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(54) Title: INTRACELLULAR ANTIBODIES

(57) Abstract: A method of identifying at least one consensus sequence for an intracellular antibody (ICS) comprising the steps of: creating a database comprising sequences of validated intracellular antibodies (VIDA database) and aligning the sequences of validated intracellular antibodies according to Kabat; determining the frequency with which a particular amino acid occurs in each of the positions of the aligned antibodies; selecting a frequency threshold value (LP or consensus threshold) in the range from 70 % to 100 %; identifying the positions of the alignment at which the frequency of a particular amino acid is greater than or equal to the LP value; and identifying the most frequent amino acid, in the positions of said alignment.



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Intracellular antibodies

The present invention relates to molecules which can function in an intracellular environment. In particular, the invention relates to the characteristics of immunoglobulin molecules which can bind selectively to a ligand within an intracellular environment. Uses of these molecules are also described.

Background to the Invention

Intracellular antibodies or intrabodies have been demonstrated to function in antigen recognition in the cells of higher organisms (reviewed in Cattaneo, A. & Biocca, S. (1997) *Intracellular Antibodies: Development and Applications*. Landes and Springer-Verlag). This interaction can influence the function of cellular proteins which have been successfully inhibited in the cytoplasm, the nucleus or in the secretory pathway. This efficacy has been demonstrated for viral resistance in plant biotechnology (Tavladoraki, P., *et al.* (1993) *Nature* **366**: 469-472) and several applications have been reported of intracellular antibodies binding to HIV viral proteins (Mhashilkar, A.M., *et al.* (1995) *EMBO J* **14**: 1542-51; Duan, L. & Pomerantz, R.J. (1994) *Nucleic Acids Res* **22**: 5433-8; Maciejewski, J.P., *et al.* (1995) *Nat Med* **1**: 667-73; Levy-Mintz, P., *et al.* (1996) *J. Virol.* **70**: 8821-8832) and to oncogene products (Biocca, S., Pierandrei-Amaldi, P. & Cattaneo, A. (1993) *Biochem Biophys Res Commun* **197**: 422-7; Biocca, S., Pierandrei-Amaldi, P., Campioni, N. & Cattaneo, A. (1994) *Biotechnology (N Y)* **12**: 396-9; Cochet, O., *et al.* (1998) *Cancer Res* **58**: 1170-6). The latter is an important area because enforced expression of oncogenes often occurs in tumour cells after chromosomal translocations (Rabbitts, T.H. (1994) *Nature* **372**: 143-149). These proteins are therefore important intracellular therapeutic targets (Rabbitts, T.H. (1998) *New Eng. J. Med* **338**: 192-194) which could be inactivated by binding with intracellular antibodies. Finally, the international efforts at whole genome sequencing will produce massive numbers of potential gene sequences which encode proteins about which nothing is known.

Functional genomics is an approach to ascertain the function of this plethora of proteins and the use of intracellular antibodies promises to be an important tool in this endeavour as a conceptually simple approach to knocking-out protein function directly by binding an antibody inside the cell.

Simple approaches to derivation of antibodies which function in cells are therefore necessary if their use is to have any impact on the large number of protein targets. In normal circumstances, the biosynthesis of immunoglobulin occurs into the endoplasmic reticulum for secretion as antibody. However, when antibodies are expressed in the cell cytoplasm (where the redox conditions are unlike those found in the ER) folding and stability problems occur resulting in low expression levels and the limited half-life of antibody domains. These problems are most likely due to the reducing environment of the cell cytoplasm (Hwang, C., Sinskey, A.J. & Lodish, H.F. (1992) *Science* **257**: 1496-502), which hinders the formation of the intrachain disulphide bond of the VH and VL domains (Biocca, S., Ruberti, F., Tafani, M., Pierandrei-Amaldi, P. & Cattaneo, A. (1995) *Biotechnology (N Y)* **13**: 1110-5; Martineau, P., Jones, P. & Winter, G. (1998) *J Mol Biol* **280**: 117-127) important for the stability of the folded protein. However, some scFv have been shown to tolerate the absence of this bond (Proba, K., Honegger, A. & Pluckthun, A. (1997) *J Mol Biol* **265**: 161-72; Proba, K., Worn, A., Honegger, A. & Pluckthun, A. (1998) *J Mol Biol* **275**: 245-53) which presumably depends on the particular primary sequence of the antibody variable regions. No rules or consistent predictions until the present invention, been made about those antibodies which will tolerate the cell cytoplasm conditions. A further problem is the design of expression formats for intracellular antibodies and much effort has been expended on using scFv in which the VH and VL segments (i.e. the antibody combining site) are linked by a polypeptide linker at the C-terminus of VH and the N-terminus of VL (Bird, R.E., *et al.* (1988) *Science* **242**: 423-6). While this is the most successful form for intracellular expression, it has a drawback in the lowering of affinity when converting from complete antibody (e.g. from a monoclonal antibody) to a scFv. Thus not all monoclonal antibodies can be made as scFv and maintain function in cells. Finally, different scFv fragments have distinct

properties of solubility or propensity to aggregate when expressed in this cellular environment.

The antigen binding domain of an antibody comprises two separate regions: a heavy chain variable domain (V_H) and a light chain variable domain (V_L : which can be either V_{kappa} or V_{lambda}). The antigen binding site itself is formed by six polypeptide loops: three from V_H domain (H1, H2 and H3) and three from V_L domain (L1, L2 and L3). A diverse primary repertoire of V genes that encode the V_H and V_L domains is produced by the combinatorial rearrangement of gene segments. The V_H gene is produced by the recombination of three gene segments, V_H , D and J_H . In humans, there are approximately 51 functional V_H segments (Cook and Tomlinson (1995) *Immunol Today*, **16**: 237), 25 functional D segments (Corbett *et al.* (1997) *J. Mol. Biol.*, **268**: 69) and 6 functional J_H segments (Ravetch *et al.* (1981) *Cell*, **27**: 583), depending on the haplotype. The V_H segment encodes the region of the polypeptide chain which forms the first and second antigen binding loops of the V_H domain (H1 and H2), whilst the V_H , D and J_H segments combine to form the third antigen binding loop of the V_H domain (H3). The V_L gene is produced by the recombination of only two gene segments, V_L and J_L . In humans, there are approximately 40 functional V_H segments (Schäble and Zachau (1993) *Biol. Chem. Hoppe-Seyler*, **374**: 1001), 31 functional V_L segments (Williams *et al.* (1996) *J. Mol. Biol.*, **264**: 220; Kawasaki *et al.* (1997) *Genome Res.*, **7**: 250), 5 functional J_{kappa} segments (Hieter *et al.* (1982) *J. Biol. Chem.*, **257**: 1516) and 4 functional J_{lambda} segments (Vasicek and Leder (1990) *J. Exp. Med.*, **172**: 609), depending on the haplotype. The V_L segment encodes the region of the polypeptide chain which forms the first and second antigen binding loops of the V_L domain (L1 and L2), whilst the V_L and J_L segments combine to form the third antigen binding loop of the V_L domain (L3). Antibodies selected from this primary repertoire are believed to be sufficiently diverse to bind almost all antigens with at least moderate affinity. High affinity antibodies are produced by "affinity maturation" of the rearranged genes, in which point mutations are generated and selected by the immune system on the basis of improved binding.

Analysis of the structures and sequences of antibodies has shown that five of the six antigen binding loops (H1, H2, L1, L2, L3) possess a limited number of main-chain conformations or canonical structures (Chothia and Lesk (1987) *J. Mol. Biol.*, **196**: 901; Chothia *et al.* (1989) *Nature*, **342**: 877). The main-chain conformations are determined by (i) the length of the antigen binding loop, and (ii) particular residues, or types of residue, at certain key position in the antigen binding loop and the antibody framework. Analysis of the loop lengths and key residues has enabled us to predict the main-chain conformations of H1, H2, L1, L2 and L3 encoded by the majority of human antibody sequences (Chothia *et al.* (1992) *J. Mol. Biol.*, **227**: 799; Tomlinson *et al.* (1995) *EMBO J.*, **14**: 4628; Williams *et al.* (1996) *J. Mol. Biol.*, **264**: 220). Although the H3 region is much more diverse in terms of sequence, length and structure (due to the use of D segments), it also forms a limited number of main-chain conformations for short loop lengths which depend on the length and the presence of particular residues, or types of residue, at key positions in the loop and the antibody framework (Martin *et al.* (1996) *J. Mol. Biol.*, **263**: 800; Shirai *et al.* (1996) *FEBS Letters*, **399**: 1).

Recently, the present inventors have devised a technique for the selection of immunoglobulins which are stable in an intracellular environment, are correctly folded and are functional with respect to the selective binding of their ligand within that environment. This is described in WO00/54057. In this approach, the antibody-antigen interaction method uses antigen linked to a DNA-binding domain as a bait and the scFv linked to a transcriptional activation domain as a prey. Specific interaction of the two facilitates transcriptional activation of a selectable reporter gene. An initial in-vitro binding step is performed in which an antigen is assayed for binding to a repertoire of immunoglobulin molecules. Those immunoglobulins which are found to bind to their ligand in vitro assays are then assayed for their ability to bind to a selected antigen in an intracellular environment, generally in a cytoplasmic environment.

The present inventors found that often, a significant number of those immunoglobulins which bind in vitro fail to bind specifically to their ligand in vivo. Therefore, there

remains a need in the art for methods and procedures for predicting whether a given antibody will function within an intracellular environment.

Summary of the invention

The invention relates to a method for *a priori* identification of stable antibodies, capable of functioning as intracellular antibodies in reducing intracellular environments and for the design of libraries enriched with intracellular antibodies. In particular, the invention describes consensus sequences for intracellular antibodies (intrabody consensus sequences, ICS) that characterise the intracellular antibodies and the use of these ICS consensus sequences for the design and construction of libraries that are enriched with intracellular antibodies.

The presence of ICS sequences in an antibody is diagnostic of its property of being a functional intracellular antibody, without having to undertake experimental selection with IACT or intracellular expression in other systems, with a considerable saving of experimental work. The ICS sequence can be used for the optimisation of antibodies of interest, as well as for the design and construction of libraries that are enriched with intracellular antibodies.

Thus in a first aspect the present invention provides a method of identifying at least one consensus sequence for an intracellular antibody (ICS) comprising the steps of:

- a) creating a database comprising sequences of at least a proportion of a variable heavy chain domain and/or variable light chain domain of validated intracellular antibodies (VIDA database) and aligning the sequences of the variable heavy chain domains or variable light chain domains of validated intracellular antibodies;
- b) determining the frequency with which a particular amino acid occurs in each of the positions of the aligned antibodies;
- c) selecting a frequency threshold value (LP or consensus threshold) in the range from 70% to 100%;

- d) identifying the positions of the alignment at which the frequency of a particular amino acid is greater than or equal to the LP value;
- e) identifying the most frequent amino acid, in the positions of the alignment defined in d).

According to the above aspect of the invention, advantageously the sequences of the variable heavy chain domains or variable light chain domains of validated intracellular antibodies present in the VIDA database are aligned according to Kabat.

As used herein, the term 'database' means any collection of data. Those skilled in the art will appreciate that there are many ways in which such data may be stored. Suitable methods include but are not limited to storage in electronic form and in paper form. Those skilled in the art will be aware of other suitable methods of data storage.

According to the present invention, the term 'creating' means generating or producing.

As herein defined, The VIDA database contains all the sequences of antibodies selected with IACT, in particular the sequences of the anti-TAU antibodies described herein. In addition, it comprises those antibodies reported in the literature to bind specifically to one or more antigen/ligand/s within an intracellular environment.

By 'aligning the (amino acid) sequences', it is meant that the (amino acid) sequences are arranged or lined up such that those amino acid residues which are the same or similar between the sequences are apparent. Thus 'aligning the sequences' as herein defined permits the simple and efficient comparison of the residue similarities and differences between two or more amino acid sequences. Sequences are advantageously aligned as set forth in Kabat, "Sequences of Proteins of Immunological Interest", US Department of Health and Human Services, using the Kabat numbering system which is known to those skilled in the art.

It should be appreciated that although reference is made throughout to the Kabat database, other databases of antibody sequences could be used as an alternative, or in addition to this database.

As used herein the term 'frequency' denotes the frequency (that is the number of times) with which a specific amino acid occurs in each of the residue positions of the aligned sequences. The % frequency means the percentage of identical amino acid residues at any given residue position in the sequence and is calculated as a percentage of the total number of amino acids at that position to be compared (that is the total number of sequences to be compared). Thus, for example, if 10 amino acid sequences are to be compared and at amino acid residue number 1, 7 out of 10 of the residues are arginines, then the percentage frequency of arginine at that position is 70%.

As used herein the term 'frequency threshold value' or 'LP' value refers to a selected minimum % frequency as herein defined for each amino acid position within the aligned sequences. Advantageously, the frequency threshold value or (LP) value selected is the same for each and every residue within the aligned sequences. The selection of a 'frequency threshold value' creates a cut-off point at each residue position for the allocation of a consensus residue at that position. That is, the % frequency of one or more identical amino acids at any given position is compared with the 'frequency threshold value' or (LP) value at that position, and if the % frequency of one or more identical or similar amino acids at any given residue position is at least the same as the selected frequency threshold value, then that residue will be assigned the 'consensus residue' for that residue position.

Those skilled in the art will appreciate that the higher the 'frequency threshold value' or 'LP value' selected, then the greater the '% frequency' (as herein defined) is required to be for a given residue at any given residue position, for it to be assigned a 'consensus residue' and hence part of the consensus sequence.

Analysis of the antibody sequences contained in VIDA makes it possible to identify a subset of the amino acid residues that are conserved in human and murine intracellular antibodies. This subset of residues is designated ICS (intrabody consensus sequence), and enables us to define an ICS for the VL chain and one for the VH chain for each species (human ICS-VH, human ICS-VL, mouse ICS-VH, mouse ICS-VL). Comparative analysis of the ICSs of different species for the same chain made it possible to identify the amino acids in common and therefore an ICS-VH hxm (man mouse) and an ICS-VL hxm, i.e. (minimum) general ICSs. Obviously the ICSs will be different depending on the threshold of homology between all the antibodies present in the VIDA database (absolute consensus, 90% consensus, etc.).

The present invention also provides a procedure for finding the optimum ICS for each reference group. The optimum ICS is obtained with an algorithm, described below, which changes the threshold of homology between the antibodies of the VIDA dataset iteratively and defines an optimum homology threshold.

Thus, in a further aspect the present invention provides a method of identifying at least one optimum consensus sequence for an intracellular antibody (optimum ICS) comprising the steps of:

- a) identifying different ICSs for different LP values;
- b) for each of said ICSs: constructing a frequency distribution of the number of identical amino acids between that particular ICS and each of the antibodies making up the VIDA database (VIDA distribution);
- c) for each of the ICSs, constructing a frequency distribution of the number of identical amino acids between that particular ICS and each of the antibodies that make up the Kabat database (Kabat distribution);
- d) defining a "distance" D between the VIDA distributions and the Kabat distribution corresponding to a value of LP;
- e) for each LP value, determining the value of the "distance" D between the VIDA distributions and the Kabat distribution corresponding to that value of LP;

f) identifying the optimum ICS as the ICS corresponding to the value of LP for which the calculated value of the distance D defined in d) is maximum.

According to the above aspect of the invention, advantageously, the ICSs are generated according to one or more methods described herein.

Analysis of the antibody sequences contained in VIDA makes it possible to identify a subset of the amino acid residues that are conserved in human and murine intracellular antibodies. This subset of residues is designated ICS (intrabody consensus sequence), and enables us to define an ICS for the VL chain and one for the VH chain for each species (human ICS-VH, human ICS-VL, mouse ICS-VH, mouse ICS-VL). Comparative analysis of the ICSs of different species for the same chain made it possible to identify the amino acids in common and therefore an ICS-VH hxm (man mouse) and an ICS-VL hxm, i.e. (minimum) general ICSs. Obviously the ICSs will be different depending on the threshold of homology between all the antibodies present in the VIDA database (absolute consensus, 90% consensus, etc.).

A procedure is described for finding the optimum ICS for each reference group. The optimum ICS is obtained with an algorithm, described below, which changes the threshold of homology between the antibodies of the VIDA dataset iteratively and defines an optimum homology threshold.

Accordingly, an intracellular antibody is identified as an antibody that has an optimum ICS, defined as above, on the positions of the chain where the said ICS is defined, whereas hypotheses are not made regarding other positions, nor are constraints placed.

Comparison between the ICS and the Kabat consensus sequence (for the same group) shows that the ICS is highly homologous (but not completely identical) to the Kabat consensus, in those positions of the chain where the ICS itself is defined.

The ICS is used for predicting the property of any given antibody of being a functional intracellular antibody. In particular, the analysis described predicts that a percentage of about 10% of the antibodies present in the Kabat database are intracellular antibodies.

The ICS can be employed for constructing antibody libraries that are greatly enriched in functional intracellular antibodies. The libraries will preferably express scFv fragments based on ICS.

According to the above aspect of the invention, the term ICS denotes 'intracellular consensus sequence' and is a consensus sequence for an immunoglobulin molecule capable of binding to its ligand within an intracellular environment. ICS's as herein described are generated using the methods of the present invention. One skilled in the art will appreciate that the amino acid residues and their sequence comprising each ICS will depend upon the number of sequences compared in order to generate the ICS, the nature of the sequences compared and the frequency threshold value selected.

As herein described a 'VIDA' denotes a 'validated intracellularly binding antibody'. That is, it denotes an antibody which has been shown by functional studies to bind specifically to one or more ligands within an intracellular environment. VIDAs as herein defined include those antibodies which have been shown by the present inventors to function within an intracellular environment as well as those antibody molecules which are reported in the literature as binding to one or more ligands specifically within an intracellular environment.

Accordingly, a 'VIDA database' includes the sequences of those antibodies which have been shown by the present inventors to function within an intracellular environment as well as those antibody molecules which are reported in the literature as binding to one or more ligands specifically within an intracellular environment.

As used herein the term 'frequency distribution' refers to a representation of the relationship between two or more characteristics. Advantageously, the representation is a

graphical representation. Specifically, the term 'VIDA distribution' of a particular ICS refers to a representation of the relationship between the number of identical amino acids between that particular ICS and each of the antibodies making up the VIDA database as herein defined.

Likewise the term 'Kabat' distribution of a particular ICS refers to a representation, preferably a graphical representation of the number of identical amino acids between that particular ICS and each of the antibodies which make up the Kabat database.

As defined herein, the 'D distance' is that graphically defined distance between a given VIDA (validated intracellular antibody) distribution value and a given Kabat distribution value for a given LP value (threshold value), as herein defined.

According to the present invention, the term 'optimum ICS' refers to the ICS (intracellularly binding antibody consensus sequence) corresponding to the LP (threshold value) for which the calculated distance D as herein defined is a maximum.

Advantageously, the consensus sequence is one of the consensus sequences for VH and/or VL comprising:

a) for a VH consensus sequence, at least the following amino acids in the positions indicated according to Chothia numbering (Chothia and Lesk, (1987) J. Mol. Biol. 196:910-917):

S-21, C-22, S-25, G-26, M-32, W-36, P-41, L-45, E-46, D-72, Q81, L-82c, E-85, D-86, A-88, Y-90, C-92, W-103, G-104, G-106, T-107, T-110, V-111, S-112;

b) for a VL consensus sequence, at least the following amino acids in the positions indicated according to Chothia numbering:

G-16, C-23, W-35, G-57, G-64, S-65, S-67, I-75, D-82, Y-86, C-88, T-102, K-103.

In a preferred embodiment of this aspect of the invention, the consensus sequence is for human VH and essentially comprises the following amino acids in the positions indicated according to Chothia numbering:

Q-1, V-2, Q-3, L-4, S-7, G-8, G-9, G10, V-12, P-14, G-15, S-17, L-18, R-19, L-20, S-21, C-22, A-24, S-25, G-26, F-27, T-28, F-29, Y-31a, M-32, W-36, R-38, Q-39, A-40, P-41, G-42, K-43, G-44, L-45, E-46, W-47, V-48, S-52, G-54, Y-58, Y-59, A-60, D-61, S-62, V-63, K-64, G-65, R-66, F-67, T-68, I-69, S-70, R-71, D-72, N-73, S-74, N-76, T-77, L-80, Q81, M-82, L-82c, R-83, A-84, E-85, D-86, T-87, A-88, Y-90, C-92, A-93, W-103, G-104, G-106, T-107, L-108, V-109, T-110, V-111, S-112, S-113;

In a further preferred embodiment, the consensus sequence represents human VL, and essentially comprises the following amino acids in the positions indicated according to Chothia numbering:

T-5, P-8, G-16, I-21, C-23, W-35, Y-36, Q-37, P-40, G-41, P-44, I-48, S-56, G-57, S-63, G-64, S-65, S-67, G-68, L-73, T-74, I-75, D-82, A-84, Y-86, C-88, T-102, K-103.

In an especially preferred embodiment of this aspect of the invention an immunoglobulin of the present invention comprises a consensus sequence which comprises the following amino acids in the positions indicated according to Chothia numbering:

Q-1, V-2, Q-3, L-4, S-7, G-8, G-9, G10, V-12, P-14, G-15, S-17, L-18, R-19, L-20, S-21, C-22, A-24, S-25, G-26, F-27, T-28, F-29, Y-31a, M-32, W-36, R-38, Q-39, A-40, P-41, G-42, K-43, G-44, L-45, E-46, W-47, V-48, S-52, G-54, Y-58, Y-59, A-60, D-61, S-62, V-63, K-64, G-65, R-66, F-67, T-68, I-69, S-70, R-71, D-72, N-73, S-74, N-76, T-77, L-80, Q81, M-82, L-82c, R-83, A-84, E-85, D-86, T-87, A-88, Y-90, C-92, A-93, W-103, G-104, G-106, T-107, L-108, V-109, T-110, V-111, S-112, S-113 and a variable light chain which comprises the following amino acids in the positions indicated according to Chothia numbering:

T-5, P-8, G-16, I-21, C-23, W-35, Y-36, Q-37, P-40, G-41, P-44, I-48, S-56, G-57, S-63, G-64, S-65, S-67, G-68, L-73, T-74, I-75, D-82, A-84, Y-86, C-88, T-102, K-103.

In a further aspect the present invention provides an intracellularly binding immunoglobulin molecule comprising at least one variable chain which is described by at least one of the consensus sequences described in figs 11a and 11b and depicted SEQ ID no 41 and 42 respectively.

Advantageously, an immunoglobulin molecule of the present invention comprises,

(a) for the heavy chain, at least the following amino acids in the positions indicated according to Chothia numbering (Chothia and Lesk, (1987) *J. Mol. Biol.* 196:910-917): S-21, C-22, S-25, G-26, M-32, W-36, P-41, L-45, E-46, D-72, Q81, L-82c, E-85, D-86, A-88, Y-90, C-92, W-103, G-104, G-106, T-107, T-110, V-111, S-112; and

(b) for the light chain at least the following amino acids in the positions indicated according to Chothia numbering:
G-16, C-23, W-35, G-57, G-64, S-65, S-67, I-75, D-82, Y-86, C-88, T-102, K-103.

In a further aspect still, the present invention provides the use of an immunoglobulin molecule comprising at least one consensus sequence described in fig 11a and/or 11b and depicted SEQ 41 and SEQ 42 respectively in the selective binding of a ligand within an intracellular environment.

As herein defined, the term 'selective binding' (of a ligand within an intracellular environment) means that the interaction between the immunoglobulin and the ligand are specific, that is, in the event that a number of molecules are presented to the immunoglobulin, the latter will only bind to one or a few of those molecules presented. Advantageously, the immunoglobulin ligand interaction will be of high affinity. The interaction between immunoglobulin and ligand will be mediated by non-covalent interactions such as hydrogen bonding and Van der Waals interactions. Generally, the interaction will occur in the cleft between the heavy and the light chains of the immunoglobulin.

In a further aspect still, the present invention provides a method for predicting whether an antibody is a functioning intracellular antibody comprising the steps of:

- a) aligning the sequence of the antibody with the sequences of the antibodies of the reference VIDA database;
- b) aligning the sequence of the antibody with the optimum ICS sequence of the reference VIDA database;

- c) constructing VIDA and KABAT distributions corresponding to the optimum value of LP (frequency threshold value) ;
- d) determining the corresponding distance D;
- e) determining the identity number N between the sequence of the antibody and the ICS reference sequence;
- f) calculating the difference between the mean value of the VIDA distribution and the product between D and the standard deviation of the VIDA distribution, obtaining the parameter S_{intra} ;
- g) if the identity number N is greater than or equal to S_{intra} , identifying the antibody as intracellular antibody.

In a further aspect the present invention provides a method for conferring upon an immunoglobulin molecule the ability to function within an intracellular environment, comprising the steps of:

- a) identifying the optimum ICS reference sequence
- b) optionally, modifying, by site-specific mutagenesis, the amino acid residues that are located in the positions defined by the optimum ICS, or a subset of these residues, in such a way that they are those identified by the optimum ICS.

According to this aspect of the invention, advantageously the aligning step is performed as described herein. Advantageously, the ICS generation is performed using the methods herein described.

The present inventors have found that by performing a functional binding assay (that is by performing a yeast two-hybrid based IACT assay) to all of the antibodies proposed to be included in a database, and only using those antibodies which are found using to IACT to bind specifically to antigen within such an environment when generating consensus sequences, then more complete consensus sequence/s for the antibody variable domains may be obtained.

Thus, in a further aspect, the present invention provides a method for identifying at least one consensus sequence for an intracellular antibody (ICS) comprising the steps of:

- (a) selecting and aligning the sequences of antibody light or heavy chain variable regions which are shown using IACT to bind specifically to antigen/ligand within an intracellular environment, and
- (b) identifying the most frequent amino acid, in each position of the alignment.

Advantageously, the consensus sequence/s identified using the method of this aspect of the invention are those described in figures 5a and depicted SEQ 3 and 4. The consensus sequence/s identified using the above listed approach share all of the amino acids present within the less complete consensus sequence shown in figs 11a and 11b and identified as SEQ no 41 and 42, which use the VIDA database (which includes those antibodies reported in the literature to bind to their ligand/antigen within an intracellular environment, and which have not necessarily been shown by IACT to do so). Details of this approach are given in strategy A, Example 10.

In a further aspect still, the present invention provides a method for selecting one or more antibodies capable of binding specifically to its one or more antigens/ligands within an intracellular environment comprising the steps of:

- (a) comparing at least a proportion of the variable heavy chain of one or more antibodies with at least the consensus sequence shown in fig 5a and depicted SEQ 3, and
- (b) selecting those one or more antibodies whose variable heavy chain is at least 85% identical with the consensus sequence of step (a).

In yet a further aspect, the present invention provides a method for selecting one or more antibodies capable of binding specifically to its one or more antigens/ligands within an intracellular environment comprising the steps of:

- (a) comparing at least a proportion of the variable light chain of one or more antibodies with at least the consensus sequence shown in fig 5a and depicted SEQ 4, and
- (b) selecting those one or more antibodies whose variable light chain is at least 85% identical with the consensus sequence of step (a).

According to the above aspects of the invention, advantageously, the intracellular environment is a mammalian intracellular environment. More advantageously, the mammal is a human being.

Advantageously, a further *in vivo* binding step (c) is performed in order to test whether the selected one or more antibodies binds specifically to their one or more antigens. Advantageously, this *in vivo* binding step is performed using IAC technology described herein and detailed in WO 000/54057.

According to the above aspects of the invention, advantageously, the immunoglobulin has a V_H amino acid sequence which shows at least 86% identity with the V_H consensus sequence identified by SEQ ID no 3 and shown in fig 5. In an especially preferred embodiment, the immunoglobulin molecule shows at least 87% identity. Advantageously the immunoglobulin has a V_H amino acid sequence which shows at least 88% identity, 89% identity, 90% identity or 91, 92, 93, 94, 95, 96, 97, 98% identity with the V_H consensus sequence identified by SEQ ID no 3. Most advantageously, the immunoglobulin has a V_H amino acid sequence which shows at least 99% identity with the V_H consensus identified by SEQ ID No 3 and shown in fig 5. In a most preferred embodiment it shows at least 100% identity with the V_H consensus sequence depicted by SEQ ID no 3 and shown in fig 5.

In a preferred embodiment of the above aspects of the invention, both the variable light chain and the variable heavy chains of the antibodies for selection are compared with the consensus sequences as detailed above.

One skilled in the art will appreciate that, the greater the degree of identity the variable light and/or variable heavy chain of the antibody whose intracellular binding ability is to be determined is to the one or more consensus sequences shown in figs 5a, then the greater the probability that the antibody in question will be capable of binding specifically to its ligand within an intracellular environment. In addition, the IACT based binding step

provides further guidance on whether the antibody in question will be capable of binding selectively to its ligand within a mammalian intracellular environment.

In a further aspect still, the present invention provides an intracellularly binding immunoglobulin molecule comprising a variable heavy chain which exhibits 85% homology to the consensus sequence shown in fig 5a and depicted as SEQ ID No 3 and at least one variable light chain.

According to the above aspect of the invention, advantageously, the immunoglobulin molecule comprises a heavy chain variable domain (VH) which is a member of the VHIII subgroup of immunoglobulin heavy chains.

In yet a further aspect, the present invention provides an intracellularly binding immunoglobulin molecule comprising a variable light chain which exhibits 85% homology to the consensus sequence shown in fig 5a and depicted as SEQ ID No 4 and at least one variable heavy chain.

In a further aspect still, the present invention provides the use of an immunoglobulin molecule comprising at least one variable heavy chain domain (VH) exhibiting at least 85% identity with the consensus sequence shown in fig 5a and depicted SEQ 3, and at least one light chain domain (VL) , in the selective binding of a ligand within an intracellular environment.

In a preferred embodiment of the above aspect of the invention, the use is of an immunoglobulin molecule comprising at least one heavy chain variable domain (V_H) which is a member of the VHIII subgroup of immunoglobulin heavy chains, in the selective binding of a ligand within an intracellular environment.

In a further preferred embodiment of the above aspect of the invention the use is of an immunoglobulin molecule comprising at least one light chain variable domain (V_L) which

is a member of the $V_{\kappa I}$ subgroup of immunoglobulin light chains, in the selective binding of a ligand within an intracellular environment.

The present inventors realised that using the knowledge of the consensus sequences for intracellular antibodies, then libraries may be generated which are enriched in antibody molecules or fragments thereof which are capable of functioning within an intracellular environment.

Thus in a further aspect, the present invention provides a library, wherein the library is generated using any one or more of the variable heavy domain amino acid sequences (VH) selected from the group consisting of: a VH amino acid sequence showing at least 85% identity with the consensus sequence depicted as SEQ 3 and shown in fig 5a, a VH sequence which is described by the consensus sequence depicted in SEQ 41 and shown in fig 11a,

According to the above aspect of the invention, advantageously, the immunoglobulin has a V_H amino acid sequence which shows at least 86% identity with the VH consensus sequence identified by SEQ ID no 3 and shown in fig 5. In an especially preferred embodiment, the immunoglobulin molecule shows at least 87% identity. Advantageously the immunoglobulin has a VH amino acid sequence which shows at least 88% identity, 89% identity, 90% identity or 91, 92, 93, 94, 95, 96, 97, 98% identity with the VH consensus sequence identified by SEQ ID no 3. Most advantageously, the immunoglobulin has a VH amino acid sequence which shows at least 99% identity with the VH consensus identified by SEQ ID No 3 and shown in fig 5. In a most preferred embodiment it shows at least 100% identity with the VH consensus sequence depicted by SEQ ID no 3 and shown in fig 5.

Thus, in a further aspect the present invention provides a method for the construction of an antibody library enriched with antibodies capable of functioning within an intracellular environment comprising the steps of:

- a) selecting an antibody framework from those that are intracellularly functionally stable, from the Kabat database;
- b) on each framework, mutagenizing the amino acids present in the positions of the sequence defined by the optimum ICS according to the invention, changing them into the amino acid residue that is located in that position in the ICS sequence; and
- c) on each of the frameworks, randomising the CDR regions of the antibody sequence.

According to the above aspect of the invention, the term 'intracellularly functionally stable' (antibodies) refers to those antibodies which are capable of functioning within an intracellular environment, that is, those antibodies which are stable with respect to specific antigen binding and advantageously eliciting an immune response.

As herein described the term 'mutagenizing' (one or more amino acid residues) refers to a change of one or more amino acid residues. Such change may involve a substitution, deletion, inversion or an insertion. Advantageously, the 'mutagenizing' as herein described involves a substitution. Methods for 'mutagenizing' amino acid sequences will be familiar to those skilled in the art and are described herein.

Furthermore, according to the present invention, the term 'randomising' (one or more CDR regions) means to 'shuffle', rearrange or otherwise change by amino acid insertion, deletion or substitution some or all the amino acid residues comprising those one or more CDR regions so as to create a repertoire of immunoglobulin molecules sharing a common framework region and comprising CDR regions which comprise the same amino acid constituents as the other immunoglobulin molecules within the library but wherein the identity of at least some of those residues differs between the individual immunoglobulin molecules. Those skilled in the art will appreciate that the randomisation of CDR residues enables the generation of libraries comprising immunoglobulin molecules having a number of different antigen binding specificities.

According to the above aspects of the invention, preferably the ICSs are generated according to the methods described herein.

In yet a further aspect, the present invention provides a method for the construction of an antibody library enriched with functional intracellular antibodies capable of functioning within an intracellular environment, comprising the steps of:

- a) selecting an antibody framework, on the basis of the homology with an optimum ICS sequence;
- b) mutagenizing all the remaining residues of the antibody framework, limited, for each position, to the amino acids that are located, in that position, in antibodies of the Kabat database; and
- c) on each of the frameworks, randomising the CDR regions of the antibody sequence,

In the above aspect of the invention, advantageously an antibody framework is selected on the basis of maximal homology with an optimum ICS sequence according to the invention. That is, a framework region is selected which shows maximum homology with an ICS generated according to the present invention.

In a final aspect, the present invention provides the use of a library as herein described for producing immunoglobulin molecules, wherein a substantial proportion of those molecules expressed are capable of selectively binding to a ligand within an intracellular environment.

Definitions

Immunoglobulin molecules, according to the present invention, refer to any moieties which are capable of binding to a target. In particular, they include members of the immunoglobulin superfamily, a family of polypeptides which comprise the immunoglobulin fold characteristic of antibody molecules, which contains two beta sheets and, usually, a conserved disulphide bond. Members of the immunoglobulin superfamily are involved in many aspects of cellular and non-cellular interactions *in vivo*, including widespread roles in the immune system (for example, antibodies, T-cell

receptor molecules and the like), involvement in cell adhesion (for example the ICAM molecules) and intracellular signalling (for example, receptor molecules, such as the PDGF receptor). The present invention is applicable to all immunoglobulin superfamily molecules which are capable of binding to target molecules. Preferably, the present invention relates to antibodies or scFv molecules.

Antibodies as used herein, refers to complete antibodies or antibody fragments capable of binding to a selected target, and including Fv, scFv, Fab' and F(ab')₂, monoclonal and polyclonal antibodies, engineered antibodies including chimeric, CDR-grafted and humanised antibodies, and artificially selected antibodies produced using phage display or alternative techniques. Small fragments, such as Fv and scFv, possess advantageous properties for diagnostic and therapeutic applications on account of their small size and consequent superior tissue distribution. Preferably, the antibody is a single chain antibody or scFv.

Heavy chain variable domain refers to that part of the heavy chain of an immunoglobulin molecule which forms part of the antigen binding site of that molecule. The VHIII subgroup describes a particular sub-group of heavy chain variable regions (the VHIII). Generally immunoglobulin molecules having a variable chain amino acid sequence falling within this group possess a VH amino acid sequence which can be described by the VHIII consensus sequence in the Kabat database .

Light-chain variable domain refers to that part of the light chain of an immunoglobulin molecule which forms part of the antigen binding site of that molecule. The VKI subgroup of immunoglobulin molecules describes a particular sub-group of variable light chains. Generally immunoglobulin molecules having a variable chain amino acid sequence falling within this group possess a VL amino acid sequence which can be described by the VKI consensus sequence in the Kabat database.

Framework region of an immunoglobulin heavy and light chain variable domain. The variable domain of an immunoglobulin molecule has a particular three-dimensional

conformation characterised by the presence of an immunoglobulin fold. Certain amino acid residues present in the variable domain are responsible for maintaining this characteristic immunoglobulin domain core structure. These residues are known as framework residues and tend to be highly conserved.

CDR (complementarity determining region) of an immunoglobulin molecule heavy and light chain variable domain describes those amino acid residues which are responsible for the specificity antigen binding, and are as defined by Kabat (*op. Cit.*). The CDR residues are mainly, but not exclusively, contained within the hypervariable loops of the variable regions as defined by Chothia and Lesk (*op. cit.*). The CDRs and the hypervariable loops are directly involved with the interaction of the immunoglobulin with the ligand. Residues within these loops tend to show a lower degree of conservation than those in the framework region.

Intracellular means inside a cell, and the present invention is directed to those immunoglobulins which will bind to ligands/targets selectively within a cell. The cell may be any cell, prokaryotic or eukaryotic, and is preferably selected from the group consisting of a bacterial cell, a yeast cell and a higher eukaryote cell. Most preferred are yeast cells and mammalian cells. As used herein, therefore, "intracellular" immunoglobulins and targets or ligands are immunoglobulins and targets/ligands which are present within a cell. In addition the term 'Intracellular' refers to environments which resemble or mimic an intracellular environment. Thus, "intracellular" may refer to an environment which is not within the cell, but is *in vitro*. For example, the method of the invention may be performed in an *in vitro* transcription and/or translation system, which may be obtained commercially, or derived from natural systems.

The **KABAT database** is an exhaustive collection of antibody sequences on which a sequence of interest can be tested for discovering its characteristics (subgroup to which it belongs, position of each amino acid residue, variability (<http://immuno.bme.nwu.edu>) (Johnson, G. et al. 2000). This database also contains citations from scientific journals and links to the PubMed archive of scientific information. Using the Kabat database, it is

possible to obtain a diagram of the distribution of the amino acids in the subgroups to which they belong. It is also possible to obtain a distribution of the residues in each position of the sequence tested and hence obtain its variability.

Consensus sequence of V_H and V_L chains in the context of the present invention refers to the consensus sequences of those V_H and V_L chains from immunoglobulin molecules which can bind selectively to a ligand in an intracellular environment. The residue which is most common in any one given position, when the sequences of those immunoglobulins which can bind intracellularly are compared is chosen as the consensus residue for that position. The consensus sequence is generated by comparing the residues for all the intracellularly binding immunoglobulins, at each position in turn, and then collating the data. In this case the sequences of 11 immunoglobulins was compared. In the context of the present invention, a consensus residue is only conferred if a residue occurred greater than 5 times at any one position. For the avoidance of doubt, the terms V_H and V_L consensus sequences does not include the sequences of the J regions. In addition, the first two residues (methionine and alanine) are not part of the consensus. They are derived from an NCI1 restriction site.

As herein defined, The **VIDA database** contains all the sequences of antibodies selected with IACT, in particular the sequences of the anti-TAU antibodies described. In addition, it comprises those antibodies reported in the literature to bind specifically to one or more antigen/ligand/s within an intracellular environment. A '**validated intracellular antibody**' as herein described refers to those antibodies which are found using IACT or reported in the literature to be functional within an intracellular environment. By the term 'functional' it is meant that those validated antibodies are capable of binding selectively to their specific antigen within such an environment.

The term '**frequency threshold value**' or 'LP' value refers to a selected minimum % frequency as herein defined for each amino acid position within the aligned sequences. Advantageously, the frequency threshold value or (LP) value selected is the same for each and every residue within the aligned sequences. The selection of a 'frequency threshold

value' creates a cut-off point at each residue position for the allocation of a consensus residue at that position. That is, the % frequency of one or more identical amino acids at any given position is compared with the 'frequency threshold value' or (LP) value at that position, and if the % frequency of one or more identical or similar amino acids at any given residue position is at least the same as the selected frequency threshold value, then that residue will be assigned the 'consensus residue' for that residue position.

\underline{P}_s

$$P_s = \sqrt[n]{\prod_{i=1}^n P_i(A_{s,i})}$$

Selective binding in the context of the present invention, means that the interaction between the immunoglobulin and the ligand are specific, that is, in the event that a number of molecules are presented to the immunoglobulin, the latter will only bind to one or a few of those molecules presented. Advantageously, the immunoglobulin ligand interaction will be of high affinity. The interaction between immunoglobulin and ligand will be mediated by non-covalent interactions such as hydrogen bonding and Van der Waals. Generally, the interaction will occur in the cleft between the heavy and the light chains of the immunoglobulin.

A **repertoire** in the context of the present invention refers to a set of molecules generated by random, semi-random or directed variation of one or more template molecules, at the nucleic acid level, in order to provide a multiplicity of binding specificities. In this case the template molecule is one or more of the VH and/or VL domain sequences herein described. Methods for generating repertoires are well characterised in the art.

A **library** according to the present invention refers to a mixture of polypeptides or nucleic acids. The library is composed of members. Sequence differences between library members are responsible for the diversity present in the library. The library may take the form of a simple mixture of polypeptides or nucleic acids, or may be in the form organisms or cells, for example bacteria, viruses, animal or plant cells and the like,

transformed with a library of nucleic acids. Typically, each individual organism or cell contains only one member of the library. In certain applications, each individual organism or cell may contain two or more members of the library. Advantageously, the nucleic acids are incorporated into expression vectors, in order to allow expression of the polypeptides encoded by the nucleic acids. In a preferred aspect, therefore, a library may take the form of a population of host organisms, each organism containing one or more copies of an expression vector containing a single member of the library in nucleic acid form which can be expressed to produce its corresponding polypeptide member.

Brief Description of the figures.

Figure 1 describes the isolation of intracellular antibodies directed against BCR or ABL using the IAC technology..

A. The flow chart (left) shows the steps involved in the IAC technology (Visintin, M., Tse, E., Axelson, H., Rabbitts, T.H. and Cattaneo, A. (1999) Proc. Natl. Acad. Sci. USA **96** 11723-11728)

Step 1: An scFv phage display library was used to screen antigen *in vitro*; the antigen in these experiments was made using bacterial expression systems. Step 2: All phage binding to antigen were recovered, plasmid DNA prepared and scFv fragments cloned into the yeast prey vector pVP16 to generate a sub-library of scFv enriched for antigen binding. Step 3: The yeast scFv sub-library was screened in yeast expressing antigen fused to a DNA binding domain as a bait and colonies growing on histidine selection plates were recovered and assayed for beta-gal activation. Step 4: Clones which grow in the absence of histidine and activate beta-gal were used to prepare plasmid DNA and the various selected clones finger-printed with BstNI digestion to identify groups of scFv. Step 5: Members of each group are subsequently re-tested in yeast with the original bait clone to identify those scFv which genuinely bind to antigen. These scFv are further characterised in mammalian cells.

B IAC of anti-BCR scFv

C. IAC of anti-ABL scFv

In B and C, 2×10^{13} clones from a phage library were screened with antigen using in vitro methods (Sheets, M.D., Amersdorfer, P., Finnern, R., Sargent, P., Lindqvist, E., Schier, R., Hemingsen, G., Wong, C., Gerhart, J.C. and Marks, J.D. (1998) Proc. Natl. Acad. Sci. USA **95** 6157-6162) and (Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C., McCafferty, J., Hodits, R. A., Wilton, J. & Johnson, K. S. (1996). Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. Nature Biotechnol. **14**, 309-314.). Around 10^5 phage were recovered, cloned into the yeast vector pVP16 to make sub-libraries of 3.2 and 1.3×10^5 for anti-BCR and anti-ABL respectively. Approximately 8.5×10^5 yeast were screened (Visintin, M., Tse, E., Axelson, H., Rabbitts, T.H. and Cattaneo, A. (1999) Proc. Natl. Acad. Sci. USA **96** 11723-11728) with BCR-ABL bait yielding 117 and 37 clones. These were sub-divided further by sequence analysis into 6 and 12 anti-BCR and anti-ABL respectively.

Figure 2 shows β -galactosidase filter assay showing interaction between anti-BCR-specific scFv and BCR-ABL protein in yeast.

The L40 yeast strain was transformed with either a DNA binding domain bait fused to BCR-ABL (pBTM/E1A2) (A.) or the AMCV plant virus p41 coat-protein (B.) These host strains were transformed with plasmids coding for individual scFv-VP16 activation domain fusions and streaked onto the YC medium lacking tryptophan and leucine to select for the plasmids. Interaction of scFv with bait protein was monitored by activation of β -galactosidase in the beta-gal filter assay shown. B3, 9, 10, 21, 33 and 89 are anti-BCR scFv and F8 is an scFv specific for the AMCV p41 protein (Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. & Galeffi, P. (1993) Nature (London) **366**, 469-472). Interaction of the B series scFv only occurs with the BCR-ABL bait and not with AMCV, whereas F8 only interacts with AMCV.

Figure 3 shows the characterisation of the ICABs by filter binding to antigen. scFv were cloned into the bacterial expression vector, pHEN2 and used to transform HB2151 *E. coli*. Periplasmic expression of scFv was obtained for anti-ABL (A) and anti-

BCR (B) ICAs. scFv were subjected to SDS-PAGE followed by Western blotting. The scFv were tagged with c-myc epitope and visualised using 9E10 monoclonal anti-myc tag antibody and secondary HRP-conjugated anti-mouse antibody. (C, D) The specificity of the scFv was evaluated *in vitro* by testing their ability to recognise filter-bound antigen (SH2 domain of ABL (C) and SH2-binding domain of BCR (D)). The antigens were separated by SDS-PAGE and transferred to filters which were incubated with scFv (bacterial periplasmic extracts) followed by 9E10 and HRP-conjugated anti-mouse antibodies for detection. Each scFv was tested against both ABL (designated A) and BCR (designated B) antigens.

Size marker positions are shown on the LHS of each panel.

Figure 4 shows a Mammalian antibody-antigen *in vivo* interaction assay.

CHO cells were transfected with a luciferase reporter, an antigen bait and an anti-BCR scFv fused to VP16 activation domain. As a negative control the non-relevant scFv F8 was used and a control bait was beta-gal in B.

(A) CHO cells were transiently transfected with a Firefly luciferase reporter plasmid together with an internal Renilla luciferase control plasmid pRL-CMV, the bait plasmid pM3-E1A2 and one of the anti-BCR scFv-VP16 expression plasmids, as indicated. The luciferases levels were assayed 48 hours after transfection using the Dual Luciferase Assay System (Promega) and a luminometer. Firefly luciferase level from each transfection was normalised to the Renilla luciferase level (internal control for the transfection efficiency). As a non-specific control, scFvF8-VP16, a non-relevant anti-AMCV scFv, expression plasmid was co-transfected with pM3-E1A2, plus the luciferase reporter. The relative Firefly luciferase level for each scFv tested was shown in the histogram with the level for scFvF8 taken as 1.

(B) The specificity of interaction of the isolated scFv was verified by using a β -gal bait (pM- β -gal) instead of BCR-ABL antigen bait in the same CHO assay system, as indicated. The normalised Firefly luciferase level for scFvF8 was taken as 1 and the (C) relative luciferase level for each BCR-specific scFv was shown in the histogram.

Figure 5 shows the Alignment of derived protein sequences of intracellular scFv.

The nucleotide sequences of the scFv were obtained and the derived protein translations (shown in the single letter code) were aligned. The complementarity determining regions (CDR) are shaded. Framework residues for SEQ no 1 to 40 are those which are underlined. The consensus sequence at a specific position was calculated for the most frequently occurring residue but only conferred if a residue occurred greater than 5 times at that position.

A. Sequences of VH and VL from anti-BCR (designated as B3-B89) and anti-ABL (designated as A5-A32). The combined consensus (Con) of the anti-BCR and ABL ICABs is indicated compared with the subgroup consensus for VH3 and VKI from the Kabat database .

- Represents sequence identity with the intracellular antibody binding V_H or V_L consensus (SEQ 3 and SEQ 4)

. represents gaps introduced to optimise alignment

B. A sequence comparison of randomly obtained scFv obtained from the unselected phage display library. The consensus obtained from the randomly isolated scFv (rcH and rcL) are indicated.

- represents gaps introduced to optimise alignment

- X represents positions at which no consensus could be assigned.

Fig. 6. Schematic diagram of folding, stability and tolerance to removal of the disulphide bridges of the scFvs.

Fig. 7. Schematic diagram of IACT technology. IACT is a combination of the double hybrid system and phage display technology. Initially, phage display technology is used for increasing the percentage of antibody fragments that are specific to the protein used as "bait". The double hybrid system is adapted so as to be able to isolate pairs of antigens and associated specific antibody fragments in conditions of intracellular expression. After selection in vivo, the clones that proved to be positive are isolated and the scFvs can be used for applications in vitro and in vivo.

Fig. 8. a) Results obtained in ELISA from phages isolated after the second cycle of preselection in vitro. The antigens were preadsorbed on the plate at a concentration of 10 µg/ml. Only 9 scFvs out of 96 tested turned out to be specific for the TAU protein 151-421. The specificity of the bond was compared with the signal obtained in ELISA from the same scFvs tested against other antigens, in particular against MBP protein, used for purification of the TAU fragment used for preselection in vitro. b) Interaction in vivo between the bait used (lexA-TAU-151-422) and a panel of scFvs isolated using IACT. Only scFv #2, #14 and #52 were capable of transactivating the reporter genes HIS3 and lacZ (first, second and fourth line) in contrast to the scFvs #37 and #85 (third and fourth line). The interaction pair scFvF8 + AMCVp41 was used as positive control (Visintin, M. et al. 1999) (sixth line). c) Soluble fractions of scFv #2, #14 and #52 extracted from the periplasmic space of E. coli were assayed in ELISA. The antigens were preadsorbed on the plate at a concentration of 10 µg/ml. The ELISA signals were measured at OD 450. d) Gel filtration chromatogram with Superdex-75 analytical column, of scFv #2, #14, #52 and scFvαD11. The elution volumes and the masses of the proteins used as markers are shown in the diagram: Ovalbumin (Oalb) (45 kDa), Bovine Carbonic Anhydrase (BCA) (29 kDa), Myosin (Myo) (17 kDa). The scFv in monomeric form flowed at about 12 ml. As a representative example, the chromatogram of a gel filtration analysis of scFvαD11 shows that the elution peaks of dimeric or polymeric species (aggregates) are eluted after about 10.5 ml.

Fig. 9. Immunofluorescence microscopy of the anti-TAU isolated with IACT (scFv #2, #14 and #52) and of the control scFvαD11. The scFvs are expressed in the cytoplasm of the COS fibroblasts. The cells were treated with an anti-myc tag antibody (Evan, G.I. et al. 1985), followed by incubation with a fluoresceinated anti-mouse monoclonal antibody (vector).

Fig. 10. A) Double immunofluorescence. The mislocalizations of TAU in the presence of the scFv-anti-TAU in the CHO cells were analysed. Marking of TAU (expressed in the cytoplasm) was effected using a (monoclonal) anti-TAU 7.51 antibody and using, as secondary antibody, an anti-mouse antibody conjugated with Texas-RED

(stains red). Transfection of the scFvs (scFv #2, #14 and #52) and scFvR4 (Martineau, P. et al. 1998) cloned in a nuclear expression vector, was detected with the anti-myc tag 9E10 (Evan, G.I. et al. 1985) followed by the fluoresceinated anti-mouse secondary antibody (vector). The co-expression of the scFvs together with TAU was analysed using an anti-TAU antibody (7.51) followed by an anti-mouse secondary antibody marked with Texas-RED (for localising TAU) and using a polyclonal antibody anti-myc tag, recognised in its turn by a fluoresceinated anti-rabbit. The cotransfected cells were visualised in the microscope using a variable wavelength filter (Zeiss Filter Set 25). The arrows indicate colocalization of the scFv-anti-TAU and of TAU in the nucleus. B) and C) Effect of retargeting of TAU in the CHO cells by scFv #2, #14, #52 and the control R4. The pattern of staining of TAU and of the scFvs in the cotransfected cells was subdivided into four classes, in accordance with analysis of the frequency observed for each group. Patterns 1), 2) and 3) are indicative of interaction between TAU and a scFv.

Fig. 11. Alignments of the VH (A) and VL (B) domains of the various anti-TAU selected with IACT. The CDRs are in bold type. At the base of panels (A) and (B), the ICSs at 100% of this particular set of intrabodies are shown. Panel (C) shows the optimum ICSs for the human and mouse variable chains and those extrapolated between man and mouse (hxm). The numbering is according to Chothia.

Fig. 12. Distribution of Ps for the human sequences. The infinite product in the calculation of Ps was extended just to the positions defined in the ICS.

Fig. 13. Distribution of the level of homology of the sequences in the various subgroups of the Kabat database, in relation to the corresponding consensus sequence. Analysis was restricted to the positions corresponding to the conserved residues 76 (part a) and 48 (part b) and c) for the heavy and light chain respectively for the IACT set. The number of amino acid residues homologous with the consensus sequence of the corresponding Kabat database is shown on the abscissa: a) in black, human VH (set 1, 3319 sequences), in light grey, mouse VH (set 2, 3353 sequences), in dark grey human VH 3 (set 3); b) in black, human VL (set 4, 2731 sequences), in light grey, mouse VL (set 5, 2518 sequences); c) in

black, human VK (set 6, 1330 sequences), in dark grey, human V λ (set 7, 1265 sequences). The lines that end in dots indicate the degree of homology of the IACT consensus sequence, for each subset of the database.

Fig. 14. Diagram showing how the IACT technology acts as a filter that brings a fraction of amino acids of the input sequences closer to the sequence of greatest consensus of the set.

Fig. 15. (b) Distribution of the degree of homology with ICS sequences, in the VIDA subsets and in the respective Kabat subsets. (a) Distribution of the degree of homology with sequences of maximum consensus of the Kabat, in the VIDA subsets and in the respective Kabat subsets. The abscissa shows the number of amino acids identical to the ICSs.

Detailed Description of the invention

General Techniques

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridisation techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel et al., *Short Protocols in Molecular Biology* (1999) 4th Ed, John Wiley & Sons, Inc. which are incorporated herein by reference) and chemical methods. In addition Harlow & Lane, *A Laboratory Manual* Cold Spring Harbor, N.Y, is referred to for standard Immunological Techniques.

Method of selecting immunoglobulins which bind to their ligand within an intracellular environment.

The Intracellular antibody capture technology

A suitable method for the selection of immunoglobulins which bind to their ligand within an intracellular environment is described by the present inventors and detailed in WO00/54057 which is herein incorporated by reference.

Generally, it is difficult to obtain antibody fragments which bind to antigen *in vivo* because antibodies are not equipped to function in a reducing environment such as the cell cytoplasm (Martineau, P., Jones, P. & Winter, G. (1998), *J. Mol. Biol.* 280, 117–127; Proba, K., Ge, L. & Pluckthun, A. (1995), Functional antibody single-chain fragments from the cytoplasm of *Escherichia coli* in the presence of thioredoxin reductase (TrxB). *Gene*, 159, 203±207.). The intracellular antibody capture approach described WO00/54057 constitutes a generic strategy for selection and intracellular characterisation of antigen-specific scFv antibody fragments. By employing this strategy, the present inventors have identified immunoglobulin molecules which bind specifically to a ligand within an intracellular environment.

The IAC technology described in WO00/54057, includes one round of scFv phage display library screening *in vitro* with a recombinant bacterial protein, followed by selection in a yeast *in vivo* antibody–antigen interaction screening of the *in vitro* enriched scFv repertoire (Visintin, M., Tse, E., Axelson, H., Rabbitts, T.H. and Cattaneo, A. (1999) *Proc. Natl. Acad. Sci. USA* 96 11723-11728)

Those skilled in the art will appreciate that there are other suitable methods for the selection of immunoglobulin molecules which bind selectively to their ligand within the cell.

Intracellularly binding Immunoglobulins

Immunoglobulin molecules, used according to the present invention include members of the immunoglobulin superfamily, which are a family of polypeptides which comprise the immunoglobulin fold characteristic of antibody molecules. The fold contains two beta sheets and, usually, a conserved disulphide bond. Members of the immunoglobulin superfamily are involved in many aspects of cellular and non-cellular interactions *in vivo*,

including widespread roles in the immune system (for example, antibodies, T-cell receptor molecules and the like), involvement in cell adhesion (for example the ICAM molecules) and intracellular signalling (for example, receptor molecules, such as the PDGF receptor). The present invention is applicable to all immunoglobulin superfamily molecules which are capable of binding to target molecules. Preferably, the present invention relates to antibodies, and scFv fragments.

The immunoglobulins molecules used according to the present invention all possess the requisite activity of being capable of selectively binding to a ligand within an intracellular environment.

Advantageously, immunoglobulin molecules according to the invention all share a V_H amino acid sequence which is a member of the VHIII subgroup of heavy chains. This suggests that immunoglobulins having a heavy chain which falls within the VHIII subgroup have particularly high efficacy in an in vivo environment. In addition, more advantageously, the immunoglobulin molecules according to the present invention have a VHIII subgroup joined to JH5 or J_k1 region.

The present inventors have surprisingly found however that it is not sufficient for an immunoglobulin molecule to have a heavy chain variable region which is a member of the VHIII subgroup of heavy chains in order for it to be a good intracellular antibody. In fact, it has been found in some cases that anti-TAU antibodies of the VHIII subgroup, isolated at random from the library, and with good properties of binding with TAU in vitro, are incapable of binding TAU in vivo.

Most of the variability between the sequences of the present invention is concentrated within the CDRs, consistent with the view that the framework regions of the molecules confer structural stability on the immunoglobulin molecules, whilst the CDRs are involved in ligand binding. However, a high degree of conservation is present in these regions, consistent with the view that residues within these regions also contribute to the efficacy of binding within an in vivo environment.

The immunoglobulins according to the invention are especially useful for diagnostic and therapeutic applications. Accordingly, they may be altered immunoglobulins comprising an effector protein such as a toxin or a label. Especially preferred are labels which allow the imaging of the distribution of the immunoglobulins in vivo. Such labels may be radioactive labels or radioopaque labels, such as metal particles, which are readily visualisable within the body of a patient. Moreover, they may be fluorescent labels or other labels which are visualisable on tissue samples removed from patients.

Recombinant DNA technology may be used to produce the immunoglobulins for use according to the present invention using an established procedure, in bacterial or preferably mammalian cell culture. The selected cell culture system preferably secretes the immunoglobulin product.

Multiplication of hybridoma cells or mammalian host cells in vitro is carried out in suitable culture media, which are the customary standard culture media, for example Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium, optionally replenished by a mammalian serum, e.g. foetal calf serum, or trace elements and growth sustaining supplements, e.g. feeder cells such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages, 2-aminoethanol, insulin, transferrin, low density lipoprotein, oleic acid, or the like. Multiplication of host cells which are bacterial cells or yeast cells is likewise carried out in suitable culture media known in the art, for example for bacteria in medium LB, NZCYM, NZYM, NZM, Terrific Broth, SOB, SOC, 2 x YT, or M9 Minimal Medium, and for yeast in medium YPD, YEPD, Minimal Medium, or Complete Minimal Dropout Medium.

In vitro production provides relatively pure immunoglobulin preparations and allows scale-up to give large amounts of the desired immunoglobulins. Techniques for bacterial cell, yeast or mammalian cell cultivation are known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or

immobilised or entrapped cell culture, e.g. in hollow fibres, microcapsules, on agarose microbeads or ceramic cartridges.

Large quantities of the desired immunoglobulins can also be obtained by multiplying mammalian cells *in vivo*. For this purpose, hybridoma cells producing the desired immunoglobulins are injected into histocompatible mammals to cause growth of antibody-producing tumours. Optionally, the animals are primed with a hydrocarbon, especially mineral oils such as pristane (tetramethyl-pentadecane), prior to the injection. After one to three weeks, the immunoglobulins are isolated from the body fluids of those mammals. For example, hybridoma cells obtained by fusion of suitable myeloma cells with antibody-producing spleen cells from Balb/c mice, or transfected cells derived from hybridoma cell line Sp2/0 that produce the desired antibodies are injected intraperitoneally into Balb/c mice optionally pre-treated with pristane, and, after one to two weeks, ascitic fluid is taken from the animals.

The foregoing, and other, techniques are discussed in, for example, Kohler and Milstein, (1975) *Nature* 256:495-497; US 4,376,110; Harlow and Lane, *Antibodies: a Laboratory Manual*, (1988) Cold Spring Harbor, incorporated herein by reference. Techniques for the preparation of recombinant antibody molecules is described in the above references and also in, for example, EP 0623679; EP 0368684 and EP 0436597, which are incorporated herein by reference.

The cell culture supernatants are screened for the desired immunoglobulins, preferentially by immunofluorescent staining of cells expressing the desired target by immunoblotting, by an enzyme immunoassay, e.g. a sandwich assay or a dot-assay, or a radioimmunoassay.

For isolation of the immunoglobulins, those present in the culture supernatants or in the ascitic fluid may be concentrated, e.g. by precipitation with ammonium sulphate, dialysis against hygroscopic material such as polyethylene glycol, filtration through selective membranes, or the like. If necessary and/or desired, the antibodies are purified by the

customary chromatography methods, for example gel filtration, ion-exchange chromatography, chromatography over DEAE-cellulose and/or (immuno-)affinity chromatography, e.g. affinity chromatography with the target molecule or with Protein-A.

The invention employs recombinant nucleic acids comprising an insert coding for a heavy chain variable domain and/or for a light chain variable domain of antibodies. By definition such nucleic acids comprise coding single stranded nucleic acids, double stranded nucleic acids consisting of said coding nucleic acids and of complementary nucleic acids thereto, or these complementary (single stranded) nucleic acids themselves.

Furthermore, nucleic acids encoding a heavy chain variable domain and/or for a light chain variable domain of antibodies can be enzymatically or chemically synthesised from nucleic acids having the authentic sequence coding for a naturally-occurring heavy chain variable domain and/or for the light chain variable domain, or a variant or derivative thereof as herein described. Preferably said modification(s) are outside the CDRs of the heavy chain variable domain and/or of the light chain variable domain of the antibody.

Identity/homology

It will be understood that polypeptide sequences of the invention are not limited to the particular sequences set forth in SEQ. ID. No. 1 to 40 or fragments thereof, but also include homologous sequences obtained from any source, for example related cellular homologues, homologues from other species and variants or derivatives thereof.

Thus, the present invention encompasses variants, homologues or derivatives of the amino acid sequences set forth in SEQ. ID. No. 1 to SEQ 40 as long as when said variants, homologues or derivatives of the amino acid sequences set forth in SEQ. ID. No. 1 to SEQ 40 are one or more components of a immunoglobulin molecule, they possess the requisite activity of binding selectively to a ligand within an intracellular environment.

In the context of the present invention, a homologous sequence is taken to include an amino acid sequence which is at least 94, 95, 96, 97, 98, 99% identical at the amino acid level over at least 30, preferably 50, 70, 90 or 100 amino acids. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues (for example less than 50 contiguous amino acids).

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each

subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, *Nucleic Acids Research* 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Atschul *et al.*, 1990, *J. Mol. Biol.*, 403-410) and the GENWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program.

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

Method for conferring upon an immunoglobulin molecule the ability to selectively bind to a ligand within an intracellular environment.

In a further aspect, the present invention provides a method for conferring upon an immunoglobulin molecule the ability to function within an intracellular environment comprising the steps of:

- a) identifying the optimum ICS reference sequence;
- b) optionally, modifying, by site-specific mutagenesis, the amino acid residues that are located in the positions defined by the optimum ICS, or a subset of these residues, in such a way that they are those identified by the optimum ICS.

Ligands

Potential ligands include polypeptides and proteins, particularly nascent polypeptides and proteins or intracellular polypeptide or protein precursors, which are present in the cell. Advantageously, the ligand is a mutant polypeptide or protein, such as a polypeptide or protein generated through genetic mutation, including point mutations, deletions and chromosomal translocations. Such polypeptides are frequently involved in tumourigenesis. Examples include the gene product produced by the spliced BCR-ABL genes. The invention is moreover applicable to all mutated oncogene products, all chromosomal translocated oncogene products (especially fusion proteins), aberrant proteins in expressed in disease, and viral or bacterial specific proteins expressed as a result of infection.

The ligand may alternatively be an RNA molecule, for example a precursor RNA or a mutant RNA species generated by genetic mutation or otherwise.

The ligand may be inserted into the cell, for example as described below, or may be endogenous to the cell.

Delivery of immunoglobulins and ligands to cells

Generally the immunoglobulin will be delivered to the cell. The ligand as herein defined may be a native component of the cell as described above, or may also be delivered to the cell.

In order to introduce immunoglobulins and ligands/target molecules into an intracellular environment, cells are advantageously transfected with nucleic acids which encode the immunoglobulins and/or their ligands.

Nucleic acids encoding immunoglobulins and/or ligands can be incorporated into vectors for expression. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for expression thereof. Selection and use of such vehicles are well within the skill of the artisan. Many vectors are available, and selection of appropriate vector will depend on the intended use of the vector, the size of the nucleic acid to be inserted into the vector, and the host cell to be transformed with the vector. Each vector contains various components depending on its function and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: an origin of replication, one or more marker genes, an enhancer element, a promoter, a transcription termination sequence and a signal sequence.

Moreover, nucleic acids encoding the immunoglobulins and/or targets according to the invention may be incorporated into cloning vectors, for general manipulation and nucleic acid amplification purposes.

Both expression and cloning vectors generally contain nucleic acid sequence that enable the vector to replicate in one or more selected host cells. Typically in cloning vectors, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses.

The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2m plasmid origin is suitable for yeast, and various viral origins (e.g. SV 40, polyoma, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors unless these are used in mammalian cells competent for high level DNA replication, such as COS cells.

Most expression vectors are shuttle vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another class of organisms for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells even though it is not capable of replicating independently of the host cell chromosome. DNA may also be replicated by insertion into the host genome. However, the recovery of genomic DNA is more complex than that of exogenously replicated vector because restriction enzyme digestion is required to excise the nucleic acid. DNA can be amplified by PCR and be directly transfected into the host cells without any replication component.

Advantageously, an expression and cloning vector may contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available from complex media.

As to a selective gene marker appropriate for yeast, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker gene. Suitable markers for yeast are, for example, those conferring resistance to antibiotics G418, hygromycin or bleomycin, or provide for prototrophy in an auxotrophic yeast mutant, for example the URA3, LEU2, LYS2, TRP1, or HIS3 gene.

Since the replication of vectors is conveniently done in *E. coli*, an *E. coli* genetic marker and an *E. coli* origin of replication are advantageously included. These can be obtained from *E. coli* plasmids, such as pBR322, Bluescript© vector or a pUC plasmid, e.g. pUC18 or pUC19, which contain both an *E. coli* replication origin and an *E. coli* genetic marker conferring resistance to antibiotics, such as ampicillin.

Suitable selectable markers for mammalian cells are those that enable the identification of cells expressing the desired nucleic acid, such as dihydrofolate reductase (DHFR, methotrexate resistance), thymidine kinase, or genes conferring resistance to G418 or hygromycin. The mammalian cell transformants are placed under selection pressure which only those transformants which have taken up and are expressing the marker are uniquely adapted to survive. In the case of a DHFR or glutamine synthase (GS) marker, selection pressure can be imposed by culturing the transformants under conditions in which the pressure is progressively increased, thereby leading to amplification (at its chromosomal integration site) of both the selection gene and the linked nucleic acid. Amplification is the process by which genes in greater demand for the production of a protein critical for growth, together with closely associated genes which may encode a desired protein, are reiterated in tandem within the chromosomes of recombinant cells. Increased quantities of desired protein are usually synthesised from thus amplified DNA.

Expression and cloning vectors usually contain a promoter that is recognised by the host organism and is operably linked to the desired nucleic acid. Such a promoter may be inducible or constitutive. The promoters are operably linked to the nucleic acid by removing the promoter from the source DNA and inserting the isolated promoter sequence into the vector. Both the native promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of nucleic acid encoding the immunoglobulin or target molecule. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

Promoters suitable for use with prokaryotic hosts include, for example, the beta-lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (*trp*) promoter system and hybrid promoters such as the *tac* promoter. Their nucleotide sequences have been published, thereby enabling the skilled worker operably to ligate them a desired nucleic acid, using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems will also generally contain a Shine-Delgarno sequence operably linked to the nucleic acid.

Preferred expression vectors are bacterial expression vectors which comprise a promoter of a bacteriophage such as phage λ or T7 which is capable of functioning in the bacteria. In one of the most widely used expression systems, the nucleic acid encoding the fusion protein may be transcribed from the vector by T7 RNA polymerase (Studier et al, *Methods in Enzymol.* 185; 60-89, 1990). In the *E. coli* BL21(DE3) host strain, used in conjunction with pET vectors, the T7 RNA polymerase is produced from the lysogen DE3 in the host bacterium, and its expression is under the control of the IPTG inducible *lac* UV5 promoter. This system has been employed successfully for over-production of many proteins. Alternatively the polymerase gene may be introduced on a lambda phage by infection with an int- phage such as the CE6 phage which is commercially available (Novagen, Madison, USA). other vectors include vectors containing the lambda PL promoter such as PLEX (Invitrogen, NL) , vectors containing the *trc* promoters such as pTrcHisXpressTm (Invitrogen) or pTrc99 (Pharmacia Biotech, SE) , or vectors containing the *tac* promoter such as pKK223-3 (Pharmacia Biotech) or PMAL (new England Biolabs, MA, USA).

Suitable promoting sequences for use with yeast hosts may be regulated or constitutive and are preferably derived from a highly expressed yeast gene, especially a *Saccharomyces cerevisiae* gene. Thus, the promoter of the TRP1 gene, the ADHI or ADHII gene, the acid phosphatase (PH05) gene, a promoter of the yeast mating pheromone genes coding for the α - or alpha-factor or a promoter derived from a gene encoding a glycolytic enzyme such as the promoter of the enolase, glyceraldehyde-3-

phosphate dehydrogenase (GAP), 3-phospho glycerate kinase (PGK), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triose phosphate isomerase, phosphoglucose isomerase or glucokinase genes, the *S. cerevisiae* GAL 4 gene, the *S. pombe* nmt 1 gene or a promoter from the TATA binding protein (TBP) gene can be used. Furthermore, it is possible to use hybrid promoters comprising upstream activation sequences (UAS) of one yeast gene and downstream promoter elements including a functional TATA box of another yeast gene, for example a hybrid promoter including the UAS(s) of the yeast PH05 gene and downstream promoter elements including a functional TATA box of the yeast GAP gene (PH05-GAP hybrid promoter). A suitable constitutive PH05 promoter is e.g. a shortened acid phosphatase PH05 promoter devoid of the upstream regulatory elements (UAS) such as the PH05 (-173) promoter element starting at nucleotide -173 and ending at nucleotide -9 of the PH05 gene.

Gene transcription from vectors in mammalian hosts may be controlled by promoters derived from the genomes of viruses such as polyoma virus, adenovirus, fowlpox virus, bovine papilloma virus, avian sarcoma virus, cytomegalovirus (CMV), a retrovirus and Simian Virus 40 (SV40), from heterologous mammalian promoters such as the actin promoter or a very strong promoter, e.g. a ribosomal protein promoter, and from promoters normally associated with immunoglobulin sequences.

Transcription of a nucleic acid by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are relatively orientation and position independent. Many enhancer sequences are known from mammalian genes (e.g. elastase and globin). However, typically one will employ an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270) and the CMV early promoter enhancer. The enhancer may be spliced into the vector at a position 5' or 3' to the desired nucleic acid, but is preferably located at a site 5' from the promoter.

Advantageously, a eukaryotic expression vector may comprise a locus control region (LCR). LCRs are capable of directing high-level integration site independent expression of transgenes integrated into host cell chromatin, which is of importance especially where the gene is to be expressed in the context of a permanently-transfected eukaryotic cell line in which chromosomal integration of the vector has occurred.

Eukaryotic expression vectors will also contain sequences necessary for the termination of transcription and for stabilising the mRNA. Such sequences are commonly available from the 5' and 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the immunoglobulin or the target.

Particularly useful for practising the present invention are expression vectors that provide for the transient expression of nucleic acids in mammalian cells. Transient expression usually involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector, and, in turn, synthesises high levels of the desired gene product.

Construction of vectors according to the invention may employ conventional ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required. If desired, analysis to confirm correct sequences in the constructed plasmids is performed in a known fashion. Suitable methods for constructing expression vectors, preparing in vitro transcripts, introducing DNA into host cells, and performing analyses for assessing gene product expression and function are known to those skilled in the art. Gene presence, amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA, dot blotting (DNA or RNA analysis), or in situ hybridisation, using an appropriately labelled probe which may be based on a sequence provided herein. Those skilled in the art will readily envisage how these methods may be modified, if desired.

Immunoglobulins and/or ligands may be directly introduced to the cell by microinjection, or delivery using vesicles such as liposomes which are capable of fusing with the cell membrane. Viral fusogenic peptides are advantageously used to promote membrane fusion and delivery to the cytoplasm of the cell.

Preparation of a library using V_H and V_L sequences of the present invention

In yet a further aspect, the present invention provides a library, wherein the library is generated using any one or more of the variable heavy domain amino acid sequences (V_H) selected from the group consisting of: a V_H amino acid sequence showing at least 85% identity with the consensus sequence depicted as SEQ 3 and shown in fig 5a, a V_H sequence which is described by the consensus sequence depicted in SEQ 41 and shown in fig 11a,

These libraries may encode, express, and/or present immunoglobulin molecules or fragments thereof which may be tested for their ability to interact with a ligand within an intracellular environment. Advantageously, the library of the present aspect of the invention may be tested for the binding of immunoglobulin molecules expressed using the intracellular antibody capture method described in WO00/54057. Advantageously, libraries of the present invention will encode or express V_H and V_L chains which when incorporated within an immunoglobulin molecule will bind selectively to a ligand within an intracellular environment. Preferably the immunoglobulin is scFv.

Systems, in which diverse peptide sequences are displayed on the surface of filamentous bacteriophage (Scott and Smith (1990 *supra*), have proven useful for creating libraries of antibody fragments (and the nucleotide sequences that encoding them) for the *in vitro* selection and amplification of specific antibody fragments that bind a target antigen. The nucleotide sequences encoding the V_H and V_L regions are linked to gene fragments which encode leader signals that direct them to the periplasmic space of *E. coli* and as a result the resultant antibody fragments are displayed on the surface of the bacteriophage, typically as fusions to bacteriophage coat proteins (e.g., pIII or pVIII). Alternatively, antibody fragments are displayed externally on lambda phage capsids (phagebodies). An

advantage of phage-based display systems is that, because they are biological systems, selected library members can be amplified simply by growing the phage containing the selected library member in bacterial cells. Furthermore, since the nucleotide sequence that encode the polypeptide library member is contained on a phage or phagemid vector, sequencing, expression and subsequent genetic manipulation is relatively straightforward.

Methods for the construction of bacteriophage antibody display libraries and lambda phage expression libraries are well known in the art (McCafferty *et al.* (1990) *supra*; Kang *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.*, **88**: 4363; Clackson *et al.* (1991) *Nature*, **352**: 624; Lowman *et al.* (1991) *Biochemistry*, **30**: 10832; Burton *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.*, **88**: 10134; Hoogenboom *et al.* (1991) *Nucleic Acids Res.*, **19**: 4133; Chang *et al.* (1991) *J. Immunol.*, **147**: 3610; Breitling *et al.* (1991) *Gene*, **104**: 147; Marks *et al.* (1991) *supra*; Barbas *et al.* (1992) *supra*; Hawkins and Winter (1992) *J. Immunol.*, **22**: 867; Marks *et al.*, 1992, *J. Biol. Chem.*, **267**: 16007; Lerner *et al.* (1992) *Science*, **258**: 1313, incorporated herein by reference).

Alternative library selection technologies include bacteriophage lambda expression systems, which may be screened directly as bacteriophage plaques or as colonies of lysogens, both as previously described (Huse *et al.* (1989) *Science*, **246**: 1275; Caton and Koprowski (1990) *Proc. Natl. Acad. Sci. U.S.A.*, **87**; Mullinax *et al.* (1990) *Proc. Natl. Acad. Sci. U.S.A.*, **87**: 8095; Persson *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.*, **88**: 2432) and are of use in the invention. Whilst such expression systems can be used to screening up to 10^6 different members of a library, they are not really suited to screening of larger numbers (greater than 10^6 members). Other screening systems rely, for example, on direct chemical synthesis of library members. One early method involves the synthesis of peptides on a set of pins or rods, such as described in WO84/03564. A similar method involving peptide synthesis on beads, which forms a peptide library in which each bead is an individual library member, is described in U.S. Patent No. 4,631,211 and a related method is described in WO92/00091. A significant improvement of the bead-based methods involves tagging each bead with a unique identifier tag, such as an

oligonucleotide, so as to facilitate identification of the amino acid sequence of each library member. These improved bead-based methods are described in WO93/06121.

Another chemical synthesis method involves the synthesis of arrays of peptides (or peptidomimetics) on a surface in a manner that places each distinct library member (e.g., unique peptide sequence) at a discrete, predefined location in the array. The identity of each library member is determined by its spatial location in the array. The locations in the array where binding interactions between a predetermined molecule (e.g., a receptor) and reactive library members occur is determined, thereby identifying the sequences of the reactive library members on the basis of spatial location. These methods are described in U.S. Patent No. 5,143,854; WO90/15070 and WO92/10092; Fodor *et al.* (1991) *Science*, **251**: 767; Dower and Fodor (1991) *Ann. Rep. Med. Chem.*, **26**: 271.

Other systems for generating libraries of polypeptides or nucleotides involve the use of cell-free enzymatic machinery for the *in vitro* synthesis of the library members. In one method, RNA molecules are selected by alternate rounds of selection against a target ligand and PCR amplification (Tuerk and Gold (1990) *Science*, **249**: 505; Ellington and Szostak (1990) *Nature*, **346**: 818). A similar technique may be used to identify DNA sequences which bind a predetermined human transcription factor (Thiesen and Bach (1990) *Nucleic Acids Res.*, **18**: 3203; Beaudry and Joyce (1992) *Science*, **257**: 635; WO92/05258 and WO92/14843). In a similar way, *in vitro* translation can be used to synthesise polypeptides as a method for generating large libraries. These methods which generally comprise stabilised polysome complexes, are described further in WO88/08453, WO90/05785, WO90/07003, WO91/02076, WO91/05058, and WO92/02536. Alternative display systems which are not phage-based, such as those disclosed in WO95/22625 and WO95/11922 (Affymax) use the polysomes to display polypeptides for selection. These and all the foregoing documents also are incorporated herein by reference.

Practically the major advantage in the generation of libraries from the consensus sequences as herein described is that their production will obviate the use of phage scFv

libraries and will reduce the necessary library size required for use in intracellular antibody capture technology.

Uses of immunoglobulins of the present invention

Immunoglobulin molecules according to the present invention, preferably scFv molecules may be employed in in vivo therapeutic and prophylactic applications, in vitro and in vivo diagnostic applications, in vitro assay and reagent applications, in functional genomics applications and the like.

Therapeutic and prophylactic uses of immunoglobulins and compositions according to the invention involve the administration of the above to a recipient mammal, such as a human. Preferably they involve the administration to the intracellular environment of a mammal.

Substantially pure immunoglobulins of at least 90 to 95% homogeneity are preferred for administration to a mammal, and 98 to 99% or more homogeneity is most preferred for pharmaceutical uses, especially when the mammal is a human. Once purified, partially or to homogeneity as desired, the immunoglobulin molecules may be used diagnostically or therapeutically (including extracorporeally) or in developing and performing assay procedures using methods known to those skilled in the art.

In the instant application, the term "prevention" involves administration of the protective composition prior to the induction of the disease. "Suppression" refers to administration of the composition after an inductive event, but prior to the clinical appearance of the disease. "Treatment" involves administration of the protective composition after disease symptoms become manifest.

The selected immunoglobulin molecules of the present invention can perturb protein function in vivo and thus will typically find use in preventing, suppressing or treating inflammatory states, allergic hypersensitivity, cancer, bacterial or viral infection, and autoimmune disorders (which include, but are not limited to, Type I diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease and

myasthenia gravis), and in preventing transplant rejection. For instance, one application of the intracellular immunoglobulins of the present invention is in perturbing the function of oncogenic proteins, in particular fusion molecules which result chromosomal translocations. These molecules are of particular interest as they are tumour-specific proteins only occurring in the progeny of cell which acquired the chromosomal translocation. A notable example is the BCR-ABL hybrid fusion protein found in CML (Chronic myelogenous leukaemia) and a proportion of ALL (Acute lymphoblastic leukaemia) carrying translocation t(9;22)(q34;q11) (de Klein, A. et al. (1982) A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. *Nature*, **300**, 765–767. Bartram, C.R. et al. (1983) Translocation of c-abl oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. *Nature*, **306**, 277–280.)

The present inventors have selected scFv directed to the SH2 domain of ABL or the SH2 binding domain of the BCR protein, which are found in both p190 and p210 BCR-ABL fusion proteins. A panel of different scFv which bind BCR-ABL in vivo have been found by the present inventors..

The antigen-specific scFv according to the present invention may be used as therapeutic agents in Philadelphia chromosome positive leukaemias. It has been shown that the SH2 binding domain of the BCR protein is essential for the transforming properties of the BCR-ABL oncogenic protein BCRsh2bd. Therefore blocking the function of this domain may neutralise the oncogenicity of BCR-ABL. In addition, the scFv have the potential to be employed, in combination with anti-ABL scFv, in an intracellular antibody mediated cell killing approach (Eric Tse and Terence H. Rabbitts Intracellular antibody-caspase-mediated cell killing: An approach for application in cancer therapy *Proc. Natl. Acad. Sci. USA* 97: 12266-12271.. Using this approach, cells carrying the BCR-ABL proteins could be specifically killed, sparing the normal ones.

Animal model systems which can be used to screen the effectiveness of the selected immunoglobulins of the present invention in protecting against or treating disease are available. Methods for the testing of systemic lupus erythematosus (SLE) in susceptible

mice are known in the art (Knight et al. (1978) *J. Exp. Med.*, 147: 1653; Reinersten et al. (1978) *New Eng. J. Med.*, 299: 515). Myasthenia Gravis (MG) is tested in SJL/J female mice by inducing the disease with soluble AchR protein from another species (Lindstrom et al. (1988) *Adv. Immunol.*, 42: 233). Arthritis is induced in a susceptible strain of mice by injection of Type II collagen (Stuart et al. (1984) *Ann. Rev. Immunol.*, 42: 233). A model by which adjuvant arthritis is induced in susceptible rats by injection of mycobacterial heat shock protein has been described (Van Eden et al. (1988) *Nature*, 331: 171). Thyroiditis is induced in mice by administration of thyroglobulin as described (Maron et al. (1980) *J. Exp. Med.*, 152: 1115). Insulin dependent diabetes mellitus (IDDM) occurs naturally or can be induced in certain strains of mice such as those described by Kanasawa et al. (1984) *Diabetologia*, 27: 113. EAE in mouse and rat serves as a model for MS in human. In this model, the demyelinating disease is induced by administration of myelin basic protein (see Paterson (1986) *Textbook of Immunopathology*, Mischer et al., eds., Grune and Stratton, New York, pp. 179-213; McFarlin et al. (1973) *Science*, 179: 478: and Satoh et al. (1987) *J. Immunol.*, 138: 179).

Generally, the selected immunoglobulins of the present invention will be utilised in purified form together with pharmacologically appropriate carriers. Typically, these carriers include aqueous or alcoholic/aqueous solutions, emulsions or suspensions, any including saline and/or buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride and lactated Ringer's. Suitable physiologically-acceptable adjuvants, if necessary to keep a polypeptide complex in suspension, may be chosen from thickeners such as carboxymethylcellulose, polyvinylpyrrolidone, gelatin and alginates.

Intravenous vehicles include fluid and nutrient replenishers and electrolyte replenishers, such as those based on Ringer's dextrose. Preservatives and other additives, such as antimicrobials, antioxidants, chelating agents and inert gases, may also be present (Mack (1982) *Remington's Pharmaceutical Sciences*, 16th Edition).

The selected immunoglobulins of the present invention may be used as separately administered compositions or in conjunction with other agents. These can include various immunotherapeutic drugs, such as cyclosporine, methotrexate, adriamycin or cisplatinum, and immunotoxins. Pharmaceutical compositions can include "cocktails" of various cytotoxic or other agents in conjunction with the chemokines, or binding proteins thereof, or T-cells of the present invention or even combinations of selected chemokines, or binding proteins thereof, according to the present invention.

The route of administration of pharmaceutical compositions according to the invention may be any of those commonly known to those of ordinary skill in the art. For therapy, including without limitation immunotherapy, the selected antibodies, receptors or binding proteins thereof of the invention can be administered to any patient in accordance with standard techniques. The administration can be by any appropriate mode, including parenterally, intravenously, intramuscularly, intraperitoneally, transdermally, via the pulmonary route, or also, appropriately, by direct infusion with a catheter. The dosage and frequency of administration will depend on the age, sex and condition of the patient, concurrent administration of other drugs, counterindications and other parameters to be taken into account by the clinician.

The selected immunoglobulins of the present invention can be lyophilised for storage and reconstituted in a suitable carrier prior to use. Known lyophilisation and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilisation and reconstitution can lead to varying degrees of functional activity loss and that use levels may have to be adjusted upward to compensate.

The compositions containing the present selected immunoglobulins of the present invention or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In certain therapeutic applications, an adequate amount to accomplish at least partial inhibition, suppression, modulation, killing, or some other measurable parameter, of a population of selected cells is defined as a "therapeutically-effective dose". Amounts needed to achieve this dosage will depend upon the severity of the disease and the general

state of the patient's own immune system, but generally range from 0.005 to 5.0 mg of selected immunoglobulin per kilogram of body weight, with doses of 0.05 to 2.0 mg/kg/dose being more commonly used. For prophylactic applications, compositions containing the present selected immunoglobulin molecules or cocktails thereof may also be administered in similar or slightly lower dosages.

A composition containing one or more selected immunoglobulin molecules according to the present invention may be utilised in prophylactic and therapeutic settings to aid in the alteration, inactivation, killing or removal of a select target cell population in a mammal. In addition, the selected repertoires of polypeptides described herein may be used extracorporeally or in vitro selectively to kill, deplete or otherwise effectively remove a target cell population from a heterogeneous collection of cells. Blood from a mammal may be combined extracorporeally with the selected antibodies, cell-surface receptors or binding proteins thereof whereby the undesired cells are killed or otherwise removed from the blood for return to the mammal in accordance with standard techniques.

The invention is further described, for the purposes of illustration only, in the following examples.

Examples

Strategy (A)

Example 1

Bacterial protein expression and purification of antigens

BCR antigen: A plasmid for expression of histidine tagged SH2 binding domain of BCR in bacteria, pRSET-BCRSH2BD, was made by amplifying the sequences encoding amino acids 185 – 417 of BCR and cloning the PCR product into mini pRSET vector (a gift from O. Perisic) as BamHI – EcoRI fragment.

ABL antigen: A plasmid for bacterial expression of histidine tagged ABL protein SH2 domain (amino acid 26 – 348) (pRSET-ABL) was constructed by PCR of the corresponding sequences and subcloning the PCR product as BamHI – EcoRI fragment

into mini pRSET vector. PCR conditions were 30 cycles of 1 minute each at 95°C, 50°C and 72°C using specific primers

(5'cagggatccgagcgcggcctggtgaag3'/5'caggaattcatcggtgggccagatctg3' for pRSET-BCRSH2BD and 5'cagggatccgaagcccttcagcggcca3'/5'caggaattccgagatctgagtgccat3' for pRSET-ABL) and pE1A2 (BCR-ABL p190, a gift from Dr. G. Grosveld) as template.

The plasmids were transformed into *E. coli* C41 bacteria (Tse, E., & Rabbitts, T. H (2000) Proc. Nat. Acad. Sci. USA 97, 12266-12271) and induction of protein was performed by adding 1mM IPTG to the exponentially growing bacterial culture (O.D.₆₀₀ 0.6) and by growth at 30°C for 4 hours. The histidine tagged proteins were purified using Ni-NTA agarose according to manufacturer's instructions (Qiagen). Concentration of the purified proteins was determined by using Bio-Rad Protein Assay Kit (BIO-RAD).

Example 2

In vitro scFv phage display library screening and preparation of specific scFv-VP16 yeast library

A detailed protocol for the IAC methodology is described elsewhere (Tse, E., Chung., G & Rabbitts, T., H K, Turksen, Editor, 2000, Humana Press: Totawa). Purified His-tagged SH2 binding domain of BCR protein or the SH2 domain of ABL protein were coated onto immunotubes (Nunc) at a concentration of 50µg/ml in PBS for overnight at 4°C. 2×10^{13} phage displaying scFv (Sheets, M. D., et al (1998) Proc. Nat. Acad Sci, USA 95, 6157-6162) were incubated with the antigen-coated tubes and the bound phage were eluted with 100mM triethylamine, neutralised by 1M Tris, pH 7.4 and were used to infect *E. coli* TG1 bacteria. The transduced bacteria were amplified by plating onto ampicillin supplemented agar plate from which phagemid DNA was extracted. The collected phagemid DNA was digested with SfiI and NotI restriction enzyme and the 700-800bp scFv DNA fragment was gel purified. The purified scFv (SfiI/NotI) DNA fragment was ligated to pVP16 vector and transformed into *E. coli* DH5α bacteria. 40 ligations were performed to obtain around 10^5 bacteria colonies after transformation. This generated the primary BCR-SH2BD and ABL-SH2 specific yeast scFv-VP16 libraries which were

amplified by plating onto ampicillin supplemented agar plate. DNA was extracted from the bacteria and used for yeast screening.

Example 3

In vivo antibody – antigen interaction screening in yeast

L40 Yeast (Statagene) was grown at 30°C for 3-4 days, in YAPD medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose, and 0.1% mg/ml adenine buffered at pH5.8) or in synthetic minimal YC medium (0.12% yeast nitrogen base, without amino acids and 0.5% ammonium sulphate, 0.1% succinic acid, 0.6% NaOH, 2% glucose and, as required, 2% agar) containing 0.075% amino acid supplements (lacking Trp, Leu, Ura, Lys, and His; 0.1% each of adenine sulphate, Arg, Cys, Thr; 0.05% each of Asp, Ile, Met, Phe, Pro, Ser, and Tyr) buffered at pH5.8. When necessary, 0.01% each of Trp, Ura, Lys, Leu and 0.005% His were supplemented to the media.

The bait antigen expressing plasmid comprised LexA linked to BCR-ABL (pBTM/E1A2) was made by subcloning the 4kb blunted EagI fragment of pE1A2 into BamHI blunted pBTM/116 vector. For yeast *in vivo* scFv library screening, 1mg of pBTM/E1A2 and 500µg of the yeast scFv-VP16AD (where AD is activation domain) library DNA were co-transformed into *Saccharomyces cerevisiae* L40 by lithium acetate transformation protocol (Gietz, D., St. Jean, A., Woods, R. A. & Schiestl, R. H. (1992). *Nucleic Acids Res.* **20**, 1425; Tse, E., Chung, G., & Rabbitts, T, H, K, Turksen, Editor, 2000, Humana Press: Totawa). Positive clones were selected by using auxotrophic markers for both plasmids and for histidine prototrophy. Histidine independent colonies were picked, re-streaked onto YC agar plates lacking Trp and Leu and assayed for β-gal activity by filter assay (Breedon, L. & Nasmyth, K. (1985) *Cold Spring Harb. Symp. Quant. Biol.* **50**, 643–650. .For re-testing the isolated scFv, individual scFv-VP16AD plasmids (200ng) were cotransformed with pBTM/E1A2 (500ng) into L40 yeast, and histidine prototrophy and β-gal activity were assayed. pBTM/AMCV (comprising the LexA DNA binding domain fused to the AMCV viral coat protein) used as a negative “bait” control was described previously (Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. & Galeffi, P. (1993) *Nature (London)* **366**, 469–472; Viscintin, M., Tse, E., Axelson, H.,

Rabbitts, T.H & Cattaneo, A., (1999) Proc. Nat. Acad. Sci, USA 96, 11723-11728).

Example 4

In vitro characterisation of ICABs

scFv was expressed as soluble bacterial periplasmic protein and used as primary antibodies for Western immunodetection. scFv DNA fragments were isolated from the scFv-VP16AD plasmid by SfiI – NotI restriction enzyme digestion and subcloned into pHEN2 vector (see [www. Mrc-cpe.cam.ac.uk](http://www.Mrc-cpe.cam.ac.uk)) to make pHEN2-scFv for bacterial periplasmic expression. pHEN2-scFv plasmids were transformed into *E. coli* HB2151 and induction of protein was performed by adding 1mM IPTG to 50ml exponentially growing bacterial culture (O.D.₆₀₀ 0.6) and by further growing at 30°C for 4 hours. The cells were pelleted and resuspended in 400 µl of ice-cold 1x TES buffer (0.2M Tris-HCl; 0.5mM EDTA; 0.5M sucrose). 600 µl of 1:5 TES buffer (ice-cold) was added, mixed gently by inversion and placed on ice for 30 minutes. The supernatant containing the periplasmic soluble scFv was collected after centrifugation. The periplasmic protein was used fresh for immunodetection at a dilution of 1:50. 9E10 anti-myc tag mouse monoclonal antibody and HRP-conjugated anti-mouse antibody were used as the secondary antibodies at 1:1000 and 1:2500 dilution respectively.

Example 5

Mammalian in vivo antibody-antigen interaction assay

The expression vector pEF-BOS-VPHS3 allows cloning of scFv in-frame with the VP16 transcriptional activation domain for mammalian expression. Individual anti-BCR scFv DNA fragments were cloned into the SfiI/NotI site of pEF-BOS-VPHS3. Expression plasmids for scFvF8 (anti-AMCV virus coat protein) and scFvR4 (anti-beta-galactosidase (Martineau, P., Jones, P., & Winter, G, (1998, J.Mol Biol 280, 117-127)) was constructed by inserting the appropriate PCR products into the Sfi-NotI site of pEF-BOS-VPHS3 (Tavliadoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. & Galeffi, P. (1993) Nature (London) **366**, 469–472.. The mammalian BCR-ABL expression plasmid, expressing Gal4DBD linked to BCR-ABL (pM3-E1A2) was made by subcloning the 4kb EagI fragment of pE1A2 REF into the SmaI site of pM3 (Sadowski, I., Bell, B., Broad, P

& Hollis, M., (1992), *Gene* 118, 137-141). The control bait pM1- β gal has been described previously (Visintin, M., Tse, E., Axelson, H., Rabbitts, T. H., & Cattaneo, A., (1999), *Proc. Nat. Acad. Sci, USA* 96 11723-11728).

Chinese hamster ovary (CHO) cells were maintained in α minimal essential medium (GIBCO/BRL) with 10% foetal calf serum, penicillin and streptomycin. One day prior to transfection, 2×10^5 CHO cells were seeded onto a single well of 6 well-plate. CHO cells were transiently transfected with 0.5 μ g of bait plasmid and pEF-BOS-scFv-VP16, together with 0.5 μ g of G5-Luciferase reporter plasmid (de Wet, J. R, Wood, K.V., DeLuca, M., Helinski, D. R., & Subramani, S., (1987) *Mol Cell Biol* 7, 725-37). and 50ng of pRL-CMV internal control plasmid (Promega), using Lipofectamine (GIBCO/BRL, according to manufacturer's instructions). 60 hours after transfection, luciferase assays were performed on the CHO cell extracts using the Dual-Luciferase Reporter Assay System (Promega) and a luminometer. Transfection efficiency was normalised with the Renilla luciferase activity measured. Each transfection was performed twice.

Example 6

Isolating specific intracellular scFv against BCR and ABL proteins by in vivo antibody-antigen interaction screening

The genetic screening approach to the isolation of intracellular antibodies comprised of yeast expression of a "bait" antigen fused to the LexA DNA binding domain (DBD) and a library of scFv fused to the VP16 transcription activation domain (AD) (Visintin, M., Tse, E., Axelson, H., Rabbitts, T.H. and Cattaneo, A. (1999) *Proc. Natl. Acad. Sci. USA* 96 11723-11728. Interaction between the antigen bait and a specific scFv in the yeast intracellular environment results in the formation of a complex which can activate yeast chromosomal reporter genes, such as *HIS3* and *LacZ*. This facilitates the identification and thus isolation of the yeast carrying the DNA vectors encoding the scFv. The main limitation of this approach is the number of scFv-VP16 fusion clones that can be screened in yeast antibody-antigen interaction system (conveniently up to $2-5 \times 10^6$). This figure is well below the size of scFv repertoires displayed on phage (Sheets, M.D., Amersdorfer, P., Finnern, R., Sargent, P., Lindqvist, E., Schier, R., Hemingsen, G., Wong, C., Gerhart,

J.C. and Marks, J.D. (1998) Proc. Natl. Acad. Sci. USA **95** 6157-6162; McCafferty et al (1990), Nature 348, 552-554). Thus to limit the numbers of scFv to be screened *in vivo* in yeast, we have used one round of *in vitro* phage scFv library screening using recombinant protein as antigen, prior to the *in vivo* yeast antibody-antigen interaction screening. A flow chart of our overall strategy to obtain antigen specific intracellular antibodies to BCR and ABL is shown in Figure 1.

The protein antigens for the *in vitro* screening were made by expressing either the SH2 binding domain of the BCR protein (BCR) or the SH2 domain of ABL (ABL) as recombinant protein. The purified antigens were used for screening an scFv phage display library (Sheets, M.D., Amersdorfer, P., Finnern, R., Sargent, P., Lindqvist, E., Schier, R., Hemingsen, G., Wong, C., Gerhart, J.C. and Marks, J.D. (1998) Proc. Natl. Acad. Sci. USA **95** 6157-6162,) (a gift from Dr. James Marks) *in vitro*. The library was derived from spleen cells and peripheral blood lymphocytes of human origin and had an initial diversity of 6.7×10^9 . A total of 2×10^{13} phage from the amplified library were screened with the purified protein fragments. After one round of *in vitro* phage screening, about 10^5 antigen- bound phage were recovered (Fig. 1). These sub-libraries had a reduced complexity because of the enrichment of antigen-specific scFv. Phagemid DNA encoding the scFv was extracted, DNA fragments encoding scFv were subcloned into the yeast prey expression vector to create yeast scFv-VP16AD libraries of 3.2×10^5 and 1.3×10^5 for BCR and ABL respectively (i.e. about 3 times the original size of the enriched phage sub-library size). *In vivo* yeast antibody-antigen interaction screening was performed (Visintin, M et al (1999) Proc. Nat. Acad. Sci, USA 96, 11723-11728; Tse, E et al, K.Turksen, Editor, 2000, Humana Press: Totawa) by co-transforming *Saccharomyces cerevisiae* L40 with a bait plasmid expressing BCR-ABL p190 (pBTM/E1A2) and the BCR or ABL scFv-VP16AD library. A total of approximately 8.5×10^5 yeast colonies were screened and 117 (anti-BCR) or anti ABL yeast colonies were selected, and confirmed using beta-galactosidase (beta-gal) filter assays (Fig. 1), indicating an interaction between the scFv and the BCR-ABL protein in the yeast cytoplasm.

The scFv-VP16AD plasmids were isolated from the yeast clones and into sorted

into different groups according to BstNI DNA fingerprinting patterns, yielding 45 (anti-BCR) and 24 (anti-ABL) clones. Verification of the intracellular binding of scFv with antigen was determined using representatives of the groups in re-transfections with the original antigen bait and assay by histidine-independent growth and beta-gal activation. In this way, ten anti-BCR and 12 anti-ABL scFv were verified by activation of beta-gal. Examples of this are displayed in Figure 2 in which interaction of anti-BCR scFv with BCR-ABL in yeast is shown. The specificity of the scFv binding to BCR-ABL was further verified by the lack of interaction between them and non-relevant antigen (a plant virus coat protein antigen AMCV) in the yeast *in vivo* assay (Fig 2B) and by the lack of binding of the non-relevant scFv F8 to the BCR-ABL bait (Fig. 2A) (Tavloraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. & Galeffi, P. (1993) *Nature* (London) **366**, 469–472).

Example 7

Expression of antigen-specific scFv in bacteria

The levels of expression of individual scFv were initially examined by bacterial periplasmic expression. The captured scFv were sub-cloned into the expression vector, pHEN2, which has the PelB leader sequence 5' to the scFv allowing periplasmic expression of soluble scFv protein and in-frame with histidine and myc epitope-tags. Periplasmic scFv extracts were Western blotted using anti-myc tag antibody, 9E10 for immuno-detection (Fig. 3A, B). Variable levels of protein are expressed. The best anti-ABL scFv (Fig. 3A) levels were found for A32 whereas much lower levels of protein could be detected for A6, for instance. Similarly anti-BCR scFv exhibited variability in expression, B10 was the highest expressed antibody fragment whereas B9 and B33 were present at lower levels (Fig. 3B). These variations may reflect differences in folding characteristics and may additionally be due to codon preferences for the human scFv in bacteria.

Example 8

In vitro interaction of ICABs with antigen

Antibody specificity was investigated by comparing the ability of the scFv to discriminate between BCR and ABL antigens when these were immobilised on membranes. Various periplasmic scFv were tested for binding to bacterially synthesised BCR and ABL antigens of comparable size and levels (Fig 3C, D). The binding of scFv to the relevant antigens paralleled the level of periplasmic expression. In the anti-ABL panel, scFv A32 had quantitatively the best interaction with ABL protein in keeping with its efficient expression, whereas A6 weakly detected antigen presumably due to the low level of scFv. In most cases, we observed discrimination by the scFv between the two antigens except for A20 which appears to cross-react with both ABL and BCR antigens, although the BCR antigen may be a degraded form given the size is around 16Kda rather than around 30Kda for intact antigen.

Like the anti-ABL scFv, there was a clear relationship between degree of binding of anti-BCR scFv to BCR SH2 binding domain protein and the level of periplasmic expression (Fig. 3D). The specificity was demonstrated by the lack of binding of the scFv to the SH2 domain of the ABL protein (amino acids 26 – 348) on the same blot (Fig 3D). Therefore when periplasmic scFv is prepared from the scFv selected using the ICA technology, most of the antibodies exhibit specific antigen recognition *in vitro* as well as *in vivo*.

Example 9

In vivo interaction of the isolated scFv with target antigen in mammalian cells

Our data show that the IAC technology can be used to develop a set of ICABs which show *in vivo* antigen binding and specificity for antigen. In our previous work in which we developed the antibody-antigen interaction screen (Visintin, M., Tse, E., Axelson, H., Rabbitts, T.H. and Cattaneo, A. (1999) Proc. Natl. Acad. Sci. USA 96 11723-11728; Tse, E, Chung, G & Rabbitts, T. H; K. Turksen, Editor, 2000. Humana Press: Totawa), we also showed that the mammalian reporter assays could be used to assess the efficacy of ICABs. This was emulated by testing the interaction of anti-BCR scFv with antigen measured by

activation of a luciferase reporter gene. The scFv were cloned into the mammalian prey luciferase vector and the BCR-ABL bait cloned into pM3 (Sadowski, I. Bell, B Broad, P & Hollis, M (1992), Gene 118, 137-141). These were co-transfected into CHO cells with a standard Renilla luciferase to control for transfection levels. Variable levels of activation was observed when the target antigen used was BCR-ABL (Fig. 4A) but none when a non-relevant antigen beta -gal was used (Fig. 4B). The best activation level was consistently found with the scFv B10. No activation was observed with the BCR-ABL bait and an irrelevant scFv F8 (Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. & Galeffi, P. (1993) Nature (London) 366, 469–472).

Example 10

Sequence comparison of the selected intracellular antibodies

A comparison of the panels of scFv selected by the IAC technology was made by determination of the nucleotide sequences and translating into the corresponding amino acid sequence (Fig. 5). Eighteen ICABs are aligned (six anti-BCR and twelve anti-ABL) and consensus sequences were derived for both the heavy chain (V_H) and light chain (V_L) variable regions. These sequence data were compared.

Most of the V_H segments in the ICAB panel fall in the VHIII subgroup joined to the JH5 region. This in part reflects the initial bias in the phage library (Sheets, M. D et al (1998). Proc. Nat Acad Sci USA 95, 6157-6162) although the random scFv, but not selected ones, have representatives of other sub-groups (Fig. 5 A and B and MNLC unpublished). It was possible to obtain a consensus for both the selected and random scFv in the complementarity determining regions CDR1 and CDR2 but CDR3 differed strongly in sequence and length, reflecting the known importance of CDR3 in antibody combining sites (Xu, J. L & Davis, M. M.. (2000), Immunity 13, 37-45). The IAC VHIII consensus matches the Kabat consensus (Kabat, E, A Wu, T.T., Perry, H. M., Gottesman, K.S, & Foeller, C., Sequences of Proteins of Biological Interest, 5th Ed, 1991. Bethesda: National Institute of Health) at all positions in the frameworks, except residue 3 (residue 1 in the Kabat consensus) which is a glutamine rather than glutamic acid residue (Fig. 5A). The residues at each framework position which vary amongst the IACBs are more restricted

than in individual VH genes²⁹ and further the CDR1/2 conservation argues for limited acceptance of changes at this position compatible with intracellular activity. Indeed, we have isolated scFv with identical frameworks in antigen-specific ICAbs which differ by only three residues in CDR1. The VHIII framework is therefore amenable for intracellular expression, solubility and function and the contribution of non-randomised CDR1 and CDR2 is also apparent. Detailed mutagenesis studies could reveal additional changes which might facilitate greater intracellular efficacy but the VHIII consensus discussed here provides at least one backbone on which to build CDR variability for future IAC use. The L chain variable region in the anti-BCR and anti-ABL ICAb set also allows derivation of a consensus, in this case a match to the VkI subgroup (Fig. 5A) linked to JkI. Unlike the VH, we were able to obtain consensus for all three CDR regions. Comparison of the ICAb VL consensus with that obtained from random scFv from the library (Fig. 5B) shows that the latter display greater overall variability. Each residue differing between the two are the same in the ICAb VkI consensus as in the VkI consensus according to the Kabat database, indicating that the ICAb consensus is conserved and can provide the backbone for scFv VL sequences for intracellular use.

Strategy (B)

Example 11. Selection of functional intracellular antibodies by IACT

Methods

Deletion mutants of the protein associated with the TAU microtubules were engineered as described in (Fasulo, L. et al. 1996 and Fasulo, L. et al. 2000) and cloned in the pBTM116 vector in the EcoRI-BamHI restriction sites. The non-immune library of antibody fragments displayed on the surface of the M13 filamentous phage was used for selecting antibodies (Sheets et al. 1998). The 151-421 fragment of the TAU protein was cloned in the pMAL-c2 vector (NEB) downstream of the malE gene. The same vector pMAL-c2 was used for producing the protein TAU 151-422. The proteins were purified according to protocols suggested by the manufacturer on an affinity column as described in (Kellerman, O.K. et al. 1982). The TAU human protein was purified from the bacterial strain BL21(DE3) as described in Kontsekova, E. et al. 1995.

The non-immune library described in Sheets et al. 1998 was assayed with TAU 151-421 preadsorbed on the solid phase used for selection, according to a protocol described in Sblattero, D. et al. 2000. After 1 and 2 rounds of selection, some clones were isolated, and characterised by DNA amplification and fingerprinting of the gene coding for the scFv by digestion with BstNI. The DNA isolated was then sequenced to confirm the diversity obtained.

The phagemid DNA was then isolated and cloned successively at the SfiI-NotI sites of the VP16 vector (Vojtek, A.B. et al. 1993) previously digested with the same restriction enzymes. The scFv-anti-TAU/VP16 ADI and ADII libraries were assayed successively against lexA-TAU as described in Visintin, M. et al. 1999 and Visintin, M. et al. 2001. 90% of the clones obtained grew in a histidine-free medium and became blue after the β -gal test. About a hundred clones coding for scFv anti-TAU were isolated from yeast following the method described in Visintin, M. et al. 2001, and the DNA isolated was analysed by BstNI fingerprinting and sequencing as described above. The individual clones were further tested against TAU by IACT.

The scFvs that proved positive after the latter analysis were then cloned in a phagemid expression vector, for the purpose of expressing the protein in soluble form. The proteins obtained were purified on an affinity column and analysed in gel filtration by means of a Superdex 75 column. The scFvs were then cloned successively in eukaryotic expression vectors, scFvexpress and nuclear scFvexpress (for expression of the scFvs in the nucleus) (Persic et al. 1997). The COS and CHO cell lines were transfected transiently according to the protocol described in Fasulo, L. et al. 1996 or following the protocol of the FuGENE6 reagent (Roche), used for transfecting the CHO cells. The cells were analysed 30-48 hours after transfection in immunofluorescence.

Sixteen scFvs of the original library Sheets et al. 1998 and twenty-five anti-TAU selected by IACT were sequenced, using an Epicentre Sequitherm Excel II kit. The Li-Cor 4000L automatic sequencer was used for automatic sequencing of the DNAs analysed.

Results

The technology developed by Visintin et al. 1999, IACT (intracellular antibody capture technology) (Fig. 2) and described in patent application PCT WO00/54057 was used for selecting antibodies for intracellular use against a panel of numerous different antigens. The example of selections against human TAU protein is described in detail here. TAU is a neuronal protein that belongs to the family of proteins that bind together the microtubules, and is a pathologic marker for Alzheimer's disease.

For the purpose of isolating antibodies using the IACT method, a fusion protein was engineered between a deletion mutant of human TAU protein (Ile151-Ser422) and maltose binding protein MBP (TAU-MBP). The protein expressed in *E. coli* is purified on a NiNTA affinity column, and is then used for in vitro preselection of a library of human antibody fragments (scFvs) expressed on phage (Sheets, M.D. et al. 1998) (Fig. 2, step 1). This step proved necessary for generating a heterogeneous population enriched in anti-TAU scFvs that might be compatible with the low efficiency of transformation of yeast. TAU-MBP was used for 2 successive rounds of in vitro preselection of the non-immune library of antibody fragments. The diversity of the library enriched in the first cycle of preselection and in the second cycle was quantified by analysis (100 clones per cycle) of the fingerprint after amplification of the DNAs coding for scFv with degenerated oligonucleotides (Sblattero, D. et al. 2000) and by sequencing some scFvs. After the first cycle, about 90% of the clones were found to be different, whereas after the second cycle only 13 clones out of 100 were found to be different (Table 1). Some clones of the first and of the second cycle were isolated and assayed in vitro in ELISA to verify the interaction with TAU. Three clones of the first cycle and 9 of the second were found to react with TAU in ELISA. These clones tested against other antigens (Fig. 3a) (MBP and BSA) were found to be TAU specific.

After this step, the library enriched in anti-TAU scFvs of the first and of the second cycle was cloned in the yeast expression vector VP16 in order to create two libraries fused at the 5' of the activation domain of transcription, VP16 (anti-

TAU/VP16ADI and anti-TAU/VP16ADII). The anti-TAU/VP16ADI library consisted of 2.2×10^6 scFvs whereas the anti-TAU/VP16ADII library was formed from 6×10^4 scFvs.

For isolating anti-TAU scFvs in vivo, the libraries thus created were tested against a fusion protein between TAU (Ile151-Ser422) and the DNA-binding domain of the *lexA* protein (*lexA*-151-422TAU) (Fig. 2, step 2). The yeast transformed with these two sublibraries was first assayed for its prototrophy with histidine. 10^5 clones grown in the absence of histidine were assayed successively for their ability to activate the *lacZ* gene (Visintin, M. et al. 2001). The DNA of some clones that were found to have a *HIS3*⁺ and *lacZ*⁺ phenotype were isolated and analysed by fingerprinting and then sequenced. In the screening of the anti-TAU/VP16ADI library, 31 different clones were isolated, whereas in the screening of the anti-TAU/VP16ADII library only 5 were isolated. Of these clones, only 17 (of the anti-TAU/VP16ADI library) and 3 (of the anti-TAU/VP16ADII library) (Fig. 3b, and Table 1) were confirmed positive after a second IACT screening. The specificity of these ICABs for TAU was evaluated by co-transforming the anti-TAU scFvs with other antigens. It was found that the anti-TAUs selected with IACT did not cross-react with any of the antigens tested. Three anti-TAU scFvs were also expressed in *E. coli* and the soluble proteins isolated from the periplasm were tested in ELISA against TAU and two deletion mutants of TAU (Fig. 3c). All three scFvs were capable of interacting in vitro against TAU and its deletion mutants.

Table 1. Results of selection in vitro and in vivo. Number of positive clones of the monoreactive scFvs.

	Diversity of the anti-TAU polyclonal library	ELISA screening	Number of positive interactions after 1 screening with IACT	Number of different scFvs	Number of positive interactions after 2 screenings with IACT
Round 1	90/100	3/96	$\sim 10^4$	31/100	17/31
Round 2	13/100	9/96	$\sim 10^5$	5/100	3/5

Four anti-TAU scFvs isolated at random from the starting library were tested in vivo with IACT against the TAU protein. These antibodies belong to the VHIII family: It was observed that none of these scFvs was capable of interacting in vivo with TAU protein. This result emphasises the fact that selection in vivo is necessary for obtaining intrabodies. Three anti-TAU's selected and validated with IACT were then analysed by biochemical techniques and studies of cell biology, for the purpose of verifying their solubility, tendency to aggregate and stability (Worn, A. et al. 1999). Analysis of the antibodies purified by gel filtration demonstrated that the three scFvs analysed occur for the most part in the elution peak corresponding to the monomeric form of a scFv (Fig. 3d). The affinity of these three scFvs was calculated using competitive ELISA and in Biacore, and the K_D has a value that varies in the range 100-350 nM. The three scFvs expressed in vivo, in different cell lines (CHO, COS, PC12) are highly soluble (Fig. 4), bind TAU and when the antibody fragments are expressed as fusion protein with a nuclear localisation signal, are capable of mislocalizing TAU in the nucleus, whenever this protein could be expressed as cytoplasmic protein (Fig. 5). It was found, moreover, that one of these scFvs recognises endogenous TAU in the PC12 neuronal cell line, and inhibits axon growth mediated by neurotrophin NGF (neuronal growth factor) (Melchionna et al. 2001).

Example 12. Analysis of the sequences of antibodies isolated with IACT

Methods

The alignments and the analyses of the scFvs described above were evaluated in accordance with the Kabat database. A database was set up containing all the sequences of antibodies selected with IACT (VIDA, validated intrabody database). VIDA contains in particular the sequences of the anti-TAU antibodies described above. Furthermore, VIDA contains the sequences of validated intracellular antibodies described in the literature. The numbering of the amino acids of the antibodies (both of the VIDA sequences and of the sequences of the Kabat databank) was effected according to the Kabat scheme (Martin AC 1996; Deret S 1995), which was used for aligning all the sequences with one another. Numbering of the amino acids was effected automatically by

means of the SeqTest program (Martin A. 1996). The frequency of each amino acid, for each position of the antibody chain, was determined.

The "consensus" (or the "consensus sequence"), for a given set of sequences, and limited to a certain subset of positions along the said sequences, is defined as the sequence in which, in the defined positions of the subset as above, the most frequently occurring amino acid is present. No amino acid is defined in the remaining positions.

For the purpose of carrying out an analysis of the sequences of antibodies present in VIDA, the said sequences are classified according to the species (mouse or human) from which they are derived, treating the VL and VH domains separately. The following 4 subsets are identified:

human VH: composed of 24 sequences

human VL: composed of 24 sequences

mouse VH: composed of 12 sequences

mouse VL: composed of 12 sequences.

The same type of subdivision was applied to the sequences of antibodies present in the Kabat databank (which are not generally tested with respect to intracellular expression) for the purpose of making, subsequently, comparisons between homogeneous sets.

Analysis of the antibody sequences contained in each subset of VIDA made it possible to identify a group of amino acid residues that are conserved. This group of residues is designated ICS (intrabody consensus sequence), and enables us to define 4 different ICSs (human ICS-VH, human ICS-VL, mouse ICS-VH, mouse ICS-VL). It is possible to define different ICSs, on the basis of a threshold of homology between the antibodies of each VIDA subset (absolute consensus, 90% consensus, etc.). For each reference group, the optimum ICS is obtained with the algorithm described below.

On the basis of the VIDA sequences, by induction, a procedure was elaborated that makes it possible to distinguish sequences of intrabodies from generic sequences of antibodies (separately for each subset of antibodies):

- a) Calculate the consensus sequence for each VIDA subset, limiting the calculation to the positions in which the probability of the most frequent amino acid being found exceeds a predetermined threshold value LP (consensus threshold). These represent the Intrabody Consensus Sequences (ICS) and there is one for each VIDA subset.
- b) Draw up two distributions for each of the four subsets of sequences identified previously. These distributions represent the identity number of each antibody of the VIDA dataset (green) or of the Kabat dataset (red) with respect to the ICS consensus sequence relating to that group (Fig. 10, column on right).
- c) For each pair of distributions (red and green) if necessary introduce a measure of their "relative diversity". It is possible to use various definitions for measuring the degree of diversity D between the two distributions. We chose to measure D as the absolute value of the ratio between the difference of the mean values and the sum of the standard deviations of the two distributions in question. When this ratio is greater than 1 the distributions are considered to be different.
- d) Repeat the procedure iteratively for different values of LP, determining the new distributions. Repeating the procedure with variation of LP (for example from a consensus threshold LP of 70% to a consensus threshold LP of 100%, in steps of 1-2%), for each of the four classes, it is possible to find the optimum value of LP that gives rise to the maximum value of D, i.e. that permits the best differentiation of the various distribution pairs (red and green). Hence, in general, we shall have a different LP value for each of the four VIDA subsets.

The procedure for identifying whether a given antibody, whose sequence does not already form a part of VIDA, is or is not an intrabody, is thus as follows: the sequences that differ from the ICS for a number of sites "compatible" with the VIDA distribution and not with the Kabat (Fig. 10 column on right) are intrabodies (intracellularly binding antibodies) (provided that the two distributions can actually be distinguished). The criterion of "compatibility" is established as follows: the number of different sites is compatible with VIDA and not with Kabat (b) when it is found to be lower than the mean value of the VIDA distribution to which the product between D and the standard deviation of VIDA is added. In practice, those sequences that differ from the ICS by less

than the sequences already contained in VIDA differ from it, are considered to be intrabodies.

In general, it is to be expected that the optimum ICS depends on the size of the VIDA database. The procedure is in fact being refined progressively with increase in the number of sequences contained in the various VIDA subsets. It is possible, on the basis of the sequences already in our possession, to estimate the number of sequences required for reaching convergence on the optimum ICSs. It is thus a matter of estimating the number of sequences present in VIDA such that the optimum ICS obtained should not vary when new sequences are added. The estimate can be made on the assumption that new VIDA sequences conserve the same degree of variability of the sequences in our possession as at present. In particular, it is always possible to calculate ICS using just one part of the VIDA sequences. Assume that VIDA is composed of N sequences. It is possible to calculate ICS using just $m < N$ sequences, and it can be done in Binomial (N, m) different ways. Then the average degree of homology can be measured between the ICSs calculated for each fixed m . This procedure enables us to understand whether the set of VIDA sequences at our disposal is large enough to saturate the average degree of homology between the ICSs as a function of m to an asymptotic value. This estimate calculated on the dataset currently available made it possible to conclude already that with the present size of VIDA we are not far from the asymptotic value.

Another way of observing the distribution of the antibody sequences consists of evaluating the probability of finding them by chance, assuming that the probability $P_i(A)$ of finding a certain amino acid A in position i does not depend on which amino acids there are in the other positions and is determined on the basis of the frequency of finding A in position i in the databank. For a given sequence s this value is written:

$$P_s = \sqrt[n]{\prod_{i=1}^n P_i(A_{s,i})}$$

in which $A_{s,i}$ is the amino acid in position i of sequence s . The infinite product can be extended to all the sites of the sequence, or can be limited to a subset.

Results

The 17 sequences of ICABs selected with IACT (VIDA set) were compared with 16 scFv sequences extracted at random from the starting library (control set). All the VH domains of the VIDA set belong to the subgroup VH III (Deret, S. 1995) (Martin, A.C. 1996), whereas 13 VL domains belong to the subgroup kappa I and 4 VL domains to kappa IV. In the control set as well, many of the sequences belong to the subgroup VH III (just one is different, belonging to VH II). In the VL domain, on the other hand, 10 sequences belong to the subgroup kappa I and 6 to lambda (mainly lambda IV). The average degree of homology between the sequences of the control set is 69% for the VH domain and 59% for the VL domain. The average homology within the VIDA set (85% for VH and 77% for VL) is greater than in the control set. Within the VIDA set, 76 amino acids in the VH domain (2 belonging to CDR 1, 2 belonging to CDR 2, 1 belonging to CDR 3), and 44 of the VL domain are conserved. The conserved amino acids define a consensus sequence that is characteristic of the intracellular antibodies (consensus ICS) both for the VH domain and for the VL domain (Fig. 11).

The first analysis undertaken gave a surprising and unexpected result. We assigned, to each VIDA and Kabat sequence, the P_s calculated on the basis of the frequencies $P_i(A)$ in the Kabat subset (see Methods); at this point it became possible to compare the VIDA distributions with those of the corresponding Kabat subsets. If the comparison (and hence the infinite product present in the formula for P_s) is extended to the entire sequence, the two distributions VIDA and Kabat cannot be distinguished; the VIDA sequences in fact have values comparable with those obtained in the corresponding Kabat subset. If, however, we limit the comparison to just the sites of the respective ICS, then surprisingly the VIDA sequences are all disposed in the tail of the Kabat distribution with the highest probability (Fig. 12). This shows that the subset of residues defined by the ICS identifies a well-defined subpopulation of all the antibodies present in the Kabat database. This shows that the ICS is a good marker of the property of being an intracellular antibody.

This result was confirmed with another type of analysis. We measured the degree of homology between the ICS consensus sequences for the VH and VL domains and consensus sequences extracted from the Kabat databank (Kabat consensus sequences). Comparison was effected for the various subgroups defined in Table 2. All except 2 of the 76 amino acids conserved in the VH domain of the VIDA set coincide with the amino acids found with greatest frequency in the analogous position of the sequences of set No. 1 extracted from the Kabat databank. Furthermore, the two positions where coincidence is not confirmed between the two sets, are occupied in the VIDA set by amino acids (PHE in position 67 and ASN in position 73) that display a frequency only slightly lower than the most frequent ones in Kabat set No. 1 (VAL in position 67 and THR in position 73). Comparison of the ICS sequence with Kabat set No. 3 (subgroup VH III) shows that only GLN in position 1 is not found as frequently as GLU, whereas the agreement is perfect on all the other sites. On the other hand, 27 amino acids of the 76 conserved in the VIDA set do not coincide with the most frequent ones in Kabat set No. 2 (mouse VH), reflecting the species difference.

For the sequences of the VL domain, 47 of the 48 positions conserved in the VIDA set coincide with the most frequent amino acids of Kabat set No. 4 (formed from human VL sequences). Only 4 of the 48 residues belonging to the ICS sequence for the VL domain differ from those that are most frequent in Kabat set No. 5 (VL domains of mouse antibodies), whereas all 48 amino acids coincide with those found most frequently in Kabat set No. 6 (VL domains of subgroup Vk of human antibodies). Finally, 10 amino acids of the 48 of the ICS sequence do not coincide with those that are most frequent in Kabat set No. 7 (sequences of VL domains belonging to subgroup Vlambda of human antibodies).

Table 3.

VH	
No. 1 (human)	2
No. 2 (mouse)	27
No. 3 (human, subgroup VH III)	1
VL	

No. 4 (human)	1
No. 5 (mouse)	3
No. 6 (human, subgroup Vk)	0
No. 7 (human, subgroup Vλ)	10
Number of amino acids in the VIDA set not coinciding with the consensus sequence of the Kabat subset shown in the column on the left (analysis limited to the positions defined by ICS).	

Accordingly, the analysis described makes it possible to define a partial consensus sequence ICS for each variable antibody domain, which, a posteriori, in the positions where it is defined (i.e. in the positions where there is total conservation in the VIDA set), is found to coincide with the Kabat consensus sequences of the VL and VH domains of human antibodies.

It is interesting to clarify to what extent this characteristic (i.e. considerable homology with the sequence of greatest consensus in a significant, but not total, portion of the amino acids of the human VL and VH domains) occurs among the sequences of antibodies chosen at random from the Kabat databank. For this purpose we analysed the degree of homology of the sequences contained in the various subsets extracted from the Kabat relative to the respective consensus sequence, limiting the analysis to the positions in which the ICS consensus sequence defined above is defined (being 76 for the VH domain and 48 for the VL domain, see Fig. 13). This analysis shows that in the regions where there is greatest homology with the Kabat sequence of maximum consensus, the sequence density is limited, i.e. there are few antibodies in the Kabat database that are very similar to the Kabat consensus sequence. (It should be recalled that the Kabat consensus sequence is a virtual sequence.)

The degree of homology of the sequences of the VIDA set relative to the Kabat consensus sequences, evaluated for the appropriate subsets, is shown in Fig. 13, but only for the positions defined by ICS (see dots in Fig. 13). Surprisingly, the sequences of the VIDA set fall in the furthest tail of these distributions, where there are few antibodies

in the database itself. Accordingly, the sequences of the VIDA set (intracellular antibodies) show a high degree of homology with the consensus sequences of the Kabat subsets, a characteristic that is very rare for any antibody of the said database. Subsets No. 2 and No. 7 do not comply with this rule. This can be ascribed to the species difference (the VIDA set used for this analysis was derived from human antibodies) and the abundance of sequences of subgroup Vk in the VIDA set.

This analysis confirms that IACT leads to the selection of antibodies that are rare in the natural population of the said antibodies. The analysis also led to the identification of a subset of residues that appear to distinguish these antibodies from all the others (set of residues ICS). Furthermore, these intracellular antibodies have the remarkable property, which is defined operatively for the first time here, of having maximum similarity with the Kabat consensus sequence, if the analysis is restricted to the amino acid positions defined by ICS (Fig. 14, right).

This same analysis was performed on an extension of the VIDA set, to which were added sequences that derive from intracellular antibodies for different antigens. These analyses confirm the concept of capture of the consensus sequence by means of the IACT technique.

The set of intracellular antibodies selected with IACT, or in other words, which constitute VIDA, is subdivided into the following families (Table 2).

Table 2. List of subgroups of intrabodies

scFv	VH	VL
Anti- β -gal	III	λ II
1 ABL-BCR	III	λ III
2 ABL-BCR	III	λ IV
3 ABL-BCR	III	K I
4 ABL-BCR	III	K I
5 ABL-BCR	III	K IV

6 ABL-BCR	III	K I
anti-tTG 2.18	V	K I
anti-tTG 2.8	V	K I
anti-tTG 3.7	I	K I
anti-TAU #a	III	K I
anti-TAU #b	III	K I
anti-TAU #c	III	K I
anti-TAU #d	III	K IV
anti-TAU #e	III	K I
anti-TAU #f	III	K I
anti-TAU #g	III	K I
anti-TAU #k	III	K IV
anti-TAU #m	III	K IV
anti-TAU #n	III	K IV
anti-TAU #o	III	K I
anti-TAU #q	III	K I
anti-TAU #s	III	K I
anti-TAU #t	III	K I
anti-TAU #v	III	K IV
anti-TAU #x	III	K I
anti-TAU #y	III	K I

Two important properties can be deduced from Table 2:

- 1) The VHIII family is far and away the most represented family. Obviously this reflects a bias in the libraries used, but more generally it reflects a higher average stability for antibodies of this family (Soderlind, E. et al. 2000). It demonstrates that in order to maximise the chances of a particular immunoglobulin to be capable of functioning within an intracellular environment, then an immunoglobulin of the VHIII family should be selected.

- 2) However, it is not sufficient to be VHIII in order to be a good intracellular antibody. In fact, it has been seen before that anti-TAU antibodies of this family, isolated at random from the library, and with good properties of binding with TAU in vitro, are incapable of binding TAU in vivo.

The additional analysis, described in the Methods, was performed on the extension of the VIDA set. This analysis employs a new procedure for identifying a new intrabody. The results of the new procedure for identifying the intrabodies are presented in the following table.

Table 4

Set	(number of sequences recognised as intrabody) / (total number of sequences in the set)
VH III human (ICS 81 sites, LP 90%, D 1.15)	
IACT	23/24
Kabat Subgroup VHIII	122/1872
VL human (ICS 29 sites, LP 96%, D 1.27)	
IACT	24/24
Kabat VL human	299/2731
VH mouse (ICS 28 sites, LP 94%, D 1.34)	
IACT	12/12
Kabat VH mouse	328/3353
VL mouse (ICS 25 sites, LP 94%, D 1.15)	
IACT	12/12
Kabat VL mouse	492/2518

This procedure, which leads to the identification of an optimum ICS and so limits the analysis to the positions identified by the ICSs themselves, was used first for identifying each antibody belonging to the said VIDA. By means of this procedure, constructed on the basis of the VIDA sequences in our possession, it is possible for all the

VH sequences, and all but one of the VL sequences, to be identified as "intrabody". This provides a first validation of the procedure, which was then applied to the identification of potential intracellular antibodies in the Kabat database (Fig. 15, right). The procedure leads to the identification, as intracellular antibodies (identification defined in Methods), of a fraction that amounts to about 10% of the sequences of the various Kabat subsets (Table 4). These antibodies are potentially good intracellular antibodies, identified by the procedure in question.

A sample of these antibodies was assayed by IACT, as described above, to ascertain its effective stability in the intracellular environment. The results of this experimental verification demonstrated that effectively all the antibodies predicted to be good intracellular antibodies passed this experimental test. This result provides further validation of the ICS concept and of the procedure based on ICS for the identification of intracellular antibodies.

Next we verified the degree of homology of the ICS sequences that we had extracted (see Table 4) with the Kabat sequence of maximum consensus in the corresponding subset. The results are shown in Table 5.

Table 5

Homology of the ICS relative to the Kabat consensus				
Maximum Consensus ICS	VH III human	VH mouse	VL human	VL mouse
ICS VH III human (81 sites)	80	52		
ICS VH mouse (29 sites)	27	29		
ICS VL human (28 sites)			28	28
ICS VL mouse (25 sites)			23	25

Finally we evaluated whether it is also possible to identify the VIDA sequences solely from their degree of homology relative to the Kabat sequence of maximum consensus, without limiting the analysis to the ICS positions, but on the entire sequence (Fig. 15, left). Fig. 15 shows that the procedure of the present invention is more selective compared with what can be done using an analysis of homology relative to the Kabat consensus, on the entire sequence. In fact, limiting the analysis to just the ICS sites

(Fig. 15, right), it is found that a much lower number of Kabat sequences is compatible with the VIDA distribution of sequences.

The intrabody recognition procedure is robust with respect to insertion of new sequences in the VIDA set if LP is less than 100%. In fact, insertion of a new sequence would only alter the probability of finding the various amino acids in the various positions to an extent equal to $1/n$, where n is the number of sequences in the VIDA set.

Fig. 15, right, shows another significant aspect: there is a significant number of sequences in the Kabat database having a homology relative to the Kabat consensus that is higher than the VIDA population. This means that if all the amino acid positions are analysed, the intracellular antibodies do not have maximum similarity with the Kabat consensus (Fig. 14, left), which is however the case if the analysis is restricted to just the sites defined by ICS (Fig. 14, right). It can be concluded from this that an antibody can be very similar to the Kabat consensus (maximum homology) and yet not be a good intracellular antibody (Knappik et al. 2000).

In order to be a good intracellular antibody it is necessary to have good homology with the Kabat consensus on the correct sites. The present invention, in addition to demonstrating this, has identified those sites on which the consensus should be calculated.

In conclusion, the description of this invention describes:

- i) the development of a procedure for identifying an intracellular antibody, without the need to verify it experimentally, on the basis of the sequence alone.
- ii) the use of this procedure on a database of sequences of experimentally validated intracellular antibodies led to the discovery of a set of key positions on the molecule of antibodies on which an ICS is defined, this discovery also being a part of this invention.
- iii) the optimum ICS can be employed for designing and constructing a library that is very rich in functional intracellular antibodies.

All publications mentioned in the above specification, and references cited in said publications, are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the

invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A method of identifying at least one consensus sequence for an intracellular antibody (ICS) comprising the steps of:
 - a) creating a database comprising sequences of at least a proportion of a variable heavy chain domain and/or variable light chain domain of validated intracellular antibodies (VIDA database) and aligning the sequences of the variable heavy chain domains or variable light chain domains of validated intracellular antibodies;
 - b) determining the frequency with which a particular amino acid occurs in each of the positions of the aligned antibodies;
 - c) selecting a frequency threshold value (LP or consensus threshold) in the range from 70% to 100%;
 - d) identifying the positions of the alignment at which the frequency of a particular amino acid is greater than or equal to the LP value;
 - e) identifying the most frequent amino acid, in the positions of the alignment defined in d).

2. A method according to claim 1 wherein the sequences of the variable heavy chain domains or variable light chain domains of validated intracellular antibodies present in the VIDA database are aligned according to Kabat.

3. A method of identifying at least one optimum consensus sequence for an intracellular antibody (optimum ICS) comprising the steps of:
 - a) identifying different ICSs for different LP values;
 - b) for each of the different ICSs, constructing a frequency distribution of the number of identical amino acids between that particular ICS and each of the antibodies making up the VIDA database (VIDA distribution);
 - c) for each of the ICSs, constructing a frequency distribution of the number of identical amino acids between that particular ICS and each of the antibodies that make up the Kabat database (Kabat distribution);

- d) defining a "distance" D between the VIDA distributions and the Kabat distribution corresponding to a value of LP;
- e) for each LP value, determining the value of the "distance" D between the VIDA distributions and the Kabat distribution corresponding to that value of LP;
- f) identifying the optimum ICS as the ICS corresponding to the value of LP for which the calculated value of the distance D defined in d) is maximum.

4. A method according to Claim 3 in which the consensus sequence is one of the consensus sequences for VH and/or VL comprising:

- a) for a VH consensus sequence, at least the following amino acids in the positions indicated according to the Chothia numbering:

S-21, C-22, S-25, G-26, M-32, W-36, P-41, L-45, E-46, D-72, Q81, L-82c, E-85, D-86, A-88, Y-90, C-92, W-103, G-104, G-106, T-107, T-110, V-111, S-112;

- b) for a VL consensus sequence, at least the following amino acids in the positions indicated according to the Chothia numbering:

G-16, C-23, W-35, G-57, G-64, S-65, S-67, I-75, D-82, Y-86, C-88, T-102, K-103.

5. A method according to Claim 3 in which the consensus sequence is one of the consensus sequences for human VH and/or VL comprising:

- a) for human VH, essentially the following amino acids in the positions indicated according to the Chothia numbering:

Q-1, V-2, Q-3, L-4, S-7, G-8, G-9, G10, V-12, P-14, G-15, S-17, L-18, R-19, L-20, S-21, C-22, A-24, S-25, G-26, F-27, T-28, F-29, Y-31a, M-32, W-36, R-38, Q-39, A-40, P-41, G-42, K-43, G-44, L-45, E-46, W-47, V-48, S-52, G-54, Y-58, Y-59, A-60, D-61, S-62, V-63, K-64, G-65, R-66, F-67, T-68, I-69, S-70, R-71, D-72, N-73, S-74, N-76, T-77, L-80, Q81, M-82, L-82c, R-83, A-84, E-85, D-86, T-87, A-88, Y-90, C-92, A-93, W-103, G-104, G-106, T-107, L-108, V-109, T-110, V-111, S-112, S-113;

- b) for human VL, essentially the following amino acids in the positions indicated according to the Chothia numbering:

T-5, P-8, G-16, I-21, C-23, W-35, Y-36, Q-37, P-40, G-41, P-44, I-48, S-56, G-57, S-63, G-64, S-65, S-67, G-68, L-73, T-74, I-75, D-82, A-84, Y-86, C-88, T-102, K-103.

6. A method of predicting whether an antibody is a functioning intracellular antibody, comprising the steps of:
- a) aligning the sequence of the antibody with the sequences of the antibodies of the reference VIDA database;
 - b) aligning the sequence of the antibody with the optimum ICS sequence of the reference VIDA database;
 - c) constructing the VIDA and KABAT distributions corresponding to the optimum value of LP (frequency threshold value);
 - d) determining the corresponding distance D ;
 - e) determining the identity number N between the sequence of the antibody and the ICS reference sequence;
 - f) calculating the difference between the mean value of the VIDA distribution and the product between D and the standard deviation of the VIDA distribution, obtaining the parameter S_{intra} ; and
 - g) if the identity number N is greater than or equal to S_{intra} , identifying the antibody as intracellular antibody.
7. A method for conferring upon an immunoglobulin molecule the ability to function within an intracellular environment comprising the steps of:
- a) identifying the optimum ICS reference sequence;
 - b) optionally, modifying, by site-specific mutagenesis, the amino acid residues that are located in the positions defined by the optimum ICS, or a subset of these residues, in such a way that they are those identified by the optimum ICS.
8. An intracellularly binding immunoglobulin molecule comprising at least one variable chain which is described by at least one of the consensus sequences described in figs 11a and 11b and depicted SEQ ID no 41 and 42.

9. The use of an immunoglobulin molecule comprising at least one consensus sequence described in fig 11a and/or 11b and depicted SEQ 41 and SEQ 42 respectively in the selective binding of a ligand within an intracellular environment.
10. A method for identifying at least one consensus sequence for an intracellular antibody (ICS) comprising the steps of:
 - (a) selecting and aligning the sequences of antibody light or heavy chain variable regions which are shown using IACT to bind specifically to antigen/ligand within an intracellular environment, and
 - (b) identifying the most frequent amino acid, in each position of the alignment.
11. A method for selecting one or more antibodies capable of binding specifically to its one or more antigens/ligands within an intracellular environment comprising the steps of:
 - (b) comparing at least a proportion of the variable heavy chain of one or more antibodies with at least the consensus sequence shown in fig 5a and depicted SEQ 3, and
 - (c) selecting those one or more antibodies whose variable light chain is at least 85% homologous with the consensus sequence of step (a).
12. An intracellularly binding immunoglobulin molecule comprising a variable heavy chain which exhibits 85% homology to the consensus sequence shown in fig 5a and depicted as SEQ ID No 3 and at least one variable light chain.
13. The use of an immunoglobulin molecule comprising at least one variable heavy chain domain (VH) exhibiting at least 85% identity with the consensus sequence shown in fig 5a and depicted SEQ 3, and at least one light chain domain (VL) , in the selective binding of a ligand within an intracellular environment.
14. A library, wherein the library is generated using any one or more of the variable heavy domain amino acid sequences (VH) selected from the group consisting of: a VH amino acid sequence showing at least 85% identity with the consensus sequence depicted

as SEQ 3 and shown in fig 5a, a VH sequence which is described by the consensus sequence depicted in SEQ 41 and shown in fig 11a,

15. A method of constructing an antibody library enriched with antibodies capable of functioning within an intracellular environment comprising the steps of:

- a) selecting an antibody framework from those that are intracellularly functionally stable from the Kabat database;
- b) on each framework, mutagenizing the amino acids present in the positions of the sequence defined by the optimum ICS, changing them into the amino acid residue that is located in that position in the ICS sequence; and
- c) on each of the frameworks, randomising the CDR regions of the antibody sequence.

16. A method of constructing an antibody library enriched with antibodies capable of functioning within an intracellular environment comprising the steps of:

- a) selecting an initial antibody framework, on the basis of the maximum homology with an optimum ICS sequence;
- b) mutagenizing all the remaining residues of the antibody framework, limited, for each position, to the amino acids that are located, in that position, in antibodies of the Kabat database;
- c) on each of the frameworks, randomising the CDR regions of the antibody sequence.

17. The use of a library according to claim 16 for producing immunoglobulin molecules, wherein a substantial proportion of those molecules expressed are capable of selectively binding to a ligand within an intracellular environment.

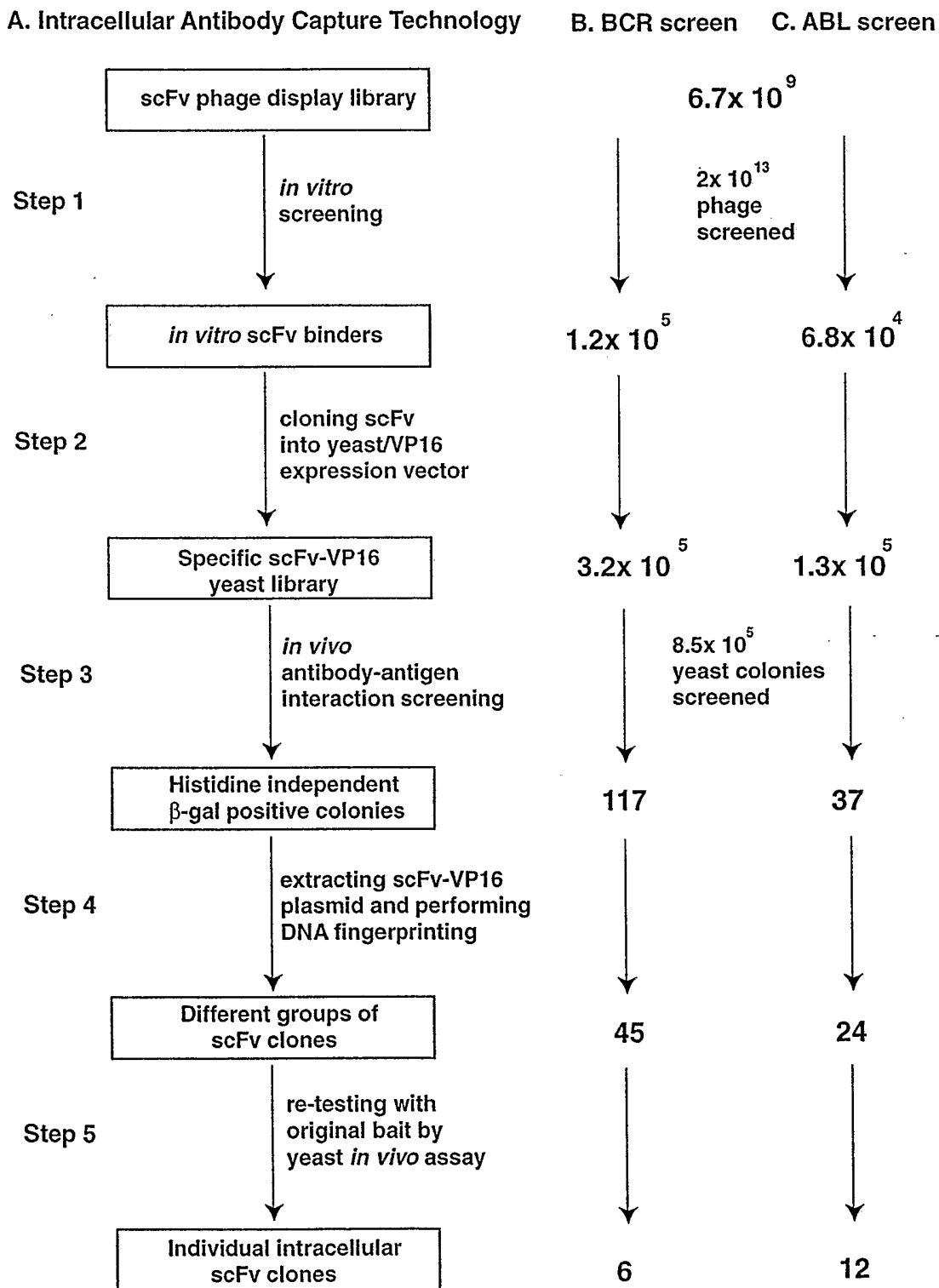


FIG. 1

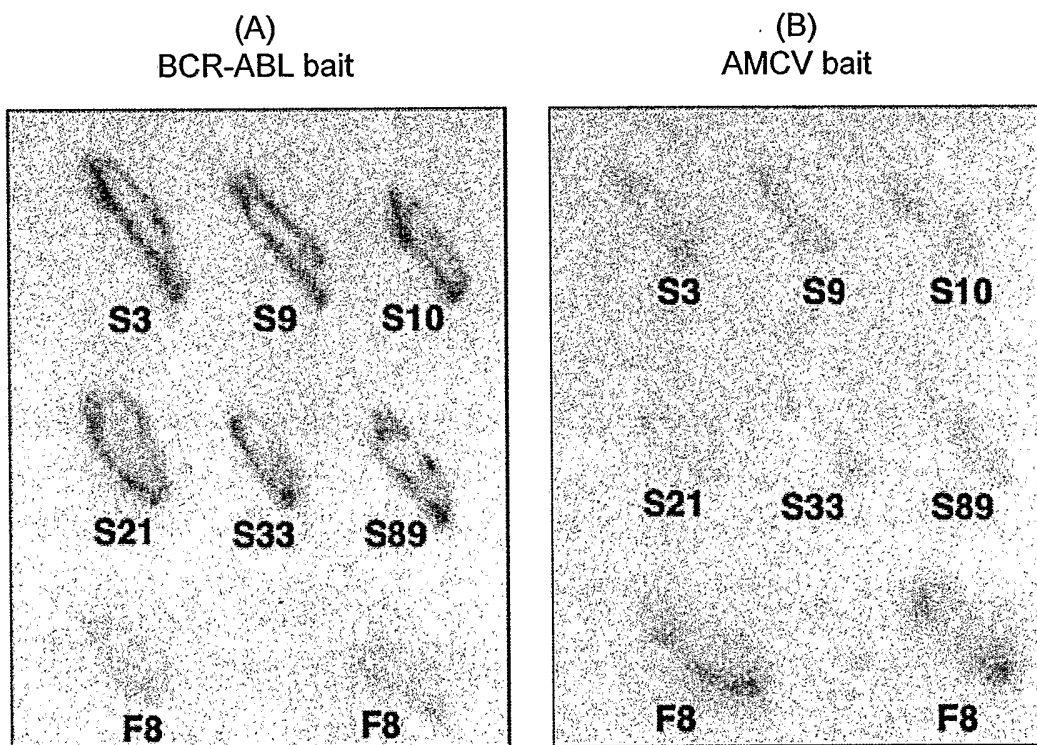


FIG. 2

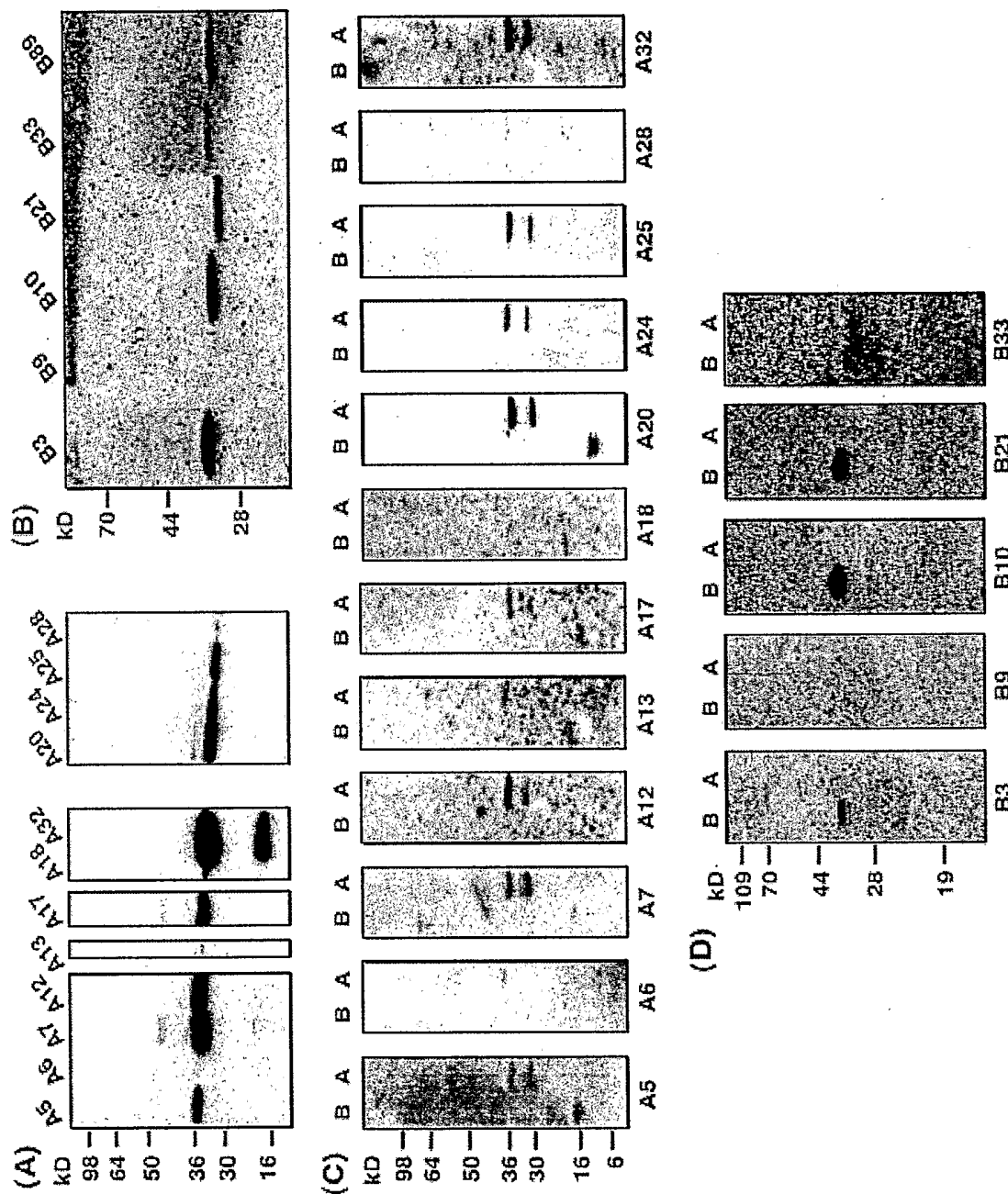


FIG. 3

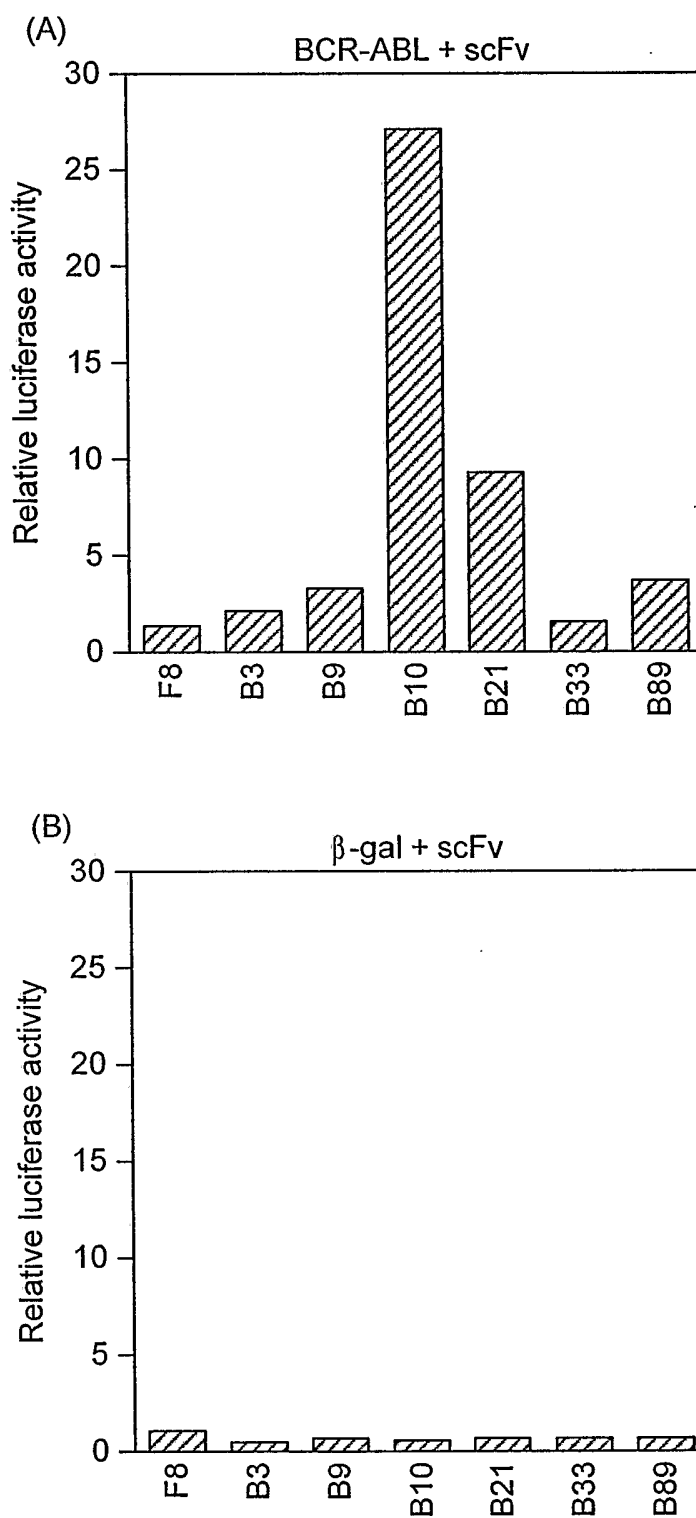


FIG. 4

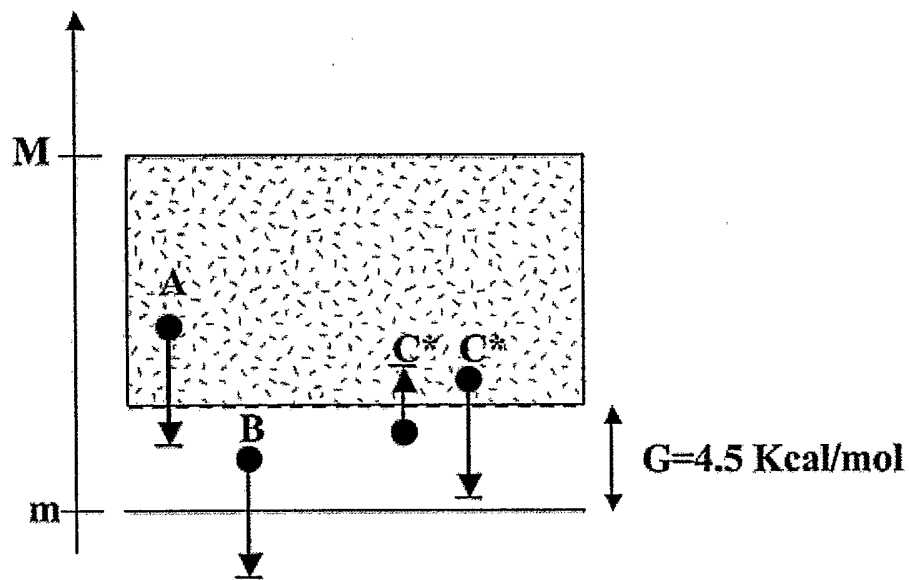


FIG. 6

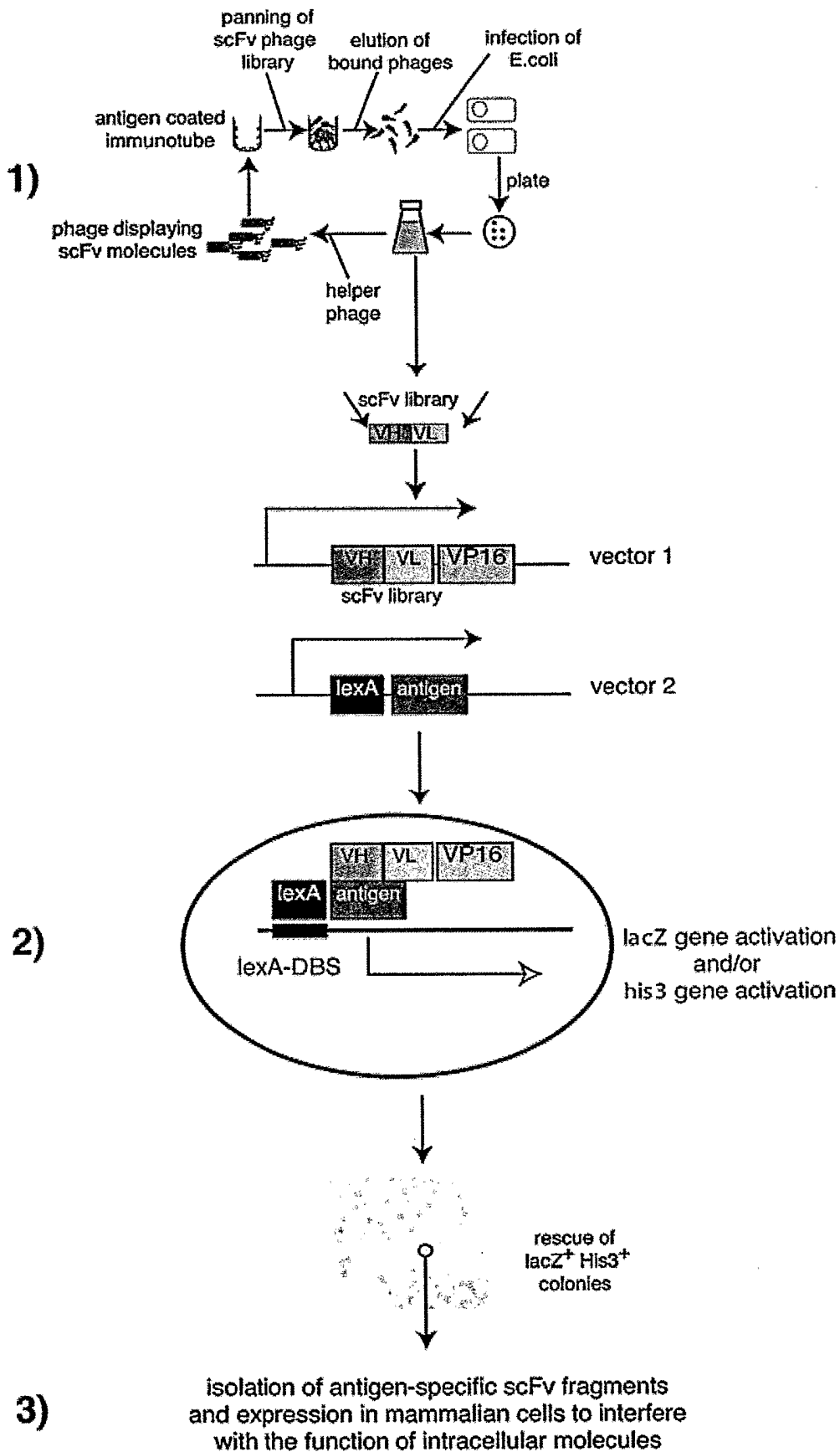


FIG. 7

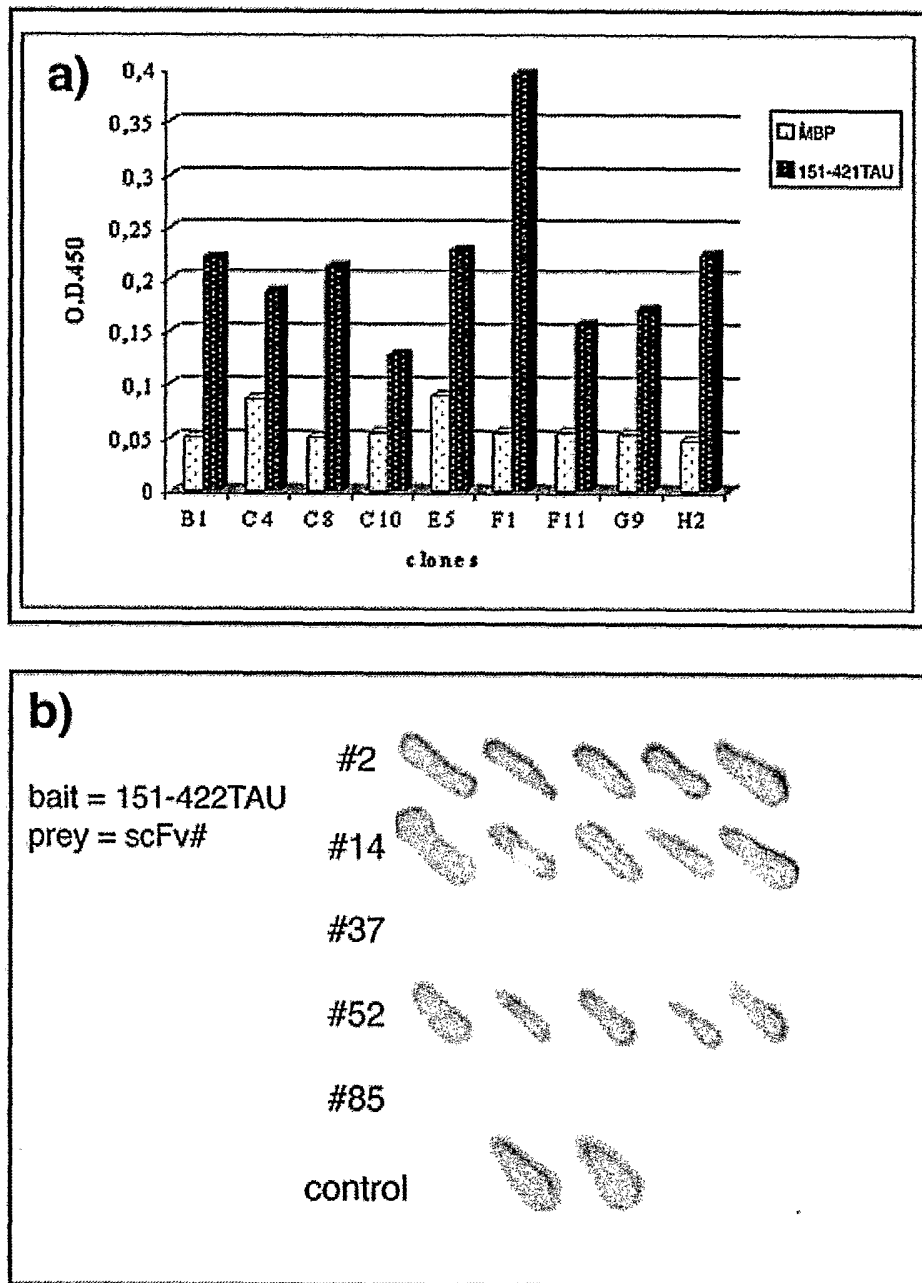


FIG. 8

10 / 21

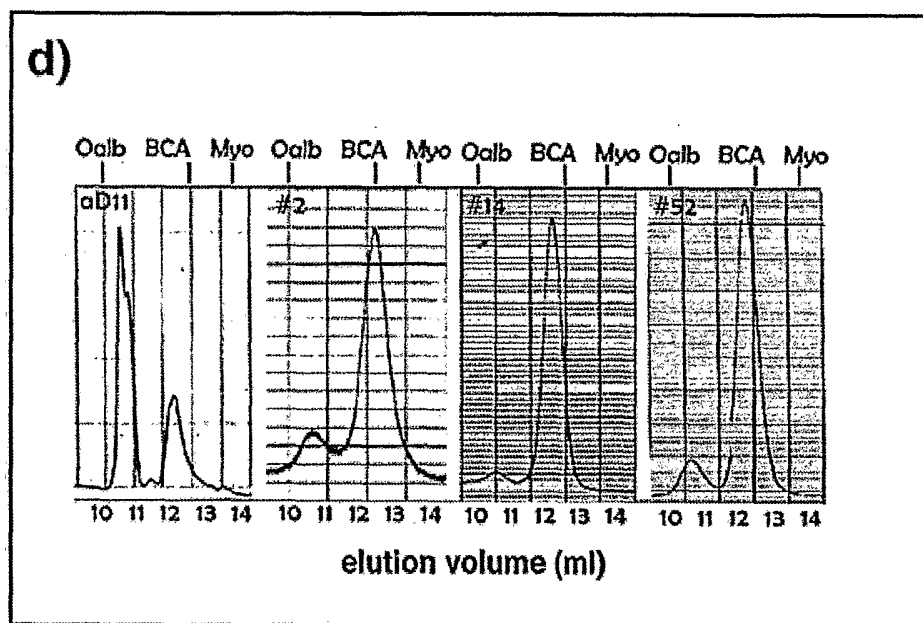
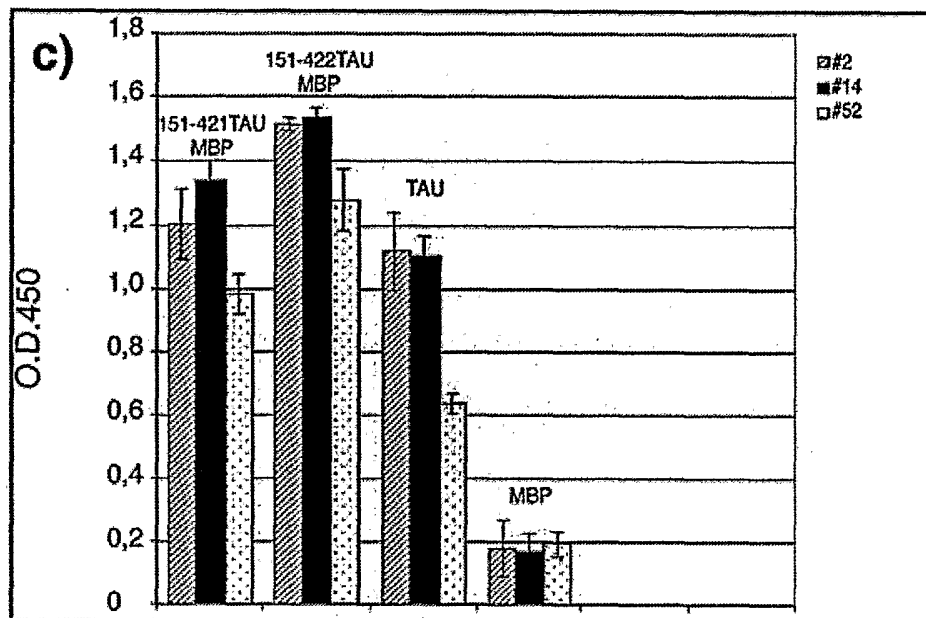


FIG. 8 CONT'D

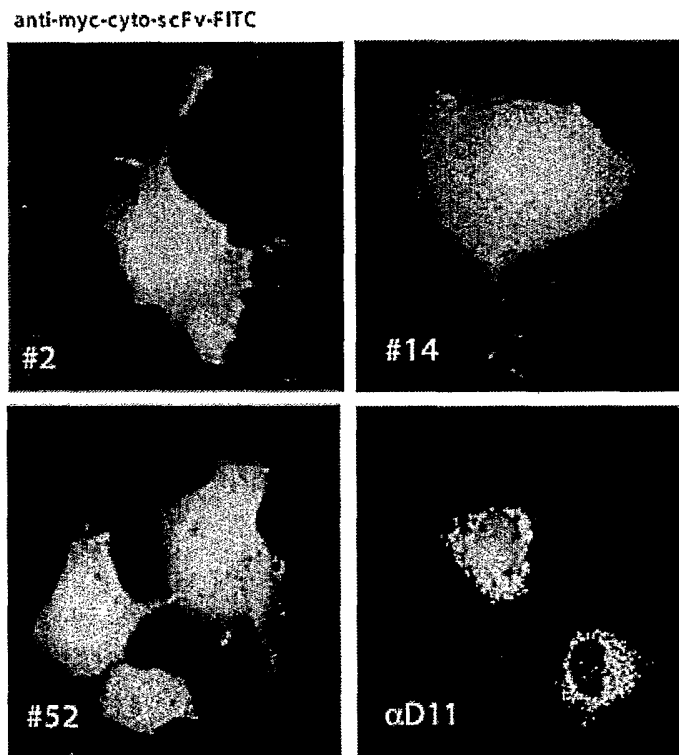


FIG. 9

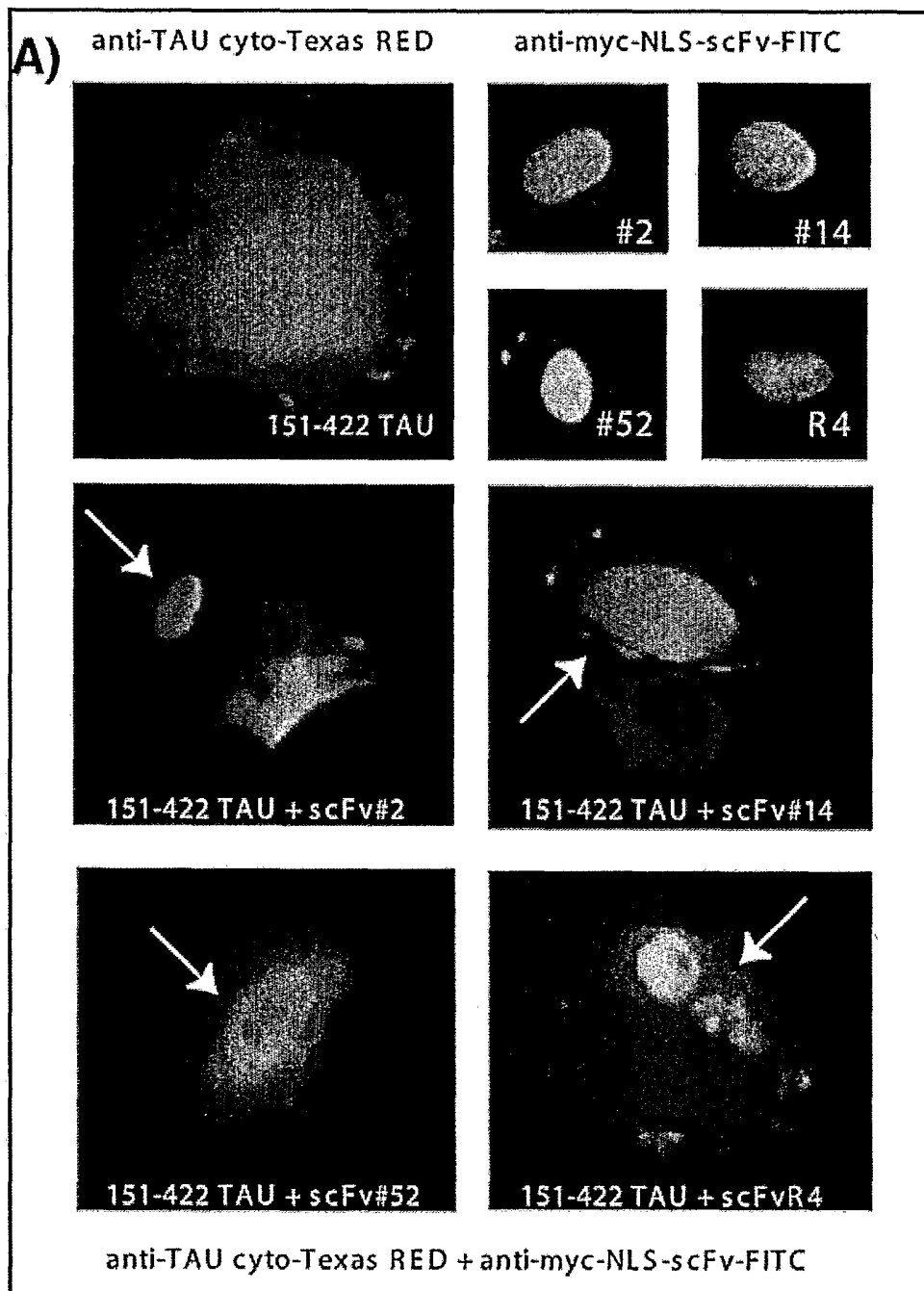


FIG. 10

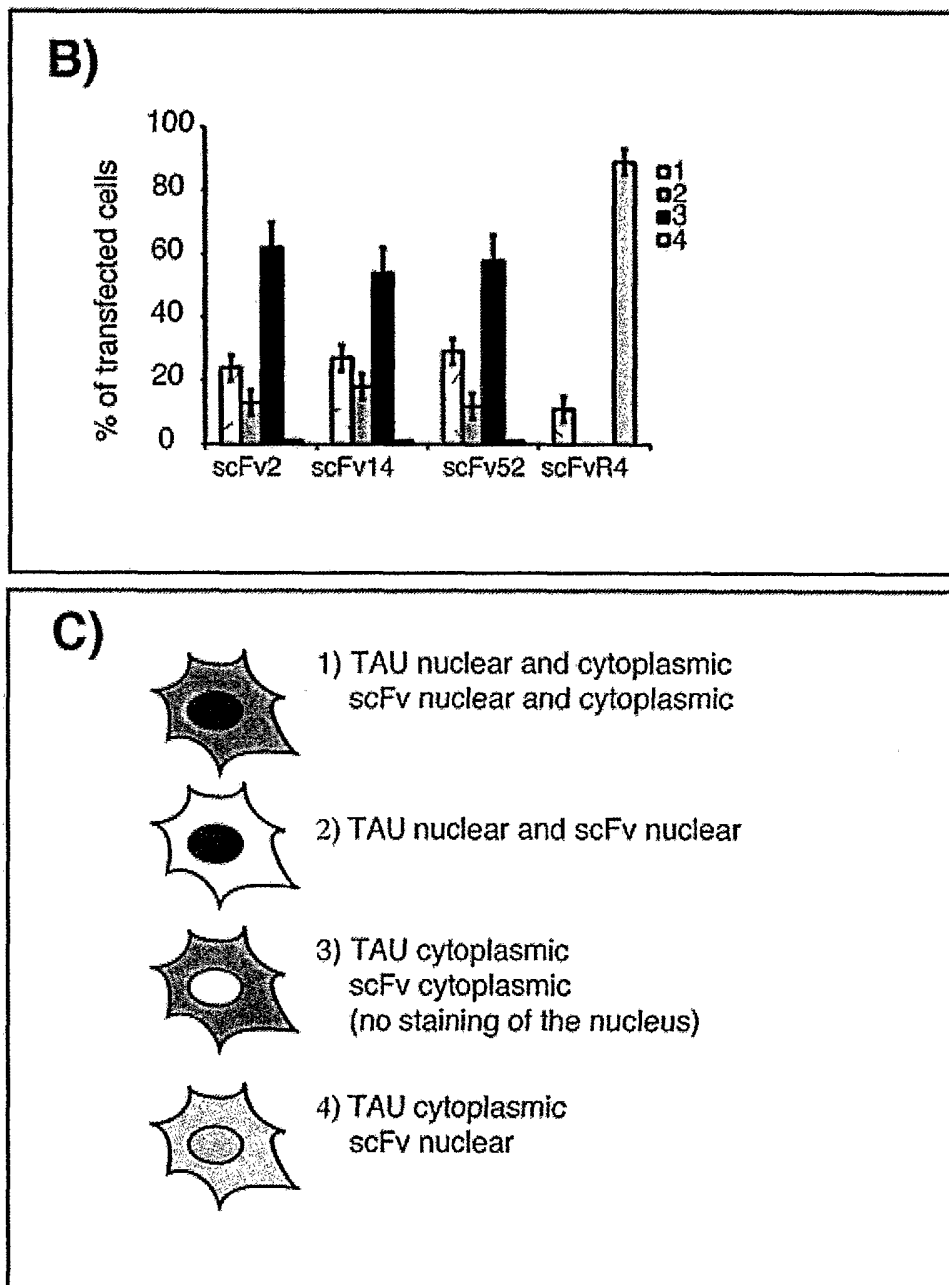


FIG. 10 CONT'D

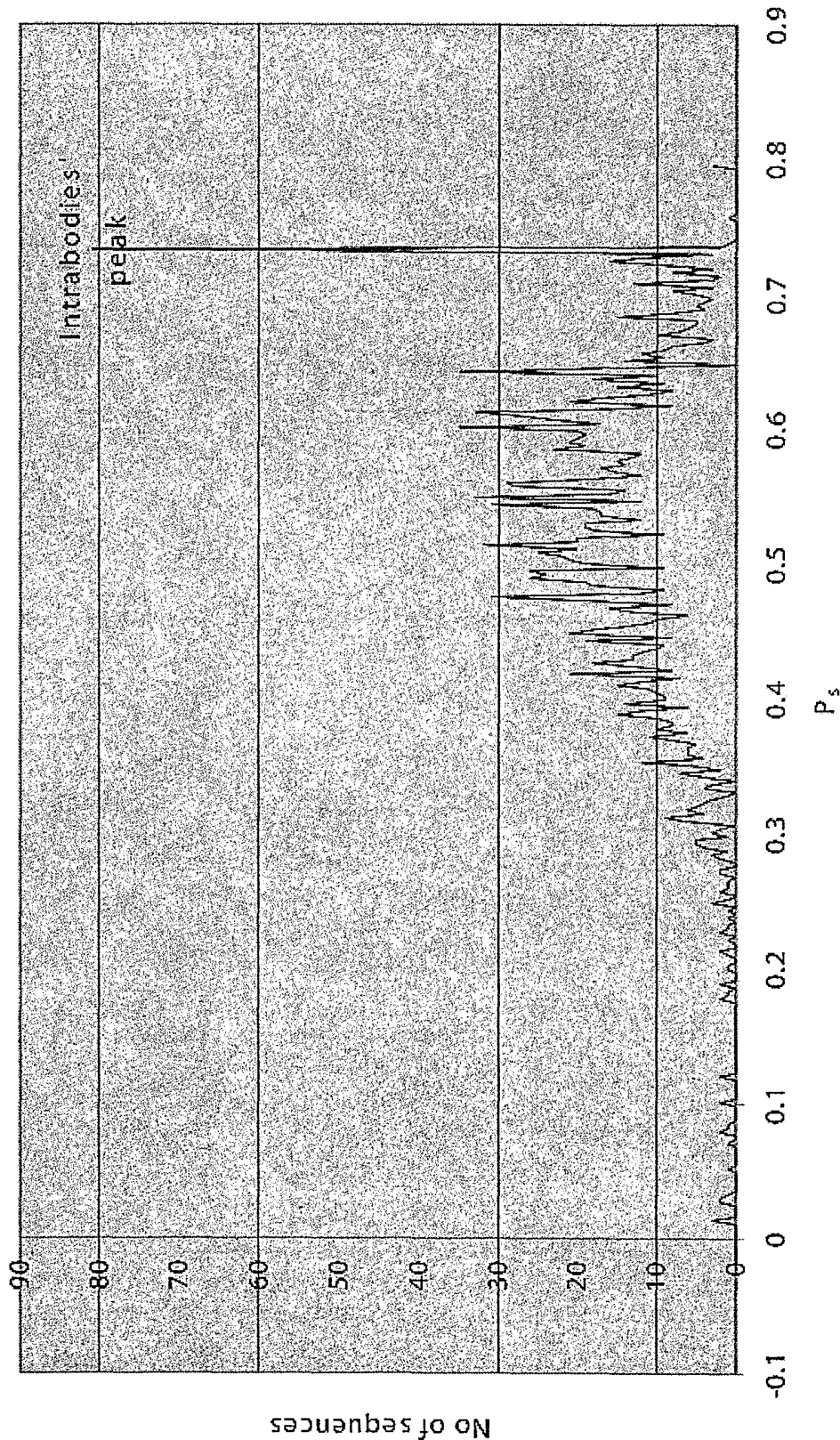


FIG. 12

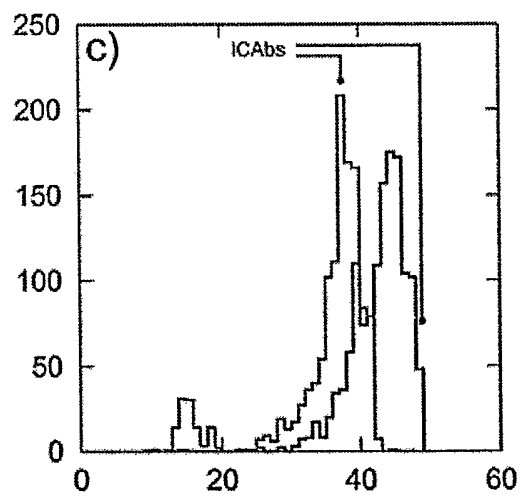
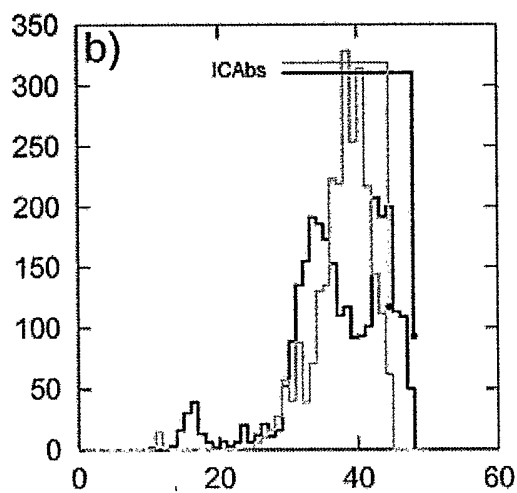
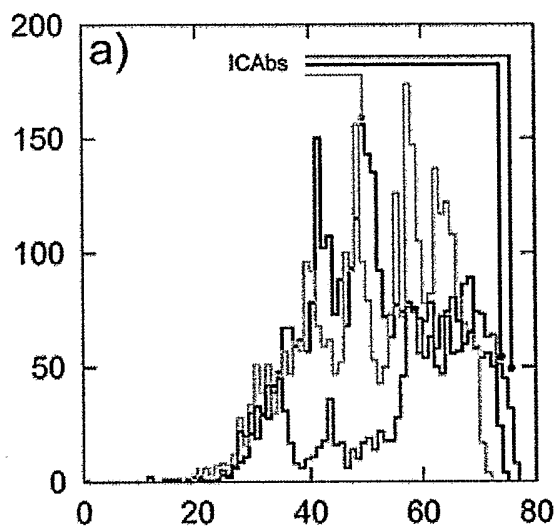


FIG. 13

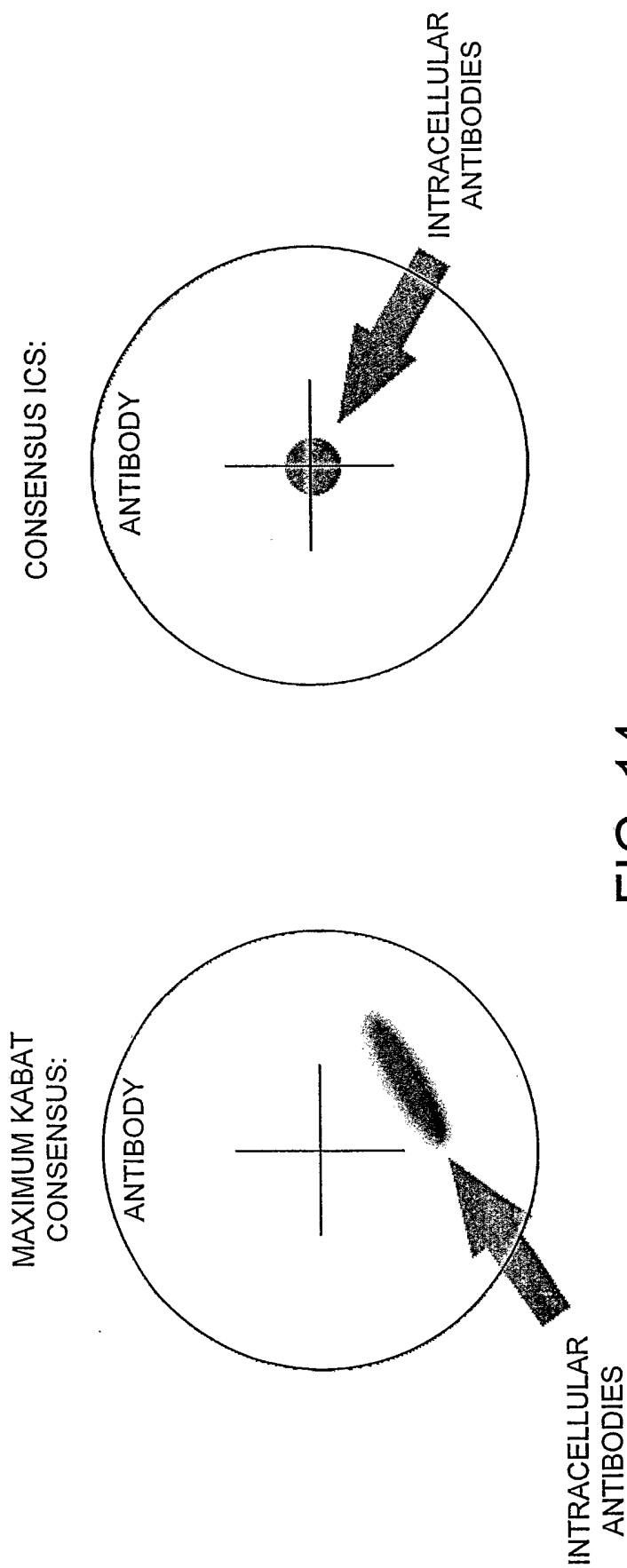


FIG. 14

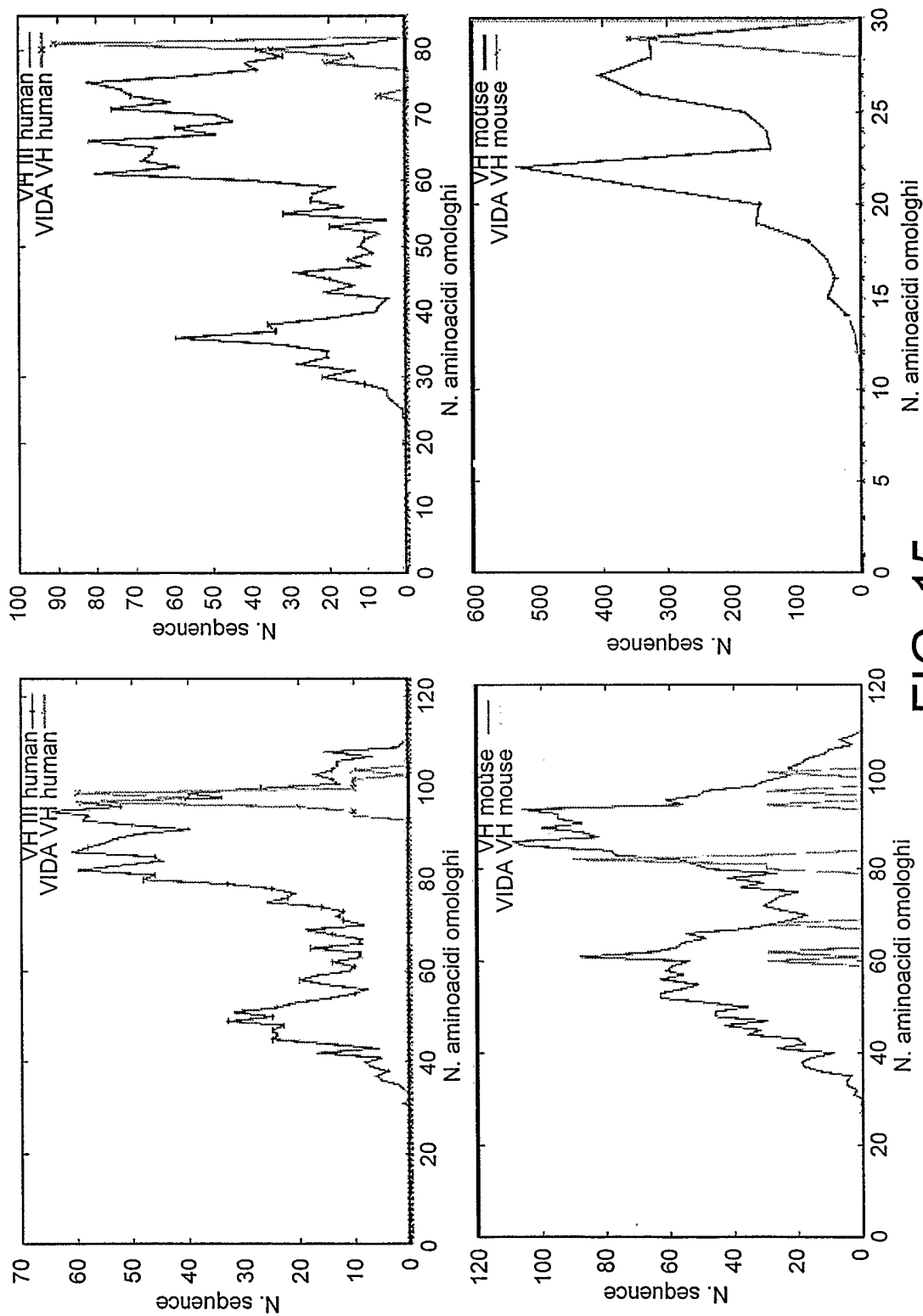


FIG. 15

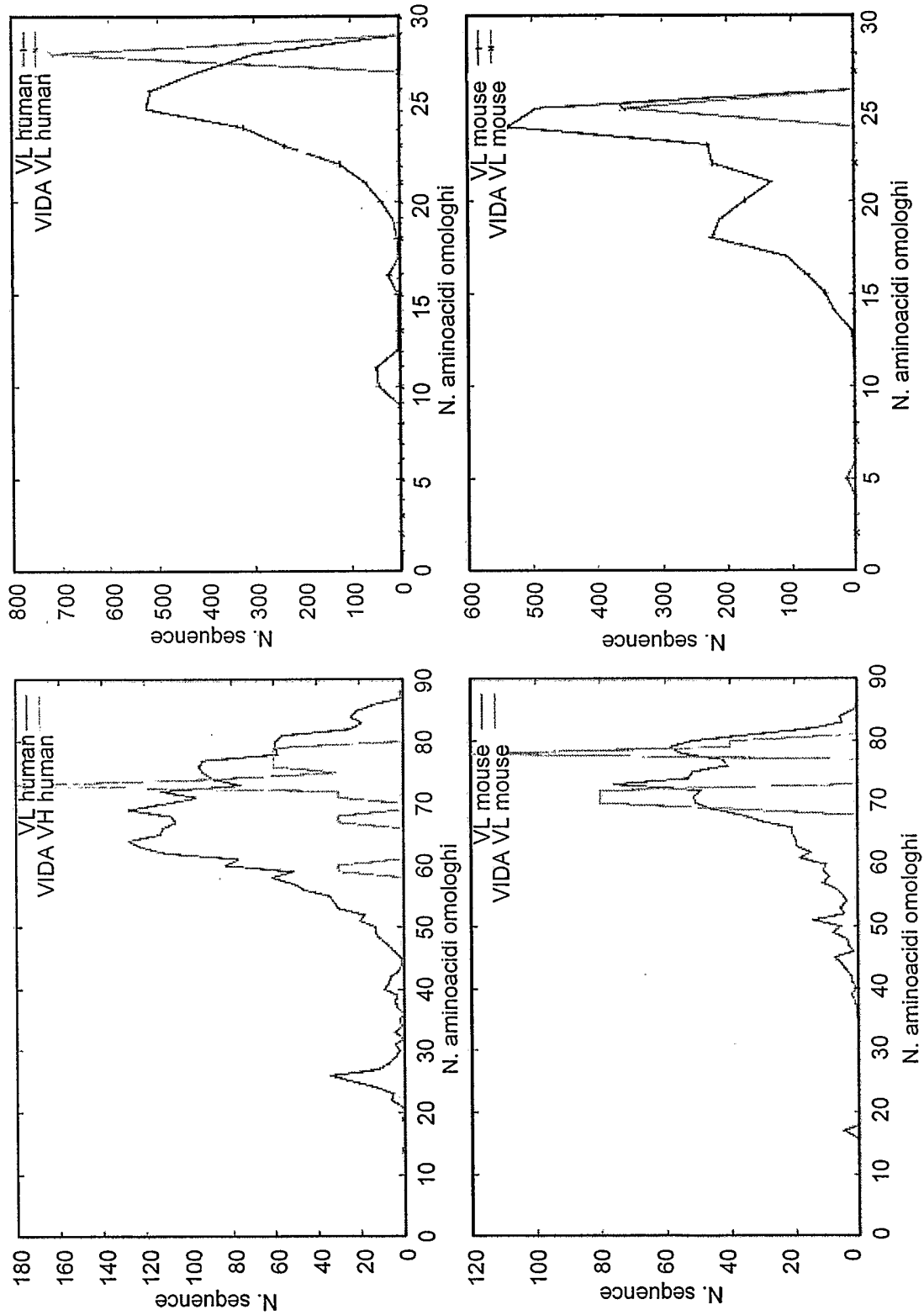


FIG. 15 CONT'D