Recognition of Sphingomyelin by Lysenin and Lysenin-Related Proteins†

Etsuko Kiyokawa,‡,§ Asami Makino,‡ Kumiko Ishii,‡ Naomi Otsuka,‡ Akiko Yamaji-Hasegawa,‡ and Toshihide Kobayashi*,‡,⊥

RIKEN, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan, Precursory Research for Embryonic Science and Technology (PRESTO), JST, Japan, and UMR585, INSERM, Institut National des Sciences Appliquees (INSA)-Lyon, 20 Ave A. Einstein, 69621 Villeurbanne, France

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ABSTRACT: Lysenin is a sphingomyelin (SM)-specific toxin isolated from the coelomic fluid of the earthworm *Eisenia foetida*. Lysenin comprises a family of proteins together with lysenin-related protein 1 (LRP-1, lysenin 2) and LRP-2 (lysenin 3). In the present study, we characterized LRP-1 and LRP-2 together with lysenin using maltose-binding-protein-tagged recombinant proteins. LRP-2 specifically bound SM and induced hemolysis like lysenin. In contrast the binding and hemolytic activities of LRP-1 were 10 times less than those of lysenin and LRP-2. Lysenin and LRP-2 share 30 common sites of aromatic amino acids. Among them, only one position, phenylalanine 210, is substituted for isoleucine in LRP-1. The activity of LRP-1 was dramatically increased by introducing a single amino acid substitution of isoleucine 210 to phenylalanine, suggesting the importance of this aromatic amino acid in biological activities of lysenin and LRPs. The importance of aromatic amino acids was further indicated by a systematic tryptophan to alanine mutation of lysenin. Lysenin contains six tryptophan residues of which five are conserved in LRP-1 and -2. We showed that the conserved tryptophans but not the nonconserved one were required both in the recognition of SM and in the hemolytic activity of lysenin. Our results suggest the importance of tryptophan in the toxin function likely due to a direct recognition of SM or in maintaining the protein structure.

Lysenin is a novel 297 amino acid protein isolated from the coelomic fluid of the earthworm *Eisenia foetida* (*1*). Originally isolated as a protein that causes contraction of rat vascular smooth muscles, lysenin induces hemolysis and has cytotoxicity to vertebrate spermatozoa as well as cultured mammalian cells (*2*−*5*). Whereas the coelomic fluid of *E. foetida* contains a number of cytotoxic proteins (*6*, *7*), lysenin is unique in that it specifically recognizes sphingomyelin (SM)[1] (*2*, *8*). SM attracts much attention as a reservoir for lipid-derived second messengers (*9*), as well as being a major component of lipid rafts, which are membrane microdomains enriched with cholesterol and sphingolipids (*10*−*12*). It is suggested that the lipid raft provides a platform for the assembly of a number of signaling molecules and thus plays important roles in various cellular functions (*13*, *14*). Specific recognition of SM made use of this toxin to examine cellular distribution of SM (*2*, *15*−*17*), as well as to study the biosynthesis and transport of SM (*3*, *18*).

Together with two additional proteins in coelomic fluid, referred to as lysenin related protein 1 (LRP-1, lysenin 2) and LRP-2 (lysenin 3), lysenin comprises a family of proteins sharing sequences of high homology, which are completely different from other proteins available in protein databanks (*1*, *7*, *8*). Amino acids coded by LRP-1 cDNA are 76% identical to those of lysenin cDNA (89% for LRP-2 cDNA). The cDNA sequence of LRP-2 is identical to that of fetidin (*19*, *7*, *8*). It is not known whether LRPs/fetidin recognize SM. In the present study, we prepared maltose-binding-protein (MBP)-tagged recombinant proteins of LRPs and compared their lipid specificity and hemolytic activity to those of recombinant lysenin. Our results indicate that LRP-2/fetidin binds SM, just like lysenin. LRP-2 was also hemolytic. In contrast, LRP-1 showed 10 times lower affinity for SM and low hemolytic activity. Lysenin and LRP-2 share 30 common sites of aromatic amino acids. Among them, only one position, phenylalanine 210 is substituted by an isoleucine in LRP-1. The activity of LRP-1 was brought to levels similar to those observed in lysenin by the introduction of a single amino acid substitution of the isoleucine 210 by a phenylalanine. Lysenin contains six tryptophan residues of which five are conserved in LRP1 and 2. We showed that the conserved tryptophans but not the nonconserved one were important both in the recognition of SM and in the hemolytic activity of lysenin. These results indicate that aromatic amino acids are important in the biological activity of lysenin and LRPs.

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* To whom correspondence should be addressed. Tel: +81-48-467-9612. Fax: +81-48-467-8693. E-mail: kobayasi@riken.jp.

‡ RIKEN.

§ JST.

⊥ INSERM.

[1] Abbreviations: SM, sphingomyelin; LRP, lysenin-related protein; MBP, maltose-binding protein; PBS, phosphate-buffered saline; PC, phosphatidylcholine; NPA, Niemann−Pick type A.

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EXPERIMENTAL PROCEDURES

Materials. Anti-maltose-binding-protein (MBP) antisera, pMAL-c2X vector, and amylose resin were purchased from New England Biolabs (Beverly, MA). Brain sphingomyelin (SM), egg phosphatidylcholine (PC), liver phosphatidylethanolamine, brain phosphatidylserine, liver phosphatidylinositol, phosphatidic acid, brain ceramide, and brain cerebroside were purchased from Avanti Polar Lipids (Alabaster, AL). Sphingosine, D-erythro-sphingosine-1-phosphate, and sphingosylphosphorylcholine were from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA).

Expression and Isolation of Recombinant Lysenin and Lysenin-Related Proteins. The cDNAs corresponding to lysenin or lysenin-related proteins (LRPs) and polyclonal anti-lysenin antibody were generous gifts from Drs. Y. Sekizawa and H. Kobayashi of Zenyaku Kogyo Co. Ltd. (Tokyo, Japan). The cDNA fragments were amplified by polymerase chain reaction (PCR) and subcloned into pMAL-c2X. Recombinant proteins fused to MBP were expressed in *Escherichia coli* JM 109 or BL 21 and purified using amylose resin according to the manufacture's instruction (*5*). Amino acid substitutions of lysenins were introduced by an overlap extension using PCR with mutant oligonucleotides or by the use of Chamelon TM double-stranded, site-directed mutagenesis (Strategene). Amplified cDNA sequences from wild-type and mutants were confirmed using BigDye Terminator and Genetic Analyzer ABI Prism 310 (PE Applied Biosystems).

Enzyme-Linked Immunosorbent Assay (ELISA). Binding of lysenin to SM was evaluated by ELISA as described previously (*5*, *20*). In brief, 50 μL of lipid (10 μM) in ethanol was added to the well of a microtiter plate (Immulon 1, Thermo Labsystems, Franklin, MA). After the solvent was evaporated at room temperature, 200 μL of 30 mg/mL bovine serum albumin (fraction V, Sigma) in Tris-buffered saline (10 mM Trsi-HCl, pH 7.4, 150 mM NaCl) was added to each well. The wells were then washed with Tris-buffered saline and were incubated with 50 μL of various concentrations of MBP−lysenin or MBP−LRPs in the same buffer containing 10 mg/mL BSA for 2 h at room temperature. The bound protein was detected by adding anti-MBP antiserum followed by the incubation with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and peroxidase-conjugated streptavidin (Zymed, San Francisco, CA). The intensity of the color developed with *o*-phenylenediamine as a substrate was measured with a microplate reader model 550 (BIO-RAD, Hercules, CA) reading the absorption at 490 nm with reference at 630 nm.

Measurement of Hemolysis. Hemolytic activity of lysenin and LRPs was measured as described previously (*5*). Sheep erythrocytes were prepared by washing sheep whole blood (Nippon Bio-Supp. Center, Tokyo, Japan) with phosphate-buffered saline (PBS, 137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4). Erythrocyte suspensions in PBS (3×10^7 cells/mL) were incubated with various concentrations of lysenin or LRPs for 30 min at 37 °C and then centrifuged at 500 × *g* for 5 min to precipitate the erythrocytes. Aliquots of the supernatants were taken, and the optical densities at 405 nm were measured using a microplate reader model 550 (BIO-RAD) to determine the percentage of hemoglobin released from the erythrocytes. Total hemoglobin contents were determined by measuring hemoglobin released after freezing and thawing of the erythrocytes.

Cell Culture. Cultured skin fibroblasts from patients with Niemann−Pick type A (NPA) and from healthy subjects were established and maintained as described (*2*). Diagnosis was confirmed by the typical clinical manifestations and by lysosomal enzyme assays. Glycosphingolipid-deficient mouse melanoma cell mutant GM95 (*21*) was cultured as described (*17*).

Immunofluorescence. Cells grown on coverslips were fixed with 3% paraformaldehyde in PBS for 20 min, quenched with 0.1 M NH_4Cl, and then blocked with 0.2% gelatin in PBS. Cells were then permeabilized by treating with 50 μg/mL digitonin in PBS for 10 min. The permeabilized cells were labeled with MBP-conjugated proteins for 30 min at room temperature, followed by rabbit anti-MBP antiserum and Alexa 488 or Alexa 546 conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR). Cell surface labeling of GM95 cells was performed as described previously (*17*). The specimens were mounted with Mowiol and examined under confocal laser microscope LSM510 (Carl Zeiss) equipped with C-Apochromat 63XW Korr (1.2 numerical aperture (na)) or Plan-Apochromat 100× (1.4 na) objectives. For sphingomyelinase treatment, cells were incubated with 10 mU/mL of *Bacillus cereus* sphingomyeliase (Sigma, St. Louis, MO) in PBS for 1 h at 37 °C.

CD Spectra. CD spectra of 2.5−3.5 μM MBP−lysenin and lysenin mutants in elution buffer (20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA, 10 mM maltose) were measured on a Jasco J-720 apparatus using 0.5-mm path-length quartz cell (*20*) at room temperature. Results from five scans were averaged. The averaged blank spectrum of buffer was subtracted.

Intrinsic Tryptophan Fluorescence. Tryptophan fluorescence of MBP−lysenin and lysenin mutants was measured using a FP-6500 spectrofluorometer equipped with a thermostatic cell holder (Jasco, Tokyo, Japan) (*5*). The fluorescence intensity of 0.6 μM protein in the elution buffer was measured at 20 °C with an excitation wavelength at 285 nm. The averaged blank spectrum of buffer was subtracted.

RESULTS

Characterization of MBP−Lysenin. When lysenin (lysenin 1) cDNA was expressed in *E. coli*, the recombinant lysenin easily formed inclusion bodies in the cytoplasm. We have tested several tags to avoid the aggregation of the protein. Among histidine, glutathione S-transferase (GST), and MBP tags, MBP−lysenin gave the highest recovery (data not shown). In Figure 1A, binding specificity of MBP−lysenin was examined by ELISA (*2*, *5*, *20*). Preliminary ELISA result was described elsewhere (*5*). MBP−lysenin specifically bound to sphingomyelin (SM). Other phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and phosphatidic acid did not bind to MBP−lysenin. MBP−lysenin also did not bind to glycosphingolipids such as glucosylceramide, galactosylceramide, and lactosylceramide, as well as gangliosides, GM1, GM2, and GM3. Intermediates of SM synthesis and sphingomyelin metabolites, such as sphingosine, sphingosin-1-phosphate, sphingosylphosphorylcholine, and ceramide,

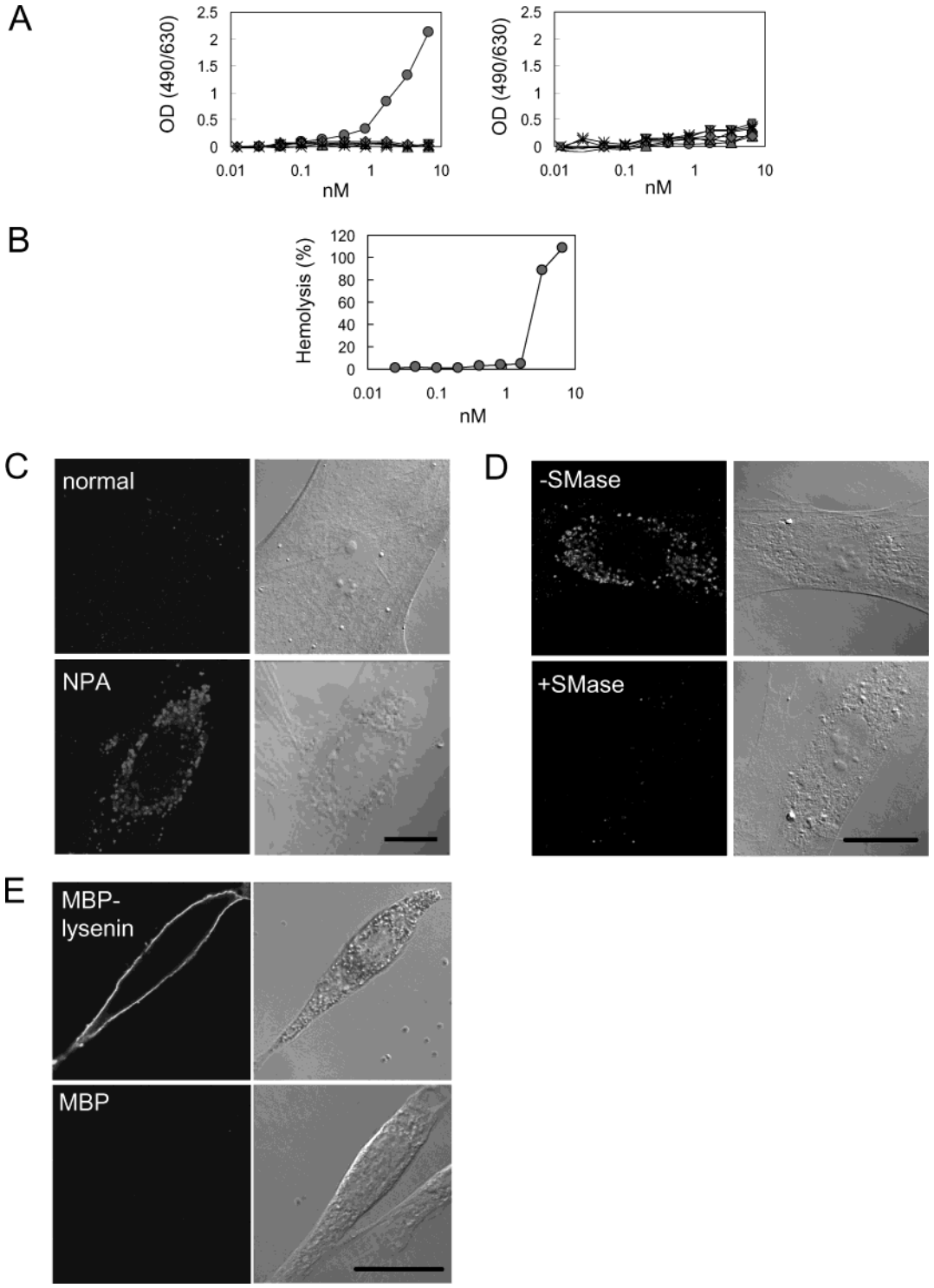

FIGURE 1: MBP−lysenin specifically recognizes SM and induces hemolysis. In panel A, binding of MBP−lysenin to various lipids was determined by ELISA as described in Experimental Procedures: (left) SM (●), ceramide (■), sphingosine (▲), sphingosine-1-phosphate (◆), cerebroside (∗), sphingosylphosphorylcholine (×), and blank (+); (right) phosphatidylcholine (●), phosphatidylethanolamine (■), phosphatidylserine (▲), phosphatidylinositol (◆), phosphatidic acid (∗), and cardiolipin (×). In panel B, sheep red blood cells (3×10^7 cells/mL) were incubated with the indicated concentration of MBP−lysenin for 30 min at 37 °C. Hemolysis was measured as described in Experimental Procedures. In panel C, normal and NPA fibroblasts were fixed and permeabilized as described in Experimental Procedures. Cells were then labeled with 10 nM MBP−lysenin followed by anti-MBP antiserum and Alexa-conjugated anti-rabbit IgG. In panel D, fixed and permeabilized NPA cells were treated with (+SMase) or without (−SMase) sphingomyelinase as described in Experimental Procedures. Cells were then labeled with MBP−lysenin as described in panel C. In panel E, GM95 cells were fixed and labeled with 10 nM MBP−lysenin or MBP alone followed by anti-MBP antiserum and Alexa-conjugated anti-rabbit IgG. The bar represents 20 μm.

also did not bind MBP−lysenin. Like native lysenin (*2*, *5*), recombinant lysenin induced hemolysis (Figure 1B).

Niemann−Pick type A (NPA) cells accumulate SM intracellularly. In Figure 1C, normal and NPA human skin fibroblasts were fixed, permeabilized, and stained with MBP−lysenin. Whereas lysenin labeled small intracellular vesicles in normal cells, NPA cells represented bright perinuclear MBP−lysenin labeling as observed using native

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                          20                  40                  60                  80
Lysenin  MSAKA--AEGYEQIEVDVVAVWKEGYVYENRGSTSVDQKITITKGMKNVNSETRTVTATHSIGSTISTGDAFEIGSVEVSYS
LRP-1      SSTVM D F E      S      A     NS  Q    M      L    K L    TL R LKV  P   AS     T
LRP-2      SR GI                           E   K     R L    K L  S          L   AT D

                         100                 120                 140                 160
Lysenin  HSHEESQVSMTETEVYESKVIEHTITIPPTSKFTRWQLNADVGGADIEYMYLIDEVTPIGGTQSIPQVITSRAKIIVGRQII
LRP-1    F  QK      Q    S Q     V    NK     K       TG           A  ADLT  E NK     L     H
LRP-2    Y                  E                                      L      K     L   E Y

                         180                 200                 220                 240
Lysenin  LGKTEIRIKHAERKEYMTVVSRKSWPAATLGHSKLFKFVLYEDWGGFRIKTLNTMYSGYEYAYSSDQGGIYFDQGTDNPKQR
LRP-1      E                 I           N N      F  SS I          P   W              ESS
LRP-2      E        D                        Y       MY                               S

                         260                 280                 297
Lysenin  WAINKSLPLRHGDVVTFMNKYFTRSGLCYDDGPATNVYCLDKREDKWILEVVG-
LRP-1      LS AM          R NF  N  M             E           NT
LRP-2                                  -    D                KP
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FIGURE 2: Sequence alignment of the lysenin family. Accession numbers at GenBank database are D85846 (lysenin), D85847 (LRP-1), and D85848 (LRP-2), respectively. Protein names are given on the left. Sequence numbers for lysenin are shown. The tryptophan residues are indicated bold. Other aromatic amino acids are underlined. Shadow indicates phenylalanine 209 of lysenin that is substituted for isoleucine 210 in LRP-1.

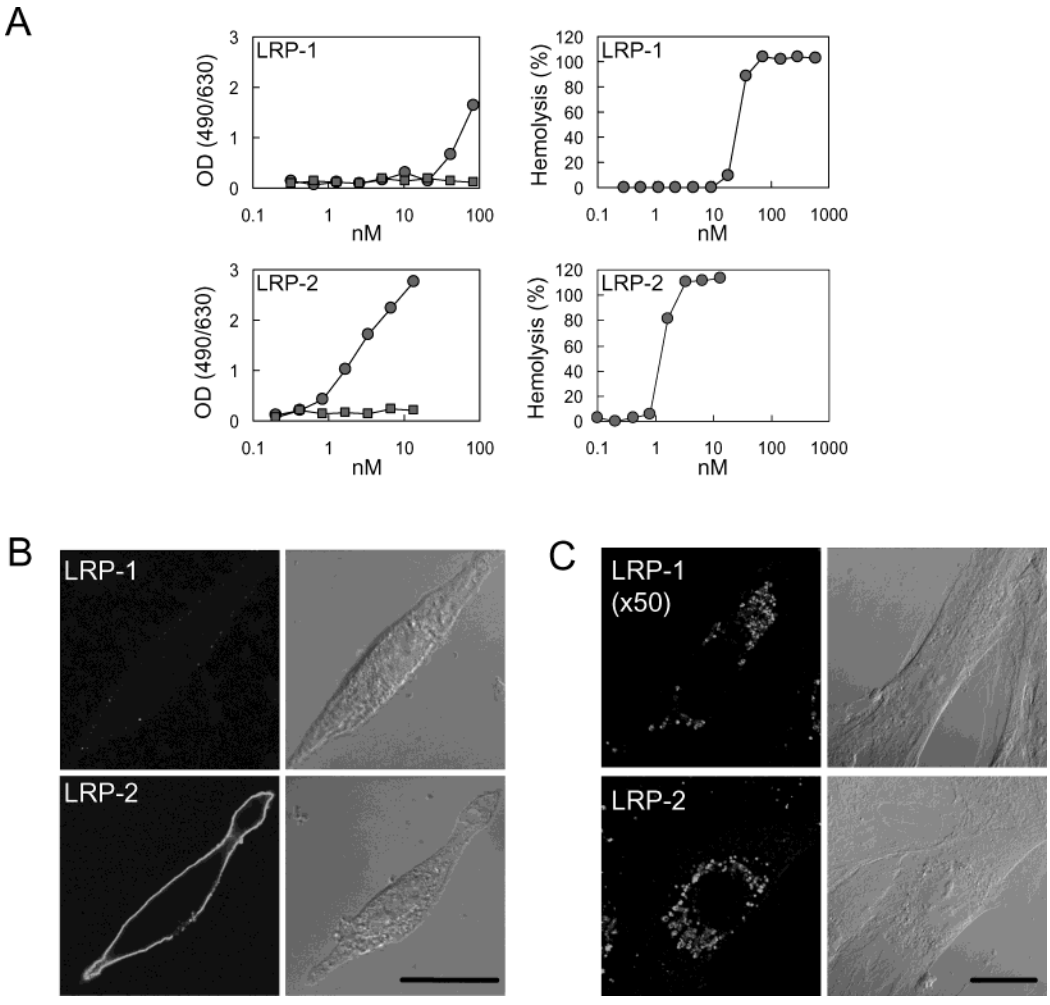

FIGURE 3: Binding to SM and hemolytic activity of LRP-1 are 10 times lower than those observed for lysenin and LRP-2. Panel A shows binding (left) to SM (●) and to PC (■) and hemolytic activity (right) of MBP−LRP-1 and MBP−LRP-2. Binding to the lipids and hemolysis were measured as described in the legend of Figure 1. Binding experiment was performed up to protein concentration 100 nM. Higher concentration of proteins gave high background in ELISA measurement. In panel B, GM95 cells were fixed and labeled as described in the legend of Figure 1 with 10 nM LRP-1 or LRP-2. Panel C, NPA cells were fixed, permeabilized, and labeled as described in the legend of Figure 1 with 500 nM (×50) LRP-1 or 10 nM LRP-2. Bar represents 20 μm.

lysenin (*2*). When fixed and permeabilized NPA cells were treated with sphingomyelinase, MBP−lysenin did not stain cells (Figure 1D), indicating that MBP−lysenin recognizes SM in NPA cells. Labeling of cell surface SM by lysenin is affected by plasma membrane glycolipids (*17*). Recently we showed that native lysenin effectively labels cell surface of

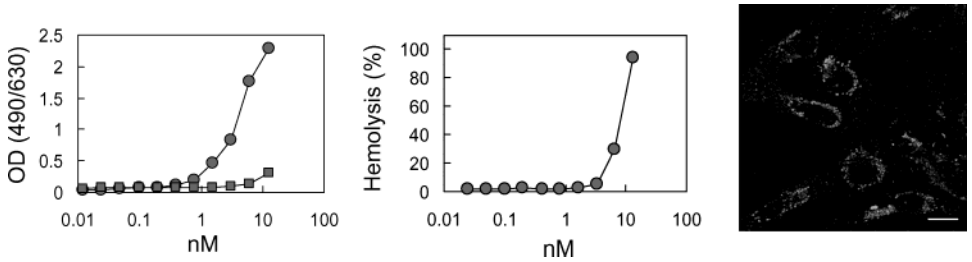

FIGURE 4: I210F mutant of LRP-1 increases both the sphingomyelin binding and the hemolytic activity of LRP-1: (left) binding to SM (●) and to PC (■); (center) hemolytic activity; (right) binding to NPA cells. Ten nanomolar protein was employed for immunolabeling. Methods are described in the legend of Figure 1. Bar represents 20 μm.

glycolipid-deficient melanoma cell mutant GM95 (*17*). In Figure 1E, GM95 cells were labeled with MBP−lysenin. MBP−lysenin clearly stained cell surface, whereas MBP alone did not label cells. These results indicate that MBP−lysenin retains characteristic properties of native lysenin.

Characterization of Lysenin-Related Proteins (LRPs). Figure 2 shows the sequence alignment for members of the lysenin family. The amino acid sequence of LRP-1 is 76% identical and 88% similar to that of lysenin, whereas the sequence of LRP-2 shows 89% identity and 94% similarity to the lysenin sequence. We then characterized lysenin-related proteins (LRPs) using the recombinant MBP conjugate proteins. We measured (1) binding of proteins to SM and PC by ELISA, (2) hemolytic activity, and (3) immunostaining of GM95 and NPA cells to evaluate the activity of LRPs. Figure 3A shows the specific binding of MBP−LRPs to SM and the hemolytic activities of these proteins. Whereas MBP−LRP-2 was as active as MBP−lysenin, both the SM binding and hemolytic activities of MBP−LRP-1 were 10 times lower than those observed for lysenin. However, both LRP-1 and LRP-2 retained binding specificity for SM. Immunofluorescence results confirmed this observation (Figure 3B,C); 10 nM LRP-2 stained the cell surface of GM95 cells, whereas the same concentration of LRP-1 hardly labeled cells (Figure 3B). However, perinuclear labeling of NPA cells was observed when 50 times more LRP-1 was employed (Figure 3C).

Conversion of Isoleucine 210 to Phenylalanine Dramatically Increased the Activity of LRP-1. Lysenin and LRP-2 share 30 common aromatic amino acid sites. Among them, only one position, phenylalanine 210, is substituted for the nonaromatic amino acid isoleucine in LRP-1. We prepared a single amino acid substitution mutant of MBP−LRP-1 with isoleucine 210 to phenylalanine (I210F) (Figure 4). I210F increased both the affinity for SM and the hemolytic activity of LRP-1 by 10-fold to the levels observed for lysenin and LRP-2 (Figure 4, compare with Figure 3A). I210F illuminated the perinuclear region of NPA cells (Figure 4), indicating that the specificity of binding was retained in this mutant. These results suggest the importance of this aromatic amino acid in the activity of lysenin and LRPs.

Relationship between Tryptophan Residues and the Recognition of Sphingomyelin and Induction of Hemolysis by Lysenin. The importance of aromatic amino acids in lysenin activity was further indicated by systematic individual mutations of all tryptophans in lysenin to alanines. Lysenin contains six tryptophan residues at positions 20, 116, 187, 206, 245, and 291. Recently, we have shown that the intrinsic tryptophan fluorescence of lysenin increases and the wavelength of maximum emission undergoes blue shift in the presence of SM (*5*). This result indicates that tryptophan residues of lysenin migrate to a less polar environment in the presence of SM. Mutations were introduced into lysenin replacing individual tryptophans with alanines. The resulting mutants were designated W20A, W116A, W187A, W206A, W245A, and W291A. These mutants were expressed in *E. coli* as MBP conjugates. One protein W116A was not expressed in *E. coli*.

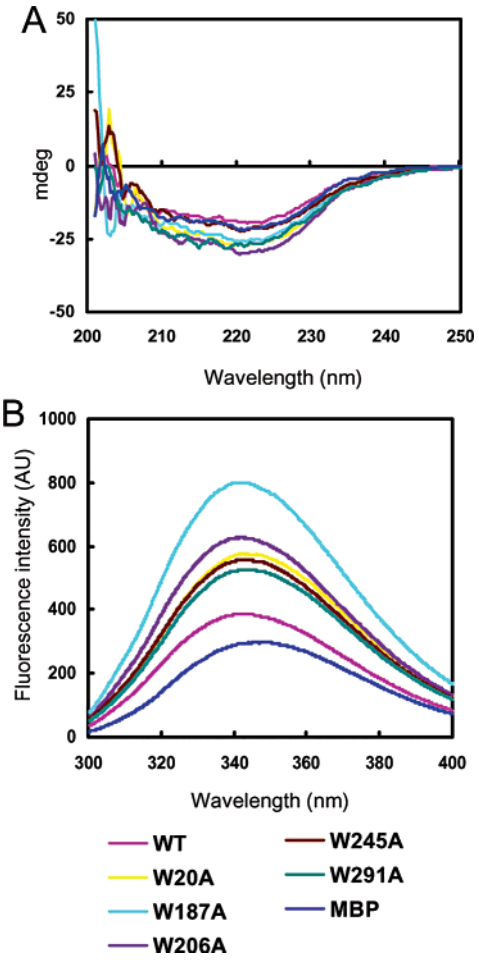

FIGURE 5: Introduction of tryptophan to alanine mutation did not significantly alter tertiary structure of MBP−lysenin: (A) CD spectra and (B) intrinsic tryptophan fluorescence of MBP−lysenin and its tryptophan mutants in elution buffer (20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA, 10 mM maltose). Spectra were measured as described in Experimental Procedures.

CD spectra and intrinsic tryptophan fluorescence of MBP−lysenin and tryptophan mutants are shown in Figure 5. The CD spectrum of MBP−lysenin was not significantly altered by the introduction of tryptophan to alanine mutation,

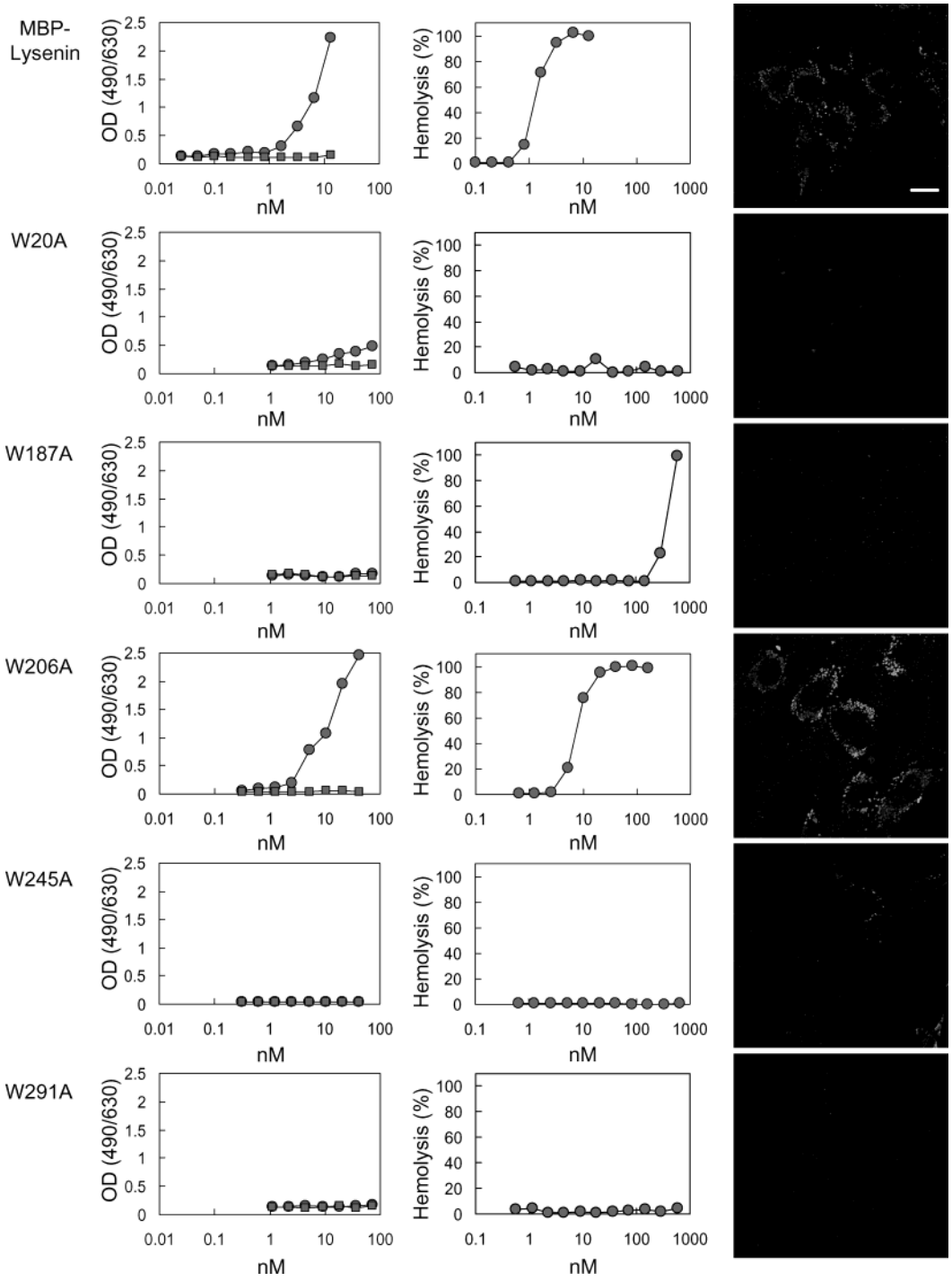

FIGURE 6: Involvement of tryptophan residues in SM binding and hemolysis by lysenin. Binding to SM (●) and to PC (■) (left), hemolytic activity (center), and the staining of NPA cells (right) were examined for MBP−lysenin and tryptophan mutants. Ten nanomolar MBP−lysenin and W206A mutant and 500 nM solutions of other mutants were employed for labeling of NPA cells. Bar represents 20 μm.

indicating that only minor changes are observed in the tertiary structure of the protein. The similar spectral shapes of intrinsic tryptophan fluorescence were observed with MBP−lysenin and tryptophan mutants. However, the fluorescence intensity varied among proteins. Introduction of tryptophan to alanine mutation induced increase rather than decrease of tryptophan fluorescence. These results suggest the migration of the residual tryptophans to a less polar environment in the mutants. We then measured the binding of the mutants to SM and the hemolysis induced by these mutants (Figure 6). W20A, W187A, W245A and W291A lost their ability to bind SM, whereas the W206A mutation did not affect binding. W206A stained NPA cells as MBP−lysenin did. W206A also retained the hemolytic activity, although the activity was 10 times less than that of MBP−lysenin. Interestingly tryptophan 206 is the only tryptophan residue that is not conserved among lysenin and LRPs. The W187A mutant induced hemolysis at high concentration, while this mutant did not bind SM. Binding to SM was not observed even with 600 nM protein (data not shown). Our results suggest the involvement of tryptophan residues in the binding of SM and in the induction of hemolysis by lysenin.

DISCUSSION

Lysenin is a unique toxin in that it specifically recognizes SM, a major lipid constituent of the plasma membranes of most of the mammalian cells (*2*, *5*, *17*). Together with LRP-1 and LRP-2, lysenin comprises a family of proteins sharing sequences of high homology. The amino acid sequence of LRP-1 is 76% identical and 88% similar to that of lysenin, whereas the sequence of LRP-2 shows 89% identity and 94% similarity to the lysenin sequence. Different residues are scattered along the entire polypeptide chain except positions 4−7 where the residues are accumulated. The cDNA sequence of LRP-2 is identical to that of fetidin (*19*, *7*, *8*). Fetidin is a glycoprotein known to have hemolytic and antibacterial properties (*22*, *19*). However, it is not known whether LRPs/fetidin recognize SM. In the present study, we measured the binding and hemolytic activities of LRPs. LRP-2 was as active as lysenin, whereas LRP-1 was 10 times less active than lysenin. Recently the primary structures of lysenin and LRPs were analyzed (*8*). Lysenin and LRPs share putative *N*-glycosylation and *N*-myristoylation sites. Since recombinant MBP conjugates of lysenin and LRPs expressed in *E. coli* bound SM and induced hemolysis, these post-translational modifications seem not to be essential for the biological activities of the proteins. Lysenin and LRP-1 are homologous with a part of the HypA domain (hydrogenase nickel incorporation protein domain). Lysenin has an additional homology with a part of the cellulose-binding domain, which is not detected in LRPs (*8*). These domains are not important in lysenin activity because both lysenin and LRPs bound SM and showed hemolytic activity. Our results indicated that recombinant LRP-2/fetidin specifically bound SM. It is interesting that recombinant fetidin retains antibacterial activity (*19*), although the presence of SM in bacterium (*B. megaterium*) is not reported. The cytotoxicity of lysenin is abolished when cell surface SM was removed (*3*). These results suggest that LRP-2 recognizes other bacterial surface components in addition to SM.

Lysenin and LRP-2 share 30 common aromatic amino acid sites. Among them, only one position, phenylalanine 210, is substituted by an isoleucine in LRP-1. Single amino acid substitution of isoleucine 210 to phenylalanine increased activities of LRP-1, suggesting the importance of aromatic amino acids in the activity of lysenin and LRPs. The importance of aromatic amino acids in lysenin was further indicated by systematic single mutations of all tryptophans to alanines. Tryptophan to alanine mutation did not significantly affect the tertiary structure of the protein as revealed by CD spectra. Among the five tryptophan residues of lysenin tested, four affected both SM binding and hemolytic activity of lysenin. It is interesting and perhaps significant that the noneffective tryptophan 206 position is the only tryptophan residue that is not conserved among the lysenin family. These results suggest the importance of conserved tryptophans for both SM binding and hemolysis. It is noteworthy that, although very weak, W187A retained hemolytic activity whereas this mutant did not bind SM under our experimental conditions. We cannot exclude the possibility that W187A weakly interacts with SM and this interaction is not detected by ELISA. However, this result suggests that the hemolytic activity and specific binding to SM could be separated.

Several proteins are reported to recognize SM. These are neutral sphingomyelinase, acidic sphingomyelinase, equinatoxin II from sea anemone (*23*), sticholysin I and II from *Stichodactyla helianthus* (*24*), and eiseniapore derived from earthworm (*25*). The amino acid sequence of eiseniapore has not been reported. The amino acids 20−167 from lysenin showed 20% similarity to acid sphingomyelinase. This domain in acid sphingomylinase is shown to be homologous to saposin-type sequences (*26*). It is supposed that key amino acids in saposin-type sequences are six cysteines that are required for proper protein folding. None of the cysteine residues is conserved in lysenin family, suggesting that lysenin possesses a novel sequence or three-dimensional structure for SM binding.

Recently we have shown that lysenin is a pore-forming toxin. Lysenin bound membranes and assembled to SDS-resistant oligomers in a SM-dependent manner, leading to the formation of pores with a hydrodynamic diameter of ∼3 nm (*5*). Tryptophan residues are shown to be involved in the interaction between several pore-forming toxins and membranes (*27*−*29*). Recently we observed that the tryptophan fluorescence of lysenin increased and the wavelength of the maximum emission underwent a blue shift from 332.8 to 330.3 nm after incubation with SM containing liposomes (*5*). In contrast, PC-containing liposomes did not alter tryptophan fluorescence of lysenin. These results suggest the migration of the tryptophan residues to a less polar environment in the presence of SM. Using differential scanning calorimetery, we also showed that lysenin interacts with the hydrophobic tails of SM in addition to the headgroup (*5*). Results presented here suggest that conserved tryptophans play important roles in the interaction of lysenin with SM containing membranes.

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