide variety of systemic inflammatory disorders (O'Hara et al., 2006). Mass spectrometry (MS)-based differential proteomics methods provide information about the alterations in global protein profiles that contribute to the disease phenotype state. This information can aid in the diagnosis, prognosis and therapeutic response prediction in various clinical conditions (Azad et al., 2006; Rocken et al., 2008; Nicholas et al., 2009). However, global proteomic approaches such as 2D gel electrophoresis or isotope labeling do not differentiate between proteins that are newly synthesized (nascent) in response to a stimulus and those from pre-existing pools, because they are chemically identical. A recent study demonstrated the enrichment of nascent proteins following metabolic incorporation of the unnatural amino acid L-azidohomoalanine (AHA), which is an analogue of methionine (Dieterich et al., 2006, 2007).

AHA allows for non-toxic labeling of biomolecules in living mammalian cells and does not affect global rates of protein synthesis or degradation (Doerr, 2006; Dieterich et al., 2007). Metabolic labeling with AHA results in nascent proteins with an azide-bearing functional group, thus making

Abbreviations: AHA, L-azidohomoalanine;BONCAT, bio-orthogonal non-canonical amino acid tagging;iTRAQ, isobaric tags for relative and absolute quantitation;MS, mass spectrometry.

* Corresponding author at: Department of Internal Medicine and Immunology, University of Manitoba, Winnipeg, MB, Canada. Tel.: +1 204 272 3115; fax: +1 204 480 1362.
E-mail address: mookherj@cc.umanitoba.ca (N. Mookherjee).

0022-1759/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2012.06.003

these molecules distinct from the pool of pre-existing cellular proteins. A biotin alkyne reagent can be covalently coupled to the reactive azide group of the AHA-modified proteins and subsequently used for enrichment of the nascent proteins by affinity purification. Even though the profiling of nascent proteins using this technology will be limited to proteins that contain at least one methionine, it has been shown that at least 94% of all human proteins will be amenable to AHA labeling using this approach (Dieterich et al., 2007). The distinct advantage of this method is the ability to specifically enrich and identify the subpopulation of proteins that are actively expressed only in the presence of the intended stimuli, thus obtaining a physiologically relevant insight into the cellular processes that constitute the stimulus response.

In this study, we used AHA labeling to selectively enrich nascent proteins synthesized in response to pro-inflammatory cytokines TNF-α and IL-1β in monocytic THP-1 cells. Further, we determined the differential expressions of the enriched AHA-labeled nascent proteins from the different stimuli using iTRAQ reagents (isobaric Tags for Relative and Absolute Quantitation) and MS . No study to date has used a combination of bio-orthogonal non-canonical amino acid tagging (Dieterich et al., 2006, 2007) along with proteomics using iTRAQ labeling to define the nascent proteome following cytokine stimulation. We identified common as well as unique proteins induced following stimulation with pro-inflammatory cytokines TNF-α and IL-1β. We further showed the induction of transcriptional message for several of the identified nascent protein candidates. The identification of common protein candidates that are induced by stimulation with both TNF-α and IL-1β provides candidates that may be targeted for immune-mediated chronic inflammatory disorders, especially for non-responders of current anti-TNF therapy. Moreover, the identification of unique responses to TNF-α and IL-1β demonstrates the potential of this approach to uncover biomarkers aimed at tailored interventions in processes with overlapping signalling pathways or molecular outcomes.

2. Materials and methods

2.1. Cell culture

Human monocytic THP-1 (ATCC® TIB-202) cells were cultured in RPMI-1640 medium containing 2 mM L-glutamine, 1 mM sodium pyruvate, supplemented with 10% (v/v) FBS and maintained in a humidified incubator at 37°C and 5% CO_2 as previously described (Mookherjee et al., 2006). Cellular cytotoxicity was evaluated by monitoring the release of lactate dehydrogenase employing a colorimetric detection kit (Roche Diagnostics, Laval, QC, Canada).

2.2. AHA labeling of nascent proteins

THP-1 monocytic cells were washed with pre-warmed D-PBS (Gibco, Invitrogen Corporation, Burlington, ON, Canada) and incubated in methionine-free RPMI-1640 medium (catalog #050001DJ, Invitrogen) without serum for 1 h at 37°C to deplete methionine reserves. Depletion of methionine reserves by incubating the THP-1 suspension cells in methionine-free RPMI-1640 medium without serum for 1 h made the cells plastic adherent, similar to macrophage-like THP-1 cells (Mookherjee et al., 2006). After 1 h incubation, the medium was removed and replaced with methionine-free RPMI medium without serum containing 100 μM of Click-iT® AHA reagent (catalog #C10102, Invitrogen), in the presence or absence of recombinant human cytokines either 10 ng/ml TNF-α or IL-1β (eBioscience, Inc., San Diego, CA, USA). The cells were further incubated for 4 h in a humidified incubator at 37°C and 5% CO_2. The tissue culture (TC) supernatants were centrifuged at 1500 ×*g* for 7 min to obtain cell-free samples, aliquoted and stored at −20 °C. The cells were washed with D-PBS and lysed with 50 mM Tris–HCl containing HALT™ protease and phosphatase inhibitor cocktail (Thermo Scientific), 250 U/ml benzonase nuclease and 1% SDS. Total protein content was estimated in each cell lysate by the micro BCA assay (Pierce; Thermo Scientific, Rockford, IL, USA) with a bovine serum albumin (Sigma-Aldrich) standard curve. The cell lysates were stored at −20 °C until required.

2.3. Biotinylation and affinity purification

Equal amounts of cell lysate (between 100 and 150 μg) were treated with an alkyne-biotin reagent (catalog #B10185, Invitrogen) as per the manufacturer's instructions. The two-step 'click' reaction involved a copper catalyzed triazole formation between the azide group of the AHA-modified nascent proteins and the alkyne-biotin reagent. The biotinylated-AHA-modified nascent proteins were subsequently enriched by affinity purification using Ultralink® Immobilized NeutrAvidin™ resin (Pierce), and the bound proteins were eluted with 6 M guanidinium hydrochloride. The samples eluted from affinity purification were precipitated overnight at −20°C in 50% (v/v) acetone/methanol solution. The pellets were washed with ice cold 50% (v/v) acetone/methanol solution and stored at −80°C until required.

2.4. Western blots

The biotinylated AHA-containing proteins were electrophoretically resolved on 4–12% NuPAGE® Bis-Tris gels (Invitrogen), followed by transfer to nitrocellulose membranes (Millipore). The membranes were subsequently blocked with TBST (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% skim milk powder and probed with anti-biotin HRP-linked antibody (Cell Signaling Technologies) in TBST containing 3% skim milk powder. The membranes were developed with the Amersham ECL detection system (GE Healthcare, Baie d'Urfe QC, Canada) according to the manufacturer's instructions.

2.5. Proteomics employing isobaric tags for relative and absolute quantitation (iTRAQ)

The amine-modifying iTRAQ™ reagents multiplex kit (Applied Biosystems, Foster City, CA, USA) was employed for determining the differential expression of proteins obtained by affinity purification. Acetone precipitated cell pellets were dissolved in 20 μl of iTRAQ dissolution buffer (Applied Biosystems) and further processed as per the manufacturer's instructions. Proteins were reduced and cysteines blocked using the reagents in the kit, followed by digestion with