

Description

Title of Invention: MULTISPECIFIC ANTIBODY FOR LYSOSOMAL DEGRADATION

Technical Field

- [1] The present invention relates to a multispecific antibody for the degradation of a target protein using lysosomes in the body and a method for producing the same.

[2]

Background Art

- [3] Since the possibility of using antibodies targeting specific antigens as therapeutic agents was suggested, bi- or multispecific antibodies capable of simultaneously binding to two or more different antigens have also been developed. Such antibodies capable of targeting multiple proteins like this have high potential for use in the treatment of diseases caused by the complex action of multiple proteins, and are expected to be used in various forms for the development of therapeutic agents (KR 10-2023-0163305 A).
- [4]
- [5] Meanwhile, in the development of therapeutic agents for various diseases including cancer, studies have been continuously conducted on a technology (Targeted Protein Degradation) that induces the degradation of disease-causing proteins by utilizing the intracellular protein degradation system. Targeted protein degradation technology is a technology that directly degrades and eliminates disease-causing proteins, and can be expected to have a high therapeutic effect since the proteins that cause diseases are fundamentally eliminated. To date, PROTAC (Proteolysis-Targeting Chimera), LYTAC (Lysosome Targeting Chimera), AUTAC (Autophagy-targeting Chimera) and the like have been reported as the targeted protein degradation technology.
- [6] Among these, LYTAC is complex of antibody directly conjugated to mannose-6-phosphate (M6P) polymer chains; a degradation system in which LYTAC binds to both target protein and the M6P receptors on the cell membrane to form endosomes, and the endosomes mature to lysosomes to degrade target proteins present in the cell membrane or outside of the cell. LYTAC technology uses antibodies and has the advantage of high selectivity for target proteins but has the problem that the synthetic process of linking sugar chains to antibodies is complex and takes a long time.
- [7]

Disclosure of Invention

Technical Problem

- [8] There is a need to develop a drug that is easily produced, has excellent proteolytic capacity, and effectively transport target proteins to lysosomes for degradation.

[9]

Solution to Problem

- [10] An object of the present invention is to provide a multispecific antibody comprising a lysosomal transporting receptor binding region and a target protein binding region.

- [11] Another object of the present invention is to provide a polynucleotide encoding the multispecific antibody.

- [12] Still another object of the present invention is to provide an expression vector comprising the polynucleotide.

- [13] Still another object of the present invention is to provide a cell transformed with an expression vector comprising the polynucleotide.

- [14] Still another object of the present invention is to provide a method for producing the multispecific antibody.

- [15] Still another object of the present invention is to provide a use of the multispecific antibody for the degradation of a target protein.

[16]

Advantageous Effects of Invention

- [17] The multispecific antibody according to the present invention transports target proteins to lysosomes and induces the degradation thereof, and can be used to treat diseases associated with the target proteins.

[18]

Brief Description of Drawings

- [19] FIG. 1 is a diagram illustrating three types of multispecific antibodies constructed in the present invention;

- [20] FIG. 2 is a diagram illustrating multispecific antibodies prepared by altering the lysosomal transporting receptor binding region and target protein binding region in three types of multispecific antibodies according to the present invention;

- [21] FIG. 3 is a diagram illustrating multispecific simultaneous binding to a lysosomal transporting receptor and a target protein;

- [22] FIG. 4 is a data showing a decrease in the level of cell surface EGFR by a multispecific antibody;

- [23] FIG. 5 is a data showing the degradation of EGFR by a APLP2 targeting multispecific antibody;

- [24] FIG. 6 is a data for demonstrating that a APLP2 targeting multispecific antibody degrades EGFR in a lysosome dependent manner;

- [25] FIG. 7 is a data showing a decrease in the level of cell surface EGFR by a M6PR targeting multispecific antibody;
- [26] FIG. 8 is a data showing the preparation of B-type Lportbodies with different APLP2 binding sequences and their EGFR degradation abilities thereof;
- [27] FIG. 9 is a data showing the preparation of B-type Lportbodies with different M6PR binding sequences and their EGFR degradation abilities thereof;
- [28] FIG. 10 is a data for demonstrating that B-type APLP2-Lportbodies degrade EGFR in a lysosome-dependent manner;
- [29] FIG. 11 is a data for demonstrating that B-type M6PR-Lportbodies degrade EGFR in a lysosome-dependent manner;
- [30] FIG. 12 is a data showing the cross-reactivity with human and mouse APLP2;
- [31] FIG. 13 illustrates the results of epitope binning;
- [32] FIG. 14a shows a comparison for the degradation efficiency of A, B, and C types of APLP2-Lportbodies against the target protein;
- [33] FIG. 14b shows the comparison for the degradation efficiency of A and B types of APLP2-Lportbodies against their target protein;
- [34] FIG. 15 shows the comparison for the degradation efficiency of A and B types of M6PR-Lportbodies in their target protein;
- [35] FIG. 16 is a data showing the dose-dependent responsiveness to degradation of B type of APLP2-Lportbodies;
- [36] FIG. 17 is a diagram illustrating the tissue versatility of B type of APLP2-Lportbodies multispecific;
- [37] FIG. 18 is a diagram illustrating the soluble protein internalization of three types of APLP2-Lportbodies;
- [38] FIG. 19 is a diagram illustrating the lysosome-dependent soluble EGFR degradation of a APLP2-Lportbody; and
- [39] FIG. 20 is a diagram illustrating two kinds of type B multispecific antibodies according to the present invention.
- [40]

Best Mode for Carrying out the Invention

- [41] An aspect embodying the present invention is a multispecific antibody comprising a lysosomal transporting receptor binding region and a target protein binding region.
- [42] In a specific embodiment, one of the lysosomal transporting receptor binding region or target protein binding region of the multispecific antibody includes a first heavy chain variable region, while the other includes a second heavy chain variable region, a heavy chain constant region, a light chain constant region and a light chain variable

region, and the first heavy chain variable region is connected to the second heavy chain variable region.

- [43] In another specific embodiment, the lysosomal transporting receptor binding region includes a heavy chain variable region, a heavy chain constant region, a light chain constant region, and a light chain variable region, and the target protein binding region is connected to the heavy chain variable region of the lysosomal transporting receptor binding region.
- [44] In still another specific embodiment, the target protein binding region includes a heavy chain variable region, a heavy chain constant region, a light chain constant region, and a light chain variable region, and the lysosomal transporting receptor binding region is connected to the heavy chain variable region of the target protein binding region.
- [45] In a specific embodiment according to any one of the preceding specific embodiments, the first heavy chain variable region includes or consists of a human-derived VH domain or an animal-derived VHH domain such as a camelid.
- [46] In a specific embodiment according to any one of the preceding specific embodiments, the lysosomal transporting receptor binding region and the target protein binding region include a heavy chain variable region.
- [47] In a specific embodiment according to any one of the preceding specific embodiments, in the multispecific antibody, a heavy chain variable region of the lysosomal transporting receptor binding region and a heavy chain variable region of the target protein binding region are connected to each other.
- [48] In a specific embodiment according to any one of the preceding specific embodiments, the heavy chain constant region includes an Fc region.
- [49] In a specific embodiment according to any one of the preceding specific embodiments, the heavy chain constant region includes a hinge region.
- [50] In a specific embodiment according to any one of the preceding specific embodiments, the multispecific antibody has a structure in which monomers in which a lysosomal transporting receptor binding region and a target protein binding region are connected symmetrically form a dimer.
- [51] In a specific embodiment according to any one of the preceding specific embodiments, in the multispecific antibody, a lysosomal transporting receptor binding region and a target protein binding region are symmetrically connected by a disulfide bond in the hinge region.
- [52] In a specific embodiment according to any one of the preceding specific embodiments, the lysosomal transporting receptor binding region includes a heavy chain variable region (VH or VHH) of an antibody against a lysosomal transporting receptor, a fragment thereof, or an antigen-binding site thereof.

- [53] In a specific embodiment according to any one of the preceding specific embodiments, the lysosomal transporting receptor binding region binds to mannose 6-phosphate receptor (M6PR) or amyloid precursor-like protein 2 (APLP2).
- [54] In a specific embodiment according to any one of the preceding specific embodiments, the target protein binding region binds to a target protein.
- [55] In a specific embodiment according to any one of the preceding specific embodiments, the target protein binding region includes a heavy chain variable region (VH or VHH) of an antibody against a target protein, a fragment thereof, or an antigen-binding site thereof.
- [56] In a specific embodiment according to any one of the preceding specific embodiments, the target protein is epidermal growth factor receptor (EGFR).
- [57] In a specific embodiment according to any one of the preceding specific embodiments, the heavy chain variable region of the lysosomal transporting receptor binding region and the heavy chain variable region of the target protein binding region are connected via a linker.
- [58] In a specific embodiment according to any one of the preceding specific embodiments, the multispecific antibody induces endocytosis and degradation by a lysosome of a target protein.
- [59] Another aspect embodying the present invention is a multispecific antibody comprising an antibody or an antigen-binding fragment thereof that specifically binds to amyloid precursor-like protein 2 (APLP2).
- [60] Still another aspect embodying the present invention is a multispecific antibody comprising an antibody or an antigen-binding fragment thereof that specifically binds to mannose 6-phosphate receptor (M6PR).
- [61] Still another aspect embodying the present invention is a polynucleotide encoding the multispecific antibody.
- [62] Still another aspect embodying the present invention is an expression vector comprising the polynucleotide.
- [63] Still another aspect embodying the present invention is a cell transformed with the expression vector.
- [64] Still another aspect embodying the present invention is a method for producing a multispecific antibody, comprising culturing the cell to express a multispecific antibody.
- [65] In a specific embodiment, the production method further comprises recovering the multispecific antibody.
- [66] In another specific embodiment, the production method further comprises purifying the multispecific antibody.

[67] Still another aspect embodying the present invention is a composition comprising the multispecific antibody.

[68] In a specific embodiment, the composition is a pharmaceutical composition.

[69] Still another aspect embodying the present invention is a use of the multispecific antibody for the degradation of a target protein.

[70]

Mode for the Invention

[71] The specific details for carrying out the present invention are as follows.

[72] Meanwhile, each description and each embodiment disclosed in the present disclosure can also be applied to another description and another embodiment, respectively. In other words, all combinations of the various elements disclosed herein fall within the scope of the present invention. Additionally, the scope of the present invention is not limited by the specific description described below.

[73] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific aspects of the present invention described in this application. Additionally, such equivalents are intended to be included in the present invention.

[74]

[75] An aspect embodying the present invention provides a multispecific antibody comprising a lysosomal transporting receptor binding region and a target protein binding region.

[76] The multispecific antibody of the present invention has a form such as Type B in FIG. 1. In the multispecific antibody having the form of Type B, the lysosomal transporting receptor binding region and the target protein binding region are connected to be adjacent to each other.

[77]

[78] In a specific aspect, the lysosomal transporting receptor binding region includes a heavy chain variable region, a heavy chain constant region, a light chain constant region, and a light chain variable region, and the target protein binding region is connected to the heavy chain variable region of the lysosomal transporting receptor binding region. At this time, the target protein binding region may include a heavy chain variable region of an antibody that binds to a target protein, an antigen-binding site thereof, or a fragment thereof. Here, the heavy chain variable region of the target protein binding region may be in the form of a single domain (VH or VHH) (FIG. 20A).

[79] In another specific aspect, the target protein binding region includes a heavy chain variable region, a heavy chain constant region, a light chain constant region, and a

light chain variable region, and the lysosomal transporting receptor binding region is connected to the heavy chain variable region of the target protein binding region. At this time, the lysosomal transporting receptor binding region may include a heavy chain variable region of an antibody that binds to a lysosomal transporting receptor, an antigen-binding site thereof, or a fragment thereof. Here, the heavy chain variable region of the lysosomal transporting receptor binding region may be in the form of a single domain (VH or VHH) (FIG. 20B).

[80]

[81] In a specific embodiment, one of the lysosomal transporting receptor binding region or target protein binding region of the multispecific antibody of the present invention may include a first heavy chain variable region, while the other may include a second heavy chain variable region, a heavy chain constant region, a light chain constant region and a light chain variable region, and the first heavy chain variable region may be connected to the second heavy chain variable region.

[82] In a more specific embodiment, one of the lysosomal transporting receptor binding region or target protein binding region of the multispecific antibody of the present invention may consist of a first heavy chain variable region, while the other may include a second heavy chain variable region, a heavy chain constant region, a light chain constant region and a light chain variable region, and the first heavy chain variable region may be connected to the second heavy chain variable region.

[83] The first heavy chain variable region may consist of, but is not limited to, a VH or VHH domain.

[84]

[85] In an embodiment, the multispecific antibody of the present invention may comprise:

[86] (i-a) a lysosomal transporting receptor binding region including or consisting of a first heavy chain variable region; and

[87] (i-b) a target protein binding region including or consisting of a second heavy chain variable region, a heavy chain constant region, a light chain constant region, and a light chain variable region, or

[88] (ii-a) a target protein binding region including or consisting of a first heavy chain variable region; and

[89] (ii-b) a lysosomal transporting receptor binding region including or consisting of a second heavy chain variable region, a heavy chain constant region, a light chain constant region and a light chain variable region, in which

[90] the first heavy chain variable region may be connected to the second heavy chain variable region.

[91] In another specific embodiment, the multispecific antibody of the present invention may comprise:

- [92] (i-a) a lysosomal transporting receptor binding region including or consisting of a first heavy chain variable region; and
- [93] (i-b) a target protein binding region including or consisting of a second heavy chain variable region, a heavy chain constant region including an Fc region, a light chain constant region, and a light chain variable region, or
- [94] (ii-a) a target protein binding region including or consisting of a first heavy chain variable region; and
- [95] (ii-b) a lysosomal transporting receptor binding region including or consisting of a second heavy chain variable region, a heavy chain constant region including an Fc region, a light chain constant region, and a light chain variable region, in which
- [96] the first heavy chain variable region may be connected to the second heavy chain variable region.
- [97] In still another specific embodiment, the multispecific antibody of the present invention may comprise:
- [98] (i-a) a lysosomal transporting receptor binding region including or consisting of a first heavy chain variable region; and
- [99] (i-b) a target protein binding region including or consisting of a second heavy chain variable region, a heavy chain constant region including an Fc region including a CH1 domain, a hinge region, a CH2 domain, and a CH3 domain, a light chain constant region, and a light chain variable region, or
- [100] (ii-a) a target protein binding region including or consisting of a first heavy chain variable region; and
- [101] (ii-b) a lysosomal transporting receptor binding region including or consisting of a second heavy chain variable region, a heavy chain constant region including an Fc region including a CH1 domain, a hinge region, a CH2 domain, and a CH3 domain, a light chain constant region, and a light chain variable region, in which
- [102] the first heavy chain variable region may be connected to the second heavy chain variable region, and
- [103] monomers in which the lysosomal transporting receptor binding region and the target protein binding region are connected may symmetrically form a dimer. At this time, the dimer may be due to a disulfide bond in the hinge region, but is not limited thereto.
- [104] In still another specific embodiment, the multispecific antibody of the present invention may have a structure such as Type B in FIG. 1, but is not limited thereto.
- [105]
- [106] The multispecific antibody provided in the present invention comprises a site that specifically binds to a lysosomal transporting receptor and a site that specifically binds to a target protein to induce the degradation of the target protein through lysosomes, and is thus capable of achieving the degradation of a target protein in lysosomes. In

particular, the multispecific antibody of the present invention has a structure in which a lysosomal transporting receptor binding region and a target protein binding region are adjacently connected, which offers not only a higher production yield compared to multispecific antibodies in other forms, but also excellent simultaneous binding ability to a lysosomal transporting receptor and a target protein and excellent target protein degradation, and thus provides a novel lysosomal transporting antibody-based multispecific antibody platform that eliminates a target protein by complete the degradation of the target protein by a lysosome.

[107]

[108] The term "antibody" in the present invention refers to a protein molecule capable of specifically recognizing an antigenic site, including an immunoglobulin or a portion thereof that immunologically has reactivity with a specific antigen. The antibody of the present invention includes all of polyclonal antibodies, monoclonal antibodies, whole antibodies, and antibody fragments. The antibody of the present invention includes mouse, human, rabbit, and rat antibodies, and the origin is not limited. The antibody of the present invention includes chimeric antibodies (for example, humanized murine antibodies), humanized antibodies, and minibodies. The antibody of the present invention additionally includes single-chain antibodies having a binding function to FcRn (neonatal Fc receptor), scaffolds, derivatives of antibody constant regions, and artificial antibodies based on protein scaffolds. A whole antibody has a structure that includes two full-length light chains (LC) and two full-length heavy chains (HC), and each light chain may be connected to a heavy chain due to a disulfide bond. The whole antibody includes IgA, IgD, IgE, IgM and IgG, and IgG includes IgG1, IgG2, IgG3 and IgG4 as subtypes. Such an antibody may be produced by cloning each gene into an expression vector by a conventional method, obtaining a protein encoded by the marker gene, and producing the antibody from the obtained protein by a conventional method, but is not limited thereto. Specifically, the multispecific antibody of the present invention may have an IgG-like structure and additionally include a target protein binding region based on the structure of IgG, but is not limited thereto.

[109] In the present invention, the term "fragment" or "antibody fragment" means an arbitrary part of an antibody, and the antibody fragment may be scFv, dsFv, Fab, Fab', F(ab')₂, Fc, Fd, sdAb, and nanobody, and combinations thereof, and the antibody fragment may include, but is not limited to, an antigen-recognizing site.

[110] The term "antigen-binding site" or "antigen-binding site fragment" in the present invention means a fragment possessing an antigen binding function, and the antigen here may be a lysosomal transporting receptor or a target protein. In the present invention, the antigen-binding site may be a fragment that includes a site capable of recognizing an antigenic site.

[111]

[112] Typically, immunoglobulins have a heavy chain and a light chain, and each heavy chain and each light chain include a constant region and a variable region. The variable regions of the light chain and heavy chain include three variable regions called complementarity determining regions (CDRs) and four framework regions (FRs).

[113] The CDRs mainly play a role in binding to the antigenic determinant of an antigen. The CDRs of each chain are typically called sequentially CDR1, CDR2, and CDR3, starting from the C-terminus, and are also identified by the chain on which a particular CDR is located. The complementarity determining regions are located between relatively conserved regions, called constant regions (FR). Each VH and each VL consist of three CDRs and four FRs, arranged in the following order from amino-terminus to carboxy-terminus: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy chain and light chain contain binding domains that interact with an antigen. The CDRs of the heavy chain variable region may be referred to as CDR-H1, CDR-H2, and CDR-H3, the CDRs of the light chain variable region may be referred to as CDR-L1, CDR-L2, and CDR-L3, the FRs of the heavy chain variable region may be referred to as HFR1, HFR2, HFR3, and HFR4, and the FRs of the light chain variable region may be referred to as LFR1, LFR2, LFR3, and LFR4.

[114] In the present invention, the heavy or light chain variable region may be in the form of a domain including three CDRs, but is not limited thereto.

[115]

[116] The "multispecific antibody" of the present invention means an antibody having binding specificity to two or more different antigens. Specifically, the multispecific antibody may be in a form that does not exist in nature and is produced by genetic engineering or an arbitrary method.

[117] The multispecific antibody of the present invention may include a bi-specific or higher antibody, a tri-specific or higher antibody, for example, bi-specific antibodies, tri-specific antibodies, tetra-specific antibodies, or antibodies targeting more than four targets.

[118] Specifically, the multispecific antibody of the present invention may simultaneously bind to a lysosomal transporting receptor and one or more, two or more, or three or more target proteins, but is not limited thereto.

[119]

[120] The multispecific antibody of the present invention may be, but is not limited to, a bi-specific antibody comprising a target protein binding region that can bind to a lysosomal transporting receptor and one target protein.

[121]

- [122] In the present invention, the term "bi-specific antibody" means a protein capable of binding to two different kinds of antigens.
- [123] In a specific example, the bi-specific antibody of the present invention may bind to a target protein (EGFR or the like) and a lysosomal transporting receptor (APLP2, M6PR) that transports the target protein to lysosomes.
- [124] The "bi-specific antibody" of the present invention may be used interchangeably with the terms "dual targeting antibody", "dual antibody" or "dual antibody protein". In a specific aspect, the bi-specific antibody of the present invention may have a lysosomal transporting receptor (for example, APLP2 or M6PR) and a target protein as antigens. The form of the bi-specific antibody of the present invention is not particularly limited thereto, but includes a form constructed based on an IgG form. In the present invention, the bi-specific antibody is named "Lportbody".
- [125]
- [126] The bi-specific antibody refers to a molecule whose antigen-binding sites are connected directly or via a linker or that can form a heterodimer by electrostatic interaction.
- [127]
- [128] The "valent" refers to the presence of a specified number of antigen-specific binding sites in a molecule. Therefore, the terms "monovalent", "bivalent", "tetravalent", and "hexavalent" refer to the presence of one, two, four, and six binding sites, respectively, specific to antigens in a molecule.
- [129]
- [130] The bi-specific antibody according to the present invention may be a bi-specific antibody having a domain that specifically binds to a lysosomal transporting receptor and a domain that specifically binds to a target protein, the domains that specifically bind to the respective antigens are typically VH/VL pairs or VH(H) domains, and bi-specific antibodies may be divided into monovalent or bivalent depending on the VH/VL pair or VH(H) domain that binds to each antigen.
- [131]
- [132] The bi-specific antibody according to the present invention comprises a lysosomal transporting receptor binding region and a target protein binding region, the lysosomal transporting receptor binding region may include a VH(H) domain, the target protein binding region may include a VH(H) domain, and the VH(H) domain of the target protein binding region may be connected to the VH(H) domain of the lysosomal transporting receptor binding region.
- [133]
- [134] In the present invention, the term "lysosomal transporting receptor (LTR)" refers to a receptor that functions to transport a target substance to a lysosome and degrade

the target substance, and in the present invention, plays a role in transporting a target protein to a lysosome by binding to the multispecific antibody according to the present invention.

[135]

[136] For the purpose of the present invention, the lysosomal transporting receptor according to the present invention may be recycled back to the cell surface after endocytosis or degraded together with a target protein, and may be expressed in various tissues and used as a therapeutic agent for various diseases. Specifically, the lysosomal transporting receptor of the present invention may be, but is not limited to, mannose 6-phosphate receptor (M6PR) or amyloid precursor-like protein 2 (APLP2).

[137]

[138] In the present invention, the term "lysosomal transporting receptor binding region" means a site in the multispecific antibody according to the present invention that can specifically bind to a lysosomal transporting receptor, and may specifically mean a site capable of binding to M6PR and/or APLP2, but is not limited thereto.

[139] For the purpose of the present invention, the lysosomal transporting receptor binding region may include, but is not limited to, a substance capable of specifically binding to a lysosomal transporting receptor, for example, an antibody, a variable region including an antigen (lysosomal transporting receptor) binding site of the antibody or a fragment thereof, an antigen-binding site, a peptide capable of specifically binding to a lysosomal transporting receptor, a nanobody, a single domain antibody, a nucleic acid, and an aptamer.

[140] In a specific example, the lysosomal transporting receptor binding region of the present invention may include a heavy chain variable region.

[141] In another example, the lysosomal transporting receptor binding region of the present invention may include, but is not limited to, a heavy chain variable region (VH or VHH) of an antibody against a lysosomal transporting receptor, a fragment thereof, or an antigen-binding site thereof.

[142] The lysosomal transporting receptor binding region comprised in the multispecific antibody of the present invention may be, but is not limited to, a single domain (VH or VHH) of the heavy chain variable region of an antibody against a lysosomal transporting receptor. Such a single domain may be a heavy chain variable region having three CDRs.

[143]

[144] Alternatively, the lysosomal transporting receptor binding region of the present invention may include, but is not limited to, a heavy chain variable region, a heavy chain constant region, a light chain constant region, and a light chain variable region. In a more specific example, the heavy chain constant region of the lysosomal

transporting receptor binding region of the present invention may additionally include an Fc region, more specifically, the heavy chain constant region or Fc region may additionally include, but is not limited to, a hinge region.

- [145] In the present invention, the "Fc region" refers to a site including the heavy chain constant region 2(CH2) and/or the heavy chain constant region 3(CH3) portion, excluding the heavy and light chain variable regions of an immunoglobulin. In the present invention, the "Fc region" may be used interchangeably with "immunoglobulin Fc region". The Fc region may be an Fc region derived from IgG, IgA, IgD, IgE, or IgM or by a combination thereof or a hybrid thereof, but is not limited thereto.
- [146] For the purpose of the present invention, the immunoglobulin Fc region is a natural immunoglobulin Fc region, or a variant in which at least one or more amino acids have undergone variation selected from the group consisting of substitution, addition, deletion, modification, or a combination thereof, and a variant may be included in the present invention without limitation as long as it exhibits biological activity equivalent to that of the natural immunoglobulin Fc.
- [147] Such an Fc region may be obtained from a natural form isolated from the living body of animals such as humans, cows, goats, pigs, mice, rabbits, hamsters, rats or guinea pigs, or may be a recombinant obtained from a transformed animal cell or microorganism or a derivative thereof. Here, the method for obtaining the Fc region from a natural form may be a method in which the entire immunoglobulin is isolated from the living body of a human or animal and then treated with a protease. The entire immunoglobulin is cleaved into Fab and Fc when treated with papain and is cleaved into pF'c and F(ab)2 when treated with pepsin. This may be subjected to size-exclusion chromatography or the like to separate Fc or pF'c.
- [148] The immunoglobulin Fc region of the present invention may be a monomer or may be a dimer formed to include a hinge region in the heavy chain constant region, but is not limited thereto.
- [149] In the present invention, the immunoglobulin Fc region of the monomer may include an amino acid sequence of SEQ ID NO: 141, but is not limited thereto.
- [150] In the present invention, the "hinge region" refers to a site located in the heavy chain to form a dimer of the immunoglobulin Fc region through an inter disulfide bond.
- [151] Specifically, the multispecific antibody of the present invention may comprise 1) a CH1 domain, a CH2 domain, a CH3 domain and a CH4 domain, 2) a CH1 domain and a CH2 domain, 3) a CH1 domain and a CH3 domain, 4) a CH2 domain and a CH3 domain, or 5) a combination of one or more of a CH1 domain, a CH2 domain, a CH3 domain or a CH4 domain with an immunoglobulin hinge region (or a part of the hinge region), but is not limited thereto. More specifically, the Fc region may include, but is not limited to, a hinge region, a CH2 domain, and a CH3 domain.

- [152] In the multispecific antibody of the present invention, the Fc regions may form a dimer with each other due to a disulfide bond by the hinge region included in the Fc region. More specifically, the multispecific antibody may be, but is not limited to, a dimer formed symmetrically by monomers in which a lysosomal transporting receptor binding region and a target protein binding region are connected to each other.
- [153] In the present invention, the hinge region may include an amino acid sequence of SEQ ID NO: 142, but is not limited thereto.
- [154]
- [155] The term "target protein binding region" in the present invention refers to a region that binds to a target protein to be degraded by the multispecific antibody of the present invention, and may include a target protein binding site that can bind to a target protein. Specifically, the target protein binding region of the present invention may specifically bind to a target protein, but is not limited thereto.
- [156] For the purpose of the present invention, as the target protein, a protein may be included in the present invention without limitation as long as it is a protein that is to be reduced/eliminated in the body through lysosome-mediated degradation. For example, the target protein may be an extracellular protein or a soluble protein. In a more specific example, proteins that affect the onset and progression of diseases, such as extracellular proteins, enzymes, hormones, cytokines, chemokines, receptor proteins, growth factors, cell surface antigens, membrane proteins, water-soluble proteins, and insoluble proteins may be the target protein, but the target protein is not limited thereto. In an example, the target protein may be, but is not limited to, epidermal growth factor receptor (EGFR). The multispecific antibody of the present invention is capable of binding to and degrading a target protein even in the aqueous state, and thus has utility for various forms of target proteins.
- [157]
- [158] The target protein binding region of the present invention may include, but is not limited to, a target protein binding site that can bind to a target protein. The Examples of the target protein binding site include, but are not limited to, an antibody capable of binding to a target protein, a variable region or fragment thereof including an antigen (target protein) binding site of the antibody, an antigen-binding site, a peptide capable of specifically binding to a target protein, a nanobody, a single domain antibody, a nucleic acid, and an aptamer. In a specific example, the target protein binding region of the present invention may include, but is not limited to, a heavy chain variable region (VH or VHH) of an antibody against a target protein, a fragment thereof, or an antigen-binding site thereof. In another specific example, the target protein binding region of the present invention may include, but is not limited to, a heavy chain variable region of an antibody against a target protein, a heavy chain constant region, a light

chain constant region, and a light chain variable region. In a more specific example, the heavy chain constant region of the target protein binding region of the present invention may additionally include an Fc region, and more specifically, the heavy chain constant region may additionally include a hinge region, but is not limited thereto. The Fc region and the hinge region are as described previously.

[159]

[160] The multispecific antibody of the present invention may comprise two or more target protein binding regions that can specifically bind to two or more target proteins. Specifically, when there are two or more target proteins, the multispecific antibody may comprise two or more target protein binding regions that can specifically bind to each target protein, and each target protein binding region may be simultaneously located at the C-terminus of the heavy chain constant region by being connected to the lysosomal transporting receptor binding region via a linker, or may be located separately at the N-terminus by being separated, but the multispecific antibody is not limited.

[161]

[162] In the multispecific antibody of the present invention, a lysosomal transporting receptor binding region and a target protein binding region are connected. Such a multispecific antibody may have two forms:

[163] (i) a multispecific antibody in which the lysosomal transporting receptor binding region includes a heavy chain variable region, a heavy chain constant region, a light chain constant region and a light chain variable region (a lysosomal transporting receptor binding region in the form of Fab) and the target protein binding region includes a heavy chain variable region of a VH or VHH domain (FIG. 20A), or

[164] (ii) a multispecific antibody in which the lysosomal transporting receptor binding region includes a heavy chain variable region of a VH or VHH domain (a lysosomal transporting receptor binding region in the form of a domain) and the target protein binding region includes a heavy chain variable region, a heavy chain constant region, a light chain constant region and a light chain variable region (FIG. 20B).

[165] In the two types of Type B multispecific antibodies, the heavy chain variable regions of the lysosomal transporting receptor binding region and target protein binding region are connected to each other.

[166] A multispecific antibody having a structure in which the lysosomal transporting receptor binding region and the target protein binding region are connected to be adjacent to each other in this way is distinguished from a structure in which the heavy chain variable region and target protein binding region are not adjacent to each other but are located at both termini of the antibody.

- [167] In this specification, the multispecific antibody of the present invention, in which the lysosomal transporting receptor binding region and the target protein binding region are adjacently connected, is referred to as a "Type B multispecific antibody" or a "B-type Lportbody", and an example thereof includes Type B in FIG. 1, but the multispecific antibody is not limited thereto. The Type B multispecific antibody of the present invention may refer to the two forms (i) and (ii).
- [168]
- [169] In a specific embodiment, the heavy chain variable region of the lysosomal transporting receptor binding region and the heavy chain variable region of the target protein binding region may be directly connected or connected via a linker or may additionally include other protein moieties, but are not limited thereto. As the method and location of connection between the heavy chain variable region of the lysosomal transporting receptor binding region and the heavy chain variable region of the target protein binding region, any method and any location known in the art may be used without limitation as long as the structure or activity of the protein being connected are not altered.
- [170]
- [171] For the purpose of the present invention, the lysosomal transporting receptor binding region and the target protein binding region may be directly connected by a covalent bond, but are not limited thereto.
- [172] Alternatively, the lysosomal transporting receptor binding region and the target protein binding region may be connected via a linker, but are not limited thereto. As the linker connecting the lysosomal transporting receptor binding region and the target protein binding region, any linker may be included in the present invention without limitation as long as it is a peptide linker or non-peptide linker used in the art.
- [173] The peptide linker may include one or more amino acids, for example, 1 to 1000 amino acids, specifically 1 to 100 amino acids, more specifically 1 to 50 amino acids, but is not particularly limited thereto. The peptide linker according to the present invention may be a peptide linker composed of glycine (G), serine (S) and threonine (T), and may have a sequence of GGGGSGGGGT (SEQ ID NO: 1), GGGGSGGGGS (SEQ ID NO: 2), GGGGSGGGSGGGT (SEQ ID NO: 3), or GGGGSGGGSGGGGS (SEQ ID NO: 4) as a specific example, but is not limited thereto. Alternatively, the peptide linker may include any peptide linker known in the art, for example, a [GS]*x* linker, a [GGGS]*x* linker, and a [GGGGS]*x* linker, where *x* may be a natural number 1 or more (for example, 1, 2, 3, 4, 5, or more). Additionally, the peptide linker of the present invention may include, but is not limited to, a place recognizable by a restriction enzyme (for example, KpnI enzyme).

- [174] The non-peptide linker is not limited in kind as long as it can connect the heavy chain variable region and the target protein binding region. For example, the non-peptide linker may be selected from, but is not limited to, lipid polymers, biodegradable polymers, chitins, or oligonucleotides.
- [175]
- [176] The multispecific antibody of the present invention may bind to a lysosomal transporting receptor and a target protein, respectively, to induce endocytosis and degradation by a lysosome of a target protein, but is not limited thereto. In particular, the multispecific antibody of the present invention, which is in a form in which the lysosomal transporting receptor binding region and the target protein binding region are connected, can stably bind to a lysosomal transporting receptor and simultaneously stably bind to a target protein like a lysosomal transporting receptor, and ultimately has high target protein degradation.
- [177]
- [178] Still another aspect of the present invention provides a multispecific antibody comprising an antibody or an antigen-binding fragment thereof that specifically binds to amyloid precursor-like protein 2 (APLP2).
- [179] Specifically, the antibody or antigen-binding fragment thereof may include, but is not limited to, the following complementarity determining regions (CDRs):
- [180] (i) CDR-H1 including an amino acid sequence of SEQ ID NO: 45, CDR-H2 including an amino acid sequence of SEQ ID NO: 46, CDR-H3 including an amino acid sequence of SEQ ID NO: 47, CDR-L1 including an amino acid sequence of SEQ ID NO: 48, CDR-L2 including an amino acid sequence of SEQ ID NO: 49, and CDR-L3 including an amino acid sequence of SEQ ID NO: 50;
- [181] (ii) CDR-H1 including an amino acid sequence of SEQ ID NO: 51, CDR-H2 including an amino acid sequence of SEQ ID NO: 52, and CDR-H3 including an amino acid sequence of SEQ ID NO: 53;
- [182] (iii) CDR-H1 including an amino acid sequence of SEQ ID NO: 54, CDR-H2 including an amino acid sequence of SEQ ID NO: 55, and CDR-H3 including an amino acid sequence of SEQ ID NO: 56;
- [183] (iv) CDR-H1 including an amino acid sequence of SEQ ID NO: 57, CDR-H2 including an amino acid sequence of SEQ ID NO: 58, and CDR-H3 including an amino acid sequence of SEQ ID NO: 59;
- [184] (v) CDR-H1 including an amino acid sequence of SEQ ID NO: 60, CDR-H2 including an amino acid sequence of SEQ ID NO: 61, and CDR-H3 including an amino acid sequence of SEQ ID NO: 62;

- [185] (vi) CDR-H1 including an amino acid sequence of SEQ ID NO: 63, CDR-H2 including an amino acid sequence of SEQ ID NO: 64, and CDR-H3 including an amino acid sequence of SEQ ID NO: 65;
- [186] (vii) CDR-H1 including an amino acid sequence of SEQ ID NO: 66, CDR-H2 including an amino acid sequence of SEQ ID NO: 67, and CDR-H3 including an amino acid sequence of SEQ ID NO: 68;
- [187] (viii) CDR-H1 including an amino acid sequence of SEQ ID NO: 69, CDR-H2 including an amino acid sequence of SEQ ID NO: 70, and CDR-H3 including an amino acid sequence of SEQ ID NO: 71;
- [188] (ix) CDR-H1 including an amino acid sequence of SEQ ID NO: 72, CDR-H2 including an amino acid sequence of SEQ ID NO: 73, and CDR-H3 including an amino acid sequence of SEQ ID NO: 74;
- [189] (x) CDR-H1 including an amino acid sequence of SEQ ID NO: 75, CDR-H2 including an amino acid sequence of SEQ ID NO: 76, and CDR-H3 including an amino acid sequence of SEQ ID NO: 77;
- [190] (xi) CDR-H1 including an amino acid sequence of SEQ ID NO: 78, CDR-H2 including an amino acid sequence of SEQ ID NO: 79, and CDR-H3 including an amino acid sequence of SEQ ID NO: 80;
- [191] (xii) CDR-H1 including an amino acid sequence of SEQ ID NO: 81, CDR-H2 including an amino acid sequence of SEQ ID NO: 82, and CDR-H3 including an amino acid sequence of SEQ ID NO: 83;
- [192] (xiii) CDR-H1 including an amino acid sequence of SEQ ID NO: 84, CDR-H2 including an amino acid sequence of SEQ ID NO: 85, and CDR-H3 including an amino acid sequence of SEQ ID NO: 86;
- [193] (xiv) a CDR-H1 including an amino acid sequence of SEQ ID NO: 87, a CDR-H2 including an amino acid sequence of SEQ ID NO: 88, and a CDR-H3 including an amino acid sequence of SEQ ID NO: 89;
- [194] (xv) CDR-H1 including an amino acid sequence of SEQ ID NO: 90, CDR-H2 including an amino acid sequence of SEQ ID NO: 91, and CDR-H3 including an amino acid sequence of SEQ ID NO: 92;
- [195] (xvi) CDR-H1 including an amino acid sequence of SEQ ID NO: 93, CDR-H2 including an amino acid sequence of SEQ ID NO: 94, and CDR-H3 including an amino acid sequence of SEQ ID NO: 95;
- [196] (xvii) CDR-H1 including an amino acid sequence of SEQ ID NO: 96, CDR-H2 including an amino acid sequence of SEQ ID NO: 97, and CDR-H3 including an amino acid sequence of SEQ ID NO: 98;

- [197] (xviii) CDR-H1 including an amino acid sequence of SEQ ID NO: 99, CDR-H2 including an amino acid sequence of SEQ ID NO: 100, and CDR-H3 including an amino acid sequence of SEQ ID NO: 101;
- [198] (xix) CDR-H1 including an amino acid sequence of SEQ ID NO: 102, CDR-H2 including an amino acid sequence of SEQ ID NO: 103, and CDR-H3 including an amino acid sequence of SEQ ID NO: 104;
- [199] (xx) CDR-H1 including an amino acid sequence of SEQ ID NO: 105, CDR-H2 including an amino acid sequence of SEQ ID NO: 106, and CDR-H3 including an amino acid sequence of SEQ ID NO: 107;
- [200] (xxi) CDR-H1 including an amino acid sequence of SEQ ID NO: 108, CDR-H2 including an amino acid sequence of SEQ ID NO: 109, and CDR-H3 including an amino acid sequence of SEQ ID NO: 110;
- [201] (xxii) CDR-H1 including an amino acid sequence of SEQ ID NO: 111, CDR-H2 including an amino acid sequence of SEQ ID NO: 112, and CDR-H3 including an amino acid sequence of SEQ ID NO: 113;
- [202] (xxiii) CDR-H1 including an amino acid sequence of SEQ ID NO: 114, CDR-H2 including an amino acid sequence of SEQ ID NO: 115, and CDR-H3 including an amino acid sequence of SEQ ID NO: 116;
- [203] (xxiv) CDR-H1 including an amino acid sequence of SEQ ID NO: 117, CDR-H2 including an amino acid sequence of SEQ ID NO: 118, and CDR-H3 including an amino acid sequence of SEQ ID NO: 119;
- [204] (xxv) CDR-H1 including an amino acid sequence of SEQ ID NO: 120, CDR-H2 including an amino acid sequence of SEQ ID NO: 121, and CDR-H3 including an amino acid sequence of SEQ ID NO: 122;
- [205] (xxvi) CDR-H1 including an amino acid sequence of SEQ ID NO: 123, CDR-H2 including an amino acid sequence of SEQ ID NO: 124, and CDR-H3 including an amino acid sequence of SEQ ID NO: 125;
- [206] (xxvii) CDR-H1 including an amino acid sequence of SEQ ID NO: 126, CDR-H2 including an amino acid sequence of SEQ ID NO: 127, and CDR-H3 including an amino acid sequence of SEQ ID NO: 128; or
- [207] (xxviii) CDR-H1 including an amino acid sequence of SEQ ID NO: 129, CDR-H2 including an amino acid sequence of SEQ ID NO: 130, and CDR-H3 including an amino acid sequence of SEQ ID NO: 131.
- [208]
- [209] CDR-H1, CDR-H2, and CDR-H3 sequentially represent the CDRs included in the heavy chain variable region, and CDR-L1, CDR-L2, and CDR-L3 sequentially represent the CDRs included in the light chain variable region.

- [210] The multispecific antibody may additionally comprise an antibody, antibody fragment or antigen-binding fragment that binds to a target protein. The target protein may be an extracellular protein or a soluble protein, and examples thereof include epidermal growth factor receptor (EGFR), but the target protein is not limited thereto.
- [211] The target protein is as described previously.
- [212]
- [213] In the present invention, even if a sequence is defined by a specific sequence number, the sequence does not exclude meaningless sequence additions before and after the amino acid sequence of the corresponding sequence number, mutations that may occur naturally, or silent mutations thereof as long as it has the same or corresponding activity as a peptide consisting of the amino acid sequence of the corresponding sequence number, and it is clear that the sequence falls within the scope of the present invention in a case of having such sequence additions or mutations as well. In other words, even if there are differences in a part of the sequence, the sequence may fall within the scope of the present invention as long as it exhibits homology or identity at a certain level or higher and exhibits binding activity to a lysosomal transporting receptor and/or a target protein.
- [214] For example, the amino acid sequence defined in the present invention may have or include an amino acid sequence having 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more homology or identity with the amino acid sequence of the corresponding sequence number, or may consist of or essentially consist of an amino acid sequence having 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more homology or identity with the amino acid sequence of the corresponding sequence number, but is not limited thereto, and a sequence may fall within the scope of the present invention as long as it exhibits binding activity to a lysosomal transporting receptor and/or a target protein.
- [215]
- [216] In the present invention, the term 'homology' or 'identity' means the degree of similarity between two given sequences and may be expressed as a percentage. The terms homology and identity are often used interchangeably.
- [217] Methods for determining sequence similarity or identity between two or more amino acid sequences are known in the art.
- [218] For example, the sequence similarity or identity may be determined using a well-known computer algorithm such as the "FASTA" program using default parameters as in Pearson et al(1988) [Proc. Natl. Acad. Sci. USA 85]: 2444. Alternatively, as

performed by the Needleman program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends Genet. 16: 276-277) (version 5.0.0 or later), the sequence similarity or identity may be determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453) (including the GCG program package (Devereux, J., et al, Nucleic Acids Research 12: 387(1984)), BLASTP, BLASTN, FASTA (Atschul, [S.] [F.,] [ET AL, J MOLEC BIOL 215]: 403(1990); Guide to Huge Computers, Martin J. Bishop, [ED.,] Academic Press, San Diego, 1994, and [CARILLO ETA/.(1988) SIAM J Applied Math 48: 1073). For example, homology or identity may be determined using BLAST or ClustalW from the National Center for Biotechnology Information Database.

[219]

[220] In a specific aspect, the multispecific antibody of the present invention may comprise any one of the amino acid sequences of SEQ ID NOS: 11 to 39, but is not limited thereto.

[221]

[222] Still another aspect of the present invention provides a multispecific antibody comprising an antibody or an antigen-binding fragment thereof that specifically binds to mannose 6-phosphate receptor (M6PR).

[223] Specifically, the antibody or antigen-binding fragment thereof may include, but is not limited to, the following complementarity determining regions (CDRs):

[224] (i) CDR-H1 including an amino acid sequence of SEQ ID NO: 132, CDR-H2 including an amino acid sequence of SEQ ID NO: 133, and CDR-H3 including an amino acid sequence of SEQ ID NO: 134;

[225] (ii) CDR-H1 including an amino acid sequence of SEQ ID NO: 135, CDR-H2 including an amino acid sequence of SEQ ID NO: 136, and CDR-H3 including an amino acid sequence of SEQ ID NO: 137; or

[226] (iii) CDR-H1 including an amino acid sequence of SEQ ID NO: 138, CDR-H2 including an amino acid sequence of SEQ ID NO: 139, and CDR-H3 including an amino acid sequence of SEQ ID NO: 140.

[227]

[228] CDR-H1, CDR-H2, and CDR-H3 sequentially represent the CDRs included in the heavy chain variable region.

[229] The multispecific antibody may additionally comprise an antibody, antibody fragment or antigen-binding fragment that binds to a target protein. The target protein may be an extracellular protein or a soluble protein, and examples thereof include epidermal growth factor receptor (EGFR), but the target protein is not limited thereto.

[230] The target protein has been described above.

[231]

- [232] In a specific aspect, the multispecific antibody of the present invention may comprise any one of the amino acid sequences of SEQ ID NOs: 40 to 42, but is not limited thereto.
- [233]
- [234] Another aspect embodying the present invention provides a polynucleotide encoding the multispecific antibody.
- [235] The multispecific antibody is as described previously.
- [236]
- [237] The polynucleotide encoding the multispecific antibody of the present invention can be readily isolated and sequenced using conventional procedures. As an example, the polynucleotide encoding the multispecific antibody of the present invention may be, but is not limited to, codon optimized. However, considering codon degeneracy or preferred codons in organisms, it is clear that a polynucleotide sequence in which a part is deleted, modified, substituted, conservatively substituted or added is also included within the scope of the polynucleotide of the present invention as long as it is a polynucleotide sequence capable of encoding the sequence of the multispecific antibody according to the present invention or a polypeptide having homology or identity therewith.
- [238]
- [239] Still another aspect embodying the present invention provides a vector comprising a polynucleotide encoding the multispecific antibody.
- [240] Still another aspect embodying the present invention provides a cell comprising the multispecific antibody of the present invention; a polynucleotide encoding the same; or a vector encoding the polynucleotide; or a cell transformed with the vector.
- [241] The multispecific antibody and polynucleotide encoding the same are as described previously.
- [242]
- [243] The expression vector comprising a polynucleotide encoding the multispecific antibody according to the present invention is not particularly limited thereto, and any vector known in the art can be used. For example, the expression vector may be a vector capable of replicating and/or expressing the polynucleotide in eukaryotic or prokaryotic cells, including mammalian cells (for example, human, monkey, rabbit, rat, hamster, and mouse cells), plant cells, yeast cells, insect cells, or bacterial cells (for example, *E. coli*), and specifically may include plasmids, cosmids, viruses and bacteriophages, either natural or recombinant. For example, pWE15, M13, MBL3, MBL4, IXII, ASHII, APII, t10, t11, Charon4A, and Charon21A may be used as phage vectors or cosmid vectors, and pDZ vectors, pBR vectors, pUC vectors, pBluescriptII vectors, pGEM vectors, pTZ vectors, pCL vectors, and pET vectors may be used as

plasmid vectors. Specifically, pDZ, pDC, pDCM2, pACYC177, pACYC184, pCL, pECCG117, pUC19, pBR322, pMW118, and pCC1BAC vectors may be used.

[244]

[245] The vector according to the present invention may be a vector that is operably linked to an appropriate promoter so that the polynucleotide can be expressed in a host cell, and includes at least one selectable marker. The selectable marker is used to select cells transformed with a vector, that is, to examine whether the target nucleic acid molecule has been inserted, and markers that confer selectable phenotypes, such as drug resistance, nutrient requirements, resistance to cytotoxic agents, or expression of surface polypeptides, may be used. In an environment treated with a selective agent, only cells expressing the selectable marker survive or exhibit other phenotypic traits, so transformed cells can be selected.

[246]

[247] The expression vector comprising a polynucleotide encoding the multispecific antibody may be an expression vector that includes a polynucleotide encoding the lysosomal targeting receptor binding region of the multispecific antibody and a polynucleotide encoding the target protein binding region of the multispecific antibody, but is not limited thereto.

[248]

[249] The cell transformed with the expression vector of the present invention is not limited thereto, but may be bacterial cells such as *E. coli*, *Streptomyces*, or *Salmonella typhimurium*; yeast cells; fungal cells such as *Pichia pastoris*; insect cells such as *Drosophila* and *Spodoptera Sf9* cells; animal cells such as CHO (Chinese hamster ovary cells), ExpiCHO cells, SP2/0 (mouse myeloma), human lymphoblastoid, COS, NSO (mouse myeloma), Bowes melanoma cells, HT-1080, BHK (baby hamster kidney cells), HEK (human embryonic kidney cells), PER.C6 (human retinal cells); or plant cells, which are transformed by introducing the expression vector.

[250]

[251] The term "introduction" in the present invention means delivering the vector comprising a polynucleotide encoding the multispecific antibody to a cell. Such introduction may be performed by various methods known in the art, such as calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofectamine and protoplast fusion. Additionally, vectors may be introduced into cells by gene bombardment and the like. In the present invention, introduction may be used interchangeably with transfection and transformation.

[252]

- [253] Still another aspect of the present invention provides a composition comprising the multispecific antibody of the present invention.
- [254] The multispecific antibody is as described previously.
- [255]
- [256] Specifically, the composition may be a pharmaceutical composition, and may be a pharmaceutical composition comprising the multispecific antibody in a pharmaceutically effective amount.
- [257] In the present invention, the term "pharmaceutically effective amount" means an amount sufficient to treat a disease at a reasonable benefit/risk ratio applicable to medical treatment, and the effective dose level may be determined by factors including the subject kind and severity, age, sex, drug activity, drug sensitivity, time of administration, route of administration and excretion rate, duration of treatment, and concomitant drugs, and other factors well known in the medical field. The composition of the present invention may be administered as an individual therapeutic agent or in combination with other therapeutic agents, and may be administered sequentially or simultaneously with conventional therapeutic agents. The composition of the present invention may be administered in single or multiple doses. It is important to administer the composition in an amount that can achieve the maximum effect with the minimum amount without side effects, taking all of the factors into consideration, and this may be easily determined by those skilled in the art.
- [258]
- [259] The pharmaceutical composition according to the present invention may be applied to different target diseases and patients depending on the kind of target protein targeted by the multispecific antibody. Those skilled in the art can determine the multispecific antibody by appropriately selecting the target protein and target protein binding region depending on the disease to be treated and patient. As an example, the pharmaceutical composition of the present invention may be a pharmaceutical composition for preventing or treating cancer, but is not limited thereto. The multispecific antibody of the present invention can function in various tissues and has versatility.
- [260]
- [261] The pharmaceutical composition may additionally contain a pharmaceutically acceptable excipient, but is not limited thereto.
- [262] The term "pharmaceutically acceptable excipient" in the present invention refers to a carrier or diluent that does not stimulate a living organism and does not inhibit the biological activity and properties of the administered compound. As the acceptable pharmaceutical carrier for compositions to be prepared as liquid solutions, saline solution, sterile water, Ringer's solution, buffered saline, albumin injection solution, dextrose solution, maltodextrin solution, glycerol, ethanol, and a mixture of one or

more of these ingredients may be used as a sterile and biocompatible substance, and other common additives such as antioxidants, buffers, and bacteriostatic agents may be added if needed. The pharmaceutical composition may be prepared as an injectable formulation such as an aqueous solution, suspension, or emulsion, pills, capsules, granules, or tablets by additionally adding diluents, dispersants, surfactants, binders and lubricants.

- [263] The pharmaceutical composition may be various oral or parenteral formulations. In a case where the pharmaceutical composition is formulated into a preparation, the preparation is prepared using diluents or excipients such as fillers, bulking agents, binders, wetting agents, disintegrants, and surfactants usually used. Solid preparations for oral administration include tablets, pills, powders, granules, and capsules, and these solid preparations are prepared by mixing one or more compounds with at least one or more excipients, such as starch, calcium carbonate, sucrose or lactose, and gelatin. In addition to simple excipients, lubricants such as magnesium stearate and talc are also used. Liquid preparations for oral administration include suspensions, solutions, emulsions, and syrups, and various excipients, such as humectants, sweeteners, flavoring agents, and preservatives, may be contained in addition to the commonly used simple diluents, such as water and liquid paraffin. Preparations for parenteral administration include sterile aqueous solutions, non-aqueous solutions, suspensions, emulsions, lyophilized preparations, and suppositories, and propylene glycol, polyethylene glycol, vegetable oils such as olive oil, injectable esters such as ethyl oleate and the like may be used as non-aqueous solvents and suspending agents. As suppository bases, Witepsol, Macrogol, Tween 61, cocoa butter, laurin butter, and glycerogelatin may be used.
- [264] The pharmaceutical composition may have any one formulation selected from the group consisting of tablets, pills, powders, granules, capsules, suspensions, oral liquids, emulsions, syrups, sterile aqueous solutions, non-aqueous solutions, suspensions, emulsions, lyophilized preparations and suppositories.
- [265]
- [266] Still another aspect of the present invention provides a method for producing a multispecific antibody, comprising culturing a cell that comprises any one or more of the multispecific antibody of the present invention; a polynucleotide encoding the same; or a vector comprising the polynucleotide or is transformed with a vector comprising the polynucleotide in a medium. With regard to culture in the production method, the culture process, medium, culture temperature, pH and the like may be appropriately controlled by suitable methods known in the art.
- [267] The multispecific antibody, polynucleotide, vector, cell, and transformation are as described previously.

[268]

[269] In a specific example, the method for producing a multispecific antibody of the present invention may comprise preparing the cell and preparing a medium for culturing the cell.

[270] The method for producing a multispecific antibody of the present invention may further comprise recovering the multispecific antibody from the medium or cell.

[271] The method for producing a multispecific antibody of the present invention may further comprise purifying the multispecific antibody.

[272] The recovery and purification may also be to collect the multispecific antibody by a suitable method known in the art (for example, centrifugation, filtration, various kinds of chromatography, or a combination of these methods).

[273] For example, the purifying step may be performed by any one or more of column chromatography, affinity chromatography, anion exchange chromatography, cation exchange chromatography, hydrophobic interaction chromatography, size exclusion chromatography, reversed phase column chromatography, ultrafiltration, diafiltration, salting out (examples: ammonium sulfate precipitation and sodium phosphate precipitation), solvent precipitation (examples: protein fraction precipitation using acetone, ethanol and the like), dialysis, or gel filtration, but is not limited thereto.

[274]

[275] In the production method of the present invention, the purifying step may be performed two or more times if necessary, and purification may be performed by different methods in the respective purification steps.

[276]

[277] Still another aspect of the present invention provides a use of the multispecific antibody for the degradation of a target protein.

[278] The multispecific antibody is as described previously.

[279]

[280] Duplicate contents are omitted in consideration of the complexity of this specification, and it is clear that the description described in one aspect also applies to other aspects. Terms not otherwise defined herein have the meaning commonly used in the art to which the present invention pertains.

[281]

[282] Hereinafter, the present invention will be described in more detail with reference to the following Examples. However, the following Examples are only intended to illustrate the present invention, and the scope of the present invention is not limited only to Examples.

[283]

[284] **Example 1: Design of multispecific antibodies**

[285]

[286] As lysosomal transporting receptors (LTRs), APLP2 and M6PR that were systemically expressed and capable of transporting target proteins to lysosomes were selected. EGFR, a cell membrane protein overexpressed in cancer cells, was selected as the target protein, and TNF α , a water-soluble protein, was selected as a negative control for the target protein.

[287]

[288] The multispecific antibodies according to the present invention were constructed into Types A, B, and C having three different characteristics (FIG. 1).

[289]

[290] Type A has an asymmetric structure, containing one binding region for each target. For this purpose, the Ajou University KiH technology, in which antibody HC mutations were introduced, was used (Choi, et al. (2015). Mol Immunol, 65:377-83.). LC (light chain) is composed of Fab and VH (H) domains.

[291]

[292] Type B and Type C have a symmetric structure based on wild-type Fc. Types B and C are similar in that they contain two binding regions for each target, but differ in the directionality of the binding regions (proximal or distal).

[293]

[294] **Example 2: Construction of multispecific antibodies**

[295]

[296] Example 2-1: Construction of Type A multispecific antibodies

[297] The VH(H) and Fab sequences that constituted the multispecific antibodies of Type A were synthesized by gene synthesis and then PCR was performed. An insert encoding a sequence consisting of [NotI-signal sequence-VH(H)-Linker-Hinge-CH2-CH3A-stop codon-BamHI] was created, and an insert encoding a sequence consisting of [NotI-signal sequence-VH-CH1-Hinge-CH2-CH3B-BamHI] and [NotI-signal sequence-VL-CL] was created. The pCIW vector and insert were treated with NotI-HF (NEB, R3189) and BamHI-HF (NEB, R3136), respectively, for digestion. After the restriction enzyme treatment, the insert and pCIW vector were treated with T4 DNA ligase (Invitrogen, 15224017) to construct pCIW-VH(H)-CH2-CH3(A) and pCIW-VH-CH1-hinge-CH2-CH3(B), pCIW-VL-CL.

[298]

[299] Example 2-2: Construction of Type B multispecific antibodies

[300] The VH(H) and Fab sequences that constituted the multispecific antibodies of Type B were synthesized by gene synthesis and then PCR was performed. An insert encoding a sequence consisting of [NotI-signal sequence-VH(H)1-Linker-VH2-ApaI] and [NotI-signal sequence-VL-CL] was created. To construct the heavy chain, the

pCIW vector and insert were treated with NotI-HF (NEB, R3189) and ApaI (NEB, R0114), respectively, for digestion. To construct the light chain, the pCIW vector and insert were treated with NotI-HF (NEB, R3189) and BamHI-HF (NEB, R3136), respectively, for digestion. After the restriction enzyme treatment, the insert and pCIW vector were treated with T4 DNA ligase (Invitrogen, 15224017) to construct pCIW-VH(H)1-Linker-VH2-CH1-hinge-CH2-CH3, pCIW-VL-CL.

[301]

[302] Example 2-3: Construction of Type C multispecific antibodies

[303] The VH(H) and Fab sequences that constituted the multispecific antibodies of Type C were synthesized by gene synthesis and then PCR was performed. An insert encoding a sequence consisting of [NotI-signal sequence-VH1-CH1-hinge-CH2-CH3-Linker-VH(H)2-stop codon-BamHI], [NotI-signal sequence-VL-CL] was created. To construct the heavy and light chains, the pCIW vector and insert were treated with NotI-HF (NEB, R3189) and BamHI-HF (NEB, R3136), respectively, for digestion. After the restriction enzyme treatment, the insert and pCIW vector were treated with T4 DNA ligase (Invitrogen, 15224017) to construct pCIW-VH1-CH1-hinge-CH2-CH3-Linker-VH(H)2, pCIW-VL-CL.

[304]

[305] The 12 kinds of multispecific antibodies according to the present invention produced in Examples 2-1 to 2-3 are summarized and illustrated in FIG. 2. The multispecific antibodies produced in the present invention may be represented in a format such as ¹A-²M³E, where 1 indicates the type of multispecific antibody, that is, a multispecific antibody of Type A, B, or C, 2 indicates the kind of lysosomal transporting receptor (M: M6PR, A: APLP2), and 3 indicates the kind of target protein (E: EGFR, T: TNFα). For example, A-ME is a Type A multispecific antibody, which means a multispecific antibody that includes regions targeting M6PR and EGFR.

[306] The sequences of the lysosomal transporting receptor binding region and target protein binding region included in the 12 kinds of multispecific antibodies in FIG. 2 are as shown in Table 1 below.

[307]

[308] [Table 1]

SEQ NO:	ID	Explanation	Sequence
5		EGFR targeting Ab (VHH)	QVKLEESGGGVQQTGGSLRLTCAASGRTSRSYGMGWFRQAPGKEREFEVSGISWRGDSTGYA DSYKGRFTISRDNNAKNTVDLQMNSLRPEDTAIYYCAAAAGSAWGTLYEYDVGQGTQVTV SS
6		TNF α targeting Ab (VHH)	EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYWMYVVRQAPGKLEWVSEINTGLITKYP DSYKGRFTISRDNNAKNTLYLQMNSLRPEDTAVYYCARSPSGFNRGQGTLVTVSS
7		APLP2 targeting Ab (VH-CH1)	EVQLVESGGGLVQPGGSLRLSCVASGFTFADYAMHWVRQAPGKLEWVSGISWNSGNIDYA DSYKGRFTISRDNNAKNSLYLQMNSLRTEDTALYYCAKVRIVVAGYIIYYGMDVWGQGTTV TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKV
8		APLP2 targeting Ab (VL-CL)	DIQMTQSPSSLSASVGDRTTITCRASQSISSYLNWYQQKPKGAPKLLIYAASLSQGVPSR FSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPPITFGQGTREIKRTVAAPSVEFP DEQLKSGTASVCLLNFPYPREAKVQWKVDNALQSGNSQESVTEQDSKDSYSLSSLTLS KADYEKKHKVYACEVTHQGLSSPVTKSFNRGEC
9		M6PR targeting Ab (VH-CH1)	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKLEWVAFIDPIFGDTRYA DSYKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYANDYWCQGTLVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY SLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKV
10		M6PR targeting Ab (VL-CL)	DIQMTQSPSSLSASVGDRTTITCRASQDVNTAVAWYQQKPKGAPKLLIYASFLVSGVPSR FSGSRSGTDFTLTISSLQPEDFATYYCQAYFFHHFPPTFGQGTKEIKRTVAAPSVEIFP PSDEQLKSGTASVCLLNFPYPREAKVQWKVDNALQSGNSQESVTEQDSKDSYSLSSLT LSKADYEKKHKVYACEVTHQGLSSPVTKSFNRGEC

[309]

[310] **Example 3: Expression of multispecific antibodies**

[311]

[312] The multispecific antibodies of Type A, B, and C constructed in Example 2 were expressed and produced by the following method.

[313]

[314] Specifically, Expi293 cells (Gibco) were passaged 3 to 4 times in Expi293 Expression Medium (Gibco) and then passaged at 3×10^6 cells/mL the day before expression. On the day of expression, it was examined whether the cells were at 4.5

to 5.5×10^6 viable cells/mL or more. Type A was prepared at a ratio of HC1 (VH(H)-CH2-CH3(A)) : HC2 (VH-CH1-hinge-CH2-CH3(B)) : LC of 1 : 1 : 1, and Type B and Type C were prepared at a ratio of HC : LC of 1 : 1.

[315]

[316] Transient expression of multispecific antibodies was performed using the ExpiFectamine 293 Transfection Kit (Gibco) according to the manufacturer's instructions. First, two tubes containing Opti-MEM (Gibco) were prepared, DNA and expifectamine were added, respectively, and incubation was performed at room temperature for 5 minutes. The two mixtures were mixed, and then incubated for an additional 20 to 30 minutes. The mixed mixture was added to the prepared cells. After 16 to 20 hours of expression, enhancers 1 and 2 were added respectively, and expression was then performed in a shaking incubator at 37°C for 5 to 7 days.

[317]

[318] **Example 4: Primary purification of multispecific antibodies**

[319]

[320] The culture solution expressed in Example 3 was centrifuged under the conditions of 4000 rpm, 20 min, and 4°C, and the supernatant was then purified using a bottle-top vacuum filter (Corning). Purification was performed at room temperature (15°C to 25°C) using AKTA AVANT 150 (Cytiva) equipment. Mabselect VL resin (Cytiva, 17542001) that bound to the kappa light chain was used for Type A multispecific antibodies having an asymmetric structure, and Mabselect sure resin (Cytiva, 17542001) was used for Type B and C multispecific antibodies. The column was equilibrated with PBS (Lonza). When equilibrium was completed, the culture filtrate was loaded and adsorbed onto the resin, and the unadsorbed liquid was collected in a collection container. When adsorption was completed, re-equilibration with PBS was performed 5 CV. When re-equilibration was completed, 10 CV of elution buffer containing 50 mM sodium citrate at pH 3.5 was allowed to flow for elution of the target protein. The eluate was collected in 1 CV increments, and the pooling section was determined according to the chromatogram. The pooled eluate was neutralized by adding an eluate neutralizing solution (0.2 M Tris, pH 8.0) in a volume to be 1.5 times the volume of the pooled eluate. When elution was completed, the column was washed (5 CV) with a column washing buffer containing 100 mM citric acid. When the process was completed, a 0.1 N sodium hydroxide solution was allowed to flow for 15 minutes to perform CIP after the process.

[321]

[322] **Example 5: UF/DF (ultrafiltration and diafiltration) for secondary purification of multispecific antibodies**

[323]

[324] According to the first column purification result in Example 4, the primary column elution neutralization solution of multispecific antibodies that did not satisfy a purity of 90% or more was subjected to concentration and buffer exchange in order to perform the secondary column purification. Concentration and buffer exchange were performed using Vivaspin20 (Sartorius, VS2022) made of a PES material with a cut off size of 30 kDa. The exchange solution used was 10 mM sodium phosphate at pH 7.0, a secondary column equilibration buffer. The primary column elution neutralization solution was added to Vivaspin20 and centrifuged under the conditions of 3000 g, 30 min, and 4°C. The permeate that passed through the membrane was removed, an exchange solution was added to the retentate, and then centrifugation was performed under the conditions of 3000 g, 30 min, 4°C. After buffer exchange was performed with an exchange solution in a volume to be about 7 times the volume of the primary column elution neutralization solution, the pH and conductivity of the permeate were measured, and the buffer exchange was terminated when the pH and conductivity were at the same levels as the pH and conductivity of the exchange solution.

[325]

[326] **Example 6: Secondary purification of multispecific antibodies**

[327]

[328] Purification was performed using a CHT type II (40 µm, Biorad) column at room temperature (15°C to 25°C). The column was equilibrated with equilibration buffer containing 10 mM sodium phosphate at pH 7.0. When equilibrium was completed, the primary column elution neutralization solution undergone UF/DF was loaded and adsorbed onto the resin, and the unadsorbed solution was collected in a collection container. When adsorption was completed, re-equilibration with equilibration buffer was performed 5 CV. When re-equilibration was completed, 10 CV of elution buffer 1 (10 mM sodium phosphate, 200 mM NaCl, pH 7.0) and 10 CV of elution buffer 2 (10 mM sodium phosphate, 250 mM NaCl, pH 7.0) were allowed to flow to elute the target protein. The elute was collected in 1 CV increments, and the pooling section was determined according to the chromatogram. When elution was completed, the column was washed (5 CV) with column washing buffer containing 400 mM sodium phosphate at pH 7.5. When the process was completed, 5 CV of 1 N sodium hydroxide solution was allowed to flow to perform CIP after the process.

[329]

[330] **Example 7: UF/DF (ultrafiltration and diafiltration) for purified solution**

[331]

[332] Concentration and buffer exchange were performed using Vivaspin20 made of a PES material with a cut off size of 30 kDa. PBS was used as the exchange solution. A purified solution (primary column elution neutralization solution or secondary column

eluate) having a purity of 90% or more was added to Vivaspin20 and centrifuged under the conditions of 3000 g, 30 min, and 4°C. The permeate that passed through the membrane was removed, an exchange solution was added to the retentate, and centrifugation was performed under the conditions of 3000 g, 30 min, and 4°C. After buffer exchange was performed with an exchange solution in a volume to be about 7 times the volume of the loaded purified solution, the pH and conductivity of the permeate were measured, and the buffer exchange was terminated when the pH and conductivity were at the same levels as the pH and conductivity of the exchange solution. Considering the amount and yield of the loaded purified solution, additional concentration was performed to satisfy the target concentration of 4 mg/mL or more, and the completed solution was recovered.

[333]

[334] **Example 8: Analysis for purity measurement**

[335]

[336] Example 8-1: Measurement of protein concentration

[337] In a case where the expected concentration was 2 mg/mL or more, the sample was diluted with formulation buffer and mixed homogeneously. At least 200 µL of test solution was prepared for each specimen. Into a 96-well plate for UV measurement (ThermoFisher, Cat. No. 8404 or equivalent or higher), 200 µL of the test solution and 200 µL of formulation buffer were added. Formulation buffer was used as blank. The absorbance was measured at wavelengths of 280 nm, 330 nm, 977 nm, and 900 nm. In a case where the plate reader program was provided with a protein measurement protocol, the provided protocol was used.

[338] - Path (d) = (A977 - A900)/0.18

[339] The protein concentration is calculated using the equation below.

[340] - Protein concentration (mg/mL) = ((Sample A280/d - Blank A280/d) - (Sample A330/d - Blank A330/d))/Extinction coefficient X Dilution factor

[341]

[342] Example 8-2: SDS-PAGE for protein analysis

[343] A protein sample was loaded onto a Novex 4-12% Bis-tris gel (Thermo, NP0321BOX). The protein sample was prepared by mixing 3 µg of protein per well with LDS sample buffer (Invitrogen, NP0007) and performing culture at 75°C for 10 minutes. A sample under reducing conditions was prepared by adding 2-mercaptoethanol (Sigma) and performing culture at 95 °C for 10 minutes. The gel was separated at 200 V for 45 minutes or at 110 V for 90 minutes, stained with a staining solution (LPS solution, SG501) for 30 minutes or more, and then destained using triple distilled water, and then the size and purity of the protein were examined.

[344]

[345] Example 8-3: SE-HPLC for purity measurement

[346] Purity was analyzed using an HPLC (Waters, e2695) instrument equipped with TSKgel G3000SWXL (Tosoh Bioscience, 08541) and guard column (Tosoh Bioscience, 808543). After the mobile phase (PBS) to be used for analysis was connected to the HPLC instrument, a wet prime for the corresponding line was performed. At a flow velocity of 0.5 mL/min, 20 µg of protein was injected and allowed to flow for 40 min, and the elution pattern was observed at 280 nm. The purity was calculated using the equation below.

[347] $\text{- \%Area} = (\text{Multispecific antibody peak area}) / \text{Total peak area} \times 100$

[348]

[349] According to the Examples, the purity and yield of the multispecific antibodies of the present invention are summarized in Table 2 below.

[350]

[351] [Table 2]

Multispecific antibody	Yield (mg, based on 1 L culture solution)			Purity (%)
	Primary column purification	Secondary column purification	Note	
A-AE	31.4	N/A	VL purification resin	97.7
A-AT	96			100.0
B-AE	130.8		Fc purification resin	100.0
B-AT	112.9			100.0
C-AE	240			100.0
C-AT	299.7	59.6	Fc purification resin + IEC	96.5
A-ME	116.6	N/A	VL purification resin	100.0
A-MT	163.2			100.0
B-ME	306.8		Fc purification resin	96.8
B-MT	654.8	360.7	Fc purification resin + IEC	96.9
C-ME	128.3	N/A	Fc purification resin	97.5

[352]

[353] **Example 9: Affinity analysis of multispecific antibodies**

[354]

[355] To measure the affinity of multispecific antibodies, an anti-human Fc captured (AHC) sensor (sartorius) was used to bind antibodies to antigens in an immobilized state. A multispecific antibody was constructed based on an antibody sequence in which the Fab antigen-binding site of the multispecific antibody targeted APLP2 or M6PR, a lysosomal transporting receptor (LTR), and the VH (H) antigen-binding site targeted EGFR as a target protein. Anti-APLP2 antibodies (SEQ ID NOs: 7 and 8), anti-M6PR antibodies (SEQ ID NOs: 9 and 10), and an anti-EGFR antibody (SEQ ID NO: 5) were used as controls.

[356]

[357] Affinity was measured using a bio-layer interferometry instrument (Fortebio). First, the multispecific antibody (Ligand) was diluted with PBS to a concentration of 2 µg/ml. The antigen (analyte) was prepared by being diluted with PBS at least 5 points for every concentration. The antigens used were recombinant human EGFR (sino bio), APLP2 (Acro bio), and M6PR (creative biomart). The AHC biosensor was hydrated by being immersed in PBS for 10 minutes. Into a black 96-well plate (VWR), 200 µl of the prepared buffer and 200 µl of the prepared sample were added. First, an equilibration process in PBS was performed for 60 seconds, and then the ligand was immobilized to 1 nm. After an equilibration process in PBS was performed again for 120 seconds, association to the analyte was performed for 300 seconds, and dissociation in PBS was performed for 600 seconds.

[358]

[359] [Table 3]

Clone	K_D (nM)	k_{on} (1/Ms)	k_{dis} (1/s)	R^2
APLP2 Ab	19.6 ± 0.15	$(1.37 \pm 0.01) \times 10^5$	$(2.69 \pm 0.01) \times 10^{-3}$	0.99
A-AE	26.0 ± 0.02	$(1.16 \pm 0.01) \times 10^5$	$(3.00 \pm 0.01) \times 10^{-3}$	0.99
B-AE	17.7 ± 0.02	$(1.05 \pm 0.01) \times 10^5$	$(1.87 \pm 0.01) \times 10^{-3}$	0.99
C-AE	19.2 ± 0.01	$(1.28 \pm 0.01) \times 10^5$	$(2.47 \pm 0.01) \times 10^{-3}$	0.99

[360]

[361] As presented in Table 3 above, APLP2-Ab had an affinity of 19.6 nM for rhAPLP2, and A-AE, B-AE, and C-AE had an affinity of 26.0 nM, 17.7 nM, and 19.2 nM, respectively, and were found to maintain binding affinity similar to that of the parental Ab.

[362]

[363] [Table 4]

Clone	K_D (nM)	k_{on} (1/Ms)	k_{dis} (1/s)	R^2
M6PR Ab	2.53 ± 0.03	$(1.07 \pm 0.01) \times 10^5$	$(2.60 \pm 0.03) \times 10^{-4}$	0.99
A-ME	3.18 ± 0.05	$(6.06 \pm 0.03) \times 10^4$	$(1.93 \pm 0.03) \times 10^{-4}$	0.99
B-ME	2.55 ± 0.04	$(1.08 \pm 0.01) \times 10^5$	$(2.74 \pm 0.04) \times 10^{-4}$	0.99
C-ME	3.38 ± 0.04	$(7.15 \pm 0.04) \times 10^4$	$(2.42 \pm 0.03) \times 10^{-4}$	0.99

[364]

[365] As presented in Table 4, M6PR-Ab had an affinity of 2.53 nM for rhM6PR, and A-ME, B-ME, and C-ME had an affinity of 3.18 nM, 2.55 nM, and 3.38 nM, respectively, and were found to maintain binding affinity similar to that of the parental Ab.

[366]

[367] For the measurement of affinity for EGFR, an AR2G biosensor (sartorius), which immobilized antibodies by amine coupling, was used. For analysis, the AR2G reagent kit (sartorius) was used. First, the antibody (Ligand) was diluted with 10 mM acetate buffer at pH 5 to 6 to a concentration of 2 μ g/ml. The antigen (analyte) was prepared by being diluted with PBS at least 5 points for every concentration. A 20 mM EDC/10 mM s-NHS (sartorius) mixture was prepared. The AR2G biosensor was hydrated by being immersed in water for 10 minutes. Into a black 96-well plate, 200 μ l of the prepared buffer and 200 μ l of the prepared sample were added. First, an equilibration process in PBS was performed for 60 seconds, and then activation was performed in an EDC/s-NHS mixture for 300 seconds. The multispecific antibody was immobilized on the biosensor until saturation. Quenching was performed in 1 M ethanolamine at pH 8.5 (sartorius) for 300 seconds. After an equilibration process in PBS was performed again for 120 seconds, association to the analyte was performed for 300 seconds, and dissociation in PBS was performed for 600 seconds.

[368]

[369] [Table 5]

Clone	K_D (nM)	k_{on} (1/Ms)	k_{dis} (1/s)	R^2
EGFR-Ab	2.55 ± 0.01	$(5.34 \pm 0.02) \times 10^5$	$(1.36 \pm 0.01) \times 10^{-3}$	0.99
A-AE	2.37 ± 0.02	$(7.46 \pm 0.04) \times 10^5$	$(1.77 \pm 0.01) \times 10^{-3}$	0.99
B-AE	3.12 ± 0.02	$(7.20 \pm 0.04) \times 10^5$	$(2.25 \pm 0.01) \times 10^{-3}$	0.99
C-AE	42.78 ± 1.48	$(3.46 \pm 0.11) \times 10^5$	$(1.48 \pm 0.02) \times 10^{-2}$	0.97
A-ME	2.68 ± 0.01	$(4.20 \pm 0.01) \times 10^5$	$(1.13 \pm 0.04) \times 10^{-3}$	0.99
B-ME	2.86 ± 0.01	$(4.14 \pm 0.01) \times 10^5$	$(1.18 \pm 0.01) \times 10^{-3}$	0.99
C-ME	115.3 ± 3.93	$(3.07 \pm 0.10) \times 10^5$	$(3.54 \pm 0.04) \times 10^{-2}$	0.98

[370]

[371] As presented in Table 5 above, the affinity of EGFR-Ab for rhEGFR was found to be 2.55 nM, and it was found that Type A multispecific antibodies, A-AE and A-ME, had an affinity at levels of 2.37 nM and 2.68 nM, respectively, and Type B multispecific antibodies, B-AE and B-ME, had an affinity of 3.12 and 2.86 nM, respectively, which were similar levels to that to the parental Ab, EGFR-Ab. However, Type C multispecific antibodies, C-AE and C-ME, had an affinity of 42.78 nM and 115.3 nM, respectively, and the affinity of Type C multispecific antibodies for EGFR tended to decrease compared to that of the parental Ab.

[372]

[373] **Example 10: Affinity and avidity analysis of multispecific antibodies**

[374]

[375] Considering the properties of Type B and Type C multispecific antibodies having 2+2 binding sites to antigens, the avidity effect was also examined. An AR2G biosensor was used to immobilize the antigen by amine coupling. Lysosomal transporting receptors (LTR) (Ligand; APLP2, M6PR) were diluted with 10 mM acetate buffer at pH 4 or 6 to a concentration of 5 µg/ml. The antigen (analyte) was prepared by being diluted with PBS at least 5 points for every concentration. A 20 mM EDC/10 mM s-NHS (sartorius) mixture was prepared. The AR2G biosensor was hydrated by being immersed in water for 10 minutes. Into a black 96-well plate, 200 µl of the prepared buffer and 200 µl of the prepared sample were added. First, an

equilibration process in PBS was performed for 60 seconds, and then activation was performed in an EDC/s-NHS mixture for 300 seconds. The multispecific antibody was immobilized on the biosensor until saturation. Quenching was performed in 1 M ethanolamine at pH 8.5 (sartorious) for 300 seconds. After an equilibration process in PBS was performed again for 120 seconds, association to the analyte was performed for 300 seconds, and dissociation in PBS was performed for 600 seconds.

[376]

[377] [Table 6]

Clone	K_D	k_{on} (1/Ms)	k_{dis} (1/s)	R^2
EGFR-Ab	171.8 ± 2.46 pM	$(6.62 \pm 0.02) \times 10^5$	$(1.14 \pm 0.02) \times 10^{-4}$	0.99
A-AE	4.82 ± 0.03 nM	$(6.21 \pm 0.04) \times 10^5$	$(3.00 \pm 0.01) \times 10^{-3}$	0.99
B-AE	94.03 ± 4.34 pM	$(3.87 \pm 0.01) \times 10^5$	$(3.64 \pm 0.17) \times 10^{-5}$	0.99
C-AE	765.1 ± 7.03 pM	$(5.94 \pm 0.04) \times 10^5$	$(4.54 \pm 0.03) \times 10^{-4}$	0.99
A-ME	5.61 ± 0.04 nM	$(5.85 \pm 0.04) \times 10^5$	$(3.28 \pm 0.01) \times 10^{-3}$	0.99
B-ME	103.2 ± 2.03 pM	$(7.41 \pm 0.02) \times 10^5$	$(7.65 \pm 0.15) \times 10^{-5}$	0.99
C-ME	3.04 ± 0.03 nM	$(4.00 \pm 0.04) \times 10^5$	$(1.22 \pm 0.01) \times 10^{-3}$	0.98

[378]

[379] As shown in Table 6 above, it was found that the avidity effect was exhibited and the affinity of EGFR-Ab for rhEGFR was improved from 2.55 nM to a level of 171.8 pM. In the case of Type A multispecific antibodies, A-AE and A-ME, there was no avidity effect, the affinities thereof were at levels of 4.82 nM and 5.61 nM, respectively, which were not significantly different from the affinity measured by the AHC biosensor. In the case of Type B multispecific antibodies, B-AE and B-ME, the avidity effect was exhibited, and the affinities thereof were at levels of 94.03 pM and 103.2 pM, respectively, which were higher than the affinity measured by the AHC biosensor. In the case of Type C multi-antibodies, C-AE and C-ME, it was found that the affinities thereof were at levels of 765.1 pM and 3.04 nM, respectively, and Type C multi-antibodies had higher binding ability to EGFR than Type A multispecific antibodies when the avidity effect was exhibited.

[380]

[381] Considering the analysis results of the affinity and avidity comprehensively, it has been found that multispecific antibodies having the form of Type B have superior target binding ability compared to multispecific antibodies of Type A and Type C.

[382]

[383] **Example 11: Analysis of simultaneous target binding ability of multispecific antibodies**

[384]

[385] It was attempted to verify that the multispecific antibodies according to the present invention could simultaneously bind to a target protein and a lysosomal transporting receptor (FIG. 3).

[386] LTR (Ligand; APLP2, M6PR) was diluted with 10 mM acetate buffer at pH 4 or 6 to a concentration of 5 µg/ml and added to each black 96-well plate by 200 µl. The antibody (analyte 1) was prepared by being diluted with PBS and added by 200 µl each. POI (analyte 2, rhEGFR) was prepared by being diluted with PBS to 200, 400, or 800 nM and added by 200 µl. EDC and s-NHS stocks were dissolved to prepare a 20 mM EDC/10 mM s-NHS solution.

[387] First, the AR2G biosensor was hydrated by being immersed in water for 10 minutes. Afterwards, the AR2G biosensor was equilibrated with PBS for 60 seconds and then activated by being immersed in an EDC/NHS mixture to immobilize the ligand (APLP2 or M6PR), and then quenching was performed in 1 M ethanolamine at pH 8.5 for 300 seconds. After an equilibration process in PBS was performed again for 120 seconds, association to the analyte 1 for 300 seconds and association to the analyte 2 for 300 seconds were successively performed.

[388]

[389] Binding of multispecific antibodies to lysosomal transporting receptors (APLP2, M6PR) was observed in the first 300 seconds and to rhEGFR in the subsequent 300 seconds. The gray graph indicated by the dotted line represents the buffer state without rhEGFR. Type A multispecific antibodies, A-AE and A-ME, showed a tendency that binding thereof to 200 nM rhEGFR increased but was not maintained and dissociation proceeded again. Type B multispecific antibodies, B-AE and B-ME, showed a tendency that binding thereof to 200 nM rhEGFR was maintained. Type C multispecific antibodies, C-AE and C-ME, bound to 400 nM and 800 nM of rhEGFR, respectively. There was a tendency that the degree of binding was low due to steric hindrance, but binding was maintained and dissociation did not proceed once binding was formed.

[390]

[391] Types A, B, and C all showed simultaneous binding ability to a lysosomal transporting receptor and a target protein EGFR, but the avidity effect was absent

and the binding state was not maintained in the case of Type A, and steric hindrance to EGFR occurred and the level of binding was low in the case of Type C. On the other hand, it was found that Type B bound well to EGFR while maintaining binding by the avidity effect on LTR, and thus the excellent simultaneous binding ability of multispecific antibodies was verified.

[392]

[393] **Example 12: Target protein degradation of multispecific antibodies**

[394]

[395] The target protein, EGFR degradation of the multispecific antibodies according to the present invention was evaluated from the following two perspectives.

[396]

[397] Specifically, firstly, it was attempted to evaluate whether the expression level of EGFR on the cell surface decreased after treatment with the multispecific antibodies according to the present invention, and secondly it was attempted to examine whether total EGFR protein levels decreased. It was attempted to compare the target protein degrading efficiency of the multispecific antibodies of the present invention according to these two test methods.

[398]

[399] Example 12-1: Examination of decrease in surface EGFR expression level by multispecific antibodies mediated by APLP2

[400]

[401] First, H1299, DLD-1, SW48, and A549 cell lines were cultured in culture media (H1299, DLD-1, SW48; RPMI1640 Gibco A10491-01, A549; DMEM Gibco 11995-065, each supplemented with 10% fetal bovine serum (Gibco, 16000-044) and anti-anti 1X), and then treated with the test substances when the confluency reached 70%. The concentrations of the test substances were 2 and 100 nM for each of A-AE, B-AE, and C-AE (test groups) and 100 nM for each of APLP2-Ab and EGFR-Ab (controls), media were a single control, and the respective substances were mixed with the culture medium and co-cultured for 24 hours. Cells were detached with trypsin-EDTA and washed with PBS. The cells were stained with a solution of Ghost Dye™ Violet 510 (TONBO, 13-0870-T500) diluted with PBS at 1 : 1,000 for 30 minutes at 4°C in a light-blocked environment. The cells were washed with FACS buffer (prepared to contain 0.5% FBS and 0.09% sodium azide in PBS, stored at 4°C, and the maintained at 4°C throughout the cell staining process), and then stained with PE-anti-human EGFR antibody (biolegend, 352904) diluted with FACS buffer at 1 : 200. Staining was performed at 4°C for 30 minutes in a light-blocked environment. After the cells were washed with FACS buffer, flow cytometry was performed using

LSRFortessa, and the results were compared using the fluorescence MFI value as the geometric median (FIG. 4).

[402]

[403] As a result of two repeated tests, it was found that A-AE, B-AE, and C-AE decreased the cell surface EGFR level compared to the control EGFR-Ab. In particular, the cell surface EGFR level treated with B-AE was the lowest.

[404]

[405] Example 12-2: Examination of decrease in total EGFR protein level by multispecific antibodies mediated by APLP2

[406]

[407] It was attempted to compare the target degrading efficiency by treatment with multispecific antibodies (A-AE, B-AE, and C-AE) by comparing the band intensities of EGFR protein in H1299, DLD-1, SW48, and A549 cell lines through Western blot. For this purpose, the day before the test, the cell number was adjusted to 1×10^5 cells/well, and the cells were mixed with a culture medium containing 10% FBS, added into a 12-well plate, and cultured. The next day, multispecific antibodies were diluted with a culture medium to 100 nM and the cells cultured in 12 wells were treated with the multispecific antibodies. After 24 hours, all the culture solution was removed, and the cells were washed with PBS. The cells were lysed using RIPA Lysis and Extraction Buffer (Thermo scientific, 89901) and protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, roche, 11697498001), and the supernatant was transferred to a new 1.5 mL tube. Into the running kit, 4% to 12% Bis-Tris Gel (Novex, NP0321BOX) was loaded, and the running kit was filled with MOPS SDS running buffer (Novex, NP0001) diluted 1X with triple distilled water. The standard maker (Bio-rad, 1610373) and cell lysate were prepared by being diluted with the sample buffer (Novex, NP0007), and then loaded into each well of the gel. Once running was completed, the gel was separated from the instrument and the protein was transferred to the membrane using iBlot™2 Transfer Stacks (invitrogen, IB24001). At this time, transfer was performed using the iBlot 2 gel transfer device under the conditions of 20 V and 7 minutes. Only the membrane was separated from the transfer device and washed with 0.1% PBST. The membrane was placed in the blocking solution (5% skin milk, BD 232100), and the reaction was conducted while stirring was performed at room temperature for 1 hour. The blocking solution was discarded, the primary antibody was diluted with the blocking solution as shown in Table 7 below and placed in the container containing the membrane, and the reaction was conducted while stirring was performed at room temperature for 1 hour.

[408]

[409] [Table 7]

Antibody	Supplier	Cat #	Dilution
EGF Receptor (D38B1) XP® Rabbit mAb	Cell signaling technology	4267	1:1,000
β-actin (13E5) Rabbit mAb (HRP Conjugate)	Cell signaling technology	5125	1:20,000

[410]

[411] After the reaction, the solution was discarded, and washing was performed with 0.1% PBST for 5 minutes three times. The multispecific antibodies (for EGFR, anti-rabbit IgG-HRP, 1:5,000) were diluted with the blocking solution and then placed in a membrane container and the reaction was conducted while stirring was performed at room temperature for 1 hour. The solution was all discarded, and washing was performed with 0.1% PBST for 5 minutes three times. After washing was completed, the ECL solution (Cytiva, GERPN2232) (prepared by mixing A with B at 1 : 1) was evenly sprayed on the membrane, the reaction was conducted, and the measurement was performed using a Chemidoc device about 1 minute after the reaction.

[412]

[413] As a result of two repeated tests, it was found that the degree of the EGFR degradation increased compared to EGFR-Ab by the treatment with A-AE, B-AE, and C-AE. It was found that B-AE among these had the highest degradation efficiency (FIG. 5).

[414]

[415] Example 12-3: Examination of lysosomal degradation by multispecific antibodies mediated by APLP2

[416]

[417] It was attempted to examine whether the EGFR degradation by the multispecific antibody B-AE was lysosome-dependent. For this purpose, the day before the test, the cell number was adjusted to 1×10^5 cells/well, and the cells were mixed with a culture medium containing 10% FBS, added into a 12-well plate, and cultured. The next day, 10 nM of multispecific antibody and 50 nM of Bafilomycin A1 (sigma, 5.08409) were evenly mixed with the culture medium, and treated to the cells (DLD-1, HT1299) in the 12 well plates. After 24 hours, all the culture solution was removed, and the cells were washed with PBS. The cells were lysed using RIPA Lysis and Extraction Buffer (Thermo scientific, 89901) and protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, roche, 11697498001), and the supernatant was transferred to a new 1.5 mL tube. Into the running kit, 4% to 12% Bis-Tris Gel (Novex, NP0321BOX) was loaded,

and the running kit was filled with MOPS SDS running buffer (Novex, NP0001) diluted 1X with triple distilled water. The standard maker (Bio-rad, 1610373) and cell lysate were prepared by being diluted with the sample buffer (Novex, NP0007), and then loaded into each well of the gel. Once running was completed, the gel was separated from the instrument and the protein was transferred to the membrane using iBlot™2 Transfer Stacks (Invitrogen, IB24001). At this time, transfer was performed using the iBlot 2 gel transfer device under the conditions of 20 V and 7 minutes. Only the membrane was separated from the transfer device and washed with 0.1% PBST. The membrane was placed in the blocking solution (5% skin milk, BD 232100), and the reaction was conducted while stirring was performed at room temperature for 1 hour. The blocking solution was discarded, the primary antibody was diluted with the blocking solution as shown in Table 7 above and placed in the container containing the membrane, and the reaction was conducted while stirring was performed at room temperature for 1 hour.

[418]

[419] As a result of two repeated tests, it was found that the inhibition of lysosomal activity impaired the targeted protein degradation activity via B-AE treatment, indicating that APLP2 targeting multispecific antibody degrades EGFR in a lysosome dependent manner (FIG. 6).

[420]

[421] Example 12-4. Examination of decrease in surface EGFR expression level by multispecific antibodies mediated by M6PR

[422]

[423] To examine the EGFR degradation by M6PR-mediated multispecific antibodies, H1299, DLD-1, SW48, and A549 cell lines were cultured in a culture medium (H1299, DLD-1, SW48; RPMI1640 Gibco A10491-01, A549; DMEM Gibco 11995-065, each supplemented with 10% fetal bovine serum (Gibco, 16000-044) and anti-anti 1X) and treated with test substances when the confluency reached 70%. The test substances and treatment conditions are as shown in Table 8 below, and each substance was mixed with the culture.

[424]

[425] [Table 8]

Purpose	Antibody	Concentration (nM)	Culture time (h)
Type A/B/C	M6PR-Ab, EGFR-Ab	100	24
	A-ME, B-ME, C-ME	100, 1	
Concentration dependency	M6PR-Ab, EGFR-Ab	100, 10, 1, 0.1, 0.01	24
	B-ME	100, 10, 1, 0.1, 0.01	
Time dependency	EGFR-Ab	10	6, 24, 48
	B-ME	10	

[426]

[427] To analyze the surface EGFR expression level, cells were detached with Trypsin-EDTA and washed with PBS, and reacted with a solution of Ghost Dye™ Violet 510 (TONBO, 13-0870-T500) diluted with PBS at 1 : 1,000 for 30 minutes at 4°C in a light-blocked environment. The cells were washed with FACS buffer (prepared to contain 0.5% FBS and 0.09% sodium azide in PBS, stored at 4°C, and then maintained at 4°C throughout the cell staining process for use), and then stained with PE-anti-human EGFR antibody (biolegend, 352904) diluted with FACS buffer at 1 : 200. Staining was performed at 4°C for 30 minutes in a light-blocked environment. After the cells were washed with FACS buffer, flow cytometry was performed using LSRFortessa, and the results were compared using the fluorescence MFI value as the geometric median.

[428]

[429] As a result of two repeated tests, it was found that all the M6PR-mediated multispecific antibodies, A-ME, B-ME, and C-ME, decreased the surface EGFR level compared to EGFR-Ab. Especially, the reduction of the surface EGFR level was the greatest in the Type B multispecific antibody treated group, and it was found that a decrease in surface EGFR level was induced in a time- and dose-dependent manner (FIG. 7).

[430]

[431] From the Examples described above, it was found that the Type B multispecific antibody according to the present invention had targetability for lysosomal transporting receptors (APLP2, M6PR) and target proteins and was capable of inducing the degradation of target proteins by lysosomes through this. This suggests that the

multispecific antibody platform of the present invention can be utilized to develop therapeutic agents through the degradation of target proteins.

[432]

[433] Hereinafter, several kinds of Type B multispecific antibodies were produced, and the binding activity and degradation efficiency of the Type B multispecific antibodies for a target protein were examined again.

[434]

[435] **Example 13: Production of APLP2 and M6PR protein antigens**

[436]

[437] For the production of antibodies targeting lysosomal transporting receptors (APLP2, M6P6), the extracellular domain (ECD) portion of human APLP2 (G32-S692) or mouse APLP2 (G32-S636) and the domain 11-13 (M1508-L1992) portion of human M6PR were used as antigens. Each antigen was fused with a His-Avi or Myc-His-Avi tag.

[438]

[439] Example 13-1. Transformation and protein expression

[440] To competent cells, 100 ng of human or mouse APLP2 ECD or human M6PR gene expression vector (pcIW) was added, and mixing was performed. After the mixture was transferred to a cuvette for electroporation, electric shock was applied at a gap of 2 mm and a voltage of 2.5 kV. Recovery was allowed in a shaking incubator at 37°C for 30 minutes with 1 mL of LB media. This was spread on an SB plate containing carbenicillin (50 µg/mL), and transformation was performed. The acquired plasmid was sequenced to examine whether the desired gene was cloned into the vector.

[441] Transient expression was performed using the ExpiFectamine 293 Transfection Kit (Gibco) according to the manufacturer's instructions. First, two tubes containing Opti-MEM (Gibco) were prepared, DNA and expifectamine were added to the two tubes, respectively, and incubation was performed at room temperature for 5 minutes. The two mixtures were mixed together, and the mixture was incubated for an additional 20 to 30 minutes. The mixed mixture was added to the prepared cells. After 16 to 20 hours of expression, enhancers 1 and 2 were added respectively, and expression was performed in a shaking incubator at 37°C for 5 to 7 days.

[442]

[443] Example 13-2. Protein purification and concentration

[444] To obtain the target expressed and secreted in Expi CHO cells (Gibco) or Expi 293 cells (Gibco), centrifugation was performed at 4,000 g for 20 minutes at 4°C. After the cell pellet was removed, the supernatant including the medium was filtered through a filter having a 0.22 µm pore size (Corning), the batch to be purified was used immediately, and the batch to be used later was stored at -20°C. To proceed with

the first purification process, affinity chromatography, in order to bind the filtered supernatant to a HisTrap HP column (Cytiva), the resin was previously filled with Ni using NiSO_4 , and the resin was previously equilibrated with 20 mM NaPi buffer at pH 7.5 to be used in column work. The supernatant was bound to the HisTrap HP column with 5 mL resin volume at a flow rate of 5.0 to 10.0 ml/min using the AKTA prime system, and washing was thoroughly performed with the equilibrium buffer in a volume to be 20 times (100 mL) or more the column volume. To further remove nonspecific binding (impurities) from the resin, washing was performed using a 20 mM NaPi buffer containing 20 mM imidazole at pH 7.5 as the washing buffer in a volume to be 10 times (50 mL) the column volume. The elution buffer prepared by containing 300 mM imidazole in the equilibration buffer at pH 7.5 was allowed to flow in a volume to be 5 times (25 mL) the column volume and collected, and elution of the target was examined at a wavelength of 280 nm.

[445] For buffer exchange and concentration, PBS buffer was added into a viva spin tube (30K MWCO, Sartorius), and the membrane was equilibrated by centrifugation at 3500 rpm for 5 minutes. PBS and the eluted sample in the first purification process were added together in the same volume, centrifugation was performed at 3500 rpm for 10 minutes, and the sample was repeatedly concentrated to be about 2 mL, and collected. To proceed with the second purification process, gel filtration, the Hiload 16/600 superdex 200 column (Cytiva) was equilibrated with PBS buffer in a volume to be 1 time (120 mL) or more the column volume at a flow rate of 1.0 ml/min using the AKTA prime system. The concentrated sample was allowed to pass through the column, and the profile of FPLC in the sample, containing the targets to be separated by size, was analyzed. The profile curve observed at a wavelength of 280 nm in the gel filtration was analyzed and the peak corresponding to the target was fractionated by comparison with the reference molecular size. The fractions of the first and second purification processes were confirmed using SDS-PAGE. The sample and the 4x reducing sample buffer were mixed at a 3 : 1 volume ratio, heated at 90°C for 5 minutes, and then cooled, then the prepared sample was loaded by 10 μL per well on a 4% to 12% Bis-Tris gel, and electrophoresis was performed at 130 V for 60 minutes. The gel was separated and stained with SunGel Staining solution for 30 minutes or more, then destaining was performed using DW, and then the size and purity of the target were determined by comparison with the reference molecule within the size marker. Only the fractions whose target purity was confirmed within PAGE were selected, concentrated, aliquoted under a concentration condition of 1.0 mg/mL or more, and stored at -20°C.

[446]

[447] **Example 14: Screening of antibody against APLP2**

[448]

[449] General antibody production technologies were used to produce antibodies against the APLP2 protein. Specifically, a synthetic library of mouse immune and human domain antibodies were constructed and screened. After sorting using the commonly used hybridoma formation and phage display panning techniques, screening was performed by a single clone ELISA method. By the single clone ELISA screening method, the culture solution expressed from a single clone was mixed with the immobilized APLP2 protein to examine whether the antibody specifically bound to the APLP2 protein. The heavy and light chain sequences of the antibodies acquired through sequence analysis were determined (Table 9). However, in the clones acquired from the human domain antibody synthetic library, the synthetic library itself was a library consisting only of heavy chain sequences, and only heavy chain sequence information was included.

[450]

[451] [Table 9]

Clone ID	Sequence	SEQ ID NO:
A3-05	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYAMGWVRQAPGKGP EWVSLISYDGSSTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARSFIPFYQLDYWGQGLTVSS	11
A3-09	EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYIMGWVRQAPGKGP EWVSLISYGGSTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARNSINAPI SHFIDYWGQGLTVSS	12
A3-10	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYAMGWVRQAPGKGP EWVSLISYDGSATWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARSLLPFKALDYWGQGLTVSS	13
A3-23	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMGWVRQAPGKGP EWVSLISYSGGSTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARNMIPFEPLDYWGQGLTVSS	14
A3-38	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYAMGWVRQAPGKGP EWVSLISYDGSSTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARSFYPLHIDYWGQGLTVSS	15
A3-39	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYAMGWVRQAPGKGP EWVSLISYDGSATWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARDLSPSHSFIDYWGQGLTVSS	16
A3-59	EVQLVESGGGLVQPGGSLRLSCAASGFTFTNYSMGWVRQAPGKGP EWVSLISSSGSTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARHMKINNLVTMDYWGQGLTVSS	17
A3-62	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYAMGWVRQAPGKGP EWVSLISYDGSNTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARLLKPTDLHLDYWGQGLTVSS	18
A3-64	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYAMGWVRQAPGKGP EWVSLISYDGGATWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARTLVPF TALDYWGQGLTVSS	19
A3-66	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYAMGWVRQAPGKGP EWVSLISYDGSATWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARHFLPFIALDYWGQGLTVSS	20
A3-71	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMGWVRQAPGRGP EWVSLISYDGGNTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCAREVYITKNATTLDYWGQGLTVSS	21
A3-76	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYIMGWVRQAPGKGP EWVSLISSSSNTWYDYSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARSI SYVNPTLFDYWGQGLTVSS	22

A3-92	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYAMGWVRQAPGKGP EWVSLISYDGSSTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCAR <u>SFIPKVL</u> DYWGQGLVTVSS	23
A3-95	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYAMGWVRQAPGKGP EWVSLISYDGSSTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARN <u>LLPF</u> MLDYWGQGLVTVSS	24
A4-05	EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYAMGWVRQAPGKGP EWVSLISYDGSNTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCAK <u>NNDS</u> PASTLDYWGQGLVTVSS	25
A4-08	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYAMGWVRQAPGKGP EWVSLISYSGSATWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARD <u>LSD</u> HRIDYWGQGLVTVSS	26
A4-23	EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYAMGWVRQAPGKGP EWVSLISSDSGSTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCAK <u>ERKPT</u> GALDYWGQGLVTVSS	27
A4-27	EVQLVESGGGLVQPGGSLRLSCAASGFTFTNYDMGWVRQAPGKGP EWVSLISGDSSNTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCAK <u>SNIR</u> SAFLDYWGQGLVTVSS	28
A4-38	EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYAMGWVRQAPGKGP EWVSLISGDGGNTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARY <u>RVH</u> GILDYWGQGLVTVSS	29
A4-49	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYAMGWVRQAPGKGP EWVSLISGDGGNTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCAK <u>QTW</u> WKGMSLDYWGQGLVTVSS	30
A4-52	EVQLVESGGGLVQPGGSLRLSCAASGFTFTNYAMGWVRQAPGKGP EWVSLISGSGSSTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCAK <u>EVYHNT</u> SDIDYWGQGLVTVSS	31
A4-55	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYMGWVRQAPGKGP EWVSLISSDGGNTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARR <u>PHAS</u> RGPYLDYWGQGLVTVSS	32
A4-69	EVQLVESGGGLVQPGGSLRLSCAASGFTFTNYMGWVRQAPGKGP EWVSLISSGGNTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYSCAK <u>F</u> SNYDGLLDYWGQGLVTVSS	33
A4-74	EVQLVESGGGLVQPGGSLRLSCAASGFTFTAYSMGWVRQAPGKGP EWVSLISSGGSSTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCAK <u>SPYHS</u> SYQYIDYWGQGLVTVSS	34
A4-75	EVQLVESGGGLVQPGGSLRLSCAASGFTFTNYMGWVRQASGKGP EWVSLISSDGSNTWYDDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCAR <u>FSDYH</u> GTLDYWGQGLVTVSS	35

A4-80	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYMGWVRQAPGKGP EWVSLISSGGSN TWYDDSVKGRFTISRDN S KNTLYLQMNSLRAEDTAVYYCAKRPHADPDPLYVDYWGQGLTVTVSS	36
A4-82	EVQLVESGGGLVQPGGSLRLSCAASGFTFTNYIMGWVRQAPGKGP EWVSLISYDSSN TWYDDSVKGRFTISRDN S KNTLYLQMNSLRAEDTAVYYCARRPYSPGPYVDYWGQGLTVTVSS	37
A9-02 heavy chain	QIQLVQSGPELKKPGETVKISCKASGYTFTNYAMNWKQAPGKGLKWMGWINTYTGEPT YADDFKGRFAFSLET S ASTAYLQINSLKNDMATYFC SREGGFDWYFDVWGAGTTTVTVSS	38
A9-02 light chain	DIVLTQSPASLAVSLGQRATISCRASKSVSTSVVNYMHWYQKPGQPPKLLIYLASNLES GVPARFSGSGSGTDF TLNIHPVEEEDAATYYCQHSRELPRTFGGGTKLEIK	39

[454]

[455] The underlined portions in the sequences correspond to the complementary determining regions (CDRs).

[456]

[457] **Example 15: Screening of antibody against M6PR**

[458]

[459] To produce antibodies against the M6PR domain 11-13 protein, a domain antibody synthetic library was constructed and screened. After sorting using the commonly used phage display panning technique, screening was performed by a single clone ELISA method. By the single clone ELISA screening method, the culture solution expressed from a single clone was mixed with the immobilized M6PR protein to examine whether the antibody specifically bound to the M6PR protein. The heavy chain sequences of the antibodies acquired through sequence analysis were determined (Table 10).

[460]

[461] [Table 10]

Clone ID	Sequence	SEQ ID NO:
M3-10	EVQLVESGGGLVQPGGSLRLSCAASGFTFTNYDMGWVRQAPGKGPWWSLISSGSSNTWYDDSVKGRFTISR NSKNTLYLQMNSLRAEDTAVYYCARIYEQLIDYWGQGLTVTVSS	40
M3-54	EVQLVESGGGLVQPGGSLRLSCAASGFTFTGYDMGWVRQAPGKGPWWSLISYDGSSTWYDDSVKGRFTISR NSKNTLYLQMNSLRAEDTAVYYCARVFAEILIDYWGQGLTVTVSS	41
M7-23	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYDMGWVRQAPGKGPWWSLISSDSSNTWYDDSVKGRFTISR NSKNTLYLQMNSLRAEDTAVYYCARATLHLVDYWGQGLTVTVSS	42

[462]

[463] The underlined portions in the sequences correspond to the complementary determining regions (CDRs).

[464]

[465] **Example 16: Expression and purification of Type B multispecific antibodies**

[466]

[467] As described in Example 2, the sequences of the lysosomal transporting receptor binding regions (SEQ ID NOs: 11 to 42) and the target protein binding regions (including the heavy chain variable region of SEQ ID NO: 43 and the light chain variable region of SEQ ID NO: 44) were subcloned into pCIW and produced in a Type B multispecific antibody format. Expi293F cells were prepared at a concentration of 3×10^6 cells/mL in the Expi293 expression medium (37°C, 8% CO₂, 125 rpm, viability $\geq 95\%$). These cells were transformed with 1 μ g of DNA per 1 ml according to the manufacturer's protocol. The expressed supernatant was filtered and purified using Protein A PhyTip Columns (Biotage). Bound antibodies were eluted using an amine-based elution buffer at pH 2.8 or a sodium citrate elution buffer at pH 3.5. The eluted solution was neutralized by adding 1 M Tris-HCL solution at pH 8.8. The concentration was measured at a wavelength of 280 nm, and the purity was determined using SE-HPLC. As a result, a sample having a purity of 70% or more based on the main peak was used.

[468]

[469] Type B multispecific antibodies produced at this time exist in two types.

[470]

[471] i) First, a multispecific antibody in which the lysosomal transporting receptor binding region includes a heavy chain variable region, a heavy chain constant region, a light chain constant region and a light chain variable region (lysosomal transporting receptor

in the form of Fab) and the target protein binding region includes a VH or VHH domain in the form as illustrated in FIG.2 (FIG. 20A), or

[472] ii) a multispecific antibody in which the lysosomal transporting receptor binding region includes a VH or VHH domain (lysosomal transporting receptor binding region in the form of a domain) and the target protein binding region includes a heavy chain variable region, a heavy chain constant region, a light chain constant region and a light chain variable region (FIG. 20B).

[473] In the two types of Type B multispecific antibodies, the heavy chain variable regions of the lysosomal transporting receptor binding region and target protein binding region are connected to each other.

[474]

[475] The sequence of the Fc region of the monomer used in the multispecific antibody of the present invention is SEQ ID NO. 141, the sequence of the hinge region is SEQ ID NO. 142, and the sequences of the heavy chain constant region and light chain constant region are SEQ ID NOs: 143 and 144, respectively.

[476]

[477] In the Example described above, it was attempted to examine whether the Type B multispecific antibody containing a lysosomal transporting receptor in the form of Fab had excellent ability to degrade a target protein, as well as to examine whether the Type B multispecific antibody containing a lysosomal transporting receptor in the form of a domain also had excellent ability to degrade a target protein.

[478]

[479] **Example 17: Type B multispecific antibody screening**

[480]

[481] To screen for B-type Lportbodies in this example, the residual protein amount for the target protein EGFR was calculated and selected.

[482]

[483] Example 17-1. Screening of EGFR degradation of B-type Lportbody

[484]

[485] First, the DLD-1 cell line was prepared to 4×10^4 cells/300 μ L in a culture medium (RPMI1640 (Gibco, A10491-01) supplemented with 10% fetal bovine serum (Gibco, 16000-044) and anti-anti 1X), at the 48-well plate . After 24 hours of incubation, the Lportbody were treated at 10 nM in culture medium. After 24 hours, all the culture solution was washed out via cold PBS, and then the cells were lysed using RIPA Lysis and Extraction Buffer (Thermo scientific, 89901) and protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, roche, 11697498001). The supernatant cell lysate was transferred to a new 1.5 mL tube. The EGFR protein concentration in the cell lysate was measured by Human EGFR DuoSet ELISA (R&D, DY231) and the total protein

concentration in the cell lysate was measured by BCA assay (according to the protocol provided by ThermoFisher 23225). The amount of EGFR was quantified by correcting for the deviation in cell count between samples according to the following formula;

[486]
$$\text{Amount of EGFR protein } \left(\frac{\text{ng}}{\text{mg}} \right) = \frac{\text{human EGFR (ng/mL) from ELISA}}{\text{total protein (mg/mL) from BCA assay}}$$

[487]

[488] The efficiency of the degradation was evaluated by converting the relative EGFR amount in the test substance treatment group based on the amount of EGFR protein in the culture solution treatment group (= 1).

[489]

[490] As a result of the test, it was found that a number of Lportbodies constructed as B type against APLP2 degraded total EGFR in cells compared to the control substance cetuximab (CTX) (FIG. 8).

[491]

[492] In FIG. 8, A9-2 is an Lportbody in which a lysosomal transporting receptor binding region (lysosomal transporting receptor binding region in the form of Fab) including a heavy chain variable region of an antibody against APLP2, a heavy chain constant region, a light chain constant region and a light chain variable region and a target protein (EGFR) binding region are connected, and other B-type Lportbodies are Lportbodies in which a lysosomal transporting receptor binding region (a lysosomal transporting receptor binding region in the form of a domain) including a heavy chain variable region of an antibody against APLP2 and a target protein (EGFR) binding region including a heavy chain variable region, a heavy chain constant region, a light chain constant region, and a light chain variable region are connected.

[493]

[494] As a result, it has been found that total EGFR in cells can be degraded compared to cetuximab (CTX) regardless of the form of the lysosomal transporting receptor, that is, either a Fab or domain form.

[495]

[496] Example 17-2. Screening of B-type Lportbody mediated by M6PR

[497]

[498] The EGFR degradation of a test substance of B-Type Lportbody against M6PR was evaluated. For this purpose, the ability was evaluated in the same manner as the APLP2 B-type test substance screening of Example 17-1. However, cells were cultured for 24 hours in a 6-well plate at 2×10^5 cells/1000 μL , and then treated with the test substance at 10 nM. The test method and analysis method other than this were applied identically.

[499] As a result of the screening, it was found that the total EGFR in cells was degraded in a number of test substances constructed as B type against M6PR compared to media (FIG. 9).

[500]

[501] In all B-Type Lportbodies against M6PR in FIG. 9, a lysosomal transporting receptor binding region (a lysosomal transporting receptor binding region in the form of a domain) including the heavy chain variable region of an antibody against M6PR and a target protein (EGFR) binding region including a heavy chain variable region, a heavy chain constant region, a light chain constant region, and a light chain variable region are connected.

[502]

[503] Example 17-3. Examination of lysosome-dependent target protein degradation of B-type Lportbody mediated by APLP2 and M6PR

[504]

[505] It was attempted to examine whether the EGFR degradation by a multispecific antibody was lysosome dependent. For this purpose, cells were treated with the Lportbodies in the same manner as in the screening test, and also treated with 50 nM of Bafilomycin A1 (sigma, 5.08409), a lysosomal activity inhibitor. The degradation efficiency was evaluated by quantifying the total EGFR protein amount by BCA assay and Human EGFR DuoSet ELISA, the same analysis methods as above and comparing the amount, or converting the relative EGFR amount based on the amount of EGFR protein in the culture solution treatment group (= 1).

[506] As a result of the test, it was found that the degradation of EGFR observed in the Lportbodies treated group disappeared when lysosomal activity was inhibited. Through this, it has been found that the APLP2-mediated or M6PR-mediated B-type Lportbody has superior degradation efficiency through lysosomes (FIGS. 10 and 11).

[507]

[508] As found in Example 17-1, through A9-2, it has been found that the Type B multispecific antibody could degrade a target protein in a lysosome-dependent manner regardless of the binding format to the lysosomal transporting receptor.

[509]

[510] **Example 18: Analysis of B-type Lportbody properties**

[511]

[512] Example 18-1. Analysis of mouse APLP2 binding ability of B-type Lportbody

[513]

[514] ELISA was performed to measure the cross-reactivity of 30 species having high EGFR degradation with mouse APLP2. ELISA plates were coated with 50 µl of human APLP2 and mouse APLP2 antigens at 2 µg/ml at 4°C overnight. The next day, the

antigens on the ELISA plates were removed and washing was performed one time with PBS + 0.05% Tween20. Each antigen-coated well was blocked with 300 µl of PBS + 4% skim milk for 2 hours at room temperature. After 2 hours, washing was performed three times with PBS + 0.05% Tween 20. The 30 antibodies were diluted to 100 nM and the concentrations thereof were measured. The diluted antibody was added to each well by 50 µl and allowed to bind for 1 hour at room temperature. Washing was performed again three times with PBS + 0.05% Tween 20. Anti-human Fc-HRP (Invitrogen, 15000:1) was added to each well by 50 µl and incubation was performed at room temperature for 1 hour. Washing was performed three times with PBS + 0.05% Tween 20. TMB solution was added to each well by 50 µl and incubation was performed for 10 minutes. The reaction was stopped by adding 50 µl of stop solution to each well. The degree of color development was measured using an ELISA reader.

[515] It was found that 15 species of antibodies also bound to mouse APLP2 and had cross-reactivity (FIG. 12).

[516]

[517] Example 18-2. Epitope binning of B-type Lportbody

[518]

[519] Epitope binning was performed in three steps using a BLI system (Octet Qke). Step #1 involved antigen immobilization; step #2 involved primary antibody binding; and step #3 involved secondary antibody binding. A baseline step for 60 seconds was set between the respective steps and the baseline signal was identified. Before step 1 was performed, the purified recombinant antigen human APLP2 was biotinylated to the Avi tag contained in the construct using the BirA enzyme kit (Avidity) according to the manufacturer's protocol. Biotinylation was confirmed using the Biotinylated APLP2 HABA Biotin Quantification Kit (Thermo).

[520] The compositions of the buffers used in steps #1 to 3 were all based on the PBS composition containing 1% BSA and 0.05% tween. In step #1, biotinylated APLP2 having a concentration of 1.0 mg/mL was diluted to 5 ng/mL and immobilized on streptavidin (SA) biosensor (Sartorius) previously hydrated at 1,000 rpm for 10 minutes. In step #2, different primary binding Abs diluted to 200 nM or 600 nM were bound for 600 seconds. In step #3, one secondary binding Ab (at 200 nM or 600 nM) was bound for 300 seconds to determine the self-association and the degree of competition.

[521] In the experimental order of step #1, step #2, and step #3, a condition of using the same antibody in step #1 and step #2 (self-blocking) was included as a reference to determine that the primary antibody binding was saturated in step #2, and a condition in which the immobilized antigen was treated with only a primary or secondary antibody was included as a value to quantify the degree of secondary antibody binding.

The values that increased sensorgram were excluded in self-blocking, the results showing that the sensorgram did not increase sufficiently when the immobilized antigen was treated with only a primary or secondary antibody were also excluded, and this was optimized by changing the conditions to increase the antibody concentration being titrated.

- [522] The degree of competition was defined as 100% when only the secondary antibody bound to the immobilized antigen, expressed as a percentage (%). A binding level of less than 33% was defined as full competition, a binding level between 33% and 66% was defined as moderate competition, and a binding level of 66% or more was defined as non-competition. The relationship was identified that simultaneously indicated all 29 antibodies to be compared.
- [523]
- [524] As a result of binning among a total of 29 antibodies excluding the reference antibody, the 29 antibodies can be distinguished in the matrix into four groups (Groups #1 to 4, FIG. 13) based on whether human/mouse cross-reactivity with APLP2 was determined to be present (15 species) or absent (14 species) in ELISA.
- [525] Group #1 is a relation diagram between antibodies having human/mouse cross-reactivity in ELISA, and indicates the correlations among a total of 15 species, including A3-09, A3-39, A3-92, A3-62, A3-38, A4-55, A3-10, A3-23, A3-05, A4-82, A4-08, A4-80, A3-95, A3-64, and A4-74.
- [526] Group #4 is a relation diagram between antibodies that do not have human/mouse cross-reactivity in ELISA, and indicates correlations among a total of 14 species, including A9-02, A3-76, A4-52, A4-87, A4-49, A4-75, A4-05, A4-88, A3-59, A4-38, A4-23, A4-27, A4-69, and A3-71. At this time, it is considered that A9-02 is Fab.
- [527] Group #2 indicates the correlation between the case of saturating 15 species having human/mouse cross-reactivity in ELISA with a primary antibody and the case of titrating 14 species not having human/mouse cross-reactivity in ELISA with a secondary antibody.
- [528] Group #3 indicates the correlation between the case of saturating 14 species not having human/mouse cross-reactivity in ELISA with a primary antibody and the case of titrating 15 species having human/mouse cross-reactivity in ELISA with a secondary antibody.
- [529] It can be inferred that A4-80, A4-55, and A4-74 in Group #1 within the epitope binning result matrix have different tendencies.
- [530] It can be inferred that A3-09, A9-02, and A3-59 in Group #4 within the epitope binning result matrix have different tendencies.
- [531] It can be inferred that A3-76 in Groups #2 and #3 within the epitope binning result matrix has a different tendency.

- [532] The epitope binning result matrix and the four Groups #1 to 4 were distinguished into Bin groups Bin #1 to #9 (FIG.13). The Bin groups reflect the case where the results differ depending on the directionality for steps 1 and 2 (uni-direction), and are classified into groups of antibodies having a tendency for similar epitopes.
- [533] Bin #1 contains A3-09.
- [534] Bin #2 contains A3-39, A3-92, A3-62, A3-38, A3-10, A3-23, A3-05, A4-82, A4-08, A3-95, and A3-64.
- [535] Bin #3 contains A4-55.
- [536] Bin #4 contains A4-80.
- [537] Bin #5 contains A4-74.
- [538] Bin #6 contains A9-02.
- [539] Bin #7 contains A3-76.
- [540] Bin #8 contains A4-52, A4-87, A4-49, A4-75, A4-05, A4-88, A4-38, A4-23, A4-27, A4-69, and A3-71.
- [541] Bin #9 contains A3-59.
- [542] It reflects a competitive result when the areas in the bin chart representing Bins #1 to #9 overlap, and it reflects a non-competitive result when the areas do not overlap.
- [543]
- [544] Example 18-3. Affinity analysis of B-type Lportbody
- [545]
- [546] Affinity was measured using bio-layer interferometry equipment (Fortebio). First, kinetic buffer was prepared by adding 1% BSA and 0.05% Tween 20 to PBS. Multispecific antibodies (Ligand) were diluted with kinetic buffer to a concentration of 2 to 4 µg/ml. The antigen (analyte) was prepared by being diluted with kinetic buffer at least 5 points for every concentration.
- [547] For the analysis of affinity for human APLP2 antigen, the AHC biosensor was immersed in PBS for 10 minutes. To a black 96-well plate (VWR), 200 µl of the prepared buffer and 200 µl of the prepared sample were added. First, the ligand was immobilized to a level of 1 nm after an equilibration process in the kinetic buffer was performed for 60 seconds. After an equilibration process in the kinetic buffer was performed again for 120 seconds, binding to the analyte was performed for 600 seconds, and then dissociation was performed in the kinetic buffer for 600 seconds (Table 11).
- [548]
- [549] [Table 11]

Clone	K_D (nM)	k_{on}	k_{off}	R^2
A3-05	29.2	2.26E+04	6.61E-04	0.993
A3-10	41.6	1.55E+04	6.47E-04	0.992
A3-23	27.5	1.99E+04	5.46E-04	0.988
A3-38	24.9	1.95E+04	4.87E-04	0.987
A3-39	18.7	2.26E+04	4.22E-04	0.992
A3-62	29.6	2.65E+04	7.82E-04	0.986
A3-64	30.0	2.74E+04	8.22E-04	0.985
A3-92	161.9	2.99E+04	4.84E-03	0.989
A4-08	55.6	2.76E+04	1.53E-03	0.977
A4-55	95.7	8.88E+03	8.49E-04	0.990
A4-74	89.5	4.01E+04	3.59E-03	0.978
A4-80	319.5	4.61E+04	1.47E-02	0.963
A4-82	33.5	5.15E+04	1.73E-03	0.988
A9-02	0.33	3.82E+05	1.25E-04	0.996

[550]

[551] For the analysis of affinity for human M6PR antigen, the AHC biosensor was immersed in PBS for 10 minutes. To a black 96-well plate (VWR), 200 μ l of the prepared buffer and 200 μ l of the prepared sample were added. First, the ligand was immobilized to a level of 1 nm after an equilibration process in the kinetic buffer was performed for 60 seconds. After an equilibration process in the kinetic buffer was performed again for 120 seconds, binding to the analyte was performed for 300 seconds, and then dissociation was performed in the kinetic buffer for 300 seconds (Table 12).

[552]

[553] [Table 12]

Clone	K_D (nM)	k_{on}	k_{off}	R^2
M3-10	13.8	$6.31E+05$	$8.73E-03$	0.996
M3-54	4.01	$5.19E+05$	$2.08E-03$	0.983
M7-23	199	$3.03E+05$	$6.01E-02$	0.986

[554]

[555] **Example 19: Comparison of EGFR degradation of A/B/C-type Lportbodies**

[556]

[557] Example 19-1. Examination of superior target protein degradation of APLP2-mediated B-type Lportbody

[558]

[559] It was attempted to compare the EGFR degradation of A/B/C-type Lportbody antibodies mediated by APLP2. For this purpose, A/B/C-type Lportbody antibodies were produced using the clone sequences identified in Example 14, and the EGFR degradation was evaluated.

[560]

[561] As a result, as illustrated in FIG. 14a, it was found that among the produced A/B/C-type Lportbody antibodies, the B-type Lportbody antibody had superior EGFR degradation compared to the control, Cetuximab (CTX), and also had the great degradation efficiency among all types of Lportbodies.

[562]

[563] As the B-type Lportbody was found to have excellent target protein degradation as described above, A/B-type Lportbodies were additionally prepared using different sequences and the target protein degradation was examined. As a result, as illustrated in FIG. 14b, it was found that the B-type Lportbody not only had superior EGFR degradation compared to the control, CTX, but also had superior degradation compared to A-type Lportbody.

[564]

[565] Example 19-2. Examination of excellent target protein degradation of M6PR-mediated B-type Lportbody

[566]

[567] It was attempted to compare the EGFR degradation of B-type Lportbody antibodies mediated by M6PR with that of A-type Lportbody antibodies. For this purpose, A/B-type Lportbody antibodies were produced using the clone sequences identified in Example 15, and the EGFR degradation was examined.

[568]

[569] As a result, as illustrated in FIG. 15, it was found that all B-type Lportbody antibodies had superior EGFR degradation compared to the control, CTX, and had superior degradation compared to A-type Lportbody antibodies.

[570]

[571] Through the Examples described above, it has been found once again that all the multispecific antibodies produced as B-type of the present invention can have excellent target protein targeting ability.

[572]

[573] **Example 20: Evaluation of EGFR degradation of B-type Lportbody**

[574]

[575] Example 20-1. Evaluation of B-type Lportbody concentration

[576]

[577] The dose-dependent degrading efficacy of APLP2-mediated B-type Lportbody was evaluated. First, the MDA-MB-231 cell line was prepared at 4×10^4 cells/300 μ L in a culture medium (RPMI1640 (Gibco, A10491-01) supplemented with 10% Fetal bovine serum (Gibco, 16000-044) and anti-anti 1X) to a 48-well plate, and cultured for 24 hours. Lportbody mixed with the culture medium at 10 nM. Were treated, after 24 hours, the culture solution was washed with PBS two times, and then the cells were lysed using RIPA Lysis and Extraction Buffer (Thermo scientific, 89901) and protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, roche, 11697498001). The supernatant cell lysate was transferred to a new 1.5 mL tube. The EGFR protein concentration in the cell lysate was measured by Human EGFR DuoSet ELISA (R&D, DY231) and the total protein concentration in the cell lysate was measured by BCA assay (according to the protocol provided by ThermoFisher 23225). As calculated in Example 17-1, the amount of EGFR was quantified by correcting for the deviation in cell count between samples, and the degradation rate was evaluated by converting the relative EGFR degradation amount in the Lportbody treatment group to that in the culture medium treatment group. The detailed formula is as follows.

[578] *Relative EGFR degradation compared to media (%)*

$$= \left(1 - \frac{\text{Amount of EGFR protein in Lportbody group (}^{ng}/mg)}{\text{Amount of EGFR protein in media group (}^{ng}/mg)} \right) \times 100$$

[579]

[580] As a result of the test, it was found that the tested B-AE, A3-39, A3-64, A4-55, A4-82, and A9-2 degraded EGFR in a concentration-dependent manner (FIG. 16).

[581]

[582] Example 20-2. Evaluation of B-type Lportbody by cell line

[583]

[584] The target tissue versatility of APLP2-mediated B-type Lportbody was evaluated. For this purpose, EGFR degradation was evaluated using various tissue-derived cancer cell lines, including MDA-MB-231, DLD-1, Hur7, and A549. First, each cell line was prepared according to the following conditions. MDA-MB-231 and DLD-1 cell lines were adjusted to 4×10^4 cells/300 μ L in a culture medium (RPMI1640 (Gibco, A10491-01) supplemented with 10% Fetal bovine serum (Gibco, 16000-044) and anti-anti 1X), and Hur7 and A549 were adjusted to 2×10^4 cells/300 μ L in the same medium, then dispensed into a 48-well plate, cultured for 24 hours, and then treated with Lportbody. The concentrations of the test substances were all 10 nM and the test substances were mixed with the culture medium for treatment. After 24 hours, the cell lysate was analyzed in the same manner as in Example 17-1 and the degradation rate was evaluated using the formula. The degradation rate was evaluated by converting the relative EGFR degradation amount in the Lportbody treatment group to that in the culture solution treatment group.

[585] As a result of the study, B-type Lportbody degraded EGFR in all four cell lines tested. Through this, it has been found that target proteins can be degraded using Lportbody in various tissues (FIG. 17).

[586]

[587] **Example 21: Evaluation of soluble EGFR degradation of multispecific antibodies**

[588]

[589] It was attempted to examine whether A9-2, a multispecific antibody of the present invention, could degrade soluble proteins. For this purpose, the cell internalization ability of soluble EGFR by A/B/C-type multispecific antibodies was first compared by flow cytometry, and the degradation potential of soluble proteins according to the type was evaluated. Specifically, soluble EGFR was fluorescently labeled using Alexa Fluor™ 488 NHS Ester (Invitrogen, A20000). The detailed protocol was performed by the test method provided in the kit. 10 nM of Alexa Fluor™ 488 labeled sEGFR, 1×10^5 cells of THP-1 cell line, and 10 nM of Lportbody were dispensed to be 100 μ L into a 96-well plate containing a medium prepared by supplementing RPMI1640 (Gibco, A10491-01) with 10% fetal bovine serum (Gibco, 16000-044) and anti-anti 1X. After being cultured for 24 hours, the cells were harvested, and washed with FACS buffer (prepared to contain 0.5% FBS and 0.09% sodium azide in PBS and then maintained at 4°C for use) at 4°C, then flow cytometry analysis was performed using LSRFortessa, and the results were compared using the fluorescence MFI value as the geometric median.

- [590] As a result of the analysis, it was found that the multispecific antibodies of the present invention could induce internalization of soluble proteins, and among these, the B-type had the greatest ability to induce internalization (FIG. 18).
- [591]
- [592] It was examined whether Lportbody degraded soluble proteins in a lysosome-dependent manner. Specifically, 1×10^6 cells of THP-1 cell line and 100 nM sEGFR (not fluorescently labeled) or 100 nM Lportbody or 50 nM Bafilomycin A1 (sigma, 5.08409) were dispensed into a 6-well plate to a total volume of 1000 μ L. The sample harvesting was performed after 24 or 48 hours, or harvesting was performed after the medium was washed out, 50 nM Bafilomycin A1 was dispensed to have a final volume of 1000 μ L, and the culture was further performed for 24 hours. The harvested cells and culture medium were washed with cold PBS, then the cells were lysed using RIPA Lysis and Extraction Buffer (Thermo scientific, 89901) and protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, roche, 11697498001), and the supernatant cell lysate was transferred to a new 1.5 mL tube. The EGFR protein concentration in the cell lysate was measured by Human EGFR DuoSet ELISA (R&D, DY231) and the total protein concentration in the cell lysate was measured by BCA assay (according to the protocol provided by ThermoFisher 23225). The amount of EGFR was quantified by correcting for the deviation in cell count between samples according to the following formula. As calculated in Example 17-1, the amount of EGFR was quantified by correcting for the deviation in cell count between samples.
- [593] As a result of the experiment, it was found that the A9-2 Lportbody of B-type degraded a soluble protein in a lysosome-dependent manner (FIG. 19).
- [594]
- [595] As described above, the excellent affinity for lysosomal transporting receptors and target proteins and the ability to degrade target proteins of multispecific antibodies in the form of Type B produced using various sequences have been found, and the multispecific antibodies produced in the form of Type B according to the present invention are not limited to a specific sequence. This suggests that the multispecific antibody platform of the present invention can be utilized to develop therapeutic agents through the degradation of target proteins.
- [596]
- [597] From the above description, those skilled in the art to which the present invention pertains will understand that the present invention can be implemented in other specific forms without changing the technical idea or essential characteristics thereof. In this regard, it should be understood that the embodiments described above are illustrative in all respects but not restrictive. The scope of the present invention is defined by the appended claims rather than by the description preceding them, and all changes or

modifications derived from the meaning and scope of the claims and their equivalent concepts should be construed as being included in the scope of the present invention.

Claims

- [Claim 1] A multispecific antibody comprising a lysosomal transporting receptor binding region and a target protein binding region, wherein
one of the lysosomal transporting receptor binding region or the target protein binding region includes the first heavy chain variable region,
the other includes the second heavy chain variable region, a heavy chain constant region, a light chain constant region and a light chain variable region, and
the first heavy chain variable region is connected to the second heavy chain variable region.
- [Claim 2] The multispecific antibody according to claim 1, wherein the heavy chain constant region includes an Fc region.
- [Claim 3] The multispecific antibody according to claim 2, wherein the heavy chain constant region includes a hinge region.
- [Claim 4] The multispecific antibody according to claim 1, wherein the first heavy chain variable region includes a VHH single domain.
- [Claim 5] The multispecific antibody according to claim 3, which is a dimer symmetrically formed by monomers in which a lysosomal transporting receptor binding region and a target protein binding region are connected.
- [Claim 6] The multispecific antibody according to claim 1, wherein the lysosomal transporting receptor binding region includes a heavy chain variable region (VH or VHH) of an antibody against a lysosomal transporting receptor, a fragment thereof, or an antigen-binding site thereof.
- [Claim 7] The multispecific antibody according to claim 1, wherein the lysosomal transporting receptor binding region binds to mannose 6-phosphate receptor (M6PR) or amyloid precursor-like protein 2 (APLP2).
- [Claim 8] The multispecific antibody according to claim 1, wherein the target protein binding region includes a heavy chain variable region (VH or VHH) of an antibody against a target protein, a fragment thereof, or an antigen-binding site thereof.
- [Claim 9] The multispecific antibody according to claim 1, wherein the target protein is an extracellular protein or a soluble protein.

- [Claim 10] The multispecific antibody according to claim 1, wherein the target protein is epidermal growth factor receptor (EGFR).
- [Claim 11] The multispecific antibody according to claim 1, wherein the first heavy chain variable region and the second heavy chain variable region are connected via a linker.
- [Claim 12] The multispecific antibody according to claim 1, which induces endocytosis and degradation by a lysosome of a target protein.
- [Claim 13] A polynucleotide encoding the multispecific antibody according to any one of claims 1 to 12.
- [Claim 14] An expression vector comprising the polynucleotide according to claim 13.
- [Claim 15] A cell transformed with the expression vector according to claim 14.
- [Claim 16] A method for producing a multispecific antibody, comprising culturing the cell according to claim 15 to express a multispecific antibody.
- [Claim 17] The method for producing a multispecific antibody according to claim 16, wherein the method of producing further comprises recovering the multispecific antibody.
- [Claim 18] The method for producing a multispecific antibody according to claim 16, wherein the method of producing further comprises purifying the multispecific antibody.
- [Claim 19] A composition comprising the multispecific antibody according to any one of claims 1 to 12.
- [Claim 20] A multispecific antibody comprising an antibody or an antigen-binding fragment thereof that specifically binds to amyloid precursor-like protein 2 (APLP2),
the antibody or antigen-binding fragment thereof is an antibody or an antigen-binding fragment thereof including the following complementarity determining regions (CDRs):
(i) CDR-H1 including an amino acid sequence of SEQ ID NO: 45,
CDR-H2 including an amino acid sequence of SEQ ID NO: 46,
CDR-H3 including an amino acid sequence of SEQ ID NO: 47,
CDR-L1 including an amino acid sequence of SEQ ID NO: 48,
CDR-L2 including an amino acid sequence of SEQ ID NO: 49,
and CDR-L3 including an amino acid sequence of SEQ ID NO: 50;

- (ii) CDR-H1 including an amino acid sequence of SEQ ID NO: 51, CDR-H2 including an amino acid sequence of SEQ ID NO: 52, and CDR-H3 including an amino acid sequence of SEQ ID NO: 53;
- (iii) CDR-H1 including an amino acid sequence of SEQ ID NO: 54, CDR-H2 including an amino acid sequence of SEQ ID NO: 55, and CDR-H3 including an amino acid sequence of SEQ ID NO: 56;
- (iv) CDR-H1 including an amino acid sequence of SEQ ID NO: 57, CDR-H2 including an amino acid sequence of SEQ ID NO: 58, and CDR-H3 including an amino acid sequence of SEQ ID NO: 59;
- (v) CDR-H1 including an amino acid sequence of SEQ ID NO: 60, CDR-H2 including an amino acid sequence of SEQ ID NO: 61, and CDR-H3 including an amino acid sequence of SEQ ID NO: 62;
- (vi) CDR-H1 including an amino acid sequence of SEQ ID NO: 63, CDR-H2 including an amino acid sequence of SEQ ID NO: 64, and CDR-H3 including an amino acid sequence of SEQ ID NO: 65;
- (vii) CDR-H1 including an amino acid sequence of SEQ ID NO: 66, CDR-H2 including an amino acid sequence of SEQ ID NO: 67, and CDR-H3 including an amino acid sequence of SEQ ID NO: 68;
- (viii) CDR-H1 including an amino acid sequence of SEQ ID NO: 69, CDR-H2 including an amino acid sequence of SEQ ID NO: 70, and CDR-H3 including an amino acid sequence of SEQ ID NO: 71;
- (ix) CDR-H1 including an amino acid sequence of SEQ ID NO: 72, CDR-H2 including an amino acid sequence of SEQ ID NO: 73, and CDR-H3 including an amino acid sequence of SEQ ID NO: 74;
- (x) CDR-H1 including an amino acid sequence of SEQ ID NO: 75, CDR-H2 including an amino acid sequence of SEQ ID NO: 76, and CDR-H3 including an amino acid sequence of SEQ ID NO: 77;
- (xi) CDR-H1 including an amino acid sequence of SEQ ID NO: 78, CDR-H2 including an amino acid sequence of SEQ ID NO:

79, and CDR-H3 including an amino acid sequence of SEQ ID NO: 80;

(xii) CDR-H1 including an amino acid sequence of SEQ ID NO: 81, CDR-H2 including an amino acid sequence of SEQ ID NO: 82, and CDR-H3 including an amino acid sequence of SEQ ID NO: 83;

(xiii) CDR-H1 including an amino acid sequence of SEQ ID NO: 84, CDR-H2 including an amino acid sequence of SEQ ID NO: 85, and CDR-H3 including an amino acid sequence of SEQ ID NO: 86;

(xiv) a CDR-H1 including an amino acid sequence of SEQ ID NO: 87, a CDR-H2 including an amino acid sequence of SEQ ID NO: 88, and a CDR-H3 including an amino acid sequence of SEQ ID NO: 89;

(xv) CDR-H1 including an amino acid sequence of SEQ ID NO: 90, CDR-H2 including an amino acid sequence of SEQ ID NO: 91, and CDR-H3 including an amino acid sequence of SEQ ID NO: 92;

(xvi) CDR-H1 including an amino acid sequence of SEQ ID NO: 93, CDR-H2 including an amino acid sequence of SEQ ID NO: 94, and CDR-H3 including an amino acid sequence of SEQ ID NO: 95;

(xvii) CDR-H1 including an amino acid sequence of SEQ ID NO: 96, CDR-H2 including an amino acid sequence of SEQ ID NO: 97, and CDR-H3 including an amino acid sequence of SEQ ID NO: 98;

(xviii) CDR-H1 including an amino acid sequence of SEQ ID NO: 99, CDR-H2 including an amino acid sequence of SEQ ID NO: 100, and CDR-H3 including an amino acid sequence of SEQ ID NO: 101;

(xix) CDR-H1 including an amino acid sequence of SEQ ID NO: 102, CDR-H2 including an amino acid sequence of SEQ ID NO: 103, and CDR-H3 including an amino acid sequence of SEQ ID NO: 104;

(xx) CDR-H1 including an amino acid sequence of SEQ ID NO: 105, CDR-H2 including an amino acid sequence of SEQ ID NO: 106, and CDR-H3 including an amino acid sequence of SEQ ID NO: 107;

(xxi) CDR-H1 including an amino acid sequence of SEQ ID NO: 108, CDR-H2 including an amino acid sequence of SEQ ID NO: 109, and CDR-H3 including an amino acid sequence of SEQ ID NO: 110;

(xxii) CDR-H1 including an amino acid sequence of SEQ ID NO: 111, CDR-H2 including an amino acid sequence of SEQ ID NO: 112, and CDR-H3 including an amino acid sequence of SEQ ID NO: 113;

(xxiii) CDR-H1 including an amino acid sequence of SEQ ID NO: 114, CDR-H2 including an amino acid sequence of SEQ ID NO: 115, and CDR-H3 including an amino acid sequence of SEQ ID NO: 116;

(xxiv) CDR-H1 including an amino acid sequence of SEQ ID NO: 117, CDR-H2 including an amino acid sequence of SEQ ID NO: 118, and CDR-H3 including an amino acid sequence of SEQ ID NO: 119;

(xxv) CDR-H1 including an amino acid sequence of SEQ ID NO: 120, CDR-H2 including an amino acid sequence of SEQ ID NO: 121, and CDR-H3 including an amino acid sequence of SEQ ID NO: 122;

(xxvi) CDR-H1 including an amino acid sequence of SEQ ID NO: 123, CDR-H2 including an amino acid sequence of SEQ ID NO: 124, and CDR-H3 including an amino acid sequence of SEQ ID NO: 125;

(xxvii) CDR-H1 including an amino acid sequence of SEQ ID NO: 126, CDR-H2 including an amino acid sequence of SEQ ID NO: 127, and CDR-H3 including an amino acid sequence of SEQ ID NO: 128; or

(xxviii) CDR-H1 including an amino acid sequence of SEQ ID NO: 129, CDR-H2 including an amino acid sequence of SEQ ID NO: 130, and CDR-H3 including an amino acid sequence of SEQ ID NO: 131.

[Claim 21]

A multispecific antibody comprising an antibody or an antigen-binding fragment thereof that specifically binds to mannose 6-phosphate receptor (M6PR),
the antibody or antigen-binding fragment thereof, wherein
the antibody or antigen-binding fragment thereof includes the
following complementarity determining regions (CDRs):

(i) CDR-H1 including an amino acid sequence of SEQ ID NO: 132, CDR-H2 including an amino acid sequence of SEQ ID NO: 133, and CDR-H3 including an amino acid sequence of SEQ ID NO: 134;

(ii) CDR-H1 including an amino acid sequence of SEQ ID NO: 135, CDR-H2 including an amino acid sequence of SEQ ID NO: 136, and CDR-H3 including an amino acid sequence of SEQ ID NO: 137; or

(iii) CDR-H1 including an amino acid sequence of SEQ ID NO: 138, CDR-H2 including an amino acid sequence of SEQ ID NO: 139, and CDR-H3 including an amino acid sequence of SEQ ID NO: 140.

- [Claim 22] The multispecific antibody according to claim 20 or 21, further comprising an antibody, antibody fragment or antigen-binding site thereof that binds to a target protein.
- [Claim 23] A polynucleotide encoding the multispecific antibody according to claim 20 or 21.
- [Claim 24] An expression vector comprising the polynucleotide according to claim 23.
- [Claim 25] A cell transformed with the expression vector according to claim 24.
- [Claim 26] A composition comprising the multispecific antibody according to claim 20 or 21.