Secretion of Endoplasmic Reticulum Aminopeptidase 1 Is Involved in the Activation of Macrophages Induced by Lipopolysaccharide and Interferon-γ*

Received for publication, March 13, 2011, and in revised form, April 27, 2011 Published, JBC Papers in Press, April 29, 2011, DOI 10.1074/jbc.M111.239111

Yoshikuni Goto1,5, Kenji Ogawa1, Akira Hattori6, and Masafumi Tsujimoto5,1

From the 1Laboratory of Cellular Biochemistry, RIKEN, Wako, Saitama, 351-0198, Japan, the 5Faculty of Pharmaceutical Sciences, Teikyo-Heisei University, Ichihara, Chiba, 290-0193, Japan, and the 1Department of System Chemotherapy and Molecular Sciences, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan

Endoplasmic reticulum aminopeptidase 1 (ERAP1) is a multifunctional enzyme with an important role in processing antigenic peptides presented to class I major histocompatibility complex in the endoplasmic reticulum. In this study, we found that endoplasmic reticulum-retained ERAP1 was secreted from macrophages in response to activation by treatment with lipopolysaccharide (LPS) and interferon (IFN)-γ and enhanced their phagocytic activity. Enhancement of the phagocytic activity of murine macrophage RAW264.7 cells induced by LPS/IFN-γ was inhibited by a potent aminopeptidase inhibitor, amastatin. The addition of recombinant wild-type but not inactive mutant ERAP1 to culture medium enhanced phagocytosis. These results suggest that enhancement of phagocytic activity is at least in part mediated by secreted ERAP1 through the generation of active peptides processed by the enzyme. Our data reveal ERAP1-mediated activation of macrophages for the first time and will provide new insights into the role of this enzyme in innate immunity.

It is well known that endoplasmic reticulum aminopeptidase 1 (ERAP1) is a multifunctional enzyme belonging to the M1 family of aminopeptidases with roles in the regulation of blood pressure, angiogenesis, ectodomain shedding of several cytokine receptors, and processing of antigenic peptides presented to MHC class I molecules (1–4). Its cDNA was initially cloned as adipocyte-derived leucine aminopeptidase (5). Based on its multifunctional properties, adipocyte-derived leucine aminopeptidase is also designated ERAP1, ERAAP (endoplasmic reticulum aminopeptidase), and ARTS-1 (aminopeptidase regulator of TNFR1 shedding), PILSAP (puromycin-insensitive leucine-specific aminopeptidase), and ARTS-1 (aminopeptidase regulator of TNFR1 shedding) (6–9) (in this paper, ERAP1 is used hereafter). Although it is evident that ERAP1 plays important roles in several pathophysiological processes, its subcellular localization is still under debate. Although several reports have presented evidence showing its localization in the ER (6, 7) or cytoplasm (10) as a soluble protein, others have shown it on the cell surface as a type II membrane-spanning protein (9).

ERAP1 is a monomeric zinc-metallopeptidase that shows a preference for leucine when measured by synthetic substrates (5). On the other hand, it shows relatively broad substrate specificity toward natural peptide hormones, such as angiotensin II, kallidin, and neurokinin A, which may reflect its role in the processing of various precursors of antigenic peptides presented to MHC class I molecules (11). On the basis of a preference for substrates of a specific length and C-terminal hydrophobic amino acid, the “molecular ruler” mechanism was proposed for the processing of antigenic peptides by the enzyme (12).

Because ERAP1 inactivates angiotensin II and converts kallidin to bradykinin, it was initially speculated that it might regulate blood pressure (11). Subsequently, by screening for polymorphisms in the human ERAP1 gene, Yamamoto et al. (13) identified an association of K528R variant enzyme with essential hypertension. Indeed, K528R mutant ERAP1 had less enzymatic activity than the wild type (14); therefore, it is conceivable that although ERAP1 is usually retained in the ER,2 the enzyme is secreted into the extracellular milieu in response to specific stimuli and in turn regulates blood pressure by processing peptide substrates in blood vessels. It was shown that the enzyme was secreted when overexpressed in COS-7 cells, also making it plausible that ERAP1 is secreted under particular conditions (5).

Although it is well established that ERAP1 in the ER is involved in antigenic peptide processing, the role of secreted ERAP1 has not been elucidated. Here, we demonstrated that enzymatically active ERAP1 is secreted from RAW264.7 cells in response to LPS/IFN-γ-induced macrophage activation. In addition, ERAP1 secreted from activated macrophages is involved in the enhancement of their Fc receptor-dependent but not opsonin-independent phagocytic activity. The data presented in this study will provide new insights into the significance of ERAP1 in the innate and acquired immune system.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant ERAP1—Sf9 insect cells were transfected with bacmid DNA using Cellfectin® reagent (Invitrogen) and after a 72-h incubation, recombinant baculoviruses were harvested. For the expression of

© 2011 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

21906 JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 24, pp. 21906 –21914, June 17, 2011

2 The abbreviations used are: ER, endoplasmic reticulum; MCA, 4-methylcoumaryl-7-amide; BFA, brefeldin A; P-LAP, placental leucine aminopeptidase; APN, aminopeptidase N; APQ, aminopeptidase Q.

1 To whom correspondence should be addressed: Faculty of Pharmaceutical Sciences, Teikyo-Heisei University, 4-1 Uruido-minami, Ichihara, Chiba, 290-0193, Japan. E-mail: tsujimoto@thu.ac.jp.

1 This work was supported in part by grants-in-aid from the Ministry of Education, Science, Sports and Culture of Japan and a grant from Suzuken Memorial Foundation.
recombinant ERAP1, SF9 cells (2.0 × 10⁶ cells/ml) infected with the recombinant baculovirus (multiplicity of infection 1–3) were cultured for 72 h in 100 ml of SF-900 III medium (Invitrogen) at 27 °C.

Culture medium containing recombinant ERAP1 was collected by centrifugation and loaded onto a hydroxyapatite column (bed volume 10 ml) pre-equilibrated with 5 mM phosphate buffer (pH 7.5) and eluted with 100 mM phosphate buffer (pH 7.5). The eluate was applied to a Co²⁺-chelating Sepharose column (bed volume 1 ml) (GE Healthcare) pre-equilibrated with 10 mM phosphate buffer containing 0.1 M NaCl and then eluted with 150 mM imidazole. The ERAP-1-containing fractions were extensively dialyzed against 25 mM Tris/HCl buffer (pH 7.5), containing 0.125 M NaCl, concentrated with an ultrafiltration membrane, and stored at −20 °C prior to use. After purification, ERAP1 gave a single band with a molecular mass of ~105 kDa on SDS-PAGE.

Measurement of Leucine Aminopeptidase Activity—Leucine aminopeptidase activity was determined with leucine-4-methylcoumaryl-7-amides (Leu-MCA). The reaction mixture contained 100 μM Leu-MCA and 50 μl of culture medium in 100 μl of 25 mM Tris/HCl buffer (pH 7.5) was incubated at 37 °C for 2 h. The amount of 7-amino-4-methylcoumarin released was measured by a multimicroplate reader MTP-810 Lab (Hitachi High Tech, Tokyo, Japan) at an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

Deglycosylation of ERAP1—Culture medium and cell lysate containing ERAP1 were denatured with 1 × Glycoprotein Denaturing Buffer (New England Biolabs, Ipswich, MA) at 100 °C for 10 min and then incubated with either peptide:N-glycosidase F (30 μg/ml; New England Biolabs) or endoglycosidase H (24 μg/ml; New England Biolabs) in G7 Reaction Buffer (New England Biolabs) containing 1% Nonidet P-40 or G5 Reaction Buffer (New England Biolabs) for 1 h at 37 °C.

Immunofluorescence Staining—RAW264.7 cells (5 × 10⁵ cells/ml) were plated on coverslips in a 24-well dish and treated with LPS/IFN-γ or ERAP1. Twenty-four hours after stimulation, cells were washed once with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. After washing with PBS, cells were permeabilized with 0.1% Nonidet P-40 in PBS for 20 min. The coverslips were blocked with 0.1% skim milk for 30 min at room temperature and incubated with biotinylated anti-ERAP1 antibody and either anti-EAA1 antibody (BD Biosciences) or anti-KDEL antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in PBS containing 0.1% skim milk for another 1 h. ERAP1 was detected by incubation with Dylight® 488 STREPTAVIDIN (Vector Laboratories, Burlingame, CA) for 30 min in the dark. EAA1 and KDEL were counterstained with Alexa 594-labeled anti-murine second antibody for 30 min in PBS containing 0.1% skim milk. After unbound dyes were washed off with PBS, coverslips were mounted with Thermo Scientific Shandon PermaFluor (Thermo Fisher Scientific, St. Waltham, MA) on a glass slide. Images were captured by an ECLIPS 80i microscope (Nikon, Tokyo, Japan) with ×40 objective, using 488- and 594-nm lasers as excitation light sources.

Measurement of Phagocytosis—Phagocytosis was assessed by measuring the amount of uptake of latex beads coated with FITC-labeled rabbit IgG into cells using a phagocytosis assay kit (FITC) (Cayman Chemical, Ann Arbor, MI) according to the instruction manual. In brief, cells with or without various stimulants were treated with the beads and cultured at 37 °C. The uptake of the beads into cells was captured by a microscope and quantified by measuring fluorescence intensity using Scion Image software (Scion Corp., Frederick, MD).

Filtration of the Conditioned Medium of RAW264.7 Cells—RAW264.7 cells were initially incubated in the absence or presence of 100 ng/ml ERAP1 for 24 h at 37 °C. After incubation, conditioned media were collected, filtrated through Amicon Ultra-4 10 kDa (Millipore, Billerica, MA) to remove residual ERAP1, and transferred to an equal volume of new culture. Fresh RAW264.7 cells in either unfiltrated or filtrated medium were then incubated for 24 h at 37 °C, and their phagocytic activities were measured.

Cell Culture—Murine macrophage cell line RAW264.7 cells were obtained from the ATCC (Manassas, VA). Thioglycollate medium-elicited peritoneal macrophages were obtained by lavage of the peritoneal cavity of mice injected 4 days previously with 2 ml of sterile 3% brewer thioglycolate broth (Difco). The cells were suspended at 1.5 × 10⁶ cells/ml in RPMI 1640 medium with heat-inactivated (56 °C, 30 min) FBS. The cells were seeded in 24-well plates at 2 ml/well and allowed to adhere to tissue culture plates for 2 h at 37 °C before gentle rinsing to remove non-adherent cells. Adherent cells were collected and used as murine peritoneal macrophages.

Murine peritoneal macrophages and RAW264.7 cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum. For assays, cells were transferred to and cultured in 24-well cell culture dishes (5 × 10⁵ cells/well) and activated with IFN-γ (100 IU/ml) and LPS (1 μg/ml) at 37 °C in RPMI 1640 without FBS.

Statistical Analysis—Data are presented as the mean ± S.D. Groups were compared by Student’s t test.

Materials—RPMI 1640 and BFA was obtained from Nacalai Tesque (Kyoto, Japan). Amastatin and Leu-MCA were purchased from the Peptide Institute (Osaka, Japan). Cytokines were from PeproTech (Rocky Hill, NJ). LPS from Escherichia coli 055:B5 was obtained from Sigma-Aldrich. Dexamethasone was purchased from Wako Pure Chemical Industries (Osaka, Japan).

RESULTS

Detection of ERAP1 Secretion from RAW264.7 Cells—Although ERAP1 has no distinct ER retention signal in the molecule, it is retained in the ER and acts as a final processing enzyme of antigenic peptides presented to MHC class I molecules (15). On the other hand, it has been speculated that the enzyme is a regulator of blood pressure (13); therefore, we hypothesized that the enzyme might be secreted into the extracellular milieu by particular stimuli; thus, we examined whether ERAP1 is secreted from murine macrophage cell line RAW264.7 cells in response to macrophage activation stimuli (Fig. 1). As shown in Fig. 1A, co-stimulation of RAW264.7 cells with LPS and IFN-γ induced the secretion of ERAP1. Treatment with either LPS or IFN-γ alone had little effect on secre-
Enhancement of Phagocytosis by Secreted ERAP1

**A**

| Lane No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|----------|---|---|---|---|---|---|---|---|---|
| 114 kDa  |   |   |   |   |   |   |   |   |   |
| 105 kDa  |   |   |   |   |   |   |   |   |   |

**B**

| IFN-γ (IU/ml) | 0 | 10 | 100 | 1000 |
|---------------|---|----|-----|------|
| LPS (μg/ml)   |   | 0.1 | 1 | 10 |

**C**

| Medium | 100 IU/ml IFN-γ | 1 μg/ml LPS |
|--------|----------------|-------------|
| Time (h) | 0 | 12 | 24 | 48 | 12 | 24 | 48 |
| ERAP1   | 114 kDa | 105 kDa | 114 kDa | 105 kDa |
| Cell    | 114 kDa | 105 kDa | 114 kDa | 105 kDa |

**D**

| IFN-γ/LPS | PNase F | Endo H |
|-----------|---------|--------|
| Medium    | 114 kDa | 97 kDa |
| Cell      | 114 kDa | 97 kDa |

FIGURE 1. Secretion of ERAP1 from RAW264.7 cells treated with LPS/IFN-γ. A, detection of stimulus-dependent secretion of ERAP1. RAW264.7 cells were treated with or without various macrophage stimulators at 37 °C for 24 h. Conditioned media were then collected, and secreted ERAP1 was detected by Western blot analysis. Lane 1, not treated; lane 2, IFN-γ (100 IU/ml); lane 3, LPS (1 μg/ml); lane 4, IFN-γ (100 IU/ml)/LPS (1 μg/ml); lane 5, TNF-α (100 IU/ml); lane 6, TNF-α (100 IU/ml)/IFN-γ (100 IU/ml); lane 7, TNF-α (100 IU/ml)/LPS (1 μg/ml); lane 8, IL-4 (100 IU/ml); lane 9, IL-4 (100 IU/ml)/dexamethasone (5 μM). B, dose-dependent study of LPS/IFN-γ-induced ERAP1 secretion. RAW264.7 cells were incubated with various concentrations of LPS and/or IFN-γ. After a 24-h incubation at 37 °C, conditioned media were collected, and secreted ERAP1 was detected by Western blot analysis. C, time course study of LPS/IFN-γ-induced ERAP1 secretion. RAW264.7 cells were incubated with LPS (1 μg/ml) and/or IFN-γ (100 IU/ml). After incubation for the indicated times, media were collected by centrifugation, and the cell-associated form of the enzyme was extracted from the cells by treatment with 0.1% Nonidet P-40. Secreted and cell-associated ERAP1s were detected by Western blot analysis.

tion. Other stimuli so far tested, including TNF/LPS and IL-4/dexamethasone, also had little effect.

RAW264.7 cells were then incubated with various concentrations of LPS/IFN-γ for 24 h to induce ERAP1 secretion. As shown in Fig. 1B, maximal secretion was achieved at concentrations of 100 IU/ml IFN-γ and 10 μg/ml LPS; however, we employed concentrations of 100 IU/ml and 1 μg/ml, respectively, for further analyses because a higher concentration of stimulators (especially LPS) caused apparent cell death.

Time course experiments showed that secretion of ERAP1 was observed within 24 h (Fig. 1C). Western blot analysis indicated that ERAP1 was secreted as a 114-kDa protein. Longer treatment (48 h) caused the appearance of another 105-kDa band in LPS/IFN-γ-treated cells, and treatment with either IFN-γ or LPS alone also caused the appearance of the band. Because longer incubation increased the population of dead cells, the low molecular weight band was attributable to ER-retained enzyme released from dead cells (see below). Taken together, these results indicate that ERAP1 is secreted in response to macrophage activation by LPS/IFN-γ.

Fig. 1C also shows that treatment with IFN-γ enhanced the intracellular 105-kDa ERAP1 protein band, indicating that the cytokine enhanced the expression of the enzyme without inducing its secretion (6, 7). On the other hand, in LPS/IFN-γ-treated cells, secretion of ERAP1 was observed in the absence of enhanced intracellular accumulation of the enzyme, suggesting that a significant portion of ERAP1 induced by LPS/IFN-γ was secreted into the medium.

**Characterization of Secreted ERAP1—**Next we characterized the secreted ERAP1. To estimate the contribution of ERAP1 to Leu-MCA-degrading activity in the medium, ERAP1 was immunoprecipitated with anti-ERAP1 antibody after treatment with LPS/IFN-γ (Fig. 2A). In control cells, little activity was immunoprecipitated with anti-ERAP1 antibody. Treatment with LPS/IFN-γ caused an ~1.5-fold increase in Leu-MCA-degrading activity in the medium. When immunoprecipitated with the antibody, it was apparent that almost all Leu-MCA-degrading activity enhanced by LPS/IFN-γ was attributable to ERAP1. We also measured cell surface aminopeptidase activity and found that cell surface Leu-MCA-degrading activity was unchanged after treatment with LPS/IFN-γ (data not shown).

In Western blot analysis, ERAP1 was present in the medium as a high molecular mass (114-kDa) form, whereas the cell-associated form showed a low molecular mass of 105 kDa (Fig. 2B). As mentioned above, both forms were occasionally present in the medium, which was apparent especially after a 48-h incubation.

To examine whether ERAP1 is secreted properly, conditioned medium containing both forms was treated with glycosidases. As shown in Fig. 2C, the high molecular mass 114-kDa
form was sensitive to peptide:N-glycosidase F but not to endoglycosidase H and shifted to 97 kDa, indicating that it contains complex type N-linked oligosaccharides. On the other hand, secreted and cell-associated 105-kDa forms were detected as glycoprotein containing high mannose type N-linked oligosaccharides because they were sensitive to both glycosidases. Because the sensitivity of cell-associated ERAP1 to glycosidases is consistent with its retention in the ER, it is most likely that the 105-kDa low molecular mass form in the medium was released from dead cells treated with LPS/IFN-γ.

We then examined the effect of BFA on ERAP1 secretion. As shown in Fig. 2D, BFA treatment caused a marked decrease in LPS/IFN-γ-induced secretion, further suggesting the proper ER to Golgi transport of the enzyme. Overall, the results shown in Fig. 2 suggest that almost all of the aminopeptidase activity of RAW264.7 cells released after treatment with LPS/IFN-γ is attributable to ERAP1, which is secreted properly from ER to the extracellular milieu through the Golgi apparatus and secretory pathway.

ERAP1 Enhances Phagocytosis of RAW264.7 Cells—To elucidate the pathophysiological relevance of ERAP1 secretion, we next measured the phagocytic activity of RAW264.7 cells. Under the microscope, cell-associated FITC-labeled IgG-coated latex beads were monitored as an index of macrophage activation. Total fluorescence intensity of FITC-labeled beads taken into the cells was quantified and shown as phagocytic activity. As shown in Fig. 3A, control cells had moderate activity to take up the beads. Although IFN-γ had little activity to enhance the uptake, a substantial increase (about 1.5-fold) was observed when cells were treated with LPS. Treatment with LPS/IFN-γ caused a further increase in the uptake (about 2-fold). These results suggest that LPS is primarily responsible for enhancement of the uptake of IgG-coated latex beads, and IFN-γ can act as its co-enhancer.

We then measured the LPS/IFN-γ-mediated enhancement of FITC-labeled IgG-coated latex bead uptake in the presence or absence of amastatin, a potent aminopeptidase inhibitor. As shown in Fig. 3B, amastatin suppressed the LPS/IFN-γ-mediated enhancement of IgG-coated latex bead uptake in a dose-dependent manner. In contrast, basal activity was largely unaffected in the same concentration range. These results strongly suggest that a significant portion of enhanced but not basal phagocytosis was mediated by aminopeptidases.

To examine whether ERAP1 is involved in LPS/IFN-γ-mediated uptake of IgG-coated latex beads, RAW264.7 cells were incubated with recombinant ERAP1. As shown in Fig. 4A, the direct addition of ERAP1 to the medium enhanced the uptake. Consistent with the data shown in Fig. 3B, amastatin inhibited ERAP1-mediated enhancement of the uptake in a dose-dependent manner. In contrast, when FITC-conjugated E. coli particles were employed as a target of phagocytosis, little change was observed in response to the activation by ERAP1 (data not shown). These results strongly suggest that ERAP1 alone can enhance the Fc receptor-dependent but not opsonin-independent phagocytic activity of RAW264.7 cells.

We then examined whether the enzymatic activity of ERAP1 was required for enhancement of the phagocytic activity of RAW264.7 cells (Fig. 4B). Although wild-type enzyme caused a 1.4-fold increase in the activity, enhancement was marginal when RAW264.7 cells were treated with enzymatically inactive E354Q ERAP1, the Zn2+ binding site of which was disrupted. In addition, Q181D ERAP1, which showed different substrate specificity from the wild-type enzyme and cleaved basic amino acids preferentially, also had only a marginal effect (16); thus, the enzymatic activity of wild-type ERAP1 is required for the enhancement of phagocytosis. We then examined the effects of other M1 aminopeptidases, such as placental leucine aminopeptidase (P-LAP)/insulin-regulated aminopeptidase/oxytocinase, laeverin/aminopeptidase Q (APQ), and aminopeptidase A (APA), on the uptake of IgG-coated latex beads (17–19). Because these three enzymes are membrane-bound proteins, we prepared recombinant soluble forms for this purpose. We found that P-LAP and laeverin/APQ but not APA enhanced the uptake, indicating that several M1 aminopeptidases other than ERAP1 also have phagocytosis-enhancing activity. Because P-LAP and laeverin/APQ as well as ERAP1 are the leucine aminopeptidases cleaving the leucine of the synthetic substrate most efficiently, we speculate that leucine (and other hydro-
phobic amino acid)-cleaving activity is important for the enhancement of phagocytosis.

Because both IFN-γ and LPS were required for maximal phagocytic activity, we next examined the interrelationship between ERAP1 activity and these two stimulants. As shown in Fig. 4C, IFN-γ had little effect on basal and ERAP1-mediated enhancement of phagocytic activities. On the other hand, LPS-mediated phagocytosis was further enhanced by ERAP1 to nearly the maximum level. In LPS/IFN-γ-treated cells, exogenously added ERAP1 had no enhancing activity, suggesting that in the presence of secreted ERAP1, exogenously added enzyme had no enhancing effect on phagocytosis. Thus, it is conceivable that the difference in the activation level between LPS- and LPS/IFN-γ-treated cells is attributable to secreted ERAP1. These results imply that there are two phagocytosis-enhancing modes: ERAP1-dependent and -independent. Both LPS- and IFN-γ-derived signals are required for the secretion of ERAP1.

Time course experiments showed that LPS/IFN-γ-mediated enhancement of IgG-coated latex bead uptake increased up to 24 h (Fig. 4D). In contrast, ERAP1-mediated enhancement of uptake reached its maximal level within 12 h. Considering that LPS/IFN-γ-mediated ERAP1 secretion could not be detected within 12 h, it is conceivable that at least two macrophage activation phases are induced by LPS/IFN-γ. The early phase is mediated by LPS in an ERAP1-independent manner, whereas the late phase is mediated by secreted ERAP1. The inactive E354Q mutant ERAP1 had no enhancing activity, confirming that enzymatic activity is required for the enhancement of phagocytosis. Taken together, these results indicate that ERAP1 secreted by LPS/IFN-γ treatment plays an important role in the enhancement of phagocytosis.

**Possible Generation of Phagocytosis-enhancing Peptides by ERAP** —To further examine the significance of the enzymatic activity, RAW264.7 cells were treated with ERAP1, and conditioned medium was divided into two fractions; one was filtrated to remove ERAP1, and the other was not. Fresh cells were then incubated either in filtrated or unfiltrated medium. ERAP1 was removed from conditioned medium of RAW264.7 cells efficiently by filtration (Fig. 5A), and little Leu-MCA-degrading activity attributable to the ERAP1 added to the medium remained (Fig. 5B). However, RAW264.7 cells cultured in filtrated medium still revealed enhanced phagocytosis to the same level as the cells in the medium without filtration (Fig. 5C), indicating that even after filtration, conditioned medium contained active substance(s), which had phagocytosis-enhancing activity. These results thus imply that filtrated medium contained active substance(s), which had phagocytosis-enhancing activity.
obtained after treatment of the cells with ERAP1 contains active small peptide(s) processed by the enzyme, which is responsible for the enhancement of phagocytosis. Thus, it is quite plausible that the enzymatic activity of wild-type ERAP1 is required for the generation of specific peptide(s) with phagocytosis-enhancing activity.

**Immunocytochemical Analyses of ERAP1 in RAW264.7 Cells**—

Next, we examined the subcellular localization of ERAP1 treated with either ERAP1 or LPS/IFN-γ. As shown in Fig. 6A, co-localization of ERAP1 with the ER retention signal sequence KDEL was obvious in control and ERAP1-treated cells (6, 7). In contrast, when treated with LPS/IFN-γ, RAW264.7 cells swelled, and dispersed distribution patterns of both ERAP1 and KDEL sequences were observed. In addition, their co-localization was not seen clearly, which may reflect that a significant portion of ERAP1 exited from the lumenal side of the ER and was secreted into culture medium during incubation with LPS/IFN-γ.

Given that ERAP1 enhances the phagocytic activity of RAW264.7 cells, we next examined the effects of ERAP1 and LPS/IFN-γ on the content of the endosomal fraction using EEA1 as an early endosome marker. Immunocytochemical analyses shown in Fig. 6B revealed that in control and ERAP1-treated cells, the distribution of EEA1 showed a punctate pattern, reflecting its localization in endosomes. When merged with ERAP1, co-localization of EEA1 and ERAP1 was marginal. On the other hand, activation of RAW264.7 cells with LPS/IFN-γ showed dispersed distribution of both EEA1 and ERAP1 in the cytoplasm. In addition, when merged, co-localization of EEA1 and ERAP1 is not, if present, obvious in these cells. These results imply that although ERAP1 enhances the phagocytic activity of RAW264.7 cells, the enzyme itself is not taken up into the cells via endocytosis.

When measuring the fluorescence intensity of EEA1, we found that ERAP1 and LPS/IFN-γ enhanced its intensity by about 3- and 5-fold, respectively, suggesting that these stimulants increased the content of EEA1-positive early endosomal fractions in the cytoplasm (Fig. 6C). It is noteworthy that although LPS/IFN-γ treatment was associated with the morphological change of RAW264.7 cells, no apparent change was observed when the cells were treated with ERAP1 alone, sug-
Enhancement of Phagocytosis by Secreted ERAP1

**Figure 7.** Secretion of ERAP1 from thioglycollate-elicited murine peritoneal macrophages in response to LPS/IFN-γ. A, secretion of ERAP1. Murine peritoneal macrophages were treated with LPS (1 μg/ml) and/or IFN-γ (100 IU/ml) for the indicated times at 37 °C. Secrated ERAP1 was then detected by Western blot analysis. B, ERAP1-induced enhancement of phagocytosis. Murine peritoneal macrophages treated with ERAP1 (100 ng/ml) or LPS (1 μg/ml) and/or IFN-γ (100 IU/ml) were incubated for 24 h at 37 °C. After incubation, uptake of latex beads coated with FITC-labeled rabbit IgG was measured as described under “Experimental Procedures.” As a control, the cells with vehicle treatment were also processed. Data are expressed as the mean ± S.D. (error bars) (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**DISCUSSION**

In this paper, we found that ERAP1, which was retained in the ER and acted as the final processing enzyme of antigenic peptides presented to MHC class I molecules, was secreted from macrophages in response to LPS/IFN-γ treatment and enhanced their phagocytic activity, strongly suggesting that secreted ERAP1 is involved in the activation of macrophages induced by LPS/IFN-γ. It was reported using aminopeptidase inhibitors that aminopeptidases might be involved in the activation of macrophages; thus, our data confirmed the previous observation and identified the responsible enzyme for the first time (20, 21).

Although ERAP1 had no obvious ER retention signals, it was retained in the ER of RAW264.7 cells and secreted in response to LPS/IFN-γ, indicating that inflammatory stimuli can induce secretion of the enzyme. During secretion, sugar chains attached to the enzyme changed from the high mannose type to the complex type, confirming that the enzyme was secreted properly. Sensitivity to BFA supported this notion. Because overexpression of ERAP1 in COS-7 cells caused secretion of the enzyme, it is tempting to speculate that some ERAP1-binding proteins may act as ER retention machinery for the enzyme, and saturation or disruption of this putative machinery may cause secretion (1, 4). Several reports have described the binding proteins of the enzyme, which are localized either in the plasma membrane or cytoplasm (9, 22–25). In our preliminary data, we identified a region required for ER retention of the ERAP1 molecule by constructing chimeric proteins. To elucidate the ER retention mechanism of ERAP1, we searched for proteins that bind to this region and identified a binding protein with an ER retention signal at its C-terminal end. We are now characterizing this protein in detail. Considering various proteins that bind to the enzyme with pathophysiological relevance (1), it is conceivable that, depending on its subcellular localization, ERAP1 may change binding partners and thus exert its multifunctional activities.

The treatment of RAW264.7 cells with LPS/IFN-γ caused an increase in aminopeptidase activity in culture medium. Most of the enhanced activity in the medium was attributable to secreted ERAP1. We tested whether LPS/IFN-γ may enhance cell surface aminopeptidase activities and found no significant difference between control and LPS/IFN-γ-treated cells, implying that enhancement of phagocytosis mediated by LPS/IFN-γ-enhanced aminopeptidase activity was attributable almost exclusively to secreted ERAP1.

To elucidate the pathophysiological relevance of secreted ERAP1, we analyzed its involvement in LPS/IFN-γ-mediated enhancement of phagocytosis of RAW264.7 cells and thioglycollate-elicited murine peritoneal macrophages. Measuring IgG-coated latex bead uptake, we demonstrated that secreted ERAP1 enhanced the phagocytosis of macrophages. Although several aminopeptidases other than ERAP1 (e.g., P-LAP and laeversin/APQ) could enhance phagocytosis, our data suggested ERAP1 as a major player in LPS/IFN-γ-mediated macrophage activation. Basal activity seen in control cells might have little

3 A. Hattori and M. Tsujimoto, unpublished observation.
relationship with aminopeptidase activity because it was rather resistant to amastatin, whereas LPS/IFN-γ-enhanced activity was suppressed in the same concentration range.

The phagocytic activity of RAW264.7 cells treated with ERAP1 did not reach the maximal level of the cells treated with LPS/IFN-γ, suggesting that macrophage activation phases can be divided into ERAP1-dependent and -independent phases. Time course studies (Fig. 4D) showed that whereas the activation level of ERAP1-treated cells peaked within 12 h, that of LPS/IFN-γ-treated cells increased gradually up to 24 h, suggesting that lag time was required for the maximal activation of LPS/IFN-γ-treated cells until ERAP1 was secreted. It is plausible that while LPS mediates early-phase activation in an ERAP1-independent manner, it induces ERAP1 secretion together with IFN-γ and mediates the late phase. Because amastatin inhibited the early phase response near the basal level, it is possible that membrane-bound aminopeptidases, such as P-LAP, APN, and laeverin/APQ, may contribute to the response; however, little difference was observed between aminopeptidase activities of membrane fractions prepared from control and LPS/IFN-γ-treated cells. Indeed, we found that whereas P-LAP was expressed equally in control and LPS/IFN-γ-treated RAW264.7 cells, expressions of APN and laeverin/APQ were undetectable. In this context, stimulus-dependent translocation of P-LAP is interesting. Because P-LAP was shown to be translocated from intracellular vesicles to the plasma membrane by peptide substrates, such as oxytocin and vasopressin, to enhance cell surface aminopeptidase activity (26–27), it can be speculated that P-LAP is translocated during the early stimulation phase. Alternatively, it is also possible that substances other than aminopeptidase mediate the response induced by LPS/IFN-γ. Further work is required.

Enzymatic activity was essential for the ERAP1-mediated enhancement of macrophage phagocytosis. The mutant enzyme with different substrate specificity had little effect. Because other leucine aminopeptidases also exerted phagocytosis-enhancing activity, it is reasonable to postulate a common peptide substrate(s) responsible for the enhancement. Our data shown in Fig. 5 also suggest that particular unidentified peptide substrates are processed by secreted ERAP1 and enhanced phagocytic activity, as revealed by the enhancement of EEA1 fluorescence intensity. It has been reported that several peptides, such as tuftsin, neuropeptide Y, and neuropeptide S, serve to enhance the phagocytic activity of macrophages (28–30). It is interesting to identify the putative substrates that may enhance the endosomal function via enhancement of EEA1 expression.

Growing evidence indicates that single proteins can have multiple functions depending on cellular localization, particularly proteins with intracellular and extracellular localizations (31). Fig. 8 shows the possible scheme of ERAP1 secretion from macrophages in response to LPS/IFN-γ based on this study. Signals delivered from LPS receptor (TLR4) together with that of IFN-γ receptor (IFNGR) might disrupt ERAP1 retention machinery in the ER and thus allow its secretion into the extracellular milieu. This secreted enzyme cleaves a putative peptide substrate that is responsible for the enhancement of Fc receptor-dependent phagocytosis by increasing the endosomal fraction. ERAP1 might play an important role in the inactivation of opsonized pathogens through their degradation in the lysosomes. Thus, ERAP1 might be a so-called moonlighting protein acting as a final processing enzyme of the precursors of MHC class I-presented antigenic peptides in the ER and a macrophage activation factor in the extracellular milieu (32). Despite the enhancement of phagocytosis, no apparent morphological change was detected after treatment of ERAP1, which was associated with that of LPS/IFN-γ, implying that ERAP1-mediated processes overlapped partially with LPS/IFN-γ-mediated full macrophage activation processes.

As for the mechanism of ERAP1 secretion, our initial characterization revealed that although a calcium ionophore, A23187, induced secretion, a calcium chelator, BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester), suppressed LPS/IFN-γ-induced ERAP1 secretion, implying that calcium mobilization in the cytosol is important for secretion of the enzyme. Detailed analyses of calcium-mediated ERAP1 secretion mechanism are now in progress.

Our data provide evidence that ERAP1 plays important roles in the innate immune system as well as in the acquired immune system. In a recent work (33), a significant association of hemo-lytic uremic syndrome, which is caused typically by gastrointestinal infection with E. coli species, with ERAP1 polymorphisms was reported, also suggesting the involvement of the enzyme in LPS-mediated innate immunity. Further work is required to elucidate the role of ERAP1 in diseases associated with its polymorphisms, which include hypertension (13), cervical cancer (34, 35), osteoporosis (36), and ankylosing spondylitis (37–41) with reference to macrophage function. It is also interesting to elucidate the dynamic aspects and pathophysiological function of ERAP2, another enzyme retained in the ER that trims antigenic peptides presented to MHC class I molecules (42–44).

REFERENCES

1. Tsujimoto, M., and Hattori, A. (2005) Biochem. Biophys. Acta 1751, 9–18
2. Tsujimoto, M., Goto, Y., Maruyama, M., and Hattori, A. (2008) Heart Fail. Rev. 13, 285–291

---

4 Y. Goto and M. Tsujimoto, unpublished observation.
