SELECTION OF INTRACELLULAR ANTIBODIES

CATTANEO A.

SELEKCIA INTRACELULÁRNYCH PROTILÁTOK

The intracellular expression of antibodies in mammalian cells is a strategy to inhibit in vivo the function of selected molecules. One limitation of this technology is represented by the unpredictable behaviour of antibodies, under conditions of intracellular expression. Recent advances in the field of antibody expression in E. coli show that the introduction of mutations inspired by sequence comparisons or by educated guesses can improve the properties of some antibody domains, but the general applicability of this approach to intracellular antibodies remains to be proven. As a complement to rational approaches, selection schemes whereby antibodies are selected on the basis of their performance in vivo as intracellular antibodies can be exploited. *(Fig. 3, Ref. 44.)*
Key words: intracellular antibodies, antibody folding, intrachain disulfide, single-chain antibody fragments, selection of intracellular libraries.

Bratisl Lek Listy 1998; 99:413–418

Vnútrobunková expresia protilátok v bunkách cicavcov predstavuje strategickú možnosť na inhibíciu funkcie vybraných molekúl. Obmedzením tejto technológie je nepredvídateľné správanie sa týchto protilátok v podmienkach vnútrobunkovej expresie. Posledné pokroky pri expresii protilátok v E. coli naznačujú, že zavedenie mutácií inšpirované porovnaním sekvencií alebo kvalifikovaný odhad môžu zlepšiť vlastnosti niektorých protilátkových domén, ale pre všeobecnú aplikovateľnosť a funkčnú využiteľnosť intracelulárnych protilátok treba ešte túto technológiu vylepšiť. Potrebné je najmä doriešiť racionálne postupy a selekčné schémy, pomocou ktorých by sa intracelulárne protilátky dali využiť na základe ich vlastností in vivo. *(Obr. 3, lit. 44.)*
Kľúčové slová: vnútrobunkové protilátky, pozohýnanie protilátkovej molekuly, vnútroreťazcové disulfidové väzby, fragmenty jednoreťazcových protilátok, selekcia intracelulárnych knižníc.

Bratisl. lek. Listy, 99, 1998, č. 8—9, s.413–418

The "intracellular antibodies" technology exploits the ectopic expression of recombinant antibodies to inhibit the function of selected antigens (1, 2). Antibodies can be ectopically expressed as intracellular proteins, equipped with suitable dominant and autonomous targeting signals, to neutralize intracellular gene products.

By using this technology, the function of many intracellular antigens has been successfully inhibited in the cytoplasm, the nucleus and in the secretory pathway of mammalian cells (for reviews see 1 and 2). In view of the initial successes, the technology holds promises for a variety of applications including plant biotechnology (3), human gene therapy (4) and functional genomics. Notwithstanding the success, the work performed so far has indicated quite clearly which are the directions in which improvements are needed and which are the points that need to be taken care of. These include folding stability, cellular half life, tolerance to disulfide bond absence, affinity, refinements of intracellular targeting and the introduction of effector functions (2). In this article we shall review how recent advances, in the field of antibody expression in *E. coli* and in the design of new selection schemes in higher cells, show the way to achieve substantial improvements in the properties of antibody domains for intracellular expression.

International School for Advanced Studies, Trieste
Address for correspondence: A. Cattaneo, Neuroscience Programme, International School for Advanced Studies (SISSA), Via Beirut 2/4, 34013 Trieste, Italy.
Phone: +040.37871 Fax: +040.3787249

Folding of antibodies under ectopical conditions

One of the problems encountered from the very initial phases of the intracellular antibody work was related to the limited half life of antibodies and of antibody domains expressed in the cell cytoplasm. It was initially surmised (5) that the expression of antibodies in the cell cytoplasm was made more difficult by the reducing environment, that would hinder the formation of the intrachain disulfide bonds of the VH and VK domains. Indeed, the intrachain disulfide bond does not form in scFv fragments expressed in the cytoplasm of eukaryotic cells (6). Thus, the cytoplasmic expression of antibodies is a "worst case" for this technology. The fact that antibodies targeted to the cytoplasm can exert functional effects on the corresponding antigens, under well controlled conditions (3, 7, 8, 9, 10), is noteworthy and suggests that folding in the cytoplasm, at least in these cases, is good enough to preserve antigen binding.

Higher expression levels, obtained with the use of vectors optimized for the subcellular targeting of scFv antibody fragments (11), do not necessarily lead to an increased yield of soluble antibodies, but rather, to a substantial increase of intracellular aggregates. While some scFvs show a diffuse intracellular distribution, typical of soluble cytoplasmic proteins, others are highly concen-

trated in granular structures, whose number, shape and size vary for each scFv, being typical for each antibody.

This is just one example of a general observation, that different cytoplasmic scFv fragments have very distinct properties and their performance under conditions of intracellular expression depends primarily on their aminoacid sequence, in a way that is *a priori* unpredictable, yet.

When expressing proteins ectopically, aggregation is often a problem. However, this is a natural phenomenon that also occurs during the maturation of newly synthesized native proteins in the cell and represents an off-pathway of the normal folding process (12). This side pathway, in the absence of chaperones, becomes crucially dependent on the primary sequence of the protein. The observed sensitivity of aggregating proteins to the "curing" effects of single-point mutations can be best explained by the existence of an aggregation-prone intermediate folding state. According to this model, mutations that either weaken the stability of the folded state or which slow down the folding rate, will lead to an increased concentration of the intermediate state. In some cases the folding intermediate implicated in the aggregation may have a significant native-like structure, but the generality of this fact remains to be proven. It is important, therefore, to understand how the primary sequence avoids off-pathway aggregation.

We envisage that two complementary approaches will facilitate the solution of this problem, a rational and a selection-based approach. The former is based on the introduction of mutations inspired by sequence comparisons or by educated guesses to study the requirements for the folding of the ectopically expressed antibodies. The latter exploits the availability of phage antibody libraries (recombinant polyclonal repertoires) and selection schemes whereby antibodies are selected on the basis of their performance *in vivo* as intracellular antibodies.

Rational approaches to engineering antibody domains

The heterogeneity in the solubility of cytoplasmic antibodies is reminiscent of the heterogeneity observed for antibody expression in the periplasm of *E. coli*, where the tendency of antibody fragments to aggregate limits the yield of soluble protein. Individual point mutations can lead to dramatic improvements in the solubility of scFv fragments, some of which are located in antibody frameworks (13), while others are found in CDRs (14, 15).

In the scFv format, all residues of the former variable/constant domain interface, most often of hydrophobic nature, become solvent exposed. These exposed hydrophobic residues may influence the in vivo folding pathway and promote the aggregation of folding intermediates. The V/C interface of a scFv fragment has been engineered (16), as first reported for scTCRs (17). The replacement of one of the V/C interface residues in a highly insoluble scFv fragment led to a 25 fold increase of the soluble protein, without affecting the thermodynamic stability and the antigen binding constant (16). However, many other mutations did not influence the solubility of the resulting protein. While the existence of the hydrophobic patches is preserved in all antibodies, their exact position and extent varies, and the contribution of each individual residue to the overall solubility can only be hypothesized but not predicted yet.

Also residues at the interface between the heavy and light variable domains may be the target of interface engineering, as shown in studies inspired by the naturally occurring camel VH domains (18). "Camelization" of a human VH domain, by substituting the hydrophobic residues at the VH/VL interface with the hydrophilic ones found in camel VH, improves its solubility (19, 20).

Grafting of antibody CDRs from their original framework to "acceptor" frameworks has been used as a strategy to "humanize" murine monoclonal antibodies (21). Carter et al. (22) reported that the humanized version (huMab 4D5) of a murine monoclonal antibody was 100 fold more soluble than its murine counterpart, suggesting that the framework could dictate the overall solubility properties of the protein. More recently, Jung et al. (23) used this procedure to convey stability and folding quality to a bad folder, by grafting its CDRs onto the framework of the superior folder huMab 4D5.

Intrachain disulfide bonds

Since antibody fragments do not form disulfide bonds in the cytoplasm (6), there is great interest in engineering antibody fragments that will fold and are stable under reducing conditions, and that could serve as frameworks to which other specificities could be grafted. Antibody variable domains have a conserved intrachain disulfide bridge linking the two ß-sheets of the Ig domains. The contributing cysteine residues are perfectly conserved in all known human or mouse germ line V regions. For one particular antibody variable domain (McPC603) (24), both VH and VL intrachain disulfide bonds were found to be necessary for the folding and stability. However, some naturally occurring antibodies lack one of the cysteine residues, as a result of somatic mutation. One antibody with a naturally missing disulfide (mAb ABPC48) (25) was recently investigated for folding and stability (26). In this antibody, the second half-cysteine in the VH is substituted by a tyrosine. The scFv version of the ABPC48 antibody was expressed in *E. coli.* While the ABPC48 scFv is less stable than an average scFv molecule, a mutant version in which the missing cysteine was restored was found to display a thermodynamic stability well above that of other scFv fragments, thus explaining why it tolerates the disulfide loss. In a follow-up study, focussed on the VL chain of the antibody ABPC48, Proba et al. (27) used DNA shuffling and phage display to select for the best replacements for the cysteine residues, as well as for mutations that may compensate for the loss in stability upon removal of both disulfide bonds in both VH and VL. scFv fragments without disulfide bridges were generated, that are as stable as the original one, showing that it is possible to generate a completely disulfide free scFv which is still functional.

The requirement for compensatory mutations allowing to overcome the loss of the disulfide bond confirms the results of earlier studies on the human VK domain REIv (28, 29). Two aminoacid mutations (Y23H and C23V) were introduced sequentially into this antibody domain. The first change stabilizes the folded state of the domain by 4,6 kJ/mol, while the second one, which abolishes the central disulfide bridge, destabilizes the folded domain by 17.5 kJ/mol. Introduction of the stabilizing mutation first is a necessary pre-requisite to the removal of the central disulfide bridge without collapse of the fold. The double mutant VK domain can be accumulated in a functional form in the cytoplasm of *E. coli.* X-ray crystallography of the variant without disulfide reveals a stringent conservation of the structure with respect to the wilf - type and to a stabilized variant (30).

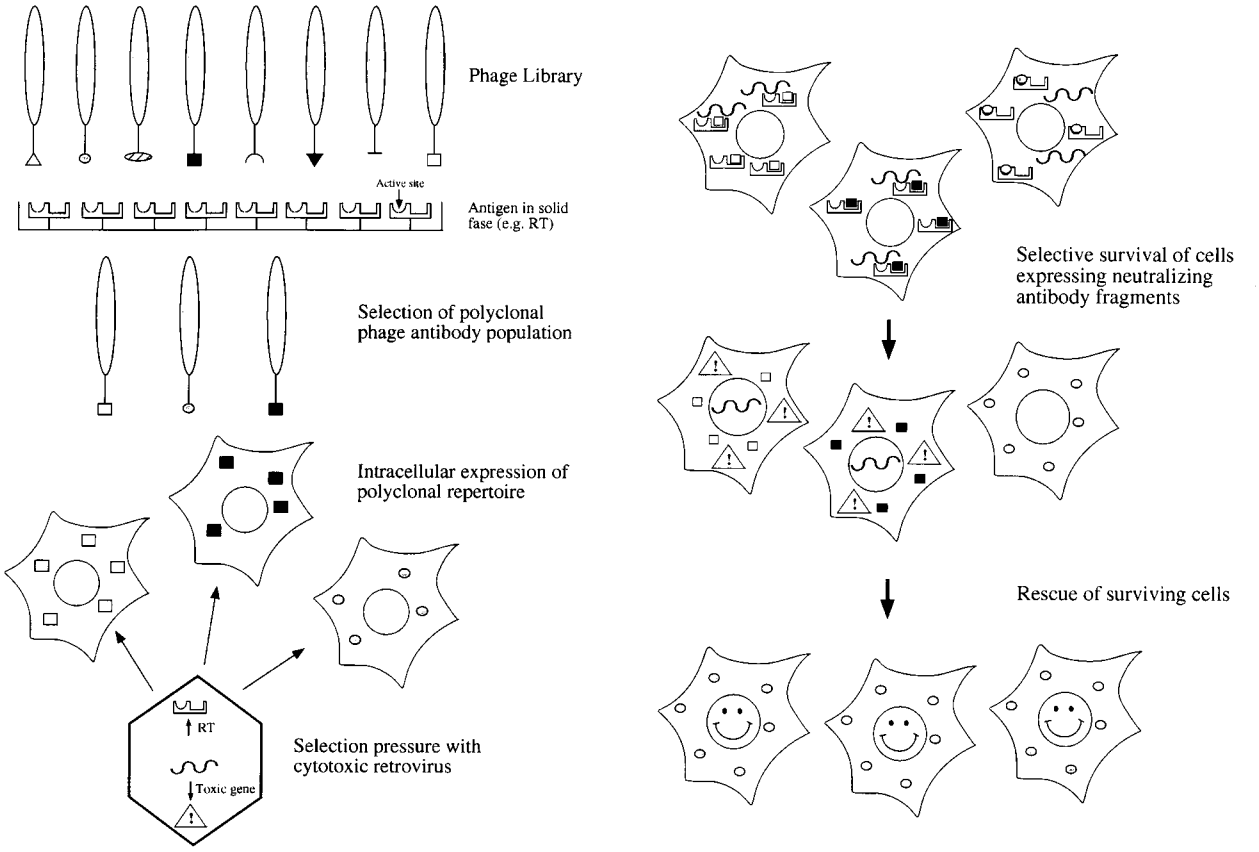

Fig. 1. From phage libraries to intracellular antibodies. Schematic representation of the phenotypic selection of intracellular antibodies.

Thus, it appears that variable domains have a range of folding stabilities and that the overall stability of the fold is contributed by many critical residues or combinations of residues in the framework regions. The loss, or the absence, of the cysteine residues will be therefore tolerated or not, according to the overall stability of each individual variable domain. Domains which are intrinsically more stable may tolerate the removal or the absence of the disulfide.

Notwithstanding the successful examples of rational improvement of the folding stability of antibodies, it is difficult to identify a priori, in a general way, those aminoacid replacements that are required for a general "superframework". Moreover, CDR residues can make significant contributions to the overall solubility and stability properties of the variable domains.

The performance of a given antibody for intracellular expression has to be optimized by many different parameters. Therefore, short of a general guiding rationale based on first principles of protein structural chemistry, a complementary possibility is to use molecular repertoire technology and genetic screens under conditions of cytoplasmic expression.

Selection of intracellular antibodies

By exploiting the phage display antibody technology is it possible to handle recombinant polyclonal repertoires of antigen binding domains that could be used for intracellular selection schemes.

Engineering repertoires of antibody domains into a format suitable for intracellular expression in eukaryotic cells, and applying appropriate selective pressures should allow us to select i) new antibody specificities, previously unknown, on the basis of the conferred phenotype, or ii) antibody frameworks more suitable for the particular ectopic expression conditions of interest, out of a pool of antibodies with a given binding specificity. Recent experiments have opened the way in this direction, and have been performed in mammalian, bacterial and, very recently, in yeast cells.

Mammalian cells

Most antibodies used so far for intracellular expression have been derived from monoclonal antibodies of predefined specificity. Antibodies are now increasingly derived from repertoires of variable regions displayed on the surface of phage.

The first "proof of principle" demonstrating the successful use of a phage-derived antibody domain for intracellular expression in mammalian cells has been recently provided (31). An antibody fragment neutralizing the RNA-dependent DNA polymerase activity of Moloney Mouse Leukaemia Virus (MMLV) –reverse transcriptase a(RT) was isolated (31) from a phage-display antibody library (D7αRT antibody fragment). Cells expressing D7αRT in

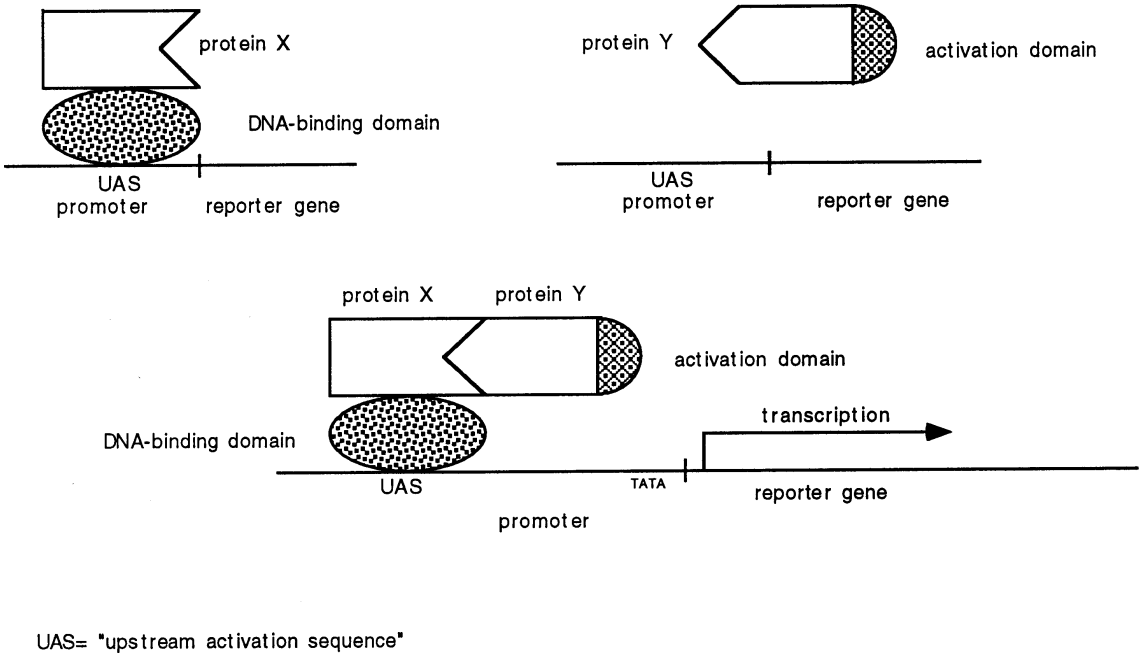

Fig. 2. Scheme of the yeast two-hybrid system, to study protein-protein interactions in vivo.

the cytoplasm were challenged with a Moloney-based recombinant virus driving the expression of herpes simplex virus thymidine kinase, a gene which confers sensitivity to the toxic action of Gancyclovir (GCV), thus leading to GCV-dependent cell death. The aintracellular expression of the D7αRT antibody fragment leads to an efficient inhibition of viral retrotranscription and thus to a selective survival of the cells (32), which are not affected by the toxic effects resulting from the MMLV-HSVtk retrovirus infection. The cell resistance to the toxic effect of the virally expressed HSV-tk correlates with an increased expression of the antibody fragment in the cell population. This experiment demonstrates a selection of cells on the basis of the function of the intracellularly expressed antibody fragment. In order to explore the possibility of rescuing an anti-viral neutralizing antibody, from an intracellularly expressed polyclonal repertoire, intracellular selection experiments were performed, using the resistance to the toxic action of the MMLV-HSVtk retrovirus as the selected phenotype. These were performed by a diluting the D7αRT DNA (up to 1/1000) with DNA encoding for a polyclonal population of anti RT scFv fragments. Cells transfected with this DNA mixture were challenged with MMLV-HSVtk retrovirus and selected with GCV. The number of asurviving clones correlates with the input D7αRT DNA in the atransfection mixture, and the number of cells containing the D7αRT DNA and expressing the corresponding antibody fragment was enriched, as the selection proceeded, at the expense of cells expressing other, less efficient, antibody fragments (33). In these amodel selections, the neutralizing properties of the D7αRT antibody fragment were known beforehand, but, in principle, this selection procedure, exploiting intracellular expression and a selectable phenotype, could be used to rescue cells expressing a previously unknown neutralizing antibody fragment against a polyclonal background of non neutralizing ones. From these cells, the corresponding intracellularly expressed antibody fragment could be isolated and characterized (Figure 1). A phage display library of antibody fragments is challenged with solid-phase coupled antigen (Ag), reverse transcriptase in this example, and an affinity purified population of phages, enriched in anti Ag specificities, is obtained after a few cycles. Only a few of these specificities, however, will be "neutralizing" ones. Bypassing the in vitro characterization of individual antibodies in this polyclonal population, this small enriched repertoire is formatted for intracellular expression in mammalian cells, using vectors now available for this purpose (11), and is transfected as a pooled DNA. Individual cells will express a small subset of antibody specificities, or even single specificities. Intracellular expression can be used to favourably select and rescue the cells expressing the neutralizing antibodies. These cells will outgrow the culture and will allow the isolation of the particular antibody expressed.

The success of this scheme will depend on the tightness and the rapidity of the selection scheme. More generally, provided a suitable selection scheme is designed, this should allow the rescue of antibody specificities providing a selection advantage to the cells expressing them intracellularly.

The nature of the polyclonal repertoire need not to be restricted to the example given, namely a polyclonal mixture of antibodies derived from a partial selection of a phage library on an antigen column. For instance, the polyclonal repertoire may be represented by a pool of mutated versions of one given lead antibody fragment. This would allow selection for higher affinity or stability to be performed in vivo.

It is clear that with mammalian cells the size of the repertoire cannot be too large, hence the need of a preselection step. The

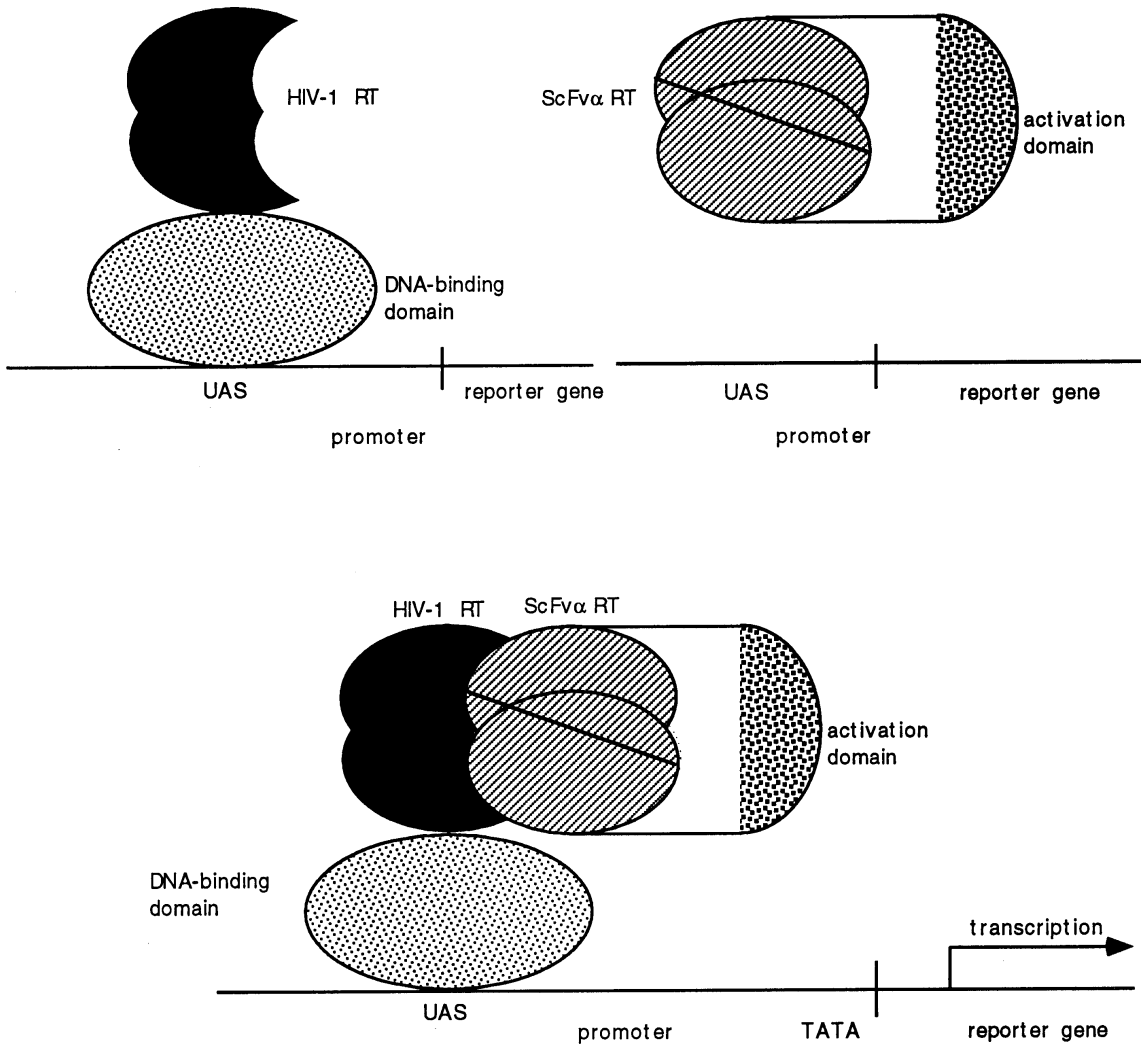

Fig. 3. Adaptation of the yeast two-hybrid system to monitor the interaction between ScFv fragments and their corresponding antigens, under conditions of intracellular expression.

polyclonal repertoire, however, can also be restricted in alternative ways (34, 35).

Bacterial and yeast cells

The cytoplasmic expression of ScFv fragments can be improved (36, 37) in strains of *E. coli* in which the thioredoxin reductase trxB gene was deleted (38) and in which the formation of disulphide bonds in the cytoplasm is enhanced. The trxB null mutation has little influence on the total amount of soluble protein, but increases the amount of disulfide-containing antibody in the cytoplasm. Thus, such systems might be attractive for screening and selection systems for cytosolic antibodies.

The concept of phenotypic selection of intracellular antibodies, as outlined above, can be extended to selection schemes based on the rescue of a vital enzymatic activity in a mutant cellular background. Initial attempts to implement this concept, based on the catalytic activity of an antibody expressed intracellularly in yeast cells (39), led however to the isolation of rescue mutations on genes other that the cytoplasmic antibody (40).

In a recent paper, Martineau et al. (41) isolated a scFv fragment that binds and weakly activates an inactive mutant ß-galactosidase. The gene encoding this scFv fragments was then subjected to random mutations in vitro and the library of mutants was coexpressed with the inactive ß-galactosidase in lac- bacteria. By plating on limiting lactose, scFv fragments are selected by virtue of their ability to bind antigen and activate its enzymatic activity in the cytoplasm. After four rounds of mutation and selection an antibody fragment with greatly improved cytoplasmic expression and binding affinity was selected. As expected, the disulfide bonds do not form, yet the fragment was able to fold.

The two hybrid system (42, 43), or interaction trap, provides an experimental system to monitor intracellular protein-protein interactions (44) (Figure 2), whereby a successful interaction between protein X and protein Y reconstitutes a functional transcrip-

tion factor, that can therefore activate the expression of a reporter gene. An intrinsic characteristics of the yeast two hybrid system is that the protein-protein interactions ocurr in an intracellular environment, namely the cytoplasm and the nucleus. For this reason, if scFv fragments could be successfully expressed in a yeast-two hybrid format, monitoring their interaction with a corresponding antigen should allow to isolate those scFv fragments that bind successfully to the antigen under intracellular expression conditions. We have recently been able to demonstrate that a positive interaction can be obtained between antigen-antibody pairs in a two-hybrid format, in yeast (AC, M. Visintin, T. Rabbitts et al., in preparation) (Figure 3). This initial proof of antigen-antibody interaction in the two hybrid format, using known antigen-antibody pairs, could be extended in a straightforward way to the analysis, in the two-hybrid format, of polyclonal repertoires selected from phage display antibody libraries, or of libraries of mutants of a given lead scFv fragment. This should allow the isolation of antibodies binding a given antigen efficiently upon cytoplasmic expression, before their subsequent validation and exploitation in functional intracellular antibody experiments in the species of interests.

Future prospects

The requirements for antibody folding vary between different cell compartments. Here we have discussed, mostly, aspects related to the expression of antibodies in the reducing environment of the cell cytoplasm. However, also in the secretory pathway different antibodies are differentially handled by the cell (2) and similar concepts of rational optimization and/or selection can be applied. In any case, finding antibody frameworks adapted for the expression in the desired compartment will greatly enhance the potential of the intracellular antibody technology.*

Refererences

1. Biocca S., Cattaneo A.: Trends Cell Biol., 5, 1995, pp. 248—252.

2. Cattaneo A., Biocca S.: Intracellular Antibodies: development and applications. Springer Verlag 1997.

3. Tavladoraki P. et al.: Nature, 366, 1993, pp. 469—472.

4. Marasco W.A.: Immunotechnology, 1, 1995, pp. 1—19.

5. Biocca S., Neuberger M.S., Cattaneo A.: EMBO J., 9, 1990, pp. 101—108.

6. Biocca S., Ruberti F., Tafani M., Pierandrei-Amaldi P., Cattaneo A.: Bio/Technology, 13, 1995, pp. 1110—1115.

7. Biocca S., Pierandrei-Amaldi P., Cattaneo A.: Biochem Biophys. Res. Commun., 197, 1993, pp. 422—427.

8. Biocca S., Pierandrei-Amaldi P., Campioni N., Cattaneo A.: Bio/Technology, 12, 1994, pp. 396—399.

9. Duan L. et al.: Proc. Natl. Acad. Sci. USA, 91, 1994, pp. 5075—5079.

10. Mhashilkar A.M. et al.: EMBO J., 14, 1995, pp. 1542—1551.

11. Persic L., Righi M., Roberts A., Hoogenboom H.R., Cattaneo A., Bradbury A.: Gene, 187, 1997, pp. 1—8.

12. Wetzel R.: Tibtech, 12, 1994, pp. 193—198.

13. Knappik A., Pluckthun A.: Protein Engineering, 8, 1995, pp. 81—89.

14. Ullrich H.D., Patten P.A., Yang P.L., Romesberg F.E., Schulz P.G.: Proc. Natl. Acad. Sci. USA, 92, 1995, pp. 11907—11911.

15. Kipriyanov S.M., Moldenhauer G., Martin A.C.R., Kupriyanova O.A., Little M.: Protein Engineering, 10, 1997, pp. 445—453.

16. Nieba L., Honegger A. Krebber, C., Pluckthun A.: Protein Engineering, 10, 1997, 435—444.

17. Novotny J. et al.: Proc. Natl. Acad. Sci. USA, 88, 1991, pp. 8646—8650.

18. Desmyter A. et al.: Nature Struct. Biol., 3, 1996, pp. 803—811.

19. Davies J., Riechmann L.: FEBS Lett., 339, 1994, pp. 285—290.

20. Davies J., Riechmann L.: Protein Engineering, 9, 1996, pp. 531—537.

21. Riechmann L., Clark M., Waldmann H., Winter G.: Nature, 332, 1988, pp. 323—327.

22. Carter P. et al.: Bio/Technology, 10, 1992, pp. 163—167.

23. Jung S., Pluckthun A.: Protein Engineering, 10, 1997, pp. 959—966.

24. Glockshuber R., Schmidt T., Pluckthun A.: Biochemistry, 31, 1992, pp. 1270—1279.

25. Rudikoff S., Pumphrey J.G.: Proc. Natl. Acad. Sci. USA, 83, 1986, pp. 7875—7878.

26. Proba K., Honegger A., Plueckthun A.: J. Mol. Biol., 265, 1997, pp. 161—172.

27. Proba K., Worn A., Honegger A., Pluckthun A.: J. Mol. Biol., 275, 1998, pp. 245—253.

28. Frisch C., Kolmar H., Fritz H.J.: Biol. Chem. Hoppe Seyler, 375, 1994, pp. 353—356.

29. Frisch C. et al.: Fold. Des., 1, 1996, pp. 431—440.

30. Uson I., Bes M.T., Sheldrick G.M., Schneider T.R., Hartsch T., Fritz H.J.: Fold. Des., 2, 1997, pp. 357—361.

31. Gargano N., Biocca S., Bradbury A., Cattaneo A.: J. Virol., 70, 1996, pp. 7706—7712.

32. Gargano N., Cattaneo A.: FEBS Lett., 414, 1997, pp. 537—540.

33. Gargano N., Cattaneo A.: J. Gen. Virol., 78, 1997, pp. 2591—2599.

34. Stausbol-Gron B. et al.: FEBS Lett., 391, 1996, pp. 71—75.

35. de Kruif J., Terstappen L., Boel E., Logtenberg T.: Proc. Natl. Acad. Sci. USA, 92, 1995, pp. 3938—3942.

36. Proba K., Ge L., Plueckthun A.: Gene, 159, 1995, pp. 203—207.

37. He M., Hamon M., Liu H., Kang A., Taussig M.J.: Nucleic Acid Res., 23, 1995, pp. 4009—4010.

38. Derman A. I., Prinz W.A., Belin D., Beckwith J.: Science, 262, 1993, pp. 1744—1747.

39. Bowdish K., Tang Y., Hicks J.B., Hilvert D.: J. Biol. Chem., 266, 1991, pp. 11901—11908.

40. Tang Y., Hicks J.B., Hilvert D.: Proc. Natl. Acad. Sci. USA, 88, 1991, pp. 8784—8786.

41. Martineau P., Jones P., Winter G.: J. Mol. Biol., 1998, in press.

42. Fields S., Song O.: Nature, 340, 1989, pp. 245—247.

43. Chien C.T., Bartel P.L., Sternglanz R. et al.: Proc. Natl. Acad. Sci. USA, 88, 1991, pp. 9578—9582.

44. Fields S., Sternglantz R.: Trends Genet., 10, 1994, pp. 286—292.

Do redakcie došlo 22.5.1998.

* Acknowledgements. I was honoured to be invited to present the work of my group at the VIII Congress of Cech and Slovak Immunologists. Simultaneously, I would like to express my deepest gratitude to the President Prof. M. Ferencik DSc, for the opportunity to share as an active participant the unique scientific atmosphere of this exciting gathering of Slovak and Cech scientists. The research described in the text has been supported by EEC (Biotechnology), Consiglio Nazionale delle Ricerche (CNR) (PF Biotecnologie) and Ministero della Sanit (AIDS Project).