Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 338 (2005) 700–709
BBRC
www.elsevier.com/locate/ybbrc

Biotinylation of heat shock protein 70 induces RANTES production in HEK293 cells in a CD40-independent pathway ☆

Yufeng Tao, Satoshi Nishikawa, Masayo Nomura [1], Naofumi Kitabatake, Fumito Tani *

Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Goka-sho, Uji, Kyoto 611-0011, Japan

Received 25 September 2005
Available online 11 October 2005

Abstract

Biotinylated proteins and peptides have been used as popular ligands for characterization of cell surface receptors by a variety of methods including flow cytometry. The number and the location of biotin moieties incorporated could alter the structural and physicochemical properties of ligands, although biotin is thought to be such a small molecule (244 Da) that it is capable of being conjugated to most proteins without affecting their activity. Here, we demonstrate that the biotinylated HSP70 molecule via primary amines bound to epithelium-like HEK 293 cells in a saturable manner whereas the unlabeled counterparts of HSP70 other than mouse Hsp72 do not. This binding was not competed by either HSP70 or the biotin entity itself. Interestingly, the biotinylated HSP70 also elicited the production of CC-chemokine RANTES independent of CD40 signaling. This response occurred regardless of sequence diversity of HSP70 derived from different species, and neither the biotinylated ovalbumin nor the unlabeled HSP70 cross-linked with a biotinylated protein stimulated a significant level of RANTES production which was induced by biotinylated HSP70 itself. Our findings suggest that modification of HSP70 such as biotinylation may function as a biological alarm signal in the innate immune system.

Keywords: Biotinylation; CC-chemokine RANTES; Heat shock protein 70; Human embryonic kidney 293 cells

Recent advances in the development of many fluorochromes and in the identification of cell surface antigens enable us to make use of a flow cytometry in many studies of receptor–ligand interaction [1–3]. This technique usually involves the chemical modification of a ligand by FITC, PE, or biotin, which might change its structural property possibly affecting its biological activity.

Biotin is a relatively small molecule with a molecular size of 244 Da, usually found in tissues and blood. This compound specifically binds to both egg white avidin or streptavidin (SAv) with an extraordinary high affinity ($K_a = 10^{-15}$ M) [4,5]. These unique properties make biotin useful as one of the conjugates to many proteins without significantly altering their biological activity. A protein of interest can be reacted with several molecules of biotin that, in turn, can bind a molecule of avidin or SAv, thus greatly increasing the sensitivity of many assay procedures. However, attention should be paid not to inactivate the target molecule by biotinylation.

The most frequently used reagents for biotinylation are the ester forms of biotin, such as a NHS-biotin, which react with primary amines of protein, as primary amines are generally available in proteins and are accessible for active

☆ *Abbreviations:* APC, antigen presenting cells; CD40L, CD40 ligand; DC, dendritic cells; FITC, fluorescein isothiocyanate; HABA 2-(4′-hydroxyazobenzene) benzoic acid; HEK, human embryonic kidney; HSP, heat shock protein; IL, interleukin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NHS, *N*-hydroxysuccinimide; sulfo-NHS, *N*-hydroxysulfosuccinimide; OVA, ovalbumin; PE, phycoerythrin; PRR, pattern recognition receptors; RANTES, regulated upon activation, normal T expressed and secreted; RCMLA, reduced carboxymethylated α-lactalbumin; SAv, streptavidin; SMCC, succinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate; TCEP, Tris-(2-carboxyethyl)phosphine hydrochloride; TNF, tumor necrosis factor; TLR, toll-like receptor.

* Corresponding author: Fax: +81 774 33 3004.
E-mail address: tanifumi@kais.kyoto-u.ac.jp (F. Tani).
[1] Present address: Division of Feed, Fukuoka Office, Fertilizer and Feed Inspection Services, Incorporated Administrative Agency, Chihaya-3-11-15, Higashi-ku, Fukuoka 813-0044, Japan.

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.09.194

esters. The primary amines available on the molecular surface of a protein mostly derive from the ε-amino groups of lysine residues and the N-terminal α-amino group. As the molecular weight of a protein increases, the presence of primary amines will be greater. As shown in the case of an antibody molecule, the number and location of biotin moieties incorporated into a molecule can alter its physicochemical properties and impair biological activity [6–8]. Storm et al. [9] also showed, for a variety of biotinylated functionally unaltered proteins, that biotinylation by NHS-esters can induce the binding to cell surfaces of monocytic U937 cells, which is not specific for the respective unlabeled ligand. Hydrophobic interaction chromatography revealed a remarkable increase in the hydrophobicity of the biotinylated proteins compared to their unlabeled counterparts. They concluded that this biotinylation-induced binding is due to increased hydrophobicity. When we consider that hydrophobicity may be a possible factor for ligands that bind to pattern recognition receptors (PRR) [10], it seems unclear whether biotinylation of macromolecules evokes merely non-specific binding or a specific binding to induce biological responses.

Heat shock protein 70 (HSP70) is known as an immune adjuvant, delivering peptides to APC for cross-presentation via MHC class I molecules [11,12]. Although the interaction of HSP with its receptors on APC is central to an understanding of the mechanism of this important phenomenon, the molecular details remain unclear. HSP70 has also been found to function as a danger signal for a variety of cells including macrophages, DC, and epithelial cells, causing the secretion of inflammatory cytokines and chemokines, such as TNF-α, IL-12, IL-16, and RANTES [13–17]. Since HSP70 also functions as a molecular chaperone, it can sense changes in physiological conditions that surround cells. Taking this into account, we hypothesized that HSP70 works as a biological sensor through its structural susceptibility to environmental changes. Therefore, in this study, we investigated the effect of chemical modification of HSP70 on immunological properties as a biological alarm signal to the innate immune system. Following from this experiment, biotinylation of HSP70 via primary amines was found to induce the binding to HEK 293 cells through an unknown receptor and to elicit CC-chemokine RANTES production independent of the CD40 signaling pathway.

Materials and methods

Reagents. ADP-Na, ATP-Na_2, ATP-agarose (A-2767), BSA, and RCMLA were obtained from Sigma (St. Louis, MO, USA). OVA was purified from the egg white of newly laid hen eggs by crystallization in a solution of ammonium sulfate and recrystallized five times [18]. Recombinant DnaK was obtained from Stressgen (Victoria, British Columbia, Canada). CD40 Ligand/TNFSF5 was purchased from TECHNE (Minneapolis, MN, USA). LPS from *Escherichia coli* O55:B5 was obtained from Nacalai Tesque (Kyoto, Japan). EZ-Link sulfo-NHS-LC-biotin (Pierce Biotechnology, Rockford, IL, USA) was used to biotinylate proteins. A heterobifunctional cross-linker sulfo-SMCC was also obtained from Pierce. A fluorescein-EX labeling kit to fluoresceinate proteins and TCEP to reduce OVA were from Molecular Probes. (Eugene, OR, USA). Protein labeling was performed according to the manufacturer's protocol. PE-conjugated monoclonal antibody against mouse CD40 (clone 1C10) and other control isotype antibodies were from eBioscience (San Diego, CA, USA). Anti-CD16/32 antibody, FITC-conjugated anti-mouse CD40 (clone HM40-3), and FITC- or PE-conjugated SAv were purchased from Becton–Dickinson (San Jose, CA, USA).

Cell culture. Human embryonic kidney 293 (HEK 293) cells were obtained from the American Type Culture Collection (ATCC, CRL-1573) and cultured in Eagle's minimal essential medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 0.1 mg/ml streptomycin, and 10% heat-inactivated horse serum.

Transfection. The DNA fragment encoding the mature form of mouse CD40 was amplified by PCR of the plasmid containing mouse *cd40* gene in an EST clone library (#4018221; Invitrogen; Carlsbad, CA, USA), using 5′-GACAAGCTTGCGGTTACGTGCAGTGACAAACAG-3′ and 5′-TAAGATCTCAGACCAGGGGCCTCAAGGCTATGC-3′ as specific forward and reverse primers, respectively. We introduced the corresponding cDNA fragments into the N-terminal FLAG expression vector pFLAG-CMV-3 using *Hin*dIII and *Bgl*II sites (Sigma). The sequence of mouse *cd40* cDNA was verified using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit.

HEK 293 cells at 60% confluency in a 12-well plate were transfected with 7 μg of linearized *cd40* construct using TransFectin Lipid Reagent (Bio-Rad; Hercules, CA, USA) according to the manufacturer's protocol. Twenty-four hours after transfection, the medium was replaced with the selection medium containing 0.8 mg/ml G418. The medium was exchanged every 2–3 days during 3 weeks. Screening of stable transfectants was performed with flow cytometry using the anti-FLAG antibody. Surface expression of the CD40 antigen was analyzed in the stable transfectants by immunoblotting and the flow cytometry using two kinds of antibodies against mouse CD40.

Protein expression and purification. Recombinant mouse Hsp72 (mHsp72) and its variants, $mHsp72_{1–384}$, with the N-terminal ATPase domain were expressed and purified as described previously [19]. To express the C-terminal variant, $mHsp72_{385–641}$, the corresponding cDNA fragment was amplified by PCR using a set of specific primers and the plasmid containing the entire mHsp72 cDNA as a template. A plasmid containing the *E. coli dnak* gene was kindly donated by Dr. T. Yoshimura (Nagoya University). The cDNA encoding the entire sequence of *E. coli* DnaK (EC-DnaK) was amplified by PCR. For expression of *Lactobacillus acidophilus* DnaK (LA-DnaK), the genomic DNA was extracted from the bacterial colony with lysis buffer [10 mM Tris–HCl (pH 8), 150 mM NaCl, 10 mM EDTA, and 0.1% SDS] and protease K. Using the genomic DNA as a template, the cDNA fragment encoding the entire sequence of *L. acidophilus dnak* was amplified by PCR techniques using 5′-GGGGAATTCCATATGTCAAAAGTTATTGGTATTGAC-3′ containing *Nde*I restriction enzyme site and 5′-GGCCTCGAGCTTGTTAGGATCTACCTTATGGAATTC-3′ containing *Xho*I restriction enzyme site as specific forward and reverse primers, respectively. For expression of spinach cytoplasmic Hsc70-1 (sHsc70), mRNA was extracted from a spinach leaf with an mRNA purification kit (Amersham Biosciences; Piscataway, NJ, USA), and single-strand cDNA was synthesized using 0.7 μg of the mRNA by reverse transcription using an oligo-$d(T)_{12–18}$ primer and ReverTra Ace (TOYOBO, Osaka Japan). PCR amplification of the spinach *hsc70-1* gene was performed with an amount of cDNA corresponding to 45 ng of starting mRNA using 5′-GAGATATACATATGGCCGGTAAAGGAGAAGGTC-3′ containing *Nde*I restriction enzyme site and 5′-ATTCTCGAGGTCGACTTCCTCAATCTTGGG-3′ containing *Xho*I restriction enzyme site as specific forward and reverse primers, respectively. The PCR product was ligated into pCR-Blunt II-TOPO vector (Invitrogen). The products were sequenced to confirm that no base-pair substitution was introduced during PCR amplification. The corresponding cDNA fragments were trimmed with the two restriction enzymes *Nde*I and *Xho*I, and subcloned into the pET-20b(+) between these sites with the correct reading frame. The resulting plasmids were suitable for expression of the designed proteins with histidine-tagged sequence at their C-termini. After the plasmids were introduced into *E. coli* BL21(DE3), the bacteria were grown in LB media for