(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
2 July 2009 (02.07.2009)

PCT

(10) International Publication Number
WO 2009/080253 A1

(51) International Patent Classification:
C07K 16/46 (2006.01) *C07K 19/00* (2006.01)
C07K 16/22 (2006.01) *A61K 39/395* (2006.01)
C07K 16/28 (2006.01)

(21) International Application Number:
PCT/EP2008/010704

(22) International Filing Date:
16 December 2008 (16.12.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
07024865.3 21 December 2007 (21.12.2007) EP

(71) Applicant *(for all designated States except US)*: **F. HOFFMANN-LA ROCHE AG** [CH/CH]; Grenzacherstrasse 124, CH-4070 Basel (CH).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **KLEIN, Christian** [DE/DE]; Moraenenweg 5, 82393 Iffeldorf (DE). **SCHAEFER, Wolfgang** [DE/DE]; Tannhaeuserring 190, 68199 Mannheim (DE).

(74) Agent: **BURGER, Alexander**; Roche Diagnostics GmbH, Patent Department (TR-E), Postfach 11 52, 82372 Penzberg (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

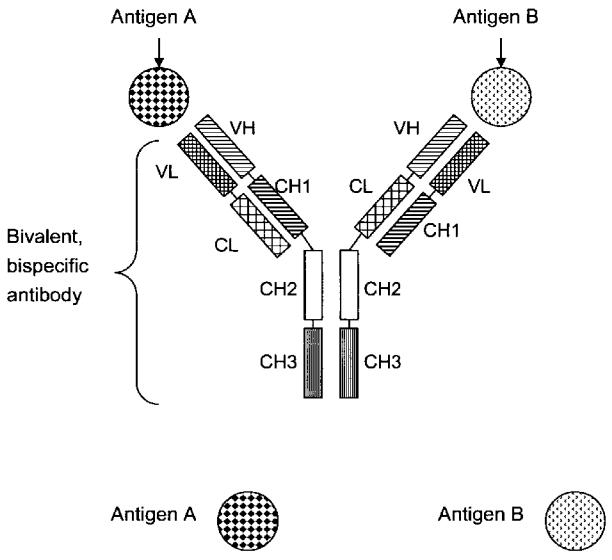
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(54) Title: BIVALENT, BISPECIFIC ANTIBODIES

Fig. 2



(57) Abstract: The present invention relates to novel domain exchanged, bivalent, bispecific antibodies, their manufacture and use.

WO 2009/080253 A1

Bivalent, bispecific antibodies

The present invention relates to novel bivalent, bispecific antibodies, their manufacture and use.

Background of the Invention

Engineered proteins, such as bi- or multispecific antibodies capable of binding two or more antigens are known in the art. Such multispecific binding proteins can be generated using cell fusion, chemical conjugation, or recombinant DNA techniques.

A wide variety of recombinant bispecific antibody formats have been developed in the recent past, e.g. tetravalent bispecific antibodies by fusion of, e.g. an IgG antibody format and single chain domains (see e.g. Coloma, M.J., et al, Nature Biotech 15 (1997) 159-163; WO 2001077342; and Morrison, S.L. , Nature Biotech 25 (2007) 1233-1234).

Also several other new formats wherein the antibody core structure (IgA, IgD, IgE, IgG or IgM) is no longer retained such as dia-, tria- or tetrabodies, minibodies, several single chain formats (scFv, Bis-scFv), which are capable of binding two or more antigens, have been developed(Holliger, P., et al, Nature Biotech 23 (2005) 1126-1136; Fischer, N., Léger O., Pathobiology 74 (2007) 3–14; Shen, J., et al, Journal of Immunological Methods 318 (2007) 65–74; Wu, C., et al, Nature Biotech 25 (2007) 1290-1297).

All such formats use linkers either to fuse the antibody core (IgA, IgD, IgE, IgG or IgM) to a further binding protein (e.g.. scFv) or to fuse e.g. two Fab fragments or scFv.(Fischer N., Léger O., Pathobiology 74 (2007) 3–14). While it is obvious that linkers have advantages for the engineering of bispecific antibodies, they may also cause problems in therapeutic settings. Indeed, these foreign peptides might elicit an immune response against the linker itself or the junction between the protein and the linker. Further more, the flexible nature of these peptides makes them more prone to proteolytic cleavage, potentially leading to poor antibody stability, aggregation and increased immunogenicity. In addition one may want to retain effector functions, such as e.g. complement-dependent cytotoxicity (CDC) or antibody dependent cellular cytotoxicity (ADCC), which are mediated through the

Fc receptor binding , by maintaining a high degree of similarity to naturally occurring.

Thus ideally, one should aim at developing bispecific antibodies that are very similar in general structure to naturally occurring antibodies (like IgA, IgD, IgE, IgG or IgM) with minimal deviation from human sequences.

In one approach bispecific antibodies that are very similar to natural antibodies have been produced using the quadroma technology (see Milstein, C. and A.C. Cuello, Nature, 305 (1983) 537-40) based on the somatic fusion of two different hybridoma cell lines expressing murine monoclonal antibodies with the desired specificities of the bispecific antibody. Because of the random pairing of two different antibody heavy and light chains within the resulting hybrid-hybridoma (or quadroma) cell line, up to ten different antibody species are generated of which only one is the desired, functional bispecific antibody. Due to the of presence of mispaired byproducts, and significantly reduced production yields, means sophisticated purification procedures are required (see e.g. Morrison, S.L. , Nature Biotech 25 (2007) 1233-1234). In general the same problem of mispaired byproducts remains if recombinant expression techniques are used.

An approach to circumvent the problem of mispaired byproducts, which is known as 'knobs-into-holes', aims at forcing the pairing of two different antibody heavy chains by introducing mutations into the CH3 domains to modify the contact interface. On one chain bulky amino acids were replaced by amino acids with short side chains to create a 'hole'. Conversely, amino acids with large side chains were introduced into the other CH3 domain, to create a 'knob'. By coexpressing these two heavy chains (and two identical light chains, which have to be appropriate for both heavy chains), high yields of heterodimer formation ('knob-hole') versus homodimer formation ('hole-hole' or 'knob-knob') was observed (Ridgway, J.B., Presta LG, Carter P; and WO 1996027011). The percentage of heterodimer could be further increased by remodeling the interaction surfaces of the two CH3 domains using a phage display approach and the introduction of a disulfide bridge to stabilize the heterodimers (Merchant, A.M., et al, Nature Biotech 16 (1998) 677-681; Atwell, S., Ridgway, J.B., Wells, J.A., Carter, P., J Mol Biol 270 (1997) 26–35). New approaches for the knobs-into-holes technology are described in e.g. in EP 1870459A1. Although this format appears very attractive, no data describing progression towards the clinic are currently available. One important constraint of this strategy is that the light chains of the two parent antibodies have to be identical

to prevent mispairing and formation of inactive molecules. Thus this technique is not appropriate for easily developing recombinant, bivalent, bispecific antibodies against two antigens starting from two antibodies against the first and the second antigen, as either the heavy chains of these antibodies an/or the identical light chains have to be optimized.

Simon T. et al, EMBO Journal, 9 (1990) 1051 -1056 relates to domain mutants of monospecific antibodies.

Summary of the Invention

The invention relates to a bivalent, bispecific antibody, comprising:

a) the light chain and heavy chain of an antibody specifically binding to a first antigen; and
b) the light chain and heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other.

A further embodiment of the invention is a method for the preparation of an a bivalent, bispecific antibody according to the invention

comprising the steps of

a) transforming a host cell with

-vectors comprising nucleic acid molecules encoding the light chain and heavy chain of an antibody specifically binding to a first antigen

-vectors comprising nucleic acid molecules encoding the light chain and heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other;

b) culturing the host cell under conditions that allow synthesis of said antibody molecule; and

c) recovering said antibody molecule from said culture.

A further embodiment of the invention is a host cell comprising

- vectors comprising nucleic acid molecules encoding the light chain and heavy chain of an antibody specifically binding to a first antigen

- vectors comprising nucleic acid molecules encoding the light chain and heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other.

A further embodiment of the invention is a composition, preferably a pharmaceutical or a diagnostic composition of the antibody according to the invention.

A further embodiment of the invention is a pharmaceutical composition comprising an antibody according to the invention and at least one pharmaceutically acceptable excipient.

A further embodiment of the invention is a method for the treatment of a patient in need of therapy, characterized by administering to the patient a therapeutically effective amount of an antibody according to the invention.

Detailed Description of the Invention

The invention relates to a bivalent, bispecific antibody, comprising:

a) the light chain and heavy chain of an antibody specifically binding to a first antigen; and
b) the light chain and heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other

Therefore said bivalent, bispecific antibody, comprises:

a) a first light chain and a first heavy chain of an antibody specifically binding to a first antigen; and
b) a second light chain and a second heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 of the second light chain and the second heavy chain are replaced by each other.

Thus for said antibody specifically binding to a second antigen the following applies:

within the light chain

the constant light chain domain CL is replaced by the constant heavy chain domain CH1of said antibody;

and within the heavy chain

the constant heavy chain domain CH1 is replaced by the constant light chain domain CL of said antibody.

The term "antibody" as used herein refers to whole, monoclonal antibodies. Such whole antibodies consist of two pairs of a "light chain" (LC) and a "heavy chain" (HC) (such light chain (LC) /heavy chain pairs are abbreviated herein as LC/HC). The light chains and heavy chains of such antibodies are polypeptides consisting of several domains. In a whole antibody, each heavy chain comprises a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region comprises the heavy chain constant domains CH1, CH2 and CH3 (antibody classes IgA, IgD, and IgG) and optionally the heavy chain constant domain CH4 (antibody classes IgE and IgM). Each light chain comprises a light chain variable domain VL and a light chain constant domain CL. The structure of one naturally occurring whole antibody, the IgG antibody, is shown e.g. in Fig.1. The variable domains VH and VL can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4 ((Janeway, C.A., Jr, et al, (2001). Immunobiology., 5th ed., Garland Publishing; and Woof, J., Burton, D., Nat Rev Immunol 4 (2004) 89-99). The two pairs of heavy chain and light chain (HC/LC) are capable of specifically binding to same antigen. Thus said whole antibody is a bivalent, monospecific antibody. Such "antibodies" include e.g. mouse antibodies, human antibodies, chimeric antibodies, humanized antibodies and genetically engineered antibodies (variant or mutant antibodies) as long as their characteristic properties are retained. Especially preferred are human or humanized antibodies, especially as recombinant human or humanized antibodies.

There are five types of mammalian antibody heavy chains denoted by the Greek letters: α, δ, ϵ, γ, and μ (Janeway, C.A., Jr, et al, (2001). Immunobiology., 5th ed.,

Garland Publishing). The type of heavy chain present defines the class of antibody; these chains are found in IgA, IgD, IgE, IgG, and IgM antibodies, respectively (Rhoades RA, Pflanzer RG (2002). Human Physiology, 4th ed., Thomson Learning). Distinct heavy chains differ in size and composition; α and γ contain approximately 450 amino acids, while μ and ϵ have approximately 550 amino acids.

Each heavy chain has two regions, the constant region and the variable region. The constant region is identical in all antibodies of the same isotype, but differs in antibodies of different isotype. Heavy chains γ, α and δ have a constant region composed of three constant domains CH1, CH2, and CH3 (in a line) , and a hinge region for added flexibility (Woof, J., Burton, D., Nat Rev Immunol 4 (2004) 89-99); heavy chains μ and ϵ have a constant region composed of four constant domains CH1, CH2, CH3, and CH4 (Janeway, C.A., Jr. et al. (2001). Immunobiology., 5th ed., Garland Publishing). The variable region of the heavy chain differs in antibodies produced by different B cells, but is the same for all antibodies produced by a single B cell or B cell clone. The variable region of each heavy chain is approximately 110 amino acids long and is composed of a single antibody domain.

In mammals there are only two types of light chain, which are called lambda (λ) and kappa (κ). A light chain has two successive domains: one constant domain CL and one variable domain VL. The approximate length of a light chain is 211 to 217 amino acids. Preferably the light chain is a kappa (κ) light chain, and the constant domain CL is preferably C kappa (κ).

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of a single amino acid composition.

The "antibodies" according to the invention can be of any class (e.g. IgA, IgD, IgE, IgG, and IgM, preferably IgG or IgE), or subclass (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2, preferably IgG1), whereby both antibodies, from which the bivalent bispecific antibody according to the invention is derived, have an Fc part of the same subclass(e.g. IgG1, IgG4 and the like, preferably IgG1), preferably of the same allotype (e.g. Caucasian).

A “Fc part of an antibody” is a term well known to the skilled artisan and defined on the basis of papain cleavage of antibodies. The antibodies according to the

invention contain as Fc part, preferably a Fc part derived from human origin and preferably all other parts of the human constant regions. The Fc part of an antibody is directly involved in complement activation, C1q binding, C3 activation and Fc receptor binding. While the influence of an antibody on the complement system is dependent on certain conditions, binding to C1q is caused by defined binding sites in the Fc part. Such binding sites are known in the state of the art and described e.g. by Lukas, T.J., et al., J. Immunol. 127 (1981) 2555-2560; Brunhouse, R., and Cebra, J.J., Mol. Immunol. 16 (1979) 907-917; Burton, D.R., et al., Nature 288 (1980) 338-344; Thommesen, J.E., et al., Mol. Immunol. 37 (2000) 995-1004; Idusogie, E.E., et al., J. Immunol. 164 (2000) 4178-4184; Hezareh, M., et al., J. Virol. 75 (2001) 12161-12168; Morgan, A., et al., Immunology 86 (1995) 319-324; and EP 0 307 434. Such binding sites are e.g. L234, L235, D270, N297, E318, K320, K322, P331 and P329 (numbering according to EU index of Kabat, see below). Antibodies of subclass IgG1, IgG2 and IgG3 usually show complement activation, C1q binding and C3 activation, whereas IgG4 do not activate the complement system, do not bind C1q and do not activate C3. Preferably the Fc part is a human Fc part.

The term "chimeric antibody" refers to an antibody comprising a variable region, i.e., binding region, from one source or species and at least a portion of a constant region derived from a different source or species, usually prepared by recombinant DNA techniques. Chimeric antibodies comprising a murine variable region and a human constant region are preferred. Other preferred forms of "chimeric antibodies" encompassed by the present invention are those in which the constant region has been modified or changed from that of the original antibody to generate the properties according to the invention, especially in regard to C1q binding and/or Fc receptor (FcR) binding. Such chimeric antibodies are also referred to as "class-switched antibodies.". Chimeric antibodies are the product of expressed immunoglobulin genes comprising DNA segments encoding immunoglobulin variable regions and DNA segments encoding immunoglobulin constant regions. Methods for producing chimeric antibodies involve conventional recombinant DNA and gene transfection techniques are well known in the art. See, e.g., Morrison, S.L., et al., Proc. Natl. Acad. Sci. USA 81 (1984) 6851-6855; US 5,202,238 and US 5,204,244.

The term "humanized antibody" refers to antibodies in which the framework or "complementarity determining regions" (CDR) have been modified to comprise the CDR of an immunoglobulin of different specificity as compared to that of the

parent immunoglobulin. In a preferred embodiment, a murine CDR is grafted into the framework region of a human antibody to prepare the "humanized antibody." See, e.g., Riechmann, L., et al., Nature 332 (1988) 323-327; and Neuberger, M.S., et al., Nature 314 (1985) 268-270. Particularly preferred CDRs correspond to those representing sequences recognizing the antigens noted above for chimeric antibodies. Other forms of "humanized antibodies" encompassed by the present invention are those in which the constant region has been additionally modified or changed from that of the original antibody to generate the properties according to the invention, especially in regard to C1q binding and/or Fc receptor (FcR) binding.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germ line immunoglobulin sequences. Human antibodies are well-known in the state of the art (van Dijk, M.A., and van de Winkel, J.G., Curr. Opin. Chem. Biol. 5 (2001) 368-374). Human antibodies can also be produced in transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire or a selection of human antibodies in the absence of endogenous immunoglobulin production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge (see, e.g., Jakobovits, A., et al., Proc. Natl. Acad. Sci. USA 90 (1993) 2551-2555; Jakobovits, A., et al., Nature 362 (1993) 255-258; Bruggemann, M., et al., Year Immunol. 7 (1993) 33-40). Human antibodies can also be produced in phage display libraries (Hoogenboom, H.R., and Winter, G., J. Mol. Biol. 227 (1992) 381-388; Marks, J.D., et al., J. Mol. Biol. 222 (1991) 581-597). The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); and Boerner, P., et al., J. Immunol. 147 (1991) 86-95). As already mentioned for chimeric and humanized antibodies according to the invention the term "human antibody" as used herein also comprises such antibodies which are modified in the constant region to generate the properties according to the invention, especially in regard to C1q binding and/or FcR binding, e.g. by "class switching" i.e. change or mutation of Fc parts (e.g. from IgG1 to IgG4 and/or IgG1/IgG4 mutation.)

The term "recombinant human antibody", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant

means, such as antibodies isolated from a host cell such as a NS0 or CHO cell or from an animal (e.g. a mouse) that is transgenic for human immunoglobulin genes or antibodies expressed using a recombinant expression vector transfected into a host cell. Such recombinant human antibodies have variable and constant regions in a rearranged form. The recombinant human antibodies according to the invention have been subjected to in vivo somatic hypermutation. Thus, the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germ line VH and VL sequences, may not naturally exist within the human antibody germ line repertoire in vivo.

The "variable domain" (variable domain of a light chain (VL), variable region of a heavy chain (VH)) as used herein denotes each of the pair of light and heavy chains which is involved directly in binding the antibody to the antigen. The domains of variable human light and heavy chains have the same general structure and each domain comprises four framework (FR) regions whose sequences are widely conserved, connected by three "hypervariable regions" (or complementarity determining regions, CDRs). The framework regions adopt a β-sheet conformation and the CDRs may form loops connecting the β-sheet structure. The CDRs in each chain are held in their three-dimensional structure by the framework regions and form together with the CDRs from the other chain the antigen binding site. The antibody heavy and light chain CDR3 regions play a particularly important role in the binding specificity/affinity of the antibodies according to the invention and therefore provide a further object of the invention.

The terms "hypervariable region" or "antigen-binding portion of an antibody" when used herein refer to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from the "complementarity determining regions" or "CDRs". "Framework" or "FR" regions are those variable domain regions other than the hypervariable region residues as herein defined. Therefore, the light and heavy chains of an antibody comprise from N- to C-terminus the domains FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. CDRs on each chain are separated by such framework amino acids. Especially, CDR3 of the heavy chain is the region which contributes most to antigen binding. CDR and FR regions are determined according to the standard definition of Kabat et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991).

The "constant domains" of the heavy chain and of the light chain are not involved directly in binding of an antibody to an antigen, but exhibit various effector functions. Depending on the amino acid sequence of the constant region of their heavy chains, antibodies or immunoglobulins are divided into the classes:

The term "bivalent, bispecific antibody" as used herein refers to an antibody as described above in which each of the two pairs of heavy chain and light chain (HC/LC) is specifically binding to a different antigen, i.e. the first heavy and the first light chain (originating from an antibody against a first antigen) are specifically binding together to a first antigen, and , the second heavy and the second light chain (originating from an antibody against a second antigen) are specifically binding together to a second antigen (as depicted in Fig. 2); such bivalent, bispecific antibodies are capable of specifically binding to two different antigens at the same time, and not to more than two antigens, in contrary to, on the one hand a monospecific antibody capable of binding only to one antigen, and on the other hand e.g. a tetravalent, tetraspecific antibody which can bind to four antigen molecules at the same time.

According to the invention, the ratio of a desired bivalent, bispecific antibody compared to undesired side products can be improved by the replacement of certain domains in only one pair of heavy chain and light chain (HC/LC). While the first of the two HC/LC pairs originates from an antibody specifically binding to a first antigen and is left essentially unchanged, the second of the two HC/LC pairs originates from an antibody specifically binding to a second antigen , and is altered by the following replacement:

- light chain: replacement of the constant light chain domain CL by the constant heavy chain domain CH1 of said antibody specifically binding to a second antigen , and
- heavy chain: replacement of the constant heavy chain domain CH1 by the constant light chain domain CL of said antibody specifically binding to a second antigen.

Thus the resulting bivalent, bispecific antibodies are artificial antibodies which comprise

a) the light chain and heavy chain of an antibody specifically binding to a first antigen; and

b) the light chain and heavy chain of an antibody specifically binding to a second antigen;

wherein said light chain (of an antibody specifically binding to a second antigen) contains a constant domain CH1 instead of CL

wherein said heavy chain(of an antibody specifically binding to a second antigen) a constant domain CL instead of CH1.

In an additional aspect of the invention such improved ratio of a desired bivalent, bispecific antibody compared to undesired side products can be further improved by one of the following two alternatives:

A) First alternative (see Fig. 3):

The CH3 domains of said bivalent, bispecific antibody according to the invention can be altered by the "knob-into-holes" technology which described with in detail with several examples in e.g. WO 96/027011, Ridgway, J.B., et al, Protein Eng 9 (1996) 617–621; and Merchant, A.M., et al, Nat Biotechnol 16 (1998) 677-681. In this method the interaction surfaces of the two CH3 domains are altered to increase the heterodimerisation of both heavy chains containing these two CH3 domains. Each of the two CH3 domains (of the two heavy chains) can be the "knob", while the other is the "hole". The introduction of a disulfide bridge stabilizes the heterodimers (Merchant, A..M., et al, Nature Biotech 16 (1998) 677-681; Atwell, S., Ridgway, J.B., Wells, J.A., Carter, P., J Mol Biol 270 (1997) 26–35) and increases the yield.

Therefore in preferred embodiment the CH3 domains of a bivalent, bispecific antibody wherein the first CH3 domain and second CH3 domain each meet at an interface which comprises an original interface between the antibody CH3 domains are altered by the "knob-into-holes" technology including further stabilization by introduction of a disulfide bridge in the CH3 domains (described in WO 96/027011, Ridgway, J.B., et al, Protein Eng 9 (1996) 617-621; Merchant. A.M, et al., Nature Biotech 16 (1998) 677-681; and Atwell, S., Ridgway, J.B., Wells, J.A., Carter P., J Mol Biol 270 (1997) 26–35) to promote the formation of the bivalent, bispecific antibody.

Thus in one aspect of the invention said bivalent, bispecific antibody is characterized in that

the CH3 domain of one heavy chain and the CH3 domain of the other heavy chain each meet at an interface which comprises an original interface between the antibody CH3 domains;
wherein said interface is altered to promote the formation of the bivalent, bispecific antibody, wherein the alteration is characterized in that:

a) the CH3 domain of one heavy chain is altered,

so that within the original interface the CH3 domain of one heavy chain that meets the original interface of the CH3 domain of the other heavy chain within the bivalent, bispecific antibody,
an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance within the interface of the CH3 domain of one heavy chain which is positionable in a cavity within the interface of the CH3 domain of the other heavy chain

and

b) the CH3 domain of the other heavy chain is altered,

so that within the original interface of the second CH3 domain that meets the original interface of the first CH3 domain within the bivalent, bispecific antibody an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity within the interface of the second CH3 domain within which a protuberance within the interface of the first CH3 domain is positionable.

Preferably said amino acid residue having a larger side chain volume is selected from the group consisting of arginine (R), phenylalanine (F), tyrosine (Y), tryptophan (W).

Preferably said amino acid residue having a smaller side chain volume is selected from the group consisting of alanine (A), serine (S), threonine (T), valine (V).

In one aspect of the invention both CH3 domains are further altered the introduction of cysteine (C) as amino acid in the corresponding positions of each CH3 domain such that a disulfide bridge between both CH3 domains can be formed.

In another preferred embodiment of the invention both CH3 domains are altered by the use of residues R409D; K370E (K409D) for knobs residues and D399K; E357K for hole residues described eg. in EP 1870459A1.

or

B) Second alternative (see Figure 4):

by the replacement of one constant heavy chain domain CH3 by a constant heavy chain domain CH1; and the other constant heavy chain domain CH3is replaced by a constant light chain domain CL. The constant heavy chain domain CH1 by which the heavy chain domain CH3 is replaced can be of any Ig class (e.g. IgA, IgD, IgE, IgG, and IgM), or subclass (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2).
The constant light chain domain CL by which the heavy chain domain CH3 is replaced can be of the lambda (λ) or kappa (κ) type, preferably the kappa (κ) type.

Thus one preferred embodiment of the invention is a bivalent, bispecific antibody, comprising:

a) the light chain and heavy chain of an antibody specifically binding to a first antigen; and

b) the light chain and heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other,

and wherein optionally

c) the CH3 domain of one heavy chain and the CH3 domain of the other heavy chain each meet at an interface which comprises an original interface between the antibody CH3 domains;

wherein said interface is altered to promote the formation of the bivalent, bispecific antibody, wherein the alteration is characterized in that:

ca) the CH3 domain of one heavy chain is altered,

so that within the original interface the CH3 domain of one heavy chain that meets the original interface of the CH3 domain of the other heavy chain within the bivalent, bispecific antibody,
an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance within the interface of the CH3 domain of one heavy chain which is positionable in a cavity within the interface of the CH3 domain of the other heavy chain

and

cb) the CH3 domain of the other heavy chain is altered,

so that within the original interface of the second CH3 domain that meets the original interface of the first CH3 domain within the bivalent, bispecific antibody
an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity within the interface of the second CH3 domain within which a protuberance within the interface of the first CH3 domain is positionable;

or d)

one constant heavy chain domain CH3 is replaced by a constant heavy chain domain CH1; and the other constant heavy chain domain CH3 is replaced by a constant light chain domain CL

The terms "antigen" or "antigen molecule" as used herein are used interchangeable and refer to all molecules that can be specifically bound by an antibody. The bivalent, bispecific antibody is specifically binding to a first antigen and a second distinct antigen. The term "antigens" as used herein include e.g. proteins, different epitopes on proteins (as different antigens within the meaning of the invention), and polysaccharides. This mainly includes parts (coats, capsules, cell walls, flagella, fimbrae, and toxins) of bacteria, viruses, and other microorganisms. Lipids and nucleic acids are antigenic only when combined with proteins and polysaccharides. Non-microbial exogenous (non-self) antigens can include pollen, egg white, and proteins from transplanted tissues and organs or on the surface of transfused blood cells. Preferably the antigen is selected from the group consisting of cytokines, cell

surface proteins, enzymes and receptors cytokines, cell surface proteins, enzymes and receptors.

Tumor antigens are those antigens that are presented by MHC I or MHC II molecules on the surface of tumor cells. These antigens can sometimes be presented by tumor cells and never by the normal ones. In this case, they are called tumor-specific antigens (TSAs) and typically result from a tumor specific mutation. More common are antigens that are presented by tumor cells and normal cells, and they are called tumor-associated antigens (TAAs). Cytotoxic T lymphocytes that recognized these antigens may be able to destroy the tumor cells before they proliferate or metastasize. Tumor antigens can also be on the surface of the tumor in the form of, for example, a mutated receptor, in which case they will be recognized by B cells.

In one preferred embodiment at least one of the two different antigens (first and second antigen), to which the bivalent, bispecific antibody specifically binds to, is a tumor antigen.

In another preferred embodiment both of the two different antigens (first and second antigen), to which the bivalent, bispecific antibody specifically binds to, are tumor antigens; in this case the first and second antigen can also be two different epitopes at the same tumor specific protein.

In another preferred embodiment one of the two different antigens (first and second antigen), to which the bivalent, bispecific antibody specifically binds to, is a tumor antigen and the other is an effector cell antigen, as e.g. a T-Cell receptor, CD3, CD16 and the like.

In another preferred embodiment one of the two different antigens (first and second antigen), to which the bivalent, bispecific antibody specifically binds to, is a tumor antigen and the other is an anti-cancer substance such as a toxin or a kinase inhibitor.

As used herein, "specifically binding" or “binds specifically to” refers to an antibody specifically binding an antigen. Preferably the binding affinity of the antibody specifically binding this antigen is of KD-value of 10^{-9} mol/l or lower (e.g. 10^{-10} mol/l), preferably with a KD-value of 10^{-10} mol/l or lower (e.g. 10^{-12} mol/l). The binding affinity is determined with a standard binding assay, such as surface plasmon resonance technique (Biacore®).

The term "epitope" includes any polypeptide determinant capable of specific binding to an antibody. In certain embodiments, epitope determinant include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl, or sulfonyl, and, in certain embodiments, may have specific three dimensional structural characteristics, and or specific charge characteristics. An epitope is a region of an antigen that is bound by an antibody. In certain embodiments, an antibody is said to specifically bind an antigen when it preferentially recognizes its target antigen in a complex mixture of proteins and/or macromolecules.

An further embodiment of the invention is a method for the preparation of a bivalent, bispecific antibody according to the invention

comprising

a) transforming a host cell with

-vectors comprising nucleic acid molecules encoding the light chain and heavy chain of an antibody specifically binding to a first antigen

-vectors comprising nucleic acid molecules encoding the light chain and heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other;

b) culturing the host cell under conditions that allow synthesis of said antibody molecule; and

c) recovering said antibody molecule from said culture.

In general there are two vectors encoding the light chain and heavy chain of said antibody specifically binding to a first antigen, and further two vectors encoding the light chain and heavy chain of said antibody specifically binding to a second antigen. One of the two vectors is encoding the respective light chain and the other of the two vectors is encoding the respective heavy chain. However in an alternative method for the preparation of a bivalent, bispecific antibody according to the invention, only one first vector encoding the light chain and heavy chain of the antibody specifically binding to a first antigen and only one second vector encoding the light chain and heavy chain of the antibody specifically binding to a second antigen can be used for transforming the host cell.

The invention encompasses a method for the preparation of the antibodies comprising culturing the corresponding host cells under conditions that allow synthesis of said antibody molecules and recovering said antibodies from said culture, e.g. by expressing

-a first nucleic acid sequence encoding the light chain of an antibody specifically binding to a first antigen;

-a second nucleic acid sequence encoding the heavy chain of said antibody specifically binding to a first antigen;

-a third nucleic acid sequence encoding the light chain of an antibody specifically binding to a second antigen, wherein the constant light chain domain CL is replaced by the constant heavy chain domain CH1; and

-a fourth nucleic acid sequence encoding the heavy chain of said antibody specifically binding to a second antigen, wherein constant heavy chain domain CH1 by the constant light chain domain CL.

A further embodiment of the invention is a host cell comprising

- vectors comprising nucleic acid molecules encoding the light chain and heavy chain of an antibody specifically binding to a first antigen

- vectors comprising nucleic acid molecules encoding the light chain and heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other.

A further embodiment of the invention is a host cell comprising

a) a vector comprising a nucleic acid molecule encoding the light chain and a vector comprising a nucleic acid molecule encoding the heavy chain, of an antibody specifically binding to a first antigen

b) a vector comprising a nucleic acid molecule encoding the light chain and a vector comprising a nucleic acid molecule encoding the heavy chain, of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other.

A further embodiment of the invention is a composition, preferably a pharmaceutical or a diagnostic composition of the bivalent, bispecific antibody according to the invention.

A further embodiment of the invention is a pharmaceutical composition comprising a bivalent, bispecific antibody according to the invention and at least one pharmaceutically acceptable excipient.

A further embodiment of the invention is a method for the treatment of a patient in need of therapy, characterized by administering to the patient a therapeutically effective amount of a bivalent, bispecific antibody according to the invention.

The term "nucleic acid or nucleic acid molecule", as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

The term “transformation” as used herein refers to process of transfer of a vectors/nucleic acid into a host cell. If cells without formidable cell wall barriers are used as host cells, transfection is carried out e.g. by the calcium phosphate precipitation method as described by Graham and Van der Eh, Virology 52 (1978) 546ff. However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used. If prokaryotic cells or cells which contain substantial cell wall constructions are used, e.g. one method of transfection is calcium treatment using calcium chloride as described by Cohen, F. N, et al, PNAS. 69 (1972) 7110ff.

Recombinant production of antibodies using transformation is well-known in the state of the art and described, for example, in the review articles of Makrides, S.C., Protein Expr. Purif. 17 (1999) 183-202; Geisse, S., et al., Protein Expr. Purif. 8 (1996) 271-282; Kaufman, R.J., Mol. Biotechnol. 16 (2000) 151-161; Werner, R.G.,

et al., Arzneimittelforschung 48 (1998) 870-880 as well as in US 6,331,415 and US 4,816,567.

As used herein, "expression" refers to the process by which a nucleic acid is transcribed into mRNA and/or to the process by which the transcribed mRNA (also referred to as transcript) is subsequently being translated into peptides, polypeptides, or proteins. The transcripts and the encoded polypeptides are collectively referred to as gene product. If the polynucleotide is derived from genomic DNA, expression in a eukaryotic cell may include splicing of the mRNA.

A "vector" is a nucleic acid molecule, in particular self-replicating, which transfers an inserted nucleic acid molecule into and/or between host cells. The term includes vectors that function primarily for insertion of DNA or RNA into a cell (e.g., chromosomal integration), replication of vectors that function primarily for the replication of DNA or RNA, and expression vectors that function for transcription and/or translation of the DNA or RNA. Also included are vectors that provide more than one of the functions as described.

An "expression vector" is a polynucleotide which, when introduced into an appropriate host cell, can be transcribed and translated into a polypeptide. An “expression system" usually refers to a suitable host cell comprised of an expression vector that can function to yield a desired expression product.

The bivalent, bispecific antibodies according to the invention are preferably produced by recombinant means. Such methods are widely known in the state of the art and comprise protein expression in prokaryotic and eukaryotic cells with subsequent isolation of the antibody polypeptide and usually purification to a pharmaceutically acceptable purity. For the protein expression, nucleic acids encoding light and heavy chains or fragments thereof are inserted into expression vectors by standard methods. Expression is performed in appropriate prokaryotic or eukaryotic host cells like CHO cells, NS0 cells, SP2/0 cells, HEK293 cells, COS cells, yeast, or E.coli cells, and the antibody is recovered from the cells (supernatant or cells after lysis).The bivalent, bispecific antibodies may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. Purification is performed in order to eliminate other cellular components or other contaminants, e.g. other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, column chromatography and others well known in the art.

See Ausubel, F., et al., ed., Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987).

Expression in NS0 cells is described by, e.g., Barnes, L.M., et al., Cytotechnology 32 (2000) 109-123; and Barnes, L.M., et al., Biotech. Bioeng. 73 (2001) 261-270. Transient expression is described by, e.g., Durocher, Y., et al., Nucl. Acids. Res. 30 (2002) E9. Cloning of variable domains is described by Orlandi, R., et al., Proc. Natl. Acad. Sci. USA 86 (1989) 3833-3837; Carter, P., et al., Proc. Natl. Acad. Sci. USA 89 (1992) 4285-4289; and Norderhaug, L., et al., J. Immunol. Methods 204 (1997) 77-87. A preferred transient expression system (HEK 293) is described by Schlaeger, E.-J., and Christensen, K., in Cytotechnology 30 (1999) 71-83 and by Schlaeger, E.-J., in J. Immunol. Methods 194 (1996) 191-199.

The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, enhancers and polyadenylation signals.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading frame. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The bivalent, bispecific antibodies are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. DNA and RNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures. The hybridoma cells can serve as a source of such DNA and RNA. Once isolated, the DNA may be inserted into expression vectors, which are then transfected into host cells such as

HEK 293 cells, CHO cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of recombinant monoclonal antibodies in the host cells.

Amino acid sequence variants (or mutants) of the bivalent, bispecific antibody are prepared by introducing appropriate nucleotide changes into the antibody DNA, or by nucleotide synthesis. Such modifications can be performed, however, only in a very limited range, e.g. as described above. For example, the modifications do not alter the above mentioned antibody characteristics such as the IgG isotype and antigen binding, but may improve the yield of the recombinant production, protein stability or facilitate the purification.

The following examples, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Sequence Listing

SEQ ID NO: 1	amino acid sequence of wild type <IGF-1R> antibody heavy chain
SEQ ID NO: 2	amino acid sequence of wild type <IGF-1R> antibody light chain
SEQ ID NO: 3	amino acid sequence of the heavy chain** (HC**) of <IGF-1R> CL-CH1 exchange antibody, wherein the heavy chain domain CH1 is replaced by the light chain domain CL.
SEQ ID NO: 4	amino acid sequence of the light chain** (LC**) of <IGF-1R> CL-CH1 exchange antibody, wherein the light chain domain CL is replaced by the heavy chain domain CH1.
SEQ ID NO: 5	amino acid sequence of IGF-1R ectodomain His-Streptavidin binding peptide-tag (IGF-1R-His-SBP ECD)
SEQ ID NO: 6	amino acid sequence of wild type ANGPT2 <ANGPT2> antibody heavy chain
SEQ ID NO: 7	amino acid sequence of wild type ANGPT2 <ANGPT2> antibody light chain
SEQ ID NO: 8	amino acid sequence of CH3 domain (Knobs) with a T366W exchange for use in the knobs-into-holes technology

SEQ ID NO: 9	amino acid sequence CH3 domain (Hole) with a T366S, L368A, Y407V exchange for use in the knobs-into-holes technology
SEQ ID NO: 10	amino acid sequence of IGF-1R ectodomain His-Streptavidin binding peptide-tag (IGF-1R-His-SBP ECD)

Description of the Figures

Figure 1	Schematic figure of IgG, a naturally occurring whole antibody specific for one antigen with two pairs of heavy and light chain which comprise variable and constant domains in a typical order.
Figure 2	Schematic figure of a bivalent, bispecific antibody, comprising: a) the light chain and heavy chain of an antibody specifically binding to a first antigen; and b) the light chain and heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other.
Figure 3	Schematic figure of a bivalent, bispecific antibody, comprising: a) the light chain and heavy chain of an antibody specifically binding to a first antigen; and b) the light chain and heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other, and wherein the CH3 domains of both heavy chains are altered by the knobs-into-holes technology .
Figure 4	Schematic figure of a bivalent, bispecific antibody, comprising: a) the light chain and heavy chain of an antibody specifically binding to a first antigen; and b) the light chain and heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other, and wherein one of the constant heavy chain domains CH3 of both heavy chains is replaced by a constant heavy chain domain. CH1, and the other constant heavy chain domain CH3 is replaced by a constant light chain domain CL.
Figure 5	Protein sequence scheme of the heavy chain** <IGF-1R> HC** of the <IGF-1R> CL-CH1 exchange antibody (with a kappa constant light chain domain CL)
Figure 6	Protein sequence scheme of the light chain** <IGF-1R> LC** of the <IGF-1R> CL-CH1 exchange antibody

Figure 7 Plasmid map of heavy chain** <IGF-1R> HC** expression vector pUC-HC**-IGF-1R

Figure 8 Plasmid map of light chain** <IGF-1R> LC** expression vector pUC-LC**-IGF-1R

Figure 9 Plasmid map of the 4700-Hyg-OriP expression vector

Figure 10 Assay principle of cellular FACS IGF-1R-ANGPT2 bridging assay on I24 IGF-1R expressing cells to detect the presence of functional bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody

Figure 11 Scheme IGF-1R ECD Biacore

Figure 12 SDS-PAGE and size exclusion chromatography of purified monospecific, bivalent <IGF-1R> CL-CH1 exchange antibody (IgG1**) with HC** and LC** isolated from cell culture supernatants after transient transfection of HEK293-F cells.

Figure 13 Binding of monospecific <IGF-1R> CL-CH1 exchange antibody and wildtype <IGF-1R> antibody to the IGF-1R ECD in an ELISA-based binding assay.

Figure 14 SDS-PAGE of and size exclusion chromatography <ANGPT2-IGF-1R> CL-CH1 exchange antibody mix purified from cell culture supernatants from transiently transfected HEK293-F cells.

Figure 15 Results for Samples A to F of cellular FACS IGF-1R-ANGPT2 bridging assay on I24 IGF-1R expressing cells to detect the presence of functional bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody in purified antibody mix: Purified proteins Samples A to F:

A = I24 untreated

B = I24 + 2 µg/mL hANGPT2 + hIgG Isotype

C = I24 + 2 µg/mL hANGPT2 + Mix from co-expression of <IGF-1R> CL-CH1 exchange antibody and <ANGPT2> wildtype antibody comprising bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody

D: not present

E = I24 + 2 µg/mL hANGPT2 + <ANGPT2> wildtype antibody

F = I24 + 2 µg/mL hANGPT2 + <IGF-1R> wildtype antibody

Examples

Materials & general methods

General information regarding the nucleotide sequences of human immunoglobulins light and heavy chains is given in: Kabat, E.A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991). Amino acids of antibody chains are numbered and referred to according to EU numbering (Edelman, G.M., et al., Proc. Natl. Acad. Sci. USA 63 (1969) 78-85; Kabat, E.A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD, (1991)).

Recombinant DNA techniques

Standard methods were used to manipulate DNA as described in Sambrook, J. et al., Molecular cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The molecular biological reagents were used according to the manufacturer's instructions.

Gene synthesis

Desired gene segments were prepared from oligonucleotides made by chemical synthesis. The 600 - 1800 bp long gene segments, which are flanked by singular restriction endonuclease cleavage sites, were assembled by annealing and ligation of oligonucleotides including PCR amplification and subsequently cloned via the indicated restriction sites e.g. KpnI/ SacI or AscI/PacI into a pPCRScript (Stratagene) based pGA4 cloning vector. The DNA sequences of the subcloned gene fragments were confirmed by DNA sequencing. Gene synthesis fragments were ordered according to given specifications at Geneart (Regensburg, Germany).

DNA sequence determination

DNA sequences were determined by double strand sequencing performed at MediGenomix GmbH (Martinsried, Germany) or Sequiserve GmbH (Vaterstetten, Germany).

DNA and protein sequence analysis and sequence data management

The GCG's (Genetics Computer Group, Madison, Wisconsin) software package version 10.2 and Infomax's Vector NT1 Advance suite version 8.0 was used for sequence creation, mapping, analysis, annotation and illustration.

Expression vectors

For the expression of the described antibodies variants of expression plasmids for transient expression (e.g. in HEK293 EBNA or HEK293-F) cells based either on a cDNA organization with a CMV-Intron A promoter or on a genomic organization with a CMV promoter were applied.

Beside the antibody expression cassette the vectors contained:

- an origin of replication which allows replication of this plasmid in E. coli, and

- a ß-lactamase gene which confers ampicillin resistance in E. coli.

The transcription unit of the antibody gene is composed of the following elements:

- unique restriction site(s) at the 5' end

- the immediate early enhancer and promoter from the human cytomegalovirus,

- followed by the Intron A sequence in the case of the cDNA organization,

- a 5'-untranslated region of a human antibody gene,

- a immunoglobulin heavy chain signal sequence,

- the human antibody chain (wildtype or with domain echange) either as cDNA or as genomic organization with an the immunoglobulin exon-intron organization

- a 3' untranslated region with a polyadenylation signal sequence, and

- unique restriction site(s) at the 3' end.

The fusion genes comprising the described antibody chains as decribed below were generated by PCR and/or gene synthesis and assembled with known recombinant

methods and techniques by connection of the according nucleic acid segments e.g. using unique restriction sites in the respective vectors. The subcloned nucleic acid sequences were verified by DNA sequencing. For transient transfections larger quantities of the plasmids were prepared by plasmid preparation from transformed E. coli cultures (Nucleobond AX, Macherey-Nagel).

Cell culture techniques

Standard cell culture techniques were used as described in Current Protocols in Cell Biology (2000), Bonifacino, J.S., Dasso, M., Harford, J.B., Lippincott-Schwartz, J. and Yamada, K.M. (eds.), John Wiley & Sons, Inc.

Bispecific antibodies were expressed by transient co-transfection of the respective expression plasmids in adherently growing HEK293-EBNA or in HEK29-F cells growing in suspension as described below.

Transient transfections in HEK293-EBNA system

Bispecific antibodies were expressed by transient co-transfection of the respective expression plasmids (e.g. encoding the heavy and modified heavy chain, as well as the corresponding light and modified light chain) in adherently growing HEK293-EBNA cells (human embryonic kidney cell line 293 expressing Epstein-Barr-Virus nuclear antigen; American type culture collection deposit number ATCC # CRL-10852, Lot. 959 218) cultivated in DMEM (Dulbecco's modified Eagle's medium, Gibco) supplemented with 10% Ultra Low IgG FCS (fetal calf serum, Gibco), 2 mM L-Glutamine (Gibco), and 250 µg/ml Geneticin (Gibco). For transfection FuGENE™ 6 Transfection Reagent (Roche Molecular Biochemicals) was used in a ratio of FuGENE™ reagent (µl) to DNA (µg) of 4:1 (ranging from 3:1 to 6:1). Proteins were expressed from the respective plasmids using a molar ratio of (modified and wildtype) light chain and heavy chain encoding plasmids of 1:1 (equimolar) ranging from 1:2 to 2:1, respectively. Cells were feeded at day 3 with L-Glutamine ad 4 mM, Glucose [Sigma] and NAA [Gibco]. Bispecific antibody containing cell culture supernatants were harvested from day 5 to 11 after transfection by centrifugation and stored at -20°C. General information regarding the recombinant expression of human immunoglobulins in e.g. HEK293 cells is given in: Meissner, P. et al., Biotechnol. Bioeng. 75 (2001) 197-203.

Transient transfections in HEK293-F system

Bispecific antibodies were generated by transient transfection of the respective plasmids (e.g. encoding the heavy and modified heavy chain, as well as the corresponding light and modified light chain) using the HEK293-F system (Invitrogen) according to the manufacturer's instruction. Briefly, HEK293-F cells (Invitrogen) growing in suspension either in a shake flask or in a stirred fermenter in serumfree FreeStyle 293 expression medium (Invitrogen) were transfected with a mix of the four expression plasmids and 293fectin or fectin (Invitrogen). For 2 L shake flask (Corning) HEK293-F cells were seeded at a density of 1.0E*6 cells/mL in 600 mL and incubated at 120 rpm, 8% CO2. The day after the cells were transfected at a cell density of ca. 1.5E*6 cells/mL with ca. 42 mL mix of A) 20 mL Opti-MEM (Invitrogen) with 600 µg total plasmid DNA (1 µg/mL) encoding the heavy or modified heavy chain, respectively and the corresponding light chain in an equimolar ratio and B) 20 ml Opti-MEM + 1.2 mL 293 fectin or fectin (2 µl/mL). According to the glucose consumption glucose solution was added during the course of the fermentation. The supernatant containing the secreted antibody was harvested after 5-10 days and antibodies were either directly purified from the supernatant or the supernatant was frozen and stored.

Protein determination

The protein concentration of purified antibodies and derivatives was determined by determining the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence according to Pace et al., Protein Science, 1995, 4, 2411-1423.

Antibody concentration determination in supernatants

The concentration of antibodies and derivatives in cell culture supernatants was estimated by immunoprecipitation with Protein A Agarose-beads (Roche). 60 µL Protein A Agarose beads are washed three times in TBS-NP40 (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet-P40). Subsequently, 1 -15 mL cell culture supernatant were applied to the Protein A Agarose beads pre-equilibrated in TBS-NP40. After incubation for at 1 h at room temperature the beads were washed on an Ultrafree-MC-filter column (Amicon] once with 0.5 mL TBS-NP40, twice with 0.5 mL 2x phosphate buffered saline (2xPBS, Roche) and briefly four times with 0.5 mL 100 mM Na-citrate pH 5,0. Bound antibody was eluted by addition of 35 µl NuPAGE® LDS Sample Buffer (Invitrogen). Half of the sample was combined with NuPAGE®

Sample Reducing Agent or left unreduced, respectively, and heated for 10 min at 70°C. Consequently, 5-30 µl were applied to an 4-12% NuPAGE® Bis-Tris SDS-PAGE (Invitrogen) (with MOPS buffer for non-reduced SDS-PAGE and MES buffer with NuPAGE® Antioxidant running buffer additive (Invitrogen) for reduced SDS-PAGE) and stained with Coomassie Blue.

The concentration of antibodies and derivatives in cell culture supernatants was quantitatively measured by affinity HPLC chromatography. Briefly, cell culture supernatants containing antibodies and derivatives that bind to Protein A were applied to an Applied Biosystems Poros A/20 column in 200 mM KH2PO4, 100 mM sodium citrate, pH 7.4 and eluted from the matrix with 200 mM NaCl, 100 mM citric acid, pH 2,5 on an Agilent HPLC 1100 system. The eluted protein was quantified by UV absorbance and integration of peak areas. A purified standard IgG1 antibody served as a standard.

Alternatively, the concentration of antibodies and derivatives in cell culture supernatants was measured by Sandwich-IgG-ELISA. Briefly, StreptaWell High Bind Strepatavidin A-96 well microtiter plates (Roche) were coated with 100 µL/well biotinylated anti-human IgG capture molecule F(ab')2<h-Fcγ> BI (Dianova) at 0.1 µg/mL for 1 h at room temperature or alternatively over night at 4°C and subsequently washed three times with 200 µL/well PBS, 0.05% Tween (PBST, Sigma). 100 µL/well of a dilution series in PBS (Sigma) of the respective antibody containing cell culture supernatants was added to the wells and incubated for 1-2 h on a microtiterplate shaker at room temperature. The wells were washed three times with 200 µL/well PBST and bound antibody was detected with 100 µl F(ab')2<hFcγ>POD (Dianova) at 0.1 µg/mL as detection antibody for 1-2 h on a microtiterplate shaker at room temperature. Unbound detection antibody was washed away three times with 200 µL/well PBST and the bound detection antibody was detected by addition of 100 µL ABTS/well. Determination of absorbance was performed on a Tecan Fluor Spectrometer at a measurement wavelength of 405 nm (reference wavelength 492 nm).

Protein purification

Proteins were purified from filtered cell culture supernatants referring to standard protocols. In brief, antibodies were applied to a Protein A Sepharose column (GE healthcare) and washed with PBS. Elution of antibodies was achieved at pH 2.8 followed by immediate neutralization of the sample. Aggregated protein was

separated from monomeric antibodies by size exclusion chromatography (Superdex 200, GE Healthcare) in PBS or in 20 mM Histidine, 150 mM NaCl pH 6.0. Monomeric antibody fractions were pooled, concentrated if required using e.g. a MILLIPORE Amicon Ultra (30 MWCO) centrifugal concentrator, frozen and stored at -20°C or -80°C. Part of the samples were provided for subsequent protein analytics and analytical characterization e.g. by SDS-PAGE, size exclusion chromatography or mass spectrometry.

SDS-PAGE

The NuPAGE® Pre-Cast gel system (Invitrogen) was used according to the manufacturer's instruction. In particular, 10% or 4-12% NuPAGE® Novex® Bis-TRIS Pre-Cast gels (pH 6.4) and a NuPAGE® MES (reduced gels, with NuPAGE® Antioxidant running buffer additive) or MOPS (non-reduced gels) running buffer was used.

Analytical size exclusion chromatography

Size exclusion chromatography for the determination of the aggregation and oligomeric state of antibodies was performed by HPLC chromatography. Briefly, Protein A purified antibodies were applied to a Tosoh TSKgel G3000SW column in 300 mM NaCl, 50 mM KH2PO4/K2HPO4, pH 7.5 on an Agilent HPLC 1100 system or to a Superdex 200 column (GE Healthcare) in 2 x PBS on a Dionex HPLC-System. The eluted protein was quantified by UV absorbance and integration of peak areas. BioRad Gel Filtration Standard 151–1901 served as a standard.

Mass spectrometry

The total deglycosylated mass of crossover antibodies was determined and confirmed via electrospray ionization mass spectrometry (ESI-MS). Briefly, 100 µg purified antibodies were deglycosylated with 50 mU N-Glycosidase F (PNGaseF, ProZyme) in 100 mM KH2PO4/K2HPO4, pH 7 at 37°C for 12-24 h at a protein concentration of up to 2 mg/ml and subsequently desalted via HPLC on a Sephadex G25 column (GE Healthcare). The mass of the respective heavy and light chains was determined by ESI-MS after deglycosylation and reduction. In brief, 50 µg antibody in 115 µl were incubated with 60 µl 1M TCEP and 50 µl 8 M Guanidine-hydrochloride subsequently desalted. The total mass and the mass of the reduced

heavy and light chains was determined via ESI-MS on a Q-Star Elite MS system equipped with a NanoMate source.

IGF-1R ECD binding ELISA

The binding properties of the generated antibodies were evaluated in an ELISA assay with the IGF-1R extracellular domain (ECD). For this sake the extracellular domain of IGF-1R (residues 1-462) comprising the natural leader sequence and the LI-cysteine rich-12 domains of the human IGF-IR ectodomain of the alpha chain (according to the McKern et al., 1997; Ward et al., 2001) fused to an N-terminal His-Streptavidin binding peptide-tag (His-SBP) was cloned into a pcDNA3 vector derivative and transiently expressed in HEK293F cells. The protein sequence of the IGF-1R-His-SBP ECD is given in SEQ ID NO: 10. StreptaWell High Bind Strepatavidin A-96 well microtiter plates (Roche) were coated with 100 µL/well cell culture supernatant containing soluble IGF-1R-ECD-SBP fusion protein over night at 4°C and washed three times with 200 µL/well PBS, 0.05% Tween (PBST, Sigma). Subsequently, 100 µL/well of a dilution series of the respective antibody and as a reference wildtype <IGF-1R> antibody in PBS (Sigma) including 1% BSA (fraction V, Roche) was added to the wells and incubated for 1-2 h on a microtiterplate shaker at room temperature. For the dilution series the same amount of purified antibody were applied to the wells. The wells were washed three times with 200 µL/well PBST and bound antibody was detected with 100 µL/well F(ab')2<hFcγ>POD (Dianova) at 0.1 µg/mL (1:8000) as detection antibody for 1-2 h on a microtiterplate shaker at room temperature. Unbound detection antibody was washed away three times with 200 µL/well PBST and the bound detection antibody was detected by addition of 100 µL ABTS/well. Determination of absorbance was performed on a Tecan Fluor Spectrometer at a measurement wavelength of 405 nm (reference wavelength 492 nm).

IGF-1R ECD Biacore

Binding of the generated antibodies to human IGF-1R ECD was also investigated by surface plasmon resonance using a BIACORE T100 instrument (GE Healthcare Biosciences AB, Uppsala, Sweden). Briefly, for affinity measurements Goat-Anti-Human IgG, JIR 109-005-098 antibodies were immobilized on a CM5 chip via amine coupling for presentation of the antibodies against human IGF-1R ECD-Fc tagged. Binding was measured in HBS buffer (HBS-P (10 mM HEPES, 150 mM NaCl, 0.005% Tween 20, ph 7.4), 25°C. IGF-1R ECD (R&D Systems or in house

purified) was added in various concentrations in solution. Association was measured by an IGF-1R ECD injection of 80 seconds to 3 minutes; dissociation was measured by washing the chip surface with HBS buffer for 3 - 10 minutes and a KD value was estimated using a 1:1 Langmuir binding model. Due to low loading density and capturing level of <IGF-1R> antibodies monovalent IGF-1R ECD binding was obtained. Negative control data (e.g. buffer curves) were subtracted from sample curves for correction of system intrinsic baseline drift and for noise signal reduction. Biacore T100 Evaluation Software version 1.1.1 was used for analysis of sensorgrams and for calculation of affinity data. Figure 11 shows a scheme of the Biacore assay.

Examples 1

Production, expression, purification and characterization of monospecific, bivalent <IGF-1R> antibody, wherein the variable domains CL and CH1 are replaced by each other (abbreviated herein as <IGF-1R> CL-CH1 exchange antibody).

Example 1A

Making of the expression plasmids for the monospecific, bivalent <IGF-1R> CL-CH1 exchange antibody

The sequences for the heavy and light chain variable domains of the monospecific, bivalent <IGF-1R> CL-CH1 exchange antibody including the respective leader sequences described in this example are derived from a human <IGF-1R> antibody heavy chain (SEQ ID NO: 1, plasmid 4843-pUC-HC-IGF-1R) and a light chain (SEQ ID NO: 2, plasmid 4842-pUC-LC-IGF-1R) described in WO 2005/005635, and the heavy and light chain constant domains are derived from a human antibody (C-kappa and IgG1).

The gene segments encoding the <IGF-1R> antibody leader sequence, heavy chain variable domain (VH) and the human kappa-light chain domain (CL) were joined and fused to the 5'-end of the Fc domains of the human γ1-heavy chain constant domains (Hinge-CH2-CH3). The DNA coding for the respective fusion protein resulting from the exchange of the CH1 domain by the CL domain (CH1-CL exchange) was generated by gene synthesis and is denoted <IGF-1R> HC** (SEQ ID NO: 3) in the following.

The gene segments for the <IGF-1R> antibody leader sequence, light chain variable domain (VL) and the human γ1-heavy chain constant domain (CH1) were joined as independent chain. The DNA coding for the respective fusion protein resulting from the exchange of the CL domain by the CH1 domain (CL-CH1 exchange) was generated by gene synthesis and is denoted <IGF-1R> LC** (SEQ ID NO: 4) in the following.

Figure 5 and Figure 6 show a schematic view of the protein sequence of the modified <IGF-1R> HC** heavy chain and the modified <IGF-1R> LC** light chain.

In the following the respective expression vectors are briefly described:

Vector pUC-HC-IGF-1R**

Vector pUC-HC**-IGF-1R is an expression plasmid e.g. for transient expression of a CL-CH1 exchange <IGF-1R> heavy chain HC** (cDNA organized expression cassette; with CMV-Intron A) in HEK293 (EBNA) cells or for stable expression in CHO cells.

Beside the <IGF-1R> HC expression cassette this vector contains:**

- an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and

- a ß-lactamase gene which confers ampicillin resistance in E. coli.

The transcription unit of the <IGF-1R> HC** gene is composed of the following elements:

- the AscI restriction site at the 5'-end

- the immediate early enhancer and promoter from the human cytomegalovirus,

- followed by the Intron A sequence,

- a 5'-untranslated region of a human antibody gene,

- a immunoglobulin light chain signal sequence,

- the human <IGF-1R> mature HC** chain encoding a fusion of the human heavy chain variable domain (VH) and the human kappa-light chain constant domain (CL) fused to the 5'-end of the Fc domains of the human γ1-heavy chain constant domains (Hinge-CH2-CH3).

- a 3' untranslated region with a polyadenylation signal sequence, and

- the restriction site SgrAI at the 3'-end.

The plasmid map of the heavy chain** CL-CH1 exchange <IGF-1R> HC** expression vector pUC-HC**-IGF-1R is shown in Figure 7. The amino acid sequence of the <IGF-1R> HC** (including signal sequence) is given in SEQ ID NO: 3.

Vector pUC-LC-IGF-1R**

Vector pUC-LC**-IGF-1R is an expression plasmid e.g. for transient expression of a CL-CH1 exchange <IGF-1R> light chain LC** (cDNA organized expression cassette; with CMV-Intron A) in HEK293 (EBNA) cells or for stable expression in CHO cells.

Beside the <IGF-1R> LC expression cassette this vector contains:**

- an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and

- a ß-lactamase gene which confers ampicillin resistance in E. coli.

The transcription unit of the <IGF-1R> LC gene is composed of the following elements:**

- the restriction site Sse8387I at the 5' end

- the immediate early enhancer and promoter from the human cytomegalovirus,

- followed by the Intron A sequence,

- a 5'-untranslated region of a human antibody gene,

- a immunoglobulin heavy chain signal sequence,

- the human <IGF-1R> antibody mature LC** chain encoding a fusion of the human light chain variable domain (VL) and the human γ1-heavy chain constant domains (CH1).

- a 3' untranslated region with a polyadenylation signal sequence, and

- the restriction sites SalI and FseI at the 3'-end.

The plasmid map of the light chain** CL-CH1 exchange <IGF-1R> LC** expression vector pUC-LC**-IGF-1R is shown in Figure 8. The amino acid sequence of the <IGF-1R> LC** (including signal sequence) is given in SEQ ID NO: 4.

Plasmids pUC-HC**-IGF-1R and pUC-LC**-IGF-1R can be used for transient or stable co-transfections e.g. into HEK293, HEK293 EBNA or CHO cells (2-vector system). For comparative reasons the wildtype <IGF-1R> antibody was transiently expressed from plasmids 4842-pUC-LC-IGF-1R (SEQ ID NO: 2) and 4843-pUC-HC-IGF-1R (SEQ ID NO: 1) analogous to the ones described in this example.

In order to achieve higher expression levels in transient expressions in HEK293 EBNA cells the <IGF-1R> HC** expression cassette can be sub-cloned via AscI, SgrAI sites and the <IGF-1R> LC** expression cassette can be sub-cloned via Sse8387I and FseI sites into the 4700 pUC-Hyg_OriP expression vector containing

- an OriP element, and

- a hygromycine resistance gene as a selectable marker.

Heavy and light chain transcription units can either be sub-cloned into two independent 4700-pUC-Hyg-OriP vectors for co-transfection (2-vector system) or they can be cloned into one common 4700-pUC-Hyg-OriP vector (1-vector system) for subsequent transient or stable transfections with the resulting vectors. Figure 9 shows a plasmid map of the basic vector 4700-pUC-OriP.

Example 1B

Making of the monospecific, bivalent <IGF-1R> CL-CH1 exchange antibody expression plasmids

The <IGF-1R> fusion genes (HC** and LC** fusion genes) comprising the exchanged Fab sequences of the wildtype <IGF-1R> antibody were assembled with known recombinant methods and techniques by connection of the according nucleic acid segments.

The nucleic acid sequences encoding the IGF-1R HC** and LC** were each synthesized by chemical synthesis and subsequently cloned into a pPCRScript (Stratagene) based pGA4 cloning vector at Geneart (Regensburg, Germany). The expression cassette encoding the IGF-1R HC** was ligated into the respective E. coli plasmid via PvuII and BmgBI restriction sites resulting in the final vector pUC-HC**-IGF-1R; the expression cassette encoding the respective IGF-1R LC** was ligated into the respective E. coli plasmid via PvuII and SalI restriction sites resulting in the final vector pUC-LC**-IGF-1R. The subcloned nucleic acid sequences were verified by DNA sequencing. For transient and stable transfections larger quantities of the plasmids were prepared by plasmid preparation from transformed E. coli cultures (Nucleobond AX, Macherey-Nagel)

Example 1C

Transient expression of monospecific, bivalent IGF-1R> CL-CH1 exchange antibody, purification and confirmation of identity by mass spectrometry

Recombinant <IGF-1R> CL-CH1 exchange antibody was expressed by transient co-transfection of plasmids pUC-HC**-IGF-1R and pUC-LC**-IGF-1R in HEK293-F suspension cells as described above.

The expressed and secreted monospecific, bivalent <IGF-1R> CL-CH1 exchange antibody was purified from filtered cell culture supernatants by Protein A affinity chromatography according as described above. In brief, the <IGF-1R> CL-CH1 exchange antibody containing cell culture supernatants from transient transfections were clarified by centrifugation and filtration and applied to a Protein A HiTrap MabSelect Xtra column (GE Healthcare) equilibrated with PBS buffer (10 mM Na2HPO4, 1 mM KH2PO4, 137 mM NaCl and 2.7 mM KCl, pH 7.4). Unbound proteins were washed out with PBS equilibration buffer followed by 0.1 M sodium

citrate buffer, pH 5.5 and washed with PBS. Elution of antibody was achieved with 100 mM sodium citrate, pH 2,8 followed by immediate neutralization of the sample with 300µl 2 M Tris pH 9.0 per 2 ml fraction. Aggregated protein was separated from monomeric antibodies by size exclusion chromatography on a HiLoad 26/60 Superdex 200 prep grade column (GE Healthcare) in 20 mM Histidine, 150 mM NaCl pH 6.0 and monomeric antibody fractions were subsequently concentrated using a MILLIPORE Amicon Ultra-15 centrifugal concentrator. <IGF-1R> CL-CH1 exchange antibody was frozen and stored at -20°C or -80°C. The integrity of the <IGF-1R> CL-CH1 exchange antibody was analyzed by SDS-PAGE in the presence and absence of a reducing agent and subsequent staining with Coomassie brilliant blue as described above. Monomeric state of the <IGF-1R> CL-CH1 exchange antibody was confirmed by analytical size exclusion chromatography. (Figure 12) Characterized samples were provided for subsequent protein analytics and functional characterization. ESI mass spectrometry confirmed the theoretical molecular mass of the completely deglycosylated <IGF-1R> CL-CH1 exchange antibody.

Example 1D

Analysis of the IGF-1R binding properties of monospecific, bivalent IGF-1R> CL-CH1 exchange antibody in an IGF-1R ECD binding ELISA and by Biacore

The binding properties of monospecific, bivalent <IGF-1R> CL-CH1 exchange antibody were evaluated in an ELISA assay with the IGF-1R extracellular domain (ECD) as descried above. For this sake the extracellular domain of IGF-1R (residues 1-462) comprising the natural leader sequence and the LI-cysteine rich-12 domains of the human IGF-IR ectodomain of the alpha chain (according to the McKern et al., 1997; Ward et al., 2001) fused to an N-terminal His-Streptavidin binding peptide-tag (His-SBP) was cloned into a pcDNA3 vector derivative and transiently expressed in HEK293F cells. The protein sequence of the IGF-1R-His-SBP ECD is given in see above. The obtained titration curve showed that <IGF-1R> CL-CH1 exchange antibody was functional and showed comparable binding characteristics and kinetics as the wildtype <IGF-1R> antibody within the error of the method and thus appeared fully functional (Figure 13).

These findings were corroborated by Biacore data with the respective purified antibodies that showed that the monospecific, bivalent <IGF-1R> CL-CH1 exchange antibody with a KD value of 3.7 pM had a comparable affinity and

binding kinetics for IGF-1R ECD as the original wildtype <IGF-1R> antibody with a KD value of 3.2 nM:

Example 1G

Analysis of the IGF-1R binding properties of monospecific, bivalent IGF-1R> CL-CH1 exchange antibody by FACS with IGF-1R over-expressing I24 cells

In order to confirm the binding activity of <IGF-1R> CL-CH1 exchange antibody to the IGF-1R over-expressed on the surface of I24 cells (NIH3T3 cells expressing recombinant human IGF-1R, Roche) is studied by FACS. Briefly, 5x10E5 I24cells per FACS tube are incubated with a dilution of purified <IGF-1R> CL-CH1 exchange antibody and wildtype <IGF-1R> antibody as a reference and incubated on ice for 1 h. Unbound antibody is washed away with 4 ml ice cold PBS (Gibco) + 2% FCS (Gibco). Subsequently, cells are centrifuged (5 min at 400 g) and bound antibody is detected with F(ab')2 <hFcγ>PE conjugate (Dianova) on ice for 1 h protected from light. Unbound detection antibody is washed away with 4 ml ice cold PBS + 2% FCS. Subsequently, cells are centrifuged (5 min 400 g), resuspended in 300-500 µL PBS and bound detection antibody is quantified on a FACSCalibur or FACS Canto (BD (FL2 channel, 10.000 cells per acquisition). During the experiment the respective isotype controls are included to exclude any unspecific binding events. Binding of <IGF-1R> CL-CH1 exchange antibody and wildtype <IGF-1R> reference antibody to IGF-1R on I24 cells result in a comparable, concentration dependent shift of mean fluorescence intensity.

Examples 2:

Description of a monospecific, bivalent <ANGPT2> wildtype antibody

Example 2A

Making of the expression plasmids for the monospecific, bivalent <ANGPT2> wildtype antibody

The sequences for the heavy and light chain variable domains of a monospecific, bivalent ANGPT2 <ANGPT2> wildtype antibody including the respective leader sequences described in this example are derived from a human <ANGPT2> antibody heavy chain (SEQ ID NO: 6) and a light chain (SEQ ID NO: 7) described

in WO 2006/045049 and the heavy and light chain constant domains are derived from a human antibody (C-kappa and IgG1).

The wildtype <ANGPT2> antibody was cloned into plasmids SB04-pUC-HC-ANGPT2 (SEQ ID NO: 6) and SB06-pUC-LC-ANGPT2 (SEQ ID NO: 7) that are analogous to the vectors described in the previous example 1A.

For comparative reasons and for co-expression experiments (see example 3) the wildtype <ANGPT2> antibody was transiently (co-)expressed from plasmids SB04-pUC-HC-ANGPT2 and SB06-pUC-LC-ANGPT2.

Example 2B

Making of the monospecific, bivalent <ANGPT2> wildtype antibody expression plasmids

The nucleic acid sequences encoding the ANGPT2> HC and LC were each synthesized by chemical synthesis and subsequently cloned into a pPCRScript (Stratagene) based pGA4 cloning vector at Geneart (Regensburg, Germany). The expression cassette encoding the <ANGPT2> HC was cloned into the respective E. coli plasmid resulting in the final vector SB04-pUC-HC-ANGPT2; the expression cassette encoding the respective <ANGPT2> LC was cloned into the respective E. coli plasmid resulting in the final vector SB06-pUC-LC-ANGPT2. The subcloned nucleic acid sequences were verified by DNA sequencing. For transient and stable transfections larger quantities of the plasmids were prepared by plasmid preparation from transformed E. coli cultures (Nucleobond AX, Macherey-Nagel).

Examples 3

Expression of bispecific, bivalent <ANGPT2-IGF-1R> antibody, wherein in the heavy and light chain specifically binding to IGF-1R, the constant domains CL and CH1 are replaced by each other (abbreviated herein as <ANGPT2-IGF-1R> CL-CH1 exchange antibody)

Example 3A

Transient co-expression and purification of <IGF-1R> CL-CH1 exchange antibody and <ANGPT2> wildtype antibody in HEK293 EBNA cells to yield bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody

In order to generate a functional bispecific antibody recognizing IGF-1R via the <IGF-1R> CL-CH1 exchange antibody Fab on one side and <ANGPT2> via the <ANGPT2> wildtype Fab region on the other side the two expression plasmids coding for the <IGF-1R> CL-CH1 exchange antibody (example 1A) were co-expressed with two expression plasmids coding for the <ANGPT2> wildtype antibody. (example 2A). Assuming a statistical association of wildtype heavy chains HC and CL-CH1 exchange heavy chains HC** this results in the generation of bispecific and bivalent <IGF-1R-ANGPT2> CL-CH1 exchange antibody . Under the assumption that both antibodies are equally well expressed and without taking side products into account this should result in a 1:2:1 ratio of the three main products A) <IGF-1R> CL-CH1 exchange antibody, B) bispecific <IGF-1R-ANGPT2> CL-CH1 exchange antibody , and C) <ANGPT2> wildtype antibody. Several side products can be expected. However, due to the exchange of only the CL-CH1 domains the frequency of side products should be reduced compared to the complete Fab crossover. Please note as the <ANGPT2> wildtype antibody showed higher expression transient expression yields than the <IGF-1R> wildtype and <IGF-1R> CL-CH1 exchange antibodies the ratio of <ANGPT2> wildtype antibody plasmids and <IGF-1R> CL-CH1 exchange antibody plasmids was shifted in favour of the expression of <ANGPT2> wildtype antibody.

To generate the mix of the main products A) <IGF-1R> CL-CH1 exchange antibody, B) bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody , and C) <ANGPT2> wildtype antibody the four plasmids pUC-HC**-IGF-1R and pUC-LC**-IGF-1R and plasmids SB04-pUC-HC-ANGPT2 and SB06-pUC-LC-ANGPT2 were transiently co-transfected in suspension HEK293-F cells as described above The harvested supernatant contained a mix of the main products A) <IGF-1R> CL-

CH1 exchange antibody, B) bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody , and C) <ANGPT2> wildtype antibody and is denoted as "Bispecific CL-CH1 exchange mix". Bispecific CL-CH1 exchange mix containing cell culture supernatants, were harvested by centrifugation and subsequently purified as decribed above. Figure 14

The integrity of the antibody mix was analyzed by SDS-PAGE in the presence and absence of a reducing agent and subsequent staining with Coomassie brilliant blue as described. The SDS-PAGE showed that there were 2 different heavy and light chain presents in the preparation as expected (reduced gel). The monomeric state of the antibody mix was confirmed by analytical size exclusion chromatography and showed that the purified antibody species were in a monomeric state. Characterized samples were provided for subsequent protein analytics and functional characterization.

Example 3B

Detection of functional bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody in a cellular FACS bridging assay on I24 IGF-1R expressing cells

In order to confirm the presence of functional bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody in the purified bispecific CL-CH1 exchange mix of the main products A) <IGF-1R> CL-CH1 exchange antibody, B) bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody , and C) <ANGPT2> wildtype antibody from the transient co-expression described in example 3A, a cellular FACS IGF-1R-ANGPT2 bridging assay on I24 cells (NIH3T3 cells expressing recombinant human IGF-1R, Roche) was performed. The assay principle is depicted in Figure 10. A bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody that is present in the purified antibody mix is capable of binding to IGF-1R in I24 cells and to ANGPT2 simultaneously; and thus will bridge its two target antigens with the two opposed Fab regions.

Briefly, 5x10E5 I24cells per FACS tube were incubated with total purified antibody mix and incubated on ice for 1 h (titration 160 µg/ml mix). The respective purified antibodies wildtype <IGF-1R> and <ANGPT2> were applied to the I24 cells as controls. Unbound antibody was washed away with 4 ml ice cold PBS (Gibco) + 2% FCS (Gibco), cells were centrifuged (5 min at 400 g) and bound bispecific antibody was detected with 50 µl 2 µg/mL human ANGPT2 (R&D Systems) for 1 h on ice.

Subsequently, unbound ANGPT2 was washed away once or twice with 4 ml ice cold PBS (Gibco) + 2% FCS (Gibco), cells were centrifuged (5 min at 400 g) and bound ANGPT2 was detected with 50 µl 5 µg/mL <ANGPT2>mIgG1-Biotin antibody (BAM0981, R&D Systems) for 45 min on ice; alternatively, cells were incubated with 50 µl 5 µg/mL mIgG1-Biotin-Isotype control (R&D Systems). Unbound detection antibody was washed away with 4 ml ice cold PBS (Gibco) + 2% FCS (Gibco), cells were centrifuged (5 min at 400 g) and bound detection antibody was detected with 50 µl 1:400 Streptavidin-PE conjugate (Invitrogen/Zymed) for 45 min on ice protected from light. Unbound Streptavidin-PE conjugate was washed away with 4 ml ice cold PBS + 2% FCS. Subsequently, cells were centrifuged (5 min 400 g), resuspended in 300-500 µL PBS and bound Streptavidin-PE conjugate was quantified on a FACSCalibur (BD (FL2 channel, 10.000 cells per acquisition). During the experiment the respective isotype controls were included to exclude any unspecific binding events. In addition, purified monospecific, bivalent IgG1 antibodies <IGF-1R> and <ANGPT2> were included as controls.

The results in Fig. 15 show that the incubation with purified antibody crossover mix (<ANGPT2-IGF-1R> CL-CH1 exchange antibody) from the co-expression of a crossover antibody (<IGF-1R> CL-CH1 exchange antibody) with a wildtype antibody (<ANGPT2> wildtype antibody) resulted in a significant shift in fluorescence indicating the presence of a functional bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody that was capable of binding to IGF-1R in I24 cells and to ANGPT2 simultaneously; and thus bridges its two target antigens with the two opposed Fab regions. In contrast to this the respective <IGF-1R> and <Ang-2> control antibodies did not result in shift in fluorescence in the FACS bridging assay

Taken together these data show that by co-expressing the respective wildtype and crossover plasmids functional bispecific antibodies can be generated. The yields of correct bispecific antibody can be increased by forcing the correct heterodimerization of wildtypoe and modified crossover heavy chains e.g. using the knobs-into-holes technology as well as disulfide stabilization See examples 4)

Example 4

Expression of bivalent, bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody with modified CH3 domains (knobs-into-holes)

To further improve the yield of the bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody the knobs-into-holes technology is applied to the co-expression of < IGF-1R > CL-CH1 exchange and wildtype <ANGPT2> antibodies to obtain a homogenous and functional bispecific antibody preparation. For this purpose, the CH3 domain in the heavy chain* HC* of the <IGF-1R> CL-CH1 exchange antibody is replaced by the CH3 domain (Knobs) of the SEQ ID NO: 8 with a T366W exchange and the CH3 domain in the heavy chain of the wildtype <ANGPT2> antibody is replaced by the CH3 domain (Hole) of the SEQ ID NO: 9 with a T366S, L368A, Y407V exchange or vice versa. In addition, a disulfide can be included to increase the stability and yields as well as additional residues forming ionic bridges and increasing the heterodimerization yields (EP 1870459A1).

The transient co-expression, and the purification of the resulting bivalent, bispecific <ANGPT2-IGF-1R> CL-CH1 exchange antibody with modified CH3 domains (knobs-into-holes) is performed as described in Example 3.

It should be noted that an optimization of heterodimerization can be achieved e.g. by using different knobs-in-holes technologies such as the introduction of an additional disulfide bridge into the CH3 domain e.g. Y349C into the "knobs chain" and D356C into the "hole chain" and/or combined with the use of residues R409D; K370E (K409D) for knobs residues and D399K; E357K for hole residues described by EP 1870459A1.

Analogously to example 4 further bivalent, bispecific CH1-CL exchange antibodies with modified CH3 domains (knobs-into-holes) directed against ANGPT2 and another target antigen (using the above described ANGPT2 heavy and light chain and the CH1-CL exchange heavy and light chain** HC** and LC** of an antibody directed against said other target, whereby both heavy chains are modified by "knobs-in-holes"), or directed against IGF-1R and another target (using the heavy and light chain of an antibody directed against said other target and the above described IGF-1R CH1-CL exchange heavy and light chain** HC** and LC**, whereby both heavy chains are modified by "knobs-in-holes") can be prepared.

Patent Claims

1. A bivalent, bispecific antibody, comprising:

a) the light chain and heavy chain of an antibody specifically binding to a first antigen; and

b) the light chain and heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other.

2. The antibody according to claim 1, characterized in that

the CH3 domain of one heavy chain and the CH3 domain of the other heavy chain each meet at an interface which comprises an original interface between the antibody CH3 domains;
wherein said interface is altered to promote the formation of the bivalent, bispecific antibody, wherein the alteration is characterized in that:

a) the CH3 domain of one heavy chain is altered,

so that within the original interface the CH3 domain of one heavy chain that meets the original interface of the CH3 domain of the other heavy chain within the bivalent, bispecific antibody,
an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance within the interface of the CH3 domain of one heavy chain which is positionable in a cavity within the interface of the CH3 domain of the other heavy chain

and

b) the CH3 domain of the other heavy chain is altered,

so that within the original interface of the second CH3 domain that meets the original interface of the first CH3 domain within the bivalent, bispecific antibody
an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity within the interface of the second CH3 domain within which a protuberance within the interface of the first CH3 domain is positionable;

3. The antibody according to claim 2, characterized in that

said amino acid residue having a larger side chain volume is selected from the group consisting of arginine (R), phenylalanine (F), tyrosine (Y), tryptophan (W).

4. The antibody according to any one of claims 2 or 3, characterized in that

said amino acid residue having a smaller side chain volume is selected from the group consisting of alanine (A), serine (S), threonine (T), valine (V).

5. The antibody according to any one of claims 2 to 4, characterized in that

both CH3 domains are further altered by the introduction of cysteine (C) as amino acid in the corresponding positions of each CH3 domain.

6. The antibody according to claim 1, characterized in that

one of the constant heavy chain domains CH3 of both heavy chains is replaced by a constant heavy chain domain CH1; and the other constant heavy chain domain CH3 is replaced by a constant light chain domain CL.

7. A method for the preparation of an a bivalent, bispecific antibody according to claim 1 comprising the steps of

a) transforming a host cell with

-vectors comprising nucleic acid molecules encoding the light chain and heavy chain of an antibody specifically binding to a first antigen

-vectors comprising nucleic acid molecules encoding the light chain and heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other;

b) culturing the host cell under conditions that allow synthesis of said antibody molecule; and

c) recovering said antibody molecule from said culture.

8. A host cell comprising:
-vectors comprising nucleic acid molecules encoding the light chain and heavy chain of an antibody specifically binding to a first antigen

-vectors comprising nucleic acid molecules encoding the light chain and heavy chain of an antibody specifically binding to a second antigen, wherein the constant domains CL and CH1 are replaced by each other.

9. A composition, preferably a pharmaceutical or a diagnostic composition of the bivalent, bispecific antibody according to claims 1 to 6.

10. A pharmaceutical composition comprising a bivalent, bispecific antibody according to claims 1 to 6 and at least one pharmaceutically acceptable excipient.

Fig. 1

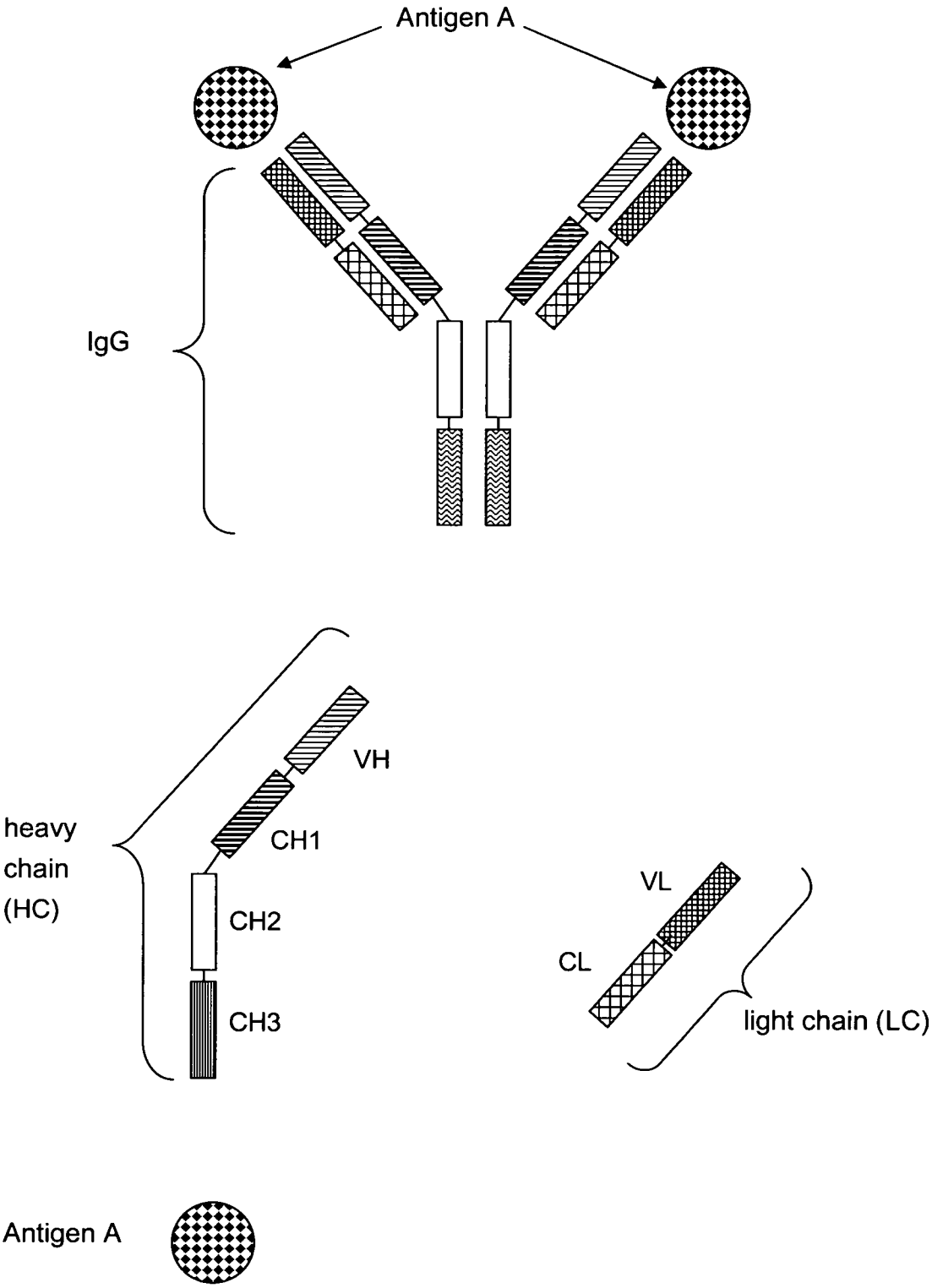

Antigen A
IgG
heavy
chain
(HC)
VH
CH1
CH2
CH3
VL
CL
light chain (LC)
Antigen A

Fig. 2

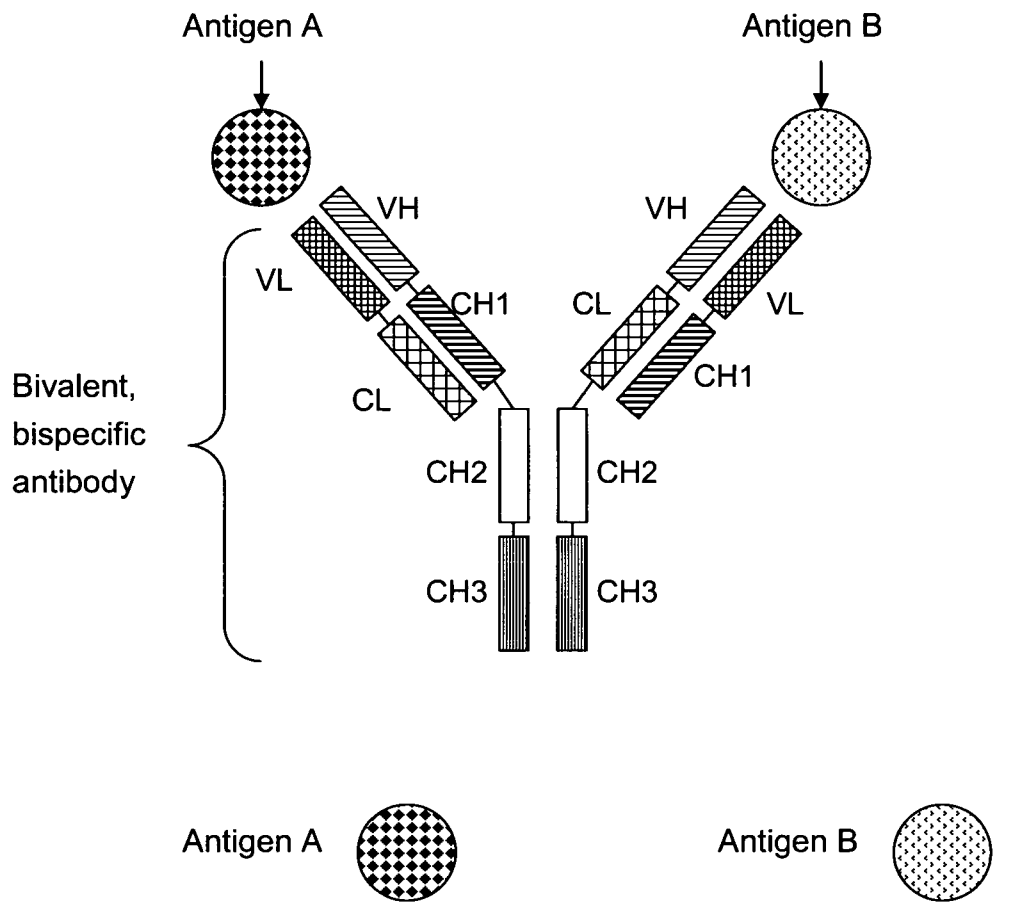

Antigen A
Antigen B
VH
VH
VL
CH1
CL
CL
VL
CH1
Bivalent,
bispecific
antibody
CH2
CH2
CH3
CH3
Antigen A
Antigen B

Fig. 3

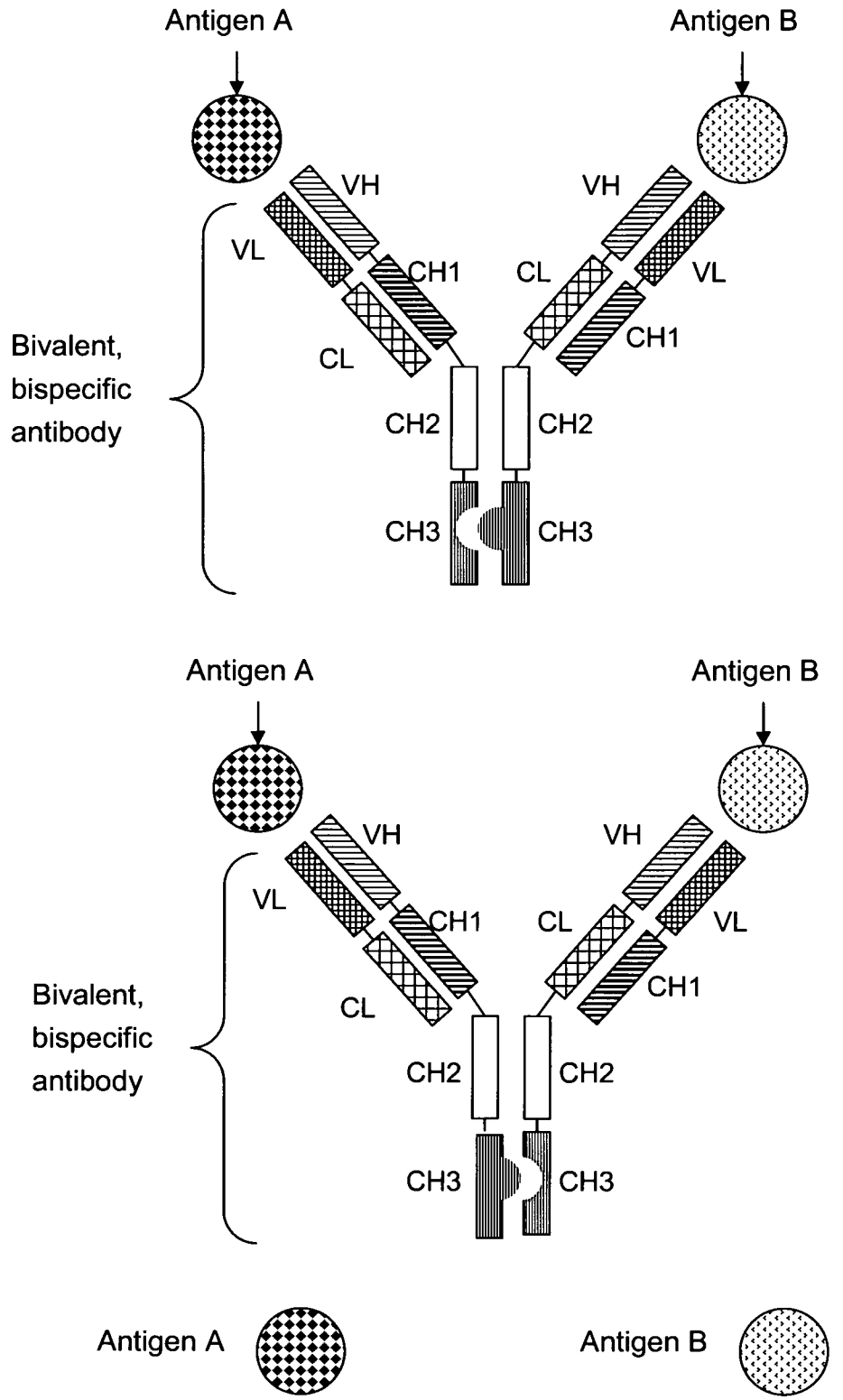

Antigen A
Antigen B
VH
VH
VL
CH1
CL
CL
VL
CH1
Bivalent,
bispecific
antibody
CH2
CH2
CH3
CH3
Antigen A
Antigen B
VH
VH
VL
CH1
CL
CL
VL
CH1
Bivalent,
bispecific
antibody
CH2
CH2
CH3
CH3
Antigen A
Antigen B

Fig. 4

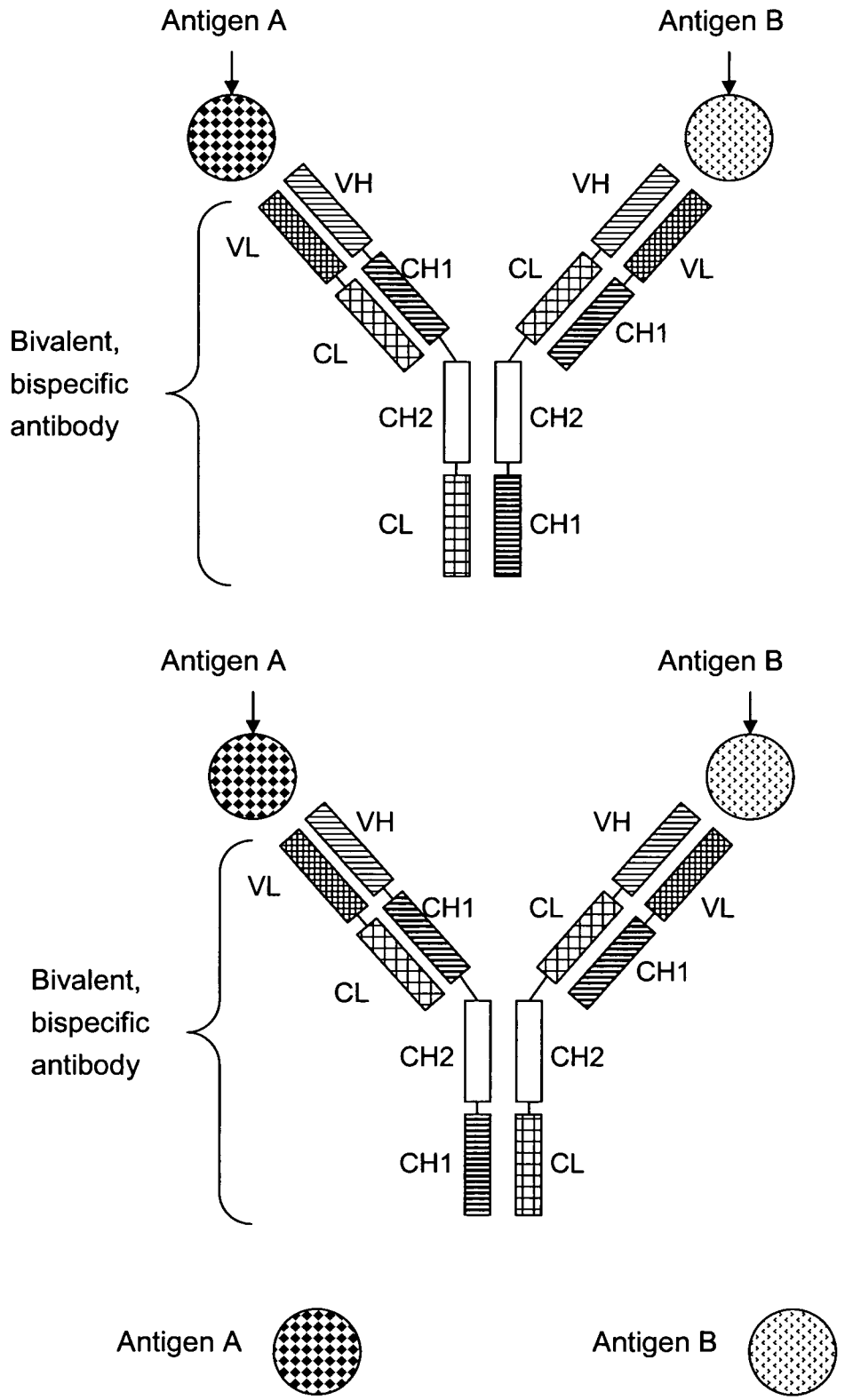
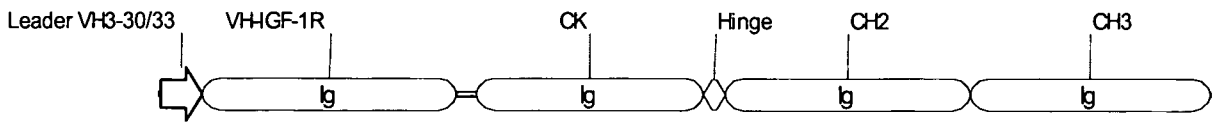

Antigen A
Antigen B
VH
VL
CH1
CL
VH
CL
VL
CH1
Bivalent,
bispecific
antibody
CH2
CH2
CL
CH1
Antigen A
Antigen B
VH
VL
CH1
CL
VH
CL
VL
CH1
Bivalent,
bispecific
antibody
CH2
CH2
CH1
CL
Antigen A
Antigen B

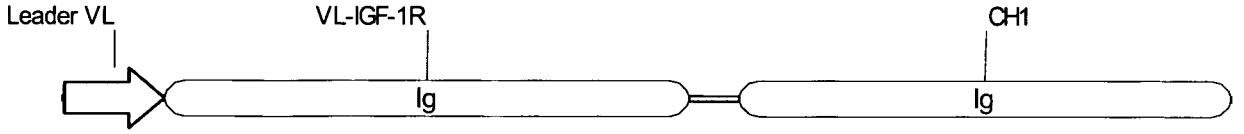
Fig. 5



<IGF-1R> HC**

471 aa

Fig. 6



<IGF-1R> LC**

233 aa

Fig. 7

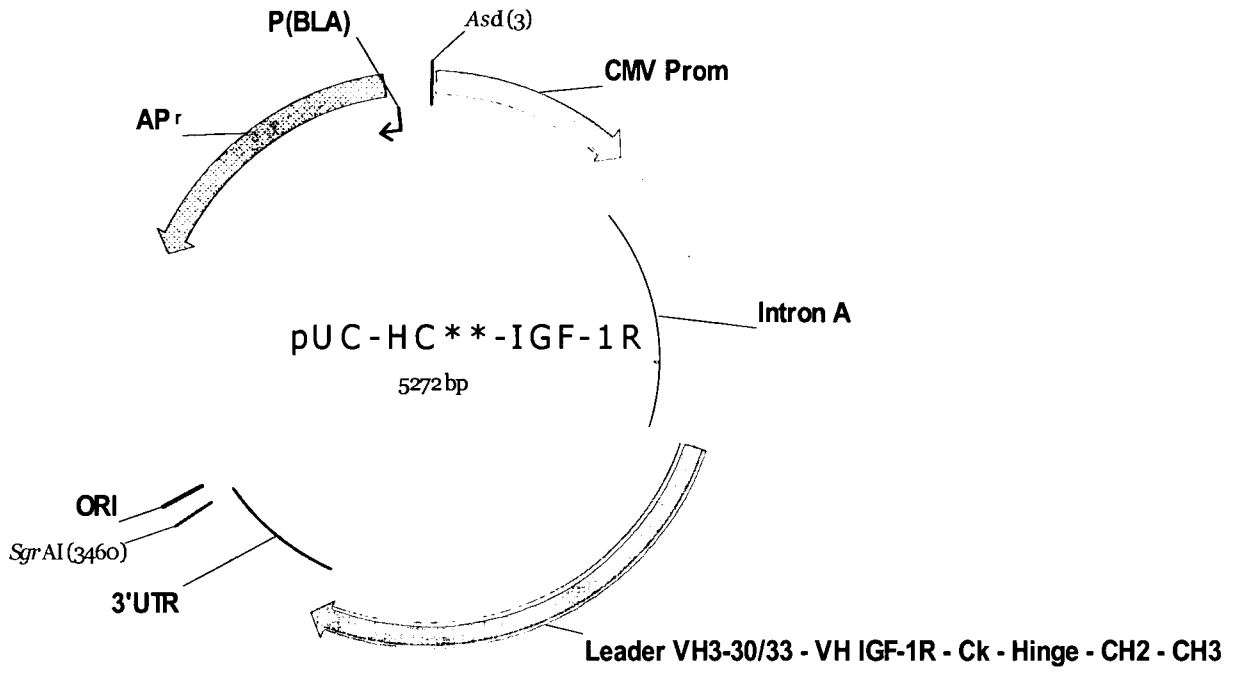

P(BLA)
Asd (3)
CMV Prom
APr
pUC-HC**-IGF-1R
5272 bp
Intron A
ORI
Sgr AI (3460)
3'UTR
Leader VH3-30/33 - VH IGF-1R - Ck - Hinge - CH2 - CH3

Fig. 8

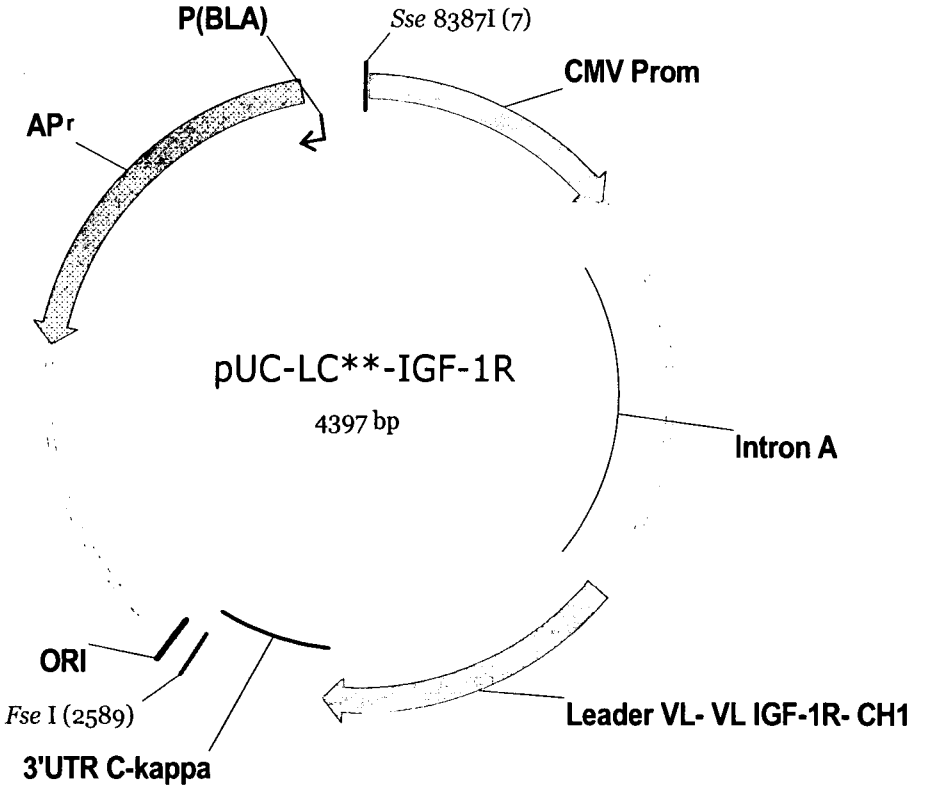

P(BLA)
Sse 8387I (7)
CMV Prom
APr
pUC-LC**-IGF-1R
4397 bp
Intron A
ORI
Fse I (2589)
3'UTR C-kappa
Leader VL- VL IGF-1R- CH1

Fig. 9

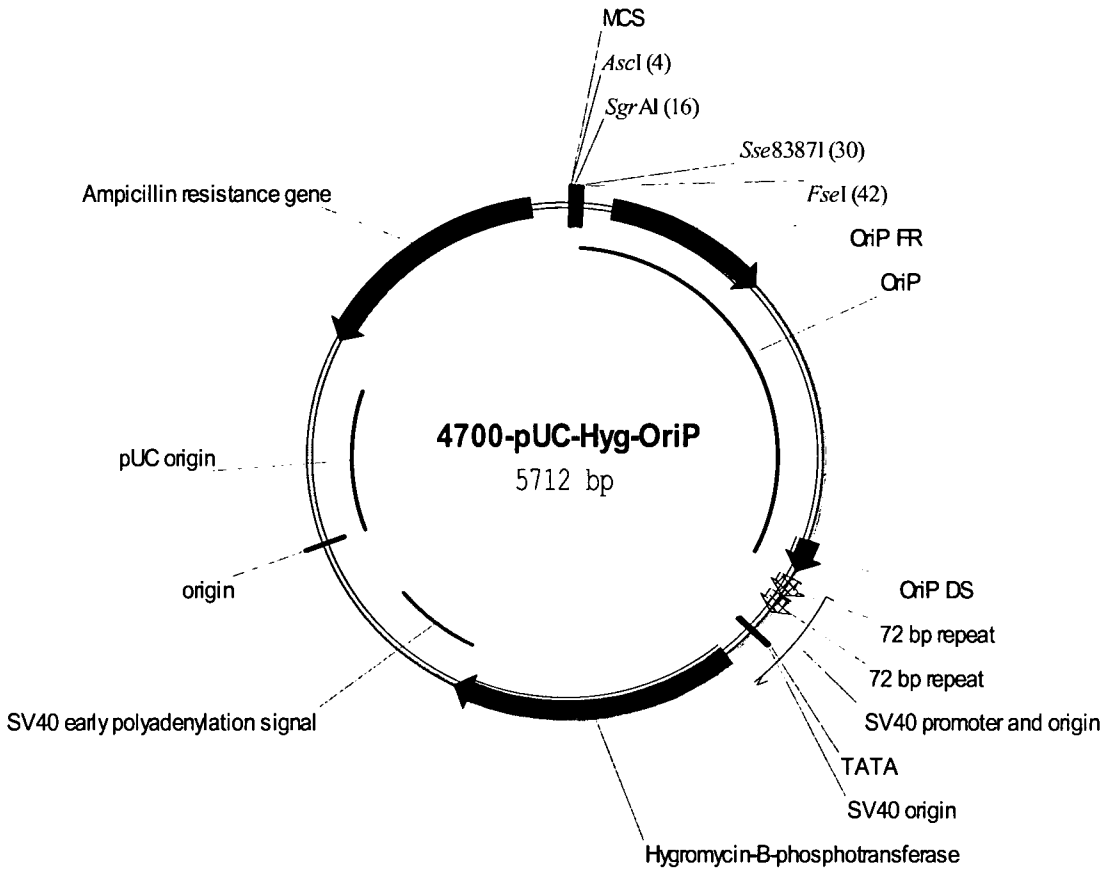

MCS
AscI (4)
SgrAI (16)
Sse8387I (30)
FseI (42)
Ampicillin resistance gene
OriP FR
OriP
4700-pUC-Hyg-OriP
5712 bp
pUC origin
origin
OriP DS
72 bp repeat
72 bp repeat
SV40 promoter and origin
SV40 early polyadenylation signal
TATA
SV40 origin
Hygromycin-B-phosphotransferase

Fig. 10

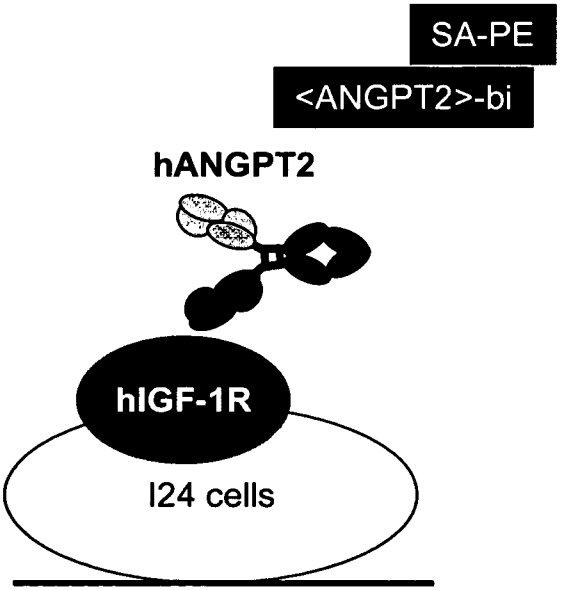

SA-PE
<ANGPT2>-bi
hANGPT2
hIGF-1R
I24 cells

Fig. 11

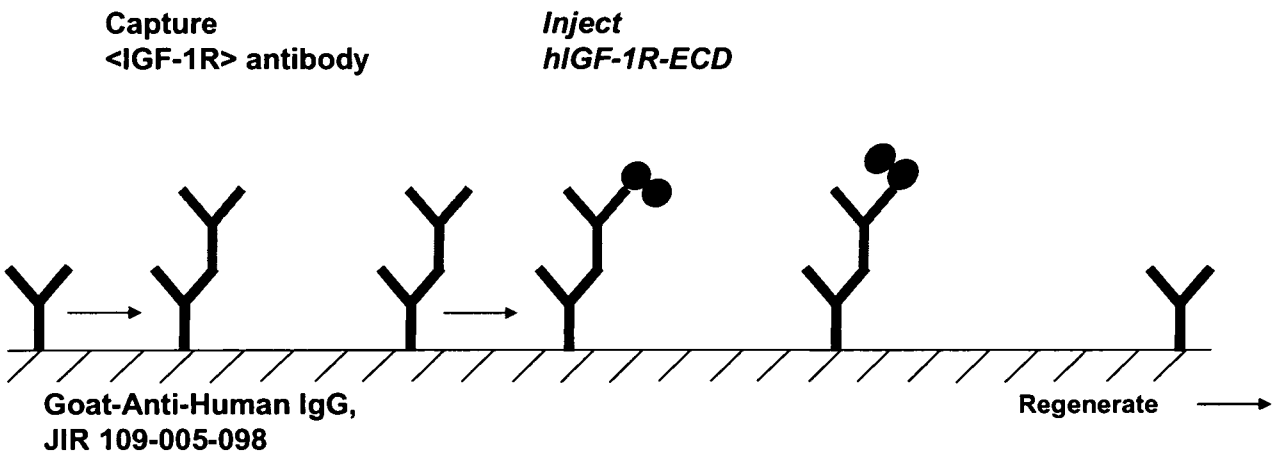
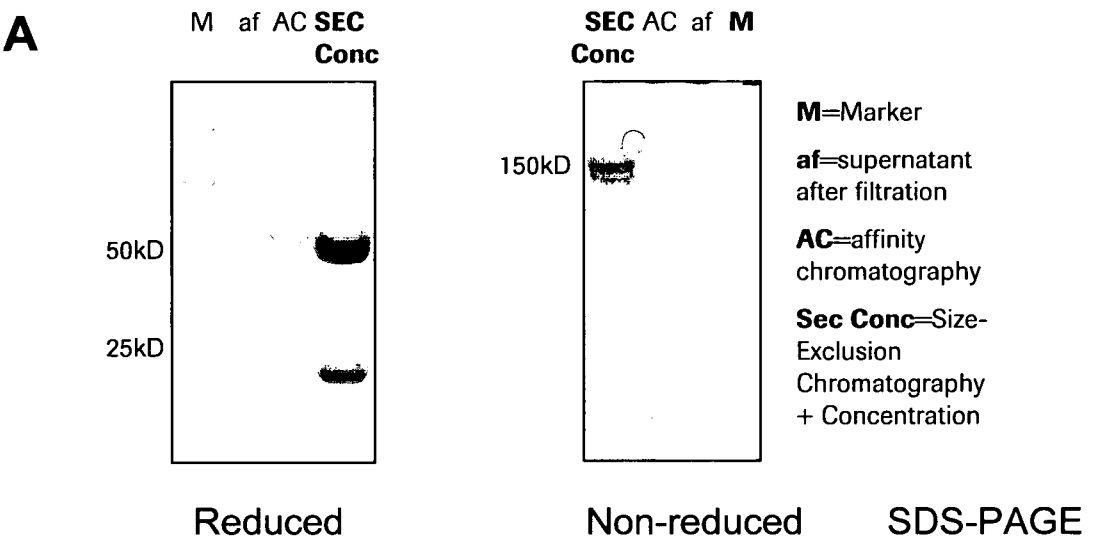

Capture
<IGF-1R> antibody
Inject
hIGF-1R-ECD
Goat-Anti-Human IgG,
JIR 109-005-098
Regenerate

Fig. 12


A
M af AC SEC Conc
SEC AC af M Conc
50kD
25kD
150kD
M=Marker
af=supernatant after filtration
AC=affinity chromatography
Sec Conc=Size-Exclusion Chromatography + Concentration
Reduced
Non-reduced
SDS-PAGE

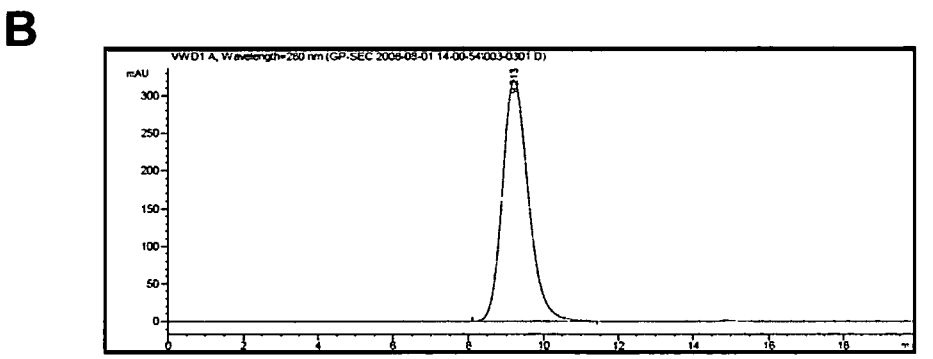
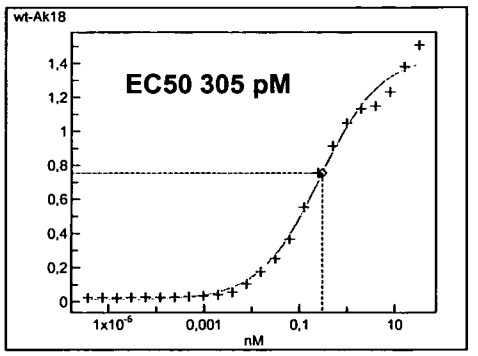
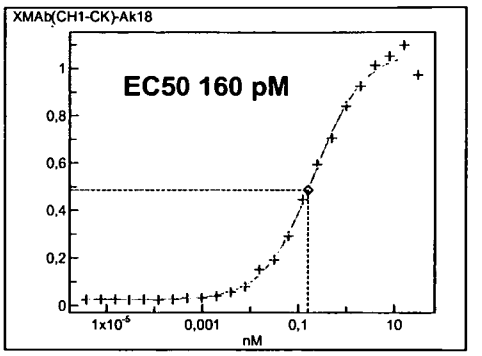

B
mAU
300
250
200
150
100
50
0

Fig. 13

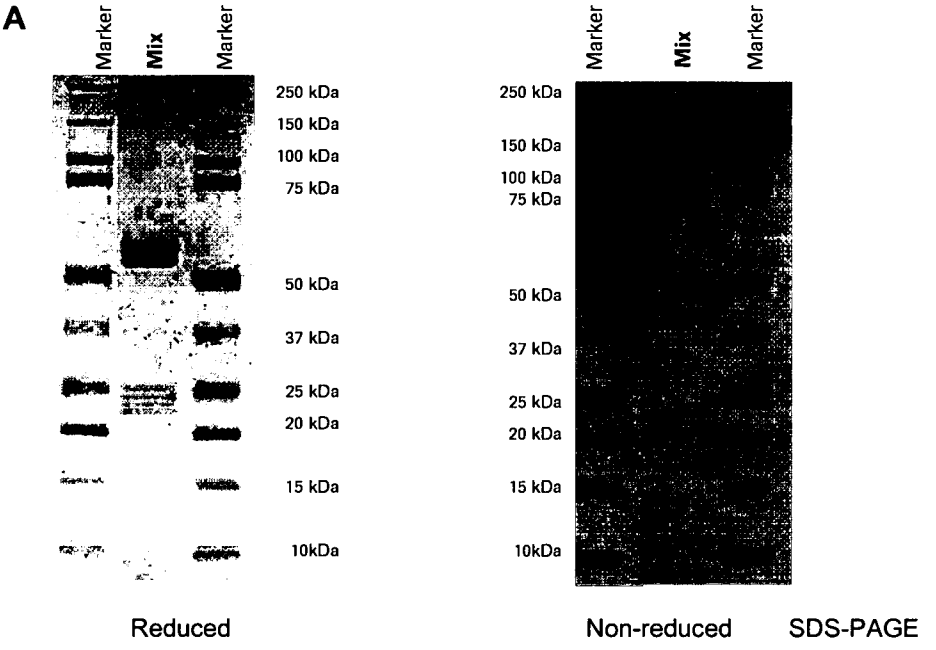


Binding of wildtype <IGF-1R> antibody to IGF-1R-ECD



Binding of <IGF-1R> CL-CH1 exchange antibody to IGF-1R-ECD

Fig. 14



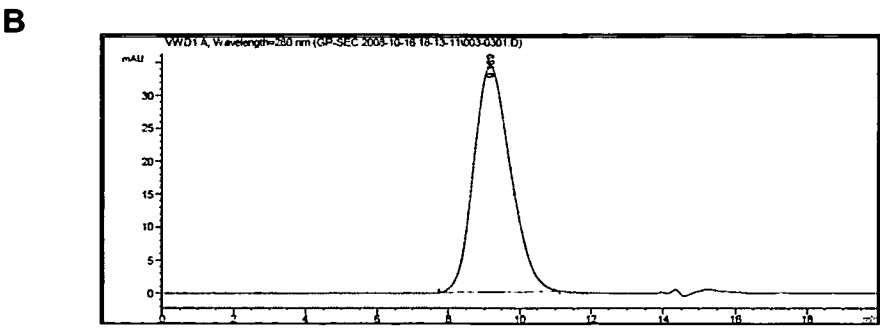
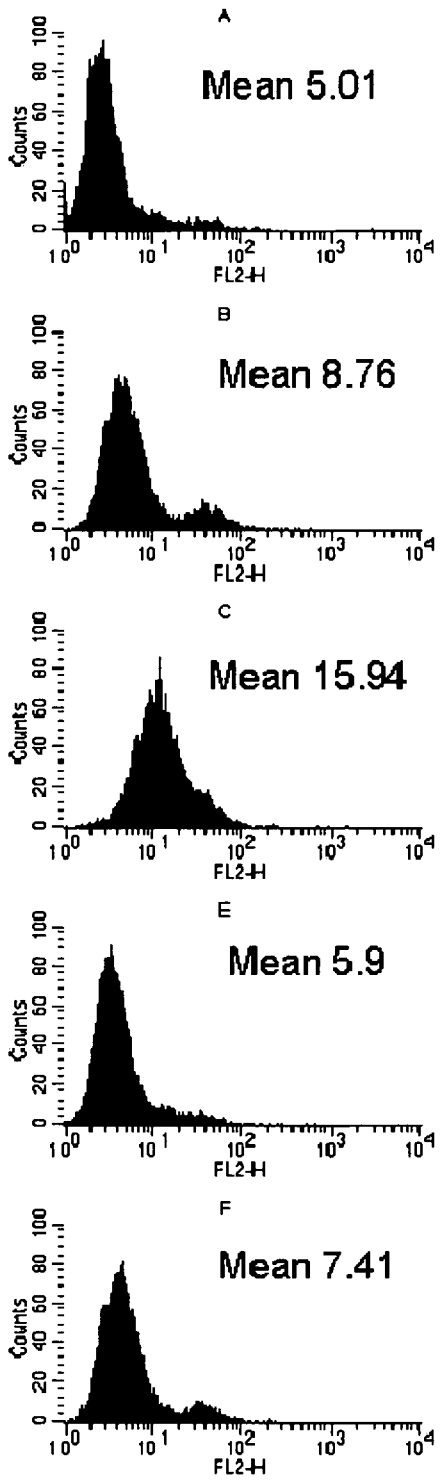


Fig. 15


A
Mean 5.01
B
Mean 8.76
C
Mean 15.94
E
Mean 5.9
F
Mean 7.41
Counts
FL2-H

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/010704

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/46
ADD. C07K16/22 C07K16/28 C07K19/00 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	A. MERCHANT ET AL.: "An efficient route to human bispecific IgG." NATURE BIOTECHNOLOGY, vol. 16, July 1998 (1998-07), pages 677-681, XP002141015 New York, USA cited in the application abstract figure 1 table 1	1-10

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 March 2009	09/04/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Nooij, Frans

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/010704

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	T. SIMON & K. RAJEWSKY: "Antibody domain mutants demonstrate autonomy of the antigen binding site." THE EMBO JOURNAL, vol. 9, no. 4, April 1990 (1990-04), pages 1051-1056, XP002492563 UK the whole document	1-10
A	L. CHAN ET AL.: "Variable region domain exchange in human IgGs promotes antibody complex formation with accompanying structural changes and altered effector functions." MOLECULAR IMMUNOLOGY, vol. 41, no. 5, July 2004 (2004-07), pages 527-538, XP002519713 U.K. page 528, left-hand column, line 31 - right-hand column, line 26 figure 1A	1-10
A	S. MORRISON: "Two heads are better than one." NATURE BIOTECHNOLOGY, vol. 25, no. 11, November 2007 (2007-11), pages 1233-1234, XP002470803 New York, USA cited in the application figure 1b	1-10
A	Z. XIE ET AL.: "A new format of bispecific antibody: highly efficient heterodimerization, expression and tumor cell lysis." JOURNAL OF IMMUNOLOGICAL METHODS, vol. 296, 2005, pages 95-101, XP004738464 Amsterdam, The Netherlands abstract figures 1,2	2-4
A	J. RIDGWAY ET AL.: "'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization." PROTEIN ENGINEERING, vol. 9, no. 7, 1996, pages 617-621, XP002084766 U.K. cited in the application abstract	2-4

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/010704

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96/27011 A (GENENTECH, INC.) 6 September 1996 (1996-09-06) cited in the application examples figures 1,4 claims	2-4
A	WO 93/06217 A (GENENTECH, INC.) 1 April 1993 (1993-04-01) example	5
A	WO 99/37791 A (VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE) 29 July 1999 (1999-07-29) example 1	6

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2008/010704

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9627011 A	06-09-1996	AU 702737 B2	04-03-1999
		AU 4973396 A	18-09-1996
		BR 9607622 A	09-06-1998
		CA 2211459 A1	06-09-1996
		CN 1176659 A	18-03-1998
		DE 69636831 T2	16-08-2007
		DK 0812357 T3	14-05-2007
		EP 0812357 A1	17-12-1997
		ES 2281080 T3	16-09-2007
		FI 973543 A	28-08-1997
		IL 117274 A	16-07-2000
		JP 3775798 B2	17-05-2006
		JP 11500915 T	26-01-1999
		NO 973982 A	03-11-1997
		NZ 303425 A	29-07-1999
		US 5731168 A	24-03-1998
		US 5807706 A	15-09-1998
		US 5821333 A	13-10-1998
		US 2007014794 A1	18-01-2007
		ZA 9601635 A	29-08-1997
WO 9306217 A	01-04-1993	CA 2116774 A1	01-04-1993
		EP 0604580 A1	06-07-1994
		EP 0861893 A2	02-09-1998
		JP 3951062 B2	01-08-2007
		JP 6510904 T	08-12-1994
		JP 2004041240 A	12-02-2004
		JP 2004337179 A	02-12-2004
		JP 2006001943 A	05-01-2006
		US 2005244929 A1	03-11-2005
		US 7018809 B1	28-03-2006
WO 9937791 A	29-07-1999	AT 283364 T	15-12-2004
		AU 2719099 A	09-08-1999
		CA 2317727 A1	29-07-1999
		DE 69922159 D1	30-12-2004
		DE 69922159 T2	01-12-2005
		ES 2234241 T3	16-06-2005
		PT 1049787 T	29-04-2005
		US 6809185 B1	26-10-2004