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INTERNATIONAL SCHOOL FOR ADVANCED STUDIES

The Development of the Intrabody Trap Technology (ITT) for Functional Genomics

Thesis submitted for the degree of
"Doctor Philosophiae"

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To my sister
whose devotion to study and knowledge is every day real paragon of virtue

*"*The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day. Never lose a holy curiosity."

Albert Einstein

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Part of the data reported in the present thesis have been published or submitted in the articles listed below:

- Fasulo, L., **Visintin, M.**, Novak, M., Cattaneo, A. 1998. Tau truncation in Alzheimer's disease: expression of a fragment encompassing PHF core tau induces apoptosis in COS cells. **Alzheimer's Reports** 1(1): 25-32

- **Visintin, M.**, Tse, E., Axelson, H., Rabbitts, T.H., Cattaneo, A., 1999. Selection of antibodies for intracellular function using a two-hybrid in vivo system. **Proc Natl Acad Sci USA** 96(21): 11723-8

- Novak, M., Ugolini, G., Fasulo, L., **Visintin, M.**, Ovecka, M., Cattaneo, A., 1999. Truncation of tau and neurodegeneration. **Alzheimer's Disease and Related Disorders** Edited by Iqbal, K., Swaab, D.F., Winblad, B. and Wisniewski, H.M., John Wiley & Sons Ltd: 281-291

- Fasulo, L.*, Ugolini, G.*, **Visintin, M.***, Bradbury, A., Brancolini, C., Verzillo, V., Novak, M., Cattaneo, A., 2000. The neuronal microtubule associated protein tau is a substrate for Caspase-3 and an effector of apoptosis. **J Neurochemistry** 75 (2): 624-633 (*These authors contributed equally to this work)

- **Visintin, M.,** Cattaneo, A., in press. Selecting intracellular antibodies using the two-hybrid system. **Antibody Engineering – Springer Lab Manual.** Kontermann/Dubel Eds.

- **Visintin, M.,** and Cattaneo, A., submitted. The Intrabody Trap Technology (ITT): development of a cell-based high throughput screening for functional genomics. **Current Genomics**

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- Cattaneo, A., **Visintin, M.** 1998. Selection of intracellular antibodies. Phage Club Meeting, Kasteel Vaeshartelt, Maastricht, The Netherlands (16)

- **Visintin, M**., Ugolini, G., Fasulo, L., Bradbury, A., Verzillo, V., Novak, M., Cattaneo, A. 1999. Tau fragments as effectors of apoptosis. Society for Neuroscience 29th Annual Meeting, Miami, USA (801.4, 2016)

- **Visintin, M**., Tse, E., Axelson H., Rabbitts, T.H., Cattaneo, A. 1999. Selection of antibodies for intracellular expression using a two-hybrid system. The Biomedical and Biotechnological Use and Further development of Phage Display**,** La Grande-Motte, France (14).

- **Visintin, M**., Tse, E., Axelson H., Rabbitts, T.H., Cattaneo, A. 1999. Selection of recombinant antibodies for intracellular expression using the two-hybrid system. : FISV Meeting, Riva del Garda, Italy (6.45)

Declaration

The work described in this dissertation was carried out at the International School for Advanced Studies, Trieste Italy, between November 1996 and August 2000. All work reported arises from my own experiments and this work has not been submitted, as a whole or in part, to any other University.

Michela Visintin
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Main abbreviations used in the text

aa: amino acids
AD: Alzheimer's disease
Ade: adenine
ADHI: alcohol dehydrogenase I
Arg: arginine
Asp: aspartic acid
C: cyteine
CNS: central nervous system
Cys: cysteine
D: aspartic acid
DEAE-dextran: di-ethyl-amino-ethyl-dextran
DEPC:
DMEM: Dulbecco's modified Eagle's medium
DMSO: dimethyl sulphoxide
DN: dystrophic neurites
DTT: dithiotreitol
E: glutamic acid
ECL: enhanced chemiluminescence
EDTA: ethylenediamine tetra acetic acid
ER: endoplasmic reticulum
ES: embrionic stem
F: phenylalanine
G: glycine
Gln: glutamine
Glu: glutamic acid
Gly: glycine
H: histidine
His: histidine
HR: homologous recombination
HRP: Horseradish peroxidase
I: isoleucine
Ile: isoleucine
IPTG: isopropylthio-β-D-galactoside
ISEL: in situ end labeling (of DNA)
ITT: Intrabody trap technology
IVEM: *in vivo* epitope mapping
K: lysine
L: leucine
Leu: leucine
LiAc: lithium acetate
Lys: lysine

M: methionine
MAP: microtubule-associated protein
MBP: maltose binding protein
Met: methionine
MPBS: PBS containing Marvel Milk
MT: microtubule
NFTs: neurofibrillary tangles
ODN: oligodeoxynucleotides
P: proline
PAGE: polyacrilamide gel electrophoresis
PDI: protein disulfide isomerase
PEG: polyethylen glycol
Phe: phenylalanine
PHFs: paired helical filaments
PO: phosphodiester oligos
Pro: proline
PSs: phosphorothioates
PTGS: post-transcriptional gene silencing
Q: glutamine
R: arginine
RNAi: RNA-mediated interference
RPM: revolution per minute
S: serine
scFv: single chain fragment variable
SDS: sodium dodecyl sulphate
Ser: serine
SFs: straight filaments
SP: senile plaques
T: threonine
Thr: threonine
TMB: 3,3',5,5'-tetra-methyl-benzidene
TRITC: tetr-methyl-rhodamine iso-thyo-cianate (isomer R)
Trp: tryptophan
TrxA: thioredoxin A
Tyr: tyrosine
U: uracil
URA: uracil
V: valine
Val: valine
W: tryptophan
X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside
Y: tyrosine

Chapter One:
General Introduction

1.1 The Aim of the Research

Antibodies can be ectopically expressed as secreted proteins in cells that do not normally express them to interfere with extracellular antigens and/or as intracellular proteins targeted to different intracellular compartments to inhibit intracellular gene products: this has lead to an effective protein knock-out technology.
Expression of antibodies inside cells has been used successfully to ablate protein function.
The performance of antibodies that are intracellularly expressed is however somewhat unpredictable because the reducing environment of the cell cytoplasm in which they are forced to work prevents some antibodies, but not others, to fold properly.
For this reason, a selection procedure for the isolation of antibodies able to fold correctly and to bind antigens under condition of intracellular expression would be highly desirable. The development of this novel selection procedure could exploit methods to monitor intracellular protein – protein interactions, for instance the yeast two-hybrid technology.
Such a system would greatly facilitate the isolation of candidate antibodies for intracellular antibody down-stream applications in the system of interest, and would lend itself to the development of cell-based high-throughput screening procedures in functional genomics applications.
In view of this, the creation of a new technology that allows the isolation of intracellular scFv fragments, based on their ability to bind antigen under conditions of intracellular expression has become an important goal both for functional genomics and for gene therapy, to fulfill the requirement of antibodies with improved thermodynamic stability and solubility properties.

1.2 Results

The following chapters will describe all the steps taken in order to achieve the final goal: the development of the intrabody trap technology (ITT), an *in vivo* selection and assay procedure for functional intracellular antibodies using a two-hybrid approach.
In the first part of the project we find that several characterized antibodies can bind their target antigen in eukaryotic cells when expressed in the two-

hybrid format and we have been able to isolate intracellular binders from panels of scFv, all of which can bind antigen *in vitro*. Furthermore, we showed a model selection in which a single chain scFv was isolated from a mixture of half a million clones, indicating that this is a robust procedure that should facilitate isolation of intrabody specificity from complex mixtures.
The results form the basis of the "intrabody trap technology", whereby many different, specific and functional antibodies can be isolated *in vivo* under condition of intracellular expression.
The second part of the thesis reports the demonstration that ITT can be effectively applied to the *de novo* selection of functional intrabodies, by performing a real selection from polyclonal scFv fragments partially enriched from a large naive phage library by antigen panning. The experimental procedure described in this thesis has allowed the selection of intrabodies against a protein antigen involved in Alzheimer's disease (AD), the microtubule associated protein tau, the main components of the paired helical filaments (PHFs), found in neurofibrillary lesions in AD brains. In particular the aim of the work was to isolate scFv fragments against a tau fragment, which displays apoptotic capacity in different cellular context.

In order to fully appreciate the different aspects of the experimental works and its underlying strategies the first chapter includes some theoretical paragraphs that provide a general background to the work performed.

1.3 Functional Genomics

1.3.1 From Genes and Proteins to Genomes and Proteomes

The completion of the sequences of the genomes of several organisms is a watershed for the new science of genomics (Smith *et al*., 1996).
Genomics refers to the analysis of the genomes and functional genomics is a component of this field that uses global approaches to understand the functions of genes and proteins.
Functional genomics is expected to predict gene function of genes identified by large-scale sequencing efforts that come from different genome projects in the public domain, biotechnology sectors, and pharmaceutical gene-discovery efforts and this is providing a framework to

allow the investigation of biological processes by the use of comprehensive global approaches.

The goal of functional genomics is to elucidate some fundamental questions of human life:

- How does the exact sequence of human DNA differ between individuals?
- What are the differences that result in disease or predisposition to disease?
- How do proteins collaborate to perform the tasks required for life?
- Which genes are used under which circumstances?
- How do this differential gene expression results in different types of cells and tissues in a multicellular organism? (Fields *et al*., 1999)
- What are the gene and the related protein functions in normal and in disease states? (Dyer *et al*., 1999)

The ultimate question of functional genomics is:

- What is the specific role of each protein synthesized by different organisms?

In the past, molecular biologists considered the coordination of cellular processes largely as the synthesis of many individual reactions. Yet it is now thought that many of these processes are performed and regulated by multiprotein complexes, otherwise termed "protein machines" (Alberts & Miake-Lye, 1992), which are helped together and exert their function through specific protein-protein interaction. With improvements in technology, new tools have become available to study the complex interactions that occur in biological systems. It is now accepted that the best approach is not the study of individual molecules, but the examination on the large-scale and high-throughput of the genes and the gene products of an organism, with the aim of understanding how that organism is assembled or how it operates (Clark, 1999) (Wiley, 1998).

One approach to reconstructing cellular machinery and inferring function is to identify protein interactions, because proteins engaged in common task (such as a signaling cascade or a macromolecular complex) often contact one another.

A number of conventional approaches to studying protein-protein interactions include biochemical techniques that are powerful tools for investigating protein complexes but are not very suitable for high throughput screening. The yeast two-hybrid system, originally developed to monitor protein-protein interactions between two given proteins (Fields & Song, 1989) was adapted to a genomic format allowing all proteins from an

organism to be assayed for interactions with all other proteins from that organism.
Interaction traps, such as two-hybrid system and modification of this system, are commonly used for the identification of interacting partners *in vivo* (Lander, 1996) and its use in high throughput screening applications allows the rapid and efficient screening of large numbers of potential interacting cDNA (Fromont-Racine *et al*., 1997) (Flajolet *et al*., 2000) or peptides.
Genome-wide two-hybrid approaches offer insight into novel interactions between proteins involved in the same biological function.
Nowadays it is possible to study molecular mechanism globally in the context of complete sets of genes, rather than analyzing genes individually (Walhout *et al*., 2000). For example, DNA microarrays and chips, which use high-density two-dimensional arrays of chemically synthesized molecules on glass surface can be used to monitor simultaneously the expression of nearly all genes of an organism or can also identify genes expressed preferentially in normal or, for example, tumor tissue.
Recently, microarray technology is also being applied for genome-wide two-hybrid approaches (Uetz *et al*., 2000).
The ability to carry out a comprehensive genetic analysis of an organism becomes more limited and difficult as the complexity of the organism increases. Complex organisms are likely to have not only more genes than simple organisms but also more elaborate networks of interactions among those genes.
The development of technologies to systematically disrupt protein networks at the genomic scale would greatly accelerate the comprehensive understanding of the cell as molecular machinery and will represent a fundamental approach for functional genomics.
In general, the classical approach to solve the problem of identifying the function of the gene is to inactivate the gene and studies what effects this has.
The function of a gene can, in principle, be inactivated at any of several levels.
The next paragraph will discuss and compare current methods of inhibiting gene function.

1.4 Genotypic and Phenotypic Knock-Out Methods for Functional Genomic

1.4.1 Gene Knock-Out by Homologous Recombination

In some organisms a wild-type gene can be replaced by a mutant version of the same gene. Gene targeting is the use of homologous recombination (HR) between the target genomic locus and DNA introduced into the cells to make defined alterations into the genome of living cells.

The principal virtue of this method is that the gene function is completely destroyed, but the present technique is only applicable at few organisms, including *Aspergillus* (Miller *et al*., 1985), *Dictyostelium* (De Lozanne & Spudich, 1987), yeast (Scherer & Davis, 1979), (Russell & Nurse, 1986), mouse (Koller & Smithies, 1992) and also in *Drosophila* (Rong & Golic, 2000).

A major obstacle to the success of this technique is the relative inefficiency of the targeting process in most mammalian cells.

Gene disruption by homologous recombination only works efficiently in embryonic stem (ES) cells. These early embryo-derived cell lines can be manipulated in tissue culture and then returned to the embryo where they participate in the normal development of the chimeric mouse (Joyner, 1991). This approach has lead to the explosion of knock-out mice technology. The gene knock-out approach allow the analysis of diverse aspects of gene function *in vivo.*

To improve the efficiency and accuracy of homologous recombination events many new approaches have been employed, including artificial double-strand breaks in both the exogenous and chromosomal DNA, a transient overproduction of an inactive recombinase and of the bacterial RecA or mammalian RecA-like proteins in mammalian cell nuclei (Lanzov, 1999).

Although this technology has broad potential for fundamental research because of the obvious advantage of the absolute knock-out of the gene in homozygous individuals, the possible lack of spatio-temporal control over the gene knock-out can pose problems of lethality or trigger the onset of compensatory mechanisms. The interpretation of results observed after development of the organism can therefore be more difficult.

Recent advances on “conditional” gene targeting technology, combined to the use of prokaryotic recombinase proteins with restricted transcriptional control has improved gene inactivation in a defined tissue and at a specific time point during development or adulthood, thereby extending the sophistication and potential of this technology (Muller, 1999).

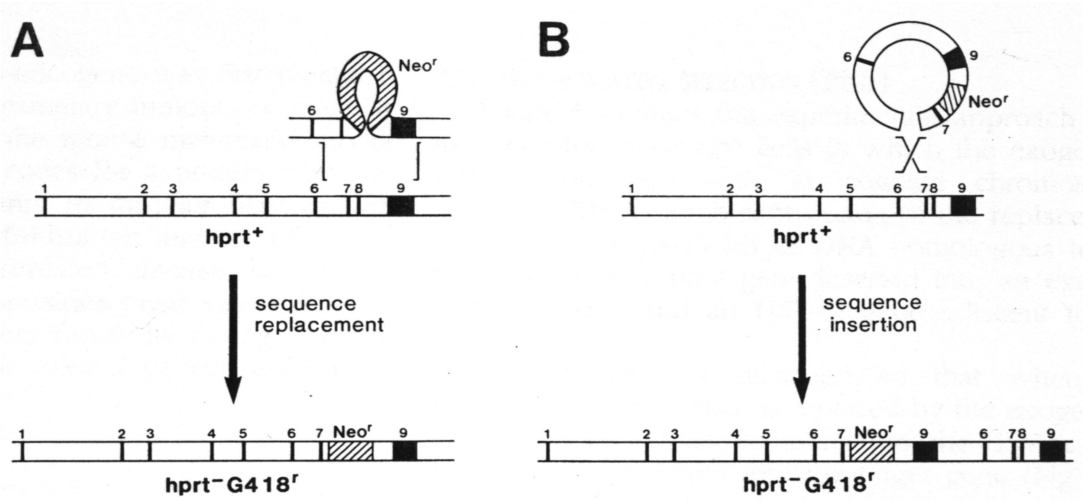

Fig.1.1 Disruption of a gene, by gene targeting

Disruption of *hprt* by gene targeting with (**A**) a sequence replacement-targeting vector or (**B**) a sequence insertion-targeting vector. Vectors of both classes contain *hprt* sequences interrupted in the eight exon with *neo* gene. With the sequence replacement vector, after homologous pairing between the vector and genomic sequence, a recombination event replaces the genomic sequence with vector sequences containing *neo*. Sequence insertion vectors are designed such that the ends of the linearized vector lie adjacent to one another on the *hprt* map. Pairing of such a vector with its genomic homologue, followed by recombination at the double-strand break, results in insertion of the entire vector into the endogenous gene. This produces a duplication of a portion of *hprt*. (Capecchi, 1989)

1.4.2 Dominant Negative Mutations

In this strategy, the cloned gene is altered so that it encodes a mutant product capable of inhibiting the wild-type gene product in a cell, thus causing the cell to be deficient in the function of that gene product. Such a mutation is "dominant" because its phenotype is manifested in the presence of the wild-type gene, and as it inactivates the wild-type gene function it is referred to as a "dominant negative" mutation.

The inhibitory variants of the wild-type product can be designed because proteins have multiple functional sites that can be mutated independently.

The dominant negative mutant proteins can retain an intact and functional subset of the domains of the parent wild-type protein, but have the complement of this subset either missing or altered so as to be non-functional.

There are large amounts of published data concerning the use of dominant-inhibitory proteins: in particular many reports using Ras mutants to suppress the activation of Ras and Ras-related GTP-hydrolyzing enzymes, or GTPases, has been published (Feig & Cooper, 1988) (Feig, 1999).

This method however, is not general and can only be applied with success in some cases. The success of this strategy requires a detailed study of structural and functional domains on the target protein and requires proper folding of the mutant protein compared to the wild-type protein.
Moreover, the properties shown using such dominant negative product suggest that there are limitations to the possible interpretations of the obtained results. The overproduction of an inactive product might have the opposite effect of increasing the activity of the wild-type protein especially when the defective form of the protein titrates a cellular inhibitor.

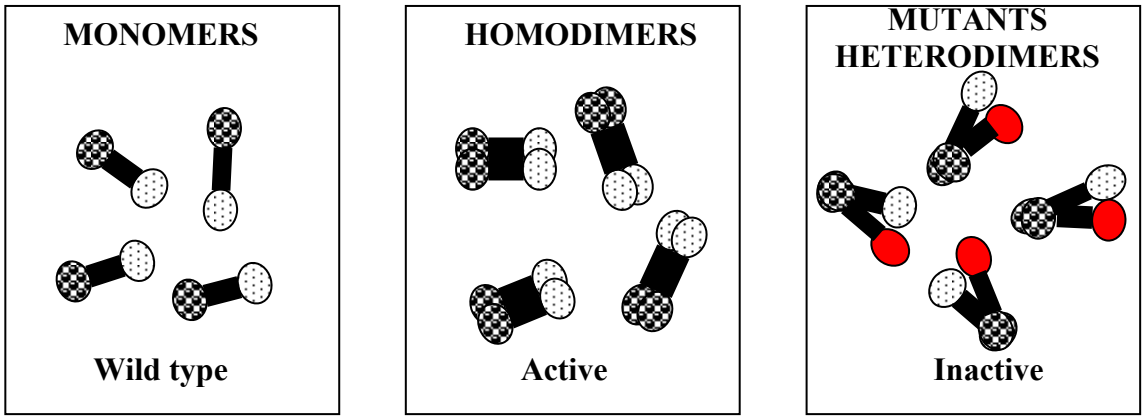

Fig.1.2 (A) Dominant negative mutants. Inhibitory polypeptides that interfere with the function of dimeric proteins. A dominant negative version of a dimeric protein may lead to an inactive dimer.

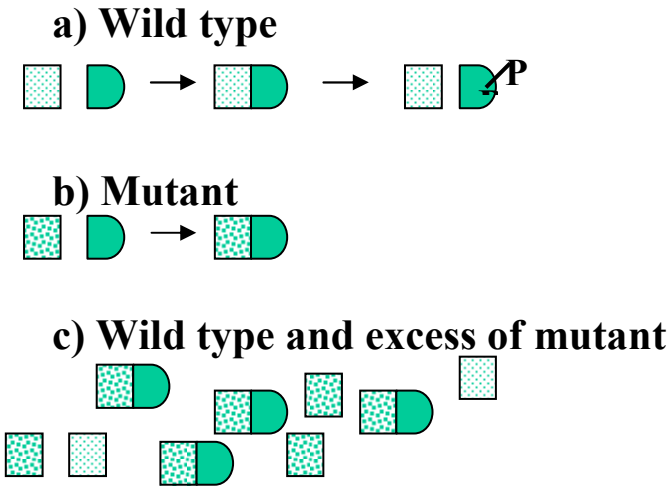

Fig.1.2 (B) Dominant negative mutants. Inhibitory polypeptide that interfere with function of monomeric proteins a) example of a monomeric enzyme that is able to phosphorylates its substrate b) the mutant form of that enzyme that has inactivated the catalytic site of the enzyme c) the mutant enzyme act as a competitive inhibitor of the wild type form (Herskowitz, 1987)

1.4.3 RNA-based methods

Experimental introduction of RNA into cells can be used in certain biological system to interfere with the function of an endogenous gene, ultimately depending on some form of antisense mechanism based on hybridization between the injected RNA and endogenous messenger RNA transcripts.

This approach might be particularly useful in species that were previously considered not to be amenable to genetic analysis.

There are four RNA-based approaches that has attracted much attention during last ten years:

- sense and antisense RNA
- antisense oligodeoxynucleotides (ODN)
- ribozymes
- double-stranded RNA-mediated interference (RNAi) or "quelling"

The expression of DNA encoded antisense RNA preparations by gene transfer methods is postulated to prevent the expression of the corresponding protein by hybridizing with the complementary nucleic acid molecules.

It was shown that sense and antisense RNA preparations are each sufficient to cause inhibition of target genes in *C.elegans* (Fire *et al*., 1991). Their effect can persist well into further generation, even though many endogenous RNA transcripts are rapidly degraded in the early embryo.

The unpredictable secondary and tertiary RNA structure, together with stability and expression levels and optimal length and locations of antisense RNA, are the most important limitation of this approach.

The promising results obtained with this technology seem to be contributed by the interference of some molecules with double-stranded character rather then the activity of individual strands (Fire *et al*., 1998).

Antisense reagents can also be designed as small oligodeoxynucleotides (ODN).

Antisense oligonucleotides affect cells by hybridizing to target sequences within mRNA, thus destabilizing the target mRNA and ultimately decreasing translation to protein.

Antisense effects of ODNs in mammalian cells have been reported in numerous tissue culture experiments and in several *in vivo* studies (Wagner, 1994). Several chemical modifications have been shown to improve the performance of the antisense ODN.

However, the phosphorothioates (PSs), which are the major antisense oligonucleotides considered for clinical therapeutic trial, induce significant non-sequence-specific effects at both the molecular and supramolecular level.

Furthermore, antisense oligonucleotides have a number of side effects related to:

- degradation: ODNs are degraded both intra- and extracellularly by serum and intracellular nucleases
- inability to enter the target cell: oligos are polyanionic molecule and cannot passively diffuse across cell membranes. The use of phosphodiester (PO) oligos can avoid this problem because they can be internalized within cells
- exocytosis. Oligos undergo exocytosis and are not retained by the target cell: the origin of this process and its effects on antisense inhibition are unknown
- ability to locate their targets. Intracellular targets for oligos (such as mRNA, pre-mRNA and genomic DNA) are protein bound, and many sites are probably not accessible for Watson-Crick base pairing
- non-specific binding with macromolecules. It has been shown that phoshorothioates bind to heparin-binding proteins with very high affinity and with cell surface proteins, producing biological consequences that may be confused with antisense inhibition
- last but not least, many studies with antisense lack appropriate controls making it difficult to judge the real effectiveness.

The potential of the antisense biotechnology with the benefits of improvements by chemical modifications are enormous but the unbalance between the number of real successful antisense inhibition and the putative reports of success is still an open question (Stein, 1999) (Stein & Cheng, 1993).

The term “ribozyme” has been introduced to described RNA molecules with enzymatic activity. The potential application of ribozymes as gene inhibitors, where a messenger RNA encoding a harmful protein would be intercepted and cleaved, is a fruitful area of research.

A variety of ribozyme catalytic motifs have been identified, all of which catalyze reaction on RNA substrate. These reactions involve site-specific strand scission and ligation reactions.

In general ribozymes require for cleavage a specific set of three nucleotides at the cleavage site; GUC is the triplet most efficiently cleaved by hammerhead ribozymes (Sigurdsson & Eckstein, 1995).

The first forms of catalytic RNA were discovered in the cellular RNA-splicing and processing machinery in the form of self-splicing group I introns and precursor tRNA-processing RNase P; other naturally occurring catalytic RNAs was subsequently discovered including group II introns, hammerhead ribozyme, hairpin ribozyme, hepatitis delta virus ribozyme and $tRNA^{Phe}$ (Scott & Klug, 1996).

The self-catalyzed RNA cleavage reactions share a requirement for divalent metal ions and neutral or higher pH (Haseloff & Gerlach, 1988).
One of the major limitations of this approach is the instability of RNA. The cleavage of ribozyme-RNA by ribonuclease can be avoided by functional-group modifications (substitution of the pyrimidines of the ribozyme with their 2'-fluoro derivatives, modifications to the 2'-O-methyl-groups, 2'-O-allyl-groups and 2'-deoxy-groups) at the 2'-site (Sigurdsson & Eckstein, 1995).
In general, the limitations shown before for antisense oligonucleotides remain the same for ribozymes with the addition of an improved selectivity of the ribozyme for its target RNA that will severely affect ribozyme efficiency when base-pairing mismatches or mutations adjacent to the site of cleavage occur.

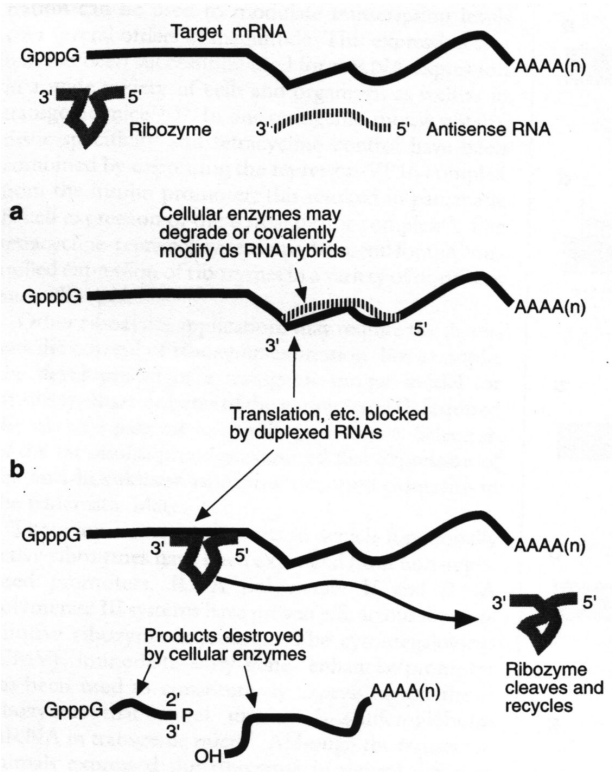

Fig.1.3 Ribozyme verus antisense-RNA-mediated inhibition of mRNA expression. The targeted messenger base pairs with either the antisense RNA (**a**) or ribozyme (**b**). The antisense inhibitory mechanisms may block cellular functions such as splicing, transport from the nucleus to the cytoplasm, or translation. In addition, the antisense-mRNA hybrid may activate cellular double-stranded ribonucleases or double-strand-specific RNA-modifying enzymes. In either case, the antisense acts stoichiometrically. The ribozyme interacts with the cleavage site by base pairing, cleaves the target and dissociates from the cleaved products; it can then recycle to cleave additional target RNAs. The ribozyme generates a 2',3'-cyclic phosphate and 5'-OH; this functionally destroys the target RNA (Rossi, 1995).

Another recent RNA-based approach for studying gene function by inhibition of RNA is the double-stranded RNA-mediated interference (RNAi) of gene expression.
This method has rapidly become a widely used method facilitating reverse genetic studies in *Cenorhabditis elegans* (Ketting & Plasterk, 2000), *Neurospora crassa* (Cogoni & Macino, 1999), *Arabidopsis thaliana* (Dalmay *et al*., 2000), *Drosophila* (Clemens *et al*., 2000), *E.coli* (Tchurikov *et al*., 2000) and mice (Wianny & Zernicka-Goetz, 2000).
In this process double strand RNA (dsRNA) silences the expression of endogenous genes after they have been transcribed by inducing sequence specific degradation of homologous messenger RNA molecules.
By exploiting post-transcriptional gene silencing (PTGS), originally called "co-suppression" by plant biologist, "RNA interference" by those studying worms and flies, and "quelling" by researchers working with fungi, specific genes has been switched off in a variety of organisms, allowing to deduce gene functions.
The mechanism by which dsRNA prevents target gene expression is not completely understood but it may represent a cellular defense against viral infection, or perhaps a post-transcriptional mechanisms for regulating gene expression in response to dsRNA formed from nuclear transcripts.
The gene silencing induced by RNAi is reversible and thus does not appear to reflect a genetic change (Fire et al., 1998).
There are several evidences that RNAi functions post-transcriptionally, including the fact that dsRNA corresponding to intron sequences does not produce RNAi and that dsRNA corresponding to exon sequences does not affect pre-mRNA levels (Tuschl *et al*., 1999).
PTGS is more efficiently induced by transgenes designed to produce the dsRNA of a gene than by either sense or antisense RNA alone.
RNAi has attracted considerable attention because it is a means of knocking-out the activity of specific genes, being particularly useful in species that were previously considered not to be amenable to genetic analysis.
RNAi process is homology-dependent, and the sequences to be targeted for gene-specific RNAi should be carefully selected to avoid cross-interferences between highly homologous sequences (Fire et al., 1998).
It was shown, for example, that the injection in *C.elegans* of dsRNA specific for the 5'part of the gene encoding the body-wall muscle myosin heavy chain generate a lethal phenotype, whereas the injection of dsRNA corresponding to other part of the same gene generates the expected paralysis phenotype (Fire et al., 1998).

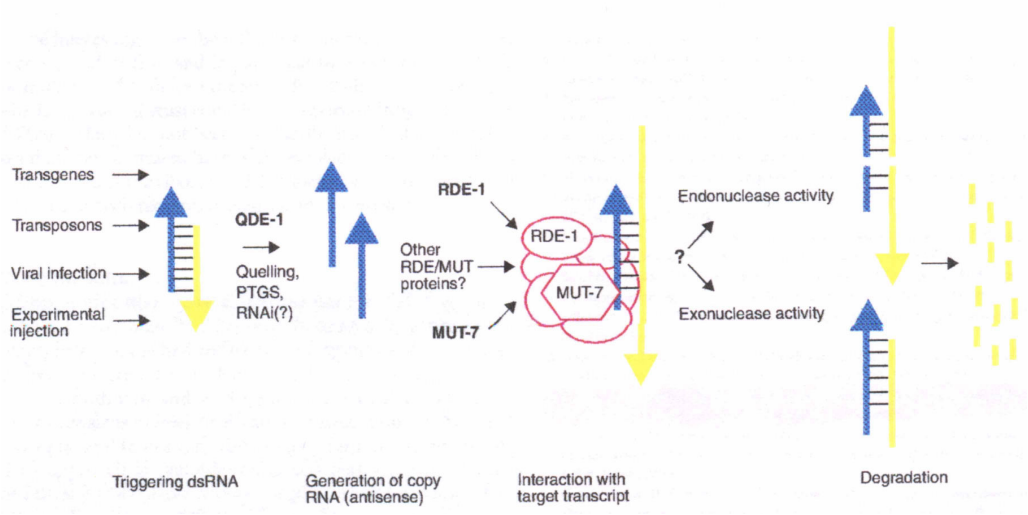

Fig.1.4 A model for RNAi/PTGS/quelling. The involvement of RNA-dependent RNA polymerase (RdRP) has been shown in quelling by the characterization of the *qde*-1 mutant. Genetic work in *C.elegans* has identified the requirement of other genes (*rde*-1, *mut*-7), but there is no evidence that they exist in a complex. A model was also proposed by Fire and coworkers in which RNAi does not involve RdRP-dependent copying step (Bosher & Labouesse, 2000).

The use of dsRNA adds to the tools available for studying gene function in many organisms. Although the effects of dsRNA-mediated interference are potent and specific, several limitations should be taken into account when designing RNA-interference-based experiments.

First, interferences between several closely related genes and sequences of gene families should be carefully evaluated. Second, it is likely that some genes and cell types resist to RNA-mediated interference effects: it is still unknown what is the active mechanism that mediate the intracellular transport of dsRNA molecule.

Third, what is the duration of the interference effect? The dilution of dsRNA through cell division should affect the expected interferences: it was shown that embrionically expressed genes are more easily knocked-out than late-acting ones (Bosher & Labouesse, 2000) (Wianny & Zernicka-Goetz, 2000).

One important limiting factor that was recently overcome in *C.elegans* is the inheritability of RNAi. The strategy of generating such inheritable dsRNA is based on the injection of a plasmid carrying inverted repeat so that the dsRNA is produced *in vivo* as a hairpin structure under the control of a strong, heat-shock-inducible promoter (Tavernarakis *et al*., 2000). The basis for this inheritance is still unclear but this approach seems to be a valid idea to investigate the late function of genes that can have also an essential early function.

Furthermore, the use of this technique is very promising because it is simple (it requires the production of dsRNA from PCR product that has T7 RNA polymerase binding sites at each end) and quick (knock-out results can be obtained within 2-3 days) (Clemens et al., 2000). Unfortunately, there are several limiting factors and mechanisms that still remain unclear: in particular the major limitation of this technique is that the target region of the RNA to be affected is still unpredictable a priori and need to be systematically scanned in order to locate the best RNAi site.

1.4.4 Protein-Based Knock-Out

Amongst the different types of molecules capable of inhibiting protein function through tight and specific binding are proteins themselves. Proteins are well suited for selection from combinatorial libraries where the binding partners can be retrieved easily by sequencing their corresponding DNA.
There are two general protein-based approaches to inhibit the function of genes at the protein level:

a) Peptide aptamers
b) Intracellular antibodies

a) Relatively small protein recognition modules that are widespread in several proteins often mediate the dynamic assembly of macromolecular complexes inside the cell. Each module family binds relatively short peptides with distinct chemical or structural characteristics.
Combinatorial peptide libraries displayed on the coat of filamentous phage are largely used to select target peptide recognition specificity.
One of the several alternatives to obtain peptide library is the design of a DNA library coding for random peptides inserted within a cellular carrier protein, which provides stability, conformational constraint, and targeting to a specific subcellular location. In general, the active site of thioredoxin A (TrxA) is chosen as carrier protein for random peptides because of its stability, ease of purification, and presence of a convenient restriction site in the region encoding the active site of the enzyme (Lu *et al*., 1995). Insertions into this site are tolerated, and tethering of both ends of the inserted peptide provides a degree of conformational constraint to the peptide not present when linear peptides have free ends (Blum *et al*., 2000). The TrxA was shown to serve as a scaffold for a combinatorial 20-mer peptide library fused to the activation domain of a yeast two-hybrid vector, also called “peptide aptamers” (Colas *et al*., 1996) and recently for the developing of the aptamer-based bacterial system (ABBIS), which allow

the conditional expression within bacterial cells of a random peptide library (Blum et al., 2000).
Although interesting examples of application of this technology were reported in yeast, with the use of two-hybrid system, and *E.coli*, the feasibility of this system in other cell system and the generality, must be proven. In particular, after the selection of a binding peptide, its use as an inhibitor of protein function in the context of the cell is not strait forward nor general.

b) Antibodies, the most popular class of molecules naturally designed as recognition molecules, provide a virtually unlimited repertoire of binding molecules for a wide range of applications. The use of ectopic antibody expression to inactivate gene products (Cattaneo & Neuberger, 1987) (Carlson, 1988) (Biocca *et al*., 1990) acts in a way that is very similar to that of the expression of a dominant negative mutant, because it is a dominant competitor, but its use is more general, since it relies on a class of diverse yet virtually similar inhibitor molecules.
Antibodies, synthesized by the cell and targeted to a particular cellular compartment, can be used to interfere in a highly specific manner with cell growth and metabolism and gene function (Cattaneo & Biocca, 1997).
The expression of antibodies inside the cell can be controlled at different levels:

1) transcriptional: the expression of inhibitory antibody molecules occurs under the use of suitable promoters in any cell or tissue of interest. The use of specific regulated promoters may also provide potential control of gene expression not only in a dose-specific but also in a time-specific manner
2) targeting: antibodies can be targeted to any cellular compartments by means of targeting signal sequences that can be inserted at different positions in the recombinant antibody

The high affinity and selective-binding properties of intracellular antibodies, or intrabodies, can be useful to modulate cellular physiology and metabolism by a wide variety of mechanisms (Richardson & Marasco, 1995) (Cattaneo & Biocca, 1997).
For example:

- they can block or stabilize macromolecular interactions
- they can stabilize macromolecular interactions
- they can modulate enzyme function by occluding an active site, sequestering substrate, or fixing the enzyme in an active or inactive conformation
- they can divert proteins from their usual cellular compartments

- they can target specifically a post-transcriptionally modified version of a given protein (including heterodimerization)
- they can specifically target the subcellular localization of a given protein
- they can selectively target non-protein antigens The results obtained to date suggest that intracellular antibodies represent a powerful alternative to the other methods of gene inactivation previously discussed, and may represent the only option in some circumstances.

The modular nature of the antibody, the completeness of antibody repertoires, the advance in antibody-library technology and the increasing knowledge in the cell and molecular biology of intracellular trafficking represent key points underlying the potential of the intracellular antibody technology.

The efficacy of this strategy depends crucially on an efficient folding of antibody domains in ectopic environments. The requirements for an efficient folding of antibody domains depend on the particular cell compartment in which the antibodies are expressed.

The next paragraph will describe recent advances in antibody engineering that allow *the novo* construction of antibodies and the selection of a desired antibody specificity by the screening of phage-display libraries, the limitation and the improvements of this technology to date, and the potentials of a designed selection schemes for the isolation of antibodies active in selected intracellular compartments.

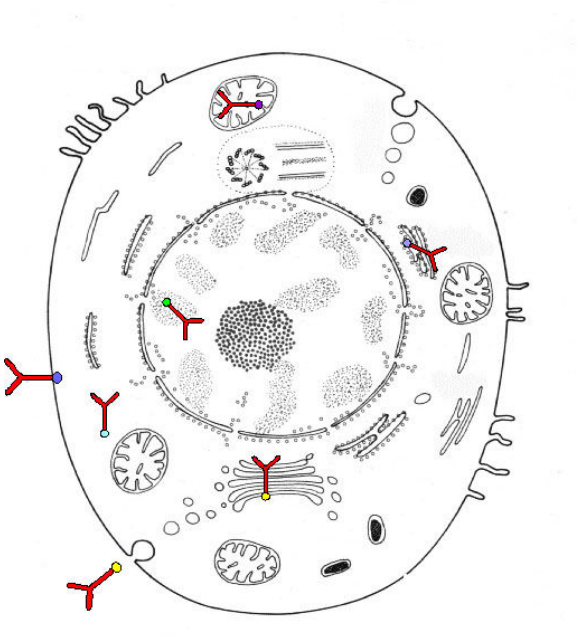

Fig.1.5 Targeting of intracellular antibodies. Illustration of how some of the known targeting signals can be exploited to redirect the intracellular trafficking of the antibodies in eukaryotic cells. For example, "intrabodies" can be directed to the nucleus, to the mitochondrion, to the plasma membrane and to the ER by means of targeting signals: see Table 1.1.

Compartment	Signal
secretory	MGWSCIILFLVATATGVHSQ (example) Hydrophobic leader sequence at N-terminal
cytoplasmic	MGWSKRRSSEETATAGVHSQ • Hydrophilic leader sequence at N-terminal None • Leader-less (Biocca *et al*., 1990) (Carlson, 1988) (Biocca *et al*., 1993) (Biocca *et al*., 1994) (Mhashilkar *et al*., 1995) (Maciejewski *et al*., 1995) (Persic *et al*., 1997)
nuclear	MGWSCPKKKRKVGGGTATVHSQ Nuclear localization sequence at N-terminal or C-terminal (Biocca et al., 1990) (Mhashilkar et al., 1995) (Persic et al., 1997) PKKKRKV: nuclear localization sequence of Large T-antigen of SV40 virus
mitochondrial	MSVLTPLLLRGLTGSARRLPVPRAK (amino terminal presequence of the mitochondrial enzyme cytochrome C oxidase) Presence of mitochondrial proteins at N-terminal (Biocca *et al*., 1995)
Endoplasmic Reticulum (ER)	Wild type leader sequence and SEKDEL (carboxy-terminal tetrapeptide sequence sufficient to cause retention in the ER) sequence at C-terminal (Biocca et al., 1995) (Graus-Porta *et al*., 1995) (Jost *et al*., 1994)
ER lumen membrane	Wild type leader sequence and μ chain transmembrane domain at C-terminal: NLWTTASTFIVLFLLSLFYSTTVTLF (Williams *et al*., 1990)
Plasma membrane	Wild type leader sequence and mutated μ chain transmembrane domain at C-terminal: NLWVVAAVFIVLFLLSLFYSTTVTLFTMD (transmembrane domain) of T cell receptor, met receptor and PDGF receptor (Williams et al., 1990) (Eshhar *et al*., 1993) (Chesnut *et al*., 1996)

Table 1.1 Peptide signals used for antibody targeting

1.5 Intracellular Immunization

1.5.1 Intrabodies: ectopic antibody expression to perturb gene products

The humoral immune system produces a vast repertoire of antibodies of high affinity and specificity by gene rearrangements. This repertoire ensures that the vast majority of invading foreign antigens can be recognized and bound, despite the enormous variety of chemical and physical structures that these entities can represent. The prospect of being able to exploit or copy this phenomenon in the laboratory, generating molecules of high binding affinity against any chosen target, has lead to the development of new technologies that allow human antibody genes to be easily manipulated (Barbas *et al*., 1991) (Nissim *et al*., 1994) (Vaughan *et al*., 1996) (McCafferty *et al*., 1990).

The advent of antibody engineering has opened up new avenues, including the possibility of producing high-affinity human antibodies against human

proteins. Antibodies derived from repertoires of variable regions displayed on the surface of filamentous phage (Winter & Milstein, 1991) (Winter *et al*., 1994) represent several advantages over classical monoclonals for many applications. The use of single-chain variable region fragments or scFv, in which the heavy- and the light-chain variable are synthesized as a single polypeptide and are separated by flexible linker peptide, is another element that has contributed to the success of recent antibody technology.
Phage antibody technology does not require immunization of humans or animals and provides large and complete repertoires containing antibodies against any specificity of interest. Single-chain antibody fragments can be produced on a large scale by isolation from bacteria (Hoogenboom *et al*., 1991) (Hoogenboom *et al*., 1992).

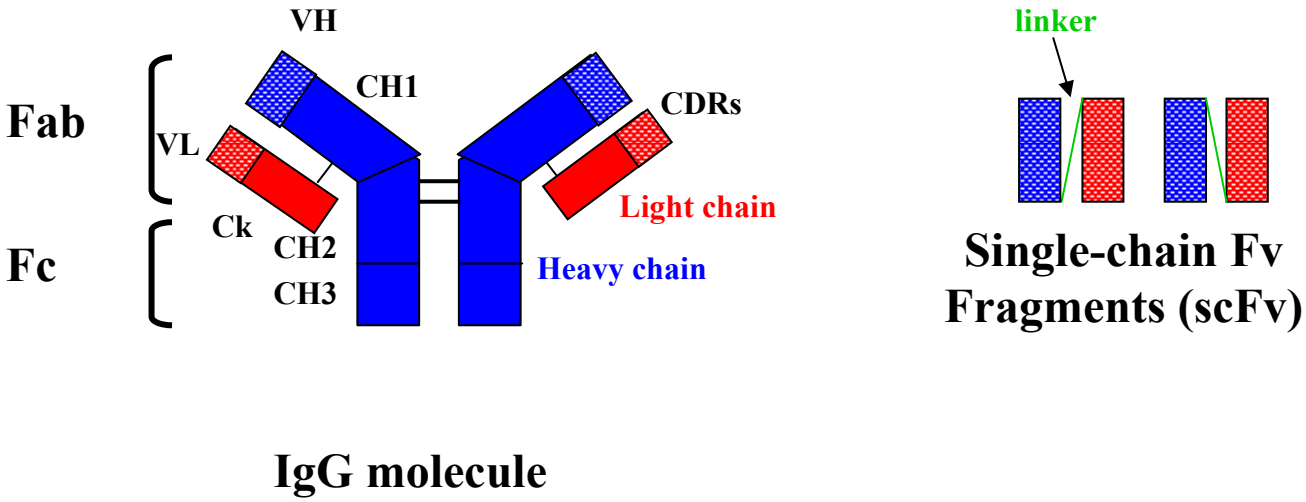

Fig.1.6 Structure of antibodies and of scFv fragment. An immunoglobulin is composed by two heavy chains and two light chains with the two antigen-binding regions made up of a heavy (VH) and light chain (VL). ScFv fragments are created by expressing the variable regions covalently joined by a polypeptide linker.

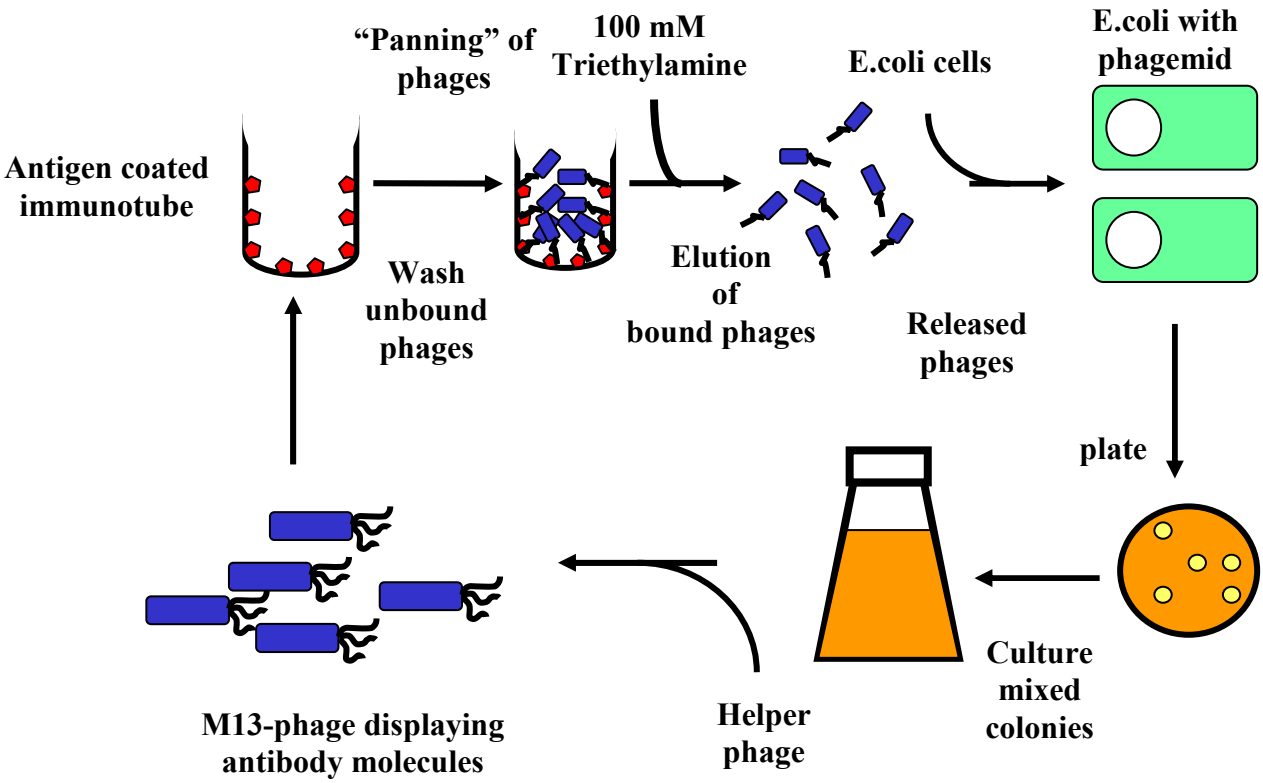

Fig.1.7 Phage Antibody Technology. Diagram showing one complete panning cycle

The phage display technology, as a source of recombinant polyclonal repertoires of antigen binding antibodies, can be used for new selection schemes whereby intracellular expression and targeting of antibodies could be exploited.
The antibody-based intracellular immunization strategy exploits the ectopic expression of recombinant antibodies to redirect antibodies to different intracellular compartments in order to inhibit the function of selected antigens in different biological systems (Cattaneo & Biocca, 1997) (Cattaneo & Biocca, 1999).
Historically the first experiment to inhibit gene function using antibody proteins by microinjection was performed by Rita Levi-Montalcini and coworkers in 1966 (Levi-Montalcini & Angeletti, 1966). In that experiment, the injection of antibodies to the nerve growth factor in newborn rats was shown to induce cell death of sympathetic neurons. This experiment provided the formal proof that NGF was a growth factor required for the survival and differentiation of these cells.

From our point of view, that experiment demonstrated that antibodies can be used in a very effective way to study the function of protein *in vivo*.
During subsequent years many other authors have used antibody injection inside cells to perturb gene function (Morgan & Roth, 1988) (Feramisco *et al*., 1985) (Hagag *et al*., 1986), but this has several limitations. The advent of recombinant antibody technology suggested that antibody expression in non-lymphoid cells could be achieved by gene transfer approaches, other than by antibody delivery. This led to the idea (Cattaneo & Neuberger, 1987) (Carlson, 1988) (Biocca et al., 1990) that the technology of the ectopic expression of antibodies can have a great potential in gene therapy, in functional studies and also (as shown in this thesis) in functional genomics (Richardson & Marasco, 1995) (Biocca & Cattaneo, 1995) (Cattaneo & Biocca, 1997) (Visintin *et al*., 1999).
The use of ectopic antibody expression exploits the virtually unlimited repertoire of the antibodies. This approach can allow the expression of inhibitory antibody molecules in the appropriate cellular compartment or extracellular space of any tissue of interest. For instance, the introduction of a moiety into a cell that leads to resistance of that cell to productive infection by viral pathogen has been entitled "intracellular immunization" by Baltimore et al. in 1988 (Baltimore, 1988) and this terminology was carried out by many authors to define the inhibition or inactivation of a function of a molecule by the ectopic intracellular expression of antibody binding domains that recognize the molecule (Biocca *et al*., 1995).
The assembly of antibodies in ectopic cellular contexts, outside of their physiological environment may results by targeting of the antibodies to different intracellular compartments by the use of suitable localization signals (Biocca et al., 1990) (Biocca et al., 1995). Many scientists have demonstrated that the antibodies can be functional and able to inhibit cellular function when expressed in different cellular context.
One of the pioneers of intracellular antibody expression was John R. Carlson: in 1988 he tested the feasibility of inactivating a protein *in vivo* through the inducible, intracellular expression of an antibody into yeast cells. The protein target chosen was the cytoplasmic enzyme the alcohol dehydrogenase I (ADHI). He introduced in a yeast expression vector the heavy and the light chain cDNA encoding a neutralizing anti-ADHI monoclonal antibody under the control of a galactose-inducible promoter (Carlson & Weissman, 1988). This "yeast intracellular antibody" was stably synthesized and co precipitated as a complex with ADHI, indicating that the antibody was capable of assembly and able to bind the antigen. This antibody was also able to produce a limited degree of neutralization *in vivo*: when cells where plated under conditions that favor the growth of cells containing reduced levels of ADH, the intracellular binding of the

antibody with the ADHI confers some degree of allyl alcohol resistance upon cells *in vivo*.
In 1990 Biocca et al., (Biocca et al., 1990) showed that by the use of suitable targeting sequences antibodies could be redirected to the desired intracellular compartments such as the nucleus.
In 1991, Hilvert and coworkers (Bowdish *et al*., 1991) (Tang *et al*., 1991) have used intracellular antibodies to alter cell metabolism: they expressed a catalytic antibody, or abzyme, to endow a yeast cell with an enzymatic activity, catalyzing a step (normally catalyzed by the product of the yeast ARO7 gene) in aromatic amino acid synthesis. Using a high-copy expression plasmid in an aro7 mutant yeast cell, they were able to recover functional antibody from yeast and the function of the antibody was detected *in vivo* by complementing the aro7 defect in yeast mutant strain, allowing the cells to grow under selective conditions.
In the same year Eugenio Benvenuto and coworkers engineered an immunoglobulin heavy-chain domain in a vector suitable for transformation of plants via *Agrobacterium* (Benvenuto *et al*., 1991). These studies represented the first alternative strategy to interfere with function of specific proteins involved in plant pathogenesis or development. Tavlodoraki et al. first reported the feasibility of plant immunoprotection against virus in 1993 (Tavladoraki *et al*., 1993). A constitutively expressed cytoplasmic scFv raised against the coat protein of the artichoke mottle crinkle virus (AMCV) was able to specifically shield transgenic plants from viral attack, reducing the infection incidence and causing a delay in symptoms development upon challenge with abnormally high titers of viral inocula.
Many other authors have reported phenotypic alterations caused by intracellular antibody expression in mammalian system.
In 1993, Marasco et al. described the intracellular expression and activity of a scFv antibody with a target signal to the endoplasmic reticulum, that recognize the envelope glycoprotein, gp120, of the HIV-1. They show that the antibody can be detected in the endoplasmic reticulum, co precipitates with the envelope protein, thereby inhibiting the processing of the envelope protein precursor and syncytia formation.
In 1993, Biocca et al. report the expression of an anti *ras* proto-oncogene scFv fragment in the cytosol of *Xenopus laevis* oocytes: this single-chain Fv fragment was able to colocalize with the endogenous p21ras protein in the cytoplasmic face of the oocyte plasma membrane and to inhibit insulin-induced meiotic maturation of the cell, a process known to be p21ras-dependent.
The efficacy of intracellular antibody expression has been reported in several applications to HIV viral proteins (Gargano *et al*., 1996) (Gargano

& Cattaneo, 1997a) (Mhashilkar *et al*., 1995) (Levy-Mintz *et al*., 1996) and also to oncogene products (Biocca *et al*., 1993) (Cochet *et al*., 1998).

1.5.2 Assembly, Folding and Stability of Intracellular Antibodies

It was very well demonstrated that antibodies for intracellular application could be directed to the relevant cellular compartments by modifying the antibody genes with N-terminus or C-terminus polypeptide extensions that encode classical intracellular-trafficking signals (Biocca et al., 1990) (Biocca & Cattaneo, 1995) (Marasco, 1995).

Functional studies have demonstrated that scFv are able to fold and assemble correctly in several intracellular environments, but when antibodies are expressed in the cell cytoplasm, folding and stability problems often occur.

One of these problems is that the cytosolic environment is not compatible with efficient disulphide bridge formation because of its reducing potential (Biocca et al., 1995).

The reducing environment (Gilbert, 1990) inhibits the formation of the conserved intradomain disulphides in antibody variable regions (Biocca et al., 1995). The intradomain disulphide contributes about 4-5 kcal/mol to the stability of antibody domains (see Fig.1.8). Therefore, antibody fragments expressed in a reducing environment are strongly destabilized. Nevertheless, a number of cytoplasmically expressed antibody fragments are active (Cattaneo & Biocca, 1997) and tolerate the absence of the disulphide bond, depending on the particular primary sequence (Proba *et al*., 1997) (Proba *et al*., 1998).

Therefore, the problem arises on how to access this subset of antibodies that tolerate the absence of the intrachain disulphide bond. This subset of antibodies is predicted to be more suitable for intracellular expression in the cell cytoplasm.

Some natural antibodies lack disulphide bond in the heavy chain variable domain (Rudikoff & Pumphrey, 1986) (Proba et al., 1997). These observations have inspired attempts to engineer antibodies that do not rely on the formation of the disulphide bond for folding in the cell cytoplasm. The most common way to do this is the introduction of designed mutations that stabilize antibody fragments lacking one or both of the key cysteine residues involved in disulphide bond formation (Worn & Pluckthun, 1999). The improvement of the *in vitro* stability in a given scFv, by concentrating engineering efforts on the weak part of the molecule may improve *in vivo* performance of scFv fragments which can be used as stable frameworks, suitable as recipients in CDR grafting experiments. Although these kinds of improvements may lead to success in individual cases, it is not possible to

apply generally such rational approaches especially in functional genomics where large networks of interacting proteins are going to be studied.

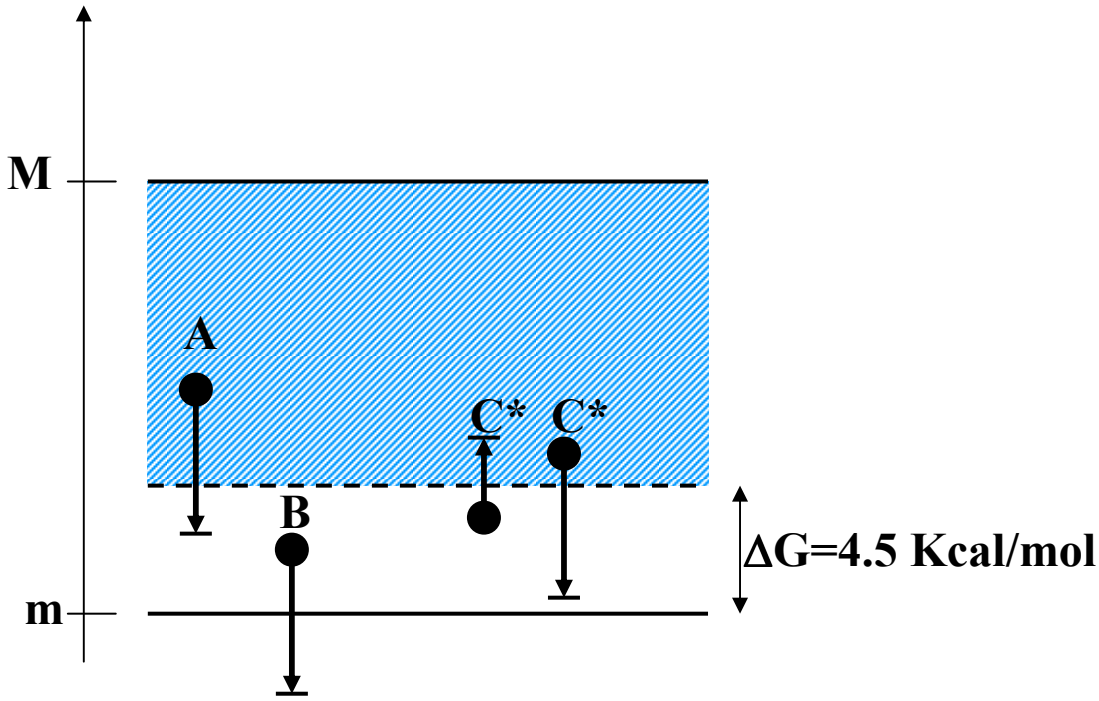

Fig.1.8 The folding of antibody domains has a range between a minimum (**m**) and a maximum (**M**) value and is contributed by many residues in the frameworks (Proba et al., 1998). Different scFv fragments have different overall stabilities. Some scFv could tolerate the removal of intrachain disulphide bond and remain folded because they are in the upper range of folding stability (**A**), while those that are in the lower range will not (**B**). Moreover, there are some mutations that could stabilize the antibody fragments rendering them tolerant to the absence of the disulphide bonds (C*).

Therefore, more general ways for selecting antibodies that perform efficiently in the different intracellular environments would be very useful.
One other problem is that the folding of newly synthesized polypeptide chains depends also on the assistance provided by two kinds of pre-existing proteins located in the endoplasmic reticulum (ER), therefore, unavailable in the cytoplasm, that are molecular chaperones, such as prolyl isomerase, protein disulphide isomerase (PDI) and other folding catalysts (Ellis & Hartl, 1999) (Hartl *et al*., 1994) (Freedman, 1995) (see Fig.1.9).

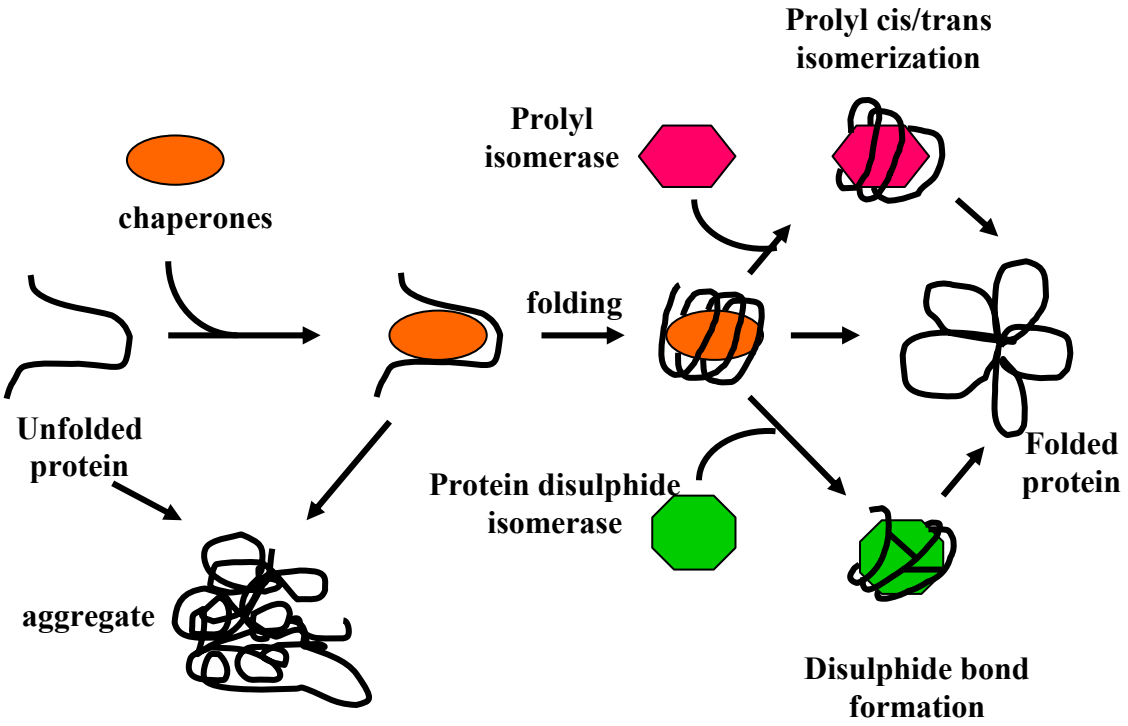

Fig.1.9 Protein Folding in *E.coli.* *E.coli* contains a number of helper proteins or "folding modulators", that catalyze certain folding steps (folding catalysts) or prevent the aggregation of the unfolded proteins.

Improper folding may prolong the interaction of the newly synthesized molecule with the folding enzymes and chaperones and can lead to degradation of the nonfunctional molecules.
The reducing environment of the cell cytoplasm hinders the formation of the intrachain disulphide bond of the VH and VL domains (Biocca et al., 1995) (Martineau *et al*., 1998) that is important for the stability of the folded protein.
The disulphide bond between the b-strand and the f-strand in the immunoglobulin domain is highly conserved and has been maintained in all known germline genes of all antibodies (see Fig.1.10)

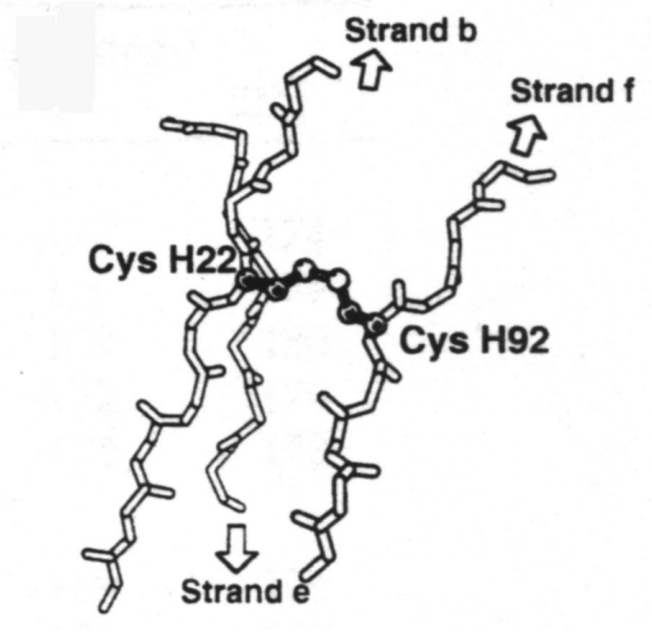

Fig. 1.10 Detailed overview of a model of a scFv fragment. In this model it is shown the disulphide-bonding between the two cysteine on the opposite b- and f-strands of the heavy chain of a scFv fragment molecule (Proba et al., 1997).

The reason for this conservation appears to be the relatively low stability of the domain, which requires additional stabilization by the disulphide.
Only more stable domains can tolerate the removal of the disulphide bond causing a conformational change in which new hydrogen bonds are formed compensating the loss of free energy from the missing disulphide (Proba et al., 1997).
Andreas Pluckthun and coworkers have extensively studied the tolerance of the disulphide loss of a natural C92Y mutation in the heavy chain of the anti-levan antibody ABPC48 (Proba et al., 1997) (Proba et al., 1998).
They demonstrated that the restored disulphide in that particular antibody increased its stability by 4.5 kcal/mol, and also showed that the original variable domain is more stable then average, explaining why it tolerates the disulphide loss. In a subsequent work they generated stable and functional cysteine-free antibody single-chain fragments by DNA shuffling and phage display obtaining new proteins that exhibit thermodynamic stability similar to the natural ABPC48 scFv fragment.
The approach taken in consideration by Pluckthun and many other authors would be the modification of the VH and VL sequences to replace the need for disulphide bonds to stabilize the scFv with either higher intrinsic stability (Worn & Pluckthun, 1998) or perhaps with differing intrachain stabilizing factors, such as metal binding residues. Such rational

approaches may lead to success in individual cases but generating generic frameworks may be difficult.
For this reason we have exploited a more general strategy in order to apply selection to the derivation of intracellular antigen-binding antibodies or fragments, allowing the isolation based on binding efficacy *in vivo* rather than a designer approach.
Our hypothesis is that the natural repertoire contains many stable antibodies that could be rescued with an appropriate method without making any molecular evolution strategy.

1.4.3 Potentials and Limitations of Intrabody Technology on a Genome-Wide Scale

In a recent review of Rogert Brent (Brent, 2000), there is an exhaustive analysis of which *ad hoc* experimental methods can be applied to functional genomics.
The ideal genomic experimental techniques should be:

- Cheap
- Accessible
- Nonproprietary
- Undertakeable
- Seeking answers to specific questions
- Generic: they should not depend on special properties
- Simple: such techniques must not require great skill in order to systematize them

At present there are several techniques that should be useful to generate affinity reagents against all encoded proteins, encoded by a given genome to knocking-out their function, and they include:

a) Antisense nucleic acids and dsRNA
b) Nucleic acid aptamers
c) Peptide aptamers
d) Intracellular antibodies

As described before, the first three tools suffer from drawbacks and limitations related to intrinsic technical problems or to experimental design. The use of intracellular antibodies can have great potential as an alternative/complementary strategy in defined situations, and the large number of careful studies showing the efficiency of this techniques, demonstrate the feasibility of this approach not only as a powerful experimental strategy in gene therapy (Marasco, 1995) and plant technology (Benvenuto & Tavladoraki, 1995), as previously suggested, but also in functional genomic as we now propose (Visintin et al., 1999).

The potential of intrabodies may be realized in the near future in functional genomics systematic screening programs and may play a central role either in conjunction with, or in place of, the other knock-out tools described in this chapter.
Notwithstanding the successes, the work performed so far has indicated quite clearly which improvement can be achieved and are required. They include above all, improvement in folding stability and cellular half-life, tolerance of disulphide bond absence, affinity and refinements of intracellular targeting.
Further improvements can be achieved by increasing selection stringency, using the selected frameworks to generate more active antibodies or engineering a new selection strategy for generating functional, soluble and stable antibody for intracellular expression.
Recent advances were also reported in antibody expression in *E.coli* and in the design of new selection schemes in mammalian cells, which show the working directions to achieve substantial improvements in the properties of intracellular expression of antibody domains.
In principle, the selection of good intrabodies may be performed on the basis of rescued phenotype and/or on the basis of binding under condition of intracellular expression.
Two studies showed the proof of principle of the formed strategy.
In 1997 Gargano et al., (Gargano & Cattaneo, 1997b) explored the possibility to rescue a neutralizing anti-viral antibody fragment from an intracellularly expressed anti-reverse transcriptase polyclonal repertoire. By exposing the cell population to the selective pressure of a cytotoxic retrovirus, they were able to show a strong inhibition of RT activity in those clones in which the expression of a specific anti-reverse transcriptase neutralizing antibody fragment was found. The results demonstrated that only the cells expressing the neutralizing antibody fragment can be efficiently rescued using as a unique selection the expression of antibody fragment.
In 1998 Martineau and coworkers, (Martineau et al., 1998) demonstrated that folded and functional antibody fragments can be isolated in the bacteria cytoplasm after successive rounds of mutation and selection on the basis of the obtained phenotype. They subjected the gene encoded the "activating" anti-β-galactosidase scFv fragments to random mutation *in vitro* by error-prone PCR, and co-expressed the mutant β-galactosidase and mutant antibody fragments in *lac*- bacteria in order to convert the cells to *lac*+.
After plating on limiting lactose, they were able to select for antibody mutants with improved expression properties.

Selection strategy purely based on intracellular antigen binding regardless of the particular phenotype generated, are however more general.
To this aim, we have exploited the yeast two-hybrid system to monitor and to select intracellular antigen-antibody interactions via reporter gene activation (Visintin et al., 1999).
The main procedure of this new selection strategy all based on *in vivo* binding is outlined in Fig. 1.11

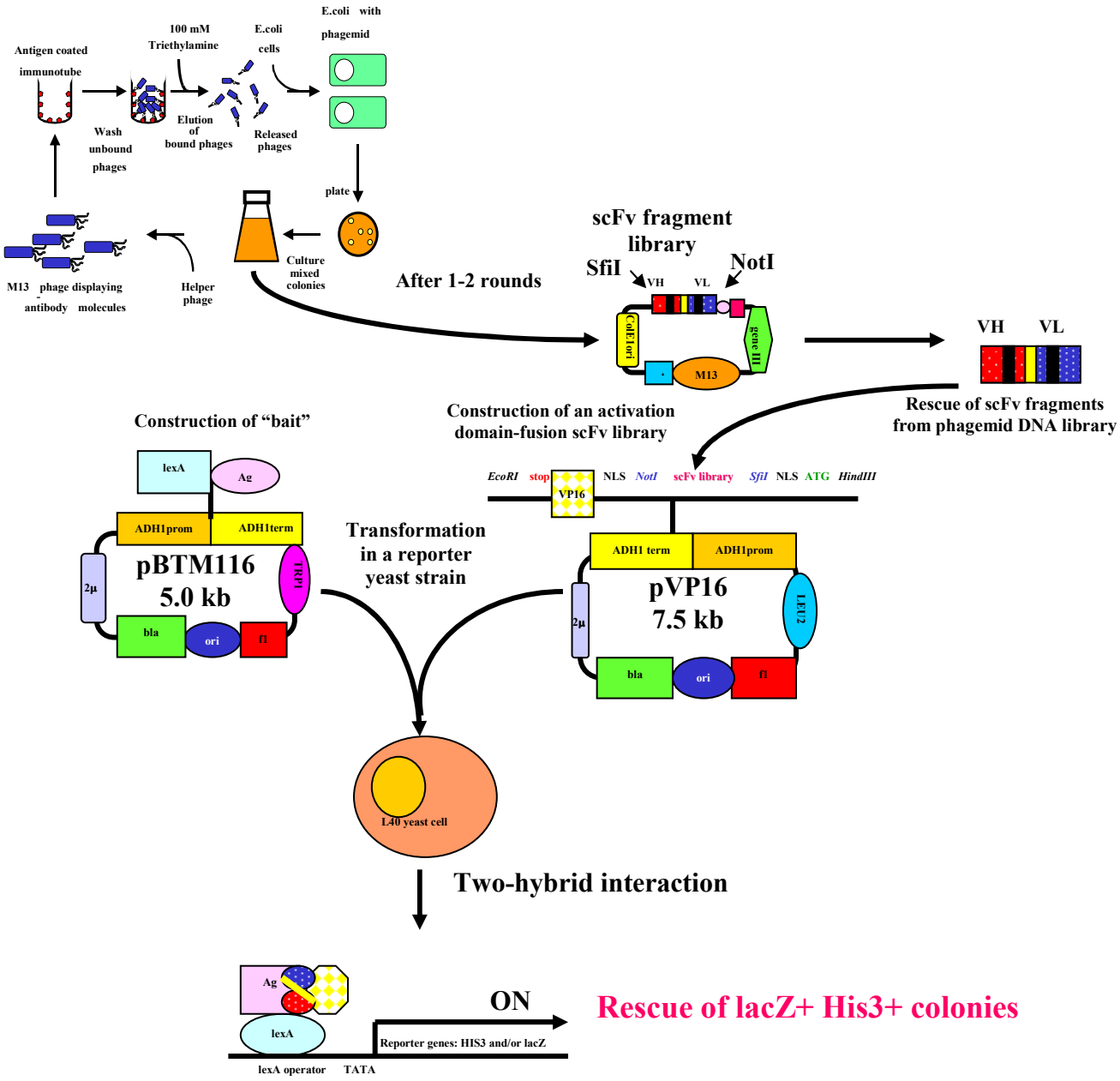

Fig.1.11 Schematic view of the *in vivo* selection with the use of the antigen-antibody two-hybrid system

The improvements yielded by this new technology are remarkable, because they fill the limitations shown so far.
The next chapter will describe all the steps taken in order to achieve the developing of this technology and the application of this method in a specific *in vivo* selection using a protein that need intracellular antibodies for both research and applicative purposes.
The potential of intrabodies in functional genomics will be described during the discussion in the next chapters.

Chapter Two: Materials and Methods

2.1 General Protocols for the Antibody-Antigen Two-Hybrid System

2.1.1 Yeast strain

The interaction system that we have used requires the yeast strain L40 (Hollenberg *et al*., 1995) which contains lexA operator-responsive reporters chromosomally integrated; the genotype of L40 is:
Mata *his*3Δ200 *trp*1-901 *leu*2-3,112 *ade*2 LYS2::$(lexAop)_4$-HIS3 URA3::$(lexAop)_8$-*lacZ* GAL4
Minimal HIS3 and GAL1 promoters fused to multimerized lexA binding sites drive the expression of the HIS3 and lacZ coding sequences, respectively. The expression of HIS3 gives a growth selection for interaction while the expression of lacZ, which encodes the enzyme β-galactosidase, can be monitored using a colorimetric assay based on the activity of β-galactosidase: the $lacZ^+$ yeasts form blue colonies in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β–D galactoside (X-gal).
This strain is deficient for TRP and LEU (auxotrophic phenotype) and cannot grow on minimal medium lacking those nutrients unless functional TRP1 and LEU2 genes are introduced. Moreover, this strain carries the ade2 mutation, which confers a red color (due to a red pigment accumulation) on medium containing limiting amounts of adenine that turns darker as the colony age.

2.1.2 Plasmids

There are several vectors for use with the two-hybrid system and in general they have a number of features in common. Not all the vectors in use can be utilized for this purpose, in particular the choice of terminal fusion for the scFv fragment can be crucial because of the steric hindrance of the molecule.
The two-hybrid vectors described here are plasmids that contain replicators and genetic markers that allow their selection and maintenance both in *S.cerevisiae* and in *E.coli*. The drug resistance markers and replicators that allow selection and maintenance in *E.coli* are standard (*bla* and *ori*

sequences respectively), whereas those that allow use in yeast are more specialized.

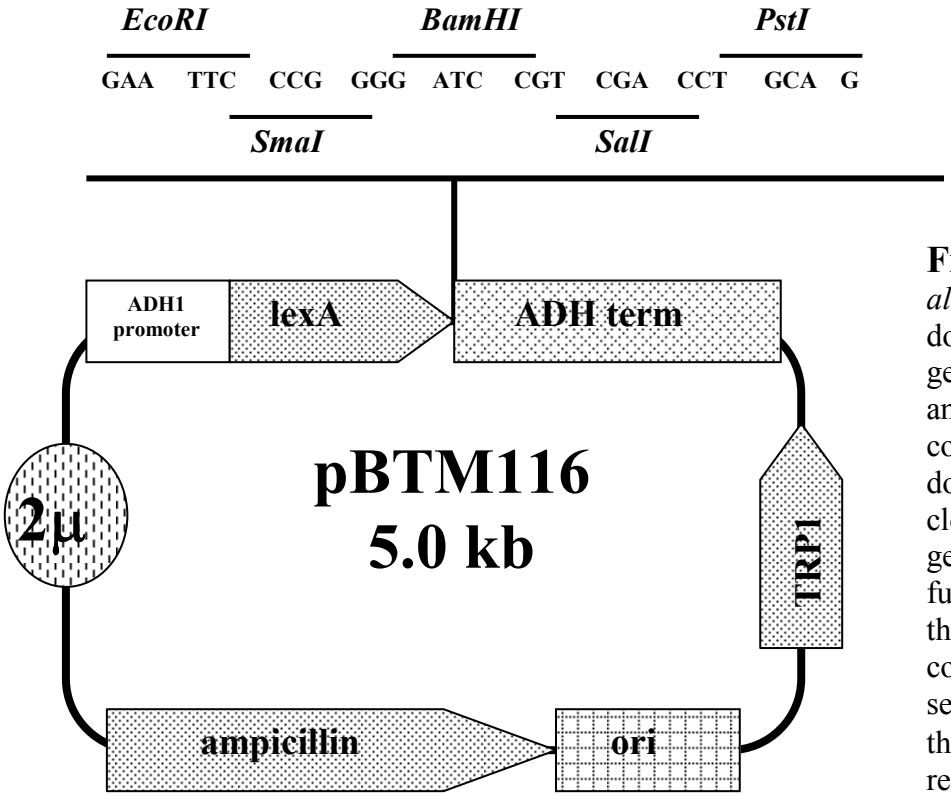

Fig. 2.1 pBTM116 (Bartel *et al*., 1993) 5-kb DNA-binding domain vector; is used to generate fusions of the target antigen with the complete lexA coding sequence, with downstream polylinker for cloning genes of interest to generate in-frame protein fusions to lexA, expressed from the yeast ADH1 promoter. It contains TRP1 gene, a selectable marker for yeast and the yeast 2µ origin of replication.

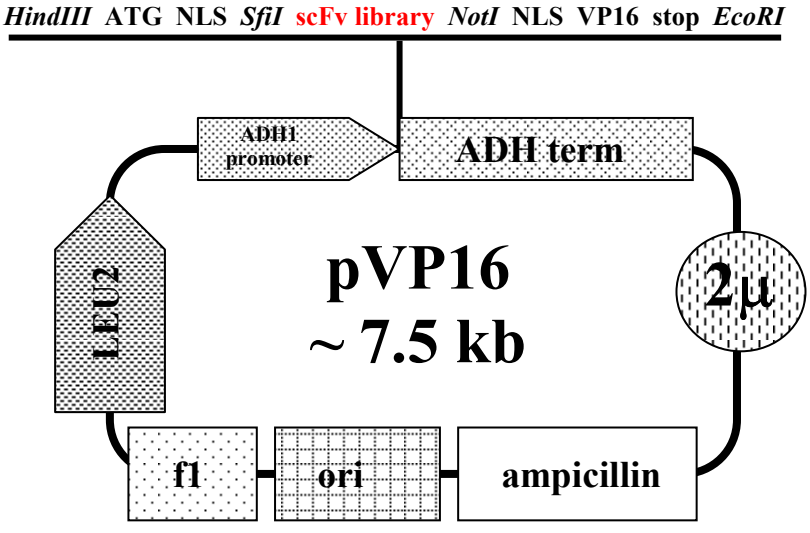

Fig. 2.2 pVP16* (Hollenberg et al., 1995) 7.5-kb activation domain vector, is used to generate fusions of a known scFv or a scFv library with the VP16 activation domain, with upstream polylinker for cloning genes of interest to generate in-frame fusions to VP16, expressed from the yeast ADH1 promoter. It contains LEU2 gene, a nutritional marker that must be used in conjunction with special host strains, as L40, that carry the appropriate complementable mutions. It contains also two SV40 large antigen nuclear localization sequences (NLS) fused to the VP16 acidic activation domain and the yeast 2µ origin of replication. ATG indicates the initial of transcription of the polylinker of the activation domain VP16 vector. The pVP16* polylinker was engineered for suitable cloning from library, which has SfiI-NotI cloning sites for scFv fragments as for example, Vaughan 96 (Vaughan et al., 1996) and Sheets 98 (Sheets *et al*., 1998).

Both plasmids contain ADH1 transcription terminators, downstream from the promoter and the cloning-fusion sites.

2.1.3 Preparation of Media and Reagents

Yeast strains, which employ auxotrophic mutations as markers, are grown on nutrient-rich media at 30°C to minimize selections for revertants.
Strains to be preserved are grown to logarithmic phase on YPD plates; the yeast is then scraped up with sterile inoculation loop and suspended in a 15-30% (v/v) glycerol-YPD medium; yeast can be stored now indefinitely at -70°C (the vials should be vortex briefly before freezing at -70°C to avoid cells settling to the bottom of the tube).
When needed, the yeast strain can be revived by transferring a small portion of the frozen sample onto YPD plates (yeast L40 colonies should appear slightly pink onto YPD plates and grow to >2 mm in diameter); yeast can be also stored at 4°C on YPD plates for up to 2 months. To verify the phenotype of the yeast strain provided, the streaking of few colonies from the working stock onto separate YC plates is performed (see table); phenotype will appear after 3-4 days of incubation at 30°C.

YPD medium
10 g yeast extract (Difco # 0127-17)
20 g peptone (Difco # 0118-17)
20 g bacto-agar (Difco # 0140-01)
2% glucose
Add H_2O to 950 ml. Adjust pH to 5.8 then adjust to 1 liter. Autoclave 121°C for 15 min.

YPA medium
10 g yeast extract
20 g peptone
20g bacto-agar
0.1g Adenine hemisulfate salt
Add H_2O to 950 ml. Adjust pH to 5.8 then adjust to 1 liter. Autoclave 121°C for 15 min.

YC medium

1. **YNB w/o aa & $(NH_4)_2SO_4$:**

1.2 g yeast nitrogen base, w/o amino acids and ammonium sulfate (Difco # 0335-15-9)
20 g bacto-agar
Add H_2O to 800 ml. Autoclave 121°C for 15 min.

2. Salts:
5.4g NaOH
10g succinic acid (Sigma # S-7501)
5g ammonium sulfate (Sigma # A-3920)

3. Glucose:
22g D-glucose
Dissolve in 16 ml H_2O

Add H_2O to 100 ml and dissolve all components one by one. Add glucose and H_2O to make a final volume of 150 ml.

4. amino acids MIX:
5.8 g NaOH
1 g Adenine hemisulfate salts
1 g L-Arginine HCl (Sigma # A-5131)
1 g L-Cysteine (Sigma # C-8277)
1 g L-Threonine (Sigma # T-8625)
0.5 g L-Aspartic acid (Sigma # A-4534)
0.5 g L-Isoleucine (Sigma # I-7383)
0.5 g L-Methionine (Sigma # M-9625)
0.5 g L-Phenylalanine (Sigma # P-5030)
0.5 g L-Proline (Sigma # P-4655)
0.5 g L-Serine (Sigma # S-5511)

Dissolve in 80 ml H_2O

5. L-Tyrosine:
0.5 g L-Tyrosine (Sigma # T-3754)
0.2 g NaOH
Dissolve in 10 ml by heating

6. Omitted aminoacid solutions:
L-Histidine (Sigma # H-9511)**:**
- 5 g/l H_2O

Uracil (Sigma # U-0750) **:**
- 10 g/l H_2O (+2 pellets NaOH)

L-Leucine (Sigma # L-1512)**:**
- 10 g/l H_2O

L-Lysine HCl (Sigma # L-1262)**:**
- 10 g/l H_2O

L-Tryptophan (Sigma # T-0254)**:**
- 10g/lH_2O

Add to aa MIX (4.) the L-Tyrosine solution (5.) and H_2O to make a final volume of 100 ml. Filter –sterilize, aliquot and store at -20°C for up to 1 year.
Filter-sterilize and aliquot individually omitted amino acids (aa) solutions (H, W, L, K, U) and store at -20°C for up to 1 year.
Before preparing YC plates or media by mixing appropriate solutions, media must be adjusted to pH 5.8 and sterilized by filtering salts + aa final mix. All the components must be mixed as suggested in Table 2.1:

	YNB w/o aa & $(NH_4)_2SO_4$	**Salts**	**aaMIX**	**W**	**H**	**U**	**L**	**K**	**H_2O**
-W	800	150	10		10	10	10	10	
-L	800	150	10	10	10	10		10	
-WL	800	150	10		10	10		10	10
-UW	800	150	10		10		10	10	10
-WHUK	800	150	10				10		30
-WHULK	800	150	10						40

Table 2.1 Note: All the suggested volumes are expressed in ml.

2.1.4 Construction and characterization of the “bait“

The first step in a two-hybrid assay is the construction of the „bait“. A series of control experiments must be performed to establish whether the

fusion protein is suitable as such or whether it must be modified; the verifications of well-behaved bait are:

a) testing for activity
b) testing for expression
c) testing for toxicity

a) activity

A well-behaved bait should not transactivate in a non-specific way the reporter genes in the L40 strain and should not interact with either the nuclear localization signals or with the VP16 activation domain. Therefore, the L40 strain transformed with the bait alone or together with pVP16* plasmid should not grow in the absence of histidine and should not contain any detectable β–galactosidase activity. Note: the bait must be modified by using a particular domain or a deletion mutant of the same protein in frame to lexA if it does not fulfill all the proposed features.

Test the extent of non specific activation of the reporter construct by the bait plasmid with the following protocol:

1) transform the bait plasmid into L40 strain using the small-scale yeast transformation protocol
2) select for transformants on appropriate YC plates as described in Table 2.2
3) assay the bait construct for activation of HIS3 reporter gene as described in Table 2.2 and for activation of the lacZ reporter gene using the β–galactosidase colony-filter assay as described below

LiAc transformation (small-scale)

This protocol is a modification of published methods (Gietz *et al*., 1992), (Hill *et al*., 1991), (Schiestl & Gietz, 1989) made by Clontech laboratories. The expected transformation efficiency is 10^3-10^4 / μg plasmid DNA.

Materials

- 10X LiAc buffer: 1M LiAc, pH 7.5 adjusted with diluted glacial acetic acid filter-sterilized (lithium acetate dehydrate –Sigma # L-6883)

- 50% (w/v) filter-sterilized PEG 4000: polyethylene glycol, avg. mol. wt.=3350-Sigma # P-3640. (Solution must be kept in a tightly sealed glass bottle to avoid evaporation)
- 10X TE buffer: 100mM Tris, 10mM EDTA, pH7.5, filter-sterilized
- 100% DMSO: dimethyl sulfoxide
- 10 mg/ml denatured herring testes carrier DNA (Clontech # K1606)

Procedure

Day 1: Inoculate few colonies of L40 in 50 ml of YPD and incubate for 16-18 hr with shaking at 250 rpm at 30°C to place the culture at mid log phase the next day (OD_{600} > 1.5). **Note.** Use only glass flasks carefully washed with ultra pure, pyrogen-free water and sterilized by autoclaving 15 min at 121°C.

Day 2:

1) Dilute the overnight culture to OD_{600} 0.2-0.3 in 300 ml of YPD prewarmed to 30°C. Grow at 30°C for 3 hours with shaking (230 rpm)
2) Pellet the cells by centrifugation (1000 X g for 5 min) at room temperature, discard the supernatant and resuspend the pellet in 50 ml of H_2O.
3) Centrifuge the cells again as in 2), decant the supernatant
4) Resuspend the pellet in 1.5 ml of freshly prepared 1X TE/LiAc (10mM TE, 0.1M LiAc)
5) Prepare in a tube a mixture of:
- ✓ 0.1 µg lexA-Ag vector construct
- ✓ 0.1 µg scFv-VP16 vector construct (if you need to test specific antigen-antibody partners)
- ✓ 0.1 mg herring testes carrier DNA

6) Add 0.6 ml of a sterile PEG/LiAc (0.1 M LiAc, 10mM TE, PEG 4000 40%) to the tube and vortex to mix
7) Incubate 30 minutes at 30°C with shaking (230 rpm)
8) Add 70 µl of DMSO, mix gently by inversion and heat shock for 15 min in a 42°C water bath.
9) Chill cells on ice
10) Pellet cells by centrifugation (20 sec at maximum speed)
11) Remove supernatant and resuspend cell in 0.5 ml of sterile 1X TE; spread 100 µl for single transformation or 250 µl for a co-transformation on each 100 mm plate.

LiAc transformation (maxi-scale)

The following transformation protocol is a modification of published methods made by Invitrogen laboratories (Gietz et al., 1992), (Hill et al., 1991), (Schiestl & Gietz, 1989). This protocol should be applicable for cDNA library transformation when a higher efficiency of transformation (≅ 10^6/μg DNA) is needed.

Materials

- ✓ 500 μg scFv/VP16* DNA
- ✓ 150 ml YC-UW
- ✓ 2 l YPAD
- ✓ 1 l YPA
- ✓ 1.5 l YC-UWL + 10 YC-UWL plates (100mm)
- ✓ 1.5 l YC-WHULK + 100 YC-WHULK plates (100mm)
- ✓ 100 ml 10X TE
- ✓ 20 ml 10X LiAc
- ✓ 1 ml of 10 mg/ml denatured salmon sperm
- ✓ 150 ml 50% PEG 4000
- ✓ 20 ml DMSO

Procedure

1. **Day1**: grow L40 yeast containing bait plasmid in YC-UW O/N
2. **Day2**: inoculate 100 ml of YC-WU with an aliquot of the overnight culture in order to find a dilution that places the 100 ml culture to logarithmic phase the next day
3. **Day3**: transfer enough overnight culture in 1 l of prewarmed to 30°C YPAD to produce an $OD_{600} = 0.3$
4. Grow at 30°C for 3 hours
5. Centrifuge the cells at 1500 X g for 5 min at room temperature
6. Wash pellet in 500 ml of 1X TE then centrifuge again the cells at 1500 X g for 5 min at room temperature
7. Resuspend pellet in 20 ml 1X LiAc, 0.5X TE and transfer to a new flask
8. Add 500 μg DNA library and 1 ml denatured salmon sperm
9. Add 140 ml of 1X LiAc, 40% PEG 3350, 1X TE; mix and incubate for 30 min at 30ºC with gently shaking
10. Add 17.6 ml of DMSO; swirl to mix
11. Heat shock for 10 minutes at 42ºC in a water bath swirl occasionally to mix
12. Rapidly cool at room temperature cells in a water bath diluting with 400 ml YPA

13. Pellet cells by centrifugation and wash with 500 ml YPA
14. After centrifugation resuspend pellet in 1 l of prewarmed YPAD
15. Incubate at for 1 hour at 30°C with gently shaking
16. Pellet cells from 1 ml: resuspend in 1 ml YC-UWL; spread 100 µl of a 1:1000, 1:100, 1: 10 dilutions for transformation efficiency controls
17. Pellet cells from the remaining culture
18. Wash pellet with 500 ml YC-UWL
19. Resuspend in 1l of prewarmed YC-UWL and incubate O/N at 30°C with gently shaking
20. **Day4:** pellet cells and wash twice with 500 ml of YC-WHULK
21. Resuspend final pellet in 10 ml of YC-WHULK
22. Spread dilutions of the total on YC-UWL plates to compare to the number of primary transformants
23. Spread the remaining transformation suspension on YC-WHULK plate

β–galactosidase filter assay
(Breeden & Nasmyth, 1985)

Materials

- ✓ Nitrocellulose filter circles (Scheicher and Schuell BA85)
- ✓ Buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1mM $MgSO_4$, pH 7.0)
- ✓ 50 mg/ml 5-bromo-4-chloro-3-indolyl–β–D-galactoside
- ✓ Whatman filter circles
- ✓ Liquid nitrogen

Procedure

1) Patch yeast colonies to a nitrocellulose filter circle
2) Lift filter and place colony side up on a pre-cooled aluminum boat floating upon a sea of liquid nitrogen
3) After 20 seconds, immerse boat and filter for 5 seconds
4) Allow the filter to come to room temperature and place on top of Whatman filter circle that had been prewet in 3 ml of Z buffer containing 30 µl of X-gal
5) Incubate the filter for up to 5 hours. Blue coloration is indicative of β–gal activity.

Transformation controls for well-behaved „bait“

„bait“	„prey“	YC selection medium	HIS3 phenotype	lacZ phenotype
Ag/BTM116		-W	/	white
Ag/BTM116		-WHUK	/	
Ag/BTM116	VP16*	-WL		white
Ag/BTM116	VP16*	-WHULK	/	

Table 2.2

b) expression

- **Preparation of Crude Yeast Lysate for SDS-PAGE analysis**

To verify that the bait fusion protein is properly synthesized, an SDS-PAGE and immunoblot analysis on crude yeast lysate must be performed.

Materials

- ✓ master plate with bait-containing positive and control yeasts
- ✓ antibody to lexA (Invitrogen) or monoclonal/polyclonal to fusion protein
- ✓ Laemmli sample buffer 2x

Procedure

Day 1:
Incubate overnight at 30°C a 5-ml culture of the bait being tested and relative controls in the appropriate YC medium.

Day 2:

1) From each overnight culture start a new 5-ml culture at OD_{600}= 0.15. Incubate at 30°C until the culture has reached OD_{600}= 0.5-0.7.
2) Remove 1.5 ml to a tube and centrifuge cells 3 min at maximum speed
3) Remove the supernatant and working rapidly resuspend in 50 µl of 2x Laemmli sample buffer.
4) Vortex and place immediately the tube on dry ice.
5) Boil 5 min and centrifuge 1 min at maximum speed the sample before loading it on SDS-PAGE.

c) toxicity

A high level expression of the fusion protein could be toxic to the reporter strain and this could lead the transformant cells to be unable to grow. To alleviate this detrimental effect the truncation of the toxic protein or a conditional promoter on hybrid plasmid could be used.

2.1.5 Construction and characterization of „prey“

The second step in a two-hybrid assay is the construction of the „prey“. In our case, the prey is a single scFv fused in frame with VP16 or a scFv library. A series of control experiments must be performed also with prey constructs in order to verify the expression level of the protein and its specificity (see Table 2.3).
In order to evaluate the specificity of the scFv fusion protein, all scFv-VP16 fragments tested should interact specifically with their relative bait but not with a non-relevant antigen (human lamin) (Bartel et al., 1993) (Hollenberg et al., 1995).

2.1.5.1 Transformation controls for a well-behaved „prey“

„bait“	„prey“	YC selection medium	HIS3 phenotype	lacZ phenotype
Lamin/BTM116		-W		white
Lamin/BTM116		-WHUK	/	
	scFv/VP16	-L		white
	scFv/VP16	-WHUK	/	
Lamin/BTM116	scFv/VP16	-WL		white
Lamin/BTM116	scFv/VP16	-WHULK	/	
Ag/BTM116	scFv/VP16	-WHULK	+	blue

Table 2.3

To see the expression level of the scFv fusion protein a western blot must be performed using an anti VP16 polyclonal antibody (Santa Cruz Biotechnology). Note: the high background level produced by this polyclonal antibody can affect the resolution of the experiment. Therefore to have better results the use of the myc tag present at the 3’end of the scFv

does not interfere in fusion constructs. To detect in western blot these tagged proteins the monoclonal antibody 9E10 (Evan *et al*., 1985) should be used.

2.1.5.2 Plasmid segregation to remove bait plasmid

Procedure

1) Inoculate lacZ+ His+ transformants in 5 ml of YC-L and grow at 30°C with shaking for 2 days
2) Plate 100 µl of a 1:1000 dilution of the 2 days cultures onto YC-L plate and incubate for 2 days at 30°C
3) Replica plate the colonies growing on YC-L first on YC-WL and then to YC-L; incubate 2 days at 30°C
4) Isolation of colonies that grow on YC-L but not on YC-WL

2.1.5.3 Plasmid isolation from yeast

The rapid procedure for the isolation of plasmid DNA from yeast is based on the method adapted from QIAprep Spin Miniprep Kit protocol by Michael Jones, Chugai Institute for Molecular Medicine, Ibaraki, Japan. Plasmid DNA isolated from yeast is contaminated by yeast genomic DNA: the procedure described below has been successfully used for transformation of *E.coli,* for restriction analysis, PCR screening and sequencing.

Procedure

1) Inoculate single, well isolated yeast transformant colonies into 5 ml of appropriate selective media at 30°C with shaking at 250 rpm until the culture is saturated (16-24 hours)
2) Spin down the culture by centrifuging at 5000 x g and resuspend cells in 250 µl of Buffer 1 containing 0.1 mg/ml RNase A; transfer the cell suspension in a 1.5 ml microfuge tube
3) Add 0.3 g of acid-washed glass beads and vortex for 5 min at room temperature
4) Spin at maximum speed for 5 min and transfer the supernatant to a new 1.5 ml microfuge tube
5) Add 250 µl lysis buffer P2 to the supernatant and invert gently 4-6 times to mix. Incubate at room temperature for 5 min.

6) Add 350 µl neutralization buffer N3 to the tube and invert immediately 4-6 times the tube
7) Centrifuge at maximum speed for 10 min
8) Transfer the cleared lysate to QIAprep spin column placed in a 2 ml collection tube by pipetting
9) Centrifuge for 1 min at maximum speed; discard the flow-through
10) Wash QIAprep spin column by adding 0.75 ml of Buffer PE and centrifuging for 1 min
11) Discard the flow-through and centrifuge for an additional 1 min to remove residual ethanol from wash buffer
12) Place QIAprep spin column in a clean 1.5 ml microfuge tube.
13) Add 25 µl of H_2O to the center of each QIAprep spin column; let stand for 1 min, and centrifuge for 1 min at maximum speed

2.1.6 Verification of a positive Ag-scFv two-hybrid interaction

The general use of yeast cells to detect antigen-scFv interaction was assessed with scFv derived either from monoclonal antibodies or from phage display antibody libraries, and some of which had been shown to have biological activity when assayed *in vivo*. The final validation for a positive interaction was assessed testing the co-transformed yeast colonies for His and lacZ gene activation.

2.2 Experimental Protocols to Monitor Intracellular Antigen-Antibody Interactions Using The Two-Hybrid System

2.2.1 Vectors: Construction was performed according to standard molecular biology techniques (Sambrook *et al*., 1990).

To construct **pLexA-AMCVp41**, the p41 gene was amplified by RT-PCR from artichoke mottle crinkle virus (AMCV) RNA by using primers:
5' - GCCCGAATTCATGGCAATGGTAAAGAGAAATAAT- 3' (sense)
5'-TTACAGGATCCCTAAATTAAAGAGACATCGTTGT-3' (antisense).
Total RNA from AMCV was isolated using 2X extraction buffer in DEPC treated H_2O:

- 20mM Tris-HCl

- 2% SDS
- 20mM EDTA
- pH > 8.5

1) Add an equal volume of 2X extraction buffer to the virus suspension
2) Check the pH. It should be> 8.5. If not, add some NaOH diluted in bidistilled H_2O (DEPC treated)
3) Keep on ice for 10 min. Check SDS precipitation, in case transfer the tube at RT
4) Extract RNA with phenol chloroform V/V (phenol should be equilibrated in 0.1 M TRIS pH8 and the final pH should be > 7.5)
5) Precipitate RNA with 1/10 volume 3M Na Acetate and 2.5 volumes ethanol precipitation.
6) Dry the pellet (shortly in SpeedVac). Resuspend in the buffer required

cDNA was prepared using SuperScript II RNase H^- Reverse Transcriptase (Gibco BRL) with random hexamers according to the instructions of the manufacturer:

1) In an eppendorf tube add the following in order:
    - 1 µl random primers (100 ng/µl)
    - 8 µl of total RNA (1-5 µg)
    - 3 µl sterile distilled water
2) Heat to 70°C for 10 min and chill quickly on ice
3) Centrifuge briefly in eppendorf to collect contents to bottom of the tube
4) Add:
    - 4 µl 5X first strand buffer (250mM Tris-HCl pH8.3 at room temperature)
    - 2 µl 100mM DTT
    - 1 µl 10mM dNTP mix (10mM of each of dATP, dGTP, dCTP and dTTP at pH 7)
5) Add 1 µl (200 units) SuperScript II RNase H^- Reverse Transcriptase (Gibco BRL) and mix gently by pipetting up and down
6) Incubate 25°C for 10
7) Incubate 42°C for 50 min
8) Inactivate at 70°C for 15 min

PCR was carried out using 1/10 of the obtained product with PFU polymerase in a PTC100 thermal cycler (MJ Research), for 30 cycles as follows:

94°C –1 min	
60°C –1 min	30 cycles
72°C -1min 30 sec	
72°C-10 min	
4°C-24h	

Aliquots of PCR reactions were checked on 1.5% agarose gel. PCR products were gel-purified using the QiaQuick Gel Extraction Kit (Qiagen).
Purified PCR products were digested with EcoRI-BamHI, and inserted into EcoRI-BamHI sites of pBTM116.

To construct **pLexA-K-Ras**, the K-Ras B gene was amplified by PCR from pGem3Z-k-ras (gently provided by Prof. Giancarlo Vecchio, Universita' degli Studi di Napoli FedericoII) by using primers:
5'GATCGGATCCGTATGACTGAATATAAACTTGTGGTAGTTGGAG CTGGT- 3' (sense)
5' -GATCCTGCAGTTACATAATTACACACTTTG- 3' (antisense).
PCR products were digested with BamHI-PstI, and inserted into BamHI-PstI sites of pBTM116.

To construct **pLexA-Syk**, the Syk gene was amplified by PCR from Syk cDNA by using primers:
5'-GCCCGAATTCATGGCGGGAAGTGCTGTGGACAGCGCC-3' (sense)
5'-TTACAGGATCCTTAGTTAACCACGTCGTAGTAGTAATTGCG-3' (antisense).
PCR product were digested with EcoRI-BamHI, and inserted into EcoRI-BamHI sites of pBTM116.

To construct **pLexA-β-gal**, the β-gal gene was amplified by PCR from pBluescriptβ-gal7 by using primers:
5' -GAATTCCCGGGGATCCGTATGACCATGATTACG- 3' (sense)
5'-GATCCGTCGACCTGCAGGCTATTTTTGACACCAGAC-3' (antisense).
PCR product were digested with BamHI-PstI, and inserted into BamHI-PstI sites of pBTM116.

To construct **pLexA-HIV-1IN33**, the HIV-1IN33 gene was amplified by PCR from pRP1012 by using primers:
5'-GCTAGCCCGGGGATCCCAATGTTTCTAGATGGAATCGAT-3'(sense)
5'-AGCCCCGGGATCCTGCAGCTAATCCTCATCCTGTCTACT-3' (antisense).
PCR products were digested with BamHI-PstI, and inserted into BamHI-PstI sites of pBTM116.

To construct **pscFvF8-VP16***, the scFvF8 gene was amplified by PCR from pGEMscFv(F8) by using primers:
5'AATGGACTATGGCCCAGCCGGCCAATGCAGGTGCAGCTGCAG GAG-3' (sense)
5'-TCACCTGATAGCGGCCGCATTCAGATCCTCTTCTGAGAT-3' (antisense).
PCR products were digested with SfiI-NotI, and inserted into SfiI-NotI sites of pVP16*.

pscFvY13-VP16* was constructed by subcloning SfiI-NotI fragments from pHENY13 into SfI-NotI cut pVP16*.

pscFvV6C11-VP16*, **pscFvG6G2-VP16*** and **pscFvG4G11-VP16*** were constructed by subcloning NcoI-NotI fragments from pscFvexpV6C11, pscFvexpG6G2 and pscFvexpG4G11 respectively into NcoI-NotI cut pCANTAB6 and subcloning again SfiI-NotI fragments from pCANTAB6scFvV6C11, pCANTAB6scFvG6G2 and pCANTAB6scFvG4G11 respectively into SfiI-NotI cut VP16*.

To construct **pscFvIN33-VP16***, the scFvIN33 was amplified by PCR from pNLVP16 by using primers:
5'-AAAAAGAGAAAAGTGGCCCAGCCGGCCATGGGAATGGACATCC AGATGACA-3' (sense)
5'GGCGGAGCTCGAGGCGGCCGCTGAGGAGACGGTGAGGCT3' (antisense), digested SfiI-NotI, and inserted into SfiI-NotI sites of VP16*.

To construct the non-relevant **scFv/VP16* library** for the Model Selection, DNA phagemids from a phage antibody library was extracted (Qiagen midiprep kit) and scFv fragment DNA was subcloned into SfiI/NotI sites of pVP16*. Naive pscFv/VP16* library had been added into pscFvF8/VP16* DNA at the dilution of 500.000:1.

The various plasmids were transformed into L40 yeast strain using small - scale lithium acetate transformation.
Positive clones were selected using auxotrophic markers for both plasmids and for lysine and histidine prototrophy (Hollenberg et al., 1995).
Histidine-positive colonies and controls were lysed in liquid nitrogen and assayed for β-galactosidase activity on filters as described before.
All clones were sequenced to confirm in-frame fusion of the inserts with the LexA binding domain or the VP16 in the vectors.

2.2.2 Fingerprinting PCR bands

Each individual V region of scFv gives an own "fingerprint". Fingerprinting carried out on V genes will give a number of discrete bands. They can be carried out on V regions amplified from cDNA from individual clones: a good library contains different fingerprinting each clone.
Pick one clone with a toothpick and dissolve it in 100 µl ddH_2O. Use 1 µl in PCR reaction mix:

DNA clone	1	
PCR buffer	2	
dNTP 2mM	2	
Taq	1	
VHmixBACK	1	} (Sblattero & Bradbury, 1998)
VKmixFOR	1	}
ddH_2O	12	
94°C –1 min		|
55°C –1 min		| 30 cycles
72°C –1 min 30 sec		|
72°C-10 min		
4°C-24h		

Check PCR amplifications on a 1.5% agarose gel, using 3 µl each PCR reaction mix. After verification, use the other 17 µl of reaction mix for digestion:

DNAmix	17
BstNI (20U/µl)	0.2
NEB buffer 2	4

BSA	0.4
ddH_2O	18.4

Put to incubate at 60°C for 2 hours. Add 5 µl 6X gel loading buffer to each tube and load on 4% Metaphor/Nusieve TBE gel. Run 60V-120 min.

2.2.3 Western blot and analysis of intrachain disulfide bonds

Yeast protein extracts were prepared as described before; 20µl of sample were subjected to SDS-PAGE (12% acrylamide gels), in the presence or absence of β-mercaptoethanol, and gels were blotted onto nitrocellulose membranes (Schleicher & Schuell). For immunodetection, the monoclonal antibody anti-myc antibody 9E10 (Evan et al., 1985) (1:2000 in 2% MPBS) was used, followed by incubation with a polyclonal anti-mouse-peroxidase conjugate (DAKO) (1:2000 in 2% MPBS).

2.3 Construction of tau deletion mutants for the ectopic expression in COS cells

The expression vector pSG5 was used for all the constructs, directing the expression of the corresponding fragments of tau. The numbering of tau fragments is according to the longest human tau isoform tau40. Plasmids encoding tau fragments were generated by PCR using tau40/pSG5 (Fasulo et al., 1996) (Fasulo et al., 2000) as template. All the clones were sequenced using the Epicentre Sequitherm Excel II kit (Alsbyte, Mill Valley, CA) and analyzed on a Li-Cor 4000L automatic sequencer (Lincoln, NE).
All the primers contained EcoRI (5') and BamHI (3') sites for the insertion into EcoRI-BamHI cutted pSG5.

Primers:

Sense (for tau 151-274, -305, -336, -368, -402, -412, -422, -432, -441)

5'GCCCGAATTCATGATCGCCACACCGCGGGGAGCA 3'

Antisense:

Tau151-274
5'TTACAGGATCCTCACTTCCCGCCTCCCGGCTGGTG3'
Tau151-305
5'TTACAGGATCCTCAACTGCCGCCTCCCGGGACGTG3'
Tau151-336
5'TTACAGGATCCTCACTGGCCACCTCCTGGTTTATG3'
Tau151-368
5'TTACAGGATCCTCAATTTCCTCCGCCAGGGACGTG3'
Tau151-402
5'TTACAGGATCCTCAGTCCCCAGACACCACTGGCGACTTGT3'
Tau151-412
5'TTACAGGATCCTCAGGAGACATTGCTGAGATGCCG3'
Tau151-422
5'TTACAGGATCCTCACGAGTCTACCATGTCGATGCT3'
Tau151-432
5'TTACAGGATCCTCACACCTCGTCAGCTAGCGTGGC3'
Tau151-441
5'TTACAGGATCCTCACAAACCCTGCTTGGCCAGGGA3'

Construct encoding for P2dGAE were made using the following primers:

Sense: 5'GCCCGAATTCATGAGCAGCCCCGGCTCCCCAGGC3'

Antisense: 5'TTACAGGATCCTCACTCCGCCCCGTGGTCTGTCTT3'

Dr Luisa Fasulo prepared construct encoding for tau-head and Nt-dGAE (Fasulo et al., 2000).

2.3.1 Cell culture, transfection and In Situ End-labeling of DNA (ISEL) with cell cultures

COS simian fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and High Glucose. Cells were transiently transfected by DEAE-dextran method as described (Fasulo *et al*., 1996) and analyzed 48 hours after the transfection for immunofluorescence and for western blotting analysis.
MN7.51 hybridoma supernatant, which recognizes human tau (Novak *et al*., 1991) was used at 1:50 dilution. TRITC-conjugated rabbit anti-mouse (1:200 dilution, Vector) was used as secondary antibodies.

All the In Situ End-labeling of DNA (ISEL) with cell cultured were performed using the manufacturers' instructions (ApopTag Direct in situ Apoptosis Detection kit, Oncor).
Apoptotic transfected cells were counted 48 hours after transfection. 200 transfected cells were examined on each coverslip.

2.3.2 Preparation of cell extracts

48-72 hour after the transfection, cells were washed three times with PBS ($+Ca^{2+}$, $+Mg^{2+}$), scraped with 1 ml of PBS, centrifuged for 3 min at 6000. Cells were resuspended in 500 µl of lysis buffer (50mM Tris pH 7.4, 2mM $CaCl_2$, 1%NP-40 and cocktail of protease inhibitors) for 30 min at 4ºC rocking. Cells were centrifuged 5 min at 14000 rpm. 2XSDS gel-loading buffer and 10% β-mercaptethanol were added to the supernatant (and to the cellular debris). Samples were boiled before being analyzed by SDS-PAGE and western blot analysis

2.4 Experimental Protocols For The Developing of The Intrabody Trap Technology (ITT)

2.4.1 Vectors:

τ 151-422/BTM116: the τ 151-422 EcoRI/BamHI fragment from τ 151-422/pSG5 (Fasulo *et al*., 2000) was subcloned into EcoRI/BamHI sites of pBTM116.

τ 151-274/BTM116, τ 151-305/BTM116, τ 151-336/BTM116, τ 151-368/BTM116, τ 151-391/BTM116, τ 151-402/BTM116, τ 151-412/BTM116 and dGAE/BTM116, were subcloned from the respectively pSG5 constructs, as described for τ 151-422/BTM116.

τ 151-421/pMAL-c2: the DNA fragment τ 151-421 was PCR amplified from τ40/pSG5 (Fasulo et al., 1996) (Fasulo et al., 2000) using the primers:

5'GCCCGAATTCATGATCGCCACACCGCGGGGAGC3' (sense)
5'GTCTCGCTAGAATTCGTCGACTCAGTCTACCATGTCGAT3' (antisense).

The EcoRI/SalI digested fragment was cloned into pMAL-c2 (NEB) down-stream from the malE gene, which encodes maltose-binding protein (MBP). This results in the expression of an MBP-fusion protein (di Guan *et al*., 1988) (Maina *et al*., 1988).

τ 151-422/pMAL-c2: the τ 151-422 EcoRI/BamHI fragment was subcloned into EcoRI/BamHI sites of pMAL-c2.

scFv2/pUC119CAT, **scFv14/pUC119CAT** and **scFv52/pUC119CAT**: the scFv2, 14 and 52 SfiI/NotI fragment were subcloned from pVP16* plasmids into SfiI/NotI sites of pUC119 CAT.

scFv2/scFvexpress, **scFv14/scFvexpress** and **scFv52/scFvexpress**: the scFv2, 14 and 52 NcoI/NotI fragment were subcloned from pUC119 CAT plasmids into NcoI/NotI sites of scFvexpress vectors (Persic *et al*., 1997).

All the clones were sequenced using the Epicentre Sequitherm Excel II kit (Alsbyte, Mill Valley, CA) and analyzed on a Li-Cor 4000L automatic sequencer (Lincoln, NE).

2.4.2 MBP fusion protein purification.
This protocol is a modification made of published methods (Kellermann & Ferenci, 1982).
The fusion protein was purified by one-step affinity purification for MBP as described:

Day1: Inoculate 10 ml 2YT+2%glucose and 100 μg/ml ampicillin with few colonies containing the fusion plasmid

Day2:

1. Inoculate 600 ml 2YT+0.2%glucose and 100 μg/ml ampicillin with 6 ml of the overnight culture of cells containing the fusion plasmid.
2. Grow to 2-4x10^8 cells/ml ($O.D._{600}$~0.5) at 37°C, 250 rpm
3. Add IPTG to a final concentration of 0.3mM
4. Incubate the cells at 30°C, 250 rpm for 4 hours
5. Harvest the cells by centrifugation at 5000 x g for 10 min.
6. Discard the supernatant and resuspend the cells in 50 ml Column Buffer (30mM Tris-HCl pH 7.4, 200 mM NaCl, 1mM EDTA, one

tablet Complete, EDTA-free protease Inhibitor Cocktail Tablets, Boehringer Mannheim)

7. Add Lysozime (0.1 mg/ml) and leave on ice for 30 min
8. Freeze sample overnight at -20°C

Day3:

1. Thaw in cold water
2. Add Dnase I (20 μg/ml)
3. Place sample in a ice-water bath and sonicate for 2 min
4. Centrifuge at 40000 rpm for 30 min. Save the supernatant
5. Pour the amylose resin in a 2.5 x 10 cm column. Wash the column with 10 column volumes of Column Buffer
6. Load the diluted crude extract at a flow rate of 1 ml/minute
7. Wash with 20-30 column volumes of Column Buffer
8. Elute the fusion protein with Elution Buffer (Column Buffer+ 10mM maltose)
9. Collect 10 fractions of 500 μl each. The fusion protein start to elute within the first 5-6 fractions.
10. Check on 10% SDS-PAGE, Coomassie stained, the fractions. Collect required fractions and dialyze over-night in 5L PBS buffer at 4°C; change buffer more than once if possible

2.4.3 Preparation of Tau full-length protein

BL21(DE3) bacterial cells (F^- *ompT* gal [*dcm*][*lon*] *hsd*S_B ($r_B^-m_B^-$) with DE3, a λ prophage carrying the T7 RNA polymerase gene, expressing tau40/pRK172 were grown to 2-4x10^8 cells/ml ($O.D._{600}$~0.5) at 37°C in 600 ml 2YT+0.2%glucose and 100 μg/ml ampicillin. IPTG to a final concentration of 0.4 mM was added and cells were then incubated for other four hours at 37°C.

Bacterial cells were then pelleted, resuspended in phosphate buffered saline and boiled for 5 min. After centrifugation the supernatant containing thermostable tau was filtered (0.45 microns) and used in ELISA (Kontsekova *et al*., 1995).

2.4.4 Western blot analysis

Yeast and *E.coli* protein extracts were prepared as described before; samples were analyzed by SDS/PAGE followed by Western blot using the anti tau monoclonal antibody MN7.51 (Novak et al., 1991) or the anti tau

mAb Tau1 (Roche) and the polyclonal antibody anti lexA (for yeast extract only) (Clontech) as primary antibodies followed by anti-mouse-peroxidase conjugate (DAKO) and anti-rabbit-peroxidase conjugate (DAKO) respectively. The blots were developed by ECL method, according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

2.4.5 Selection of phage antibody library

A large non-immune phage antibody library kindly provided by J. Marks, Departments of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco (Sheets et al., 1998) was selected as described (Marks *et al*., 1992):

1) To a 75 x 12 mm Nunc-immunotube (Maxisorp; Cat. No. 4-44202) add 2 ml PBS and 10 mg/ml of 421MBP-purified protein. Leave overnight at 4°C to coat.
2) Next day wash the tube 3X with PBS-Tween-20 (0.1%), 3X with PBS (simply pour solution in and pour out again immediately)
3) Fill tube to brim with MPBS 2% (PBS containing 2% Marvel). Cover with parafilm and incubate at room temperature for at least 30 min to block.
4) Prepare phage mix: 100 μl of PEG concentrated phage (~10^{11}-10^{12} TU) (aliquots were stored in 30% glycerol at –80ºC)+ 100 μl MBP purified protein (200 μg/ml) + 300 μl PBS and 500 μl of 4% MPBS. Store remainder of phage prep at 4°C
5) Wash tube 2X with PBS-Tween-20 (0.1%), 2X with PBS
6) Transfer phage mix from step 4 to the Immunotube. Seal tube with parafilm. Incubate 30 min at room temperature on under and over turntable and then stand for at least a further 1.5 hours at room temperature
7) Wash tubes with 20 washes PBS-Tween-20 (0.1%) then 20 washes with PBS. Each washing step is performed by pouring buffer in and out immediately.
8) Elute phage from tube by adding 1 ml 100 mM triethylamine (make fresh: 140 μl per 10 ml water, pH 12). Rotate the tube for 8 min on an under and over turntable
9) Transfer solution to an eppendorf tube with 0.5 ml 1 M Tris-HCl, pH 7.4 and mix by inversion to neutralize the phage eluate.
10) Transfer to ice for later re-infection
11) Grow TG1 *E.coli* [K12, Δ(*lac-pro*), *sup*E, *thi*, *hsd*Δ5/F'*tra*D36, $proA^{+}B^{+}$, $lacI^{q}$, *lac*ZΔM15] to an $O.D_{600}$ of 0.5

12) Add to 5 ml ($2x10^9$ bacteria) TG1 500 μl of selected phagemid particles and let stand for 40 min at 37°C with occasional agitation
13) After the infection event, spread the infected bacteria on 2YT, 100 μg/ml ampicillin, 2% glucose plates

After the first and the second cycle of selections, 96 scFv fragments present in individual colonies were identified by BstNI fingerprinting and the same isolated clones were used in Phage ELISA as described (Sblattero & Bradbury, 2000) to identify the number of different positive 421MBP binding scFvs.

2.4.6 Growth and Rescue of Phagemid particles in 96-well microtitre plates

Day1:
1) Toothpick colonies into 150 μl 2YT, 100 μg/ml ampicillin, 2% glucose in 96-well flat-bottomed plates (Costar, Cat n. 3595) and grow with shaking (250 rpm) overnight at 30°C. This will be the master plate

Day2:
1. Transfer 2 μl (each single clone) from master plate to a fresh 96-well plate containing 150 μl 2YT, 100 μg/ml ampicillin, 2% glucose per well. Grow 2.5 hours, 37°C shaking. To the wells of the master plate, add 50 μl 60% glycerol per well and store at -70°C, after use.
2. To each well add 50 μl 2YT, 100 μg/ml ampicillin, 2% glucose containing 2 x 10^9 pfu M13K07 phage (0.1 μl of a 2 x 10^{13} pfu/ml stock per well). Let stand for 30 min at 37°C
3. Spin at 600 rpm for 10 min; then remove supernatant and resuspend pellet in 150 μl 2YT, 100 μg/ml ampicillin, 25 μg/ml kanamycin. Grow overnight at 30°C shaking

Day3:
Spin at 600 rpm for 10 min and use 50 μl supernatant for phage ELISA

2.4.7 Induction of Soluble Antibody Fragments

This method can also be applied for a 96-well microtitre plates: the recommended volumes can be referred to the previous phagemid rescue in a 96-well microtitre (see before).

The scFvs were expressed from the plasmid pUC119CAT and pHEN in HB2151 non suppressor strain [*K12, ara Δ(lac-pro), thi/F' proA+B+, lackI^qZΔ*M15]

Day1:

Inoculate clones directly from the plate in 10 ml 2YT, 100 μg/ml ampicillin, 2% glucose. Incubate in a 30°C shaker overnight at 250 rpm

Day2:

1. Inject 5 ml of the overnight stock to 500 ml 2YT, 100 μg/ml ampicillin, 0.1% glucose
2. Incubate in a 37°C shaker at 250 rpm until bacteria reach $O.D._{600}$ of 0.6
3. Induce with 0.5 mM IPTG
4. Incubate in a 30°C shaker for 4 hours
5. Collect cells by centrifuging in 500 ml plastic bottles at 6000 rpm for 10 min
6. Resuspend pellet in 12.5 ml PPB buffer (200 mg/ml Sucrose, 1mM EDTA, 30 mM Tris-HCl pH8, and one tablet Complete, EDTA-free protease Inhibitor Cocktail Tablets, Boehringer Mannheim). Keep on ice for 20 min
7. Spin down cells in centrifuge at 5000 rpm for 15 min at 4°C
8. Collect supernatant
9. Resuspend pellet in 12.5 ml of 5 mM $MgSO_4$ buffer. Incubate on ice for 20 min
10. Spin down cells in centrifuge at 5000 rpm for 15 min at 4°C
11. Collect the supernatant
12. Load supernatants into dialysis tubing and dialyze overnight in 5L PBS buffer. Change buffer more than once if possible

Day3:

Collect the dialyzed soluble samples and store on ice until Ni-NTA agarose resin column purification

2.4.8 Purification of scFv Fragments on Ni-NTA Agarose Resin Column

All the following steps are carried out at 4°C or on ice:

Materials:

- NaPi 0.1 M (233 ml/L Na_2HPO_4, 17 ml/L NaH_2PO_4)
- WB1 (500 ml/L NaPi 0.1 M, 250ml/L NaCl 1M)
- WB2 (500 ml/L NaPi 0.1 M, 500ml/L NaCl 1M)
- WB3 (WB2 + 15 mM Imidazole)
- EL (WB2+ 100 mM Imidazole)

Procedure:

1) Pre-equilibrate 2 ml 50% slurry of Ni-NTA agarose (Qiagen cat. N. 30210): add 8 ml of PBS to resuspend the resin. Centrifuge at 800 rpm for 5 min. Repeat twice this wash step.
2) Load to the dialyzed periplasmic extract (equilibrated with NaCl, 250 mM NaCl final concentration and pH 7.5) the washed resin and incubate rocking for 1-2 hours at 4°C
3) Centrifuge at 800 rpm for 10 min. Wash the resin with WB1 (50 ml)
4) Centrifuge at 800 rpm for 10 min. Resuspend the resin with 10 ml WB1
5) Pour the resin into a 1 x 10 column
6) Wash the column with 20-30 ml of WB2
7) Wash the column with 20-30 ml of WB3
8) Elute the scFv with the elution buffer EL. Collect 10 fractions of 500 µl each. The scFv usually start to elute within the 5 fractions
9) Analyze sample by SDS-PAGE and western blotting
10) Dialyze the collected fractions into PBS as described before

ScFv concentrations can be quantitated using the bicinchoninic acid kit (Pierce) according to the instructions of the manufacturer.

2.4.9 ELISA for Detection of Soluble or Phage Antibody Fragments

Day1:

Coat plate with 100 µl per well of protein antigen (10 µg/ml in PBS). Leave overnight at 4°C

Day2:

1) Rinse wells 2X with PBS-Tween-20 (0.1%), 2X with PBS

2) Block with 120 μl per well of 2% MPBS for 30 min at room temperature
3) Wash by submersing the plate into buffer and removing the air bubbles in the wells by agitation
4) Rinse wells 3X with PBS-Tween-20 (0.1%), 3X with PBS
5) Add 50 μl of 4% MPBS
6) Add 50 μl culture supernatant containing antibody fragment or phage antibody to appropriate wells. Mix by pipetting up and down and leave at room temperature for 1.5 hours
7) Wash 3X with PBS-Tween-20 (0.1%), 3X with PBS

8a) For soluble antibody detection: add 100 μl of 9E10 anti myc (Evan et al., 1985) antibody (1:50 dilution) in 2% MPBS into each well. Incubate at room temperature for 1 hour

8b) For phage antibody detection: add 100 μl of anti fd-phage HRP conjugated (1:5000 dilution) in 2% MPBS to each well. Incubate for 1 hour at room temperature

9a,b) Wash 3X with PBS-Tween-20 (0.1%), 3X with PBS

10a) Add 100 μl of anti-mouse antibody HRP in 2% MPBS. Incubate for 1 hour at room temperature

11a) Wash 3X with PBS-Tween-20 (0.1%), 3X with PBS

10b) 12a) Add 70 μl of TMB (Tecna s.r.l). Leave in dark at room temperature for 2-30 min. Quench by adding 70 μl H_2SO_4 2N. Read at 450 nm

2.4.10 Construction of an Activation Domain-Fusion anti 421MBP scFv library

DNA phagemids from the first and the second cycle of phage antibody selection was extracted (Qiagen midiprep kit) and scFv fragment DNA was subcloned into SfiI/NotI sites of pVP16*. The ligation mix was electroporated into electrocompetent DH5αF' (Gibco BRL, Rockville, MD) [*F'/endA1 hsdR17(r_K-m*K+*) supE44 thi-1 recA1 gyrA (Nal^r) relA1 Δ(lacZYA-argF)U169 deoR (ϕ80dlac*Δ(*lacZ*)*M15*] and plated on LB 100μg/ml ampicillin plates to obtain the AD-library.

The final libraries are calculated to have a diversity of approximately $2.2x10^6$ for the first cycle and $6x10^4$ for the second cycle respectively.

2.4.11 Selection of intracellular antibodies using the two-hybrid system

The AD-fusion anti 421MBP scFv library was transformed in L40 yeast containing 422/BTM116 bait plasmid following a maxi-scale LiAc transformation protocol, as described before. After the scoring of His+ yeast colonies, a X-Gal lift assay was performed as described before.
100 individual AD-fusion scFv plasmids were isolated from segregated yeast cells and scFv inserts were identify by BstNI fingerprinting and by sequencing as described before.
The isolated AD-fusion scFv plasmids were cotransformed with the LexA-422 antigen bait vector into L40 yeast strain by using a small-scale LiAc transformation protocol and reselected by using auxotrophic markers for both plasmids and for lysine and histidine prototropy and by X-Gal lift assay.

2.4.12 Analytical Gel filtration

Gel filtration was carried out on a Superdex 75 column (Amersham Pharmacia Biotech, UK). All the measurements were carried out in PBS buffer. The respective scFv fragments were injected at 50-200 μg/mL in a volume of 200 μL. The column was calibrated in the same buffer with molecular mass standards. The incubated samples were centrifuged for 5 min at 14000g before applying them to the column to remove large aggregates, which might block the column.

2.4.13 Competition ELISA

Prior to conventional ELISA 40nM of individual scFvs were incubated for one hour at room temperature with increasing amounts (100nM to 1μM) of purified 422MBP, MBP and tau40 respectively. After incubation of scFvs with antigens, the unbound scFvs are detected by ELISA using the mouse anti-myc monoclonal antibody 9E10 (Evan et al., 1985) as primary antibody and the anti-mouse-peroxidase conjugate (DAKO) as secondary antibody. The ELISA were developed by TMB method, according to the manufacturer's instructions (Tecna s.r.l).

2.4.14 Cell line, transfection and immunofluorescence analysis

CHO Chinese Hamster Hovary Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, High Glucose and Proline (17.2 mg/L final concentration).
Cells were transiently transfected by FuGENE-6 Transfection Reagent (Roche) method according to the manufacturer's instruction (Roche) and analyzed 30-48 hours after the transfection for the immunofluorescence and for western blotting analysis.
COS simian fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and High Glucose. Cells were transiently transfected by DEAE-dextran method as described (Fasulo et al., 1996) and analyzed 30-48 hours after the transfection for immunofluorescence and for western blotting analysis.
MN7.51 hybridoma supernatant, which recognizes human tau (Novak et al., 1991) was used at 1:50 dilution. mAb anti-myc antibody 9E10 (Evan et al., 1985) and polyclonal anti-myc (Clontech) were used in dilution 1:50 and 1:40 respectively. TEXAS-RED-conjugated anti-mouse (1:50 dilution, Vector) fluorescein anti-mouse conjugate (1:50 dilution, Vector) or anti-rabbit immunoglobulins fluorescein-conjugate (1:50 dilution, Vector) was used as secondary antibodies. Nuclei were stained with the fluorescent dye DAPI (Boehringer Mannheim) following the manufacturer's instructions.

Chapter Three: The development of an *in vivo* assay for functional intracellular antibodies using the two-hybrid approach

3.1 Introduction

The virtually unlimited repertoire of immunoglobulins is widely used as a source of specific reagents for biochemical or physiological studies in which specific gene products can be inhibited or modulated *in vitro*.
The advances in molecular immunology and the development of phage display library techniques provide conditions for the generation of large numbers of human antibodies with desired specificities and functional activity.
The potential of using antibodies to interfere with biological processes inside the cell in a highly specific manner has been successfully demonstrated. Intrabodies can be used in different experimental strategies to specifically interfere with function of selected intra- or extracellular gene products in mammalian cells.
Intracellular immunization approaches are receiving great attention for application to gene therapy and for functional genomics, as this thesis will demonstrate. This approach suffers however from drawbacks and limitations represented by the fact that the environment in which the antibodies are naturally selected is very different from the one in which prospective intrabodies are asked to work.
In particular, when antibodies are expressed in the cell cytoplasm, folding and stability problems often occur, resulting in low expression level and limited half-life of antibody domains.
To overcome these limitations, a general strategy for selecting antibodies that are stable and soluble under intracellular expression conditions is needed.
The first step of this work was the developing of a general antibody-antigen two-hybrid system.
In order to practically test the system, we have monitored a panel of scFv derived either from mAbs or from phage display antibody libraries, some of which had been shown to have biological activity when intracellularly expressed *in vivo*.

3.2 Results

3.2.1 The Antibody-Antigen Two-Hybrid System

The yeast two-hybrid system was adapted to monitor the interaction of scFv fragments with their corresponding antigens, under condition of intracellular expression. The procedure is outlined in Fig 3.1.

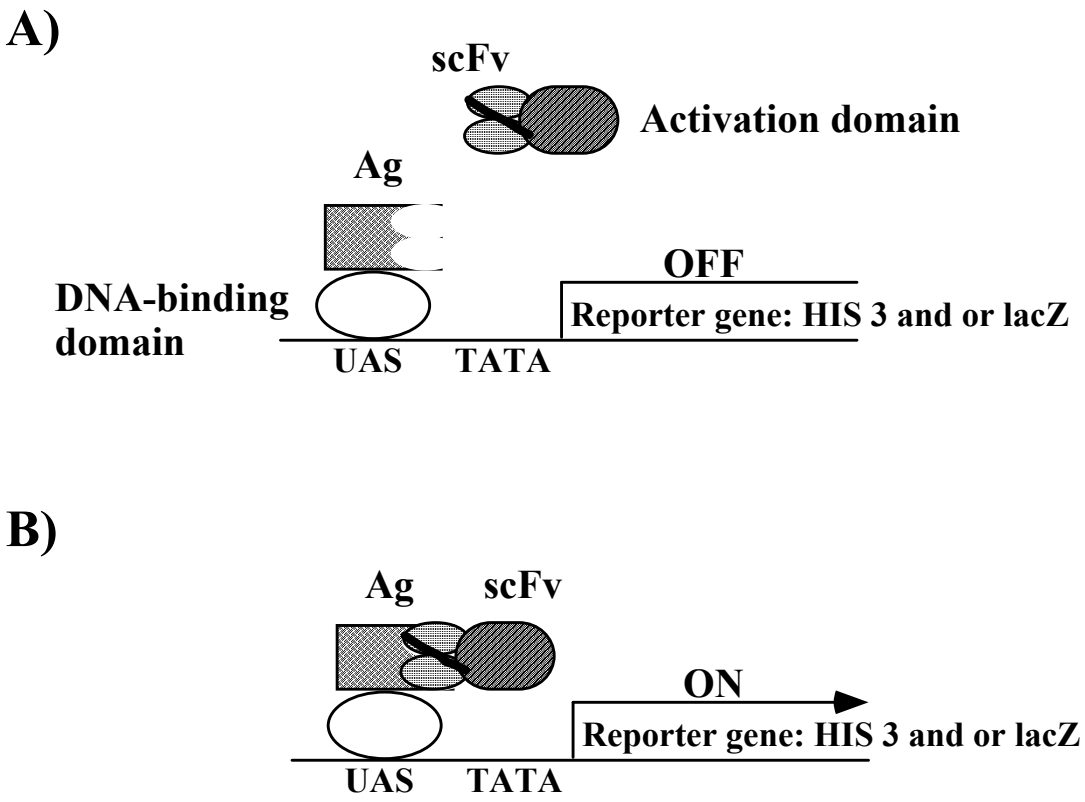

Fig.3.1 Dual selection/screen by using the two-hybrid system. **A)** The antigen (Ag) fused to the DNA-binding domain of the E.coli repressor LexA is expressed in the yeast strain L40 carrying two reporter genes, lacZ and HIS3, under control of the LexA regulatory sequence. This hybrid protein can bind to its operator site but will not activate transcription. A second plasmid, which expresses the scFv fragments fused to the herpes simplex virus VP16 transcriptional activation domain, is introduced into the same strain; this hybrid protein does not bind to the upstream activation sequence (UAS) because it lacks the DNA-binding domain. **B)** The interaction of the activation domain fusion scFv with the DNA-binding fusion Ag results in the expression of the reporter construct.

The expression vectors used for this purpose were obtained by cloning the in frame sequence of a target antigen at the 3'end of the *E.coli* repressor protein lexA (Bartel et al., 1993) to construct the DNA binding domain fusion protein and by cloning the in frame sequences of scFv at the 5'end of the activation domain of the herpes simplex virus 1 VP16 transcription factor (Hollenberg et al., 1995) (See figure 2.1 and 2.2 respectively).
Two nuclear localization signals are located on the scFv-VP16 fusion protein, whereas the antigen bait has none (Silver et al., 1986) (Hollenberg et al., 1995). Therefore, the interaction between the antigen and the scFv fragment could occur in the cytoplasm (some small lexA-fusion antigens could be small enough to diffuse into the nucleus and to locate to their operator sites), before the complex is translocated to the nucleus and activates transcription.
These vectors were cotransfected into L40 yeast cells, carrying the yeast biosynthetic gene HIS3 and the bacterial gene lacZ under the control of a minimal promoter with LexA binding site.
If the scFv antibody fragment binds to the antigen target in vivo, a complex formed between the scFv fragment and the antigen can bind to the HIS3 promoter (via the DNA-binding part of the lexA-antigen fusion protein) and activate transcription (via the activation domain of the VP16-scFv fusion protein). This interaction will restore histidine independent growth of the yeast (Fig. 3.1 B).
The selection for interacting clones was carried out by both nutritional selection for histidine and an assay for β-galactosidase activity.
As a model system, we have chosen the very well characterized scFv F8.
This scFv antibody recognizes a highly conserved site on the coat protein p41 that is involved in divalent-cation regulated swelling of the artichoke mottle crinkle virus (AMCV) (Tavladoraki et al., 1993).
It was previously shown that the constitutive expression of this monoclonal-derived scFv fragment in transgenic *Nicotiana benthamiana* plants, causes reduction of infection incidence and delay in symptom development following AMCV infection (Tavladoraki et al., 1993).
Before testing the interaction between the scFvF8 and its relative antigen, the expression level of both fusion protein was assessed in L40 yeast cells by western blot analysis using a polyclonal anti-lexA protein (Invitrogen) for bait construct and the anti-myc-tag mAb 9E10 (Evan et al., 1985) for scFv-VP16 fusion protein (Fig.3.2 a and b, respectively).
Co-expression of the lexA-AMCVp41 bait and the scFvF8-VP16 fusion protein in L40 yeast cells, results in the transcription of reporter genes that confer the His^+ and the blue phenotype on the host cells (Fig.3.3 row 4).
The transfection of the bait lexA-AMCVp41 alone or with the activation domain vector VP16 or of an unrelated antigen fusion lexA-lamin with the

scFvF8 fusion, did not result in activation of both reporter genes (Fig. 3.3, row1, 2 and 3 respectively).
These results demonstrate that the scFvF8 is able to specifically interact with its corresponding antigen under intracellular expression conditions in yeast cells.

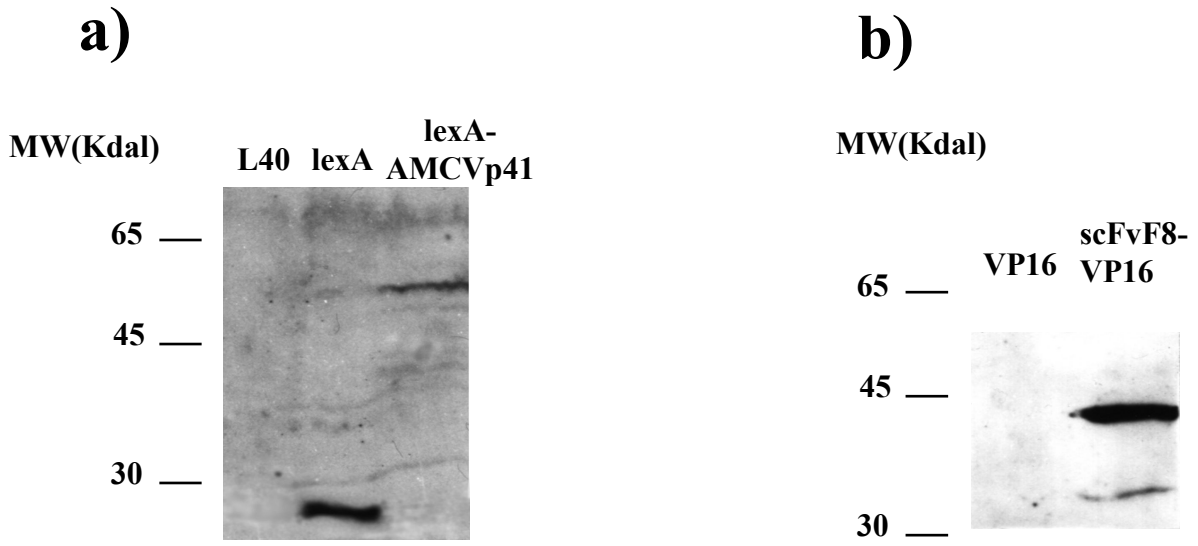

Fig.3.2 Western blot analysis of lexA-AMCVp41 bait and control extracts (a) and scFvF8-VP16 and control extract (b). Samples were prepared in SDS-PAGE loading buffer with β-mercaptoethanol as described in chapter 2. After blotting, the fusion protein was detected with the anti-lexA (a) and with the anti-myc antibody 9E10 (b).

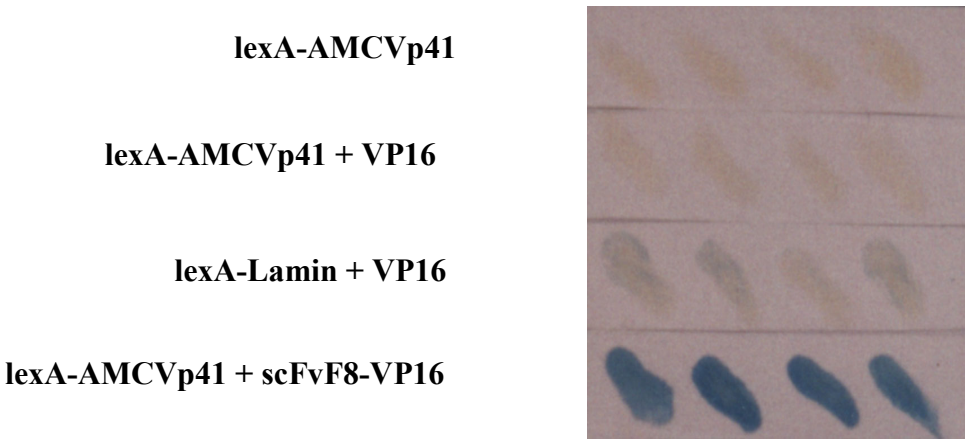

Fig.3.3 lexA-AMCVp41 bait + scFvF8-VP16 prey. L40 yeast cells were co-transformed with a lexA-AMCVp41 antigen bait vector (AMCVp41/BTM116) and scFvF8/VP16* fusion vector (row 4) or the pVP16* vector alone (row 2). β-galactosidase activation was observed only when the lexA-AMCVp41 bait was coexpressed with scFvF8/VP16* (row 4).

3.2.2 The Yeast Antibody-Antigen Two-Hybrid System Can Distinguish Intracellular Binders

In order to verify the general use of the antibody-antigen two-hybrid system, a panel of scFv fragments (see panel below) derived either from monoclonal antibodies or from phage display antibody libraries was tested.

scFv IN33:
The intracellular expression of the monoclonal-derived scFvIN33, which bind to catalytic and carboxy-terminal domains of the virus-encoded enzyme integrase (IN), results in resistance to productive HIV-1 infection. This inhibition of HIV-1 replication is observed with the scFv localized in either the cytoplasmic or nuclear compartment of the cell. The expression of anti-IN scFv fragment in human T-lymphocytic cells and peripheral blood mononuclear cells appears to specifically neutralize IN activity prior to integration and, thus, has an effect on the integration process itself (Levy-Mintz et al., 1996).

scFv V6C11, scFv G6G2, scFv G4G11:
These three scFv fragments, directed against the non-receptor protein-tyrosine kinase Syk, were isolated from a large naive phage display single-chain antibody library (Vaughan et al., 1996). They react with Syk in ELISA, immunoprecipitation, immunofluorescence and western blot (Peneff *et al*., 2000)

scFv Y13-259:
The monoclonal-derived anti-p21ras Y13-259 scFv fragment has been shown to inhibit the insulin induced meiotic maturation in *Xenopus* oocytes (Biocca et al., 1993) (Biocca *et al*., 1994), and the intratumor transduction of HCT116 colon carcinoma cells with this scFv fragment, using an adenovirus vector, leads to tumor regression in nude mice (Cochet et al., 1998). Furthermore, the expression of anti-p21ras Y13-259 scFv fragment in 3T3 fibroblast cell line neutralizes the activity of p21ras (Cardinale *et al*., 1998).

scFvs anti p21-ras:
Twelve scFv fragments, directed against p21-ras protein (Persic *et al*., 1999), were isolated from a large naive phage display single-chain library (Sheets et al., 1998). They react with the 57-76 p21-ras neutralizing epitope in ELISA and in western blot. The binding affinity of each scFvs, determined by plasmon resonance, was in the range of 5-800 nM (Lener *et al*., 2000).

All the baits used in these assays were individually assessed for auto-activation of the two reporter genes, with or without the pVP16* vector as demonstrated in Fig. 3.4 a), b) and c) (first and second row respectively).
All the baits used, were unable to grow in the absence of histidine (data not shown) and they didn't turn blue, also in the presence of the empty vector pVP16*, when β-gal assay was assessed.
When anti-HIV scFv fragment was expressed as VP16 fusion protein with the lexA-HIV-IN bait in L40 yeast cells, the activation of HIS3 reporter gene and a high level of β-gal activity was observed (Fig.3.4 a, row 4) whereas, the expression of the same scFv with the non-relevant antigen lamin, did not result in any activation (Fig.3.4 a, row 3).
To determine whether scFv selected from phage display libraries *in vitro* could function in the antibody-antigen two-hybrid assay, three phage-derived scFv fragments were tested.
Only one of these, scFv G4G11, shows a positive interaction with its relative antigen (Fig.3.4 b, row 8) while the other two failed to interact (Fig.3.4 b, row 6 and 7). The specificity of this scFv interaction with the lexA-Syk bait was established with the lamin bait (Fig.3.4 b, row 5).
This observation reinforce the idea that scFv selected *in vitro* from a phage display library, purely for their ability to bind antigen, may not necessarily function correctly as intracellular antibodies. This result point out the inadequacy of the *in vitro* selection in fishing out possible functional candidates for intracellular expression.
When scFvY13-259 was used in two-hybrid assay with the lexA-K-ras fusion bait, it failed to activate the HIS3 and lacZ reporter genes at 30°C (Fig.3.4 c, row 4) but was able to induce growth in the absence of histidine or β-gal activity at 20°C (Fig.3.4 c, row 5). This interaction was specific for K-ras and not for the lamin bait at both temperature (Fig.3.4 c, row 3), and the K-ras bait did not auto-activate the reporter genes when expressed alone or in the presence of the pVP16* empty vector (Fig.3.4 c, row 1 and 2 respectively).
Another demonstration that not all the scFv fragments isolated from a phage display library are able to bind their antigen when expressed intracellularly in two-hybrid system was assessed testing several distinct scFv fragments for p21-ras. Only 2 scFvs among 12 were able to interact with the K-ras bait even when tested at 20°C (data not shown).

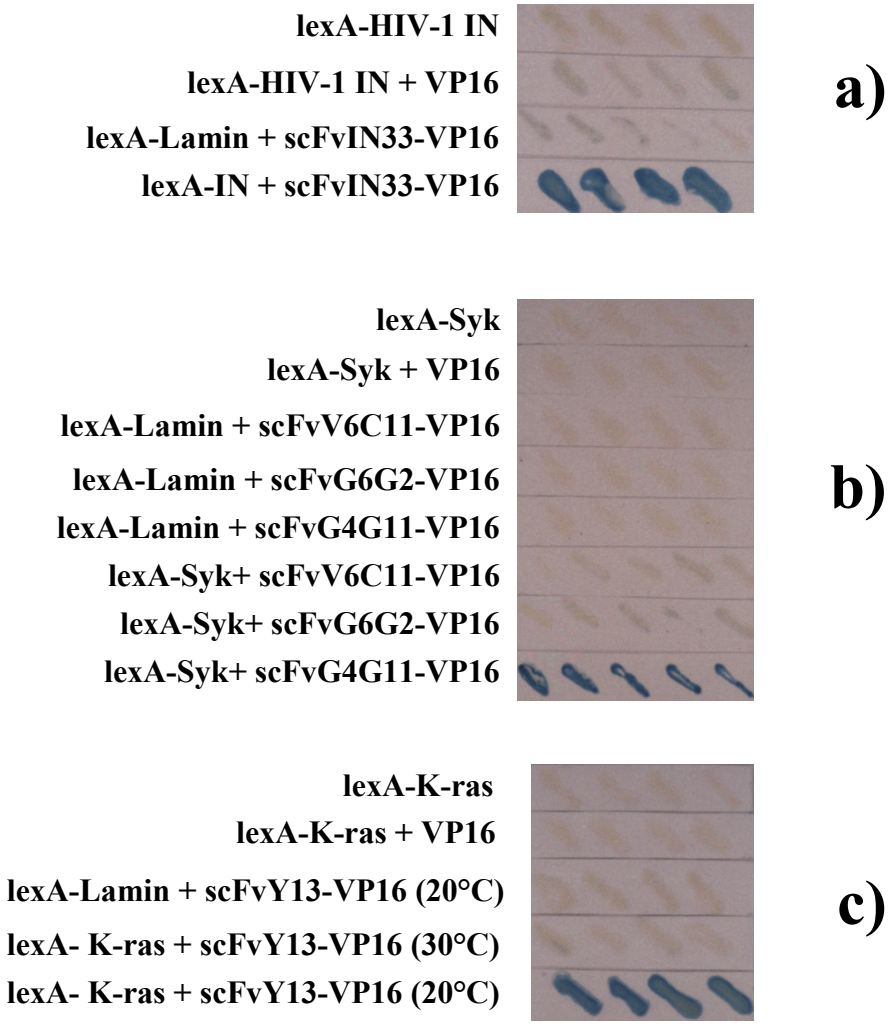

Fig.3.4 Intracellular expression of a panel of scFv fragment in the yeast two-hybrid system. L40 yeast cells were cotransformed with a panel of lexA-antigen DNA-BD and scFv-VP16 fusion transcriptional AD pairs.
Yeast were streaked and grown on his- medium and scored for β-gal activity. **a**) Transformation of lexA-HIV-1 IN alone (row1), lexA-HIV-1IN with pVP16* vector (row2), or with scFvIN33-VP16 (row4). β-gal activation was scored only when scFvIN33-VP16 was cotransformed with lexA-HIV-1IN (row4) and not when it was cotransformed with the non-relevant antigen lamin (row3). **b**) Transformation of lexA-Syk bait, alone (row1) or with pVP16* vector (row2). In rows 3-5 specificity of scFvV6C11, scFvG6G2 and scFvG4G11 was assessed with the lexA-bait lamin. In rows 6-8, the same three scFvs were cotransformed with the lexA-Syk bait. β-gal activation was observed only for scFvG4G11 (row8). **c**) Transformation of lexA-K-ras bait alone (row1) or with the pVP16* vector alone (row2). In row 3, specificity for scFvY13-VP16 was assessed with the non relevant antigen lamin at 20°C (row3) and with the lexA-K-ras bait at 30°C (row4) or 20°C (row5). β-gal activation was observed only in row 5.

3.2.3 Redox state of scFv fragments

The folding and the stability of antibodies in the cell cytoplasm is not constant, as revealed by the number of experiments performed so far in several intracellular contexts. Therefore, there is a need to isolate the subset of scFv fragments that can bind the corresponding antigen inside the cell. As demonstrated before, the two-hybrid assay for intracellular antigen-antibody interactions should allow the isolation of antibody domains that tolerate the reducing cytoplasmic and nuclear expression conditions. In order to prove that a positive outcome of antigen-antibody interaction in the two-hybrid format does indeed occur in the absence of disulphide bond formation, we have investigated the redox state of the scFvF8 fusion protein under yeast expression conditions. A gel mobility assay (Biocca et al., 1995) was performed in order to compare protein extracts under reducing or non-reducing conditions. The western blot analysis revealed no difference in the mobility of the scFvF8-VP16 fusion protein in both reducing and non-reducing conditions, while the control scFv expressed in the endoplasmic reticulum of insect cells revealed a clear gel shift between the two conditions (Fig.3.5).

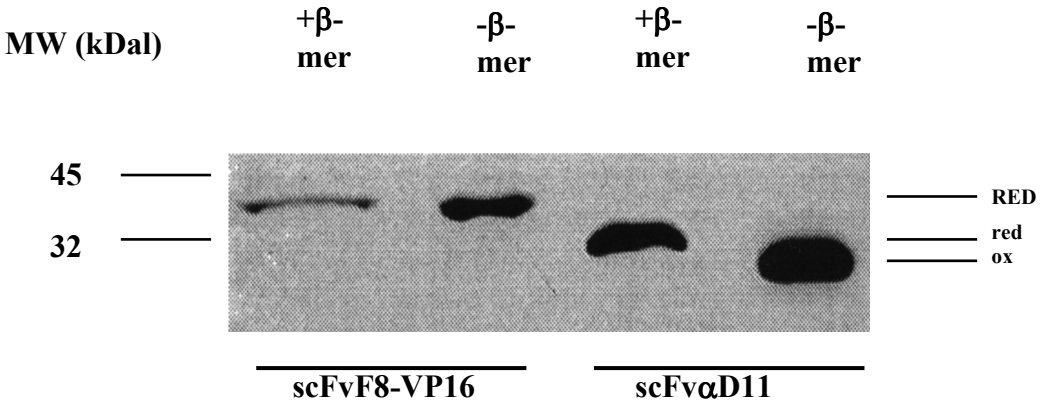

Fig.3.5 Redox state of scFv fragments. Samples were prepared in SDS-PAGE loading buffer containing β-mercaptoethanol (+ β-mer) or not β-mercaptoethanol (-β-mer). After blotting, the proteins were detected by using the anti myc-tag mAb 9E10 β-mer (Evan et al., 1985). The bars at the right of the lanes indicate the molecular mass gel shift between the oxidized (ox) and reduced (red) forms of the scFvαD11. scFvF8-VP16 fusion protein does not show a shift in electrophoretic mobility, between reducing and non-reducing conditions, indicating that no disulphide bond occurs in the yeast cytoplasm (RED).

The success of the interaction between the scFvF8 and its relative antigen in yeast cells, reinforced by the evidence that no disulphide bonds is formed, under those conditions, suggested us to consider the scFvF8 an ideal molecule with which to perform a model selection. This *in vivo*

experiment should show whether the yeast antibody-antigen two-hybrid system could be applicable to antibody selection strategies.

3.2.4 Model Selection

In order to perform this experiment, the scFvF8-VP16 fusion plasmid was diluted with DNA from a library encoding non-relevant scFvs-VP16. A DNA mixture in which 1 part of scFvF8 was added to $5x10^5$ parts of scFv library was obtained and DNA plasmids of this mixture were cotransformed in L40 yeast cells, using a LiAc maxi-scale transformation protocol. After transformation, cells containing the mixed scFvF8 + scFv library plasmids, encoding the scFvF8 interacting with AMCVp41 bait, were selected on histidine deficient plates. After 3 days, 84 independent colonies from 1/40 of the transformation mix were rescued from his- plates. These colonies were picked and replated on his-plates for screening for β-gal activity. Among 48 colonies, 22 developed a strong blue color (Fig.3.6). 10 blue colonies were taken for further DNA restriction analyses and sequences. The restriction pattern obtained after the BstNI digestion of isolated plasmid DNA from blue yeast transformed cells revealed an identical pattern with scFvF8 starting material (Fig.3.7). The sequences performed with the same 10-isolated DNA plasmid confirmed those data (data not shown).

The result obtained with this experiment demonstrate the feasibility of this system to select interacting scFv from a dilution of up to 1 in 500000, indicating that selection with even higher dilutions are feasible by scaling up the number of yeast transfected colonies.

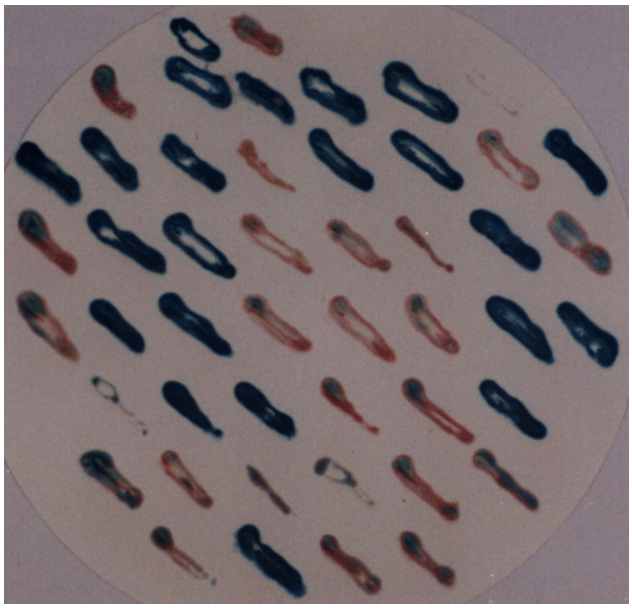

Fig.3.6 Model selection. L40 yeast cells were cotransformed with lexA-AMCVp41 bait with a mixture of DNA of a dilution of 1:500000 of scFvF8-VP16 in scFv-VP16 DNA library respectively. Yeast were plated onto –his plates and incubated for three days at 30°C. Strongly activating interactions were detected by HIS3 transactivation. His+ colonies were transferred from the master plate to a new –his plate for the β-gal filter assay. 22 colonies developed a strong blue color indicating the presence of the interaction between lexA-AMCVp41 fusion bait and its relative scFvF8-VP16 transactivation fusion protein.

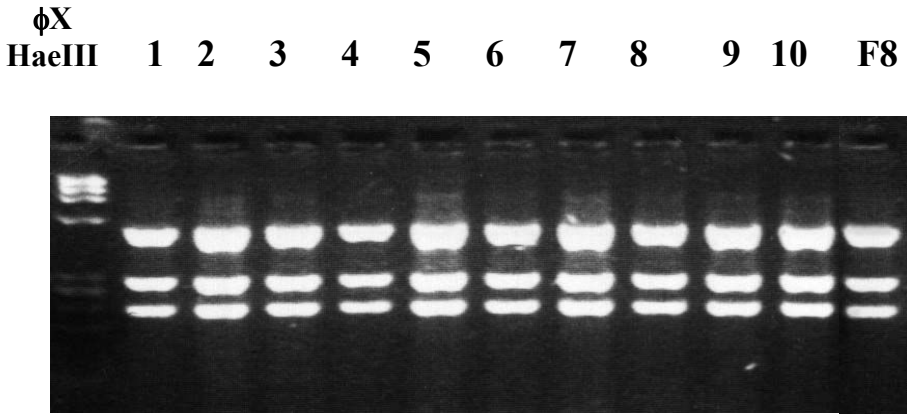

Fig.3.7 Fingerprinting analysis of isolated yeast blue colonies. Single colonies after HIS3 transactivation, were selected, and scFv were PCR amplified using a set of primers designed to amplify V genes (Sblattero & Bradbury, 1998). BstNI fingerprinting analysis shows the presence of scFvF8 in all the scored plasmids. On the right the scFv-VP16 plasmid was used as a control in the experimental conditions.

3.3 Discussion

The potential of the intracellular use of antibody fragments to inhibit protein function has been demonstrated in several experimental applications (Biocca et al., 1993) (Richardson *et al*., 1995) (Cochet et al., 1998) and the future applications of this approach in gene therapy and in functional genomics must not be underestimated.

While satisfactory scFv fragment for *in vitro* studies can be selected by using large phage antibody libraries, it is clear that not all the *in vitro* isolated scFv fragments can be specifically functional *in vivo,* particularly in some cell compartment such as the cytoplasm.

The ability to use scFv fragments *in vivo* as intracellular antibodies is restricted by their ability to fold correctly and stably in the reducing environment of the cell cytoplasm.

Many residues in the frameworks contribute to the folding stability of antibody domains. Recent quantitative studies on the stabilizing role of the intrachain disulphide bond in immunoglobulin variable regions have shown that removal of this covalent bond has a destabilizing effect in the order of approximately 4.5 kCal/mol. Therefore, those scFv fragments which are in the upper range of folding stability will tolerate the loss of intrachain disulphide bond and remain folded (Fig.1.8), while those that are in the

lower range, will unfold. The latter, can be stabilized by mutations in the framework, and thus made tolerant to the absence of disulphide bonds. These results, altogether, suggest that the antibodies in the upper part of the folding stability range are good candidates for intracellular expression.
However, folding stability under reducing conditions is not the only parameter important for a good intracellular expression, solubility versus propensity to aggregate, cellular half-life and affinity being some of the most relevant properties of antibodies that will greatly affect their performance when intracellularly expressed. It can take much work and long time to discover that a particular antibody is not able to interact efficiently with its corresponding antigen in the cytoplasm. Therefore, there is a need for a selection system that could rapidly predict the performance of antibody fragments under conditions of intracellular expression.
A proof of principle for selection schemes based on the phenotype provided by an intracellular antibody has been recently assessed for mammalian cells (Gargano & Cattaneo, 1997b). More recently, a phenotypic selection scheme for intracellular antibodies in the cytoplasm of *E.coli* cells has been described (Martineau et al., 1998) and this selection scheme was used to select a scFv variant with greatly improved expression properties. However, phenotypic selection schemes for cytoplasmic antibodies are limited to the particular system under study. The challenge is to develop more general selection schemes.
With this purpose, the first part of the project was aimed at developing a selection scheme for the isolation of intracellular antibodies, based on their ability to bind antigen under conditions of intracellular expression, in the yeast two-hybrid format.
We tested a panel of scFv fragments, some of which had been previously used as intracellular antibodies, while others not. All those fragments that were previously shown to induce a well-controlled biological phenotype when intracellularly expressed in different systems were also positive in the two-hybrid system. The only partial exception to this statement is provided by the Y13-259 scFv fragment, which, in the two-hybrid format, is only positive at 20°C, and not at 30°C. It is noteworthy that the initial intracellular expression experiments performed with this antibody were performed in *Xenopus laevis* oocytes (Biocca et al., 1993), at 18°C. We now know that this scFv fragment, at higher temperatures, forms intracellular aggregates in the cytoplasm of cells (Cardinale et al., 1998). On the other hand, not all the scFv fragments tested are positive in the two-hybrid system, on the contrary, quite a few of them are negative, even if their binding affinities are comparable to that of the scFv fragments that work. This confirms the utility of a simple and quick selection procedure for intracellular antigen-antibody interactions and the model selection

described demonstrates the feasibility of isolating, from an antigen-enriched polyclonal phage antibody population, those scFv fragments able to interact intracellularly with the antigen.
This result provided the demonstration that antigen-specific scFv fragments can be easily isolated from a large pool of scFvs specificity using this approach. This technique can obviate the need for scFv engineering, such as random or direct mutagenesis, or CDR grafting on stable frameworks, and may represent a valid alternative in functional knock-out on a wide genomic scale.

Chapter Four: Alzheimer's Disease: Mechanism of Neuronal Degeneration

The experimental procedures described in this thesis have allowed the selection of intrabodies against the protein tau (see chapter five), a protein that is involved in Alzheimer's disease.
During the first part of my work I was devoted to look at the general impact of tau fragments on cell viability (Fasulo et al., 2000). This chapter will briefly introduce the involvement of tau protein in Alzheimer's disease and summarize the work performed by myself in that project.

4.1 Introduction: Major pathological hallmarks of Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder, which affects 5 to 10% of all people over 65 years of age. Patients suffering from Alzheimer's disease develop a progressive dementia in adulthood, accompanied by three main structural changes in the brain:

- Diffuse loss of neurons in the hippocampus and neocortex
- Accumulation of intracellular protein deposits termed neurofibrillary tangles (NFTs)
- Accumulation of extracellular protein deposits termed amyloid or senile plaques (SP), surrounded by misshapen nerve terminals (dystrophic neurites or DN) (St George-Hyslop & Westaway, 1999).

The neurofibrillary tangles are considered to be a major pathological hallmark of Alzheimer's disease; NFTs develop within the pyramidal neuronal soma as filamentous inclusions and may extend into dendrites.
In AD brain the nerve cells can degenerate and it appears that they die because they contain neurofibrillary lesions. These lesions are found in nerve cell bodies and apical dendrites as NFTs, in distal dendrites as neuropil threads and in abnormal neurites, which are often, but not always, associated with amyloid plaques.
Neurofibrillary lesions consist of paired helical filaments (PHFs), paired 8-20 nm filaments tightly wound into helices with periodicity of 80 nm (Wisniewski *et al*., 1976) form of dispersed filaments (Greenberg &

Davies, 1990) (Lee *et al*., 1991), and the related straight filaments (SFs), as their minor fibrous components (Spillantini & Goedert, 1998).
PHF are highly stable structures that can withstand protease digestion: they consist of 4-8 100-micron protofilaments, appeared as twisted ribbons 15-20-nm and 21-23-nm, left handed double helices, with periodicity of 160 nm, without significant internal substructure (Tracz *et al*., 1997) containing proteins that are immunologically related to normal cytoskeletal proteins. While the dispersed filaments are soluble in guanidine or SDS, the majority of tangle fragments PHFs are insoluble in these reagents. Pronase treatment of tangle preparations removes a fuzzy coat from the PHF and leaves behind a pronase-resistant core.
The principal constituent of PHFs is tau protein (Kosik *et al*., 1986) (Grundke-Iqbal *et al*., 1986) (Ksiezak-Reding *et al*., 1987) (Nukina & Ihara, 1986), which represent the protein chosen for testing the technology developed in this study: the next paragraphs will describe the pathology in neurodegenerative disease of this protein.

4.1.1 Tau Protein: Physiological Functions

Tau is a developmentally regulated microtubule-associated protein (MAP).
Tau protein is prominent in neurons, particularly in axons, and one of its roles is thought to be the stabilization of microtubule arrays (Drubin & Kirschner, 1986).
It is encoded by a single gene located on the long arm of chromosome 17 at band position 17q21 (Neve *et al*., 1986).
The tau primary transcript contains 16 exons but 3 are not present in human brain (exon 4A, 6 and 8). They are specific to peripheral tau proteins.
In adult human brain, six tau isoforms, 352-441-residue proteins with apparent molecular masses ranging from 48 to 67 kDa have been identified. They are produced by complex alternative mRNA splicing of 11 exons (Himmler, 1989) (Andreadis *et al*., 1992).
These isoforms differ by the presence or absence of one or two (29 or 58 amino acids long, respectively) NH_2 inserts and by a COOH region with three (3Rtau) or four (4Rtau) imperfect 18-amino acid microtubule (MT)-binding repeats, each of which is separated by an interrepeat domain of 13 or 14 amino acids (Goedert *et al*., 1989).
There is also a larger tau isoform, with an additional 254 amino acid insert in the amino-terminal region, which is mainly expressed in the peripheral nervous system (Goedert *et al*., 1992).
Fetal human brain expresses only a shortest tau isoform, which lacks both amino-terminal inserts and the fourth carboxy-terminal microtubule-binding motif (Goedert et al., 1989).

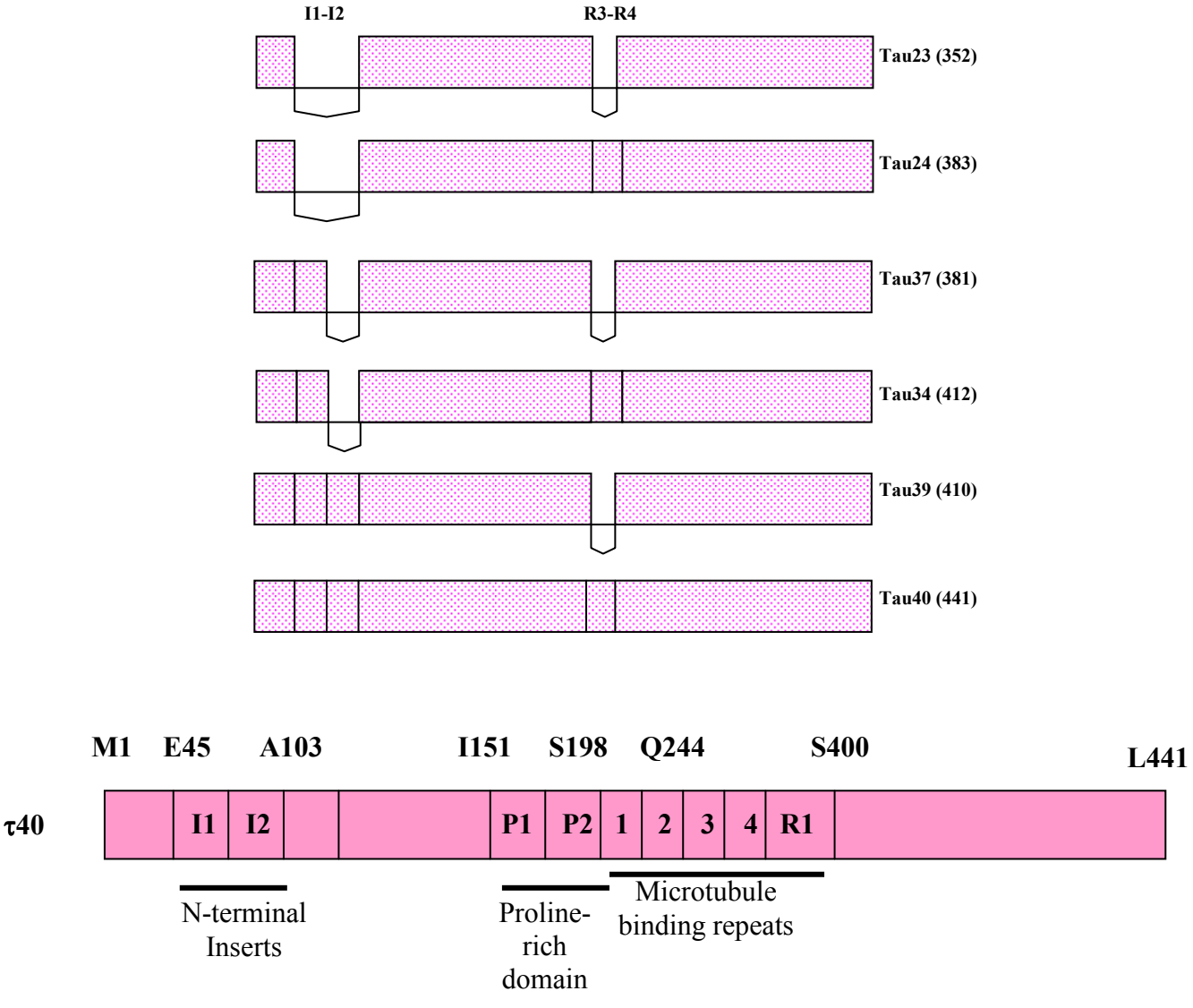

Fig.4.1 Tau Isoforms and Composition

Tau promotes tubulin assembly *in vitro* (Weingarten *et al*., 1975) and stabilizes microtubules against depolymerization *in vivo* (Drubin *et al*., 1985) (Kanai *et al*., 1989).
The presence of tau antisense in primary neuronal cell culture appears to block the development and maintenance of axon-like processes, suggesting a role in the establishment of neuronal polarity (Caceres & Kosik, 1990) (Caceres *et al*., 1991).
Tau has been considered to have a role in neurite elongation (Drubin et al., 1985) (Drubin & Kirschner, 1986) and infection of Sf9 cell with a baculovirus expressing human tau markedly alters the morphology of the host cells, inducing long axon-like processes and microtubule bundles (Knops *et al*., 1991).

On the basis of its ability to promote microtubule assembly and stability *in vitro*, tau has been proposed to promote microtubule assembly and stabilization in growing axons (Brandt & Lee, 1993).
Tau is a phosphoprotein and its mobility on SDS-PAGE is slowed by phosphorylation. Fetal tau is highly phosphorylated and shows immunoreactivity with several phosphorylation-sensitive antisera (Billingsley & Kincaid, 1997).
The binding of tau to microtubules is regulated by a "physiological" phosphorylation.
The discovery that highly phosphorylated tau protein is the major component of the paired helical filaments (PHFs) of AD and other neurodegenerative disease has focused attention on the nature and enzymology of the post-translational modification of this particular microtubule-associated protein (Goedert *et al*., 1993).

4.1.2 Tau Protein: Pathological Aberrations

Tau is the major component of the filamentous neurofibrillary lesions of AD and other tauopathies.
This protein extracted from PHF exhibits features of postranslational modifications, including:

- Phosphorilation
- Ubiquitination (Mori *et al*., 1987)
- Glycation (Ko *et al*., 1999)
- Glycosylation
- Proteolysis

This raises the possibility that accumulation of aberrant-modified tau may play a crucial role in tangle formation leading to dysfunction, degeneration and ultimate death of affected neuron in AD.
PHF that accumulate in affected neurons in AD are comprised of hyperphosphorylated tau that exhibits electrophoretic and antigenic properties distinct from that of normal adult CNS (central nervous system) tau (Goedert *et al*., 1993).
At least 25 phosphorilation sites have been identified in the PHF-tau (Iqbal *et al*., 1986) (Goedert, 1993). These sites can be classified as either proline-directed or nonproline-directed phosphorylation sites (Morishima-Kawashima *et al*., 1995) (Song & Yang, 1995). Both types of phosphorylation can affect tau's function.
It is commonly assumed that the hyperphosphorylation of tau causes its detachment from microtubules and promotes its assembly into PHFs; in fact it was shown that PHF-tau exhibits a decreased microtubule (MT)

binding activity, and dephosphorylation dissociates PHFs and restores microtubule-assembly promoting properties to tau (Iqbal et al., 1986) (Wang *et al*., 1995). A decrease in the tau phosphatase activity in AD brains has also been shown, suggesting that tau is abnormally hyperphosphorylated in AD brains, probably due to an imbalance in the protein phosphorylation/dephosphorylation systems (Gong *et al*., 1993) (Gong *et al*., 1995) (Matsuo *et al*., 1994) (Garver *et al*., 1996).

It is still impossible to confirm at present whether in AD tau is first hyperphosphorylated, which induces its dissociation from microtubules, leading in turn to their collapse, or whether MT collapse represents an initiating phenomenon, and newly-dissociated tau subsequently becomes hyperphosphorylated. Furthermore, recent studies suggest that the hyperphosphorylation of tau in AD is not directly responsible for the pathological aggregation into PHFs, (Schneider *et al*., 1999) and the results of the effect of two kinases (MAPK and GSK-3) on tau-microtubule interactions revealed that this kind of phosphorylation never enhanced aggregation but, on the contrary, protected against it. This last observation reinforces the assumption that hyperphosphorylation of tau is unlikely to be directly responsible for the pathological aggregation of tau in AD.

The aggregates of hyperphosphorylated tau and normal tau work as “seeds” for the polymerization of tau into neurofibrillary tangles in which PHF are formed after glycosylation of tau, another abnormal posttranslational modification present on AD-tau (Wang *et al*., 1996).

In AD the glycosylation of tau, a cytoplasmic protein, raises an intriguing possibility of a membrane abnormality whereby tau and a specific glycosyltransferase, normally separated in different cell compartments, become accessible to each other.

PHF retains, after digestion with pronase, its characteristic protease-resistant core, which is formed by a group of 12 Kdal tau fragments that encompass the microtubule-binding repeat regions (Wischik *et al*., 1988a; Wischik *et al*., 1988b). These fragments are unable to bind to microtubules and to induce their bundling when expressed *in vivo*, but do not affect either cell morphology or viability (Fasulo et al., 1996).

Core PHFs have been used to raise the monoclonal antibody MN423 (Wischik et al., 1988b) (Novak *et al*., 1989), which specifically stains all the main neuropathological hallmarks of AD, including intracellular granular and neurofibrillary structures (Bondareff *et al*., 1991). MN423 decorates PHFs isolated with or without pronase treatment in immunoelectronmicroscopy studies, binds to the 12 Kdal fragments of the core PHFs terminating at Glu391 and does not react with full-length tau (Novak et al., 1991). On this basis it was hypothesized that tau is endogenously truncated in AD brain at Glu391.

Ugolini et al. also investigated the distribution of MN423-immunoreactivity in AD neurons (Ugolini *et al*., 1997). Using this antibody it was shown intense granular staining inside of neurons that did not exhibit any overt neurofibrillary changes, suggesting that the proteolysis of tau represent an early pre-tangle stage in the development of neuropathology at single cell level. Moreover Ugolini et al., demonstrated a high degree of colocalization of MN423 positive neurons with apoptotic neurons suggesting a correlation between the truncation of tau at Glu 391 and cell death by apoptosis in AD brains.

4.1.3 Neuronal Death and Neurodegeneration

It is well documented that AD is a degenerative disease characterized by progressive loss of neurons. In order to investigate which could be the role of the proteolysis of tau on the morphological degeneration and profound apoptotic cell death of neurons, the ISEL technique was employed in order to verify one of the commonly used marker of cell death.

Apoptosis is a process characterized by cytoplasmic blebbing, cell shrinking, neurite degeneration, fragmentation of the nucleus and fragmentation of the genomic DNA (Oppenheim, 1991); the ISEL technique identify nuclei with fragmented DNA, as DNA fragmentation is a commonly accepted marker of cell death.

MN423/ISEL double labeling studies have demonstrated the presence of cleaved tau in AD neurons associated with the expression of markers for neuronal death (Ugolini et al., 1997). In that work, only MN423 immunoreactivity could be tested, but the presence of dying ISEL-positive cells that did not shown MN423 immunoreactivity, has suggested that intermediate truncated tau fragments could be present inside neurons that are not detectable with MN423, that only revealed those fragments whose carboxy-terminal residue is Glu391.

Recently we have demonstrated that the ectopic expression of a tau fragment (tau 151-391) induces cell death by apoptosis in COS cells (Fasulo *et al*., 1998), as well as other cell types such as 3T3 fibroblast, SH-SY5Y neuroblastoma and NT-2 teratocarcinoma cells.

In order to identify the maximum fragments still endowed with full apoptotic properties, we engineered three sets of mutants (C-terminal extension, N-terminal extensions, N- and C-terminal truncations of tau 151-391), and expressed them in COS and NT-2 cells in experiments of transient transfection.

The results of this work in which I have collaborated (Fasulo et al., 2000) will be briefly described in the next paragraph.

4.2 Results: Tau as substrate and effector of apoptosis

Figure 4.2 illustrates the set of tau constructs, starting at residue 151 (proline rich-domain) and terminating at residues 274, 305, 336, 368, 391, 402, 412, 422, 432, 441 (the natural end of tau protein).

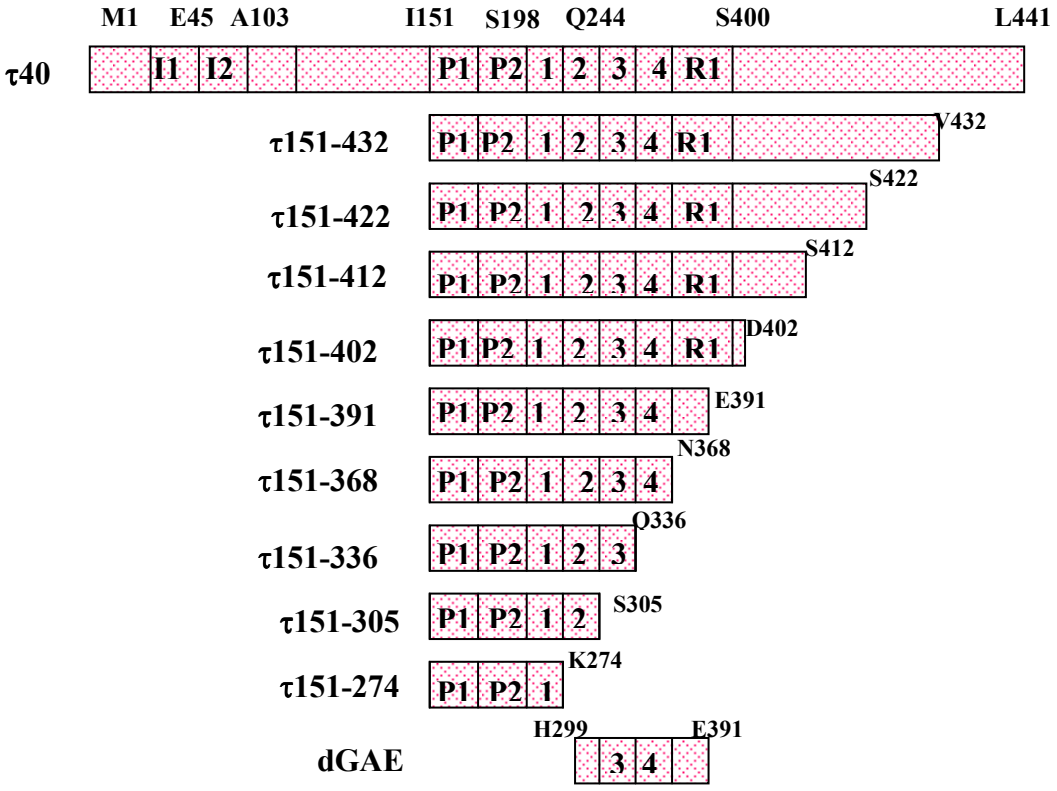

Fig. 4.2 The set of tau fragments

COS cells were transiently transfected with the tau constructs described in Fig.4.2 and analyzed after 48 hours by double labeling with the anti-tau monoclonal antibody MN 7.51 and the ISEL procedure for DNA fragmentation.
In the first set of construct (C-terminal extension of tau 151-391) the expression of fragments encompassing the proline-rich regions and terminating anywhere between residues 391 and 422 induce in a large number of cell morphological changes and DNA fragmentation typical of apoptosis: 40-45% of transfected cells expressing all fragments terminating between amino acids 391 and 422 were apoptotic, with a sharp transition

observed upon further extension into the 19 C-terminal residues (Fig. 4.3 b).

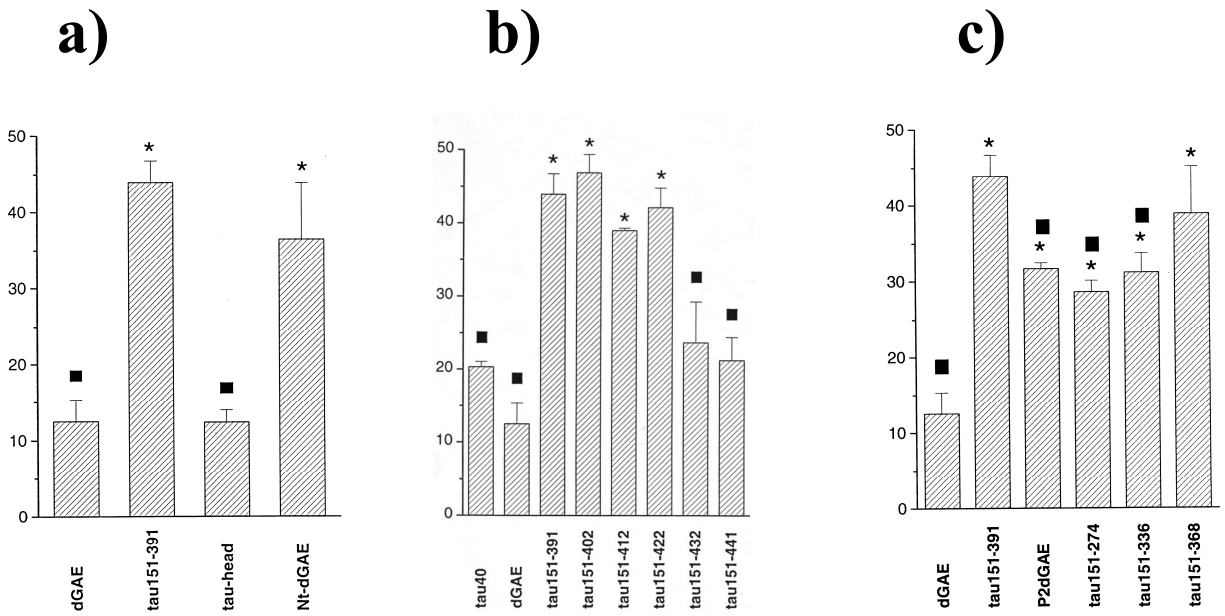

Fig.4.3 Percentage of cells transfected with tau fragments undergoing apoptosis. Percentage of transfected cells were scored for each construct. The transfected cells expressing fragments of tau40 and showing DNA fragmentation were visualized using a multiple wavelenght filter (Zeiss Filter Set 25), and the percentage of apoptotic cells of the total number of transfected cells was determined. The lowest percentage of DNA fragmentation was found in cells expressing the dGAE fragment, and for this reason this fragment was used as a negative control in all experiments. The level of apoptosis induced by dGAE fragment correspond to the backround levels induced by negative controls in similar conditions.
Bars represent standard errors. Asterisk and filled squares over the bars indicate the statistical significance with respect to the dGAE and to tau 151-391 respectively ($p<0.001$, except for tau 151-432, $p<0.05$) for b), and for a) and c) with respect do dGAE only ($p<0.001$, except for tau 151-368, $p<0.05$).
In a) N-terminal extension of tau 151-391 fragment and controls. In b) C-terminal extension and in c) contribution of the microtubule repeats and proline-rich domain to the apoptotic properties of tau fragments.
Tau-head (tau 1-150): this fragment includes the N-terminal region of tau molecule up to Lys150
Nt-dGAE (tau 1-391): this fragment includes the N-terminal region of tau molecule up to Glu391
P2dGAE (tau 198-391): this fragment includes the second proline rich domain (P2) up to Glu391

A further mapping of the apoptotic properties of tau fragments (N-terminal extension and N- and C-terminal truncation of tau 151-391) revealed that unlike the C-terminal extension, the N-terminal extension did not affect the apoptotic properties of tau fragments (Fig.4.3 a).
In other experiments, deletion mutants that are reduced at their N- and C-termini (Fig.4.3 c) revealed that the N-terminal deletion of the first proline-rich domain and a reduction at a C-terminus of one microtubule binding repeat, lowered the apoptotic capacity of the fragment to a level that is intermediate between that of tau 151-391 (maximal apoptotic capacities) and of dGAE (minimal apoptotic capacities).
In this work it was shown that the apoptotic capacity of tau fragments is comprised in the set of fragments starting from tau 151-368 up to tau 151-422.
A transition point around residue 422 was observed, located around one of the three putative caspase-3 cleavage sites (D421), beyond which the fragments lose their ability to induce apoptosis. It was also shown that only this site, in the C-terminal portion of the molecule, could be effectively used by caspase-3 *in vitro*.
This work, together with data from an *in vitro* model of neuronal apoptosis (Canu *et al*., 1998) demonstrates that tau is, at the same time, a substrate and an "effector" of apoptosis, suggesting that it could be involved in a feed forward mechanism affecting neuronal viability in AD.
On the basis of the results obtained in this work, the use of intracellular antibodies as a tool for *in vivo* studies and furthermore, as a general strategy to interfere with apoptotic stimuli induced by tau fragments, could be the final goal of my work. The ability to link the development of the intrabody trap technology, that leads to the high throughput isolation of knock-out antibodies with a real multifactorial disease, might be the demonstration that such system can be readily used to identify novel genes involved in the disease area under investigation as "targets" for new drugs and/or for gene therapy.

Chapter Five: Intrabody trap technology: selection of anti human tau intracellular antibodies

5.1 Introduction

To demonstrate whether the yeast antigen-antibody two-hybrid system can be feasible also in "real" complex situations, in which the protein under investigation has a role in a cascade of events and can be a target itself for functional studies and possibly for drug development, we have applied this technology or "intrabody trap technology (ITT)" to the isolation of intracellular antibodies of tau fragments (see preceding chapter).
The microtubule-associated protein tau seems to us a good candidate for several reasons:

1. it is the major component of the paired helical filaments (PHFs) which are found in the neurofibrillary lesions that correlate with the presence of dementia in Alzheimer's disease
2. it is known to be involved in microtubule assembly and stabilization in neurons but does not appear to be an essential protein, since inactivation of its gene by homologous recombination leads to no overt phenotype, except a reduction in the number of microtubules in some small-caliber axons (Harada *et al*., 1994)
3. abnormal posttranslational modifications occur under pathological conditions, including hyperphosphorylation (Iqbal *et al*., 1994), glycosylation (Wang et al., 1996), ubiquitination (Bondareff *et al*., 1990), glycation (Ko et al., 1999) and truncation (Mena *et al*., 1996) (Fasulo et al., 1996) (Fasulo et al., 1998). Although all these abnormalities have been extensively studied under *in vitro* conditions, few examples of *in vivo* studies were performed so far
4. the structure and self-assembly of tau protein is still unsolved and the mechanisms of PHF formation *in vitro* is not fully understood

The efforts performed so far in dissecting all the probable mechanisms involved in neurodegeneration that show a possible involvement of tau protein as effector of apoptosis (Fasulo et al., 2000) have suggested us to apply the ITT on this specific problem in order to obtain intrabodies either for studying or, as more ambitious target, for applicative purposes.
In the next paragraph I will explain all the step performed so far to achieve the goal of my project.

5.2 Results

5.2.1 Preparation of 151-422 tau fragment bait-antigen

The first step to achieve in the preparation of an intrabody trap selection was the construction of the bait. I have chosen a domain of tau protein, including amino acids 151 up to 422 to exclude the higher backgrounds due to the transactivation of the reporter construct by the C-terminal domain of the protein, that was revealed in pilot studies (data not shown). Moreover, fragment 151-421τ display apoptotic properties when expressed in mammalian cell and represent therefore a good candidate for isolations of intrabodies.

The constructed bait antigen underwent a series of control-tests as described in chapter 2. The transformation of the lexA-151-422τ fusion protein alone in L40 yeast cell, or with the VP16* vector did not result in activation of the HIS3 nor of the lacZ gene (Fig.5.1, rows 1 and 2 respectively).

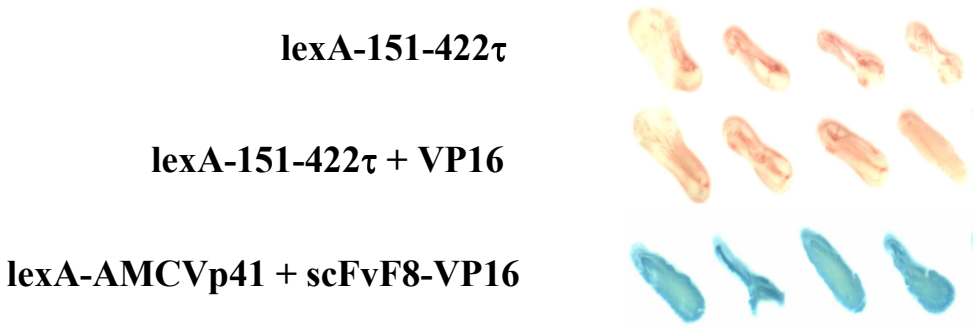

Fig.5.1 Transactivation controls for 151-422τ bait. L40 yeast cells were transfected with lexA-151-422τ (row1) and cotransformed with lexA-151-422τ and VP16* plasmid (row2). Yeast was scored for β-gal activity and streaked and grown on his-medium (data not shown). No auto-activation (3 different separated assays were performed) of HIS3 and lacZ genes was detected. In row 3 the contransformation of a positive control: lexA-AMCVp41+scFvF8-VP16.

The absence of background transactivation of the two-reporter genes has induced us to proceed in constructing the fusion protein to use for selection of the scFv antibody phage library. The purified 151-421 τ fusion with maltose binding protein, that was available in the laboratory, (Fig.5.2) was

used for the selection scFv fragments of the human non-immune antibody fragments library (Sheets et al., 1998).

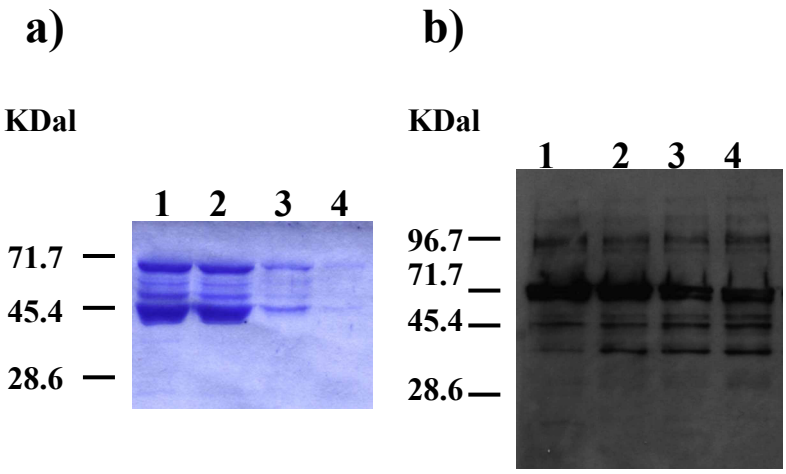

Fig.5.2 151-421 τ–MBP protein. After extraction, the soluble pool of the fusion proteins was affinity chromatographed using amilose resin column. The eluted fractions were subjected to a) coomassie SDS-PAGE and b) western blot (after blotting protein were detected with the anti-tau mAb 7.51)

5.2.2 Selection of anti -151-421 τ human antibody-fragments from a non-immune antibody fragments library

The purified 151-421 τ–MBP protein was used for the selection of human antibody fragments from the Sheets library (Sheets et al., 1998). After the 151-421 τ–MBP protein was applied to the solid phase, the selection was performed by successive rounds of panning, elution, and phage growth, and the presence of phage particles with binding specificity for the 151-421 τ–MBP protein was directly monitored after the second cycle by phage ELISA. Enrichment of 151-421 τ -positive phage particles was found after two selection rounds, as measured on the single phage clones. The results of the screening are reported in Table 5.1.

Antigen	Round of selection	Positive clones
151-421 τ–MBP	**2**	**77/96**
MBP	**2**	**68/96**
151-421 τ	**2**	**9/96**

Table 5.1 Results of the selection second cycle. The number of ELISA positive clones and the number of different monoreactive clones is reported

After two-round of selection using high stringency washes, 77 clones out of 96 to 151-421 τ–MBP were positive but polyreactive. Only 9.4% of the clones were positive and monoreactive (Fig.5.3).

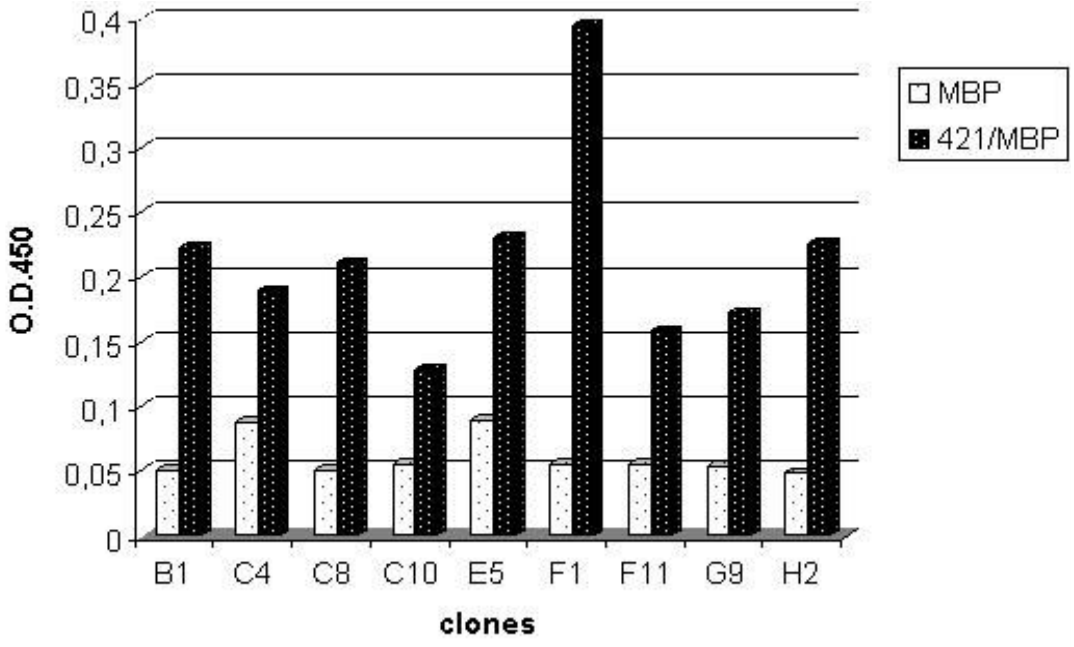

Fig.5.3 ELISA reactivity of the isolated clones against 151-421 τ–MBP and MBP. Antigens were coated at 10 μg/ml

To test the diversity of this "enriched" library, the isolated scFv were fingerprinted by PCR amplification and digestion with BstNI (Fig.5.4).

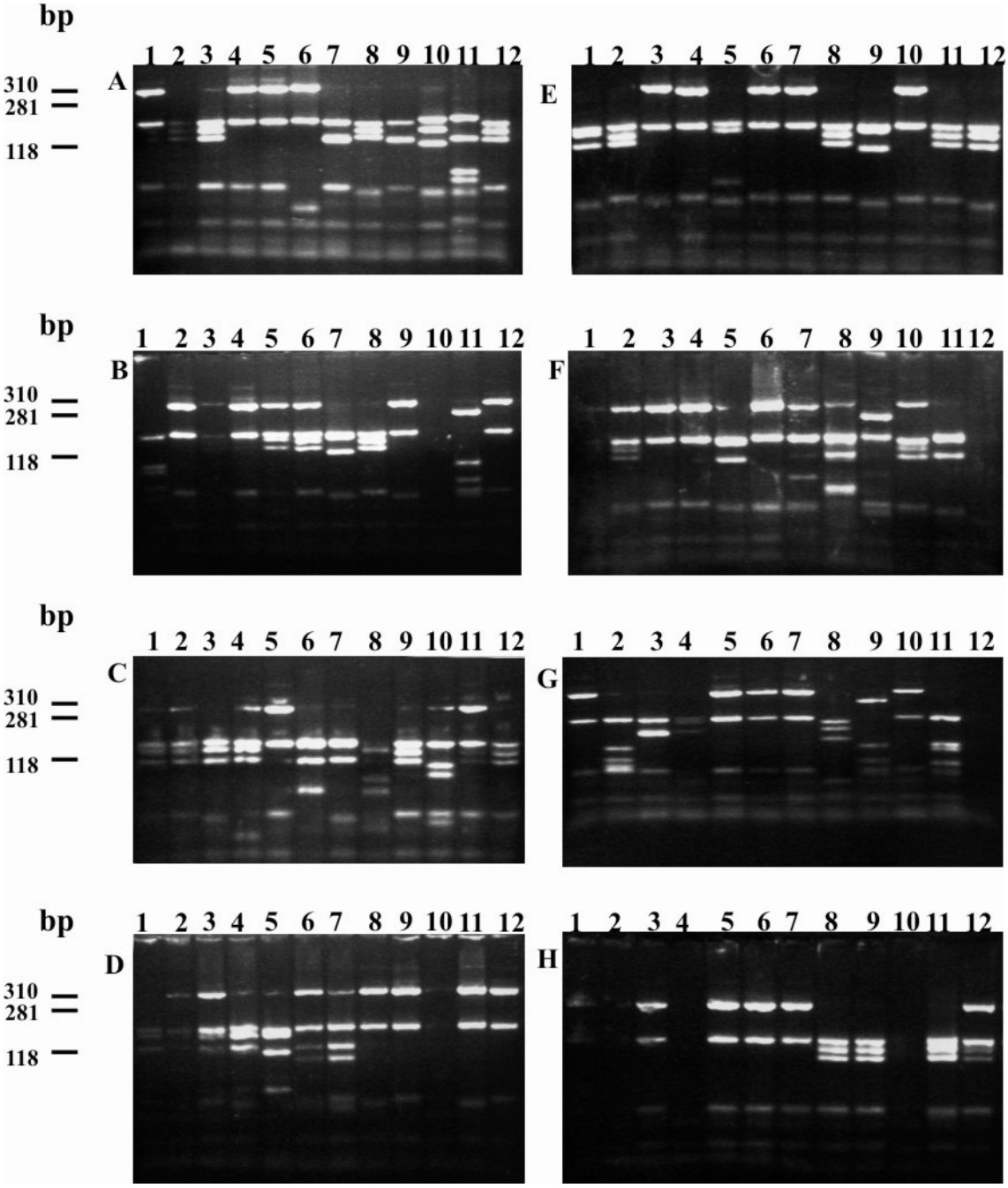

Fig. 5.4 Fingerprinting patterns of the 96 isolated clones after digestion with BstNI. 13 different fingerprints were found, indicating the efficiently enrichment of the library due to high stringency conditions.

5.2.3 Construction and selection of the activation domain-fusion anti-151-421 τ scFv antibody library

In order to rescue anti-tau neutralizing antibodies from an intracellularly expressed polyclonal repertoire, the bulk DNA encoding for the second round polyclonal scFv- anti-151-421τ fragments was subcloned into the VP16 activation domain vector to obtain the library consisting of $6x10^4$ clones. DNA fingerprinting of 20 clones from this library confirmed the diversity (data not shown). In order to isolate scFv fragments that recognize 151-421τ, the yeast bait strain was constructed by transforming lexA-151-422τ bait into L40 yeast strain. Since pBTM116 contains TRP1 as a selectable marker, transformants should grow on Trp-negative plates and express lexA-151-422τ protein. The yeast bait was transformed in large scale with the second round polyclonal scFv-anti-151-421τ –VP16 library and transformants were selected on –his medium for histidine prototropy. After 3 days of incubation at 30°C, 10^5 yeast transformants were screened by β–gal assay. 90% of the clones were identified that interact more strongly (blue-color development that take from 5 minutes to 1 hour) with the lexA-151-422τ fusion protein. 100 blue colonies were grown, DNA encoding for the scFv-anti-151-421τ–VP16 was isolated and the fingerprint of BstNI digestion products analyzed (Fig.5.5).

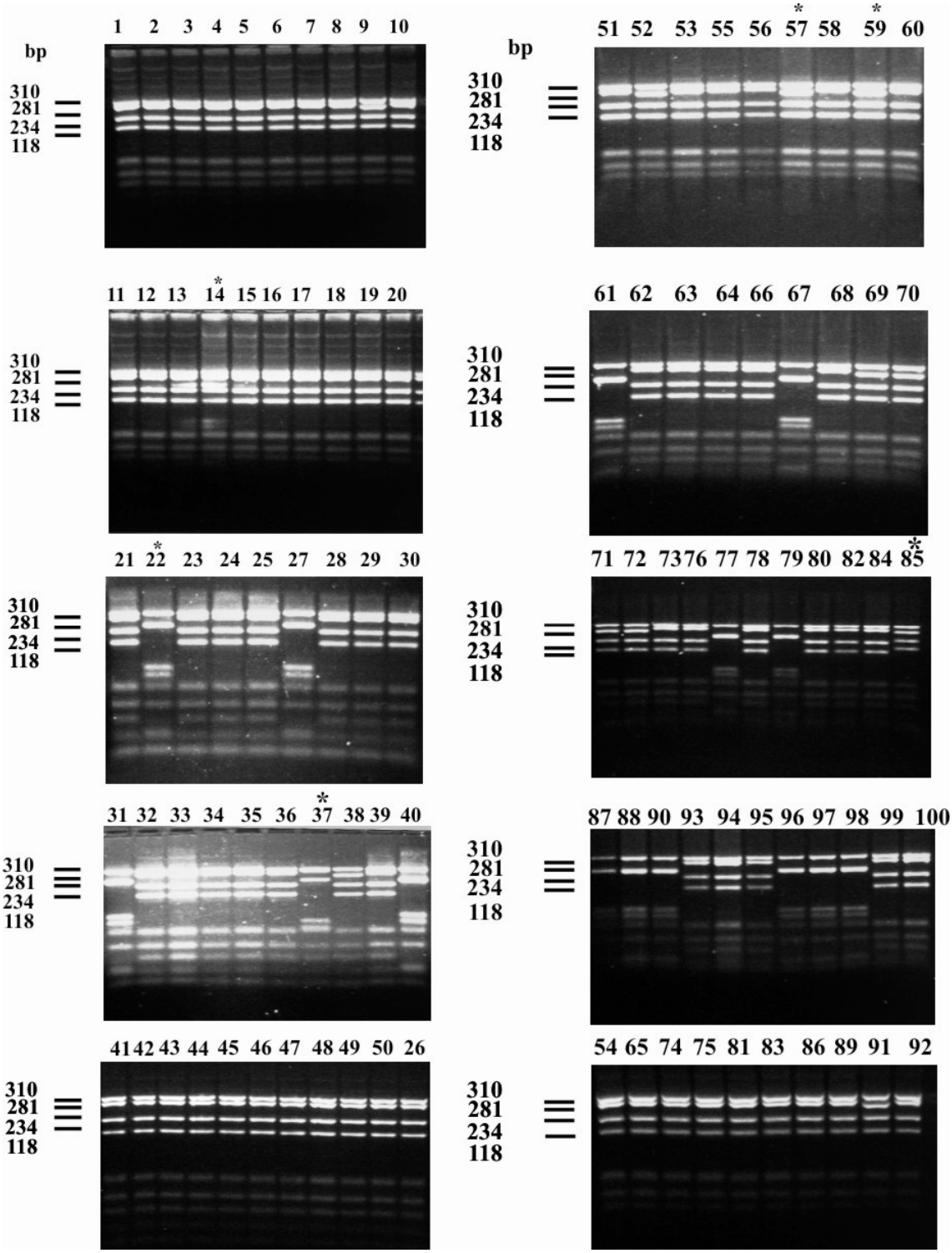

Fig.5.5 Fingerprinting patterns of the 100 isolated clones after digestion with BstNI. DNA fingerprinting analysis of the isolated clones were confirmed by other gels: clones 37 and 85 that cannot find in this gel are visible in other gels. The comparison of different gels revealed 5 different fingerprintings (Fig.5.6)

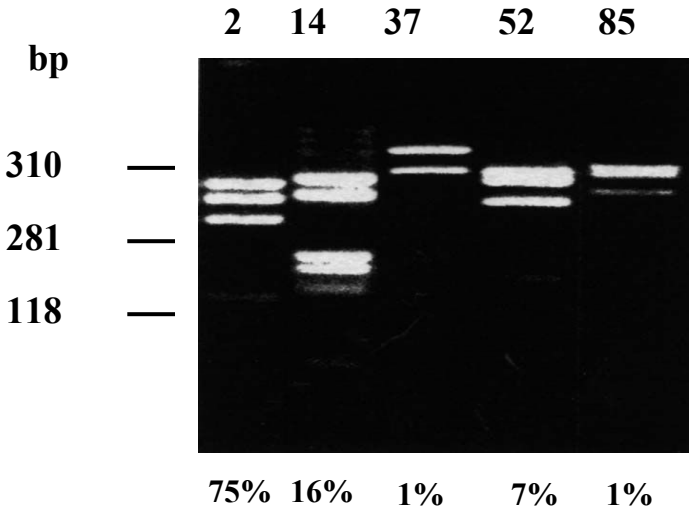

Fig.5.6 Fingerprinting analysis of the 5 different clones. The % reported indicates the frequency at which each scFv fragment is represented in the input second cycle library. Single clones may be spurious false positive, as confirmed by other analysis (see Fig.5.7).

The analysis of the hundred fingerprints revealed 5 different patterns representing 5 independent scFv-fusions to VP16 acidic activation domain. The percentage of scFv fragments that have the same fingerprint in the input library is high for type 2 and very low for the others, indicating that the *in vitro* enrichment was very stringent for the other scFv types. To control for false positives the five independent clones were further re-tested in a two-hybrid assay with the lexA-151-422τ bait. In this screen, only 3 scFv transformants containing the bait plasmid were able to transactivate the HIS3 and the lacZ genes (Fig.5.7). These clones were sequenced to confirm the diversity and to verify the correct in-frame fusion (data not shown).
In general, interactions that have affinity tighter than a Kd value of 10^{-6} will be detected by a two-hybrid system. Although there may exist a weak positive correlation between apparent strength of binding and biological significance, many apparently weak interactions are real while some strong ones are not. To be sure that antigen-antibody interactions chosen by the ITT showed a biological significance, scFv fragments that were weak and

strong positive were re-tested in a two-hybrid assay and the same antibody-antigen pairs were further verified by a different biochemical technique (see paragraph 5.2.5), while others that did not interact after a second two-hybrid screening were not chosen for use in subsequent verifications.

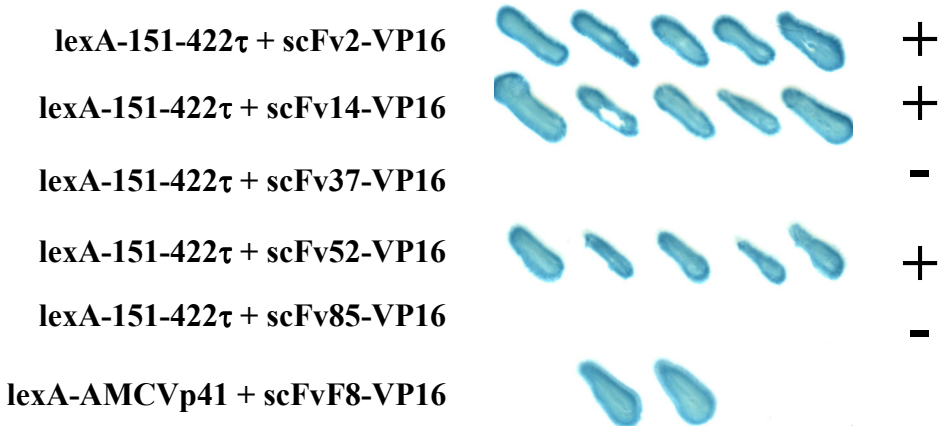

Fig.5.7 Interaction of lexA-151-422τ bait with 5 independent scFv-fusions to VP16 AD-domain. L40 yeast strain was cotransformed with lexA-151-422τ bait and the panel of the isolated scFv-VP16 plasmids. Only scFv2, scFv14 and scFv52 were able to transactivate HIS3 and lacZ genes (row1, 2 and 4) while scFv37 and scFv85 were not (row 3 and 5). scFvF8-VP16+lexA-AMCVp41 were used as positive control (row6).

The scFvs-anti-151-421τ protein were also tested by cotransfection with other baits encoding lexA DNA-binding domain alone or as fusion with lamin, and growth was detected in his- medium (data not shown). β-gal and HIS3 activations were not observed after three days of incubation at 30°C, indicating that these scFv are able to specifically interact only with their corresponding 151-421τ antigen under the intracellular conditions of this assay.

5.2.4 Is the *in vivo* selection needed?

In order to test the input library that was used for the ITT selection for isolating intrabodies without any *in vivo* selection scheme, four ELISA positive anti-151-421τ protein after the second cycle of *in vitro* selection were coexpressed with lexA-151-421τ bait as scFv-VP16 fusion proteins in yeast.

No transactivation of HIS3 and β-gal genes were observed after three days of incubation at 30°C (data not shown), confirming that there is a need for an *in vivo* selection procedure for the isolation of scFvs able to display intracellular antigen-antibody interactions.

5.2.5 scFv-anti-151-421τ protein *in vitro* characterization

To determine the binding activity of the three scFv fragments, the scFvs-anti-151-421τ protein were cloned into prokaryotic phagemid expression vector pUC119CAT. This vector displays the expressed scFv on the surface of the filamentous M13 phage by fusion with the gene III minor coat protein (g3p) that is required for host infection (McCafferty et al., 1990). Following isopropyl-thiogalactoside (IPTG) induction, soluble scFv were expressed in the periplasmic space of amber-suppressor bacteria (HB2151 *E.coli* strain) recognizing an amber mutation interposed between genes encoding the antibody fragment and the coat protein (Hoogenboom et al., 1991). To allow the immunodetection of the soluble scFv, the myc tag peptide is fused in frame at the C-terminus of the Vk domain. Preparation of pure scFv-anti-151-421τ protein from *E.coli* periplasm was achieved by affinity chromatography using the His tag.

Analysis by SDS-PAGE (coomassie staining and western blot) revealed a band at the expected size (30 kDal), with great purity after purification. The average yield of immunopurification from the periplasmic fractions was 300-900μg of scFv per liter of bacterial culture.

All three scFv fragments were tested in ELISA against the protein used for the selection *in vitro* (MBP-151-421τ), the deletion mutant used for the selection *in vivo* (MBP-151-422τ), the relative full-length protein tau and the MBP. All the soluble scFv fragments were specific for tau and for its deletion mutants and did not recognized maltose-binding protein (Fig.5.8).

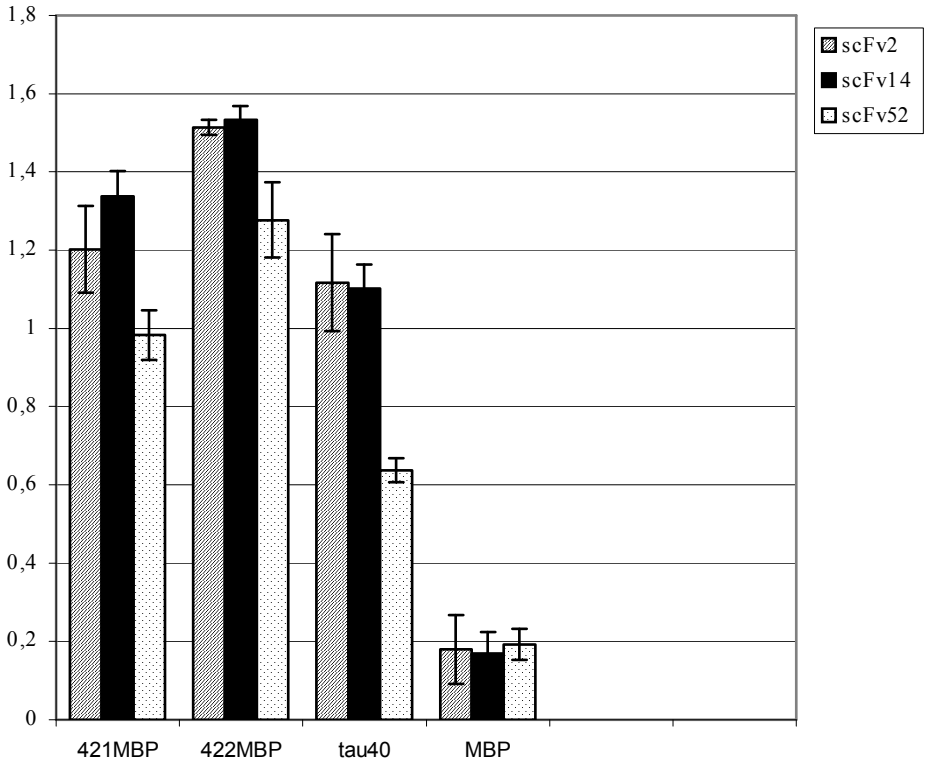

Fig.5.8 Reactivity of the isolated clones against tau protein, tau deletion mutants and MBP. The ELISA signal was measure at O.D. 450.

5.2.5.1 Analytical gel filtration

ScFv fragments often show a propensity to aggregate in to dimers or multimers.

After purification by affinity chromatography using His tag, the three scFv fragments were subjected to analytical gel filtration in order to isolate the monomeric form and to assess the gel filtration profile of the single chain antibody fragments. All the purified scFvs showed a single peak elution profile corresponding to the monomeric form of the scFv fragments. More than 90% of the protein was monomeric. (Fig.5.9). Therefore, these gel filtration data reflect the low aggregating behavior of the scFv fragments indicating the capability of these scFvs of maintaining the ability of independent structure formation also under *in vitro* conditions.

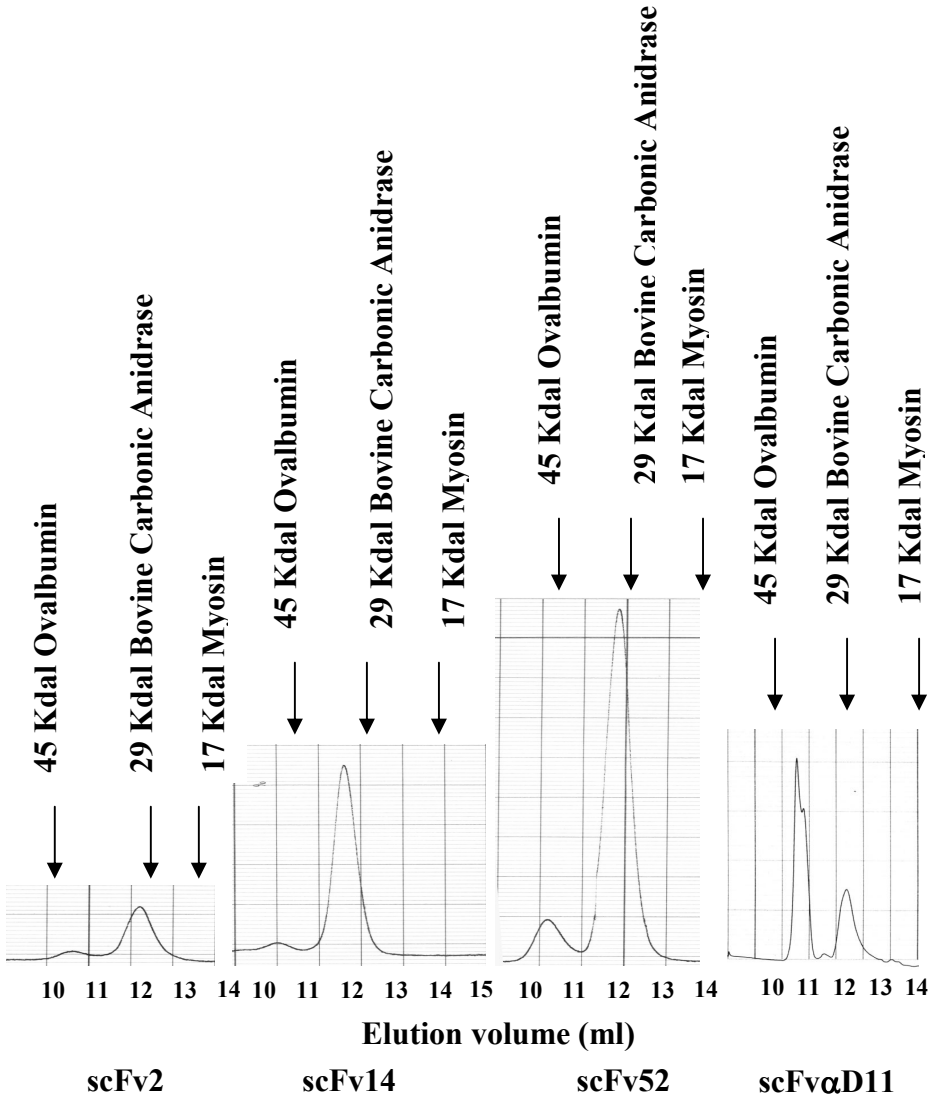

Fig.5.9 Analytical gel filtration. The amount of monomeric protein was quantified by integrating the peak representing monomeric scFv fragments, which was eluted from a Superdex-75 analytical gel filtration column. Elution volumes and molecular mass marker proteins are indicated. As a representative example, the analytical gel filtration chromatogram of scFvαD11 shows the amount of dimeric and aggregated species that were eluted at about 10.5 ml. The amount of remaining monomeric species decreased with increasing incubation time (data not shown).

5.2.5.2 Competition ELISA

In order to measure the affinity constants of the isolated scFv towards the corresponding antigens, equilibrium saturation analysis was performed by competition ELISA (Fig.5.10). After incubation of monomeric scFv fragments with the antigen, the concentration of free antibody was determined by indirect ELISA. The concentration of the scFv antibody was

chosen to be close to the estimated value of the dissociation constant. ScFv fragments supernatants were titrated with known antigen concentrations. The calibration curves shown in Fig. 5.10 demonstrate that for scFv2 and scFv14, the absorbance versus the initial concentration of antibody is linear in a range of concentration between 0.08-0.02μg/ml and 0.08-0.01μg/ml for scFv2 and scFv14 respectively. It was not possible to obtain linear calibration curve for scFv52 under the same experimental conditions probably because of the lower affinity of the interaction. A second possible explanation for this is that the scFv52 was not able to recognize the antigen in solution because of a hidden epitope due to possible interaction between the bound antigen and the one in solution.

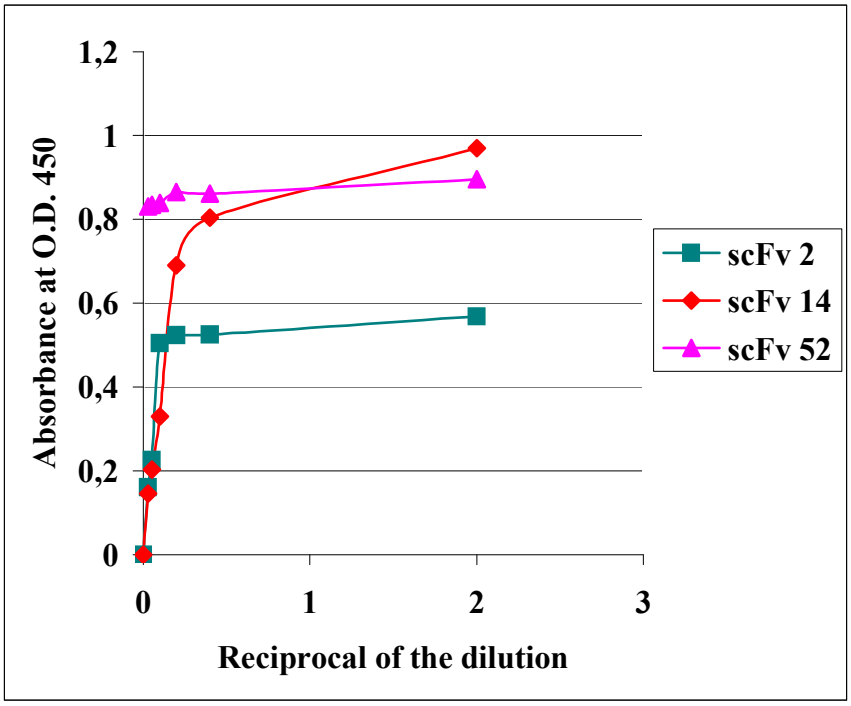

Fig.5.10 a) Calibration curves of the binding of scFv2 and scFv14. The concentration range of purified 422MBP was 100nM-1μM. Each assay was analyzed in triplicate. This figure shows that scFv2 and scFv14 have linear relation between absorbance and dilution in a wide range of antigen concentration, while scFv52 fails to display such a behavior.

The dissociation constant for scFv2 and scFv14 were determined from the corresponding equilibrium binding curves shown in Fig. 5.10 b) and c), taking into account the half of the maximum value of absorbance at O.D.450 of free antibody, yielding a Kd of 357 nM and of 143 nM for scFv2 and scFv14 respectively.

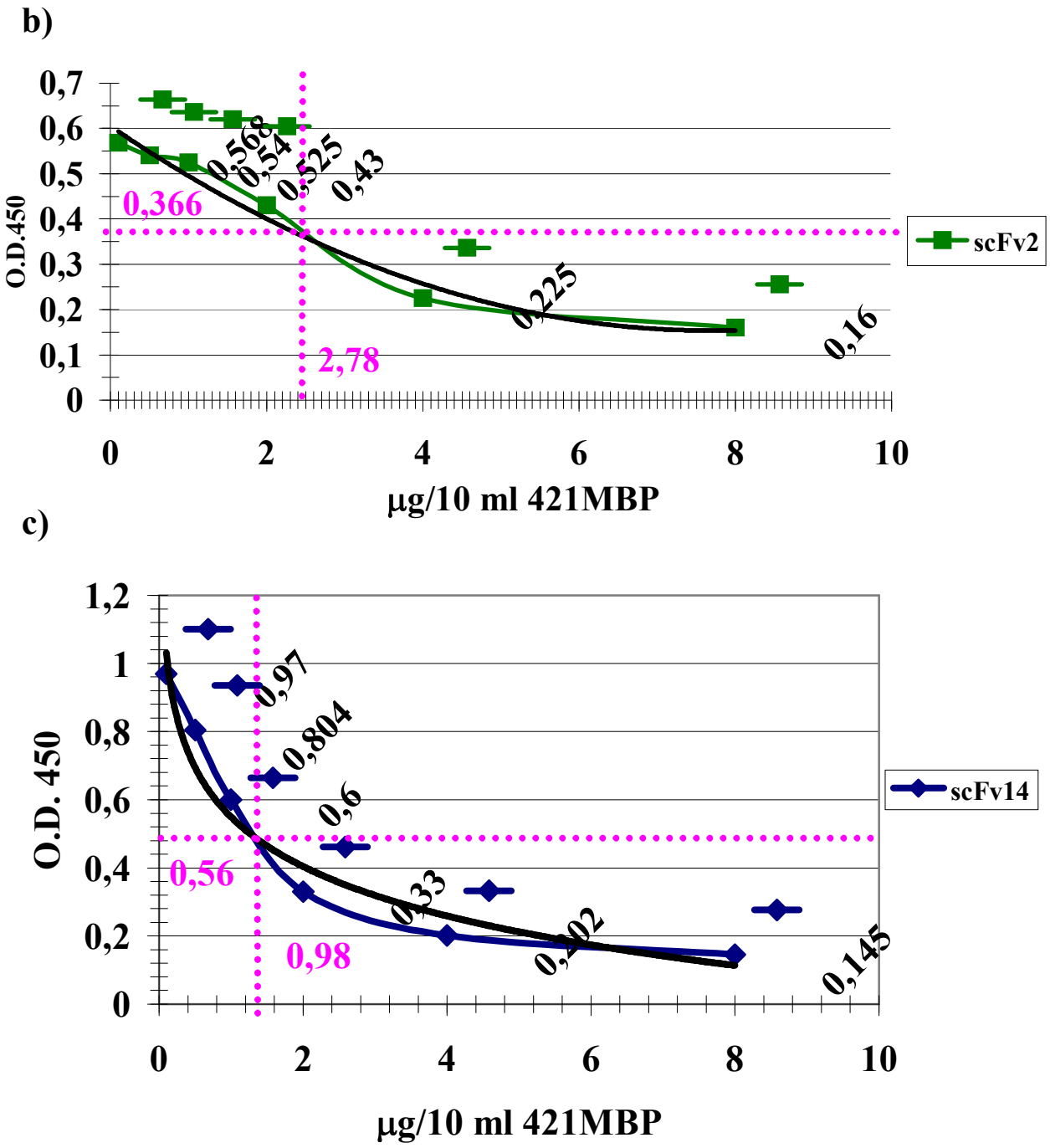

Fig.510 b) and c): Kd was derived at semisaturation of free antibody by plotting the absorbance on the ordinate versus the concentration of free ligand on the abscissa.

5.2.6 *In vivo* expression of anti-tau scFv intrabodies: intracellular retargeting of cotransfected 151-422τ by the scFv intrabodies

Using transient transfection analysis, the effect of the anti-tau scFv fragments on 151-422τ fragment was studied. Cellular expression of the anti-tau scFv intracellular antibodies was first analyzed by immunofluorescence microscopy of transiently transfected COS and CHO cells.

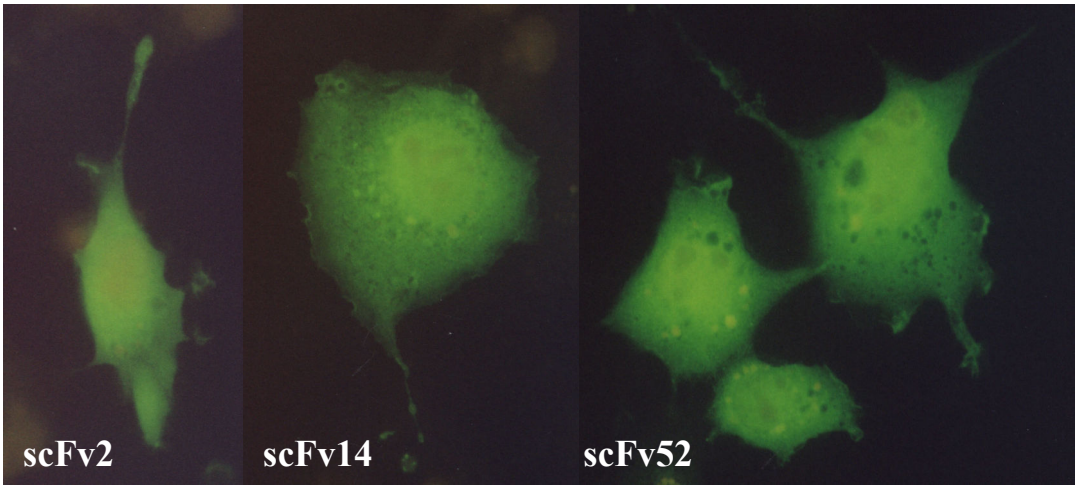

Fig.5.11 Immunofluorescence microscopy of the anti-151-422τ scFv fragments transiently transfected in COS cells. Cyto-scFv2, cyto-scFv14 and cyto-scFv52 were visualized under a fluorescence microscope; cells were reacted with the anti-myc tag antibody 9E10, followed by incubation with an anti-FITC-conjugated anti-mouse (Vector).

As demonstrated in Fig.5.11, the anti-tau scFv fragments shown a diffuse intracellular staining typical of soluble cytoplasmic proteins, confirm the good solubility properties of the isolated scFv fragments.
In order to demonstrate the interaction of antigen-antibody in mammalian cells, retargeting was thought to be one possibility. The effect of the three-scFv fragments on cotransfected 151-422τ antigen was evaluated in terms of their ability to retarget 151-422τ to a different subcellular compartment in an antibody-depending way. In order to do this, the three scFv fragments were cloned in mammalian expression vector containing a myc tag (Persic et al., 1997) and a nuclear localization signal and individually coexpressed in CHO cells with the cytoplasmic fragment 151-422τ (Fasulo et al., 2000).

The soluble scFvR4 (Martineau et al., 1998) was used as a non relevant scFv control.
After 36-40 h post-transfection, the cell were fixed, permeabilized, and stained with the anti-myc rabbit polyclonal antibody and with the mouse anti tau 7.51. Using immunofluorescence microscopy, the subcellular localization of the 151-422τ antigen and its corresponding scFv fragment were visualized (Fig.5.12).

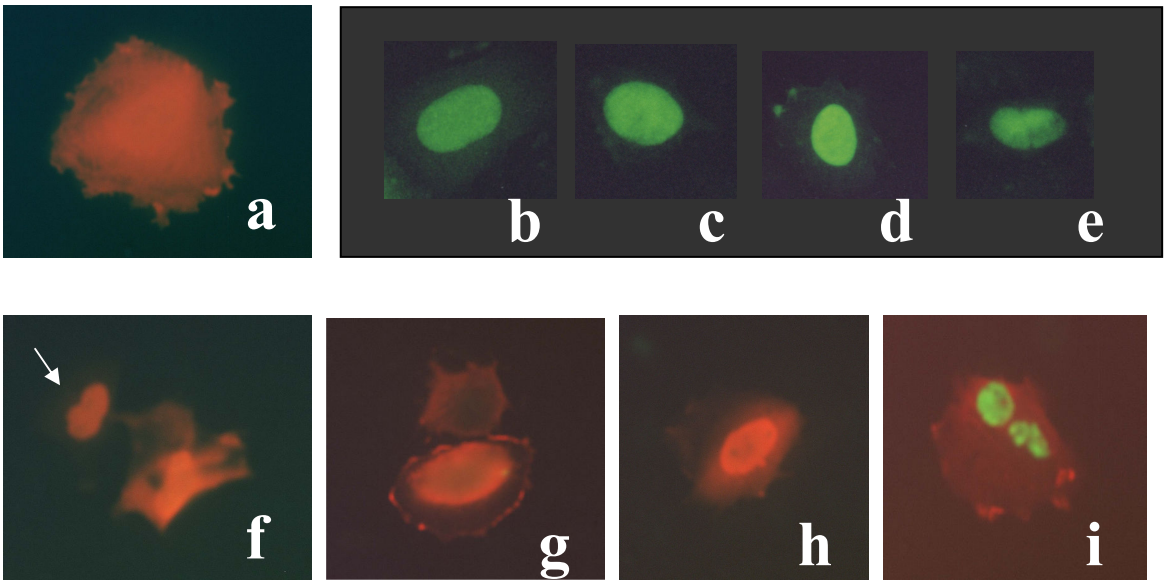

Fig.5.12 Dual immunofluorescence analysis of retargeting of 151-422τ antigen in the presence of different scFv fragments. Row1: cytoplasmic (cyto) 151-422τ antigen (a), (nuclear targeted, nls) nls-scFv2 (b), nls-scFv14 (c), nls-scFv52 (d) and nls-scFvR4 control antibody (e). Row2: coexpression of cyto-151-422τ antigen with nls-scFv2 (f), with nls-scFv14 (g), with nls-scFv52 (h) and with nls-scFvR4 (i). All the nls-scFv fragments were FITC-stained while the 151-422τ antigen was Texas-RED-stained. Transfected cells were visualized using a multiple wavelengths filter (Zeiss Filter Set 25). Only the cotransfected cells with cyto-151-422τ antigen and with nls-scFvR4 display the double different labeling which correspond to the proper targeting locations: green nucleus for the nls-scFvR4 and red cytoplasm for the cyto-tau fragment antigen. All the nls-anti-151-422τ antigen scFv fragments colocalized in the nucleus with the retargeted cyto-151-422τ antigen (red nucleus). The arrow in f) indicate a nuclear staining for the retargeted cyto-151-422τ antigen.

Fig.5.12 shows that 151-422τ was a cytoplasmic protein in CHO cells when transfected alone (row1, a) or when it was cotransfected with the non-relevant nuclear-scFvR4 (row2, i). In contrast, nuclear staining was observed when scFv2 (row2, f), scFv14 (row2, g) and scFv52 (row2, h) were coexpressed. The retargeting of 151-422τ was due to specific interactions between 151-422τ antigen and the anti-151-422τ single chain antibody fragments. This was confirmed by the coexpression of the irrelevant nuclear-scFvR4, which failed to retarget the 151-422τ antigen under the same intracellular expression conditions. This result demonstrates that a specific antigen-antibody interaction is required for retargeting.
This quality observation was quantified in the cell populations (n=100 counted cells for each experiment). The retargeting was evaluated as the ability of the nuclear scFv fragments to colocalize tau in the nucleus or as the ability of cytoplasmic tau to retain the nuclear scFv fragment in the cytoplasm. According to both criteria, antigen-antibody interactions were shown.
The effect on re-directing 151-422τ to the nucleus was less prominent that the retargeting of nuclear scFv fragments into the cytoplasm (Fig.5.13) but the overall efficiency of the anti-tau scFv antibody fragments to bind the antigen was absolutely clear with respect to the control scFvR4 to occur.

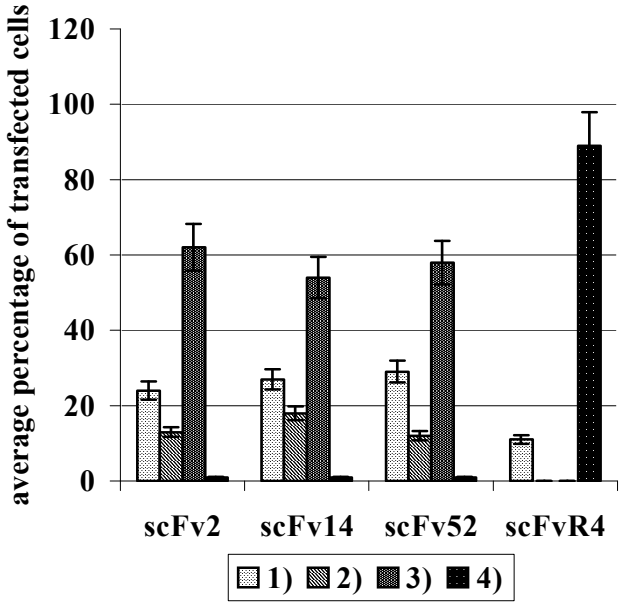

Fig.5.13 Effect on retargeting of 151-422τ in CHO cells by scFv2, scFv14, scFv52 and scFvR4.

1) τ nuclear and cytoplasmic
   scFv nuclear and cytoplasmic
2) τ nuclear
   scFv nuclear
   no staining of the cytoplasm
3) τ cytoplasmic
   scFv cytoplasmic
   no staining of the nucleus
4) τ cytoplasmic
   scFv nuclear

Pattern 1), 2) and 3) are indicative of an interaction between τ and scFv.

5.2.7 Selection from a scFv phage display library after one round of antigen panning

The affinity of the antibodies isolated using a conventional phage display library, is proportional to the initial size of the library used for the selection. The use of larger libraries has become an important goal in phage display, and in theory also in ITT the same fundamental consideration may be applied with the addition that this procedure may rescue scFv fragments not only on the basis of best affinity *in vivo* but also for best solubility.

One of the most important limitations using this technology is that the efficiency of transformation of yeast cell does not exceed 10^6/μg DNA. This important consideration has suggested us to clone, as first attempt, an enriched anti-151-421τ polyclonal phage population, to overcome this problem. The results showed so far, have demonstrated that it is possible to rescue functional and soluble antibody fragments that bind their antigen under intracellular expression conditions, after two cycles of antigen panning *in vitro*. In order to detect the sensibility of the ITT versus the diversity of the library, the first panning cycle of the polyclonal anti-151-421τ DNA was cloned as fusion library with the activation domain of the VP16 vector.

The library consisted of $2.2x10^6$ clones. DNA fingerprinting of 96 clones confirmed the diversity of this library (data not shown).

The fist cycle anti-151-421τ -VP16 DNA was cotransformed in lexA-151-422τ L40 yeast strain as described before.

After 3 days of incubation at 30°C, 10^4 yeast transformants were screened by β-gal assay and 50 blue colonies were subjected to PCR fingerprinting. 11 different fingerprints were detected in which we were able to rescue also identical fingerprinting to those of scFv2, scFv14 and scFv52 (data not shown). Six blue colonies with different fingerprinting were picked into liquid culture and plasmid DNA encoding for scFv-VP16 transactivation domain fusion was segregated. Yeast L40 was then cotransformed with the lexA-151-422τ DNA-binding bait clone together with each one of the isolated scFv-VP16 plasmids. 4 out of the original 6 clones grew on –his plates and showed a positive interaction when β-gal assay was performed (Fig.5.14), indicating that selection of anti-151-421τ scFv fragments from a library panned ones on antigen is indeed feasible and can lead to the rescue of more candidates for intracellular expression.

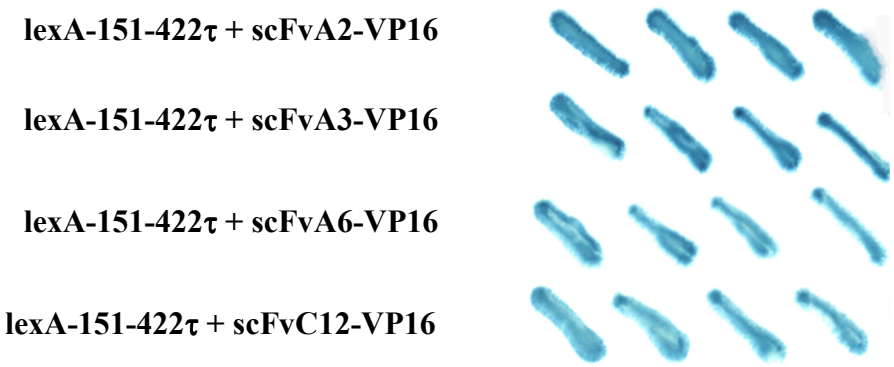

Fig.5.14 Intracellular interaction of scFvA2 (row1), scFvA3 (row2), scFvA6 (row3) and scFvC12 (row4) with target antigen lexA-151-422τ in L40 yeast cells. Cotransformed yeasts were streaked and grow on his- medium and scored for β-gal activity. β-gal activity was observed in each His+ clones.

5.2.8 *In vivo* epitope mapping (IVEM)

Epitope mapping is an important part of the characterization of an antigen. For research, diagnostic, and therapy it is desirable to have antibodies that bind to a given epitope of an antigen. Many different approaches have been used, but all explored the possibility to map the epitope *in vitro*. Epitope mapping is using a very laborious procedure. In general the most useful technique in determining the epitopes recognized by a number of antibodies is the use of peptide, which can be displayed as peptide-phage library or used as synthetic products (Bradbury & Cattaneo, 1995). This strategy is very general, and sometimes the peptide motifs have no resemblance to the primary sequence of the protein antigen and this indicates that the epitope that is recognized by the antibody is a discontinuous or conformational epitope.

The ability of the two-hybrid system to display the *in vivo* conformation of the protein and to detect conformational changes in a given protein, has induced us to explore the ability of this system to determine the epitopes recognized by the three isolated scFv fragments. To perform this, deletion mutants of the protein tau were fused in frame to lexA binding domain. The set of fragments included (see also chapter four, fig. 4.2):

- Tau 151-274: this fragment includes the proline rich domain and the R1 repeat
- Tau 151-305: this fragment includes the proline rich domain and the R1-R2 repeats

- Tau 151-336: this fragment includes the proline rich domain and the R1-R2-R3 repeats
- Tau 151-368: this fragment includes the proline rich domain and the R1-R2-R3-R4 repeats
- Tau 151-391: this fragment includes the proline rich domain and the R1-R2-R3-R4 repeats extended up to Glu 391
- Tau 151-402: this fragment includes the proline rich domain and all the repeats extended up to Asp 402
- Tau 151-412: this fragment includes the proline rich domain and all the repeats extended up to Ser 412
- Tau 151-422: this fragment includes the proline rich domain and all the repeats extended up to Ser 422
- dGAE: is one of the AD PHF core fragments (Wischik et al., 1988b)

The expression level of lexA-fusion bait proteins was assessed by western blot analysis using the polyclonal antibody anti-lexA protein (Fig.5.15 a), mAb Tau-1 (Roche) (Fig. 5.15 b) and mAb 7.51 (Fig.5.15 c) (Evan et al., 1985). The specificity of the three different scFv fragments against the panel of antigen baits is illustrated in Fig.5.16 and summarized in Table 5.2.

Antigen	151-274	151-305	151-336	151-368	151-391	151-402	151-412	151-422	dGAE
scFv2	-	-	-	-	-	-	+	+	-
scFv14	-	-	-	-	+	+	+	+	+
scFv52	-	-	-	-	-	-	+	+	-

Table 5.2 Specificities of the scFv2, scFv14 and scFv52. Binding was determined by ITT to a panel of deletion mutants of tau protein by transactivation of HIS3 and lacZ genes.

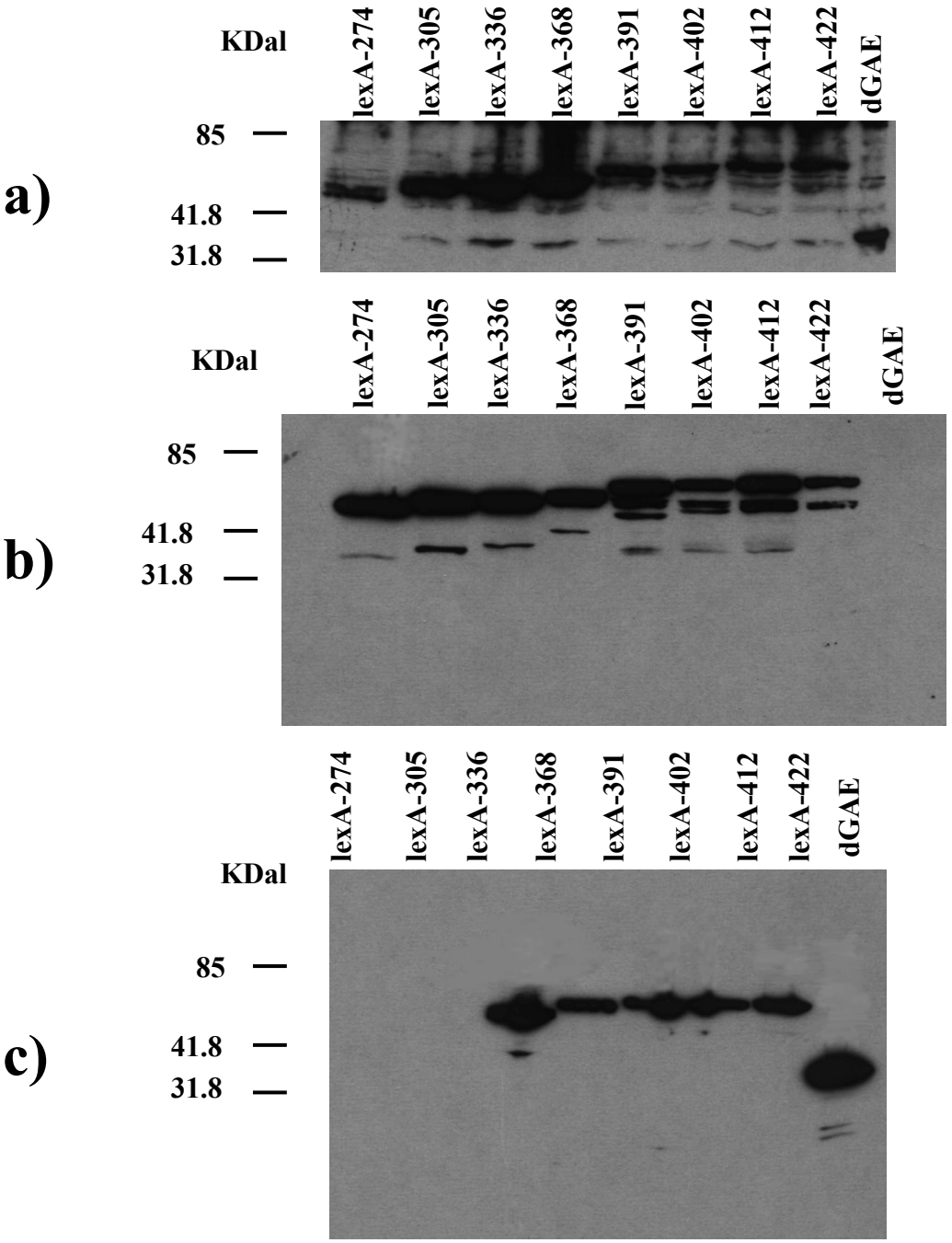

Fig.5.15 Western blot ananlysis of lexA-deletion mutants of tau fusion proteins. a) the proteins were revealed by using a polyclonal antibody anti-lexA, b) the proteins were revealed by using a generic anti-tau monoclonal antibody Tau-1, c) the proteins were revealed by using another generic anti-tau monoclonal antibody 7.51.

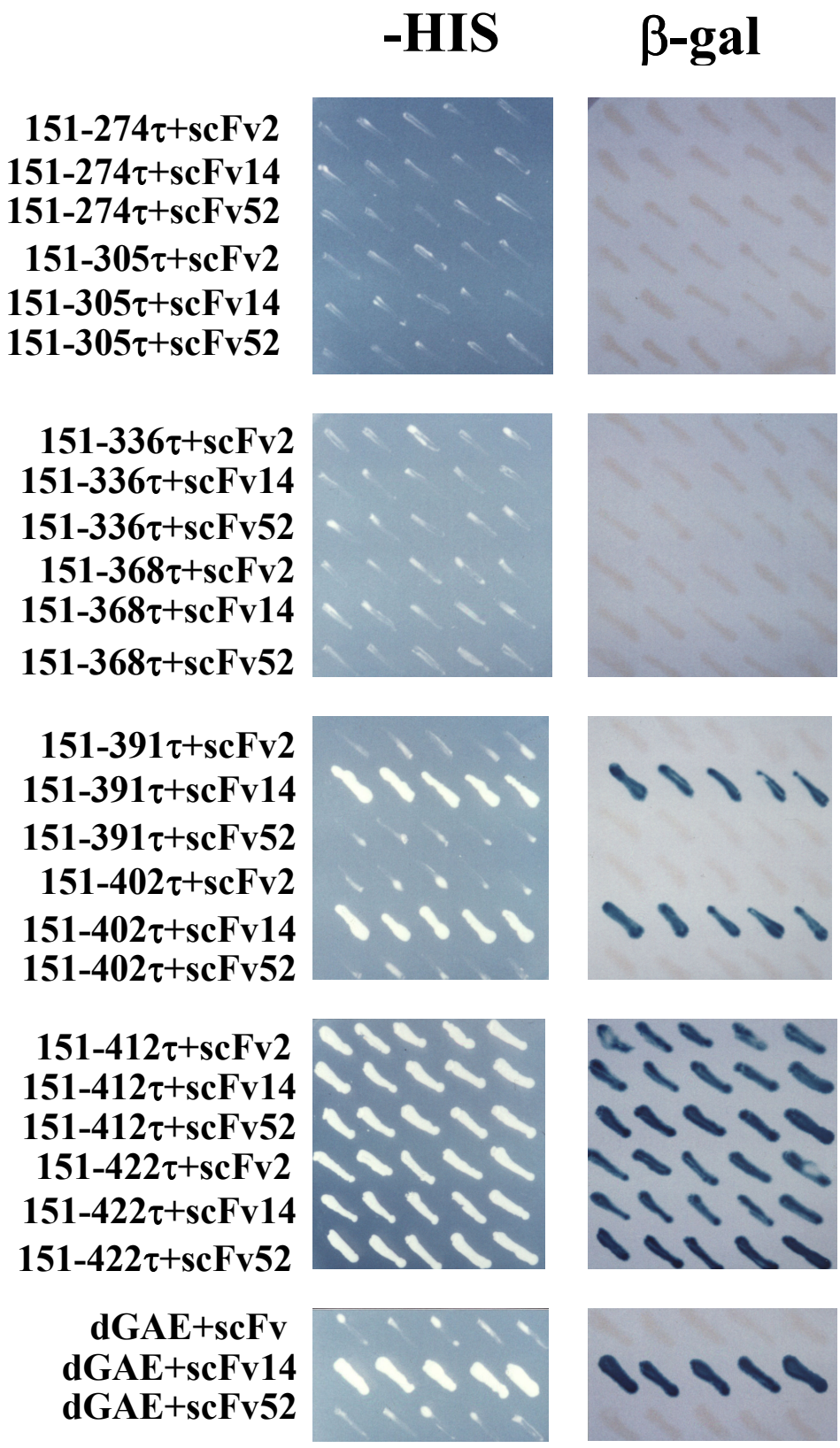

Fig.5.16 Epitope mapping of scFv2, scFv14 and scFv52. Growth (-his plates) and β-gal phenotypes conferred by the set of fragments of tau interacting with the three-scFv fragments.

This new way to map the epitope of given intracellular antibodies *in vivo* (*in vivo* epitope mapping, IVEM), is a valid alternative to laborious and expensive approaches that will always reveal the *in vitro* and not the *in vivo* properties of an intrabody. The simplicity of this approach should also be extended to a high throughput screening, were interesting intrabody specificities may also be obtained using this system.

5.3 Discussion

5.3.1 Intabody trap technology: a new selection procedure to facilitate protein knock-out at a genomic scale

The complexity of the genome of many organisms, and in particular of mammalian genomes renders the functional understanding of a new protein superficial if it is examined only with experimental genomic technologies.
The efforts to trace the intricate connections and activities of proteins have induced many laboratories to develop even more sophisticated technologies with the whole tools of cell and molecular biology, genetics, biochemistry and structural analysis. One of the strategies adopted to generate protein linkage maps of large networks of interacting proteins is the two-hybrid system. This approach is having a strong impact in functional genomics where proteins of unknown function, predicted from genomic sequences, generate complex relationships and connections among the elements, sometimes previously suspected or not.
In the last decade yeast two-hybrid system have been used to isolate interactors of many proteins of interest. The idea to use the two-hybrid system to isolate intracellular antibody-antigen pairs arises from the modular nature of the system itself and from the fact that the interaction occurs under the reducing condition of the cell cytoplasm and the nucleus.
Intracellular expression in mammalian cell has extensively demonstrated that neutralizing antibodies can confer new phenotypic traits upon interactions, but the bottleneck of using directly specific antibodies derived from monoclonals or from phage display libraries, is due to the difficulty of finding antibody that respect the requirement for good intracellular performance, that are high solubility, high stability and good affinity.
The study performed so far in the field of antigen-antibody two-hybrid system has demonstrated that this approach is not only feasible to detect this kind of interaction but provide a ‘proof of principle’ for the concept of selection of intracellular libraries, whereby a polyclonal population of

antibody fragments can be intracellularly expressed and can be an unlimited source of "high affinity intra-binders".

5.3.2 The choice of tau protein as "target antigen"

To design a suitable selection scheme using the "intrabody trap technology", the case of a multifactorial disease was evaluated.
Alzheimer's disease is one of the most common causes of late-life intellectual impairment in countries that have achieved life expectancies above 70 years. In contrast with other unsolved disease of the human brain, Alzheimer's disease presents features that make it particularly amenable to studies of pathogenesis at the molecular level. Accumulation in the brain of the classical hallmarks of the disease, neurofibrillary tangles and senile plaques are the defining feature of AD. It is still not known how the formation of these pathological structures occurs and how they contribute to the gradual decrease in the numbers of synapses and neurons that seems to be of particular clinical importance.
The abundance of neurofibrillary tangle seems to be more closely related to the extent of neuronal loss found in AD brain; furthermore the tangle formation seems to be a late event and presumably represent a final pathway towards neuronal death in subset of neurons (Morishima-Kawashima et al., 1995).
The evidence that proteins that are involved in PHF formation should be one of the key events that leads to neurodegeneration has lead us to focus our attention on one of the main component of this abnormal deposit.
The principal component of PHF, which are the main constituent of neurofibrillary tangles, is the protein tau.
Understanding of the cellular and molecular biology of the processes in which tau protein is involved is an essential prerequisite for the development of an early diagnostic assay and drugs capable of preventing steps leading to this neurodegenerative disease.
Attempts for therapeutic intervention need to focus on the primary defect which tau and other proteins are responsible for initiating the neuronal degeneration and the application of antibodies to use in *in vivo* experiments should greatly facilitate the understanding of the molecular processes which tau protein is involved.

5.3.3 From gene to antibody bypassing proteins

One of the prerequisite of the ITT is that the entire selection scheme passes through gene to the antibody without a necessary and obligatory handling of the protein.

This may have great advantages because handling of DNA is much more cost-effective and simple than handling of protein.
The application of the two-hybrid system on a large scale has increasingly broadened the application of this technique in detecting protein pairs, complexes and also DNA-protein pairs. The use of this system is becoming even more versatile. The application of the ITT in the genome sequencing projects may facilitates the research of individual gene functions but also may provide a generic tool for many functional genomic characterizations as, for example, protein localization, protein-protein interaction maps and gene knock-out.
Following the results obtained with the model selection experiment the possibility of carrying out library search with ITT was implicit in the original idea. To perform such experiment several schemes on the methodologies were becoming available in order to facilitated spread of this technology.
The first requirement to develop ITT selection scheme was to solve the problem of the size of the input scFv library that, in our case (Sheets et al., 1998) is in the order of 10^9 different clones. To deal with the very low efficiency of transformation of the yeast, that cannot exceed $10^6/\mu g$ of DNA, a need of a preselection step in order to achieve the repertoire size of 10^4-10^6 was evaluated. In general, the best approach to enrich a library is to perform affinity purification on antigen column.
The selection scheme for the isolation of intracellular scFv was initially tested using a polyclonal anti-151-421τ population from the second cycle of selection and successively a polyclonal anti-151-421τ population from the first cycle of selection.
The diversity of the polyclonal anti-151-421τ population from the second cycle of selection was evaluated to be around 13% (i.e. 13 different fingerprinting pattern were found out of a random selection of 100 clones). The number of specific anti-151-421τ scFv fragments was evaluated to be in the order of 10% after the selection of the second cycle. Furthermore, the percentages of different scFvs achieved after fingerprinting screening of 100 clones was only 5% (i.e. 5 different fingerprinting pattern of scFv in the two-hybrid selected library) and among these only 3 were positive after a second two-hybrid screening. The analysis of the fingerprinting pattern obtained from the isolated two-hybrid positive scFv fragments has indicated that two putative positive binders could be rescued analyzing 100 different scFv positive anti-151-421τ scFv fragments after the phage-ELISA. These scFvs (scFv2 and scFv52) were present at higher frequency in the polyclonal population (20% for scFv2 and 2% for scFv52). It was impossible to rescue any scFv14 fingerprinting pattern in the same polyclonal population, and this indicates that ITT is able to detect rare scFv

fragments with higher sensitivity, lower time-consuming selections schemes than phage display technology with the advantage of rescuing directly for *in vivo* specificities.
To stress the importance of the *in vivo* selection scheme, four different anti-151-421τ scFv fragments selected with antigen from phage display library were analyzed by expression in two-hybrid format. None of these exhibited binding to antigen in the cytoplasm of the yeast cell, indicating that an effective selection scheme for intracellular antigen-antibody interactions is required.
The finding obtained so far has indicated that the selective pressure arises from the *in vitro* enrichment during phage library panning cycle may lead to a loss of variability of the polyclonal repertoire. To overcome this problem a first cycle of selection on 151-421τ antigen was investigated.
The results obtained has indicated that this polyclonal population of anti-151-421τ scFv fragments is compatible with selection schemes in yeast two-hybrid format, not limiting the sensitivity of the assay to screen all possible candidates. In fact, the polyclonal repertoire was restricted enough to deal with yeast transformation but the diversity was still high enough (90% of the clones undergone to PCR-fingerprinting analysis had different pattern, data not shown) to permit selection of 4 new positive scFv after screening of only 6 different blue colonies. The diversity of the library after two-hybrid screening was restricted to 20% of the total. This result confirmed that the system is able to fish out many new intracellular candidates from the high variability of the intracellular scFv repertoire, indicating that selection of even more different specificity could be rescued by scaling up the number of the transformed yeast colonies.

5.3.4 Solubility and affinity of the selected anti-151-422τ scFv fragments

In the present study we have also investigated the interplay between *in vitro* affinity, solubility and the performance of *in vivo* expressed scFv intrabodies.
Anti-151-422τ scFv fragments have essentially very similar affinity as demonstrated by competition ELISA and all scFv were almost exclusively in the monomeric format, showing a small “diabody” peak. All the selected scFv fragments were able to bind *in vitro* not only the deletion mutant 151-421τ used in preselection screening but also the deletion mutant 151-422τ used for *in vivo* selection. Furthermore, all the scFv recognize also the full-length protein, but no irrelevant proteins were recognized by the scFvs either *in vivo* or *in vitro*. This result indicates that antibodies obtained by ITT follow all the criteria of a good monoreactive and highly specific best-

performing intrabody without the need of further protein engineering to improve stability and affinity of the isolated proteins.

5.3.5 Efficiency of intracellular antibody-mediated nuclear retargeting of 151-422τ protein

The intracellularly expressed scFv fragments, which bind in two-hybrid format with 151-422τ protein, is demonstrated in this study to have specific properties toward redirection of the localization of the antigen to a particular subcellular compartment.

The expression of several scFv fragments show distinct propensity to aggregate, when expressed in the cytoplasm of mammalian cells, depending on the overall expression levels (Persic et al., 1997). Their performance depends primarily on their amino acid sequence, in an unpredictable way (Cattaneo & Biocca, 1999).

The phenomenon of the aggregation occurs naturally during maturation of newly synthesized native proteins and represents an off-pathway of the normal folding process (Wetzel, 1994).

In order to evaluate this possibility, the expression of the three anti-151-422τ protein was compared in immunofluorescence studies. All the scFvs showed a diffuse intracellular staining typical of soluble cytoplasmic proteins. The same homogeneous staining was exhibited when scFv fragments were directed by means of an intracellular-trafficking signal, to the nucleus.

The effects of the anti-151-422τ protein on cotransfected 151-422τ protein were evaluated in terms of their ability to retarget 151-422τ protein to a different subcellular compartment.

All the three scFv fragments were able to retarget 151-422τ protein to the nucleus (the irrelevant scFvR4 did not exert the same capabilities), although the efficiency was sometimes variable due to the property of aggregation of the antigen-antibody complex. In fact, when anti tau antibodies were interacting *in vivo* with the tau antigen, it was shown that the latter was able to divert them from its normal location and to change their solubility state. This fact was less evident when the expressions of the scFv fragments were higher then the expression of tau antigen. Modulation of the expression of the antigen or expression of the antigen-antibody pairs in a dicistronic vector, may avoid this problem.

These findings demonstrate that, despite the reduced conditions of the intracellular environments, these scFv were efficiently expressed in the cytoplasm and in the nucleus of mammalian cells, and that they maintain the same binding capabilities showed in the yeast cells, confirming that ITT is a feasible technique to allow the isolation of intrabodies without a need for further scFv engineering for increased folding stability.

5.3.6 Epitope mapping

In the present study the design of a suitable selection scheme that should allow the selection of antibodies against known or unknown epitopes was presented.

Moreover, fine specificity of the anti-tau scFv fragments was investigated by testing the recognition of several deletion mutants of tau protein by using ITT. The originality of this technique is that the epitope mappings were performed *in vivo* without the use of laborious, time-consuming and expensive peptide productions and with the same accuracy of the classical methods.

These findings should greatly accelerate the detection of critical epitopes in high throughput screening, were identifying functional sites is correlated with how the protein actually works.

Moreover, the functional analysis of proteins that are multifunctional may also be remarkably accelerated by the use of a panel of intrabodies that could be rescued after an ITT selection schemes.

5.3.7 Possible exploitation of the ITT in a genome-wide study

Genome sequencing projects are predicting large numbers of novel proteins, whose interactions with other proteins must mediate the function of cellular processes. To analyze these networks, several techniques should be used on a genome-wide scale. Among these the two-hybrid system seems to be the most rapid and sensitive, yielding the DNA sequences encoding proteins to interact and allowing many searches to be conducted simultaneously (Bartel et al., 1996).

The data generated from the protein interaction analysis offer insight into the possible biological roles of genes with unknown functions by connecting them to other characterized proteins, or can provide novel interactions between proteins that are known to be involved in a common biological process, or can assign novel functions to some previously characterized proteins. Recent papers (Bartel et al., 1996) (Fromont-Racine *et al*., 1997) (Walhout *et al*., 2000) (Flajolet *et al*., 2000) (McCraith *et al*., 2000) (Uetz *et al*., 2000) (Ito *et al*., 2000) have demonstrated the success and the widespread applications of the two-hybrid system in practical approaches for the identification of interacting partners by screening methods on a genome-wide scale.

One approach that can be used to understand protein function is to disrupt and/or manipulate gene expression. The coming era will require several generic reagents and methods for the characterization of gene functions (Lander, 1996). ITT can find exciting applications in conjunction with

functional genomics programs, in particular with programs aimed at systematically studying the protein network of a cell (see Fig.5.17).

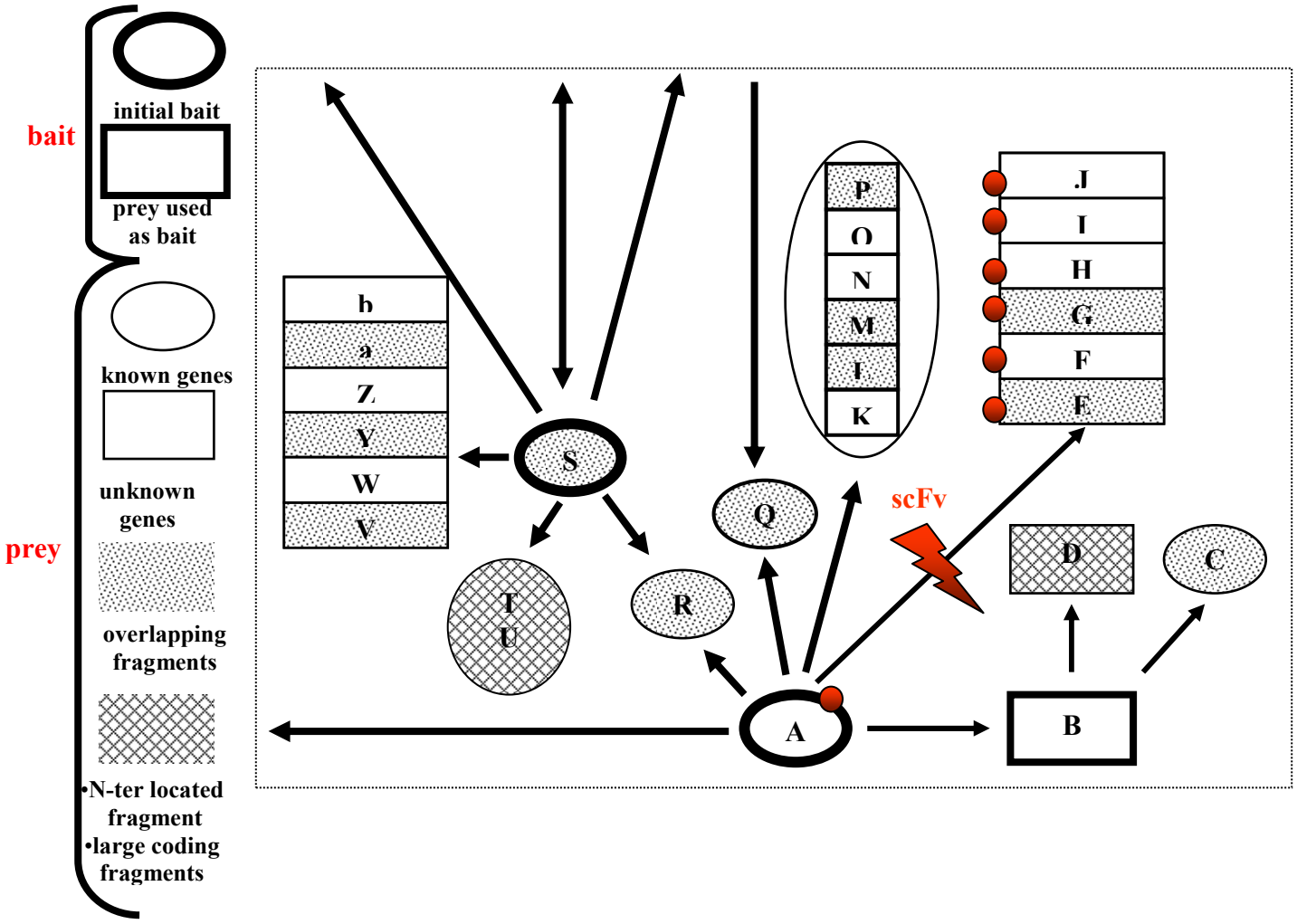

Fig.5.17 Protein interaction map. An example of a functional analysis of a genomic library screening in which for each bait are selected a limited set of interacting preys that are classified in categories of distinct heuristic values (Fromont-Racine et al., 1997). The use of intrabodies to systematically distrupt this kind of networks can get closer to the understanding of function. As shown in the figure scFvs can interfere or block interactions between a known gene product (A) and several unknown genes (E, F, G, H, I, J). scFvs can block protein interactions at the level of the known protein (red spot on A) or at several level of the unknown gene products (red spots on E, F, G, H, I, J respectively). The distruption of such interactions can perturb many other linkage in the network under investigation helping the comprehension of the associations formed during a high throughput two-hybrid screens.

Despite the need for comprehensive studies on *in vivo* protein-protein interactions, the knock-out technologies developed so far are not so general to be applied in a genome-wide studies, althought some studies using combinatorial protein reagents (i.e. aptamers and phage display

technology) have demonstrated that proteins could be the latest breakthroughs to probe regulatory networks (Geyer *et al*., 1999) (Colas, 2000) (Blum *et al*., 2000).

Antibodies are the most popular class of molecules providing molecular recognition. Their usual format for their application in a high-troughput screening is that of small binding units, for instance scFv fragments. The intracellular antibody strategy increases the spectrum of the antigens which are accesible to functional studies. Recent advances in the field of intracelluar expression of antibodies have demonstrated that the use of molecular-repertoire technology and genetic screens under conditions of cytoplasmic expression should allow the selection of new antibody specificities or antibody frameworks more suitable for the particular expression condition of interest (Worn & Pluckthun, 1999) (Worn & Pluckthun, 1998) (Visintin *et al*., 1999).

Recombinant antibody fragments expressed in the cytoplasm of the cells have considerable practical potential. However in the reducing environments of the cytoplasm, the intradomain disulphide bonds are not formed and fragments are unstable and expressed in low yields. To overcome these limitations several strategies were applied (Martineau et al., 1998) (Visintin *et al*., 1999) and we believe that the use of selection strategies in combination with the use of libraries that are constructed on very stable frameworks should be more effective and should improve expression and stability of scFv fragments.

The results obtained during these years have indicated quite clearly what are the limitations of the use of ectopic expression of antibodies. There is considerable room for improvements, however, in particular as far as the optimization of promoters, choice of new tags (i.e. GFP proteins), looking for new targeting signals.

None of the other approaches to gene- or protein- knock-out produce an effect that is general and reproducible from line to line as the ectopic expression of antibodies. Moreover, the mechanism by which antibodies prevent gene expression is quite clearly understood while this is not true for other techniques such as RNAi and antisense RNA. The dramatic recent progress in uncovering the gene-specific silencing in a number of organisms may be an additional challenge to allow high-throughput sample analysis in conjunction with other better characterized techniques.

The expression of antibodies to dissect signal transduction pathways should have several advantages over other methods and may lead to a better understanding of the regulatory events involved in normal and disease processes and offer a systematic approach for searching for effective targets for drug discovery and diagnostics.

Conclusions

The present study describes the development of a new technology, which is capable of selecting antibody-fragments under intracellular expression conditions. This technology involves the use of the yeast two-hybrid approach in which a scFv linked to a transcriptional transactivation domain can interact with a target antigen linked to a DNA-binding domain, and thereby activate a reporter gene.

It was found that several characterized antibodies can bind their target antigen in eukaryotic cells in this two-hybrid format, and that this system is able to isolate intracellular binders from among sets of scFv that can bind antigen *in vitro*.

Moreover, the application of this system to a library-selection scheme, in which a protein involved in Alzheimer's disease was chosen for investigations, has lead to the selection of several specific and soluble antibody-fragments readily folded for intracellular application.

The ability of this system to work in intracellular conditions and to provide specific binding quickly and economically, suggest that this approach can be used on a genome-wide scale to specifically knock-out each node of a protein network as identified from protein linkage maps and to understand the connections of large networks of interacting proteins.

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