Characterization of CD36/LIMPII Homologues in Dictyostelium discoideum*

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The CD36/LIMPII family is ubiquitously expressed in higher eukaryotes and consists of integral membrane proteins that have in part been characterized as cell adhesion receptors, scavenger receptors, or fatty acid transporters. However, no physiological role has been defined so far for the members of this family that localize specifically to vesicular compartments rather than to the cell surface, namely lysosomal integral membrane protein type II (LIMPII) from mammals and LmpA from the amoeba Dictyostelium discoideum. LmpA, the first described CD36/LIMPII homologue from lower eukaryotes, has initially been identified as a suppressor of the profilin-minus phenotype. We report the discovery and initial characterization of two new CD36/LIMPII-related proteins, both of which share several features with LmpA: (i) their size is considerably larger than that of the CD36/LIMPII proteins from higher eukaryotes; (ii) they show the characteristic “hairpin” topology of this protein family; (iii) they are heavily N-glycosylated; and (iv) they localize to vesicular structures of putative endolysosomal origin. However, they show intriguing differences in their developmental regulation and exhibit different sorting signals of the di-leucine or tyrosine-type in their carboxyl-terminal tail domains. These features make them promising candidates as a paradigm for the study of the function and evolution of the as yet poorly understood CD36/LIMPII proteins.

The amoeboid eukaryote Dictyostelium discoideum is an established model organism for the study of diverse aspects of cell biology, such as development, cell motility, vesicle transport, and signal transduction. Upon starvation, the unicellular amoebae undergo a simple developmental cycle that involves chemotaxis, cell differentiation, the formation of multicellular structures, and the terminal formation of fruiting bodies that carry spores. Growth phase cells very effectively take up bacteria and nutrients from the medium by phagocytosis and macropinocytosis. Actin-binding proteins like coronin, profilin, or motor molecules of the myosin I class have been shown to be involved in both processes (1). After particles or fluid are ingested by the amoebae, hydrolase-rich lysosomes are fused with the newly formed phagosomes or macropinosomes. The endosomal and phagosomal pathways in D. discoideum have been the object of thorough investigation over the last decade, so many of the key proteins have been identified, e.g. small GTPases (2, 3), vacuolin B (4), or myosin I motors (1, 5). The lipid and protein composition of several vesicular compartments involved in these pathways have been characterized (6, 7). However, the integral proteins of the lysosomal membrane were not known until very recently.

The first characterized DdLIMP protein with homology to the mammalian CD36/LIMPII superfamily (LIMPII† for lysosomal integral membrane protein II; Ref. 8) was DdLIMP (LmpA; Ref. 9). This gene family consists of cell adhesion molecules, fatty acid transporters, and scavenger receptors at the cell surface as well as lysosomal membrane proteins; its members are ubiquitously present in the animal kingdom from mammals to nematodes but are apparently missing in yeast. The LmpA gene had been identified in a screen for suppressors of the profilin-minus phenotype in our laboratory. Profilin is a small cytoplasmic protein that can bind to actin monomers (10) and regulate actin polymerization; it is also believed to play a role in vesicle transport (11). In yeast, it has been well established that rapid actin polymerization and depolymerization is necessary for the endocytic process (12). In Dictyostelium, profilin-minus mutants showed defects in endosomal trafficking (13). LmpA was found to be an integral membrane glycoprotein that is localized to the membranes of endolysosomal vesicles and macropinosomes (9, 13). The higher eukaryote homologues of the CD36/LIMPII family as well as LmpA share a common “hairpin” topology with two transmembrane domains, one hydrophobic signal anchor near the NH₂ terminus and another hydrophobic sequence close to the COOH terminus (8). Both termini are cytosolic, whereas the central domain in between the transmembrane domains is generally heavily glycosylated and localizes to the lumen of vesicles or to the cell surface. The founding member CD36 (14) has been identified as a receptor for a variety of negatively charged macromolecules such as extracellular matrix proteins (15, 16), modified low density lipoproteins (17), long chain fatty acids (18, 19), and anionic phospholipids (20, 21). The class B scavenger receptor SRBI belongs to the same superfamily, it also functions as a lipid receptor; however, the uptake mechanism is fundamentally different from that of CD36. SRBI was identified as the long

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† The abbreviations used are: LIMPII, lysosomal integral membrane protein II; kb, kilobase(s); Lmp, lysosomal integral membrane protein from Dictyostelium; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PIP₂, phosphatidylinositol 4,5-bisphosphate; mAb, monoclonal antibody; Pipes, 1,4-piperazinediethanesulfonic acid; PBS, phosphate-buffered saline; SRBI, scavenger receptor BI; TRITC, tetramethylrhodamine B isothiocyanate.

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sought high density lipoprotein receptor by a knock-out approach in mice (22). Furthermore, SRBI binds to phosphatidylinositol and phosphatidylserine liposomes with a specificity that closely resembles that of CD36 (20). The CD36 gene from rat, also known as fatty-acid translocase, has been implicated in fatty acid metabolism and insulin resistance (23).

We have previously shown that DlLIMP from *Dictyostelium* specifically binds to the anionic phospholipid PI(4,5)P₂ but not to phosphatidylcholine and only weakly to phosphatidylserine. The binding is thought to be mediated by the cytosolic carboxy-terminal tail of DlLIMP (9). The majority of the CD36/LIMPII proteins is localized to the plasma membrane, whereas LIMPII, which is ubiquitously expressed in all cell types tested so far, has been found in vesicles of endolysosomal origin (24). The specific sorting of LIMPII to lysosomes has been demonstrated to be accomplished by an interaction of its lysosomal sorting signal (di-leucine type) with the adaptor complex AP-3 (25). The *in vivo* function of mammalian LIMPII is still unknown; it has been postulated to be responsible for the uptake of hydrolytic products from the lysosomes to the cytoplasm (24). No physiological ligands have been demonstrated for LIMPII so far; however, it has been shown that LIMPII binds *in vitro* to peptides derived from the extracellular matrix component thrombospondin-1, and the binding is mediated by a domain that is conserved between CD36 and LIMPII (26). The newly discovered CD36/LIMPII homologues that are described in this study show differences in developmental regulation. They exhibit putative sorting signals of the di-leucine or tyrosine type and are located in distinct vesicle populations.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—*D. discoideum* strain AX2 (referred to as wild type) and mutant strains were cultivated at 21 °C, either on SM agar plates with *Klebsiella aerogenes* (27) or axenically in liquid nutrient medium (28) submerged in plastic culture dishes or in shaking suspension at 150 rpm. All reagents were purchased from Sigma, if not stated otherwise. Antibodies against contact site A protein (mAb 33-294-17), α-t-fucosidase (mAb 173-185-1), and golgins (mAb 275-392-5) were kindly provided by Dr. G. Gerisch (MPI for Biochemistry, Martinsried, Germany). Generation of the polyoma virus through a 5-quantum leap enhancer of DlLIMP (LmpA) is described elsewhere (9). For studies on the development of *D. discoideum*, cells were grown to a density of 2.3 × 10⁶ cells/ml and washed in phosphate buffer (14.6 mM KH₂PO₄, 2 mM Na₂HPO₄, pH 6.0), and 2 × 10⁶ cells were deposited on phosphate agar plates and allowed to develop to 21 °C. The cells were harvested immediately (0 h) or at various time points of starvation.

**Polyclonal Antisera against LmpB and LmpC**—The inserts corresponding to the *lmp* inserts of *D. discoideum* RB2 cells that contain no LmpA (9). The mounted cells were observed in an Axiopt microscope (Carl Zeiss, Oberkochen, Germany). Nuclei were stained for 1 h with 4,6-diamidino-2-phenylindole (Sigma) in PBS. The cells were washed in 4,6-diamidino-2-phenylindole-free buffer, rinsed in distilled water, and mounted in gelvatol.

For labeling of macropinosomes and endosomes, coverslips to be used were washed with 3.6% HCl followed by distilled water. Exponentially growing *K. aerogenes* cells were allowed to adhere for 15 min at room temperature. After this time the medium was replaced by medium containing 5 mg/ml TRITC-dextran (Mᵣ 155,000, Sigma) for the desired times. After two short washes in phosphate buffer the cells were either chased with normal medium again or fixed immediately. The cells were routinely fixed with 2% paraformaldehyde in 15% saturated picric acid and 10 mM Pipes, pH 6.0, but 1% formaldehyde in methanol worked as well. To avoid a subsequent major loss of fixed cells from the coverslips, the cells were treated briefly with 70% ethanol, and then washed with PBS/glycine and PBS/bovine serum albumin/gelatin according to routine procedures. Confocal images for detection of TRITC-dextran vesicles and LmpB/C-specific antibodies were taken with an inverted Leica TCS-SP laser scanning microscope equipped with an argon laser. The Alexa488 label was excited at 488 nm, and the TRITC label was excited at 514 nm. To avoid an artificial bleaching effect through all data were obtained by a sequential scanning procedure. The samples were observed with a 63× oil immersion objective, and the step sizes were usually between 0.15 and 0.25 μm.

**Protease Protection and Deglycosylation Assays**—Axenically grown cells were harvested, washed in phosphate buffer, and lysed by several passages through a 5-μm Nucleopore (Nucleopore/Costar, Tübingen, Germany) filter. The lysate was centrifuged for 1 h at 100,000 × g, and the resulting pellet was resuspended in TD buffer containing 10 mM Tris/HCl, 1 mM dithiothreitol, pH 8.0, and treated with 200 μg/ml of trypsin for 30 min at 37 °C in the absence or presence of Triton X-100. For the deglycosylation assays, 100,000 × g pellets were resuspended in PBS containing 1% SDS and boiled for 5 min. Aliquots of 10 μl were loaded on 10% SDS-PAGE and subjected to gelatinolytic digestion (1% and treated with 0.4 units of N-glycosidase F (Roche Molecular Biochemicals) at 37 °C for 12 h.

**Subcellular Fractionation by Differential Centrifugation and Enzyme Assays**—Axenically grown AX2 cells (5 × 10⁶ cells/ml) were harvested and washed twice with cold phosphate buffer. The cell pellet was resuspended at a density of 1 × 10⁶ cells/ml in HEPES buffer containing 10 mM HEPES, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, pH 7.4, and the cells were opened by a short ultrasonic pulse and several passages through a 5-μm Nucleopore filter. Complete rupture of cells was controlled by light microscopy. The homogenate was centrifuged at 15,000 × g for 30 min. The membrane pellet was resuspended in a small volume of HEPES buffer with a Dounce homogenizer and loaded on top of a discontinuous sucrose step gradient consisting of 2-ml layers of 0.88, 1.02, 1.17, 1.32, 1.47, and 2.49 M sucrose in HEPES buffer (33). The gradient was subsequently ultra centrifuged for 20 h at 110,000 × g in a SW40 Beckman swinging bucket rotor (Beckman Instruments, Muenchen, Germany). Fractions from the gradient were loaded from top to bottom and immediately assayed for enzymatic activity. Equal amounts of protein from the different fractions were subjected to SDS–PAGE, transferred to a specific antibody after immunoblotting. Colorimetric assays for acid or alkaline phosphatase activity were carried out according to Loomis and Kuspa (34) or Loomis (35), respectively, with p-nitrophenyl phosphate as substrate and measured with an Ultraspec III spectrophotometer (Amersham Pharmacia Biotech).

**Immunofluorescence Microscopy**—For immunofluorescence studies, cells were allowed to attach to coverslips for 30 min in liquid nutrient medium, washed with phosphate buffer, fixed with cold methanol (−20 °C, 10 min), air dried, and subsequently labeled and mounted essentially as described (31). The polyclonal antisera against LmpB and LmpC were used at dilutions of 1:200 to 1:500. Secondary antibodies used for immunofluorescence included goat anti-mouse IgG and goat anti-rabbit IgG coupled to fluorescein, Alexa-488, or Cy3 (Dianova, Hamburg, Germany). As an control for the specificity of the antisera, preimmune sera from all rabbits were tested under the same conditions. In addition, the antisera against LmpB and LmpC were tested on *D. discoideum* RB2 cells that contain no LmpA (9).

**The recombinant proteins carrying an NH₂-terminal His₉ tag were expressed in Escherichia coli M15 cells (LmpB) or E. coli XL1 Blue cells (LmpC) using a pQE30 (LmpB) or a pQE32 (LmpC) vector (Qiagen, Hilden, Germany) and purified from inclusion bodies by affinity chromatography on Ni²⁺-nitrilotriacetic acid columns (Qiagen) in the presence of 8 M urea.**
Southern and Northern blot analysis were prepared according to Noegel et al. (36), transferred onto nitrocellulose membranes (Schleicher & Schuell), and incubated with 32P-labeled probes generated using a random prime labeling kit (Stratagene, La Jolla, CA). Hybridization was performed at 37 °C for 12–16 h in hybridization buffer containing 50% formamide and 2X SSC. The hybridized filters were washed twice for 5 min in 2X SSC containing 0.1% SDS at 37 °C and for 60 min in a buffer containing 50% formamide and 2X SSC at 37 °C. SDS-PAGE (37) and immunoblotting (32) followed standard procedures. Secondary antibodies were used goat anti-mouse IgG and goat anti-rabbit IgG coupled to horseradish peroxidase (Dianova, Hamburg, Germany), and the bound secondary antibodies were visualized with the enhanced chemiluminescence method (ECL, Amersham Pharmacia Biotech).

**RESULTS**

Identification of Two New Dictyostelium CD36/LIMPII Genes—By sequence comparison of lmpA with the data base of the cDNA-sequencing project at Tsukuba University (Japan), several cDNA clones with significant homology were found. For the generation of the cDNAs, cells from the D. discoideum strain AX4 in the developmental finger stage (T12-T14) had been used (42). Further comparison with the data base of the German Dictyostelium Genome Sequencing Project resulted in the identification of additional genomic clones with significant homology to the known sequence of lmpA. Careful analysis showed that the sequences of all different clones were derived from three putative genes, two of them were new. According to the order of their discovery, the new genes have been named lmpB and lmpC, and their gene products LmpB and LmpC, respectively.

**lmpB and lmpC, Sequence and Structural Features**—The insert of clone SSD382 was sequenced several times with standard T3 and T7 primers on both strands. An open reading frame of 951 bases was found, and the deduced gene product showed 34% similarity and 24% identity to the COOH-terminal region of LmpA (GAP program, University of Wisconsin GCG package). The open reading frame ended with a TAA stop codon, the most frequently found termination codon in D. discoideum (43), followed by a polyadenylation signal and several other stop codons. Whereas the 5’-end was missing in this cDNA clone, it was found to overlap with two genomic clones, JAX4b23c10 and JAX4a28b05. In the genomic clones, a short single intron with conserved splice sequences 5’-GTXXGT( )-3’ (AG-3’ was present. The small size (109 bases) of the intron and the extremely high AT content of 95% are typical for Dictyostelium introns. Comparison with homologous proteins showed that a small part of the 5’-coding sequence (including the start codon and the putative first transmembrane domain) was still missing. By screening of a size-fractionated cDNA library (30) with a probe corresponding to the 5’-end of the genomic clone JAX4b23c10, 8 clones out of 4 X 105 primary clones were found to contain partial inserts of the lmpB cDNA. Phagemids were prepared from the lmpB-positive λ-ZAP phages by in vitro excision with helper phages and sequenced from both sides with T3 and T7 primers as well as lmpB-specific primers. One phagemid clone (E2.1) that showed a complete overlap with the genomic clone JAX4b23c10 was found to consist of the start codon together with an adenine-rich upstream sequence (AAA ATG) that is apparently necessary for stable expression of proteins in D. discoideum (44), as well as an in-frame stop codon 12 bases upstream of the ATG. The completed open reading frames of lmpB and lmpC encode putative proteins of 756 and 783 amino acids, respectively. Analysis with the transmembrane prediction program TM-Predict (45) revealed for both proteins two potential transmembrane domains, one very close to the NH2 terminus and another one close to the COOH terminus. In LmpB, eight consensus sites for N-glycosylation (NX(S/T)) were present, and by computer prediction (46), a single putative O-glycosylation site was found in a serine/threonine-rich motif (TSTSSST, at amino acid 223). The predicted cytosolic COOH-terminal tail consists of only 6 amino acids and is considerably shorter than the corresponding region in LmpA. Interestingly, there is an Ile-Ile sequence in this region that resembles the lysosomal sorting signal of the di-leucine type from mammalian LIMPII (Fig. 1A). Complete sequencing of the second lmpA homologous cDNA clone (SSE457) showed that the insert of about 2.5 kb contained a complete open reading frame of 783 amino acids coding for a new protein of the DlLIMP family. It partially overlapped (99% identity on the nucleotide level) with the genomic clone JAX4b12d09. By PCR on genomic DNA, two short introns with the correct 5’-GTXXGT( )-3’-splicing sequences were identified. The location of these introns close to the 5’-end of the gene as well as their small size (155 and 61 bases, respectively) and high AT content (85 and 92%) are typical for Dictyostelium. Two in-frame stop codons were located about 50 bases upstream of the start codon; the TAA stop codon was followed by several polyadenylation signals. In the lmpC sequence, 22 consensus sites for N-glycosylation (NX(S/T)) were found in between the two hydrophobic stretches. The predicted cytosolic carboxy-terminal tail consists of a putative lysosomal sorting signal of the tyrosine type (GYNI) that resembles the lysosomal sorting signal of LmpA (GYQA) but differs from the di-leucine-type motif in the corresponding region of LmpB (Fig. 1B). Fig. 2B schematically depicts the domain organization of the Dictyostelium Lmp family proteins in comparison to rat LIMPII.

The lmp Genes and the CD36/LIMPII Superfamily—Southern blot analysis of genomic DNA from AX2 cells was performed with radioactive probes that corresponded to fragments of lmpB and lmpC, as shown in Fig. 3B. For lmpC, the analysis confirmed the existence of a single gene, and there were no cross-reacting bands observed. For lmpB, however, there are additional weak signals for the restriction digest with the enzyme XbaI; these might be due to incomplete digestion. The expected second band for the restriction digest with HindIII (at 15 kb) is only weakly visible because of incomplete transfer due to the large size. The reading frames of both genes are interrupted by short introns (one for lmpB, two in the case of lmpC) in the 5’-proximal region. The position of the second intron in lmpC is homologous to the splicing site of the single intron in lmpA, whereas the positions of the first intron of lmpC and the single intron of lmpB are apparently not conserved. A comparison of the conservation of the position of the N-glycosylation sites between LmpA, LmpB, and LmpC leads to the following results: four sites are conserved among all three proteins and seven additional sites are conserved between LmpA and LmpC. Together with the overall homologies for the three gene products on the amino acid level (Table I), these findings suggest a closer evolutionary relationship between lmpA and lmpC, and a more divergent position for lmpB. A similar picture arises upon a phylogenetic analysis of the relations with the other members of the CD36/LIMPII superfamily (Fig. 2A). Four subgroups can clearly be distinguished as follows: (i) the CD36-type group (47); (ii) the LIMPII-type group (8); (iii) the SRBI-like group (scavenger receptor type B class I; see Ref. 48); and (iv) LmpA and LmpC from Dictyostelium. The CD36/LIMPII members from insect species might be regarded as a fifth group.
FIG. 1. Nucleotide sequences of the Dictyostelium lmpB and lmpC genes and deduced amino acid sequences. A, the lmpB gene. The open reading frame (capital letters) of the lmpB gene is interrupted by a short intron (lowercase letters). The 5'- and 3'-flanking sequences are shown in lowercase letters. The deduced amino acid sequence is shown below the corresponding coding sequence. The start codon with the typical D. discoideum upstream nucleotide sequence (AAA) is underlined. The consensus splice sequences GTxxG(T/A)G are shown in bold. The in-frame stop codons upstream of the coding sequence, as well as the TAA codon terminating the open reading frame are marked by an asterisk. Tandem polyadenylation signals downstream of the coding sequence are underlined. The presumed transmembrane regions are shaded in light gray. The eight predicted N-glycosylation sites (NX(S/T)) are boxed, and the single predicted O-glycosylated serine residue is boxed and marked in bold. Note the putative di-leucine type lysosomal sorting motif in the COOH-terminal tail (underlined). This sequence has been submitted to GenBank™ with accession number AF238324. B, the lmpC gene. The open reading frame (capital letters) of the lmpC gene is interrupted by two short introns (lowercase letters). The predicted amino acid sequence is shown below the corresponding coding sequence. The start codon is underlined. The consensus splice sequences GTxxG(T/A)G are shown in bold. The in-frame stop codons upstream of the coding sequence as well as the TAA codon terminating the open reading frame are marked by asterisks. Tandem polyadenylation signals downstream of the coding sequence are underlined. The presumed transmembrane regions are shaded in light gray. The 22 predicted N-glycosylation sites (NX(S/T)) are boxed. Note the putative tyrosine-based lysosomal sorting motif (GYXX) in the COOH-terminal tail (underlined). This sequence has been submitted to GenBank™ with accession number AF238325.
containing sensory membrane neuron protein-1 from the silk moth *Antheraea* (49), epithelial membrane protein from *Drosophila* (50), and DMCD36 (*croquemort* from *Drosophila*; see Ref. 51), with somewhat lower homology in the subgroup. Two open reading frames from the nematode *Caenorhabditis elegans* also belong to the CD36/LIMPII family but apparently not to any of the subgroups.

**Developmental Regulation**—Northern blots with equal amounts of total RNA from different developmental stages were hybridized with *lmpA*- and *lmpC*-specific probes. Equal loading was controlled by densitometry of the ethidium bromide-stained bands of ribosomal RNA (Fig. 4A) or by stripping the blots and subsequent hybridization with a probe for serpin. Single transcripts of about 3.5 (*lmpA*), 3.4 (*lmpB*), and 3.6 kb (*lmpC*) were labeled (Fig. 4A). The transcript of *lmpB* showed an intriguing bi-phasic developmental regulation, in contrast to the *lmpA* transcript, which was found at all stages of development in comparable quantities. The mRNA for *lmpB* was present in growth phase cells (*t*0) but showed a reduction to almost undetectable levels upon induction of development (*t*6–12). At later multicellular stages of the development (*t*18), the transcript was present again, at higher amounts than in growth phase cells, and was still detectable in significant amounts in fully developed fruiting bodies (*t*24). The *lmpC* transcript was present at all stages of development. However, a pronounced accumulation at late development (*t*18 and *t*24) could sometimes be seen. On the protein level, a significant down-regulation of LmpA was observed at the onset of aggregation (*t*6), but small amounts of LmpA were detectable also at late stages of development (Fig. 4B). LmpC is present throughout development. The intriguing two-phasic regulation of the LmpB transcript is also found on the protein level. The protein is present in growth phase cells as well as in early aggregating cells (*t*0–3), but the levels drop significantly during the slug stage (at time points *t*9–15, the expression is at 39 ± 4% of the growth phase level, *n* = 3). At late development (*t*18–24), the protein levels for LmpB rise again in a similar pattern as can be seen in the Northern analysis.

**Intracellular Distribution**—Immunofluorescence studies with polyclonal antisera raised against LmpB and LmpC re-
revealed a vesicular punctate staining in AX2 growth phase cells. The pre-immune sera showed no significant staining when tested under the same conditions. The LmpB- and LmpC-positive vesicles were distributed all over the cytoplasm, with no obvious preferential localization to specific subcellular areas. In some cases, larger, ring-like punctate structures (diameter, 2 μm) at the cell surface were also labeled and corresponded to phase-opaque vesicles (Fig. 5A). This type of staining is very reminiscent of the situation found for LmpA, where these structures could be identified as early macropinosomes that are formed upon the uptake of relatively large quantities of medium by growth phase cells (13).

LmpB and LmpC Colocalize with Endosomal Vesicles—To test a putative recruitment of LmpB and LmpC in the endocytic pathway, we incubated AX2 cells in medium containing TRITC-dextran, a fluid phase marker that is taken up by pinocytosis and macropinocytosis. Within 5 min of uptake, TRITC-dextran-positive vesicles were found to stain with both anti-LmpB and anti-LmpC antiserum (Fig. 5B), which argues for an early recruitment of the DdLIMPs in the process of fluid phase uptake. The same behavior was found for LmpA under equivalent conditions (not shown). However, the vast majority of TRITC-dextran-positive vesicles were also labeled with LmpA (ratio labeled to unlabeled vesicles 4:1), whereas LmpB and LmpC co-localized only to a fraction of the endocytic vesicles (ratio labeled to unlabeled vesicles 0.6 and 0.3, respectively). The number of double-labeled vesicles showed a rapid decrease for LmpB and LmpC after a 15-min chase and further declined after chasing for 30 min. Even after a 45-min chase we

![Southern blot and genomic organization of LmpB and LmpC. A, genomic DNA from wild-type strain AX2 was digested, and the fragments were resolved on 0.7% agarose gels. Hybridization was performed under stringent conditions with 32P-labeled probes corresponding to the fragments marked in B. Genomic DNA was digested with EcoRV, HincII, HindIII, XbaI, and BglII and labeled with a probe for LmpB. Genomic DNA was digested with HindIII, XbaI, AluI, EcoRV, and HincII and labeled with a probe for LmpC. B, schematic representation of the genomic organization of LmpB and LmpC, the arrangement of the isolated genomic and cDNA clones, and the fragments used to generate radiolabeled probes for A. The 5′- and 3′-untranslated regions are indicated by white boxes and the introns by black boxes.

**Table 1**

Identity/homology on the amino acid level (%)

The following abbreviations are used: LmpA/B/C, *D. discoideum*; LIMPII, *R. norvegicus*; CD36, *H. sapiens* (computed with GAP from the University of Wisconsin GCG package).

|          | LmpA | LmpB | LmpC | LIMPII | CD36 |
|----------|------|------|------|--------|------|
| LmpA     | 36/26| 60/51| 36/26| 37/25  |      |
| LmpB     | 36/26| 35/24| 33/26| 35/25  |      |
| LmpC     | 60/51| 33/26| 30/21| 36/26  |      |
| LIMPII   | 36/26| 35/24| 33/26| 45/36  |      |
| CD36     | 37/25| 35/25| 36/26|        |      |
observed double-labeled structures for all three DdLIMPs and TRITC-dextran. However, these constituted only a small fraction of the TRITC-dextran marked vesicles (not shown).

LmpA, -B, and -C Are Highly Glycosylated Integral Membrane Proteins—Polyclonal antisera raised against recombinant proteins for LmpB and LmpC recognized in Western blots of Dictyostelium membrane fractions bands of about 120 kDa for both LmpB (asterisk) and LmpC. Sometimes a second band with higher electrophoretic mobility could be detected for LmpB (p100, open arrowhead), which could simply be a degradation product or a differently glycosylated form (Fig. 6A). The antisera were tested for their possible cross-reactivity with LmpA (Fig. 6B), which had been purified from Dictyostelium membrane fractions by conventional chromatography (9). Clearly, anti-LmpB and anti-LmpC antisera did not show any cross-reaction with LmpA. However, the anti-LmpB antiserum recognized a distinct single band of about 100 kDa (p100, open arrowhead) in Fig. 6A. This might be an isoform of LmpB that co-purified with the LmpA protein. Both antisera for LmpB (7656 and 7657), which were raised in parallel, essentially the same staining, and the same holds true for LmpC (7654 and 7655). There was also no detectable cross-reaction observed between the anti-LmpB antiserum and recombinantly expressed hisLmpC and for the anti-LmpC antiserum and recombinant hisLmpB. These results clearly demonstrate that the specificity of the antisera is sufficiently high to distinguish between the three different CD36/LIMPII family members in Dictyostelium.

Based on computer predictions with the GCG program, it was proposed that the newly discovered Lmps were integral membrane glycoproteins. Computational analysis with the transmembrane prediction program TMPred (45) strongly predicted two transmembrane helices (not shown) connected by a large intravesicular domain; the NH2 and COOH termini were proposed to be cytosolic. To test the membrane orientation of the Dictyostelium Lmp proteins, a protease protection assay was carried out by treating vesicles from disrupted AX2 cells with trypsin. It was found that tryptic proteolysis only occurred in the presence of detergent (shown in Fig. 6C), leading to a reduced amount of the 120-kDa protein and a prominent breakdown product of about 55 kDa. For LmpA, there is a minor degradation band of about 70 kDa visible in the control (lane 1), which is due to endogenous proteases, since the preparation does not contain protease inhibitors. It is also noteworthy that a relatively high concentration of protease (100–200 μg/ml) was required to get a visible effect on DdLIMP, whereas control proteins were easily digested at lower trypsin concentrations. This observation might be due to a protection of DdLIMP by glycosylation, which would correspond to the experimentally observed protective effect of asparagine-linked oligosaccharides in the case of the lysosomal proteins LAMP-1 and LAMP-2 (52). Furthermore, the three Dictyostelium Lmp pro-
termini are cytosolic, whereas the major part of the proteins between the two membrane spanning regions is N-glycosylated and intravesicular.

**N-Glycosylation**—In the case of LmpA, a polyclonal antiserum recognized a band of about 120 kDa in Western blots of AX2 homogenates, which exceeded the calculated molecular mass of 88 kDa; after centrifugation at 100,000 × g the signal was found in the membranous pellet (9). In two-dimensional gel electrophoresis two major spots with a pl of 5.6 and 5.8 were observed for LmpA, which is less acidic than the calculated pl of 4.4 and also suggests post-translational modifications (not shown). When treated with N-glycosidase, a significant shift to higher electrophoretic mobility was observed in all three cases. For example, a mobility shift from 120 to ~105 kDa was seen for LmpA, indicating a high amount of N-glycosylation (Fig. 6D). This considerable decrease in molecular mass is in good correlation with the existence of 19 predicted N-glycosylation sites in LmpA. Interestingly, the anti-LmpB antiserum recognized two bands of 120 (asterisk) and 100 kDa (open arrowhead) that both underwent deglycosylation (Fig. 6D). However, the relative intensity of the signals for LmpB, p100 and p120, changed for different protein preparations that were analyzed, indicating that the 100-kDa protein represented a breakdown product of the full-length protein. A similar mobility shift was observed for LmpC upon treatment with N-glycosidase (Fig. 6D). The cross-reacting bands that are present in Fig. 6D but not in A or B are due to the fact that the antisera that were used for this assay were not affinity-purified.

In the extracellular domain of bovine CD36, the formation of intrachain disulfide bonds has been reported, whereas mammalian LIMPII is lacking one conserved cysteine and therefore predicted to have a different pattern of disulfide bridges (53). The position of the six cysteine residues in the extracellular part of CD36 is not conserved in LmpA, LmpB, or LmpC, due to many sequence insertions as compared with the homologous proteins from higher eukaryotes. In LmpB, there are only three cysteine residues in the putative intravesicular domain, and their position does not correspond to the pattern found in LmpA or LmpC. In LmpC, however, the position of four cysteine residues in the central domain corresponds exactly to the pattern found in LmpA (Cys-343, Cys-354, Cys-489, and Cys-503), so two intrachain disulfide bonds might be formed in these proteins. However, the electrophoretic mobility of LmpA, LmpB, and LmpC was similar under reducing and non-reducing conditions (not shown), even after previous enzymatic N-deglycosylation. For human CD36, changes in the electrophoretic behavior that indicate the existence of disulfide bonds have been reported to be difficult to observe (54).

**Subcellular Fractionation**—In order to clarify the nature of the Lmp-positive vesicles, subcellular fractionation was performed by differential centrifugation on sucrose gradients (Fig. 7). The marker enzymes acid phosphatase and alkaline phosphatase were assayed to distinguish between vesicles of lysosomal or plasma membrane origin, respectively. Whereas the acid phosphatase activity showed a peak in the first fractions (low density) containing mainly lysosomes, the alkaline phosphatase was found together with membranes of intermediate and high density, reflecting the distribution of the contractile vacuole membranes and vesicles of plasma membrane origin (33). Furthermore, by densitometric scanning of immunoblots, the distribution of marker proteins was assayed. The lysosomal protein α-L-fucosidase showed a distribution similar to the acid phosphatase and was restricted to the second and third fraction. The Golgi marker comitin and the contact site A protein, a cell adhesion protein expressed in developing cells, distributed with the alkaline phosphatase to
fractions of intermediate to high density. In the fractions of highest density, nuclei and endoplasmic reticulum membranes are reported to be localized (33). Interestingly, the presumed lysosomal protein LmpA was distinct from the lysosomal marker α-L-fucosidase but rather co-migrated with membranes of higher density, which is comparable to the localization reported for early endosomes and postlysosomes (55). Essentially the same localization was found for the newly discovered isoform LmpC. In contrast to this, LmpB comigrated with membranes of even higher density than LmpA or LmpC. This particular fraction of the gradient was also positive for golveisin, a marker for intermediate endosomes and Golgi (56). However, a major part of the golveisin staining was observed in very dense fractions at the bottom of the gradient.

DISCUSSION

LmpB and lmpC, Two New CD36/LIMPII Homologues from Dictyostelium—In a search for a complete set of CD36/LIMPII proteins from *D. discoideum*, we have identified several open reading frames that could all be attributed either to the already described gene *lmpA* or to two novel genes that have been named *lmpB* and *lmpC*, respectively. Like in the other members of this family, there is a high probability of two transmembrane regions in both proteins, one near the carboxyl terminus and the other one at the amino terminus. Between both hydrophobic sequence stretches, several consensus sites for N-glycosylation have been found. LmpB is characterized by a dileucine sequence at the COOH terminus, which is very similar to the lysosomal sorting signal for rat or human LIMPII and contains in addition one potential site for O-glycosylation. The coding sequence of *lmpC* was found to be disrupted by two short introns, and based on phylogenetic analysis and several structural features it is evident that *lmpA* and *lmpC* are more closely related and might in fact have arisen by a gene duplication event. Even though LmpB is more distantly related to both LmpA and LmpC, it shares with the other two proteins some important structural features, such as the large N-glycosylated intravesicular domain that is flanked by hydrophobic domains near the carboxyl and the amino termini. The overall topology of these proteins is apparently highly conserved and corresponds to the hairpin topology found in CD36/LIMPII family proteins from higher eukaryotes (24, 47, 49, 50). We could experimentally confirm both the N-glycosylation and the proposed topology of the products for all three different *lmp* genes. This finding clearly shows that the homology among the
CD36/LIMPII members from amoeba to man extends beyond the primary sequence and that structural features of these proteins have also been conserved in the course of evolution. The antisera that were raised against the amoeba Lmp isoforms showed cross-reaction when tested on cells from higher eukaryotes. The antisera recognized vesicular structures in immunofluorescence studies on the endothelial cell line Xth2 from the frog Xenopus (not shown), which points to the fact that some epitopes on CD36/LIMPII family proteins may have been conserved throughout evolution. However, the functional importance of the topology of the CD36/LIMPII family is still under debate, since the putative carboxyl-terminal transmembrane domain of CD36 has been shown by deletion mutants to be dispensable for its function (57). Further analysis of the newly discovered lmp genes in the genetically tractable amoeba could contribute to solve this question.

Possible Functions of LmpB and LmpC—Immunofluorescence studies revealed a presence of both LmpB and LmpC in vesicles and sometimes also in larger, ring-like structures with a diameter of 2 μm that resemble macropinosomes. Numerous punctate, vesicular structures were found to be positive for the fluid phase marker TRITC-dextran and either LmpB or LmpC, which suggests that both proteins play a role in the endosomal pathway. In fact, the presence of LmpA in macropinosomes has been observed previously (9, 13). Macropinosomes are structures that are derived from large actin-containing membrane ruffles at the dorsal surface of the cell, which circularize to large vesicles containing extracellular fluid (58), and are distinct from the small sized (0.2 μm) clathrin-coated pinosomes (59). Macropinosomes from Dictyostelium (60) have been described to be very similar in size and appearance to those observed in mammalian cells (61). The process of fluid internalization in Dictyostelium has been shown to depend both on clathrin (62) and the act cytoskeleton (60). Mutants for the act-binding protein coronin, which localizes to dynamic membrane protrusions known as “crows,” show a drastic reduction in phagocytosis (63), and disruption of the myosin IB gene was reported to result in a slower rate of particle uptake (64). The amount of DdLIMP double-labeled vesicles was strongly decreased even after a 15-min chase, but nonetheless, all three DdLIMPs were present in TRITC-dextran-positive structures even after a 45-min chase. This means that members from this protein family can be present in endosomal structures ranging from macropinosomes to lysosomes. However, there are some differences among the family members. Whereas the vast majority of TRITC-dextran-positive vesicles found inside a cell were also labeled with LmpA, both LmpB and LmpC co-localized only to a fraction of the endocytic vesicles. The strong co-localization of LmpA with TRITC-dextran-positive endosomal vesicles is in good accordance with functional data obtained with lmpA-minus cells, which indeed display defects in macropinocytosis (13).

The intracellular distribution and the overall sequence homologies of the newly discovered Lmp proteins are similar to the putative phospholipid carrier/transporter LmpA, so the question was raised whether the two new Lmp proteins would also show a functional redundancy or resemblance to LmpA. Genetic disruption of lmpA partially suppressed the defects of the profilin-minus strain, and development, cytokinesis, and endosomal trafficking defects were restored to wild-type levels (9, 13). Dictyostelium cells that harbor a genetic disruption of lmpA in a wild-type background showed, in accordance to the results obtained with the profilin/lmpA double-minus strains, a normal developmental cycle, and their growth rates on bacterial lawn or in submersion culture were equal to wild-type levels. However, they were unable to grow in shaking culture (9); the rate of pinocytosis was reduced to 25% of the wild-type levels, and the rate of efflux of fluid phase was also greatly reduced, which suggests a block in a transport step from lysosomes to postlysosomes (13). The lmpA-minus strain T2.25 and the profilin-suppressor mutant RB2 that lacks lmpA and profilin I and II (9) were analyzed for potential differences in the transcript levels of lmpB and lmpC under axenic growth conditions. Whereas the relative amount of lmpB transcript was not significantly altered as compared with AX2 wild-type cells, the lmpC-mRNA was slightly up-regulated by a factor of 1.5 (not shown). The expression levels of the LmpB and LmpC
proteins were essentially the same as in wild-type cells (not shown). The profilin-suppressor LmpA is believed to play an important role at the interface of phospholipid metabolism and the actin cytoskeleton, especially in the uptake or structural organization of phosphatidylinositol phospholipids (reviewed in Ref. 65). It has been proposed that LmpA could create clusters of PIP2 in the surrounding membrane regions, which might act like a nucleating point for aggregation of further PIP2-binding proteins, e.g., cytosolic actin-binding proteins or membrane-bound enzymes. The lack of a PIP2-binding motif in the sequences of LmpB and LmpC argues against an involvement of these two new members of the CD36/LIMPII family in the PIP2 pathway. However, the subcellular localization of LmpB and LmpC points toward a possible role in vesicle transport, may be in fusion or maturation steps of macropinocytosis.

The fact that both LmpA and LmpC are expressed at all developmental stages could be taken as indication for a housekeeping function of these gene products. However, there is a significant down-regulation of LmpA on the protein level at the onset of development, which, together with the fact that LmpA strains are able to develop normally, argues against a specific role of LmpA for Dictyostelium development. In contrast, the more divergent isoform LmpB shows a unique biphasic regulation. All three Lmp isoforms are present in growth phase, but only LmpB is strongly up-regulated in late development, which is evident both on transcript as well as on protein level. This could indicate an involvement of LmpB in late developmental events, like culmination or spore formation.

Three CD36/LIMPII Proteins, Two Different Sorting Signals—The apparently divergent endocytic sorting motifs of the LmpA/C proteins also deserve attention. Mammalian LIMPIII has been shown to interact with the recently identified non-clathrin AP-3 adaptor complex via a di-leucine signal (Leu-475 and Ile-476) near its COOH terminus (25, 66). However, LmpA shows a di-leucine motif in the short cytoplasmic tail (Ile-753 and Ile-754). It has been shown recently (67) for mammalian LIMPIII that acidic amino acid residues (Asp-470 and Glu-471) in the proximity of the di-leucine signal in the COOH-terminal tail may act like a nucleating point for aggregation of further cytosolic actin-binding proteins or -L-fucosidase, and acid phosphatase.

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