Generation and Characterization of Monoclonal Antibodies That Specifically Recognize p65/L-Plastin Isoform but Not T-Plastin Isoform

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The 65-kDa protein (p65) was previously identified as a phosphorylated protein in activated macrophages, and has turned out to be a member of a plastin protein family characterized by a series of Ca$^{2+}$-, calmodulin-, and $\beta$-actin-binding domains. In mice, two isoforms, p65/L-plastin and T-plastin, have so far been identified; p65/L-plastin is expressed in hemopoietic cells and cancer cells, and T-plastin in solid tissue cells. We generated monoclonal antibodies to p65/L-plastin, examined the isoform-specificity by using recombinant (r) T-plastin, and found that the antibodies were specific for rp65/L-plastin, whereas immune sera to rp65/L-plastin showed cross-reactions to rT-plastin. One of the antibodies, p65-7B5, was demonstrated to react to native p65/L-plastin by Western blot, flow cytometric, and immunohistochemical analysis. Furthermore, p65-7B5 has made it possible to detect p65/L-plastin-expressing cells in tissues where T-plastin is abundantly expressed. These reagents and procedures should provide specific tools to investigate the role of p65/L-plastin in leukocytes.

Key words: p65/L-plastin; monoclonal antibody; plastin family; leukocyte; cancer

The actin cytoskeleton is an essential component of all eucaryotic cells and is involved in diverse cellular functions such as cell motility, cell division, cellular adhesion, and control of cellular shape.1) It is especially important in leukocytes such as macrophages that are highly motile and move rapidly to infectious and inflammatory sites. We have purified and identified a 65-kDa cytosolic protein (p65) that was heavily phosphorylated in macrophages in response to bacterial lipopolysaccharide (LPS), and found that the protein contains two Ca$^{2+}$-, a calmodulin-, and two actin-binding domains,2–4) suggesting that p65 exerts its function on the actin cytoskeleton in a phosphorylation- and/or Ca$^{2+}$-dependent manner. Interestingly, it has turned out to be a murine homolog of human L-plastin: a protein originally identified as a novel transformation-induced protein in cancer cells.5,6) Plastins are known to be a family of actin-binding proteins that are evolutionarily conserved from animal to plant cells.2,5,7,8) In mice, two isoforms, L-plastin and T-plastin, have so far been identified. The T-plastin isoform is constitutively expressed in epithelial and mesenchymal cells of solid tissues, while the p65/L-plastin isoform is expressed in hemopoietic cell lineages and in many types of cancer cells.3,5,9–12) The molecular basis whereby these isoforms are cell type-dependently expressed is largely unknown. The two isoforms differ in 21% of amino acid sequences, and the phosphorylation site (Ser-5) of p65/L-plastin that we previously determined4) is not found in T-plastin. Jones et al. have recently shown that the Ser-5 phosphorylation of p65/L-plastin regulates $\beta_2$-integrin-mediated leukocyte adhesion.13) This leads to speculate that the transformation-dependent expression of p65/L-plastin in cancer cells may be related to their acquisition of migration and invasion, in which adhesion molecules are involved. Since leukocytes and cancer cells often migrate to or invade other tissues where T-plastin is abundantly expressed, specific monoclonal antibodies would be highly useful for investigating the biological functions of p65/L-plastin in more detail. In the present study, we generated monoclonal antibodies to murine p65/L-plastin and examined their specificity using recombinant (r) T-plastin. We also developed experimental procedures employing these reagents, and this made it...
possible to analyze specifically the p65/L-plastin isoform in cells and tissues.

Materials and Methods

Reagents. The sources of materials used in this work are as follows: chromatography-purified protein-free LPS of Salmonella typhimurium from Sigma-Aldrich (St. Louis, MO); pT7Blue T-vector, EASYPrep RNA, First-strand cDNA Synthesis Kit, and DNA Ligation Kit from Takara Bio (Tokyo); ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit from Applied Biosystems (Foster City, CA); pET-3a vector plasmid from Novagen (Madison, WI); Freund’s complete adjuvant from Difco (Detroit, MI); Seph          

Mice. Specific pathogen-free BALB/c and ICR mice (8–15 weeks old) were purchased from Clea Japan (Osaka, Japan). All mice were used in accordance with our institutional guidelines for animal experiments.

Murine T-plastin cDNA cloning. Total RNA was isolated from mouse liver using EASYPrep RNA, and cDNA was prepared using a First-strand cDNA Synthesis kit. A cDNA fragment containing the full-length coding region was amplified using the PCR method with the synthesized cDNA as a template and two primers (S’-CATATGGATGAGATGGCGACCACC-3’, including the sequences for N-terminal amino acids and an additional NdeI site, and S’-GACGTCTTACACTCTCTTATCCCTGTCG-3’, including the complementary sequences for C-terminal amino acids and an additional AarII site). The primer sequences were designed according to information in the NCBI data base. The PCR-generated DNA fragment was inserted into pT7Blue T-vector, and the construct was used to transform E. coli BL-21 (DE3), and expression was induced with isopropyl-β-d-thiogalactopyranoside. The expression of the target gene was assessed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Large-scale cultures were then performed and rT-plastin was extracted from E. coli with lysis buffer containing 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin, and 200  µg/ml lysozyme for 30 min at 30°C, followed by sonication. The rT-plastin was recovered in the supernatant of the lysates after centrifugation and further purified by sequential chromatography on Sephacryl S-200, HiPrep anion exchange, and hydroxymapatite columns according to the protocol for preparing rp65/L-plastin, as previously described.14)

Preparation of monoclonal antibodies. Recombinant mouse p65/L-plastin used as antigen was expressed in E. coli and purified as previously reported.14) BALB/c mice were immunized with rp65/L-plastin (30 µg/mouse) emulsified with Freund’s complete adjuvant. The mice were boosted with the same protein three times every 2 weeks. Splenic B cells of the mice were fused with Ag8.653 myeloma cells by the methods of Galfré and Milstein.15) Hybridomas were cultured in HAT (hypoxanthine, aminopterin, and thymidine) selection medium. Screening of culture supernatants was done by ELISA using rp65/L-plastin as coating antigen. After single-cell cloning by limiting dilution, three different clones, p65-7B5, p65-7F6, and p65-3G5, were finally established. One of these clones, p65-7B5, was cultured on a large scale in a serum-free medium, and the IgG fractions were precipitated with ammonium sulfate and further purified with a HiPrep anion exchange column.

Western blot analysis. Western blot analysis was done as described previously.14) In brief, macrophage lysates were solubilized in SDS–PAGE sample buffer and analyzed on 12% SDS–PAGE gels. Samples separated by SDS–PAGE were electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk in Tris-buffered saline, washed, and reacted with p65-7B5. After washing, the membranes were reacted with horseradish peroxidase (HRP)-coupled anti-mouse IgG and washed. Bands were detected using ECL Western blotting detection reagents.

Enzyme-linked immunosorbent assay (ELISA). An ELISA assay for detecting anti-p65/L-plastin antibodies was done as described previously.14,16) In brief, ELISA plates were coated with 50 µl of rp65/L-plastin, rT-plastin, or BSA (2.0 µg/ml) in 0.1 M carbonate–bicarbonate buffer (pH 8.5). After the plates were blocked, 100 µl of serial dilutions of monoclonal antibodies was added to each well and incubated. After washing, diluted alkaline phosphatase (AP)-conjugated anti-mouse IgG
was added. After washing, enzyme substrate (4-methylumbelliferylphosphate dilitium salt) was added to each well, and fluorescent signals were measured by Cytofluor (Perceptive Biosystems, Framingham, MA).

**Fluorescence flow cytometric (FACS) analysis.** Intracellular staining was carried out as described previously. In brief, mouse spleen cells or thymocytes were fixed with 0.1 M PBS containing 4% paraformaldehyde and 4% sucrose. The fixed cells were permeabilized with a PBS solution containing 0.1% saponin and 2% BSA, and then washed and reacted with fluorescein-labeled p65-7B5. Stained cells were analyzed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) with the CellQuest software (BD Bioscience, San Jose, CA).

**Immunohistochemical analysis.** Macrophage: Mouse macrophage J774.1 cells were allowed to adhere on cover slips in a culture medium containing 10% heat-inactivated fetal bovine serum, and stimulated with LPS (100 ng/ml) for 30 min or 6 h. Immunostaining was done as described previously. In brief, macrophages were fixed in 2% paraformaldehyde in PBS, washed, and permeabilized in 0.1% saponin in PBS. The cells were reacted with p65-7B5, washed, and reacted with Alexa 488-labeled anti-mouse IgG. Immunofluorescent signals were viewed with a confocal laser scanning microscope system (LSM 510; Carl Zeiss, Jena, Germany).

Liver: Mice were perfused with PBS and then 4% paraformaldehyde–0.1 M PB under ether anesthesia. The liver was removed and cut into pieces. The tissue samples were immersed in the same fixative at 4°C, transferred to 30% sucrose–0.1 M PB, and frozen in dry ice/acetone. Frozen sections were cut at 6μm on a cryostat, thaw-mounted on slide glasses, and stored at −80°C until use. Immunohistochemical staining was performed using biotinylated p65-7B5 and an avidin–biotin peroxidase complex (ABC) kit, as described previously.

**Results**

**Development of monoclonal antibodies against p65/L-plastin**

Although p65/L-plastin is a mouse protein, we expected that mice would produce antibodies against the protein when strongly immunized with the antigen emulsified in Freund’s complete adjuvant, since p65/L-plastin is an intracellularly sequestered antigen. Hence we thus immunized BALB/c mice with rp65/L-plastin and prepared hybridomas using their splenic B cells as described in “Materials and Methods.” After screening for their capacity to produce anti-p65/L-plastin antibodies, three clones were finally established. The monoclonal antibodies produced by the clones were designated p65-3G4, p65-7B5, and p65-7F6. The Ig subclass and light chain types were p65-3G4 (IgG1, κ), p65-7B5 (IgG1, κ), and p65-7F6 (IgG1, κ) respectively.

**Preparation of rT-plastin**

In order to assess the specificity of the monoclonal antibodies, we cloned full-length murine T-plastin gene (DDBJ accession no. AB182243) and expressed rT-plastin in E. coli. The rT-plastin was recovered in a soluble fraction of E. coli lysates and further purified by sequential chromatography on HiPrep anion exchange, Sephacryl S-200, and hydroxyapatite columns. The purified protein had an apparent molecular mass of 68 kDa as determined by SDS–PAGE (Fig. 1).

**Reactivity of monoclonal antibodies to rp65/L-plastin and rT-plastin**

Diluted hybridoma culture supernatants were added to each well of microtiter plates that had been coated with rp65/L-plastin, rT-plastin, or BSA. The bound antibodies were detected as described in “Materials and Methods.” Three monoclonal antibodies, p65-7B5, p65-7F6, and p65-3G5, were found specifically to recognize rp65/L-plastin but not rT-plastin, while polyclonal antiserum raised against rp65/L-plastin was considerably cross-reactive with rT-plastin (Fig. 2A). Since production of p65-7B5 was better than that of the others, we purified a large amount of p65-7B5 and assessed its reactivity to rp65/L-plastin and rT-plastin over a wide range of concentrations. As shown in Fig. 2B, p65-7B5 did not react to rT-plastin even at as much as 10μg/ml of the antibody, suggesting that p65-7B5 recognizes a p65/L-plastin-specific determinant.

**Reactivity of p65-7B5 to native p65/L-plastin**

Next we confirmed that p65/L-plastin reacted with...
native p65/L-plastin, as well as r-p65/L-plastin that was used as an antigen for mouse immunization and ELISA assay. As shown in Fig. 3A, it was found by Western blot analysis that p65-7B5 reacted with native p65/L-plastin in macrophage lysates. Flow cytometric analysis of intracellular p65/L-plastin in leukocytes also revealed that p65-7B5 detected native p65/L-plastin at the individual cell level (Fig. 3B). The procedure also demonstrated that the amount of p65/L-plastin in mature splenic lymphocytes was much higher than that in less differentiated thymocytes.

**Immunohistochemical analysis using p65-7B5**

The development of p65-7B5 that reacts to p65/L-plastin but not to T-plastin enabled us to perform immunohistochemical analysis of tissues containing both leukocytes and non-leukocytes cells. First we tested the stainability of macrophages with p65-7B5. When the J774 macrophage cell line stimulated with LPS for 30 min or 6 h was fixed with paraformaldehyde, permeabilized with saponin, and stained with p65-7B5 together with a second antibody, intracellular p65/L-plastin was clearly detected, as shown in Fig. 4A. It is of note that the stainability of p65/L-plastin was stronger in J774.1 cells stimulated with LPS for 6 h than in those stimulated for 30 min, and that the former cells exhibited more spread shape. Next we stained liver sections that contained parenchymal cells and non-parenchymal cells such as Kupffer cells (liver macrophages) and lymphoid cells. Liver parenchymal cells expressed T-plastin, since the mRNA used for the T-plastin gene cloning was isolated from the liver. Figure 4B clearly shows that Kupffer cells and lymphocyte-like cells were positively stained with p65-7B5 while parenchymal cells were not, even when these cells were closely adjacent to each other.

**Discussion**

In this report, we describe the development and
characterization of monoclonal antibodies that recognize p65/L-plastin. We also prepared recombinant murine T-plastin (Fig. 1), examined the specificity of the monoclonal antibodies to the two isoforms, and found that they exclusively recognized the p65/L-plastin isoform (Fig. 2). One of the monoclonal antibodies, designated p65-7B5, was prepared in a large quantity, and flow cytometric and immunohistochemical analyses using the antibody were performed (Figs. 3 and 4). The availability of p65-7B5 has for the first time made it possible specifically to detect p65/L-plastin in tissues where T-plastin is abundantly expressed (Fig. 4B).

p65/L-Plastin was identified as a 65-kDa cytosolic protein heavily phosphorylated in macrophages by bacterial stimulation and expressed in hematopoietic cells including neutrophils and lymphocytes. This protein is characterized by Ca$^{2+}$-, calmodulin, and actin-binding domains, suggesting that it exerts its function on the cytoskeleton in a phosphorylation- and/or Ca$^{2+}$-dependent manner. We further determined the phosphorylation site of p65/L-plastin (Ser-5). Regarding this, Jones et al. reported that the Ser-5 phosphorylation of the protein regulates $\beta_2$-integrin-mediated leukocyte adhesion, indicating that the protein contributes to host defense via modulation of cytoskeleton-integrin interactions. Indeed, it has been clarified recently that leukocytes of p65/L-plastin-gene disrupted mice cannot generate an adhesion-dependent respiratory burst and that the mice are susceptible to bacterial infections.

Though the molecular mechanism whereby p65/L-plastin regulates cell adhesion via the cytoskeleton is largely unknown, we observed that fiber-like structures of p65/L-plastin were generated in the extended area of LPS-stimulated macrophages (Fig. 4A). This might be related to the increased adhesion of the macrophages.

Leukocytes like macrophages are able to recruit into inflamed or infected tissues through adhesion-based cellular locomotion, and function there. Cells composing solid tissues are known to express the T-plastin isoform. Since the amino acid homology between the two isoforms is 79%, polyclonal antibodies raised against p65/L-plastin show cross-reactions to T-plastin (Fig. 2A). Thus monoclonal p65-7B5 without cross-reactivity to T-plastin should be highly useful for detecting p65/L-plastin-expressed cells in tissues (Fig. 4B).

In addition to leukocytes, many types of cancer cells express p65/L-plastin, as we reported previously. The function of p65/L-plastin in cancer cells is not known, but is supposed to be similar to that in leukocytes, that is, reorganization of the actin cytoskeleton. It is conceivable that cancer cells acquire the ability to migrate into body tissues like macrophages by expressing p65/L-plastin. Since their original epithelial cells do not express p65/L-plastin but express T-plastin, p65-7B5 has enabled us to analyze p65/L-plastin-expressing cancer cells surrounded by normal tissue cells. Such studies are currently in progress in our laboratory.

In summary, monoclonal antibodies to p65/L-plastin and experimental procedures such as ELISA and the flow cytometric and immunohistochemical methods described in this report should provide powerful tools to investigate the functions of p65/L-plastin at the molecular level.

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![Fig. 4. Immunohistochemical Analysis of p65/L-Plastin-Expressing Cells.](image)

A. J774.1 cells that had been stimulated with 100 ng/ml of LPS for 30 min (a, b) or 6 h (c, d) were fixed, permeabilized in 0.1% saponin, and blocked as described in "Materials and Methods." Then samples were reacted with p65-7B5, washed, and reacted with Alexa 488-labeled anti-mouse IgG. The stained cells were observed with a confocal laser microscope (a, c), and by phase contrast microscopy (b, d) (bar = 10 $\mu$m). B. Prepared sections of liver were stained by the avidin-biotin peroxidase complex (ABC) method with p65-7B5 and a commercial ABC kit, and counterstained with hematoxylin. Kupffer cells (▼) and lymphocyte-like cells (◇) that express p65/L-plastin show a brown color, while liver parenchymal cells (→) that express T-plastin show a blue color (bar = 10 $\mu$m).
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References


