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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.

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 be 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 V_L (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 the 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

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