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A proteomic approach for the elucidation of the specificity of ectodomain shedding

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ABSTRACT

Ectodomain shedding (shedding) is a posttranslational modification mechanism, which liberates extracellular domains of membrane proteins through juxtamembrane processing. Because shedding alters cell characteristics in a rapid and irreversible manner, it must be strictly regulated. However, the regulatory mechanisms of shedding in response to environmental changes remain obscure. To evaluate the regulatory mechanisms of endogenous shedding, we previously developed a proteomic screening system to identify shedding targets. This system revealed a comprehensive picture of membrane proteins shed under defined conditions. In this study, we have improved the screening system to compare the shedding patterns in a mouse macrophage cell line treated with two different shedding inducers, lipopolysaccharide (LPS) and 12-O-tetradecanoylphorbol 13-acetate (TPA). We show here that LPS simultaneously activates the shedding of multiple membrane proteins. We further show that TPA specifically activates the shedding of αM/β2 integrin (Mac-1), which was not shed upon LPS-stimulation of macrophages. These results clearly demonstrate that the regulation of endogenous membrane protein shedding is both stimulus- and substrate-specific.

Biological significance
The shedding targets reported to date play pivotal roles in a variety of biological phenomena, including the immunological response, cell growth, cell adhesion and cell movement. In addition, several disease-related membrane proteins are shedding targets. Thus, understanding the regulation of shedding is important for the elucidation of pathogenesis and the development of therapeutic strategies. We submit that a comprehensive characterization of endogenous shedding is indispensable for understanding the regulatory mechanisms of shedding, and thus have developed a proteomic screening system to identify shedding targets. In this study, using our screening system, we demonstrate that different extracellular stimuli

Abbreviations: ADAM, a disintegrin and metalloprotease; LPS, lipopolysaccharide; TPA, 12-O-tetradecanoylphorbol 13-acetate; GPCR, G-protein coupled receptor; TLR, Toll-like receptor; M-CSF, macrophages colony stimulating factor; 2D-DIGE, two-dimensional difference gel electrophoresis; LBP, lipopolysaccharide-binding protein; MMP, matrix metalloproteinase; SILAC, stable isotope labeling using amino acids in cell culture.

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activate different types of shedding, even in a single cell. Our results prove that this proteomic approach is quite effective for the elucidation of the regulatory mechanisms of shedding.

1. Introduction

Ectodomain shedding, also simply called shedding, is a post-translational modification mechanism for membrane proteins. Shedding liberates the extracellular domain (ectodomain) of membrane proteins through juxtamembrane processing, which is executed mainly by A Disintegrin And Metalloproteases (ADAMs), a family of membrane-bound metalloproteases [1–4]. Shedding can solubilize growth factors and cytokines that are expressed as extracellular domains of transmembrane proteins, thus drastically expanding their effective working area. On the other hand, shedding can decrease the amount of cell surface receptors and adhesion molecules, and alter the responsiveness of cells to the corresponding ligands. Taken together, shedding is an effective mechanism for regulating in a rapid and irreversible manner not only the membrane proteins to be shed (hereafter called shedding targets) but also the functions of cells expressing shedding targets on their surface.

Since shedding is such an effective and immediate mechanism, it should be tightly regulated. However, the molecular mechanisms ensuring the specificity of shedding are unclear. First of all, because shedding targets reported to date have no consensus cleavage site sequence [1,2], it is unclear how those membrane proteins are selectively shed. Secondly, while extracellular stimuli, including lipopolysaccharide (LPS), 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and G-protein coupled receptor ligands have been reported to activate shedding [2], the intracellular signaling pathways activated by these stimuli are completely different, and molecular mechanisms linking these stimuli and the activation of shedding remain obscure. Furthermore, these stimuli are assumed to activate different types of shedding (e.g. LPS, pro-inflammatory cytokine and its receptors; TPA, almost all reported shedding targets; G-protein coupled receptor ligands, epidermal growth factor family members) through the activation of different signaling pathways [2], but this assumption is mainly based on analysis using stimulus-sensitive cells ectopically expressing a shedding target of interest. It is thus unclear whether different stimuli can activate different types of endogenous shedding machineries even in a single cell.

Based on this background, comprehensive analysis of endogenous shedding is indispensable in order to evaluate the specificity of shedding. Since unbiased proteomic screening approaches have been successful in identifying endogenous substrates of several types of proteases [5–9], we have developed a proteomic screening system for shedding targets to reveal the comprehensive picture of endogenous membrane proteins shed under defined conditions. In a previous study, we screened for shedding targets in a LPS-stimulated mouse macrophage cell line and identified vesicular integral membrane protein 36 kDa (VIP36) as a new shedding target [10]. We further showed that VIP36-activated phagocytosis of macrophages is shedding-dependent, confirming that our screening system could identify a shedding target that executed a physiological function in the cell type subjected to the screening, i.e. phagocytosis in activated macrophages.

In this study, we further improved our proteomic screening system for shedding targets and succeeded in comparing the endogenous shedding occurring in macrophages stimulated with two different shedding inducers, LPS and TPA. We show that LPS simultaneously activates the shedding of multiple membrane proteins that are weakly shed even in the absence of extracellular stimuli. We further show that TPA specifically activates the shedding of Mac-1, a bimolecular complex of integrin αM and β2 [11], as well as the membrane proteins shed in LPS-stimulated macrophages. These results clearly demonstrate that different extracellular stimuli can activate different types of endogenous shedding even in a single cell, and prove that shedding of endogenous membrane proteins is regulated in a both stimulus- and substrate-specific manner.

2. Materials and methods

2.1. Antibodies, plasmids, and chemicals

Antibodies were purchased from Santa Cruz Biotechnology (anti-M-CSF receptor, sc-692), Promega (anti-HaloTag, G921A), BioLegend (anti-CD11b/integrin αM, M1/70), and SouthernBiotech (biotin conjugate anti-CD18/integrin β2, C71/16). To construct a plasmid expressing N-terminally HaloTag-fused membrane proteins of interest, the signal sequence of IL-6 and coding sequence of HaloTag 7 (Promega) were conjugated using a PCR-based method and subcloned into pcDNA3.1/Zeo(–) (Life Technologies). The coding sequences of human Sema4D (pF1KSDBB1316, Kazusa DNA Research Institute) and mouse M-CSF receptor (M-CSFR) were subcloned into the HaloTag-expressing plasmid after removal of its own signal sequence (Met1-Ala21 and Met1-Gly19, respectively) by PCR. LPS was purchased from Sigma. TPA was purchased from Calbiochem. BB94 was provided by Vernalis.

2.2. Cell line and transfection

The Raw 264.7 line was purchased from ATCC and cultured in high glucose DMEM supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and antibiotics at 37 °C with 5% CO_2. Transfections were performed using FuGENE HD (Promega).

2.3. Sample preparations for Western blotting

Cell extracts were prepared using an extraction buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS) supplemented with a protease inhibitor mixture (Sigma) and 10 μM BB94. When conditioned culture media were prepared, cells were washed twice with serum-free medium and cultured in serum-free medium with or without 20 μM BB94, 1 μg/ml LPS, or 200 ng/ml TPA for 60 min at 37 °C with 5% CO_2. Media were centrifuged at 20,400 ×*g* for 10 min to remove cells and debris, and proteins were precipitated by