

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 potential use of antibodies targeting specific antigens as therapeutic agents was proposed, bi- or multi-specific 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 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, thereby target degrading proteins existing in the cell membrane and outside the cell, and has a form in which antibodies and M6P chains are connected. 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 (LTR) 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 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 according to the present invention;

- [20] FIG. 2 is a diagram illustrating the results that multispecific antibodies of different sequences containing the M6PR binding region are prepared as A and C type and their degradation efficacy for EGFR is evaluated; and

- [21] FIG. 3 is a diagram comparing the EGFR degradation of a multispecific antibody including an M6PR binding region in the form of scFv and a multispecific antibody including an M6PR binding region in the form of an antibody VH domain.

[22]

Best Mode for Carrying out the Invention

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

- [24] In a specific embodiment, the multispecific antibody comprises a lysosomal transporting receptor binding region, a target protein binding region, and an Fc region.
- [25] In another specific embodiment, in the multispecific antibody, the lysosomal transporting receptor binding region and the target protein binding region are linked to the Fc region on the opposite side.
- [26] 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, 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 and the target protein binding region are connected to different termini of the Fc region.
- [27] In a specific embodiment according to any one of the preceding specific embodiments, the lysosomal transporting receptor binding region is not a single-chain variable fragment (scFv).
- [28] In a specific embodiment according to any one of the preceding specific embodiments, the Fc region includes a hinge region.
- [29] 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, an Fc region, and a target protein binding region are connected symmetrically form a dimer.
- [30] In a specific embodiment according to any one of the preceding specific embodiments, the multispecific antibody symmetrically forms a dimer due to a disulfide bond in the hinge region.
- [31] 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.
- [32] In a specific embodiment according to any one of the preceding specific embodiments, the lysosomal transporting receptor binding region is connected to a C-terminus of the Fc region and the target protein binding region is connected to an N-terminus of the Fc region.
- [33] In a specific embodiment according to any one of the preceding specific embodiments, the Fc region includes a hinge region, the lysosomal transporting receptor binding region is connected to a C-terminus of the Fc region, and the target protein binding region is connected to an N-terminus of the Fc region.

- [34] 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).
- [35] In a specific embodiment according to any one of the preceding specific embodiments, the target protein binding region binds to a target protein.
- [36] In a specific embodiment according to any one of the preceding specific embodiments, the target protein is epidermal growth factor receptor (EGFR).
- [37] In a specific embodiment according to any one of the preceding specific embodiments, the lysosomal transporting receptor binding region and Fc region are connected via a linker.
- [38] 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.
- [39] 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).
- [40] Still another aspect embodying the present invention is a polynucleotide encoding the multispecific antibody.
- [41] Still another aspect embodying the present invention is an expression vector comprising the polynucleotide.
- [42] Still another aspect embodying the present invention is a cell transformed with the expression vector.
- [43] Still another aspect embodying the present invention is a method for producing a multispecific antibody, comprising culturing the cell to express a multispecific antibody.
- [44] In a specific embodiment, the production method further comprises recovering the multispecific antibody.
- [45] In another specific embodiment, the production method further comprises purifying the multispecific antibody.
- [46] Still another aspect embodying the present invention is a composition comprising the multispecific antibody.
- [47] In a specific embodiment, the composition is a pharmaceutical composition.
- [48] Still another aspect embodying the present invention is a use of the multispecific antibody for the degradation of a target protein.
- [49]

Mode for the Invention

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

- [51] 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.
- [52] 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.
- [53]
- [54] An aspect embodying the present invention provides a multispecific antibody comprising a lysosomal transporting receptor binding region and a target protein binding region.
- [55] The multispecific antibody of the present invention has a form such as Type C in FIG. 1. In the multispecific antibody of the present invention having the form of Type C, the lysosomal transporting receptor binding region and the target protein binding region are not connected to be adjacent to each other but are connected through a separate moiety.
- [56]
- [57] In a specific aspect, the multispecific antibody of the present invention may comprise a lysosomal transporting receptor binding region, a target protein binding region, and an Fc region, the lysosomal transporting receptor binding region may include a heavy chain variable region (VH or VHH) of an antibody against a lysosomal transporting receptor, the target protein binding region may include 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 and the target protein binding region may be connected to different termini of the Fc region.
- [58] The lysosomal transporting receptor binding region may consist of, but is not limited to, a VH or VHH domain.
- [59] The lysosomal transporting receptor binding region is not a single-chain variable fragment (scFv).
- [60]
- [61] In an embodiment, the multispecific antibody of the present invention may comprise:
- [62] (i) a lysosomal transporting receptor binding region including or consisting of a heavy chain variable region (VH or VHH) of an antibody against a lysosomal transporting receptor;

- [63] (ii) a target protein binding region including or consisting of 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; and
- [64] (iii) an Fc region, in which
- [65] the lysosomal transporting receptor binding region and the target protein binding region may be connected to different termini of the Fc region.
- [66] In another specific embodiment, the multispecific antibody of the present invention may comprise:
- [67] (i) a lysosomal transporting receptor binding region including or consisting of a heavy chain variable region (VH or VHH) of an antibody against a lysosomal transporting receptor;
- [68] (ii) a target protein binding region including or consisting of 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; and
- [69] (iii) an Fc region including a hinge region, a CH2 domain, and a CH3 domain, in which
- [70] the lysosomal transporting receptor binding region and the target protein binding region may be connected to different termini of the Fc region.
- [71] In still another specific embodiment, the multispecific antibody of the present invention may comprise:
- [72] (i) a lysosomal transporting receptor binding region including or consisting of a heavy chain variable region (VH or VHH) of an antibody against a lysosomal transporting receptor;
- [73] (ii) a target protein binding region including or consisting of 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; and
- [74] (iii) an Fc region including a hinge region, a CH2 domain, and a CH3 domain, in which
- [75] the lysosomal transporting receptor binding region is connected to a C-terminus of the Fc region and the target protein binding region is connected to an N-terminus of the Fc region, and
- [76] monomers in which the lysosomal transporting receptor binding region, the Fc 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.
- [77] In still another specific embodiment, the multispecific antibody of the present invention may have a structure such as Type C in FIG. 1, but is not limited thereto.
- [78]

- [79] 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 degradation of the target protein through lysosomes, and is thus capable of achieving degradation of a target protein in lysosomes. In particular, it has been found that the multispecific antibody of the present invention comprises a lysosomal transporting receptor binding region in the form of a VH or VHH domain, and has higher target protein degradation compared to a multispecific antibody comprising a lysosomal transporting receptor binding region in the form of a single-chain variable fragment (scFv), and this provides a more effective lysosomal transporting antibody-based multispecific antibody platform.
- [80] Type C bi-specific antibodies have the advantage of being more easily assembled when the antibodies are produced using wild-type Fc than Type A antibodies, having 2+2 valent for each target to bind to more antigens simultaneously than Type A antibodies having 1+1 valent, and being able to bind more strongly by the avidity effect.
- [81]
- [82] 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 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 having 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.
- [83] 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.

[84] 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 including a site capable of recognizing an antigenic site.

[85]

[86] Typically, immunoglobulins have a heavy chain and a light chain, and each heavy chain and each light chain includes 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).

[87] 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.

[88] 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.

[89]

[90] 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.

[91] 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.

- [92] 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.
- [93]
- [94] 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.
- [95]
- [96] In the present invention, the term "bi-specific antibody" means a protein capable of binding to two different kinds of antigens.
- [97] 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.
- [98] 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".
- [99]
- [100] The bi-specific antibody refers to a molecule of which antigen-binding sites are connected directly or via a linker or can form a heterodimer by electrostatic interaction.
- [101]
- [102] 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.
- [103]
- [104] 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.
- [105]
- [106] 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, and the target protein binding region may include a VH(H) domain.

[107]

[108] 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.

[109]

[110] 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).

[111]

[112] 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.

[113]

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.

[114]

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

[115]

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.

[116]

[117] The lysosomal transporting receptor binding region comprised in the multispecific antibody of the present invention may be, but is not limited to, a human-derived VH domain or an animal-derived VHH domain such as a camelid 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.

[118]

[119] The multispecific antibody of the present invention comprises a lysosomal transporting receptor binding region in the form of a domain and has superior target protein degradation compared to a multispecific antibody comprising a lysosomal transporting receptor binding region in the form of a single-chain variable fragment (scFv).

[120] In the Examples of the present invention, it has been verified that the multispecific antibody comprising a lysosomal transporting receptor binding region in the form of a domain according to the present invention has remarkably superior target protein degradation compared to an antibody comprising a binding region in the form of a single-chain variable fragment (scFv), which is disclosed in WO 2024/035955 A2 (FIG. 3).

[121]

[122] The multispecific antibody of the present invention has a structure in which the lysosomal transporting receptor binding region and the target protein binding region are not adjacently connected, and has a separate moiety mediating the two binding regions. Such a moiety may be an Fc region. Therefore, in the multispecific antibody of the present invention, the target protein binding region-Fc region-lysosomal transporting receptor binding region may be connected in this order.

[123]

[124] In the present invention, the term "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.

[125] 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.

[126] 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.

- [127] 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.
- [128] In the present invention, the immunoglobulin Fc region of the monomer may include an amino acid sequence of SEQ ID NO: 17, but is not limited thereto.
- [129] In the present invention, the term "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.
- [130] 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.
- [131] In the multispecific antibody of the present invention, the Fc regions may form a dimer with each other due to a disulfide bond in 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, an Fc region, and a target protein binding region are connected to each other.
- [132] In the present invention, the hinge region may include an amino acid sequence of SEQ ID NO: 18, but is not limited thereto.
- [133]
- [134] 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.
- [135] 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 to be reduced/eliminated in the body through lysosome-mediated degradation. For example, the target protein may be an extracellularly expressed protein, or proteins that affect the

onset and progression of diseases, such as 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).

[136]

[137] 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. 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 of an antibody against a target protein, a heavy chain constant region, a light chain constant region, and a light chain variable region.

[138]

[139] 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 the respective target proteins, and the respective target protein binding regions may be connected to each other and connected to the N-terminus of the Fc region, but the multispecific antibody is not limited.

[140]

[141] In the multispecific antibody of the present invention, the lysosomal transporting receptor binding region and the target protein binding region are connected to different termini of the Fc region.

[142]

Specifically, the multispecific antibody of the present invention may be 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), 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 and the target protein binding region are connected to the C-terminus and N-terminus of the Fc region, respectively.

[143]

Such a multispecific antibody comprising a lysosomal transporting receptor binding region in the form of a domain has superior target protein degradation compared to a

multispecific antibody comprising a lysosomal transporting receptor binding region that is not in the form of a domain but in the form of a single-chain variable fragment (scFv).

- [144] 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 connected to different termini of the Fc region, is referred to as a "Type C multispecific antibody" or a C-type "Lportbody". An example of this includes, but is not limited to, Type C in FIG. 1.
- [145]
- [146] As an example of the multispecific antibody of the present invention, the lysosomal transporting receptor binding region may be connected to the C-terminus of the Fc region, the target protein binding region may be connected to the N-terminus of the Fc region, and the Fc region may include a hinge region at the N-terminus.
- [147] More specifically, in the multispecific antibody of the present invention, a lysosomal transporting receptor binding region in the form of a domain may be connected to the C-terminus of the Fc region, which includes the hinge region-CH2 domain-CH3 domain from the N-terminus, and a target protein binding region including a heavy chain variable region, a heavy chain constant region, a light chain constant region, and a light chain variable region may be connected to the N-terminal hinge region of the Fc region, but the multispecific antibody is not limited thereto.
- [148]
- [149] The lysosomal transporting receptor binding region, the Fc region, and the target protein binding region may be directly connected or connected via a linker.
- [150] In a specific aspect, the lysosomal transporting receptor binding region and the Fc region and the Fc region and the target protein binding region may be directly connected or connected via a linker or may additionally include another protein moiety, but the connection is not limited thereto. As the method and location of connection between the lysosomal transporting receptor binding region, Fc region, and 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 is not altered.
- [151]
- [152] For the purpose of the present invention, the Fc region and the target protein binding region may be directly connected by a covalent bond, but are not limited thereto.
- [153] Additionally, the lysosomal transporting receptor binding region and the Fc region of the present invention may be connected via a linker, but are not limited thereto. As the linker connecting the lysosomal transporting receptor binding region and the Fc 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.

- [154] 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), GGGGSGGGSGGT (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 site recognizable by a restriction enzyme (for example, the KpnI enzyme).
- [155] 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.
- [156]
- [157] 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 the target protein, but is not limited thereto.
- [158]
- [159] 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).
- [160] Specifically, the antibody or antigen-binding fragment thereof may include, but is not limited to, the following complementarity determining regions (CDRs):
- [161] (i) CDR-H1 including an amino acid sequence of SEQ ID NO: 8, CDR-H2 including an amino acid sequence of SEQ ID NO: 9, and CDR-H3 including an amino acid sequence of SEQ ID NO: 10;
- [162] (ii) CDR-H1 including an amino acid sequence of SEQ ID NO: 11, CDR-H2 including an amino acid sequence of SEQ ID NO: 12, and CDR-H3 including an amino acid sequence of SEQ ID NO: 13; or
- [163] (iii) CDR-H1 including an amino acid sequence of SEQ ID NO: 14, CDR-H2 including an amino acid sequence of SEQ ID NO: 15, and CDR-H3 including an amino acid sequence of SEQ ID NO: 16.
- [164]

[165] More specifically, the multispecific antibody of the present invention may comprise any one of the amino acid sequences of SEQ ID NOs: 5 to 7, but is not limited thereto.

[166]

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

[168] 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 extracellularly expressed protein, and examples thereof include epidermal growth factor receptor (EGFR), but the target protein is not limited thereto.

[169] The target protein is as described previously.

[170]

[171] 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.

[172] 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.

[173]

[174] 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.

- [175] Methods for determining sequence similarity or identity between two or more amino acid sequences are known in the art.
- [176] 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.
- [177]
- [178] Another aspect embodying the present invention provides a polynucleotide encoding the multispecific antibody.
- [179] The multispecific antibody is as described previously.
- [180]
- [181] 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 of 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.
- [182]
- [183] Still another aspect embodying the present invention provides a vector comprising a polynucleotide encoding the multispecific antibody.
- [184] 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.
- [185] The multispecific antibody and polynucleotide encoding the same are as described previously.

[186]

[187] 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.

[188]

[189] 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.

[190]

[191] 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.

[192]

[193] 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.

[194]

[195] 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.

[196]

[197] Still another aspect of the present invention provides a composition comprising the multispecific antibody of the present invention.

[198] The multispecific antibody is as described previously.

[199]

[200] Specifically, the composition may be a pharmaceutical composition, and may be a pharmaceutical composition comprising the multispecific antibody in a pharmaceutically effective amount.

[201] 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.

[202]

[203] 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 the 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.

[204]

[205] The pharmaceutical composition may additionally contain a pharmaceutically acceptable excipient, but is not limited thereto.

[206] 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.

[207] 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.

- [208] 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.
- [209]
- [210] 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.
- [211] The multispecific antibody, polynucleotide, vector, cell, and transformation are as described previously.
- [212]
- [213] 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.
- [214] The method for producing a multispecific antibody of the present invention may further comprise recovering the multispecific antibody from the medium or cell.
- [215] The method for producing a multispecific antibody of the present invention may further comprise purifying the multispecific antibody.
- [216] 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).
- [217] 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.
- [218]
- [219] 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.
- [220]

- [221] Still another aspect of the present invention provides a use of the multispecific antibody for the degradation of a target protein.
- [222] The multispecific antibody is as described previously.
- [223]
- [224] 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.
- [225]
- [226] 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 the Examples.
- [227]
- [228] **Example 1: Design of multispecific antibodies**
- [229]
- [230] 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.
- [231]
- [232] The multispecific antibodies according to the present invention were constructed into Types A, B, and C having three different characteristics (FIG. 1).
- [233]
- [234] 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.
- [235]
- [236] 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).
- [237]
- [238] **Example 2: Construction of multispecific antibodies**
- [239]
- [240] Example 2-1: Construction of Type A multispecific antibodies

[241] 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.

[242]

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

[244] 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.

[245]

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

[247] 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.

[248]

[249] The multispecific antibodies according to the present invention produced in Examples 2-1 to 2-3 are schematically illustrated in FIG. 1.

[250]

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

[252]

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

[254]

[255] 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.

[256]

[257] 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.

[258]

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

[260]

[261] 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.

[262]

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

[264]

[265] 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.

[266]

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

[268]

[269] 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 eluate 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.

[270]

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

[272]

[273] 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.

[274]

[275] **Example 8: Production of M6PR protein antigen**

[276]

[277] For the production of antibodies targeting a lysosomal transporting receptor (M6PR), the domain 11-13 (M1508-L1992) portion of human M6PR was used as an antigen. Each antigen was fused with a Myc-His-Avi tag.

[278]

[279] Example 8-1. Transformation and protein expression

[280] To competent cells, 100 ng of 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.

[281] 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.

[282]

[283] Example 8-2. Protein purification and concentration

[284] 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 NiSO₄, 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.

[285] 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.

[286]

[287] **Example 9: Screening of antibody against M6PR**

[288]

[289] 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 1).

[290]

[291] [Table 1]

Clone ID	Sequence	SEQ ID NO:
M3-10	EVQLVESGGGLVQPGGSLRLSCAASGFTFTNYDMGWVRQAPGKGPEWVSLISSGSSNTWYDDSVKGRFTISRDN NSKNTLYLQMNSLRAEDTAVYYCARIYEQILIDYWGQGLTVTVSS	5
M3-54	EVQLVESGGGLVQPGGSLRLSCAASGFTFTGYDMGWVRQAPGKGSEWVSLISYDGSSTWYDDSVKGRFTISRDN NSKNTLYLQMNSLRAEDTAVYYCARVFAEILIDYWGQGLTVTVSS	6
M7-23	EVQLVESGGGLVQPGGSLRLSCAASGFTFTTYDMGWVRQAPGKGPEWVSLISSDSSNTWYDDSVKGRFTISRDN NSKNTLYLQMNSLRAEDTAVYYCARATLHLVDYWGQGLTVTVSS	7

[292]

[293] **Example 10: Expression and purification of Type C multispecific antibodies**

[294]

[295] As described in Example 2, the sequences of the lysosomal transporting receptor binding region (SEQ ID NOs: 5 to 7) and the EGFR target protein binding region (including the heavy chain variable region of SEQ ID NO: 21 and the light chain variable region of SEQ ID NO: 22) were subcloned into pCIW and produced in a Type C 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 µg 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.

[296]

[297] The Type C multispecific antibodies produced at this time are multispecific antibodies 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. The lysosomal transporting receptor binding region and the target protein binding region are connected on opposite sides based on the heavy chain constant region. The sequence of the Fc region of the monomer used in the multispecific antibodies of the present invention is SEQ ID NO. 17, the sequence of the hinge region is SEQ ID NO. 18, and the sequences of the heavy chain constant region and light chain constant region are SEQ ID NOs: 19 and 20, respectively.

[298]

[299] **Example 11: Comparison of EGFR degradation of M6PR-mediated A/C-type Lportbodies**

[300]

[301] It was attempted to compare the EGFR degradation of C-type Lportbody antibodies mediated by M6PR with that of A-type Lportbody antibodies. For this purpose, A/C-type Lportbody antibodies were produced using the clone sequences identified in Example 9, and the EGFR degradation thereof was examined.

[302]

[303] First, the DLD-1 cell line was adjusted to 2×10^5 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), dispensed into a 6-well plate, cultured for 24 hours, and then treated with the Lportbody clone. The concentrations of the test substances were all 10 nM and the test substance was mixed with the culture medium for treatment. After 24 hours, all the culture solution was removed, washing was performed with cold 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). The amount of EGFR was quantified by correcting for the deviation in cell count between samples according to the following formula.

[304]

[305]

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

[306]

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

[308]

[309] As a result of the test, as confirmed in FIG. 2, it was found that all C-type Lportbody antibodies not only had superior EGFR degradation compared to the control, CTX, but also had superior degradation compared to A-type Lportbody antibodies.

[310]

[311] **Example 12: Comparison of EGFR degradation of C-type Lportbodies depending on form of lysosomal transporting receptor binding region**

[312]

[313] It was attempted to examine how the target protein degradation changed when the Type C multispecific antibody according to the present invention had a domain or Fab form as the lysosomal transporting receptor binding region.

[314]

[315] For this purpose, the EGFR degradation of the multispecific antibody of the present invention having a lysosomal transporting receptor binding region that binds to M6PR while having a VHH domain form and a multispecific antibody having a lysosomal transporting receptor binding region in the form of scFv was examined (FIG. 3).

[316]

[317] As a result, it was found that the multispecific antibodies of the present invention, M3-10, M3-54, and M7-23, had superior EGFR degradation compared to multispecific antibodies (Lytac-3C7, Lytac-6D1) having a lysosomal transporting receptor binding region in the form of scFv.

[318]

[319] From the Examples described above, it has been found that the Type C form of the multispecific antibody of the present invention has excellent ability to bind to both a lysosomal transporting receptor and a target protein, in particular, a multispecific antibody having a lysosomal transporting receptor binding region that binds to a lysosomal transporting receptor in the form of a domain has remarkably superior target protein degradation. This suggests that the multispecific antibody platform of the present invention can be utilized to develop therapeutic agents through degradation of target proteins.

[320]

[321] 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, a target protein binding region, and an Fc region, 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,
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 and the target protein binding region are connected to different termini of the Fc region.
- [Claim 2] The multispecific antibody according to claim 1, wherein the lysosomal transporting receptor binding region is not a single-chain variable fragment (scFv).
- [Claim 3] The multispecific antibody according to claim 1, wherein the Fc region includes a hinge region.
- [Claim 4] The multispecific antibody according to claim 3, which is a dimer symmetrically formed by monomers in which a lysosomal transporting receptor binding region, an Fc region, and a target protein binding region are connected.
- [Claim 5] The multispecific antibody according to claim 1, wherein the lysosomal transporting receptor binding region is connected to a C-terminus of the Fc region and the target protein binding region is connected to an N-terminus of the Fc region.
- [Claim 6] The multispecific antibody according to claim 5, wherein the Fc region includes a hinge region, the lysosomal transporting receptor binding region is connected to a C-terminus of the Fc region, and the target protein binding region is connected to an N-terminus of the Fc region.
- [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 is an extracellularly expressed protein.
- [Claim 9] The multispecific antibody according to claim 1, wherein the target protein is epidermal growth factor receptor (EGFR).
- [Claim 10] The multispecific antibody according to claim 1, wherein the lysosomal transporting receptor binding region and the Fc region are connected via a linker.
- [Claim 11] The multispecific antibody according to claim 1, which induces endocytosis and degradation by a lysosome of a target protein.
- [Claim 12] A polynucleotide encoding the multispecific antibody according to any one of claims 1 to 11.
- [Claim 13] An expression vector comprising the polynucleotide according to claim 12.
- [Claim 14] A cell transformed with the expression vector according to claim 13.
- [Claim 15] A method for producing a multispecific antibody, comprising culturing the cell according to claim 14 to express a multispecific antibody.
- [Claim 16] The method for producing a multispecific antibody according to claim 15, further comprising recovering the multispecific antibody.
- [Claim 17] The method for producing a multispecific antibody according to claim 15, further comprising purifying the multispecific antibody.
- [Claim 18] A composition comprising the multispecific antibody according to any one of claims 1 to 11.
- [Claim 19] A multispecific antibody comprising an antibody or an antigen-binding fragment thereof that specifically binds to mannose 6-phosphate receptor (M6PR), wherein the antibody or antigen-binding fragment thereof is an antibody or an antigen-binding fragment thereof that includes the following complementarity determining regions (CDRs):
(i) CDR-H1 including an amino acid sequence of SEQ ID NO: 8, CDR-H2 including an amino acid sequence of SEQ ID NO: 9, and CDR-H3 including an amino acid sequence of SEQ ID NO: 10;
(ii) CDR-H1 including an amino acid sequence of SEQ ID NO: 11, CDR-H2 including an amino acid sequence of SEQ ID NO: 12, and CDR-H3 including an amino acid sequence of SEQ ID NO: 13; or

(iii) CDR-H1 including an amino acid sequence of SEQ ID NO: 14, CDR-H2 including an amino acid sequence of SEQ ID NO: 15, and CDR-H3 including an amino acid sequence of SEQ ID NO: 16.

- [Claim 20] The multispecific antibody according to claim 19, further comprising an antibody, an antibody fragment or an antigen-binding site thereof that binds to a target protein.
- [Claim 21] A polynucleotide encoding the multispecific antibody according to claim 20.
- [Claim 22] An expression vector comprising the polynucleotide according to claim 21.
- [Claim 23] A cell transformed with the expression vector according to claim 22.
- [Claim 24] A composition comprising the multispecific antibody according to claim 20.